MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2020; 26: e922479
DOI: 10.12659/MSM.922479

Received: 2019.12.30
Accepted: 2020.05.04
Available online: 2020.08.13
Published: 2020.10.10

miR-106a Targets Anoctamin 1 (ANO1) to Regulate Lipopolysaccharide (LPS)-Induced Inflammatory Response in Macrophages

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

CDEF 1 Junfeng Heng
BCD 2 Dingye Wu
CDE 1 Shiqi Lu
ABCD 2 Yiming Zhao

1 Department of Emergency, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu,P.R., China
2 Department of Intensive Care Unit, Wuxi People's Hospital, Wuxi, Jiangsu, P.R. China

Corresponding Author: Shiqi Lu, e-mail: abwidu@163.com
Source of support: Departmental sources

Background: Sepsis is an organ dysfunction characterized by systemic inflammatory response. Micro(mi)ribonucleic acids take part in the regulation of the inflammatory response in many conditions. However, the role and mechanism of miR-106a and anoctamin 1 (ANO1) in the inflammatory response in sepsis remain largely unknown.

Material/Methods: The serum samples were collected from 31 sepsis patients and healthy volunteers. Lipopolysaccharide (LPS)-treated RAW264.7 cells were used for the study *in vitro*. The inflammatory response was investigated via interleukin-6 and tumor necrosis factor-alpha levels using quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay. The expression abundances of miR-106a and ANO1 were detected via qRT-PCR or western blot. The target association between miR-106a and ANO1 was explored using dual-luciferase reporter analysis.

Results: The inflammatory response was trigged in sepsis and LPS-treated RAW264.7 cells. miR-106a expression was enhanced and ANO1 declined in sepsis and LPS-treated RAW264.7 cells. Overexpression of ANO1 suppressed the inflammatory response and knockdown of ANO1 promoted the inflammatory response in RAW264.7 cells. ANO1 was directly targeted via miR-106a, and miR-106a reversed ANO1-mediated inflammatory inhibition in LPS-treated RAW264.7 cells.

Conclusions: MiR-106a regulated LPS-induced inflammatory response by targeting ANO1 in RAW264.7 cells, indicating the potential value of miR-106a for treatment of inflammatory diseases, including sepsis.

MeSH Keywords: Cell Proliferation • Pelvic Inflammatory Disease • Systemic Inflammatory Response Syndrome

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/922479

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Background

Sepsis is a deadly disease worldwide, along with systemic inflammatory response, leading to organ dysfunction in patients [1]. Great effort has been made to understand the pathogenesis of sepsis; it is still a disorder in search of a cure [2]. The activation of macrophages is associated with numerous inflammatory diseases, including sepsis [3]. Lipopolysaccharide (LPS) is a prototypical trigger of sepsis by inducing an inflammatory response [4]. Therefore, exploring the mechanism of LPS-induced inflammation in macrophages might be helpful for understanding the pathogenesis of inflammatory disorders, including sepsis.

Anoctamin 1 (ANO1) is also called transmembrane protein 16A (TMEM16A), which is vital in several malignancies [5,6]. Moreover, Zhang et al. suggest that ANO1 is regulated by LPS and decreases the secretion of tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) in LPS-treated A549 cells [7]. In addition, Dai et al. revealed that ANO1 could reduce the inflammatory response and fibroblast proliferation in idiopathic pulmonary fibrosis mice through transforming growth factor-β/Smad3 signaling [8]. Hence, ANO1 might play an anti-inflammatory role in some inflammatory-related diseases. More important, the emerging evidence demonstrates that ANO1 can decrease expression of TNF-α and IL-6 in LPS-induced sepsis [9]. However, the mechanism underlying the action of ANO1 in sepsis is largely unclear.

Microribonucleic acids (miRNAs) are a class of noncoding RNAs that target mRNA to participate in the development of sepsis [10]. Thus, we propose that miRNAs are associated with the regulation of ANO1. miR-106a is a member of the miR-17 family that plays important roles in progression of human cancers, such as gastric cancer, breast cancer, and oral carcinoma [11–14]. Importantly, miR-106a contributes to the inflammatory response *in vitro* and *in vivo* [15]; miR-106a knockdown has been found to weaken inflammation in inflammatory bowel disease [16]. Furthermore, the miR-17 family (miR-17/20a/106a) is suggested to regulate macrophage inflammatory response by signal-regulatory protein α (SIRPα) [17]. Moreover, miR-17/20a/106a are aberrantly expressed in sepsis mice induced by cecal ligation and puncture, and miR-106a level was higher than miR-17/20a in the blood of sepsis mice [18]. In addition, miR-106a could exacerbate sepsis-induced acute kidney injury by decreasing the inflammatory response [19]. Hence, we propose that miR-106a plays an important role in regulating the inflammatory response in sepsis. However, the function and mechanism of miR-106a in sepsis inflammation are largely unknown.

Bioinformatics analysis predicts the existence of potential complementary sequences of miR-106a and ANO1. Hence, we assumed that miR-106a could target ANO1 to regulate the inflammatory response. In the current research, we detected the abundances of miR-106a and ANO1 in sepsis patients and in LPS-treated RAW264.7 cells, a mouse macrophage cell line widely used for the study on sepsis *in vitro* [20,21]. Moreover, we explored the role of ANO1 in LPS-induced inflammatory response and analyzed the target relationship between miR-106a and ANO1 in RAW264.7 cells.

Table 1. Clinicopathological characteristics of the 31 healthy patients and 31 sepsis patients.

	Sepsis patients	Healthy patiens
Gender (Female/Male)	14/17	16/15
Age, year (mean±SD)	49.8±12.5	53.6±11.8
Pneumonia	10 (10/31)	
Urinary tract infection	7 (7/31)	
Central nervous system infection	9 (9/31)	
Intraabdominal infection	3 (3/31)	
Other	2 (2/31)	
WBC (10^9/L)	16.8±1.4	
CRP (mg/L)	108.6±22.5	
Leukocyte median (range)/μL	14.3×10^3	
SOFA score	7.6±2.3	

WBC – white blood cell; CRP – C-reactive protein; SOFA – sequential organ failure assessment.

Material and Methods

Sepsis patients and serum collection

Thirty-one sepsis patients (14 males and 17 females, ages 45–60 years) and 31 healthy volunteers without infection (15 males and 16 females, ages 43–55 years) were recruited from the First Affiliated Hospital of Soochow University. Information on the human subjects is shown in Table 1. Peripheral venous blood was collected from all subjects who signed the informed consents. After keeping for 1 h, the blood samples were centrifuged at 1000×g for 10 min for serum separation. The serum was collected and used for further experiments. This research was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Cell culture and treatment

Murine macrophage RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

grown at 37°C in 5% CO_2 in RPMI-1640 medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) as well as 1% penicillin-streptomycin solution (Procell, Wuhan, China). To stimulate a sepsis-like inflammatory environment, RAW264.7 cells were exposed to 1 μg/mL LPS (Solarbio. Beijing, China) for 24 h.

