

MIR-141 is Negatively Correlated With TLR4 in Neonatal Sepsis and Regulates LPS-induced Inflammatory Responses in Monocytes

Xinyu Lin

Weifang People's Hospital

Yaohui Wang (✉ sunnyma0101@163.com)

Weifang People's Hospital

Research

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Abstract

Background: Neonatal sepsis is an inflammatory system syndrome caused by bacteria or viruses, which is the main cause of neonatal morbidity and mortality. But currently there is a lack of an ideal biomarker to meet the standard. The abnormally expression of miRNAs may be a group of potential diagnostic biomarkers in neonatal sepsis.

Methods: First, qRT-PCR was used to calculate the expression of miR-141 in the serum of neonatal with sepsis and control. The diagnostic value of PCT and serum miR-141 was evaluated by receiver operating characteristic (ROC) curve. Second, the relationship between miR-141 and TLR4 was verified using luciferase report assay. An inflammation model was established using monocytes with the LPS treatment. Elisa analyzed the relationship between miR-141 and inflammatory factors.

Results: qRT-PCR found that the expression of miR-141 in neonatal sepsis was significantly lower than healthy controls. ROC curve shows that miR-141 has high diagnostic accuracy. LPS stimulation in monocytes also led to a decrease in the expression of miR-141. Luciferase report assay proves that miR-141 can target and regulate TLR4 expression. ELISA proved that overexpression of miR-141 has the effect of inhibiting the LPS-induced inflammation in monocytes.

Conclusion: In conclusion, miR-141 serves as a candidate diagnostic biomarker and is involved in the regulation of inflammatory response in the development of neonatal sepsis. Overexpression of miR-141 inhibits LPS-induced inflammation by targeting TLR4. Therefore, miR-141 is expected to be a potential diagnostic biomarker and a therapeutic target in neonatal sepsis.

Introduction

Neonatal sepsis is an inflammatory system syndrome caused by bacteria or viruses, which is the main cause of neonatal morbidity and death [1-3]. Researchers have been looking for an ideal biomarker with high sensitivity and specificity in order to diagnose and eliminate neonatal sepsis as early as possible [4]. Currently, the most commonly used biomarkers are C-reactive protein (CRP), micro-erythrocyte sedimentation rate and procalcitonin (PCT) and serum amyloid A [5-7]. However, the specificity of these indicators is poor, and their clinical application is limited. So far, no ideal biomarker can meet the standard [8, 9]. Therefore, exploring new diagnostic methods and molecular mechanisms is essential for neonatal sepsis.

MicroRNA (miRNA) is a type of highly conserved non-coding RNA, which plays an important role in gene regulation in animals and plants by pairing with the mRNA of protein-coding genes to guide its post-transcriptional suppression [10]. In sepsis, there are functional miRNAs associated with disease progression through an inflammatory response. For example, miR-150 may have diagnostic and prognostic value in neonatal sepsis [11]. miR-300 exerts a negative regulatory effect on NAMPT by activating the AMPK/mTOR signaling pathway, inhibits the inflammatory response and thus plays a role in neonatal sepsis [12]. There are literatures confirming that miR-141 is significantly down-regulated in

neonatal sepsis [13], which may be a potential clinical diagnosis and prognostic marker for neonatal sepsis. However, the mechanism of action of miR-141 in neonatal sepsis is still unclear.

This study aimed to explore the diagnostic value of miR-141 in neonatal sepsis and its regulatory role in LPS-induced inflammation. First, qRT-PCR was used to calculate the expression of miR-141 in the serum of neonates with sepsis and control neonates. The diagnostic value of PCT and serum miR-141 was evaluated by ROC curve. Second, the relationship between miR-141 and TLR4 was verified using luciferase report assay. An inflammation model was established with monocytes treated by LPS. ELISA assay was applied to evaluate the relationship between miR-141 and inflammatory factors. The results of this study may provide a novel insight into the early diagnosis and targeted treatment for neonatal sepsis.

