Association between miR-126, miR-21, inflammatory factors and T lymphocyte apoptosis in septic rats

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Abstract. MicroRNAs (miRs) serve an important role in regulating expression levels of inflammatory factors but the underlying mechanism is still unclear. The present study aimed to observe miR-126 and miR-21 expression and apoptosis in T lymphocytes and to analyze their association with cytokine release in septic rats. The septic model rats were given intraperitoneal lipopolysaccharide (LPS) and divided into 0, 12, 24, 48 and 72 h groups. Peripheral blood was collected from each group to isolate T lymphocytes. The expression levels of miR-126 and miR-21 in T lymphocytes were observed, as well as cytokine release and apoptosis. Finally, the association between miR-126, miR-21, cytokines and apoptosis in T lymphocytes was analyzed. The release of TNF-α and IL-6 in septic rats was initially elevated but then decreased. miR-126 and miR-21 levels in T lymphocytes in septic rats were lower than those of NC rats. miR-126 and miR-21 initially decreased and then increased, whereas of apoptosis of T lymphocytes increased and then decreased, in septic rats. The expression of miR-126 was positively correlated with that of miR-21 (r=0.316; P=0.029) and negatively correlated with that of TNF-α (r=-0.480; P=0.001) and IL-6 (r=-0.626; P<0.001), as well as the apoptotic rate of T lymphocytes (r=-0.377; P=0.008). Furthermore, expression levels of miR-126 were negatively corrlated with caspase-3 expression levels (r=-0.606; P<0.001) and activity (r=-0.541; P<0.001). There was a negative correlation between miR-21 and levels of TNF-α (r=-0.311; P=0.032) and IL-6 (r=-0.439; P=0.002), as well as caspase-3 expression (r=-0.398; P=0.005) and activity (r=-0.378; P=0.008). However, there miR-126 expression was not correlated with apoptotic rate of T lymphocytes. Altered expression levels of miR-126 and miR-21 reflected the severity of inflammatory response and indicated levels of T lymphocyte apoptosis in septic rats.

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Introduction

The Third International Consensus Definitions for Sepsis was released in 2016 (Sepsis 3.0) (1). Sepsis is defined as a dysfunctional host response to infection and life-threatening organ dysfunction, which can lead to septic shock, multiple organ failure and death. Sepsis is a common systemic infection in intensive care units. An international study showed that the hospital mortality rate of sepsis was 17% and that of severe sepsis was 26%; sepsis kills ~5.3 million people/year (2). Because of its high morbidity and mortality, this disease attracts worldwide medical attention.

Growing research has improved understanding of the pathogenesis of sepsis (3-5). The pathophysiological process of sepsis is complex and includes inflammation, immune and coagulation functions and changes in cell function, metabolism and microcirculation (6); the most important of these is the immune mechanism (7). Immune regulation affects the prognosis of patients with sepsis (8). The immunoregulation mechanism serves a key role in early hyperimmune responses, such as systemic inflammatory response syndrome, excessive release of inflammatory factors, late immunosuppression and T lymphocytes (9).

MicroRNAs (miRs) are important in regulation of post-transcriptional gene expression, especially regulation of cell apoptosis and proliferation (10). In recent years, miRs have been shown to regulate signaling pathways, inflammation and immune cells, such as T lymphocytes (10,11), in sepsis. miR-126 is an important member of the miR family and is expressed in endothelial cells of blood vessels, as well as the heart, lung and other tissue. miR-126 inhibits the development of the T helper (Th)2-specific immune response and regulates differentiation of T lymphocytes in the direction of Th2 or T regulatory cells (Tregs) (12). It has also been found that miR-126 enhances activation of T lymphocytes by upregulating the insulin receptor substrate-1 pathway (13). miR-21 exhibits antiapoptotic effects in cancer. For example, overexpression of miR-21 decreases 5-fluorouracil-induced apoptosis and necrosis of non-small cell lung cancer cells (14), however, further investigation is required to determine its effect on the immune system. Certain

studies have shown that miR-21 inhibits apoptosis of activated T cells (15,16). miR-21 mediates the interaction between Treg and endothelial cells via inducible T cell co-stimulator (ICOS) and ICOS ligand in B lymphocytes (17). Similarly, a study has shown that miR-21 affects the differentiation of $CD4^+$ and $CD8^+$ T cell subsets (18).

The present study aimed to investigate the effects of miR-126 and miR-21 on sepsis immune response, apoptosis of T lymphocytes and release of inflammatory factors. The expression levels of miR-126 and miR-21 and the apoptotic rate of T lymphocytes were observed and the association between these factors was analyzed.

Materials and methods

Sepsis model and groups. The experimental rats (weight, 200-250 g; age, 8 weeks) were provided by the Experimental Animal Center of Bengbu Medical College (Anhui, China). Rats were maintained at 20-25˚C, 50-65% humidity, 14/10-h light/dark cycle and free access to food and water. A total of 48 male Sprague-Dawley rats were divided into 6 groups: Normal control (NC) and sepsis (0, 12, 24, 48 and 72 h; n=8/group). NC rats received intraperitoneal injection with 0.9% saline (10 ml/kg); experimental rats were used to construct a model of sepsis and received intraperitoneal injection with lipopolysaccharide (LPS; 15 mg/kg) . Peripheral blood (100 μl) was taken at each time point (at 0, 12, 24, 48, 72 h).

Lymphocyte isolation. Lymphocytes were separated by density gradient centrifugation. Rat lymphocyte isolation solution (Sigma-Aldrich; MercK KGaA) was used and the samples were centrifuged by horizontal rotor centrifuge (4˚C, 450 x g, 25 min). The centrifuged liquid was separated into four layers. The lymphocyte layers were carefully collected by suction tube and centrifuged (4˚C, 300 x g, 10 min). The lymphocyte layers were centrifuged (4˚C, 250 x g, 10 min) and the supernatant was discarded. The lymphocyte pellet was collected for subsequent analysis.

