Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

LncRNA MEG3 expression in sepsis and its effect on LPS-induced macrophage function

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Received March 1, 2020; **Accepted** June 9, 2020; **Published** July 31, 2020

Doi: http://dx.doi.org/10.14715/cmb/2020.66.5.23

Abstract: Sepsis is a dangerous disease that is caused by an overreaction of the body's immune system to infection and gradually spreads throughout the body. This experiment was carried out to explore the expression of LncRNA MEG3 in sepsis and its effect on LPS-induced macrophage function. Methods 60 sepsis patients admitted to our hospital from February 2017 to September 2018 were selected as the sepsis group, and 50 non-septic patients diagnosed and treated in our hospital during the same period were selected as the control group. qRT-PCR was used to detect the expression level of MEG3. ROC curve was used to analyze the diagnostic value of serum MEG3 in sepsis. The human macrophage cell line U937 was cultured in vitro and randomly divided into NC group, LPS group, LPS + pcDNA group, and LPS + pcDNA-MEG3 group. Flow cytometry was applied to detect the apoptosis rate. The levels of IL-1β and TNF-α were detected by ELISA. Western blot was used to detect the expression of Bax, Bcl-2 and NF-κB signaling pathway-related proteins p65 and p-p65. Results: The expression level of serum MEG3 in the sepsis group was significantly lower than that in the control group (P <0.05). ROC curve analysis showed that the AUC area was 0.856, the sensitivity was 0.700, and the specificity was 0.883. Compared with the NC group, the macrophage apoptosis rate in the LPS group was increased (P <0.05), the levels of Bax and p-p65 protein were significantly increased (P <0.05), and the level of Bcl-2 protein was decreased (P <0.05), the levels of IL-1β and TNF-α were increased (P <0.05). Compared with the LPS + pcDNA group, the apoptosis rate of the LPS + pcDNA-MEG3 group was significantly reduced (P <0.05), the levels of Bax and p-p65 protein were reduced (P <0.05), and the level of Bcl-2 protein was significantly increased (P <0.05), the levels of IL-1β and TNF-α were reduced (P <0.05). Conclusion: The low expression of LncRNA MEG3 in the serum of patients with sepsis can predict the occurrence of sepsis. Overexpression of MEG3 can inhibit LPS-induced macrophage apoptosis and secretion of inflammatory factors by inhibiting the activation of the NF-κB signaling pathway.

Key words: LncRNA MEG3; Sepsis; Lipopolysaccharide; Macrophage; Apoptosis; Inflammatory factor.

Introduction

Sepsis is one of the clinical common disease types, mainly refers to the pathogenic bacteria that invade the blood circulation after releasing a variety of toxins that cause a systemic acute infection. It has a high fatality rate and has been a serious threat to human life safety (1-2). Sepsis occurs due to a severe reaction to the infection. To counter the threat, the body sends large amounts of chemicals into the bloodstream. This causes severe inflammation that slows blood flow over time and damages the organs. Sometimes people get severe sepsis or septic shock, which can lead to death (2-4). People who suffer from this disease have a serious infection. Fever and feeling sick, fainting, weakness or confusion are the early signs of sepsis. In this case, the heart rate and respiration become faster than usual. If left untreated, it can damage the body's organs, make it difficult to breathe, cause diarrhea and nausea, and cause mental disorders (2-4). Currently, blood culture is mainly used in the clinical diagnosis of sepsis, but the time of blood culture needs a long time and the positive rate is low, so it is of great significance to search for biomarkers of early sepsis infection)1-4(. At present, the molecular mechanism of the occurrence and development of sepsis has not been fully elucidated, thus it is helpful to improve the diagnostic efficiency and reduce the mortality of patients by actively exploring the relevant diagnostic indicators of sepsis. Lipopolysaccharide (LPS) can induce inflammatory responses in macrophages and promote the occurrence and development of sepsis)5(.