Materials And Methods

Patients and blood sample collection

All serum samples were collected from 98 neonates with sepsis and 50 neonates diagnosed with respiratory tract infection or pneumonia but without symptoms of sepsis in Weifang people's hospital from 2013 to 2018. The neonatal sepsis patients who participated in the study were diagnosed based on clinical manifestations and blood pathogen detection according to the criteria that was established by the 2003 Kunming Neonatal Sepsis Definition Conference. The study has been approved by the Research Ethics Committee of the Weifang people's hospital, and all neonatal guardians are informed and provided paper-based informed letters.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Use Trizol reagent (Invitrogen, Carlsbad, CA, USA) to extract total RNA from the serum of neonates with sepsis and neonates with respiratory tract infection or pneumonia. PrimeScript RT reagent (TaKaRa, Shiga, Japan) uses RNA as a template for reverse transcription to obtain cDNA with the program of 42°C for 30min, 85°C for 10min. The serum levels of miR-141 and TLR4 mRNA were analyzed using SYBR-Green I Master Mix kit (Invitrogen, Carlsbad, California, USA) and 7300 Real-Time PCR system (Applied Biosystems, USA). U6 and GAPDH were respectively used as the internal control gene for miR-141 and TLR4. Three replicates were set up in this study. The final expression value was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell culture and stimulation conditions

After adding 4.5% dextran to the blood sample of sepsis newborns and separating the white blood cells, mononuclear cells were isolated by density gradient centrifugation, and the purity of the cells was

confirmed to be >95% by flow cytometry based on detection of the specific cell markers CD14 and CD45. The extracted mononuclear cells were cultured in RPMI-1640 medium containing 10% PBS at 37°C and 5% CO₂. To explore the effect of MiR-141 on LPS-induced inflammation, monocytes were stimulated with 100ng/ml LPS for 4 hours.

Cell transfection

The isolated monocytes were seeded on a 48-well plate, and the monocytes were transfected with MiR-141 mimic, MiR-141 inhibitor or negative controls (mimic NC and inhibitor NC; GenePharma, Shanghai, China) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to manufacturers' protocols.

Luciferase reporter assay

Computer-aided algorithm (miRanda) was used to predict the target sequence of miR-141 in the 3'-UTR region of TLR4. To verify the relationship between MiR-141 and TLR4's 3'-UTR, a luciferase reporter assay was performed in this study. According to the predicted binding sites, the wild type (WT) and mutant (MT) TLR4 3'-UTR were ligated into pGL3 basic vector (Promega Corp.) to obtain the wild type (WT) vector pLUC-WT-TLR4 or mutant (MT) vector pLUC-MT-TLR4. miR-141 mimics, MiR-141 inhibitors or miR-NC were co-transfected into isolated monocytes with pLUC-WT-TLR4 or pLUC-MT-TLR4 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). The luciferase activity in different groups was measured using a dual luciferase reporter assay system (Promega Corp.).

ELISA assay

The protein concentration of inflammatory cytokines IL-8 and tumor necrosis factor (TNF) - α in monocyte culture supernatant was evaluated using enzyme-linked immunosorbent adsorption co-precipitation (ELISA). This experiment was performed according to the instructions of the IL-8 ELISA kit (catalog number 550999; BD Biosciences) and the TNF- α ELISA kit (catalog number 550610; BD Biosciences). Finally, using the microplate reader (Bio-Rad Laboratories, Inc.) read the absorbance at 450nm.

Statistical analysis

All statistical analyses were performed by using SPSS 21.0 software and GraphPad Prism 5.0 software (GraphPad Software, Inc., USA). Values are expressed as the mean \pm standard deviation and compared with Student's t-test, the χ^2 test or one-way analysis of variance followed by Tukey's multiple-comparisons test. A receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of MiR-141 a regarding NS. $P < 0.05$ was considered to indicate statistical significance.

Results

Clinicopathological characteristics of the patients with neonatal sepsis and the controls

The clinicopathological characteristics were summarized in **Table 1**. There was no significant difference between sepsis neonates and controls at age, gender, body weight, white blood cell (WBC) number and CRP (all $P > 0.05$). Sepsis newborns had significantly increased levels of PCT compared with the controls ($P < 0.001$).

