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MicroRNA-218 alleviates sepsis inflammation by negatively regulating VOPP1 via JAK/STAT pathway

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Abstract. – OBJECTIVE: To investigate the possible role of microRNA-218 in the pathogenesis of sepsis and its underlying mechanism.

PATIENTS AND METHODS: MicroRNA-218 expression in peripheral blood mononuclear cells (PBMCs) of 53 sepsis patients and 20 healthy controls was detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). MicroRNA-218 expression in Treg cells of sepsis patients and healthy controls was also detected. The binding condition of microRNA-218 to VOPP1 was confirmed by dual-luciferase reporter gene assay and RNA binding protein immunoprecipitation (RIP) assay, respectively. Furthermore, sepsis mouse model was constructed. MicroRNA-218 mimics or inhibitor was injected into mouse tail vein, respectively. The proportion of Treg cells was compared between sepsis mice injected with microRNA-218 mimics and inhibitor. Expressions of microRNA-218 and VOPP1 in Treg cells extracted from sepsis mouse were detected. ELISA (enzyme-linked immunosorbent assay) assay was conducted to detect serum levels of inflammatory factors (TNF-α, IL-6, TGF-β, and IL-10) in sepsis mouse. Finally, protein expressions of key genes in JAK/STAT pathway in sepsis mouse spleen were detected by Western blot.

RESULTS: MicroRNA-218 expression in sepsis patients was remarkably lower than that of healthy controls, which was gradually decreased with the deteriorating symptoms. Specifically, microRNA-218 expression was the lowest in patients who died of sepsis. Downregulated microRNA-218 was seen in Treg cells extracted from advanced sepsis patients. Both dual-luciferase reporter gene assay and RIP assay suggested that microRNA-218 can bind to VOPP1. VOPP1 expression was negatively regulated by microRNA-218. In advanced sepsis mouse, administration of microRNA-218 mimics increased expressions of TNF-α and IL-6, but decreased expressions of IL-10 and TGF-β. Western blot results indicated that microRNA-218 can inhibit the JAK/STAT pathway in sepsis mice.

CONCLUSIONS: MicroRNA-218 expression in the PBMCs of sepsis patients was remarkably reduced, which inhibited sepsis development via negatively regulating VOPP1 and suppressing JAK/STAT pathway.

Key Words

Sepsis, MicroRNA-218, VOPP1, JAK/STAT pathway.

Introduction

Sepsis is a systemic inflammatory response syndrome caused by infections. Sepsis involves multiple organs and tissues, which severely endangers affected patients. Globally, sepsis is the leading death cause in the intensive care unit (ICU). A recent epidemiological survey[1] showed that about 50% of patients in the ICU are accompanied by infection, and the mortality rate of infected patients is twice higher than that of non-infected patients. Sepsis incidence has been increased every year due to the aging of the population and the number of patients with the impaired immune system. Besides, sepsis is also related to clinical applications of chemotherapy, immunosuppressive therapy, and invasive medical treatments[2-4].

During the occurrence and progression of sepsis, the body is attacked by pathogenic microorganisms and toxins. The initial immune response exerts a vital role in defending external toxins. The dynamic balance of immune resistance and immune inhibition is impaired during the course of sepsis. The inflammatory response is also involved in the sepsis[5]. The specific mechanism of sepsis is still unclear, which requires further investigations.

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MicroRNAs are a class of non-coding, small RNA molecules, with 18-25 nucleotides in length. MicroRNAs degrade mRNA expression and inhibit translation by directly regulating the target mRNA. Functionally, microRNAs participate in various biological processes, including cell growth, proliferation, differentiation, apoptosis, tumorigenesis, and immunity[6,7]. Several studies have confirmed that microRNA is closely related to the occurrence and development of sepsis. In the early stages of severe infections (including sepsis), lymphocytes exert their biological activity to prevent inflammation. T cells exert a vital role in the regulation of immune responses and inflammatory processes. Studies have found that 6 microRNAs are remarkably downregulated in T cells, namely miR-16, miR-142-3p, miR-142-5p, miR-150, miR-15b, and let-7f. However, miR-21 is significantly overexpressed in T cells. The above-mentioned microRNAs are greatly involved in the development of T cells[8]. During LPS-induced endotoxin tolerance, miR-221, miR-125b, and miR-939 bind to the 3'UTR of tumor necrosis factor-α (TNF-α), thereby affecting TNF-α expression[9,10]. However, whether microRNA-218 can participate in sepsis has not been reported yet.