For 50 years, the term gene was synonymous with areas of the genome that were encoded by mRNAs and translated into proteins. However, recent extensive studies of the genome have shown that in the human genome, transcription and production are performed by thousands of non-coding regulatory RNAs, including Micro RNA, Small Nucleolar RNA, and Small Interfering RNA, which are functional molecules. Which fall into the category of small non-encoded RNAs and different categories of long non-encoded RNAs. These RNAs play vital roles in regulating transcription and post-transcription, gene silencing, and DNA demethylation. Evidence suggests that intergenic regions are associated with the expression of non-coding RNAs in complex diseases and that the use of non-coded RNAs as markers will be useful in diagnosing disease and therapeutic goals. These observations emphasize that it is necessary to understand the range of protein coding genes and to continue the evolutionary research and

function of non-encoded RNAs for a comprehensive understanding of human disease (6-8).

Long-chain non-coding RNA (Long non-coding RNA, LncRNA) belongs to the endogenous non-coding RNA molecules, it does not have the function of coding protein itself. However, it can regulate the expression of its target genes by regulating the expression of downstream microRNA (miRNA), thus participating in a variety of physiological and pathological processes such as inflammatory diseases and tumors. Studies have shown that the regulatory effect of LncRNA on the inflammatory response of macrophages has not been fully elucidated)6,7(. Long non-coding RNA MEG3 (LncRNA MEG3) inhibits the occurrence of rheumatoid arthritis by regulating the mir-141 /AKT/mTOR signaling pathway)8(. Silencing MEG3 expression can enhance lung cell injury induced by lipopolysaccharide by regulating the expression of mir-4262)9(. Knockdown of MEG3 expression can be involved in inflammatory damage induced by lipopolysaccharide by regulating the expression of mir-203)10(. However, the expression of MEG3 in sepsis and its effect on LPS-induced macrophage function has not been fully elucidated. Therefore, this study mainly discussed the expression of MEG3 in sepsis patients and its effect on LPS-induced macrophage apoptosis and inflammatory response, aiming to provide a new direction for the diagnosis and treatment of sepsis.

Materials and Methods

Materials and reagents

A total of 60 patients with sepsis admitted to our hospital from February 2017 to September 2018 were selected as the sepsis group. Meanwhile, 50 cases of non-sepsis patients treated in our hospital were selected as the control group. There was no statistically significant difference in age and gender between the two groups ($P > 0.05$), which was comparable (see table 1). This study was approved by the ethics committee of our hospital, and all subjects were informed and signed the consent. Human macrophage cell system U937 was purchased from Nanjing Kaiji biotechnology development co., LTD. LPS purchased from Sigma-Aldrich Company. Duchenne improved medium (DM EM) and fetal bovine serum were purchased from Gibco. Lipofectamine2000 and Trizol reagent were purchased from Invitrogen Company. PcDNA3.1 purchased from Shanghai Kray Biotechnology Company. The reverse transcription kit and real-time fluorescence quantitative PCR kit were purchased from TaKaRa Company (Japan). Annexin V - FITC/propidium iodide cell apoptosis kit was purchased from Sigma. Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α(TNF-α) ELISA kits were purchased from Ebiosci Ence Company (USA). RIPA lysate was purchased from Xi 'an Hutt Biotechnology Co., LTD. Diquinoline formic acid (Bicinchonicacid, BCA) protein quantitative detection kit, enhanced chemiluminescence reagent (ECL), sodium dodecyl sulfate (SDS) were purchased from Beijing Quanshi Gold Biotechnology Company. Rabbit anti-human bcl-2-associated X protein (Bcl-2-associated X protein, Bax) and b-cell lymphoma 2 (B-cell lymphoma-2, Bcl-2) antibodies were purchased from CST Company. Rabbit anti-human nuclear factor - kappa B (NF-κB) signaling pathway p65, p-p65 antibodies were purchased from Santa Cruz. Horseradish peroxidase (HRP) -labeled goat anti-rabbit secondary antibody was purchased from Wuhan Emmett Technology Company.

Blood samples collection

Five mL of fasting venous blood was extracted from the two groups in the morning, then stored at room temperature for 30 min, centrifuged at a speed of 3000 r/min for 10 min at 4 ℃, and the separated serum was stored in an ultra-low temperature refrigerator at -80 ℃.

Experimental grouping

Macrophages (5×10^5 / mL) vaccination within 12 holes (1 mL) were randomly divided into NC group (normal cultured macrophage), the LPS group (contain a concentration of 100 ng/mL LPS cultured macrophage, the incubation time of 24 h))11(. LPS + pcDNA group (macrophages cultured with LPS concentration of 100 ng/mL after transfection of pcDNA into macrophages, culture time was 24 h), and LPS+ pcDNA-meg3 group (macrophages cultured with LPS concentration of 100 ng/mL after transfection of pcDNA-meg3 into macrophages, culture time was 24 h). The transfection process was operated according to the specification of Lipofectamine2000 reagent.