Table 1
Comparison of clinical characteristics between NS patients and control group

Features	Controls (n = 50)	NS (n = 98)	P value
Age (days)	11.55 ± 3.63	11.78 ± 4.21	0.741
Gender (female/male)	22/28	46/52	0.734
Body weight (g)	3468.94 ± 323.57	3449.63 ± 300.82	0.719
WBC ($\times 10^9/L$)	10.80 ± 5.29	11.73 ± 5.15	0.307
CRP (mg/L)	10.85 ± 5.37	12.44 ± 5.27	0.087
PCT (ng/mL)	1.82 ± 0.70	4.39 ± 2.79	< 0.001

NS, neonatal sepsis; WBC, white blood cells; CRP, C-reactive protein; PCT, procalcitonin.

Serum miR-141 is downregulated in sepsis neonates

In order to study the role of miR-141 in neonatal sepsis, the serum levels of miR-141 in two groups were detected and compared. The results shown in **Figure 1** revealed that the expression of miR-141 was in the patient group was significantly lower than that in the control group ($P < 0.001$).

Diagnostic value of miR-141 in sepsis neonates

PCT has been considered a biomarker for sepsis diagnosis, and this study found the significantly elevated PCT levels in sepsis neonates compared with neonates with respiratory tract infection or pneumonia. A ROC curve based on serum PCT levels was shown in **Figure 1B** with an area under the curve (AUC) of 0.760. At the cutoff value of 2.325, the diagnostic sensitivity and sensitivity using PCT were 68.4% and 78.0%, respectively. By analyzing the serum expression of miR-141, the ROC curve showed that the AUC was 0.870 and the sensitivity and specificity were 76.5% and 84.0%, respectively at a cutoff value of 0.715 (**Figure 1B**). Furthermore, a ROC curve was constructed using the combination of

PCT and miR-141, which presented a high AUC of 0.930 with the sensitivity of 85.7% and specificity of 92.0%.

Expression of MiR-141 in LPS-treated monocytes

To investigate the functional role of miR-141 in LPS-induced inflammation. In this study, mononuclear cells were isolated from patients' blood samples, and LPS treatment was used to induce an inflammation model. The results were shown in **Figure 2**, the expression of miR-141 in LPS-treated cells was significantly inhibited compared with untreated cells ($P < 0.001$).

MiR-141 directly regulates the expression of TLR4

TLR4 was predicted to possess the binding site of miR-141 at its 3'-UTR (**Figure 3A**), a subsequent luciferase reporter assay was performed to confirm the relationship between miR-141 and TLR4. As shown in **Figure 3B**, the relative luciferase activity was inhibited by the overexpression of miR-141, but was enhanced by the knockdown of miR-141 in WT group (both $P < 0.05$, **Figure 3B**). However, no significant luciferase activity changes were observed in MUT group (all $P > 0.05$). These findings demonstrated the interaction between miR-141 and TLR4. Moreover, the regulatory effect of miR-141 on TLR4 in LPS-induced inflammatory cell model was analyzed. The expression of miR-141 was overexpressed in monocytes transfected with miR-141 mimic ($P < 0.001$), while was downregulated in cells with miR-141 inhibitor ($P < 0.001$, **Figure 3C**). The elevated TLR4 in monocytes induced by LPS treatment was significantly inhibited by the overexpression of miR-141, but was promoted by the knockdown of miR-141 (all $P < 0.01$, **Figure 3D**). In addition, the serum TLR4 mRNA expression levels were as expected to be upregulated in sepsis neonates compared with the controls ($P < 0.001$, **Figure 3E**), and its expression was negatively correlated with serum levels of miR-141 ($r = -0.845$, $P < 0.001$, **Figure 3F**). These findings above demonstrated that miR-141 could directly inhibited TLR4 in LPS-induced monocytes.

Effects of miR-141 on the levels of pro-inflammatory cytokines in monocytes

In order to study the relationship between miR-141 and LPS-induced inflammatory response. This study used ELISA to detect the expression of inflammatory factors TNF- α and IL-8 in the cell supernatant. The results are shown in **Figure 4**, which presented that the concentrations of inflammatory cytokines TNF- α and IL-8 increased after LPS stimulates the monocytes (all $P < 0.001$). Then, the results showed that the overexpression of miR-141 can inhibit the level of inflammatory factors, while silencing miR-141 had the opposite effect (all $P < 0.001$). This shows that miR-141 is involved in the regulation of inflammatory response in the development of neonatal sepsis. Increasing the level of miR-141 may have the effect of alleviating neonatal sepsis.