T cell counting. T cell apoptosis was detected by mixing lymphocytes ($1x10^6$ cells/ml) with 400 μl Annexin V binding solution. Then, 5 μl Annexin V-FITC (cat. no. 40302-A; Shanghai Yeasen Biotechnology Co., Ltd.) was added to the T cell suspension, gently mixed and incubated at 2-8˚C for 15 min After adding 5-10 μl PI dye solution (cat. no. 40302-B; Shanghai Yeasen Biotechnology Co., Ltd.), gently mixing and incubating at 2-8˚C for 5 min, T cells were counted by flow cytometry (FACS Calibur; BD Biosciences) and analyzed by software (FlowJo V10.0; BD Biosciences). The collected lymphocytes were resuspended in 2-5 ml cold ethanol and then fixed with 1X Binding Buffer (cat. no. 40302-C; Shanghai Yeasen Biotechnology Co., Ltd.) for 1 h at -20˚C. Cells were collected by centrifugation (4˚C, 1,000 x g, 10 min). Cells were resuspended in PBS and RNase A solution, then immersed in a water bath at 37˚C (30 min). Cells were collected by centrifugation (4˚C, 1,000 x g, 10 min). The lymphocytes were resuspended in PI dye solution and incubated at 4˚C (30 min) without light. The results were detected by flow cytometry (FACSCalibur; BD Biosciences).

Caspase-3 activity detection. Total protein of lymphocytes was extracted by protein extraction kit (cat. no. SD-001; Invent Biotechnologies, Inc.) and collected by centrifugation (4˚C, 16,000 x g, 30 sec). After extracting total protein from the collected lymphocytes, caspase-3 reaction buffer containing fluorescent substrates was added into the control and sample well. The fluorescence intensity was analyzed by fluorescence spectrophotometer (Qubit Flex; Thermo Fisher Scientific, Inc.) immediately after adding the sample. The fluorescence intensity was measured every 10 min. The monitoring time was 120 min and the detection temperature was 37˚C. The observed fluorescence intensity was caspase-3 activity.

Western blot analysis. Lymphocyte proteins were extracted from the collected lymphocytes by protein extraction kit (cat. no. SD-001; Invent Biotechnologies, Inc.). BCA protein assay kit was used to determine protein concentration and 50 μg protein/lane was sampled. A 5X SDS buffer solution was added for electrophoresis. The starting voltage was 60 V (5% concentrated gel). When the strip ran out of the gel concentrate, the voltage was increased to 120 V (12% separated gel) for the transmembrane. The transmembrane current was 250 mA. After the membrane was washed with TBST (0.05% Tween-20) for 1-2 min, the antigen was blocked. The PVDF membrane was removed and placed in blocking solution (5% skimmed milk) and shaken gently for 1 h on a shaker at room temperature. Primary antibodies (diluted with 5% skimmed milk) were as follows: Caspase-3 (1:2,000; cat. no. ab184787; Abcam)and GAPDH (1:3,000; cat. no. ab125247; Abcam). Shock incubation at 37˚C overnight followed by incubation at 4˚C for 2 h was performed. Then, the membrane was washed with TBST (0.05% Tween-20) three times times on a shaker (10 min each) and the secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG; cat. no. KGAA35; Jiangsu Kaiji Biotechnology Co., Ltd.] was added at 25˚C for 1 h. The antibody was diluted with 5% skimmed milk at 1:3,000, then added to the membrane. The membrane was washed with TBST three times (10 min each). Finally, the exposure was developed by chemiluminescence (G:BOX chemiXR5; Syngene Europe) and analyzed (Gel-Pro32 software; Media Cybernetics, Inc.).

RNA extraction and reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Then, 0.5 μg RNA was subjected to RT using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Life Science) as follows: 37˚C for 15 min, 85˚C for 5 sec and 4˚C. Relative RNA expression quantitation was performed using SYBR Premix EX Taq (Takara Bio, Inc.) according to the manufacturer's protocol and the $2^{-\Delta\Delta Cq}$ method (19). Primer sequences used for RT-qPCR were as follows: MiR-126, forward, 5'-CGC GTCGTACCGTGAGTAAT-3' and reverse, 5'-AGTGCAGGG TCCGAGGTATT-3'; miR-21 forward, 5'-CGCAACAGCAGT CGATGG-3' and reverse, 5'-AGTGCAGGGTCCGAGGTA TT-3' and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. U6 was used as an internal control for miRNA Total RNA from collected