Cell transfection

The ANO1 sequences were cloned into pcDNA3.1 vector (Thermo Fisher, Wilmington, DE, USA) to generate the overexpression vector of ANO1, with empty vector as control (pcDNA). The short interfering RNA (siRNA) for ANO1 (si-ANO1, 5'-AAGUAUAGUCCAUACUUGCAU-3'), siRNA negative control (si-NC, 5'-UUCUCCGAACGUGUCACGU-3'), miR-106a mimic (5'-CAAAGUGCUAACAGUGCAGGUAG-3'), mimic negative control (miR-NC, 5'- CGAUCGCAUCAGCAUCGAUUGC-3'), miR-106a inhibitor (in-miR-106a, 5'-UAGAACUCAAAAAGCUACCUG-3'), and inhibitor negative control (in-miR-NC, 5'-CUAACGCAUGC ACAGUCGUACG-3') were provided by GenePharma (Shanghai, China). Transfection was conducted in RAW264.7 cells via Lipofectamine 3000 (Thermo Fisher) for 24 h.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Trizol reagent (Thermo Fisher) was applied to RNA extraction from cells or serum. Complementary deoxyribonucleic acid (cDNA) was synthesized using 500 ng of RNA with PrimeScript One Step qRT-PCR kit (Takara, Tokyo, Japan) or specific miRNA reverse transcription kit (Haigene, Ha'erbin, China). The cDNA was mixed with SYBR (Thermo Fisher) as well as specific primers (Sangon, Shanghai, China) for qRT-PCR. Each sample was prepared in triplicate, and the qRT-PCR experiment was repeated 3 times. β-Actin or U6 was used as reference control. The primers were listed as: hsa-IL-6:
forward 5'-GTCCAGTTGCCTTCTCCCTGG-3',
reverse 5'-CCCATGCTACATTTGCCGAAG-3';
hsa-TNF-α: forward 5'-CTGGGCAGGTCTACTTTGGG-3',
reverse 5'-CTGGAGGCCCCAGTTTGAAT-3';
hsa-ANO1: forward 5'-AGAGGAAGAGGAGGCTGTCA-3',
reverse 5'-TGACTGTGACCCGGATGTTG-3';
hsa-miR-106a: forward 5'-AAAAGTGCTTACAGTGCAGGTAG-3',
reverse 5'-TTACCTAGCGTATCGTTGAC-3';
hsa-U6: forward 5'-CTCGCTTCGGCAGCACATATACT-3',
reverse 5'-TTACCTAGCGTATCGTTGAC-3';
hsa-β-actin: forward 5'-CTCGCCTTTGCCGATCC-3',
reverse 5'-TCTCCATGTCGTCCCAGTTG-3';
mmu-IL-6: forward 5'-CCCCAATTTCCAATGCTCTCC-3',
reverse 5'-CGCACTAGGTTTGCCGAGTA-3';
mmu-TNF-α: forward 5'-TAGCCCACGTCGTAGCAAAC-3',
reverse 5'-ACCCTGAGCCATAATCCCCT-3';
mmu-ANO1: forward 5'-ATGGGTATCACCAGCCTCCT-3',
reverse 5'-ACAGCTTCCTCCTCCTCCTC-3';
mmu-miR-106a: forward 5'-CAAAGTGCTAACAGTGCAGGTAGC-3',
reverse 5'-TTACCTAGCGTATCGTTGAC-3';
mmu-U6: forward 5'-GCGCGTCGTGAAGCGTTC-3',
reverse 5'-TTACCTAGCGTATCGTTGAC-3';
mmu-β-actin: forward 5'-CCACCATGTACCCAGGCATT-3',
reverse 5'-CGGACTCATCGTACTCCTGC-3'.
Relative RNA level was calculated by $2^{-\Delta\Delta Ct}$ method [22].

Enzyme-linked immunosorbent assay (ELISA)

The contents of IL-6, TNF-α, IL-1Ra, and IL-10 in serum or cell medium were analyzed via specific human or mouse ELISA kit (Thermo Fisher) following the protocols of the manufacturer. The absorbance was examined at 450 nm through a microplate reader (BioTek, Winooski, VT, USA) and the concentrations of IL-6, TNF-α, IL-1Ra, and IL-10 were calculated following the standard curve. This experiment was conducted 3 times with the triplicate samples.

Western blot

RAW264.7 cells were lysed in the ice-cold protein extraction buffer (Solarbio) for total protein isolation. The concentrations of protein were detected using a bicinchoninic acid assay kit (Thermo Fisher). The protein was mixed with loading buffer with a final concentration of 2 mg/mL and denatured at 100°C for 10 min. Next, 10-μL protein samples in triplicate were loaded onto sodium dodecyl sulfate-polyacrylamide gels, electrophoresed, and transferred onto polyvinylidene difluoride membranes (Solarbio). The membranes were blocked with 5% blocking buffer for 1 h and then incubated with primary antibodies against ANO1 (ab72984, 1: 1000 dilution, Abcam, Cambridge, UK) overnight and horseradish peroxidase-conjugated IgG (secondary antibody; ab6721, 1: 10 000 dilution) for 2 h. β-Actin (ab8227, 1: 3000 dilution, Abcam) was the loading control. The protein bands were visualized with ECL western blotting substrate (Solarbio) and exposed to X-ray films in the dark. The relative protein level of ANO1 was assessed by QuantityOne (Bio-Rad, Hercules, CA, USA). The entire experiment was performed 3 times.

Dual-luciferase reporter analysis

The complementary sites of miR-106a and ANO1 were predicted by DIANA Tools – microT-CDS online (*http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index*). The 3' untranslated region (UTR) sequence of ANO1 with the wild-type (WT) (UGCAGU) or mutant (MUT) (ACGUCA) miR-106a binding sites were inserted downstream of the luciferase gene in pmiR-RB-REPORT vector (RiboBio, Guangzhou, China) to generate corresponding luciferase reporter vectors ANO1 3'-UTR WT or MUT, respectively. For dual-luciferase reporter