The expression level of MEG3 was detected by quantitative real-time PCR (qRT-PCR)

The frozen serum and macrophages in each group were taken out and the total RNA in the serum or cells was extracted by the Trizol method. RNA concentration and purity were determined by Nanodr op2000c ultramicro spectrophotometer. The reaction system was configured with reference to the reverse transcription kit: RNA 2 alarm μL, 10×RT Buffer 2 alarm μL, dNTP 0.4 alarm μL, Multiscripe RT 1 alarm μL, 10×Random Primer 2 alarm μL, RNase-Free ddH2O supplement system to 20 alarm μL. Reaction conditions: storage at 25 ℃ for 10 min, 37 ℃ for 60 min, 95 ℃ for 5 min, and 4 ℃. Reverse transcription synthesizes the total RNA into cDNA. MEG3 forward primer 5 '- GGGCTTCTGGAATGAGCA TGG-3', reverse primer 5 '- TCTATGCCAGATCCTGCCTG-3'. GAPDH forward primers 5 '-AACGGATTTGGTCGT ATTG-3' and reverse primers 5 '-ggaagatggtgatggcontr-3' were designed and synthesized by Shanghai Sangon Biological Engineering (China). Reverse transcription was used to synthesize cDNA, and cDNA was used as the template for the qRT-PCR reaction. Reaction system: SYBR Green Master Mix 10 μL/ well, positive and negative primers 0.8 μL/ well, cDNA 1 μL/ well, ddH2O supplement system to 20μL. Reaction conditions: pre-denaturation at 95℃ for 5min was performed once, denaturation at 95℃ for 15s, annealing at 60℃ for the 60s, and extension at 72℃ for 30s, with a total of 40 cycles. GAPDH is the internal parameter of MEG3, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of the MEG3.

Cell apoptosis rate was detected by flow cytometry

The macrophages in each group were collected in the logarithmic phase and washed with pre-cooled PBS. The cells were centrifuged at 3000 r/min at 4 ℃ for 6

min. The supernatant was discarded. 500 L binding buffer solution was added to re-suspend the cells, followed by 5 μL Annexin v-fitc and 5 μL PI, and incubated at room temperature for 10 min. FACS Calibur flow cytometry was used to detect the apoptosis rate in each group.

Enzyme-linked immunosorbent assay (ELISA) detection

Cell culture supernatant was collected from each group, and the levels of IL-1β and TNF-α were detected by ELISA.

Bax, bcl-2, NF- κB signaling pathways p65 and p-p65 were detected by Western blot

The logarithmic macrophages in each group were collected, 500 μL of RIPA lysate was added, the ice cracked for 30 min, centrifuged for 10 min at 4 ℃ by 3000 r/min, and the supernatant was absorbed. Protein concentration was detected by the BCA method. 50 μg protein samples were added to 5×SDS loading buffer, then were boiled for 10 min. The isolated protein was transferred and sealed by SDS-PAGE, with primary antibody diluent (1:1000) added, incubated overnight at 4 ℃, washed at TBST, added with a secondary antibody diluent (1:500), incubated at room temperature for 1 hour, added with ECL, exposed and developed in a dark room, and analyzed the gray value of each strip with Image J software.

Statistical treatment

SPSS21.0 statistical software was used to analyze the data. The measurement data were expressed by (x±s) and all accorded with a normal distribution. An independent sample t-test was used for comparison between the two groups, and a single factor analysis of variance was used for comparison between multiple groups. The diagnostic value of serum meg3 on sepsis was analyzed by receiver operating characteristic (ROC) curve, and the difference was statistically significant ($P < 0.05$).

Results

Comparison of clinical data

There was no statistically significant difference in age and gender between the control group and the sepsis group, which was comparable ($P > 0.05$), as shown in table 1.

Relative expression of serum MEG3 in sepsis patients

The results of qRT-PCR (figure 1) showed that the relative expression levels of serum MEG3 in the control group and the sepsis group were 2.67±0.65 and 1.43±0.50 respectively. The expression levels of serum MEG3 in the sepsis group were significantly lower than those in the control group, with statistically significant differences ($P < 0.05$).