Patients and Methods

Patients

53 patients who were diagnosed as sepsis based on the guideline of Society of Critical Care Medicine (SCCM) and European Society of Intensive Care Medicine (ESICM) published in 2012 from July 2013 to August 2017 in ICU, Dezhou People's Hospital were enrolled[11]. Meanwhile, 20 healthy controls during the same period were selected. This study was approved by Ethics Committee of Dezhou People's Hospital. All the subjects signed the informed consent.

PBMCs Extraction

A blood sample of each subject was collected and incubated with ACD (acid citrate dextrose) anticoagulant at a ratio of 9:1. Anticoagulant blood was then added in the upper layer of lymphocyte separation solution at a ratio of 1:2, followed by centrifugation at 20°C, 400 g/min for 30 min. Cells were resuspended in RPMI-1640 (Roswell Park Memorial Institute-1640) (HyClone, South Logan, UT, USA), followed by centrifugation at 200 g/min for 10 min for three times. The extracted cells were PBMCs.

Treg Cell Sorting

The cell suspension was centrifuged at 1500 rpm/min for 5 min and the supernatant was discarded. Cells were resuspended in MACS buffer and incubated with anti-CD4 microbeads at 4°C for 15 min. The un-labeled microspheres were removed by MACS buffer, followed by another centrifugation. Resuspended cells were filtrated on the separation column, and the un-labeled splenocytes were harvested. The above separation was performed twice. Subsequently, MACS buffer MS was added in the separation column and the remaining solution was preserved in 15 mL EP tube. The remaining solution was centrifuged for cell counting and cells were incubated with flow cytometry antibody at 4°C for 20 min. Centrifuged cells were filtered by a 200 mesh filter, followed by flow cytometry detection. Treg cells were those expressed $CD4^+GFP^+$.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). 1 μL of complementary Deoxyribose Nucleic Acid (cDNA) was used for PCR detection. Primers used in the study were as follows: VOPP1, F: 5′-TCGAGGAGCCAGCCTTCAA-3′, R: 5′-TCCTGGGTCGGTGTAATAGGG-3′; GAPDH, F: 5′-ACCCACTCCTCCACCTTTGA-3′, R: 5′-CTGTTGCTGTAGCCAAATTCGT-3′; microRNA-218, F: 5′-GTGATAATGTAGCGAGATTTT-3′, R: 5′-AAAATCTCGCTACATTATCAC-3′.

Cell Transfection

One day prior to transfection, cells were seeded in the 24-well plates and cell confluence was required for 80-90%. 20 pmol microRNA-218 inhibitor, microRNA-218 mimics or miR-control was diluted in 50 μL of serum-free RPMI-1640, respectively. 1 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was diluted in serum-free RPMI-1640 as well and maintained at room temperature for 5 min. The two mixture solutions were mixed together and incubated for 15-20 min. Culture medium was replaced by the transfection mixture for 24 h incubation.

Dual-Luciferase Reporter Gene Assay

Based on the sequence of VOPP1, wild-type VOPP1 (VOPP1 wt) and mutant-type VOPP1 (VOPP1 mut) were constructed. Cells were seed-

ed in the 12-well plates and co-transfected with 50 pmol/L microRNA-218 mimics or negative control and 80 ng VOPP1 wt or VOPP1 mut, respectively. Cells were lysed and incubated for 15 min at room temperature, followed by detection of Firefly-Luc/Renilla Luc.

