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LncRNA NEAT1 promotes the progression of sepsis-induced myocardial cell injury by sponging miR-144-3p

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Abstract. – OBJECTIVE: Sepsis is a systemic inflammatory response that can lead to the dysfunction of many organs, including the cardiac one. Long noncoding RNAs (lncRNAs) have been shown to be involved in multiple organ injuries induced by sepsis. However, the regulatory effect of nuclear enriched abundant transcript 1 (NEAT1) on sepsis-induced myocardial injury remains to be explored.

MATERIALS AND METHODS: The sepsis models of myocardial cell injury were constructed using lipopolysaccharide (LPS). Cell counting kit-8 (CCK-8) assay was used to detect cell viability. Flow cytometry was performed to assess cell apoptosis. Moreover, the levels of apoptosis-related and nuclear factor-kappa B (NF-κB) signaling pathway-related proteins were evaluated by Western blot (WB) analysis. Besides, the contents of inflammatory cytokines were tested by enzyme-linked immunosorbent assay (ELISA). The expression levels of NEAT1 and microRNA-144-3p (miR-144-3p) were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). In addition, Dual-Luciferase reporter and RNA immunoprecipitation (RIP) assays were used to verify the interaction between NEAT1 and miR-144-3p.

RESULTS: LPS could induce myocardial cell injury to construct sepsis models. NEAT1 was upregulated in LPS-treated myocardial cells, and its knockdown promoted viability, suppressed apoptosis, and relieved inflammatory response in LPS-induced myocardial cell injury. MiR-144-3p was downregulated in LPS-treated myocardial cells, and the effect of its overexpression on LPS-induced myocardial cell injury was similar to the effect of NEAT1 knockdown. Besides, miR-144-3p could be sponged by NEAT1, and its inhibitor could reverse the effect of NEAT1 knockdown on LPS-induced myocardial cell injury. Moreover, NEAT1 and miR-144-3p could regulate the activity of NF-κB signaling pathway.

CONCLUSIONS: LncRNA NEAT1 could interact with miR-144-3p to regulate sepsis-induced myocardial cell injury through the NF-κB signaling pathway, which might provide a new theoretical basis for the study on the effect of sepsis treatment.

Key Words:
Sepsis, LPS, Myocardial cell injury, NEAT1, MiR-144-3p.

Introduction

Sepsis is a severe inflammatory disease caused by infection and characterized by systemic inflammatory response syndrome[1,2]. Due to its high morbidity and mortality, sepsis has become a huge obstacle in clinical treatment[3,4]. Severe sepsis can lead to multiple organ dysfunction, among which cardiac dysfunction is a common complication[5,6]. Lipopolysaccharide (LPS) is often used to induce cellular sepsis models *in vitro* due to its ability to bind to toll-like receptor 4 (TLR4) and activate inflammatory response[7]. Therefore, LPS-induced

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myocardial cell injury provides a reliable model for the study of cardiac sepsis *in vitro*.

Long noncoding RNAs (lncRNAs) are long RNA molecules that are transcribed over 200 nucleotides (nts) in length[8]. Scholars[9,10] have confirmed that many lncRNAs are involved in the regulation of sepsis; for example, lncRNA THRIL correlates with acute respiratory distress syndrome caused by sepsis, and lncRNA ITSN1-2 is associated with disease severity and inflammation in sepsis patients. Nuclear enriched abundant transcript 1 (NEAT1) is a lncRNA with high expression in many diseases, including hepatocellular carcinoma[11], breast cancer[12], and nonalcoholic fatty liver disease[13]. Besides, NEAT1 has been reported to be involved in the regulation of sepsis-induced liver, kidney, and brain injury[14-16]. However, there are few studies on the role of NEAT1 in sepsis-induced myocardial injury.

There are many molecular functions of lncRNA[17]. The most studied one is that lncRNAs act as ceRNAs to adsorb microRNAs (miRNAs) to play the regulatory role on downstream target genes[18]. MiRNAs are a small noncoding RNA with a length of about 22 nts, which has been proved to be involved in the regulation of many diseases[19,20]. MiR-149, miR-346, and miR-106a have been shown to be associated with the inflammatory responses induced by sepsis[21-23]. MiR-144-3p was lowly expressed in many diseases[24,25]. High-throughput sequencing and NanoString technology revealed that miR-144-3p has differentially expressed in aortas of sepsis mice and the control group[26]. Nevertheless, its role in sepsis and whether it is involved in the regulation of sepsis, is still unclear.

This study aimed to explore the function of NEAT1 in the sepsis model of LPS-induced myocardial injury and to determine its molecular mechanism through further bioinformatics prediction and experimental verification. Our findings provided a potential target for the treatment of sepsis-induced myocardial injury.