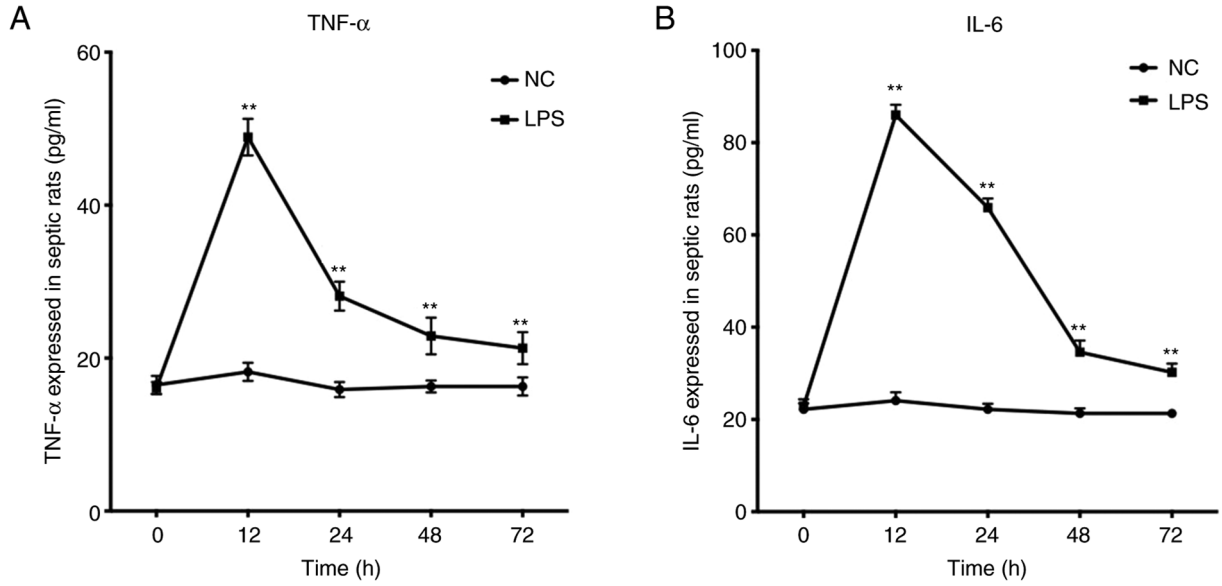

Figure 1. Expression of TNF-α and IL-6 in NC and septic rats at different time intervals. (A) TNF-α and (B) IL-6 initially increased and then decreased in septic rats, reaching the peak at 12 h. **P<0.01 vs. NC. NC, normal control; LPS, lipopolysaccharide.

lymphocytes was extracted using an RNA kit (cat. no. DP501; Tiangen Biotech Co., Ltd.). RNA concentration was determined using a spectrophotometer (NanoDrop 1000). The levels of miR-126 and miR-21 was determined by using fluorescence qPCR system (cat. no. 7900HT; Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec and 95˚C for 15 sec, then 60˚C for 1 min.

ELISA. The blank, standard and sample wells were selected and the blank well was not sampled. Different concentrations of 50 μl standard product and 100 μl horseradish peroxidase-labeled TNF-α or IL-6 antibody (cat. nos. RJ16622 and RJ15478, respectively; both Shanghai Renjie Biological Technology Co, Ltd.) were added to standard wells. The sample wells were filled with 10 μl sample, 40 μl sample diluent and 100 μl HRP-labeled antibody. The plate was incubated at 37˚C (60 min), then the detergent was shaken off and the plate was patted dry and washed five times. Following addition of chromogenic solution, the plate was incubated at 37˚C (15 min). Next, 50 μl terminating solution was added to terminate the reaction. The absorbance [optical density (OD) value] of each well was measured at 450 nm. The standard curve was drawn according to the concentration of the standard sample and the corresponding OD value and the concentration of samples was calculated by regression equation according to the OD value of each sample.

Statistical analysis. Data were statistical analyzed by SPSS 24.0 (IBM Corp.) and are presented as the mean ± SD of 3-6 independent repeats. Comparison between two groups were performed by one-way ANOVA with post hoc Bonferroni's correction or paired Student's t-test. Correlation analysis was evaluated via the Pearson method. P<0.05 was considered to indicate a statistically significant difference.

Results

Release of inflammatory factors increases in septic rats. Levels of TNF-α and IL-6 secreted in the NC and sepsis groups were measured at different time points. TNF-α and IL-6 release in the sepsis groups peaked at 12 h, then decreased. There was statistical significance in TNF-α and IL-6 secretion between the two groups (Fig. 1).

miR-126 and miR-21 expression is downregulated in T lymphocytes of septic rats. Expression levels of miR-126 and miR-21 in T lymphocytes in the NC and sepsis groups were compared. Expression of miR-126 and miR-21 in the sepsis groups were significantly below that in the NC group (Fig. 2).

Expression of miR-126 in T lymphocytes in the sepsis groups initially decreased and then increased slowly after 24 h. These changes were statistically significant (Fig. 3A). Similarly, expression of miR-21 initially decreased and then increased slowly after 24 h. Compared with the NC group, miR-21 was only significantly different in the sepsis groups at 24 h; differences were not statistically significant at 12, 48 and 72 h (Fig. 3B).

T lymphocyte apoptosis increases in septic rats. T cell counting showed that apoptosis of T lymphocytes significantly increased up to 48 h, then decreased in the sepsis groups (Fig. 4).

Expression and activity of caspase-3 in T lymphocytes is elevated in septic rats. Expression of caspase-3 in T lymphocytes in the sepsis groups significantly increased up to 48 h and then decreased (Fig. 5A and B). The change in caspase-3 activity was consistent with that of its expression levels (Fig. 5C).

Expression of miR-126 is positively correlated with that of miR-21 in T lymphocytes of septic rats. The levels of miR-126 and miR-21 in T lymphocytes of septic rats decreased up to

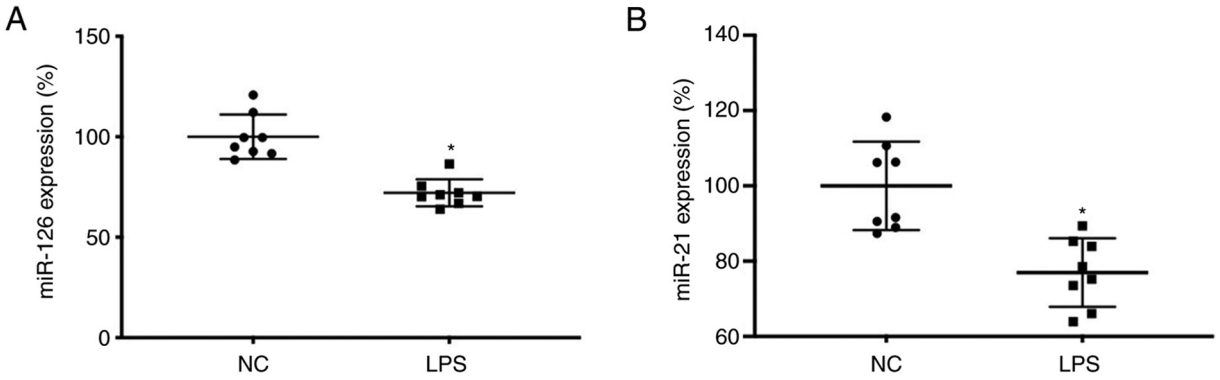

Figure 2. Expression of miR-126 and miR-21 in lymphocytes of NC and septic rats. (A) Expression levels of (A) miR-126 and (B) miR-21 were lower in septic compared with NC rats. Data are presented as the mean ± SD (n=8/group). *P<0.05 vs. NC. miR, microRNA; NC, normal control; LPS, lipopolysaccharide.