Discussion

In the past few decades, miRNA has become an essential post-transcriptional regulator in the regulation of gene expression [14-16]. Studies have shown that the abnormal expression of miRNA may be related to the inflammatory response in patients with sepsis. For example, miR-26a is down-regulated in blood monocytes and serum in neonatal sepsis [17]. As a specific biomarker, miR-1290 provides a basis for pediatricians to diagnose NEC cases and neonatal sepsis [18]. SNHG16 is able to reverse the effect of miR-15a/16 on the LPS-induced inflammation pathway [19]. But there is still a lack of an ideal biomarker for neonatal sepsis early diagnosis. Therefore, exploring new functional miRNAs is of great value for the treatment of neonatal sepsis.

This study found that there is abnormal expression of miR-141 in neonatal sepsis. Studies have shown that miR-141 has abnormal expression in various diseases, such as non-small cell lung cancer [20], bladder cancer [21], hallmark of nonalcoholic steatohepatitis [22], infantile pneumonia [23]. Therefore, this study detected and compared the expression levels of miR-141 in the serum of neonates with sepsis and controls. The results showed that the miR-141 in the patient group was significantly lower than the controls. In the inflammation model induced by LPS stimulation, the results showed that the expression of miR-141 in LPS-treated cells was significantly suppressed compared to untreated cells. These results indicate that miR-141 may be involved in the inflammation of neonatal sepsis.

Increasingly microRNAs (miRNAs) are recognized as key players in the diseases system, which regulate development, differentiation and other processes in various diseases [24-26]. For example, Wang found that miRNA-221-3p and miRNA-382-5p might be used as potential noninvasive biomarkers for the diagnosis of ischemic stroke [27]. Cui found that miRNA-23-a and miRNA-451 were potential biomarkers for early diagnosis of non-small cell lung cancer. In sepsis, otherwise there were multiple abnormally expressed miRNAs, such as miR-29a, miR-96, miR-101 and so on [13]. Therefore, given the miR-141 is abnormally expressed in the serum of neonatal sepsis. This experiment further evaluated the diagnostic value of an established diagnostic marker procalcitonin (PCT) and the serum levels of miR-141 in neonatal sepsis. The results prove that miR-141 has relatively high diagnostic accuracy and may have potency to improve the diagnostic performance of PCT in neonatal sepsis. This shows that miR-141 may be a potential biomarker for neonatal sepsis early diagnosis.

In order to further determine the biological function of miR-141 in the pathogenesis of neonatal sepsis. An inflammation model was constructed by LPS stimulation in monocytes, and the expression of miR-141 was regulated by in vitro manipulation. As a result, it was found that the overexpression of miR-141 can reverse the increase in inflammatory factor levels induced by LPS in monocytes. This shows that miR-141 may be involved in the regulation of inflammatory response in the development of neonatal sepsis, and increasing the level of miR-141 may have a role in relieving neonatal sepsis. Based on the role of miR-141 in the inflammatory response, we used bioinformatics analysis to predict the target sequence of miR-141 in the 3'-UTR region of TLR4. It is understood that TLR4 is one of the important molecules in the body's immune function and inflammatory response. TLR4 can be used as a target gene in sepsis. For example,

Zhang et al. demonstrate that lncRNA NEAT1 interacts with Let-7a, targeting TLR4 to contribute to the LPS-induced inflammatory response[28]. Ji et al. found that Sch B could increase miR-17-5p expression, promote inflammation, and decrease TLR4 expression in sepsis mice and LPS-induced macrophages [29]. The interaction between miR-141 and TLR4 3'-UTR was analyzed by luciferase reporter assay. The results show that TLR4 is the target gene of miR-141. miR-141 plays a role in neonatal sepsis by binding to the 3'-UTR region of TLR4. The mechanism of action of miR-181a in sepsis also confirms this view [30]. The results of this study uncover the potential role of miR-141 as a useful potential therapeutic agent in the treatment of sepsis and provide a basis for the application of miR-141 in neonatal sepsis. However, the specific mechanism of action of miR-141 in neonatal sepsis needs further investigation