Materials and Methods

Cell Culture

Mice myocardial cells (HL-1) were purchased from BeNa Culture Collection (BNCC, Beijing, China) and cultured in Claycomb medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; HyClone, South-Logan, UT, USA),100 U/mL penicillin, 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), 0.1 mM norepinephrine and 2 mM L-glutamine (Yuanye, Shanghai, China) at 37°C with 5% CO_2. HEK293 cells were bought from Procell (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone) containing 10% FBS (HyClone),100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2.

Cell Treatment and Transfection

HL-1 cells were treated with LPS at different concentrations (0, 1, 5, and 10 μg/mL) for 12 h to induce sepsis models. NEAT1 small interfering RNA (si-NEAT1) and its negative control (si-NC), miR-144-3p mimic and inhibitor (miR-144-3p and anti-miR-144-3p) or their negative controls (miR-NC and anti-NC) were synthesized by GeneChem (Shanghai, China). HL-1 cells were treated with 10 μg/mL LPS for 12 h, followed by transfection with above plasmid vectors for 24 h using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

After treatment with LPS or transfection, HL-1 cells were incubated with CCK-8 solution (Amyjet Scientific Inc., Wuhan, China) for 4 h. The absorbance was determined using a microplate reader (Molecular Devices, Shanghai, China) at 450 nm.

Flow Cytometry

After treatment with LPS or transfection, HL-1 cells were stained using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Vazyme, Nanjing, China). Fluorescence signals were collected using flow cytometer (Merck KGaA, Darmstadt, Germany), and the apoptosis rate of HL-1 cells was determined.

Western Blot (WB) Analysis

HL-1 cells were treated with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) to extract total proteins. Then, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After the membranes were closed with non-fat milk, they were incubated with the primary antibodies against B-cell lymphoma2 (Bcl2, 1:500, Boster, Wuhan, China), Bcl2-associated X (Bax, 1:1,000, Boster), Cleaved-caspase 3 (Cleaved-casp 3, 1:1,000, Boster), IκBα (1:1,000, Bioss, Beijing, China), phosphorylated-IκBα (p-IκBα, 1:1,000, Bioss), p65 (1:1,000, Boster),

phosphorylated-p65 (p-p65, 1:1000, Boster), GAPDH (1:2,000, Boster) at 4°C overnight. Then, the membranes were incubated with the secondary antibody (1:2,000, Boster) for 1 h. The protein signals were displayed by chemiluminescence reagents (Beyotime, Shanghai, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

After treatment with LPS or transfection, the medium of HL-1 cells was collected. After centrifuged at 1500 rpm for 15 min, the supernatant was collected. Then, the contents of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) were detected by the corresponding ELISA Kit (Renjie Bio, Shanghai, China).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

HL-1 cells were added TRIzol reagent (TaKaRa, Dalian, China) to extract total RNAs. Then, total RNAs were reverse-transcribed complementary DNA (cDNA) using the PrimeScript™ RT Reagent Kit (TaKaRa). QRT-PCR was performed using SYBRGreen (Vazyme, Nanjing, China). Relative quantification ($2^{-\Delta\Delta Ct}$ methods) was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6. The primer sequences were as follows: NEAT1: F 5'-GTAATTTTCGCTCGGCCTGG-3', R 5'-TACCCGAGACTACTTCCCCA-3'; GAPDH: F 5'-GAGCTCAGCTCGCCTGGAGAAAC-3', R 5'-TGCTGATCGTAGCCCTTTAGT-3'; miR-144-3p: F 5'-CCTCGCACCTGGAGGCTGGCTG-3', R5'-TTATCAGTTGGGAAAATAGTTA-3'; U6: F 5'-GCAGGAGGTCTTCACAGAGT-3', R 5'-TCTAGAGGAGAAGCTGGGGT-3'.

Dual-Luciferase Reporter Assay

The sequences of NEAT1 containing predicted miR-144-3p binding sites and mutant binding sites were cloned into pGL3 promoter vector (Promega, Madison, WI, USA) to build NEAT1-WT and NEAT1-MUT reporter vectors. HEK293 cells were co-transfected with miR-144-3p mimic or miR-NC and the above reporter vectors using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the Luciferase activities were detected using Dual-Luciferase Reporter Assay Kit (Beyotim, Beijing, China).

RNA Immunoprecipitation (RIP) Assay

HL-1 cells were lysed with RIP lysis buffer (Millipore, Billerica, MA, USA). Argonaute2 antibody (Anti-Ago2) and normal immunoglobulin G (IgG) antibody (Anti-IgG) were incubated with magnetic beads (Millipore, Billerica, MA, USA) for 1 h at 4°C. Next, a part of the cell lysate was used as a blank negative control (Input), and the other part of the cell lysate was incubated with magnetic beads at 4°C overnight. After purified, the enrichment of NEAT1 and miR-144-3p was detected by qRT-PCR.