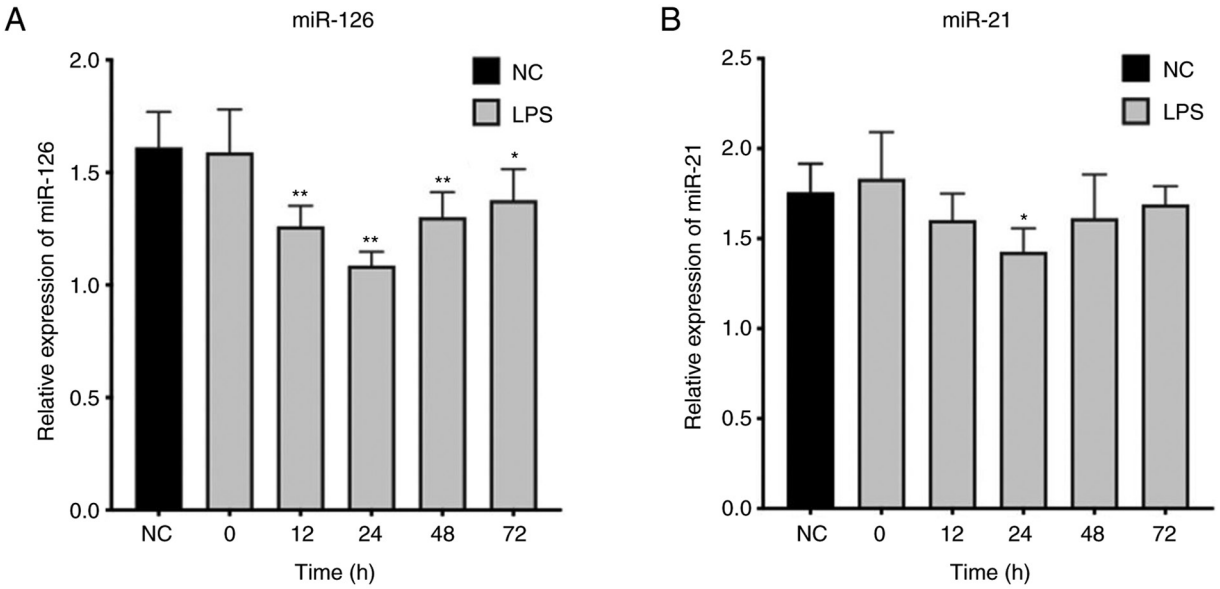

Figure 3. Expression levels of miR-126 and miR-21 in lymphocytes of NC and septic rats at different time intervals. Expression of (A) miR-126 and (B) miR-21 initially but increased after 24 h. Data are presented as the mean ± SD (n=8/group). *P<0.05, **P<0.01 vs. NC. miR, microRNA; NC, normal control; LPS, lipopolysaccharide.

24 h and then slowly increased (Fig. 6A). There was a linear positive correlation between expression levels of these two miRs (Fig. 6B).

Expression of miR-126 is correlated with inflammatory factors, apoptotic rate of T lymphocytes and expression and activity of caspase-3 in septic rats. miR-126 expression was negatively correlated with expression levels of inflammatory factors, the apoptotic rate of T lymphocytes and expression and activity of caspase-3. Levels of inflammatory factors peaked at 12 h, expression of miR-126 at 24 h and the apoptostic rate of T lymphocytes at 48 h (Fig. 7A).

miR-126 was negatively correlated with levels of TNF-α and IL-6 (Fig. 7E and F), apoptotic rate of T lymphocytes (Fig. 7B) and expression and activity of caspase-3 (Fig. 7C and D).

Expression of miR-21 is correlated with inflammatory factors and the expression and activity of caspase-3 but not T lymphocyte apoptosis in septic rats. The expression levels of miR-21 were negatively correlated with levels of inflammatory factors, apoptotic rate of T lymphocytes and expression and activity of caspase-3, but not significantly correlated with apoptotic rate of T lymphocytes (Fig. 8A).

miR-21 was negatively correlated with levels of TNF-α and IL-6 (Fig. 8E and F) and expression and activity of caspase-3 (Fig. 8C and D). However, miR-21 wasnot significantly correlated with the apoptotic rate of T lymphocytes (Fig. 8B).

Discussion

At present, the uncontrolled inflammatory response is considered to be the basis for the pathogenesis of sepsis. The release of inflammatory factors in the early stage of sepsis leads to amplification of an inflammatory cascade and tissue and organ damage (20-23). TNF-α and IL-6 are associated with occurrence and progress of inflammatory reactions in sepsis (24). TNF-α is the most important proinflammatory factor in the early stage of inflammation and a key mediator of the LPS damage effect (25). Here, levels of TNF-α and IL-6 increased gradually after LPS was injected into the abdominal cavity of