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

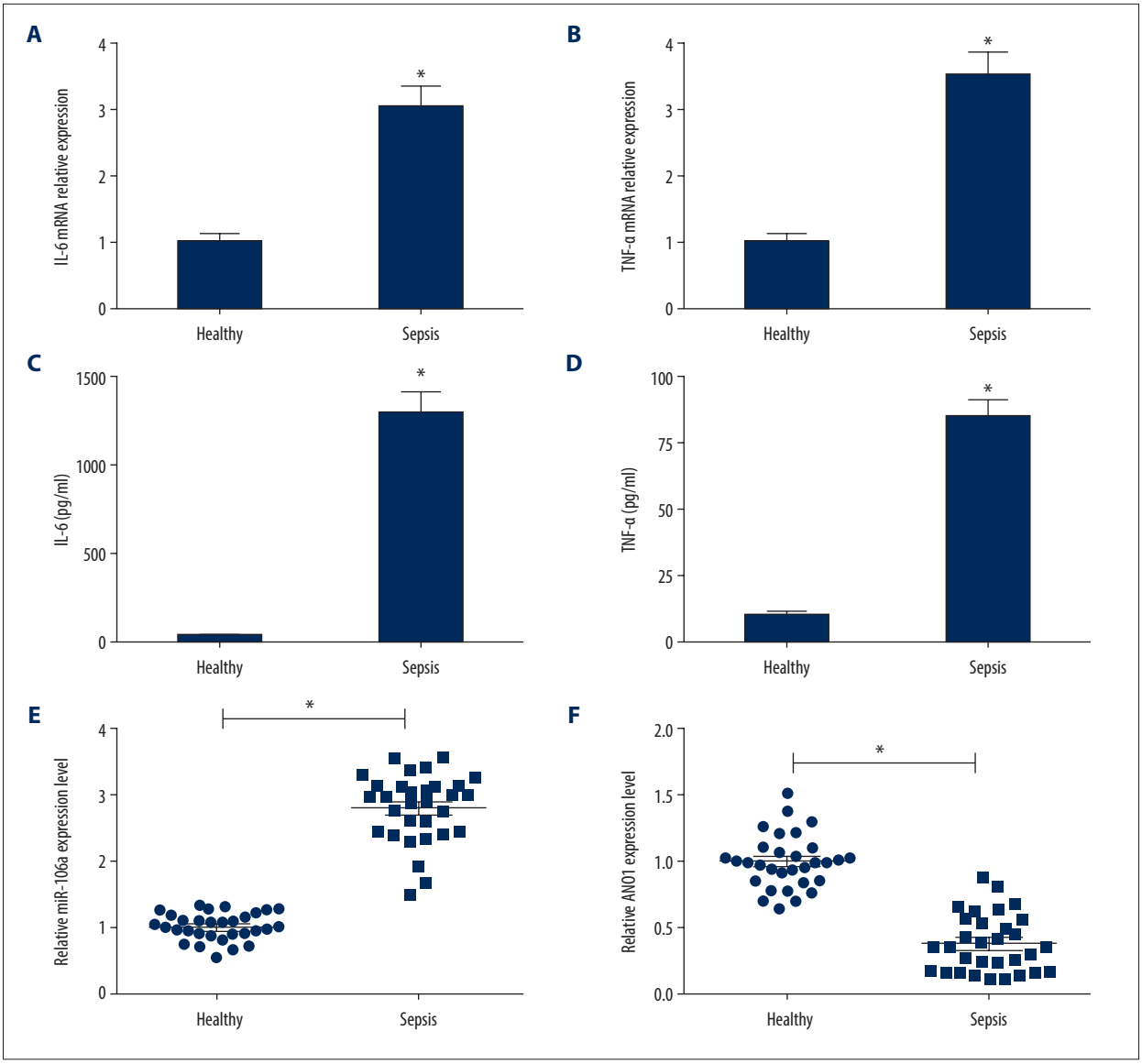


Figure 1. miR-106a expression is increased and anoctamin 1 (ANO1) expression is decreased in sepsis. (**A, B**) Messenger ribonucleic acid (mRNA) abundances of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) were detected in the serum samples of sepsis patients or healthy volunteers by quantitative real-time polymerase chain reaction. (**C, D**) Secretion levels of IL-6 and TNF-α were detected in the serum samples of sepsis patients or healthy volunteers by enzyme-linked immunosorbent assay. (**E, F**) Abundances of miR-106a and ANO1 mRNA were examined in patients' serum or that of healthy volunteers. * *P*<0.05.

assay, the constructed luciferase reporter vectors ANO1 3'-UTR WT or MUT were cotransfected with miR-NC, miR-106a mimic, in-miR-NC, or in-miR-106a into RAW264.7 cells. After 48 h, luciferase activity was examined with a specific luciferase assay system (GeneCopoeia, Rockville, MD, USA). Each sample was prepared in triplicate, and the entire experiment was repeated 3 times.

Statistical analysis

The experiments were conducted with 3 biological replicates×3 technical replicates, unless otherwise indicated. Data were shown as mean±standard deviation. The *t* test was applied to identify the difference between 2 groups. Analysis of variance followed by Tukey's test was performed to compare the difference for multiple groups. The statistical analysis was conducted via GraphPad Prism 6 (GraphPad, La Jolla, CA, USA). *P*<0.05 indicated a statistically significant difference.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