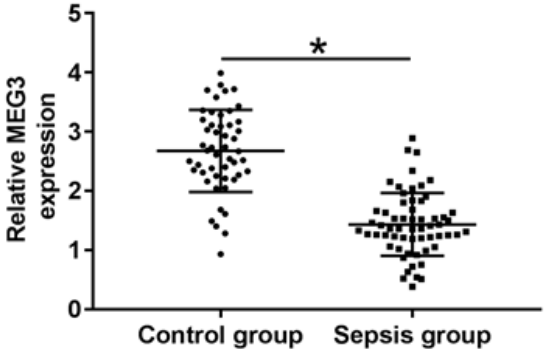

Figure 1. The relative expression of serum MEG3 in septicemia patients (x±s). Note: compared with the control group, *P <0.05.

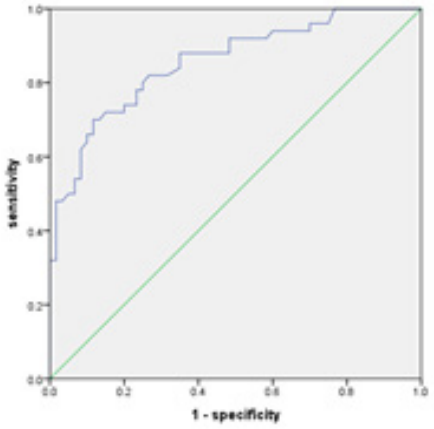

Figure 2. ROC curve of serum MEG3 in the diagnosis of sepsis.

Clinical diagnostic value of serum MEG3 in sepsis

The ROC curve analysis results (figure 2, table 2) showed that the AUC of the ROC curve for the diagnosis of sepsis of serum MEG3 was 0.856, the 95% confidence interval (CI) was 0.786 ~ 0.926, the optimal threshold value was 0.583, the sensitivity was 0.700, and the specificity was 0.883.

Effects of overexpression of MEG3 on LPS-induced macrophage apoptosis

Compared with the NC group, the apoptosis rate of macrophages in the LPS group was significantly increased ($P < 0.05$), the Bax protein level was significantly increased ($P < 0.05$), and the bcl-2 protein level was significantly decreased ($P < 0.05$).Compared with

Table 1. Comparison of clinical data (x±s).

Group	Cases	Gender		Age (years, x±s)
		Man	Women	
Control	50	30	20	54.82±10.29
Septicemia	60	37	23	54.13±11.42
X^2/t	--	0.032		0.182
P	--	0.858		0.856

Table 2. ROC curve analysis of serum MEG3 in the diagnosis of sepsis.

Test index	AUC	Standard error	95% CI	*P*	Best boundary value	Sensitivity	Specificity
MEG3	0.856	0.036	0.786~0.926	<0.001	0.583	0.700	0.883

the LPS+pcDNA group, the cell apoptosis rate of the LPS+ pcDNA-meg3 group was significantly reduced (P < 0.05), the Bax protein level was significantly reduced (P < 0.05), and the bcl-2 protein level was significantly increased (P < 0.05), as shown in figure 3.

Effects of overexpression of MEG3 on the secretion of inflammatory factors by LPS-induced macrophages

Compared with the NC group, the levels of IL-1β, TNF-α in the LPS group were significantly increased (P < 0.05). Compared with the LPS+pcDNA group, IL-1β, TNF-α levels in the LPS+ pcDNA-meg3 group were significantly reduced (P < 0.05), as shown in figure 4.