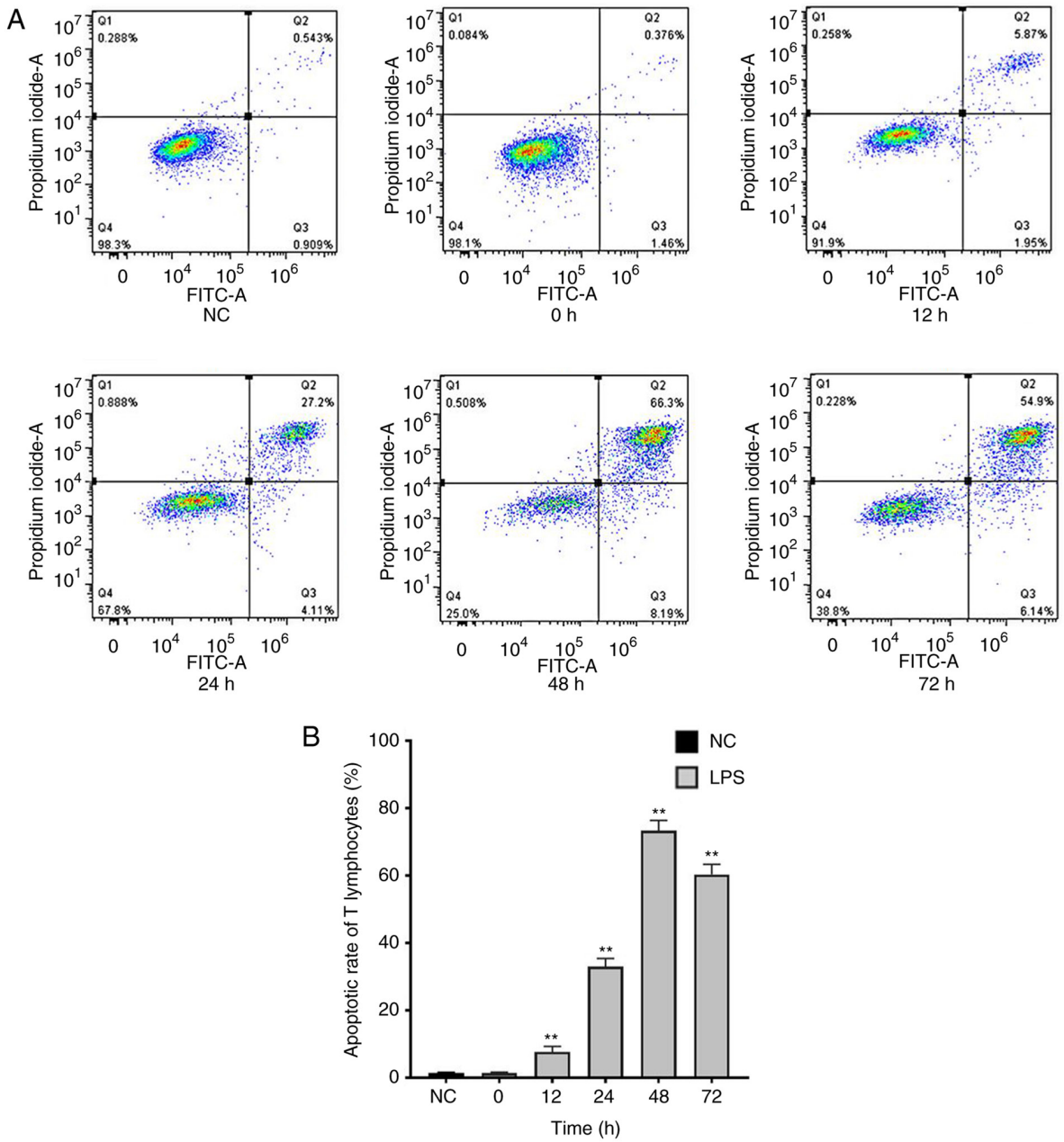

Figure 4. Apoptosis of lymphocytes in septic rats at different time periods. (A) Flow cytometry was used to determine the apoptotic rate of T lymphocytes in NC and septic rats at different time points. (B) Apoptotic rate of T lymphocytes in septic rats increased up to 48 h, then decreased. Data are presented as the mean ± SD (n=8/group). **P<0.01 vs NC. NC, normal control; LPS, lipopolysaccharide.

rats, peaked at 12 h, and then decreased over time. Although the levels of TNF-α and IL-6 decreased significantly, they were still higher than in NC rats at 72 h. This trend is consistent with a study by Sun *et al* (26), which demonstrated that levels of HMGB1, TNF-α, IL-6 in serum from patients with sepsis increased first and then decreased.

With the aggravation of inflammatory reactions, the body enters a state of immunosuppression in sepsis (27). T lymphocytes are important in the pathogenesis of sepsis and immunomodulation therapy is a focus of research (28). Numerous studies have shown that decreased levels of T cells result in increased apoptosis of T cells in sepsis (29-31). Here, apoptosis of T lymphocytes in septic rats increased, which was consistent with the aforementioned studies. The number of T lymphocytes decreased and levels of caspase-3, which reflect apoptosis, increased significantly. Apoptosis of T lymphocytes was peaked at 48 h, which appeared after the peak of the inflammatory response (TNF-α and IL-6), then gradually decreased to normal levels.

Previous attempts to decrease mortality by decreasing release of inflammatory factors and the inflammatory response in sepsis have failed. Meta-analysis has shown that high-throughput hemofiltration and other methods of eliminating inflammatory factors do not improve the prognosis of patients with sepsis (32). Studies have show that the development and prognosis of sepsis is associated with apoptosis of immune cells, particularly T lymphocytes (7,33). Regulation of T lymphocyte apoptosis improves the prognosis of sepsis (34). Regarding T lymphocyte apoptosis, various caspases are activated in sepsis; lymphocyte apoptosis is triggered by release of TNF-α, glucocorticoids,