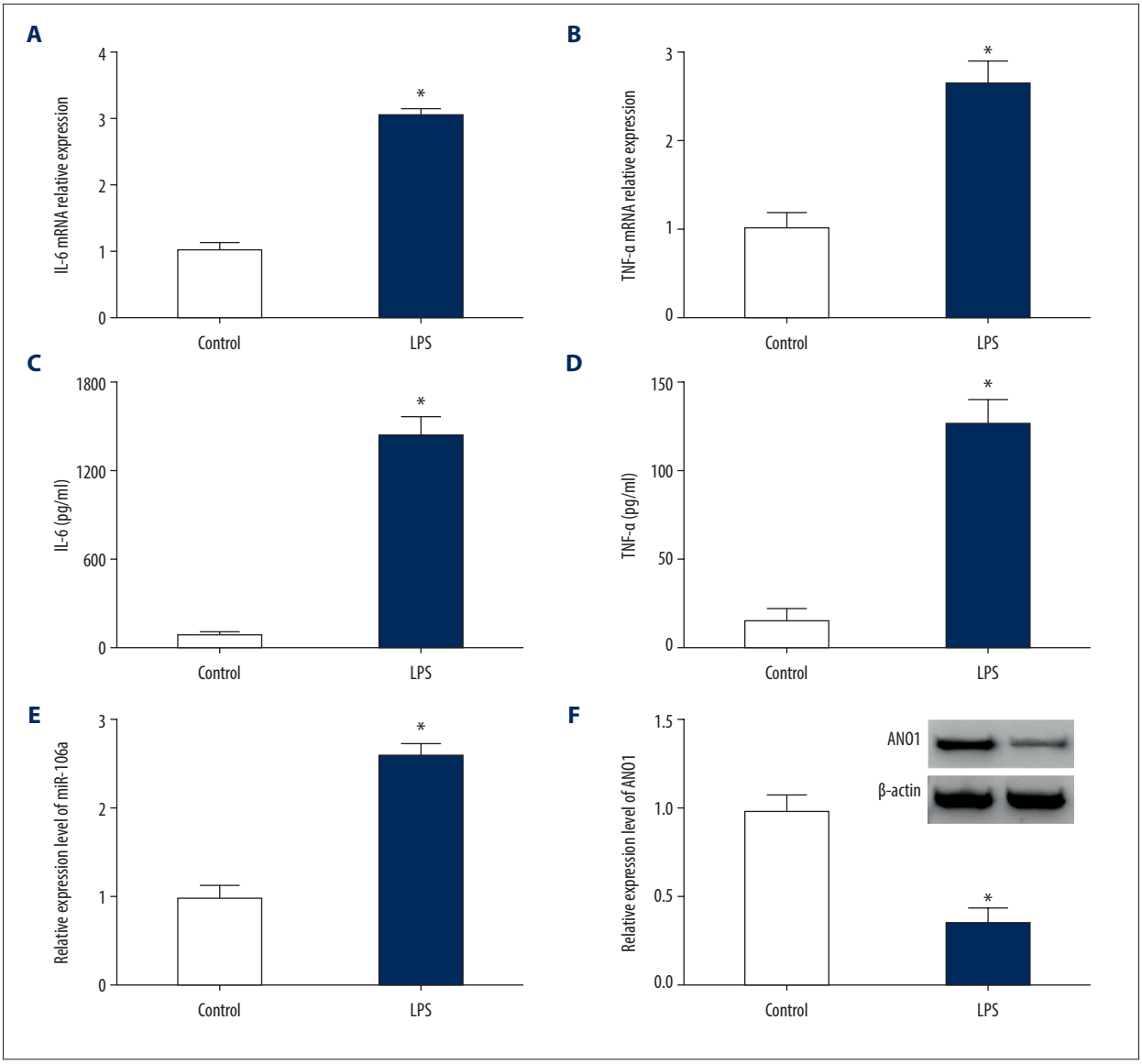


Figure 2. miR-106a expression is increased and anoctamin 1 (ANO1) expression is decreased in lipopolysaccharide (LPS)-treated RAW264.7 cells. (**A, B**) Messenger ribonucleic acid levels of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) were measured in RAW264.7 cells after treatment with LPS or not by quantitative real-time polymerase chain reaction. (**C, D**) Secretion levels of IL-6 and TNF-α were detected after treatment with LPS or not by enzyme-linked immunosorbent assay. (**E, F**) Abundances of miR-106a and ANO1 protein were detected after treatment with LPS or not. * *P*<0.05.

Results

miR-106a expression is enhanced and ANO1 expression is reduced in sepsis

To probe the roles of miR-106a and ANO1 in sepsis, the abundances of miR-106a and ANO1 were detected in the serum samples of sepsis patients. A total of 31 sepsis patients and 31 healthy volunteers was recruited and the expression of inflammatory cytokines was measured in the serum. As displayed in Figure 1A and 1B, the mRNA levels of IL-6 and TNF-α were evidently enhanced in the serum samples of sepsis patients compared with those in the healthy group. Moreover, the levels of IL-6 and TNF-α in the serum were also elevated in the sepsis group in comparison with the healthy group (Figure 1C, 1D). These data confirmed that sepsis was an inflammatory disease. Furthermore, miR-106a expression was abnormally upregulated and ANO1 mRNA abundance was markedly decreased in sepsis patients when compared with those in the healthy group (Figure 1E, 1F). These data indicated that miR-106a and ANO1 might be involved in sepsis development.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

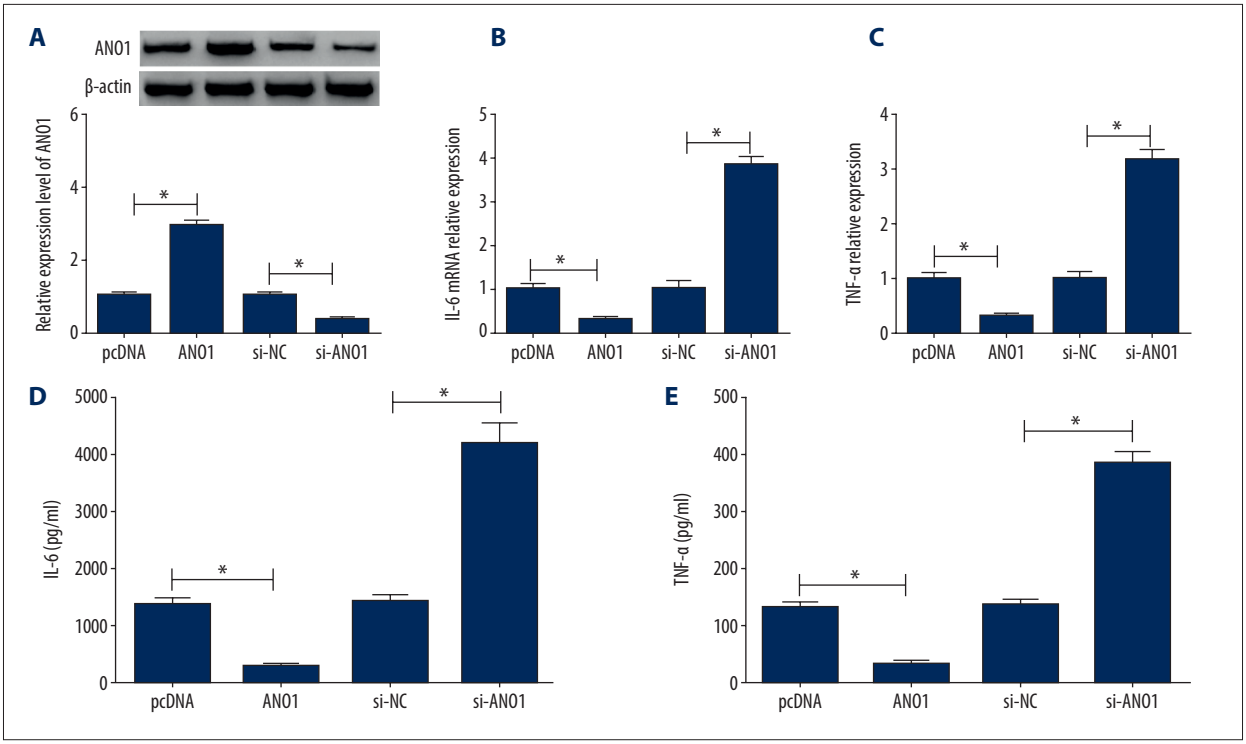


Figure 3. Overexpression of anoctamin 1 (ANO1) inhibits lipopolysaccharide (LPS)-induced inflammatory response in RAW264.7 cells. (**A**) Protein expression of ANO1 was detected in LPS-treated RAW264.7 cells with transfection of pcDNA, ANO1 overexpression vector, short interfering (si)-NC, or si-ANO1 by western blot. (**B, C**) Messenger ribonucleic acid levels of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) were examined in LPS-treated RAW264.7 cells with transfection of pcDNA, ANO1 overexpression vector, si-NC, or si-ANO1 by quantitative real-time polymerase chain reaction. (**D, E**) Secretion levels of IL-6 and TNF-α were detected in cells transfected with pcDNA, ANO1 overexpression vector, si-NC, or si-ANO1 by enzyme-linked immunosorbent assay. * $P<0.05$.

miR-106a level is increased and ANO1 level is downregulated in LPS-treated RAW264.7 cells

To establish an inflammatory model *in vitro*, RAW264.7 cells were stimulated by LPS for 24 h. After treatment with LPS, the mRNA levels of IL-6 and TNF-α were remarkably increased (Figure 2A, 2B). Furthermore, the secretion levels of IL-6 and TNF-α in the medium were evidently elevated in LPS-treated cells compared with those in the control group (Figure 2C, 2D). To explore the role of miR-106a and ANO1 in LPS-induced inflammation, their abundances were first detected in LPS-treated RAW264.7 cells. As shown in Figure 2E and 2F, treatment with LPS led to obviously increased miR-106a and decreased ANO1 protein in RAW264.7 cells. These findings indicated that miR-106a and ANO1 might be associated with LPS-induced inflammation in RAW264.7 cells.