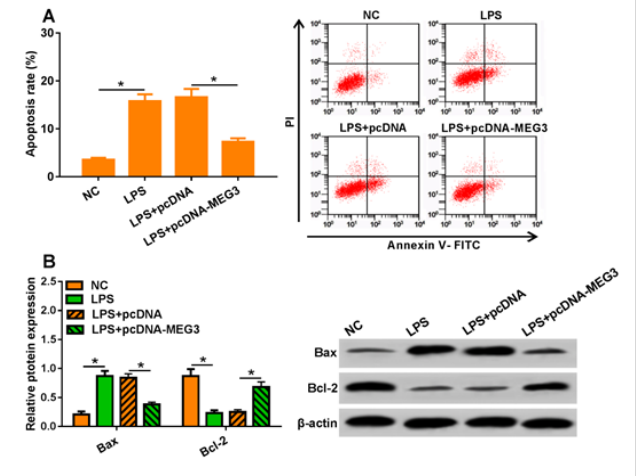

Figure 3. Effect of overexpression of MEG3 on LPS-induced macrophage apoptosis. A: Effect of MEG3 overexpression on LPS-induced macrophage apoptosis was detected by flow cytometry. B: Effect of MEG3 overexpression on the expression of LPS-induced apoptosis-related proteins in macrophages using western blot detection. Note: Compared with the NC group, *P<0.05; LPS+pcDNA group, *P<0.05.

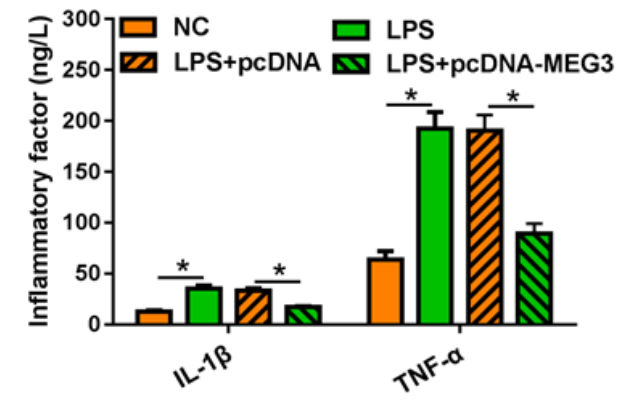

Figure 4. The effect of MEG3 overexpression on the secretion of inflammatory factors by LPS-induced macrophages using the ELISA method. Note: Compared with the NC group, *P<0.05; LPS+pcDNA group, *P<0.05.

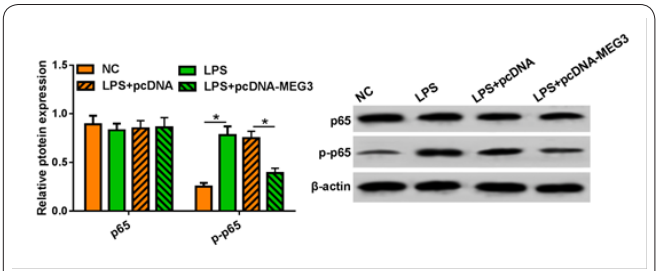

Figure 5. The effect of MEG3 overexpression on the expression of related proteins in the NF- κB signaling pathway in macrophages induced by LPS using Western blot analysis. Note: Compared with the NC group, *P<0.05; LPS+pcDNA group, *P<0.05.

Effect of MEG3 overexpression on LPS-induced macrophage NF- κB signaling pathway

Compared with the NC group, the level of p-p65 protein was significantly increased in the LPS group (p < 0.05). Compared with the LPS+pcDNA group, the p-p65 protein level in the LPS+ pcDNA-meg3 group was significantly reduced (p < 0.05), while there was no statistically significant difference in the p65 protein level in the NC group, the LPS group, the LPS+pcDNA group and the LPS+ pcDNA-meg3 group (p > 0.05), as shown in figure 5.

Discussion

Sepsis has seriously affected the quality of patients' life, based on the pathological process of the disease, inflammation response in sepsis, and play an important role in the process of development. The early inflammatory response can prompt the pathogens to produce inflammatory cytokines. The human body can remove part of the pathogens and inhibit inflammation, but with the development of the disease, inflammation aggravate can increase the severity of the disease. Excessive increase in the production of inflammatory factors can lead to the occurrence of sepsis and other diseases)12,13(. LncRNA can participate in transcriptional regulation and other processes to participate in the biological processes of cell proliferation, differentiation, and apoptosis, as well as the occurrence and development of inflammatory response, sepsis and other diseases)14,15(. However, the mechanism of LncRNA in the pathogenesis and development of sepsis has not been elucidated.