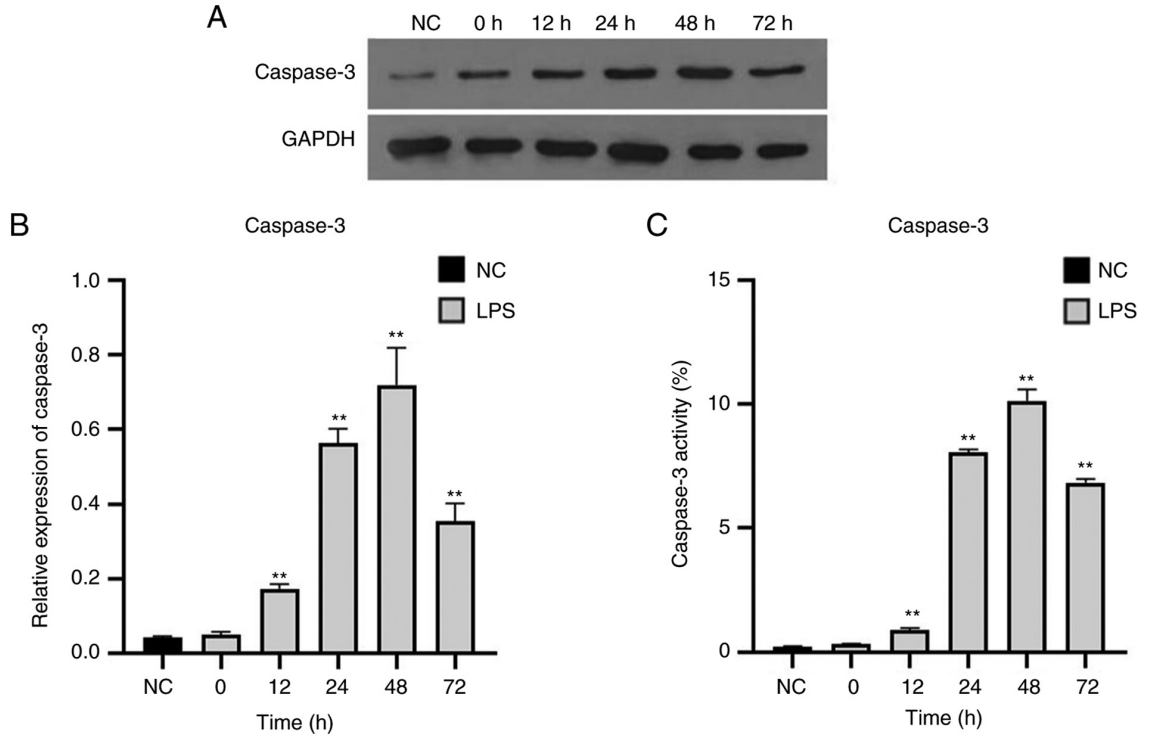

Figure 5. Changes of caspase-3 expression and activity in septic rats at different time points. (A) Western blot analysis of caspase-3 expression in NC and septic rats. (B) Expression and (C) activity of caspase-3 in T lymphocytes of septic rats increased up to 48 h, then decreased. Data are presented as the mean ± SD (n=8/group). **P<0.01 vs. NC. NC, normal control; LPS, lipopolysaccharide.

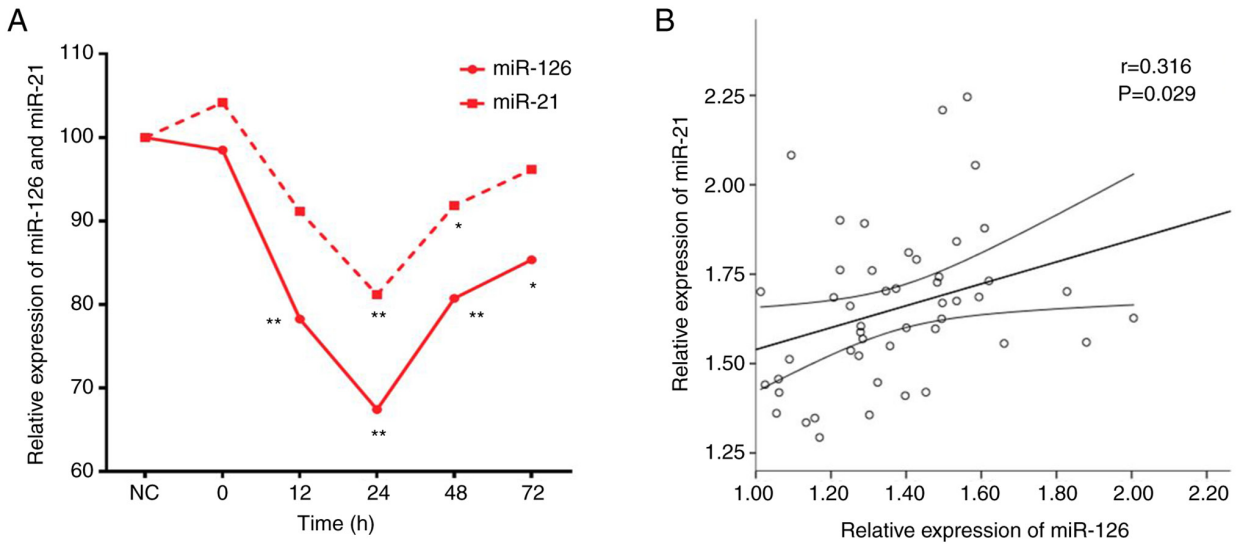

Figure 6. Correlation between expression levels of miR-126 and miR-21 in lymphocytes of septic rats. (A) Expression of miR-126 and miR-21 in lymphocytes of septic rats decreased up to 24 h, then increased. (B) Changes in expression levels of miR-126 and miR-21 exhibited a linear positive correlation. *P<0.05, **P<0.01 vs. NC. miR, microRNA; NC, normal control.

granzymes or by the absence of IL-2 (35). Animal experiments have confirmed that use of a caspase inhibitor (VX-166) increases the survival rate of septic mice from 40 to 92% (36,37). Therefore, regulation of T lymphocyte apoptosis is an important area of research. MiRNAs expression affects differentiation and proliferation of T lymphocytes (38,39). Here, miR-126 and miR-21 in T lymphocytes of septic rats decreased significantly; this trend was contrary to that of inflammatory factors and T lymphocyte apoptosis, which decreased up to 24 h, then gradually increased. Agudo *et al* (40) found that miR-126 regulates the function of plasma-like dendritic cells via the VEGFR2 axis and participates in the innate immune response initiated by microorganisms such as viruses. miR-126 regulates peripheral induction of Tregs via PI3K/AKT signaling, suggesting that miR-126 is important in the immune response (41). Recently, it has been shown that miR-126 inhibits invasion and metastasis of malignant glioma by downregulating the proliferation of mature T lymphocytes, suggesting that miR-126 exerts a regulatory