RNA Binding Protein Immunoprecipitation (RIP)

Cells were washed and cross-linked with 0.01% formaldehyde for 15 min. After centrifugation and cell lysis, cells extracted were incubated with RIP buffer containing protein A/G magnetic beads coated with anti-Ago2 or negative control anti-IgG antibody. After overnight incubation at 4°C, cells were incubated with Protein A Agarose for 1 h at 4°C, followed by the isolation of RNA. The microRNA-218 level was then detected by qRT-PCR.

Western Blot

Cells were lysed using a cell lysis buffer, shaken on ice for 30 min, and centrifuged at 4°C, 14,000 ×g for 15 min. Total protein concentration was calculated by BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The extracted proteins were separated on a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to the standard procedures.

ELISA (Enzyme-Linked Immunosorbent Assay)

A serum sample was collected for detecting cytokine dose according to the instructions of ELISA detection kit (Bio Legend, San Diego, CA, USA). The optical density at the wavelength of 450 nm was detected using a microplate reader.

Sepsis Mouse Model Construction

Sepsis mouse model was constructed by intraperitoneal injection of 20 mg/kg LPS (lipopolysaccharide) for 28 days. Mice experienced chills, increased respiratory rate, decreased activity, horripilation, and watery stool after LPS injection. Sepsis mice were randomly assigned into microRNA-218 NC group and microRNA-218 mimic group.

Splenic Cell Suspension Preparation

The mice pupils were excised and the carotid artery was cut to fully bloodletting. Mouse spleen was harvested and placed in 1×PBS (phosphate buffered saline). Spleen tissues were ground by two slides and filtered using a 200 mesh filter. The filtrate was centrifuged at 1500 rpm/min for 5 min and the precipitate was lysed using 1 ml of FACS lysing solution for 10 min. After centrifugation at 1500 rpm/min for 5 min and filtration, 10 μL of the suspension was mixed with 10 μL of trypan blue for cell counting.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Continuous variables were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

MicroRNA-218 Was Lowly Expressed in Sepsis

To investigate the role of microRNA-218 in sepsis, qRT-PCR was performed to detect microRNA-218 expression in PBMCs of sepsis patients and healthy controls. The results showed that microRNA-218 expression in sepsis patients was lower than that of healthy controls (Figure 1A). Sepsis patients were further divided into normal sepsis, severe sepsis, and sepsis shock groups based on their disease conditions. We found that microRNA-218 expression was gradually decreased with the deteriorating conditions (Figure 1B). Sepsis patients were subsequently divided into death patients and survivors, and microRNA-218 expression was remarkably reduced in dead patients (Figure 1C). Furthermore, Treg cells were extracted from sepsis patients and healthy controls for detecting microRNA-218 expression. We found that microRNA-218 was lowly expressed in Treg cells extracted from sepsis patients, especially in advanced sepsis patients (Figure 1D).

MicroRNA-218 Inhibited VOPP1 Expression

Transfection efficacies of microRNA-218 mimics and inhibitor in PBMCs were verified by qRT-PCR (Figure 2A). Subsequently, VOPP1 wt and VOPP1 mut were constructed. Dual-luciferase reporter gene assay indicated that after transfection of microRNA-218 mimics, luciferase activity was decreased in VOPP1 wt group and the opposite results were obtained after microRNA-218 inhibitor