In short, the abnormal expression of miR-141 is related to the inflammatory response of neonatal sepsis, and miR-141 has a higher diagnostic accuracy in neonatal sepsis. The expression level of miR-141 in LPS-treated cells was significantly inhibited. It was found that overexpression of miR-141 inhibited LPS-induced inflammation by targeting TLR4 in monocytes. Therefore, increasing the level of miR-141 may be alleviate neonatal sepsis. miR-141 is expected to become a potential diagnostic biomarker and therapeutic target in neonatal sepsis.

Declarations

Ethics approval and consent to participate

A signed written informed consent was obtained from each patient and the experimental procedures were all in accordance with the guideline of the Ethics Committee of Weifang people's hospital.

Consent for publication

Written informed consent for publication was obtained from each participant.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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None.

Authors' contributions

XL and YW analyzed and interpreted the data regarding , performed the examination of cell, and wrote and revised the manuscript. Both authors read and approved the final manuscript.

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Figures

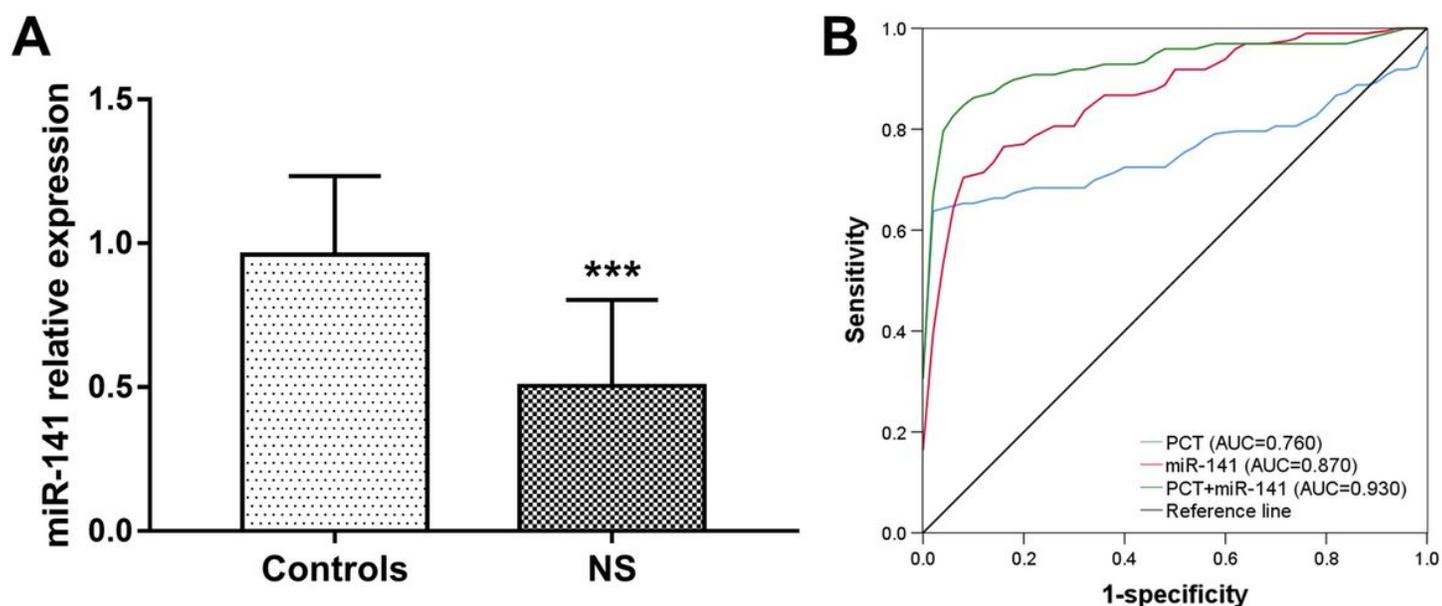


Figure 1

Serum expression of miR-141 in neonatal sepsis newborns and its diagnostic performance results. A. Serum miR-141 expression was decreased in sepsis neonates compared with controls (NS: neonatal sepsis; *** $P < 0.001$). B. ROC curves based on serum PCT and miR-141 for sepsis newborns (blue line for PCT; red line for miR-141; green line for the combination of PCT and miR-141; AUC: area under the curve).