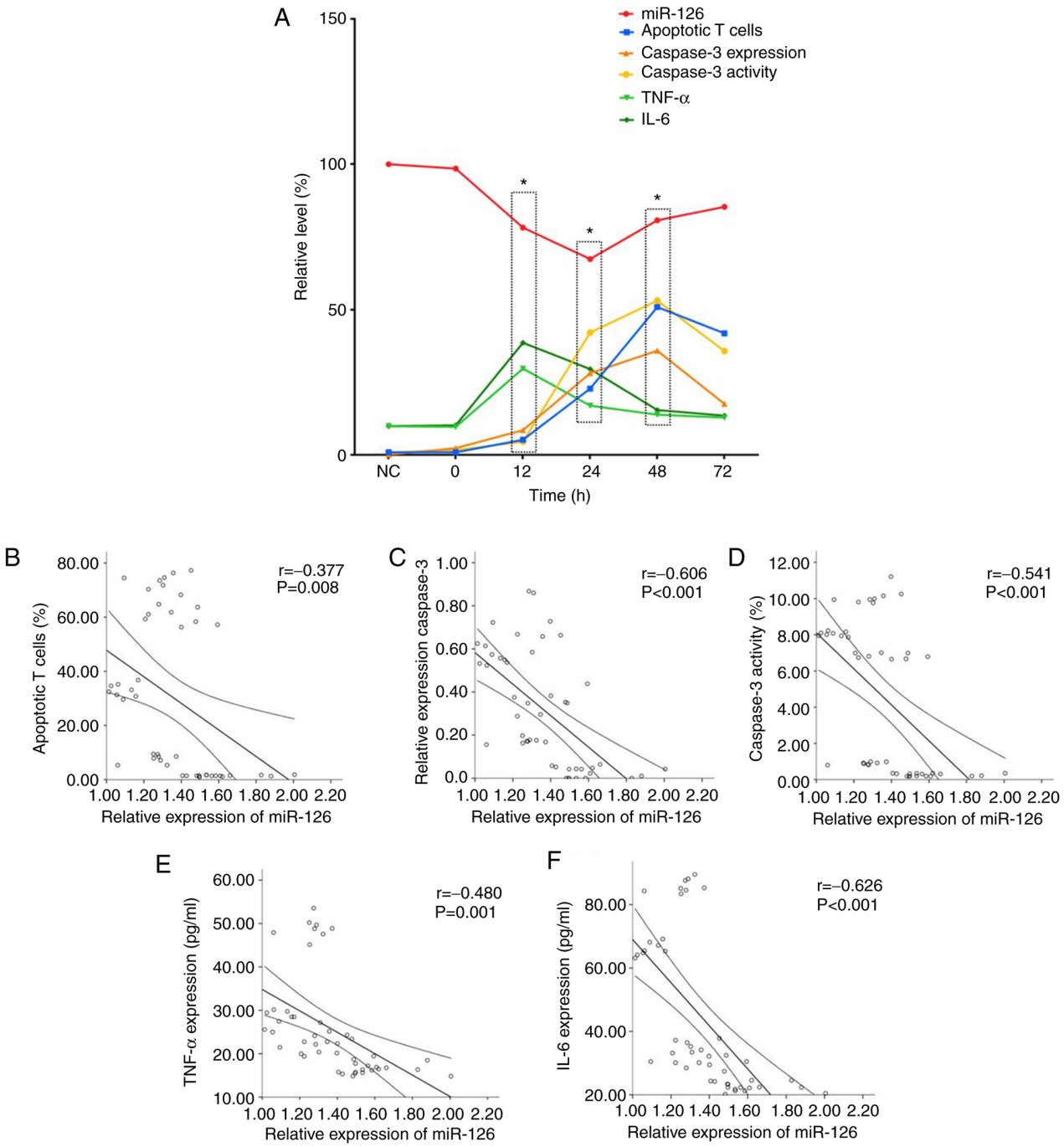

Figure 7. Correlation between expression of miR-126, apoptotic rate of T lymphocytes, expression and activity of caspase-3 and levels of TNF-α and IL-6 in septic rats. (A) Relative levels of miR-126, T lymphocyte apoptosis, caspase-3 expression and activity, TNF-α and IL-6 in septic rats. *P<0.05 vs. NC. Expression of miR-126 was negatively correlated with (B) apoptotic rate of T lymphocytes, (C) expression and (D) activity of caspase-3 and (E) TNF-α and (F) IL-6. miR, microRNA; NC, normal control.

effect on T lymphocytes (42). The results of the present study confirmed the aforementioned findings. There was a linear correlation between miR-126 and levels of TNF-α and IL-6, apoptotic rate of T lymphocytes and activity and expression of caspase-3 in T lymphocytes in septic rats. In addition, altered expression of miR-126, which preceded apoptotic changes, indicated that miR-126 may regulate apoptosis of T lymphocytes in septic rats. Previous studies have shown that miR-126 is expressed primarily in T cells and affects the activation of $CD4^+$ T cells (43-45). Inflammatory factors, such as IL-12, TGF-β and IFN-γ, show increased expression in $CD4^+$ T lymphocytes of mice with a miR-126 gene knockout (13), which supports the results of the present study. These results further indicated that the apoptosis of T lymphocytes increased and expression of miR-126 decreased in sepsis, both of which were consistent with previous research (46-48). It has also been found that miR-21 is universally expressed in T lymphocytes, especially in memory phenotype T lymphocytes (49). Inhibition of miR-21 promotes apoptosis and growth defects of memory phenotype T lymphocytes, suggesting that the survival of this type of T lymphocyte is associated with miR-21 (49). Ruan *et al* (15) found that miR-21 regulates the TNF-α-induced protein 8-like