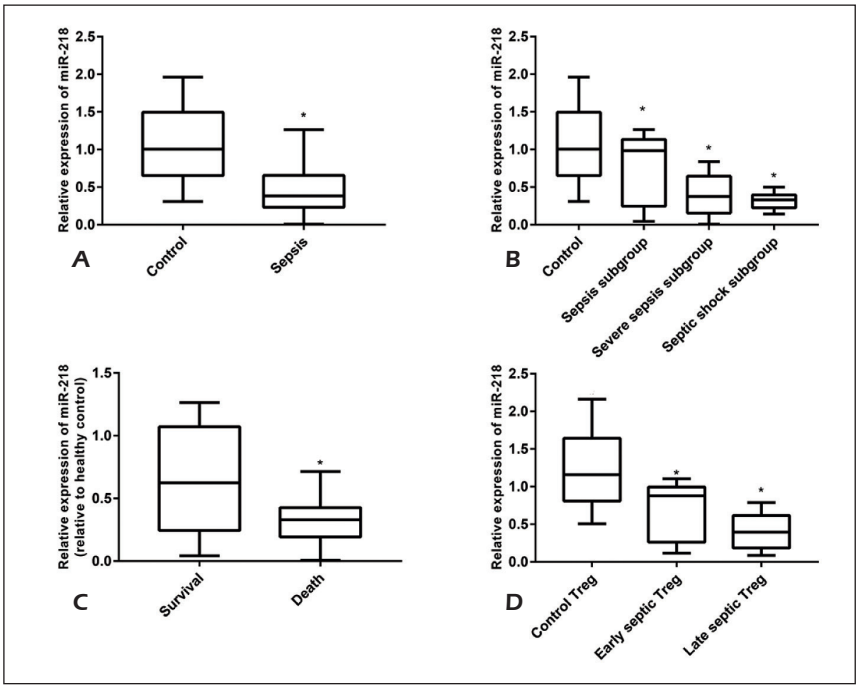

Figure 1. MicroRNA-218 was lowly expressed in sepsis. **A**, MicroRNA-218 expression in sepsis patients was lower than that of healthy controls. **B**, MicroRNA-218 expression was gradually decreased with the deteriorating disease conditions. **C**, MicroRNA-218 expression was remarkably reduced in dead patients. **D**, MicroRNA-218 was lowly expressed in Treg cells extracted from sepsis patients, especially in advanced sepsis patients.

transfection (Figure 2B). We also detected VOPP1 expression in PBMCs. Both mRNA and protein expressions of VOPP1 were negatively regulated by microRNA-218 (Figure 2C and 2D). RIP assay demonstrated that microRNA-218 expression was upregulated after VOPP1 wt transfection, whereas no significant change was found by transfecting VOPP1 mut (Figure 2E).