MEG3 alleviates hyperglycemia-induced inflammation and apoptosis of retinal epithelial cells by regulating the mir-34a/SIRT1 axis)16(. MEG3 inhibits the inflammatory response of ankylosing spondylitis by targeting miR-146a)17(. However, some studies have shown that MEG3 promotes cerebral ischemia-reperfusion injury by increasing cell apoptosis by targeting the molecular axis of mir-485 / AIM2)18(. MEG3 regulates cse-induced apoptosis and inflammatory response of 16HBE cells by regulating the expression of mir-218)19(. MEG3 can also be used as a serum biomarker to diagnose hepatitis b with liver fibrosis)20(. MEG3 can

also inhibit ox-ldl-induced inflammation and macrophage apoptosis)21(. The results of this study showed that the expression level of serum MEG3 in sepsis patients was significantly lower than that in the control group, suggesting that MEG3 may play an important regulatory role in the occurrence of sepsis. In this study, ROC was used to analyze the diagnostic value of serum MEG3 in sepsis. The results showed that the AUC area was 0.856, the 95% confidence interval (CI) was 0.786 ~ 0.926, the optimal threshold value was 0.583, the sensitivity was 0.700, and the specificity was 0.883, suggesting that MEG3 may be an important auxiliary index for the diagnosis of sepsis.

This study explores MEG3 in sepsis and possible mechanism of action, in the process of development, the LPS induced macrophage cell damage simulation was conducted. In this study, MEG3 expression vector was transfected into macrophages and then treated with LPS. The results showed that after treatment, the apoptosis rate of LPS macrophages was significantly increased, the level of Bax protein was increased, and the level of Bcl-2 protein was reduced, while After MEG3 expression, cell apoptosis was reduced. The Bax protein levels decreased, Bcl-2 protein levels increased significantly.

Studies have shown that bcl-2 is an anti-apoptotic protein, and it's up-regulated expression in cells can inhibit cell apoptosis, while Bax is a pro-apoptotic protein, and it's up-regulated expression in cells can promote cell apoptosis)22(. It is suggested that overexpression of MEG3 can inhibit LPS-induced macrophage apoptosis. Studies have shown that increased levels of inflammatory cytokines IL-1β, TNF-α can promote the occurrence of inflammatory reactions and promote the occurrence of a variety of inflammatory diseases)23,24(. The results of this study showed that the amount of IL-1β, TNF-α produced by macrophages significantly increased after LPS treatment, and the overexpression of MEG3 significantly reduced the levels of IL-1β, TNF-α, suggesting that overexpression of MEG3 could inhibit the release of inflammatory factors by LPS-induced macrophages. Studies have shown that urolithin attenuates IL-1β-induced inflammatory response and cartilage degradation by inhibiting the MAPK /NF-κB signaling pathway in the articular chondrocytes of rats)25(. Inhibition of NF- B signaling pathway can reduce OGD/r-induced HM cell inflammation and thus play a protective role on nerve cells)26(. Inhibiting the expression of NF-κB signaling pathway-related proteins p65 and p-p65, and inhibiting the occurrence of the inflammatory response and oxidative stress, can improve pulmonary ischemia-reperfusion injury)27(. Inhibition of the NF-κB signaling pathway can reduce the inflammatory response of pulp cells of lipopolysaccharide and du 'ao)28(. NF-κB signaling pathway plays an important regulatory role in the inflammatory response)29-32(. Vascular endothelial growth factor (VEGF) produced by several cell types including fibroblasts, neutrophils, endothelial cells, and peripheral blood mononuclear cells, particularly T lymphocytes and macrophages has been been linked with a number of vascular pathologies including cardiovascular diseases such ischemic heart disease, heart failure, stroke, and diabetes (33). The results of this study showed that the level of p-p65 protein in macrophages significantly increased after LPS treatment, while the level of p-p65 protein in macrophages significantly decreased after overexpression of MEG3, suggesting that overexpression of MEG3 may inhibit LPS-induced macrophage apoptosis and secretion of inflammatory factors by inhibiting the NF-κB signaling pathway. In this regard, it is necessary to use new technologies such as genome editing (34).

In summary, the expression of MEG3 in the serum of sepsis patients is down-regulated and may serve as a potential biological marker for the diagnosis of sepsis. In vitro cell experiments have demonstrated that overexpression of MEG3 can inhibit LPS-induced macrophage apoptosis and secretion of inflammatory factors. The mechanism is related to the inhibition of the activation of the NF-κB signaling pathway, which may provide a new direction for the treatment of sepsis.

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