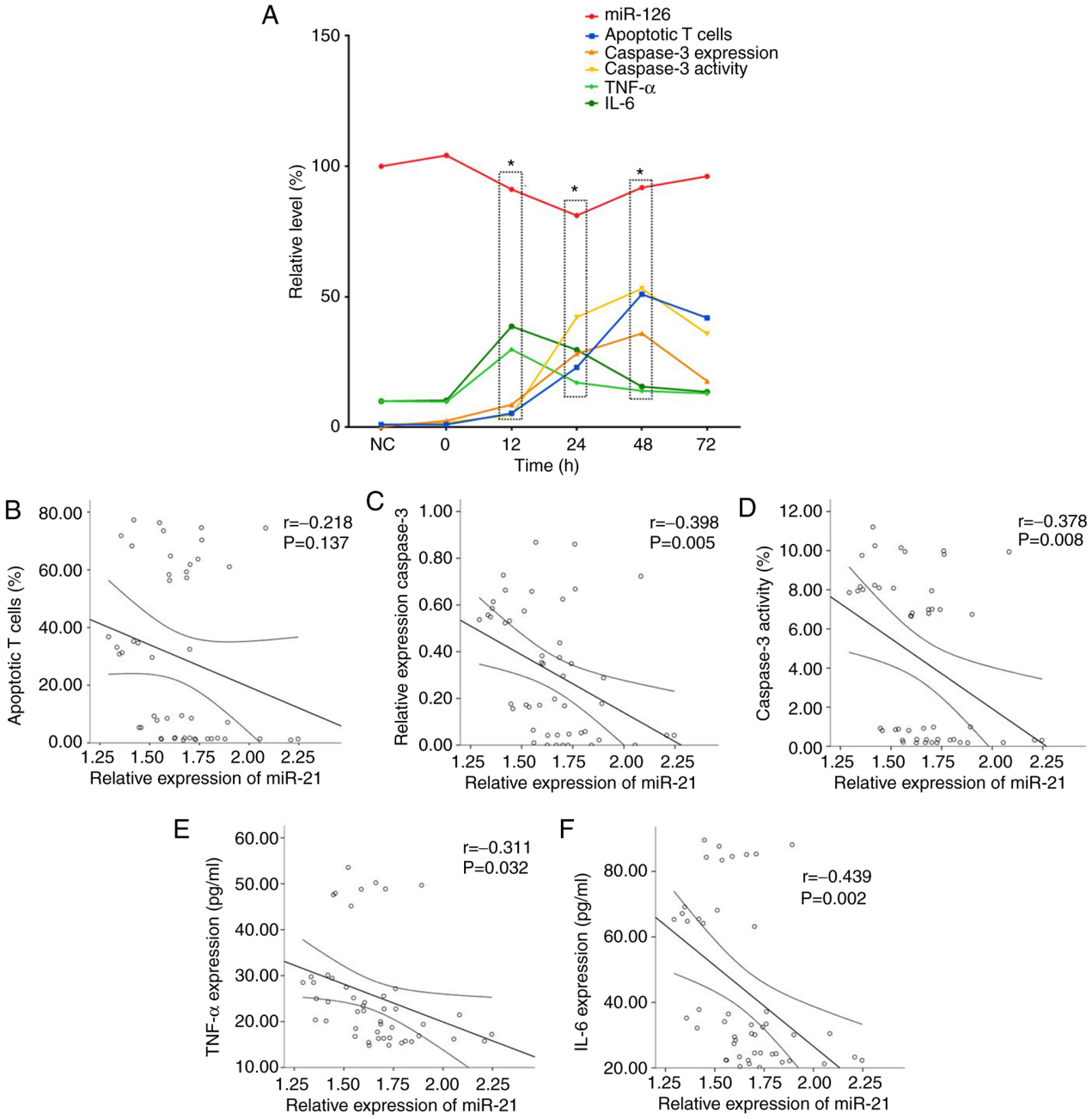

Figure 8. Correlation between expression of miR-21, apoptotic rate of T lymphocytes, expression and activity of caspase-3 and levels of TNF-α and IL-6 in septic rats. (A) Relative levels of miR-21, T lymphocyte apoptosis, caspase-3 expression and activity, TNF-α and IL-6 in septic rats. *P<0.05 vs. NC. (B) Expression of miR-126 was not significantly correlated with the apoptotic rate of T lymphocytes. There was a negative correlation between expression of miR-21 and (C) expression and (D) activity of caspase-3, (E) TNF-α and (F) IL-6. miR, microRNA; NC, normal control.

2 gene to inhibit apoptosis of T lymphocytes and that NF-κB regulates expression of miR-21. Here, levels of miR-126 and miR-21 in T lymphocytes in septic rats were similar to those in the aforementioned studies. Expression levels of miR-126 and miR-21 initially decreased, then increased and were negatively correlated with release of TNF-α and IL-6 and activity and expression of caspase-3. Moreover, the increase in TNF-α and IL-6 occurred prior to the decrease in miR-21 and was followed by an increase in activity and expression of caspase-3, which suggested that inflammatory factors such as TNF-α and IL-6 may regulate expression of miR-21 and the molecular mechanism underlying T lymphocyte apoptosis. This is consistent with the study by Ruan *et al* but requires further study to determine which signaling pathways are involved in miR-21-mediated regulation of apoptosis and release of inflammatory factors in sepsis.

In summary, the inflammatory response and apoptosis of T lymphocytes are important in sepsis. miR-126 and miR-21 expression levels in T lymphocytes in sepsis were significantly altered; the changes in miR-126 and miR-21 expression were consistent with the inflammatory response and apoptosis, indicating they may be associated with inflammatory factors and apoptosis of T lymphocytes in sepsis. Further research is required to determine whether and how miR-126 and miR-21 regulate apoptosis of T lymphocytes in sepsis to provide novel options for the diagnosis and treatment of sepsis.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ and CL analyzed patient data and wrote the manuscript. QZ and MYa collected the data and performed the experiments. MYu analyzed the experimental data. All authors read and approved the final version of the manuscript. QZ and CL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the animal ethics committee of Bengbu Medical College (approval no. 2018074).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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