ANO1 inhibits inflammatory response in LPS-treated RAW264.7 cells

To analyze the effect of ANO1 on LPS-induced inflammation, RAW264.7 cells were transfected with pcDNA, ANO1 overexpression vector, si-NC, or si-ANO1 before treatment with LPS. The transfection efficacy was confirmed by western blot. The abundance of ANO1 protein increased 2.94-fold via transfection of ANO1 overexpression vector and decreased 68% by the use of siRNA for ANO1 when compared with their corresponding controls (Figure 3A). Moreover, the analyses of qRT-PCR and ELISA revealed that overexpression of ANO1 significantly decreased the expression of IL-6 and TNF-α in LPS-treated cells, whereas knockdown of ANO1 exhibited an opposite effect on these inflammatory cytokines (Figure 3B–3E). Overexpression of ANO1 evidently increased the levels of IL-1Ra and IL-10 in LPS-treated cells (Supplementary Figure 1A, 1B). These data indicated that ANO1 played an anti-inflammatory role in RAW264.7 cells treated with LPS.

ANO1 is a target of miR-106a in RAW264.7 cells

The association between miR-106a and ANO1 was explored in RAW264.7 cells. DIANA Tools – microT-CDS online predicted that ANO1 might be a target of miR-106a (Figure 4A). To validate this prediction, ANO1 3'-UTR WT and MUT were constructed and dual-luciferase reporter assay was carried out.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

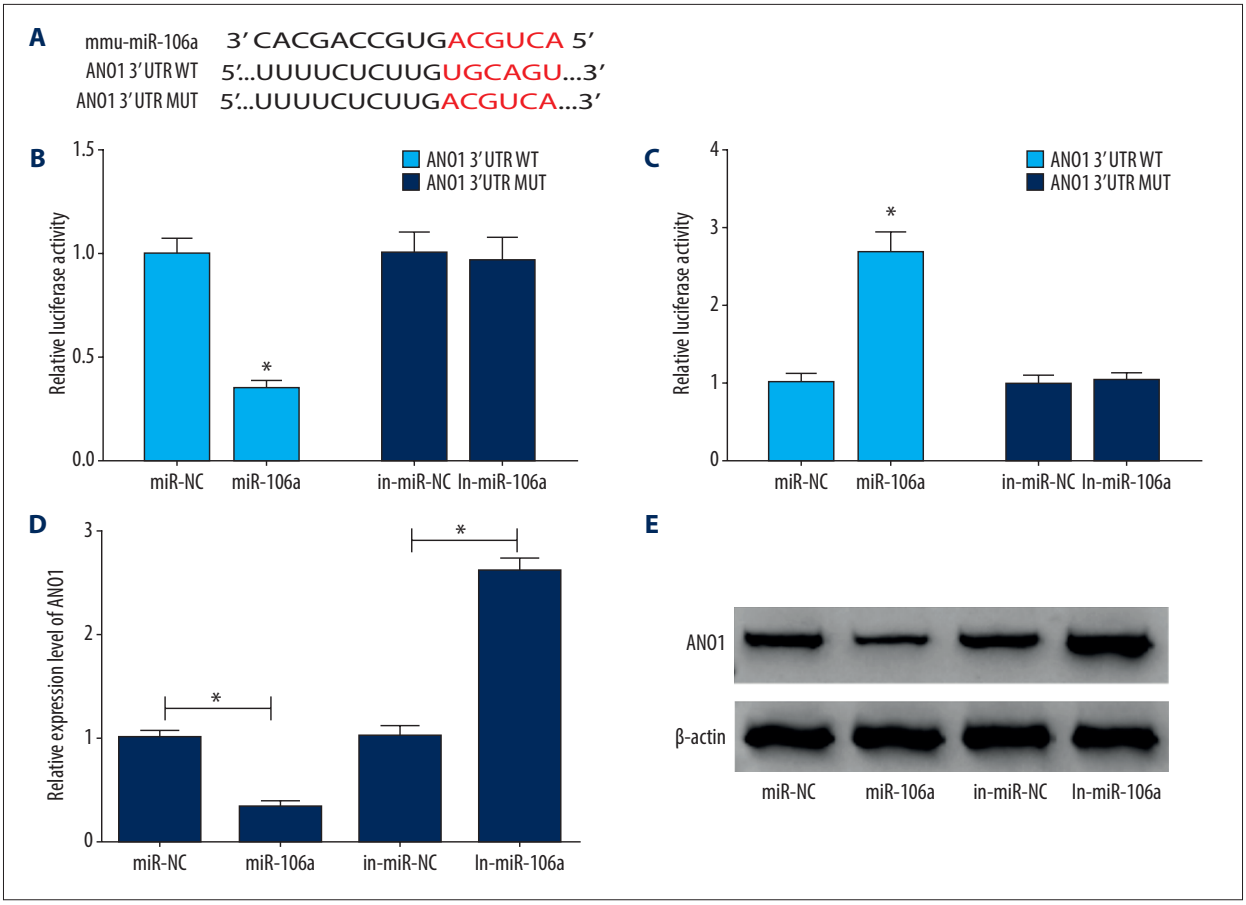


Figure 4. Anoctamin 1 (ANO1) is a target of miR-106a in RAW264.7 cells. (**A**) Binding sequence of miR-106a and ANO1 was searched via DIANA Tools – microT-CDS. (**B, C**) Luciferase activity was measured in RAW264.7 cells cotransfected with ANO1 3′-untranscribed region wild-type or mutant and miR-NC, miR-106a mimic, in-miR-NC, or in-miR-106a. (**D, E**) Protein level of ANO1 was detected in RAW264.7 cells transfected with miR-NC, miR-106a mimic, in-miR-NC, or in-miR-106a. * $P<0.05$.