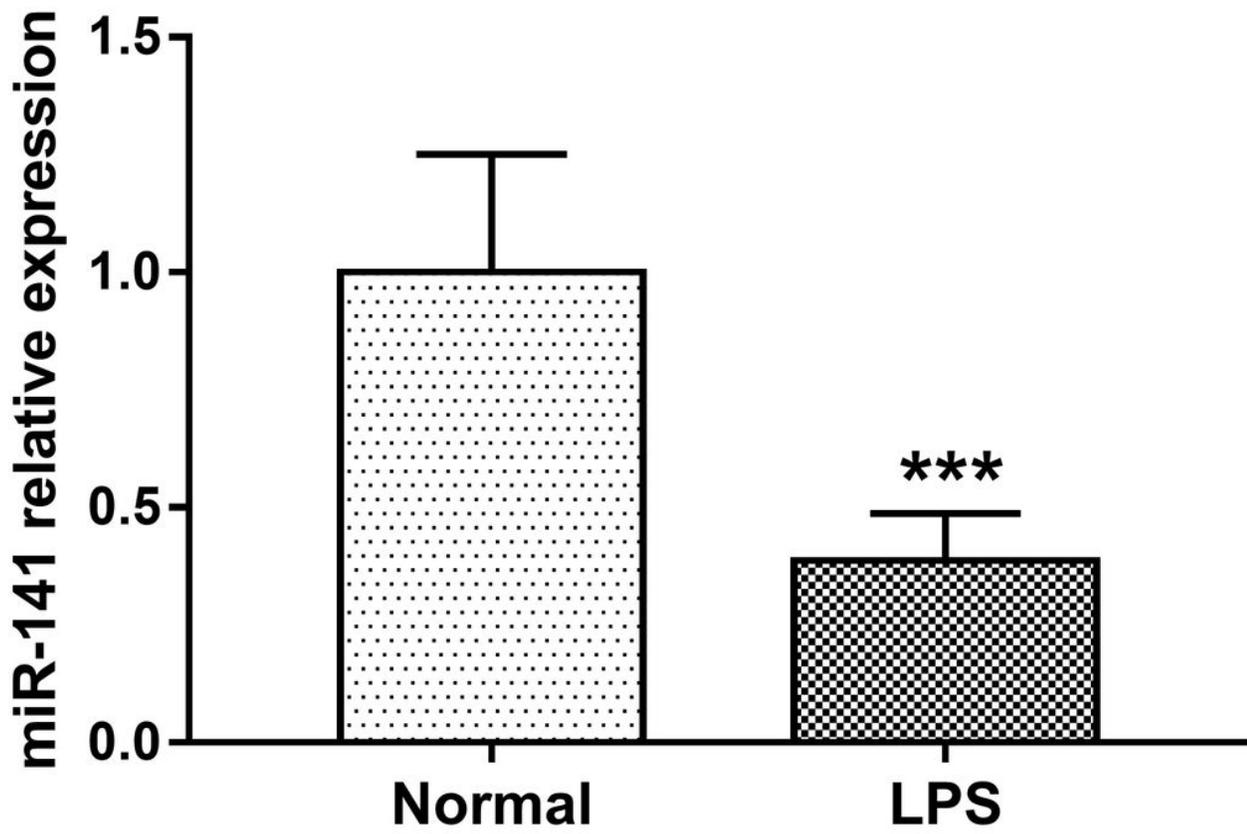


Figure 2

Inhibited expression of miR-141 in monocytes treated with LPS (***) $P < 0.001$).