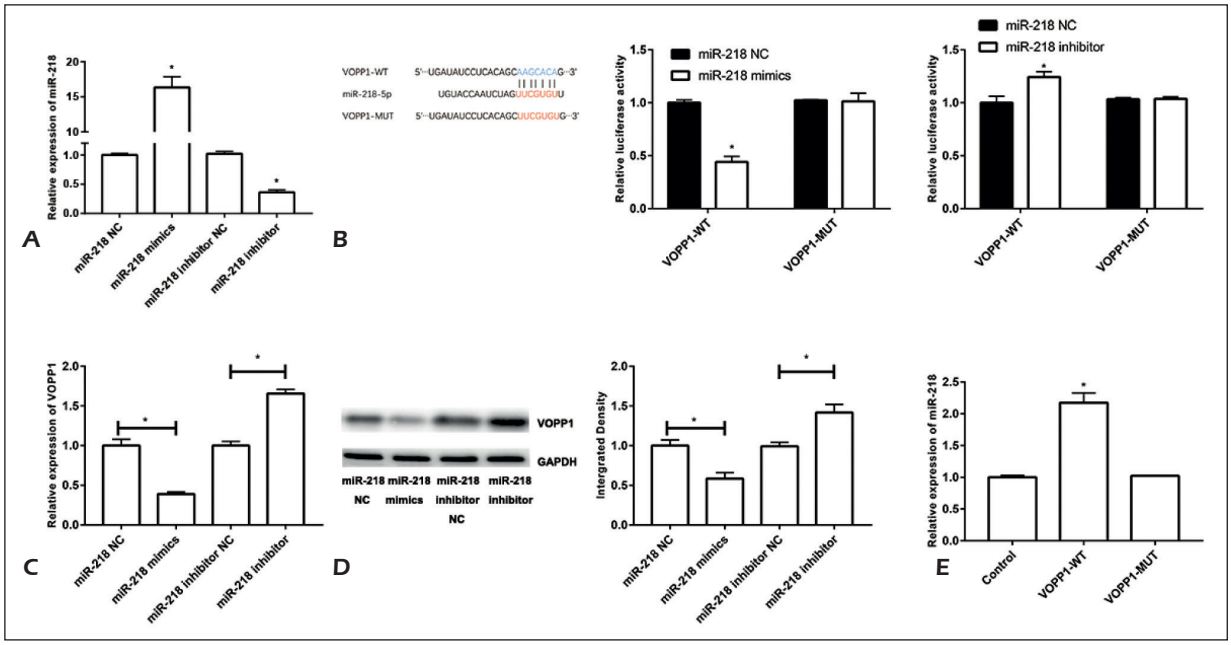

Figure 2. MicroRNA-218 inhibited VOPP1 expression. **A**, Transfection efficacies of microRNA-218 mimics and inhibitor in PBMCs were verified by qRT-PCR. **B**, Dual-luciferase reporter gene assay indicated that after transfection of microRNA-218 mimics, luciferase activity was decreased in VOPP1 wt group and the opposite results were obtained after microRNA-218 inhibitor transfection. **C**, **D**, Both mRNA (**C**) and protein (**D**) expressions of VOPP1 were negatively regulated by microRNA-218. E, RIP assay demonstrated that microRNA-218 expression was upregulated after VOPP1 wt transfection, whereas no significant change was found by transfecting VOPP1 mut.

MicroRNA-218 Alleviated Inflammation Induced by Advanced Sepsis

We further evaluated the protective role of microRNA-218 in sepsis. Previous research showed that sepsis mouse model was constructed by LPS injection[12]. By injection of microRNA-218 mimics in the tail vein of advanced sepsis mouse, Treg cell amount in mouse spleen was detected by flow cytometry. Our data indicated that microRNA-218 mimics injection in sepsis mouse reduced the amount of Treg cells and upregulated microRNA-218 expression (Figure 3A and 3B). However, the target gene of microRNA-218, VOPP1 was downregulated (Figure 3C). Due to the increased immunosuppression and the downregulation of pro-inflammatory cytokines in advanced sepsis mouse[13], we hypothesized that survived sepsis mice may present immunoreactivity and response to LPS stimulation. After sepsis induction for 28 days and 10 mg LPS treatment for 6 h, peripheral blood levels of proinflammatory factors (TNF-α and IL-6) and anti-inflammatory factors (IL-10 and TGF-b) were measured. The data showed that microRNA-218 mimics treatment upregulated levels of IL-6 and TNF-α, but downregulated IL-10 and TGF-β (Figure 3D-3G).

MicroRNA-218 Inhibited JAK/STAT Pathway

JAK/STAT pathway is closely related to immune response, which is an essential pathway in regulating the occurrence and progression of sepsis. Spleen tissues of sepsis mouse were harvested to detect protein expressions of key genes in JAK/STAT pathway. Western blot results showed that microRNA-218 mimics treatment downregulated expressions of p-JAK2, p-STAT1, p-STAT3, and p-STAT5 (Figure 4A). Injection of microRNA-218 inhibitor obtained the opposite results (Figure 4B).