As displayed in Figure 4B and 4C, luciferase activity was obviously decreased by 65% by miR-106a overexpression and increased 2.69-fold by miR-106a knockdown in the ANO1 3′-UTR WT group, but it was not altered in the ANO1 3′-UTR MUT group. Moreover, the effect of miR-106a on ANO1 expression was detected in RAW264.7 cells. The results showed that ANO1 protein level was notably reduced by miR-106a overexpression and enhanced by miR-106a knockdown (Figure 4D, 4E). These findings indicated that ANO1 was directly targeted by miR-106a in RAW264.7 cells.

miR-106a overexpression abates the effect of ANO1 on inflammatory response in LPS-treated RAW264.7 cells

To explore whether miR-106a could regulate ANO1-mediated inflammatory response, RAW264.7 cells were transfected with pcDNA, ANO1 overexpression vector, ANO1 overexpression vector+miR-NC, or miR-106a mimic before treatment with LPS. As shown in Figure 5A and 5B, the mRNA and protein abundances of ANO1 were notably enhanced by transfection of ANO1 overexpression vector, whereas it was weakened by cotransfection of ANO1 and miR-106a. Moreover, introduction of miR-106a reversed the suppressive effect of ANO1 on expression of IL-6 and TNF-α mRNA in RAW264.7 cells treated with LPS (Figure 5C, 5D). Addition of miR-106a also attenuated ANO1-mediated inhibition of IL-6 and TNF-α in the cell medium (Figure 5E, 5F). Furthermore, miR-106a overexpression markedly decreased the levels of IL-1Ra and IL-10 (Supplementary Figure 1C, 1D). These findings suggested that miR-106a regulated LPS-induced inflammatory response by ANO1 in RAW264.7 cells.

Discussion

Sepsis is a problem associated with the inflammatory response [23]. LPS-induced macrophage activation and deactivation are associated with sepsis development [24]. ANO1, also known as TMEM16A, plays an important role in human diseases [25]. A previous report showed that ANO1 mediated

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

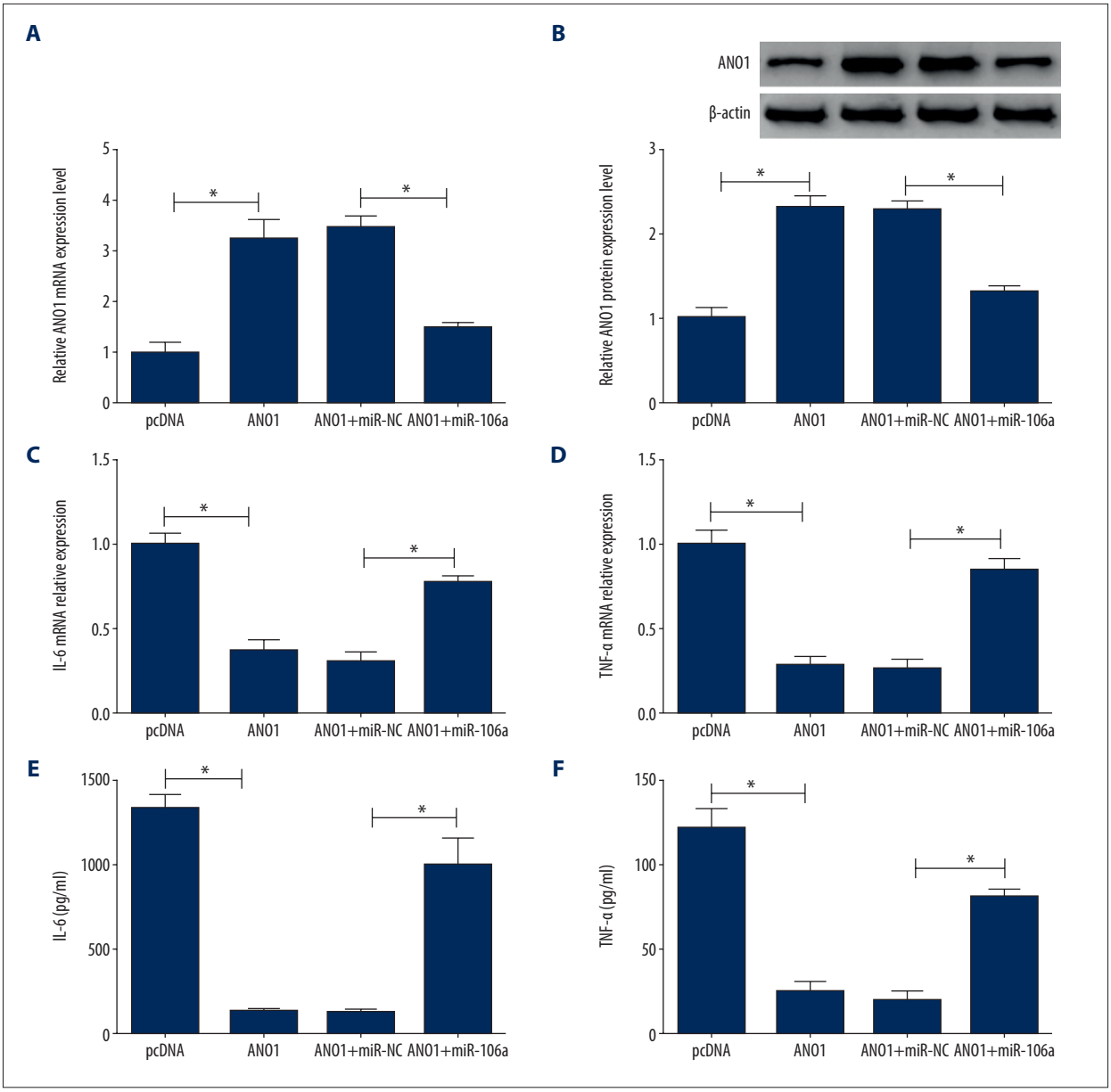


Figure 5. Overexpression of miR-106a attenuates anoctamin 1 (ANO1)-mediated inflammatory inhibition in lipopolysaccharide (LPS)-treated RAW264.7 cells. (**A, B**) Messenger ribonucleic acid (mRNA) and protein levels of ANO1 were measured in cells with transfection of pcDNA, ANO1 overexpression vector, ANO1 overexpression vector+miR-NC, or miR-106a mimic. (**C, D**) mRNA levels of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) were measured in LPS-treated RAW264.7 cells transfected with pcDNA, ANO1 overexpression vector, ANO1 overexpression vector+miR-NC, or miR-106a mimic via quantitative real-time polymerase chain reaction. (**E, F**) Secretion levels of IL-6 and TNF-α were detected in cells transfected with pcDNA, ANO1 overexpression vector, ANO1 overexpression vector+miR-NC, or miR-106a mimic by enzyme-linked immunosorbent assay. * $P<0.05$.

by LPS treatment was associated with the development of inflammatory disorders [7]. In this research, we aimed to investigate the function of ANO1 and miR-106a in the LPS-induced inflammatory response *in vitro*. Here we first investigated the anti-inflammatory role of ANO1 in LPS-treated RAW264.7 cells and confirmed that miR-106a could target ANO1 to regulate LPS-induced inflammation.