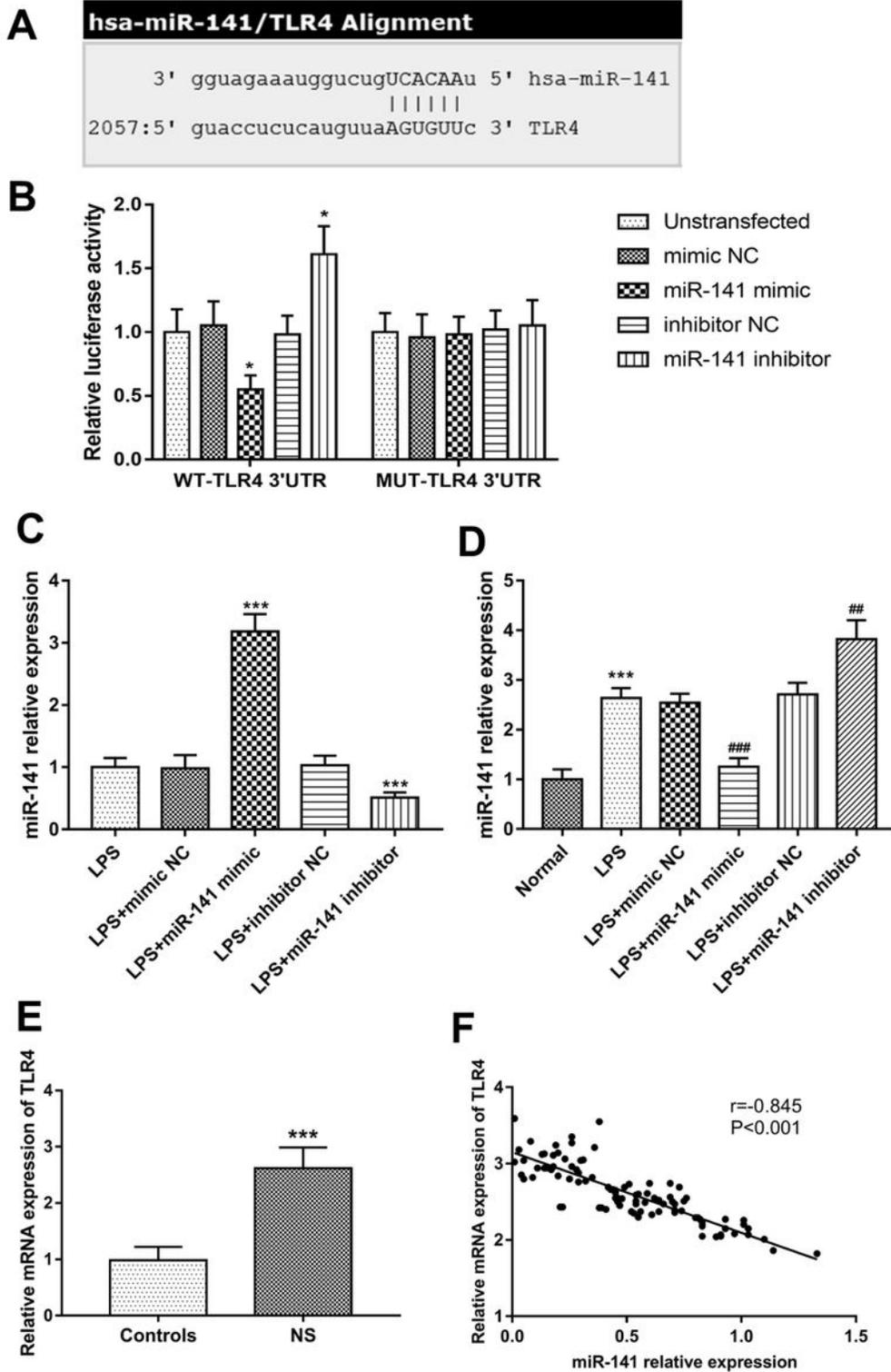


Figure 3

miR-141 directly regulated TLR4 in LPS-treated monocytes. A. The putative binding site of miR-141 at the 3'-UTR of TLR4. B. The relative luciferase activity in WT group was inhibited by the overexpression of miR-141, but was enhanced by the inhibition of miR-141 (WT: wild type; MUT: mutant type; * $P < 0.05$). C. The expression of miR-141 was successfully upregulated by miR-141 mimic, while was downregulated by miR-141 inhibitor (** $P < 0.001$). D. The promoted expression of TLR4 induced by LPS was inhibited by

the overexpression of miR-141, but was further enhanced by the knockdown of miR-141 (compared with normal group, *** $P < 0.001$; compared with LPS group, ### $P < 0.01$, ### $P < 0.001$). E. Serum relative mRNA expression of TLR4 was upregulated in sepsis neonates than that in control newborns (*** $P < 0.001$). F. Serum levels of miR-141 were negatively correlated with levels of TLR4 ($r = -0.845$, $P < 0.001$).