Discussion

Sepsis is a systemic inflammatory response that is secondary to infection. Sepsis has become one of the leading causes of death in ICU worldwide. Although comprehensive interventions and treatments have been progressed, the incidence and mortality of sepsis still remain high[14]. In the pathophysiology of sepsis, infection induces an immune response, manifesting as a large number of inflammatory and chemokines are released by inflammatory cells. The body also causes organ damage when removing pathogens. Meanwhile, negative feedback regulation during an immune response and abnormal apoptosis of immune cells lead to immunosuppression that increases the susceptibility of the organism. Imbalance of immune hyperactivity/inhibition that is secondary to infection is the key factor in the occurrence

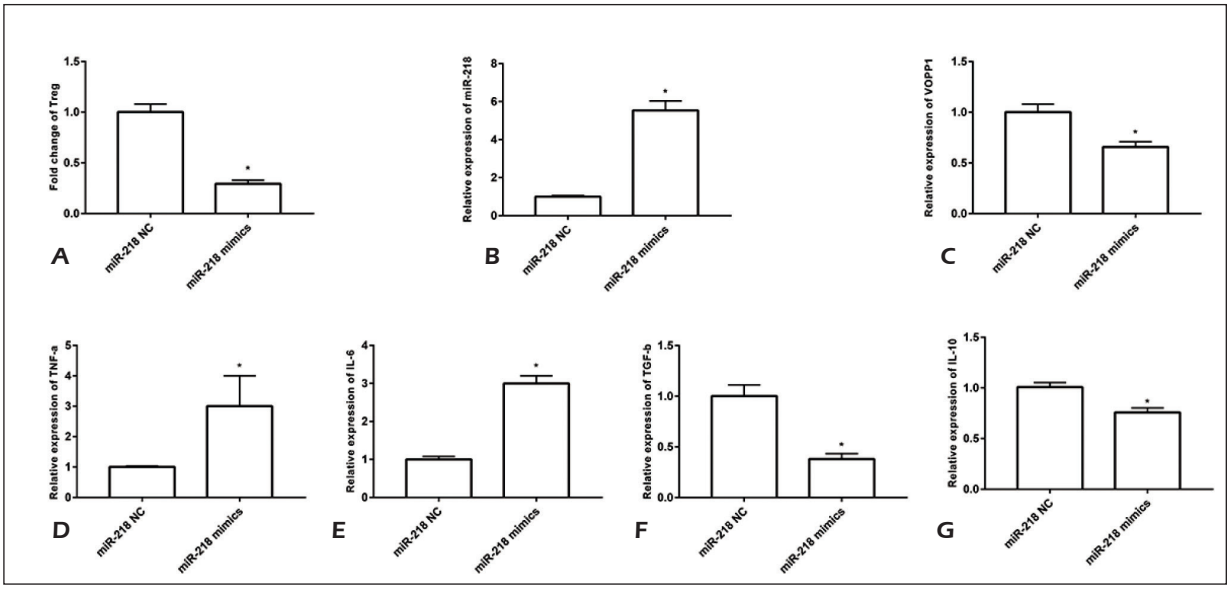

Figure 3. MicroRNA-218 alleviated inflammation induced by advanced sepsis. **A-B**, MicroRNA-218 mimics injection in sepsis mouse reduced the amount of Treg cells and upregulated microRNA-218 expression. **C**, MicroRNA-218 mimics injection in sepsis mouse downregulated VOPP1 expression. D-G, MicroRNA-218 mimics treatment upregulated levels of IL-6 (**D**) and TNF-α (**E**), but downregulated IL-10 (**F**) and TGF-β (**G**).