The increasing evidence indicated that ANO1 could inhibit the inflammatory response in some conditions [7,8,26]. Similarly, here we found that ANO1 expression was decreased in sepsis samples and LPS-treated RAW264.7 cells, indicating that low expression of ANO1 might be required for the process of inflammatory injury. There are multiple proinflammatory or anti-inflammatory cytokines implicated in sepsis development [27]. Among these,

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

IL-6 and TNF-α are 2 key proinflammatory cytokines, contributing to inflammatory injury [28,29]. Zhen et al. reported that ANO1 knockdown could attenuate knockdown of miR-9-mediated sepsis inhibition in the LPS-induced model, uncovering the anti-sepsis role of ANO1 [9]. We found that ANO1 inhibited LPS-caused inflammatory response by decreasing the expression of proinflammatory IL-6 and TNF-α and increasing the levels of anti-inflammatory IL-1Ra and IL-10, uncovering the anti-inflammatory role of ANO1 in RAW264.7 cells, which was also consistent with the report of Zhen et al. This indicated that ANO1 might be a promising therapeutic target in inflammatory diseases.

miRNAs can regulate RNA expression by targeting the 3'-UTR, which is associated with the inflammatory response [30]. Previous works showed that miR-106a could promote the inflammatory response in multiple conditions [15–17]. In our work, miR-106a expression was elevated in sepsis, indicating that high expression of miR-106a might be required for sepsis development, which was also consistent with a previous work [19]. Furthermore, here we found that miR-106a expression was enhanced in LPS-treated RAW264.7 cells. Previous studies have reported that miR-106a could mediate a LPS-triggered inflammatory response by regulating SIRPα, toll-like receptor 4, or IL-1 receptor-associated kinase 4 (IRAK4), which are associated with LPS signaling [17,31,32]. The mechanism underlying the effect of miR-106a in the inflammatory response is complex. To explore a new mechanism addressed by miR-106a, this study was the first to confirm that ANO1 was a target of miR-106a in RAW264.7 cells, suggesting that ANO1 might be important and play an additional role in miR-106a-mediated inflammatory response. miR-106a overexpression alleviated the effect of ANO1 on the LPS-induced inflammatory response, indicating that miR-106a could regulate the LPS-induced inflammatory response by targeting ANO1 *in vitro*. These data disclosed the potential role of miR-106a in inflammatory disorders including sepsis, which might be relevant to SIRPα, TLR4, IRAK4, ANO1, or another target in different signaling. This needs more exploration in the future.

Nevertheless, the *in vivo* experiments were limited in the current study. Hence, to further assess the roles of miR-106a and ANO1 in sepsis development, animal experiments should be performed in the future. Previous studies also demonstrated that nuclear factor-κB (NF-κB) signaling that was regulated by TNF-α was a major mechanism underlying inflammatory progression [33–35]. In addition, previous studies suggested that the NF-κB pathway was activated by miR-106a in glioma cells and inactivated by ANO1 in LPS-treated A549 cells [25,36]. Hence, we hypothesized that the NF-κB pathway might be responsible for the miR-106a/ANO1-mediated inflammatory response in LPS-treated RAW264.7 cells, which will be explored in a further study.

Conclusions

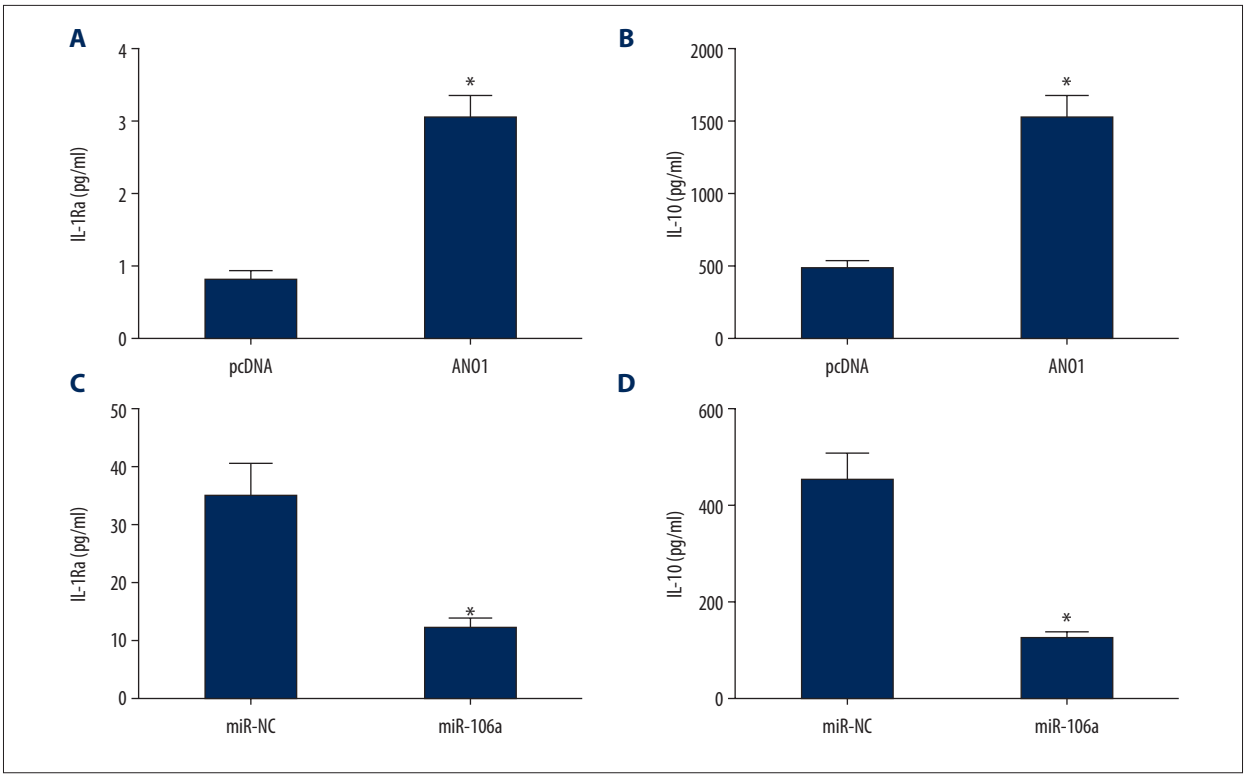
This research disclosed the anti-inflammatory role of ANO1 in LPS-treated RAW264.7 cells. Moreover, miR-106a could target ANO1 to mediate an LPS-induced inflammatory response. This research indicated that miR-106a and ANO1 might be used as promising targets for treatment of inflammatory diseases, including sepsis.

Conflicts of interest

None.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Supplementary Data



Supplementary Figure 1. Effect of anoctamin 1 (ANO1) and miR-106a on anti-inflammatory cytokine expression. (**A, B**) Levels of interleukin (IL)-1Ra and IL-10 were detected in lipopolysaccharide (LPS)-treated RAW264.7 cells transfected with pcDNA or ANO1 overexpression vector. (**C, D**) Levels of IL-1Ra and IL-10 were measured in LPS-treated RAW264.7 cells transfected with miR-NC or miR-106a mimic. * $P<0.05$.