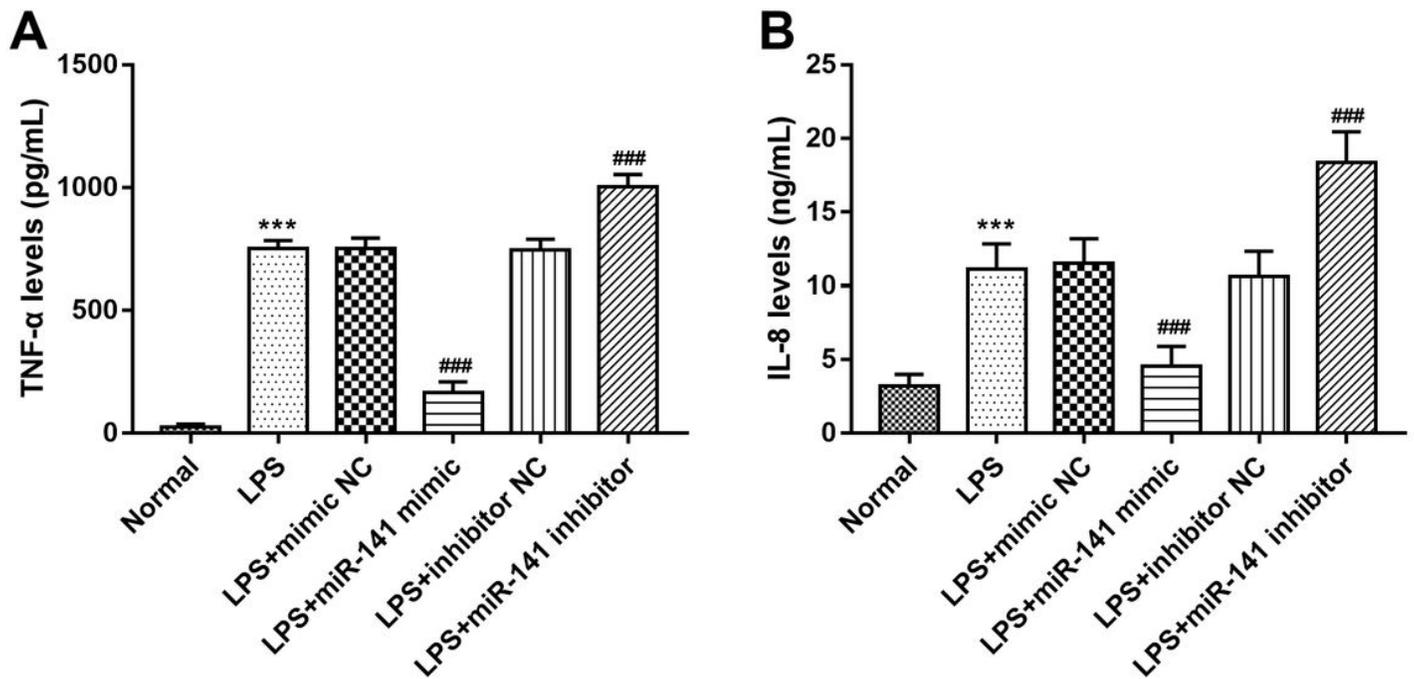


Figure 4

Effects of miR-141 on LPS-induced inflammatory response in monocytes. A and B. LPS treatment increased TNF- α (A) and IL-8 (B) levels, and this effect was weakened by the overexpression of miR-141 and enhanced by the reduction of miR-141 in monocytes (compared with normal group, *** $P < 0.001$; compared with LPS group, ### $P < 0.001$).