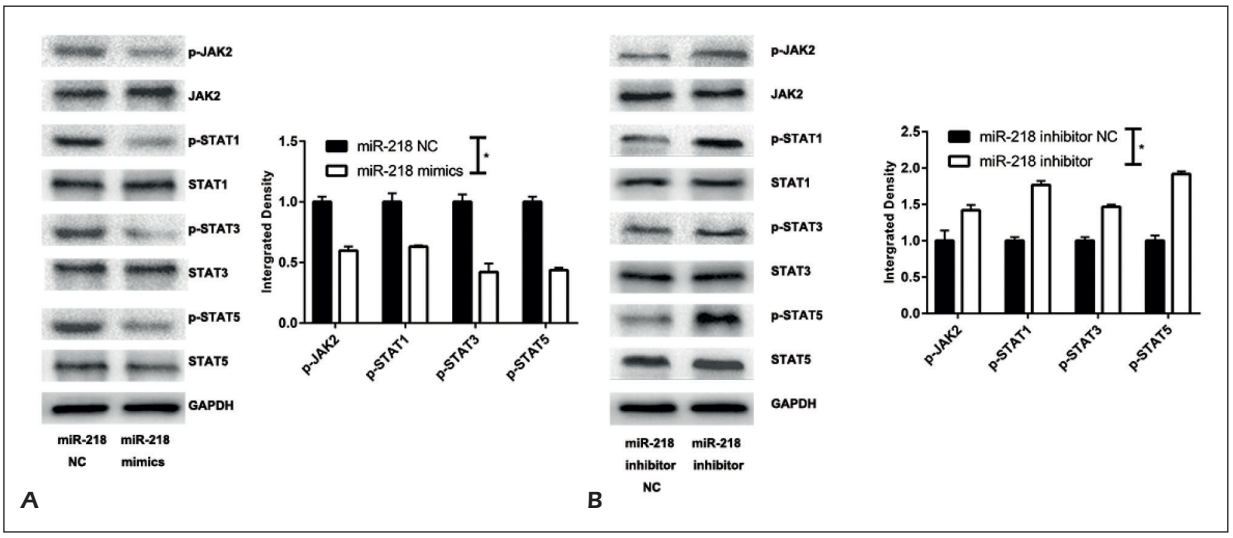

Figure 4. MicroRNA-218 inhibited JAK/STAT pathway. **A**, Western blot results showed that microRNA-218 mimics treatment downregulated expressions of p-JAK2, p-STAT1, p-STAT3, and p-STAT5. **B**, Injection of microRNA-218 inhibitor upregulated expressions of p-JAK2, p-STAT1, p-STAT3, and p-STAT5.

and progression of sepsis[15]. In addition, expression changes in tissue factors, antithrombin, and protein C result in an abnormal coagulation and hemodynamic changes, which are closely related to the development of sepsis[16].

Some studies[17,18] have shown that microRNAs are involved in sepsis through different regulations. Under the stimulation of pathogenic microorganisms, host cells rapidly produce different microRNAs that participate in immune responses and immune cell regulation. Through microarray screening of microRNAs in peripheral blood leukocyte of sepsis patients, they found that miR-150 expression was upregulated, which was negatively correlated to IL-10 and TNF-α[19]. Some studies have pointed out that miR-223 exerts a high sensitivity in predicting sepsis children[20]. MicroRNAs are confirmed to participate in the innate immunity, adaptive immunity, and immune cell function in sepsis. In our study, microRNA-218 expression was lowly expressed in PBMCs of sepsis patients *via* targeting VOPP1. Besides, microRNA-218 alleviated the inflammatory response in the advanced sepsis.

JAK/STAT pathway is closely related to immune response, which is an essential pathway in regulating the occurrence and progression of sepsis[21]. JAK/STAT pathway is a tyrosine receptor-related kinase containing Janus kinase and STAT. The JAK family has four members, namely JAK 1, JAK2, JAK3, and TYK2. The STAT family consists of seven structural and functionally related members, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Activation of JAK selectively stimulates the formation of homodimers or heterodimers by STAT that translocate in the nucleus *via* nuclear importin a-5 and Ran nuclear pathways. Nuclear STAT could regulate transcription of the target genes[22-24]. In this study, expressions of JAK/STAT pathway-related proteins in mouse spleen were downregulated after injection of microRNA-218 mimics in sepsis mouse for 28 days. Opposite results were seen in those injected with microRNA-218 inhibitor.

Conclusions

We showed that microRNA-218 expression in the PBMCs of sepsis patients was remarkably reduced, which inhibited sepsis development *via* negatively regulating VOPP1 and suppressing JAK/STAT pathway.

Conflict of Interests:

The authors declare they have no conflict of interest.

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