References:

1. Gotts JE, Matthay MA: Sepsis: Pathophysiology and clinical management. Br Med J, 2016; 353: i1585
2. Lakshmikanth CL, Jacob SP, Chaithra VH et al: Sepsis: In search of cure. Inflamm Res, 2016; 65: 587–602
3. Arora S, Dev K, Agarwal B et al: Macrophages: Their role, activation and polarization in pulmonary diseases. Immunobiology, 2018; 223: 383–96
4. Marshall JC: Lipopolysaccharide: An endotoxin or an exogenous hormone? Clin Infect Dis, 2005; 41(Suppl. 7): S470–80
5. Jiang Y, Cai Y, Shao W et al: MicroRNA144 suppresses aggressive phenotypes of tumor cells by targeting ANO1 in colorectal cancer. Oncol Rep, 2019; 41: 2361–70
6. Hu C, Zhang R, Jiang D: TMEM16A as a potential biomarker in the diagnosis and prognosis of lung cancer. Arch Iran Med, 2019; 22: 32–38
7. Zhang A, Yan X, Li H et al: TMEM16A protein attenuates lipopolysaccharide-mediated inflammatory response of human lung epithelial cell line A549. Exp Lung Res, 2014; 40: 237–50
8. Dai WJ, Qiu J, Sun J et al: Downregulation of microRNA-9 reduces inflammatory response and fibroblast proliferation in mice with idiopathic pulmonary fibrosis through the ANO1-mediated TGF-beta-Smad3 pathway. J Cell Physiol, 2019; 234: 2552–65
9. Zhen J, Chen W, Zhao L et al: A negative Smad2/miR-9/ANO1 regulatory loop is responsible for LPS-induced sepsis. Biomed Pharmacother, 2019; 116: 109016
10. Kingsley SMK, Bhat BV: Role of microRNAs in sepsis. Inflamm Res, 2017; 66: 553–69
11. Pan YJ, Zhuang Y, Zheng JN et al: MiR-106a: Promising biomarker for cancer. Bioorg Med Chem Lett, 2016; 26: 5373–77
12. Zhu M, Zhang N, He S: Transcription factor KLF4 modulates microRNA-106a that targets Smad7 in gastric cancer. Pathol Res Pract, 2019; 215: 152467
13. Wu J, Li M, Zhang Y: Long noncoding RNA HOXA-AS2 regulates the expression of SCN3A by sponging miR-106a in breast cancer. J Cell Biochem, 2019; 120: 14465–75
14. Shi B, Ma C, Liu G et al: MiR-106a directly targets LIMK1 to inhibit proliferation and EMT of oral carcinoma cells. Cell Mol Biol Lett, 2019; 24: 1
15. Omidbakhsh A, Saeedi M, Khoshnia M et al: Micro-RNAs -106a and -362-3p in peripheral blood of inflammatory bowel disease patients. Open Biochem J, 2018; 12: 78–86
16. Sanctuary MR, Huang RH, Jones AA et al: miR-106a deficiency attenuates inflammation in murine IBD models. Mucosal Immunol, 2019; 12: 200–11
17. Zhu D, Pan C, Li L et al: MicroRNA-17/20a/106a modulate macrophage inflammatory responses through targeting signal-regulatory protein alpha. J Allergy Clin Immunol, 2013; 132: 426–36.e8
18. Wu SC, Yang JC, Rau CS et al: Profiling circulating microRNA expression in experimental sepsis using cecal ligation and puncture. PLoS One, 2013; 8: e77936
19. Shen Y, Yu J, Jing Y et al: miR-106a aggravates sepsis-induced acute kidney injury by targeting THBS2 in mice model. Acta Cir Bras, 2019; 34: e201900602
20. Sharma A, Yang WL, Ochani M, Wang P: Mitigation of sepsis-induced inflammatory responses and organ injury through targeting Wnt/β-catenin signaling. Sci Rep, 2017; 7(1): 9235

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

21. Mei L, He M, Zhang C et al: Paeonol attenuates inflammation by targeting HMGB1 through upregulating miR-339-5p. Sci Rep, 2019; 9(1): 19370
22. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 2001; 25: 402–8
23. Stearns-Kurosawa DJ, Osuchowski MF, Valentine C et al: The pathogenesis of sepsis. Annu Rev Pathol, 2011; 6: 19–48
24. Fujihara M, Muroi M, Tanamoto K et al: Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: Roles of the receptor complex. Pharmacol Ther, 2003; 100: 171–94
25. Ji Q, Guo S, Wang X et al: Recent advances in TMEM16A: Structure, function, and disease. J Cell Physiol, 2019; 234: 7856–73
26. Veit G, Bossard F, Goepp J et al: Proinflammatory cytokine secretion is suppressed by TMEM16A or CFTR channel activity in human cystic fibrosis bronchial epithelia. Mol Biol Cell, 2012; 23: 4188–202
27. Chaudhry H, Zhou J, Zhong Y et al: Role of cytokines as a double-edged sword in sepsis. In Vivo, 2013; 27: 669–84
28. Blaser H, Dostert C, Mak TW et al: TNF and ROS crosstalk in inflammation. Trends Cell Biol, 2016; 26: 249–61
29. Kruttgen A, Rose-John S: Interleukin-6 in sepsis and capillary leakage syndrome. J Interferon Cytokine Res, 2012; 32: 60–65
30. O'Connell RM, Rao DS, Baltimore D: microRNA regulation of inflammatory responses. Annu Rev Immunol, 2012; 30: 295–312
31. Yang J, Chen Y, Jiang K et al: MicroRNA-106a provides negative feedback regulation in lipopolysaccharide-induced inflammation by targeting TLR4. Int J Biol Sci, 2019; 15(11): 2308–19
32. Tomar S, Nagarkatti M, Nagarkatti PS: 3,3'-Diindolylmethane attenuates LPS-mediated acute liver failure by regulating miRNAs to target IRAK4 and suppress toll-like receptor signalling. Br J Pharmacol, 2015; 172(8): 2133–47
33. Hayden MS, Ghosh S: Regulation of NF-kappaB by TNF family cytokines. Semin Immunol, 2014; 26: 253–66
34. Taniguchi K, Karin M: NF-kappaB, inflammation, immunity and cancer: Coming of age. Nat Rev Immunol, 2018; 18: 309–24
35. Mussbacher M, Salzmann M, Brostjan C et al: Cell type-specific roles of NF-kappaB linking inflammation and thrombosis. Front Immunol, 2019; 10: 85
36. Wang Q, Wang Z, Chu L et al: The effects and molecular mechanisms of miR-106a in multidrug resistance reversal in human glioma U87/DDP and U251/G cell lines. PLoS One, 2015; 10: e0125473

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]