Statistical Analysis

Data were represented as mean ±standard deviation (SD). GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analysis with Student's *t*-test or one-way analysis of variance (ANOVA). $p< 0.05$ was defined as statistically significant.

Results

LPS Could Induce Myocardial Cell Injury

To determine the effect of LPS on myocardial cells, we treated HL-1 cells with different concentrations (0, 1, 5, and 10 μg/mL) of LPS. By detecting cell viability, we found that the viability of HL-1 cells was decreased in LPS concentration-dependent manner (Figure 1A). Besides, flow cytometry results showed that the apoptosis ability of HL-1 cells was improved with the increase of LPS concentration (Figure 1B). At the same time, WB analysis results confirmed that Bax and Cleaved-casp3 protein levels were increased in LPS concentration-dependent manner, while the expression trend of Bcl2 was opposite (Figure 1C-F). To determine whether myocardial cells had an inflammatory response, we measured the contents of inflammatory cytokines. The results proved that the contents of TNF-α, IL-1β, and IL-6 were accelerated with the increase of LPS concentration, indicating the increased inflammatory response of HL-1 cells (Figure 1G-I). Therefore, all data confirmed that LPS could induce myocardial cell injury to construct the sepsis models.

NEAT1 Silencing Could Ameliorate LPS-Induced Myocardial Cell Injury

We detected NEAT1 expression in HL-1 cells treated with LPS. As shown in Figure 2A, the expression of NEAT1 was remarkably enhanced in LPS concentration-dependent manner. To verify the function of NEAT1 on LPS-induced myocardial cell injury, we transfected si-NEAT1 or si-NC into HL-1 cells treated with 10 μg/mL LPS. The results of NEAT1 expression detection showed

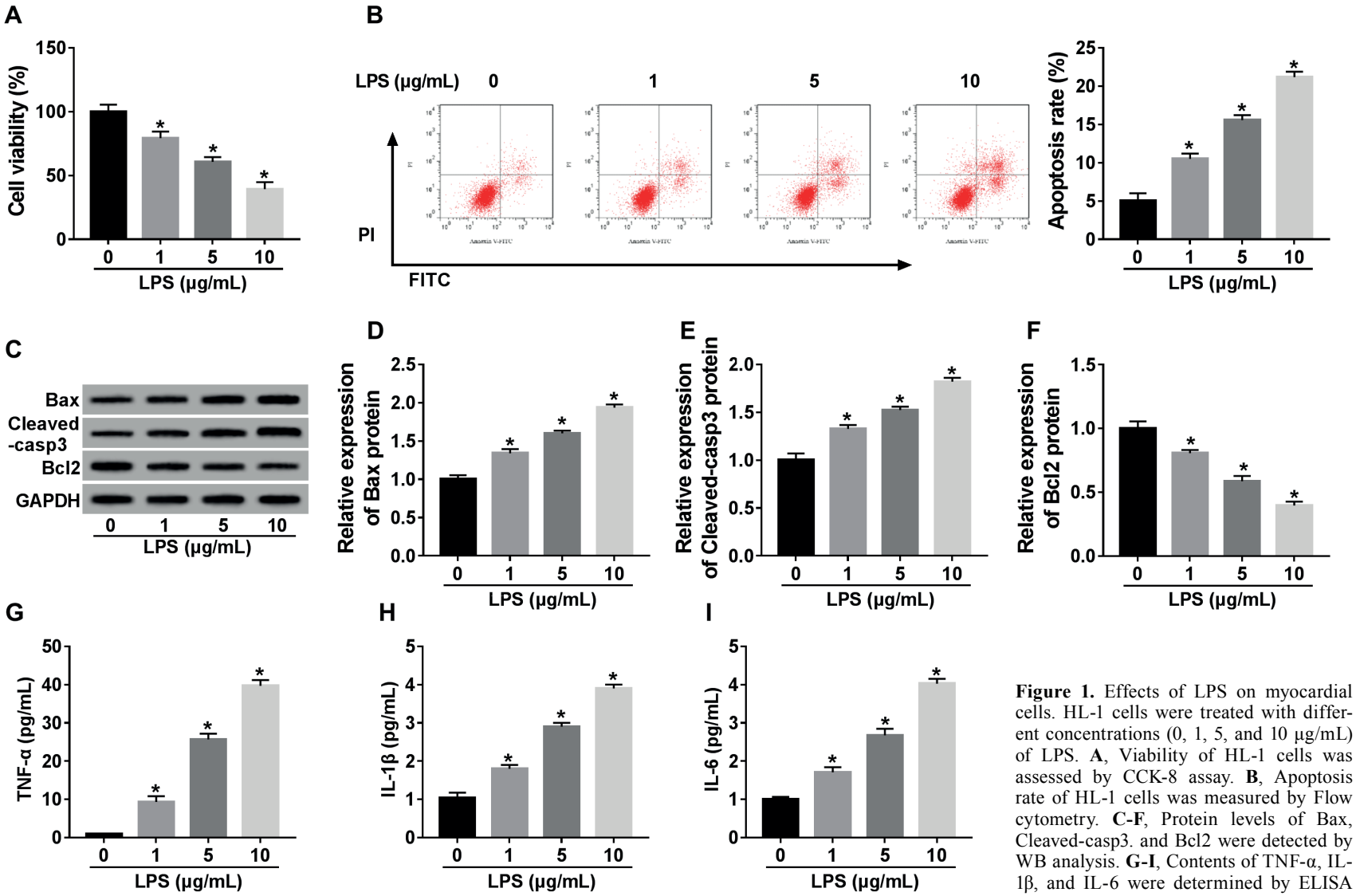

Figure 1. Effects of LPS on myocardial cells. HL-1 cells were treated with different concentrations (0, 1, 5, and 10 μg/mL) of LPS. **A**, Viability of HL-1 cells was assessed by CCK-8 assay. **B**, Apoptosis rate of HL-1 cells was measured by Flow cytometry. **C-F**, Protein levels of Bax, Cleaved-casp3. and Bcl2 were detected by WB analysis. **G-I**, Contents of TNF-α, IL-1β, and IL-6 were determined by ELISA assay. *$p < 0.05$.

that si-NEAT1 transfection was effective (Figure 2B). Besides, CCK-8 assay uncovered that the silencing of NEAT1 could improve the viability of HL-1 cells treated with LPS (Figure 2C). Furthermore, flow cytometry revealed that knockdown of NEAT1 significantly suppressed LPS-induced apoptosis in HL-1 cells (Figure 2D), which was also confirmed by enhanced protein levels of Bax and Cleaved-casp3 and decreased Bcl2 protein level (Figure 2E-H). In addition, we measured the contents of TNF-α, IL-1β, and IL-6 and found that LPS-induced inflammatory response of HL-1 cells was inhibited after silenced-NEAT1 (Figure 2I-K). These results revealed that NEAT1 played a vital role in LPS-induced myocardial cell injury.

MiR-144-3p Overexpression Could Alleviate LPS-Induced Myocardial Cell Injury

We also explored the function of miR-144-3p in LPS-induced myocardial cell injury. Through qRT-PCR, we found that miR-144-3p expression was markedly inhibited with the increase of LPS concentration (Figure 3A). Hence, we transfected miR-144-3p mimic or miR-NC into HL-1 cells treated with 10 μg/mL LPS. The detection of miR-144-3p expression showed that the transfection of miR-144-3p mimic was successful (Figure 3B). CCK-8 results revealed that overexpressed-miR-144-3p promoted the viability of HL-1 cells treated with LPS (Figure 3C). Meanwhile, through the detection of apoptosis rate and apoptosis-related protein levels, we found that miR-144-3p overexpression significantly suppressed the apoptosis of LPS-induced HL-1 cells (Figure 3D-H). In addition, we discovered that overexpressed-miR-144-3p markedly restrained the contents of TNF-α, IL-1β, and IL-6 in HL-2 cells treated with LPS (Figure 3I-K). Thus, these data indicated that miR-144-3p hindered LPS-induced myocardial cell injury.

NEAT1 Could Sponge MiR-144-3p

Through the above experiments, we found that NEAT1 knockdown and miR-144-3p overexpression had similar effects on LPS-induced sepsis model, so we wanted to know whether there was a target relationship between them. To confirm this hypothesis, we performed a bioinformatics analysis using the starBase v3.0 tool. As shown in Figure 4A, NEAT1 and miR-144-3p have complementary binding sites. Dual-Luciferase reporter assay results revealed that miR-144-3p overexpression remarkably inhibited the Luciferase activity of NEAT1-WT, while it did not affect NEAT1-MUT (Figure 4B). To further confirm the interaction between them, we performed a RIP assay. The results showed that NEAT1 and miR-144-3p were markedly enriched in Anti-Ago2, which once again proved that NEAT1 could be related to miR-144-3p (Figure 4C). Besides, we found that knockdown of NEAT1 remarkably upregulated the expression of miR-144-3p in HL-2 cells treated with LPS (Figure 4D). Hence, these results proved that miR-144-3p could be sponged by NEAT1.

MiR-144-3p Inhibitor Could Reverse the Inhibition Effect of Silenced-NEAT1 on LPS-Induced Myocardial Cell Injury

To further explore whether NEAT1 regulated LPS-induced myocardial cell injury through miR-144-3p, we co-transfected si-NEAT1 and anti-miR-144-3p into LPS-treated HL-1 cells. The decreased expression of miR-144-3p showed that the transfection efficiency of anti-miR-144-3p was good (Figure 5A). Besides, CCK-8 assay revealed that miR-144-3p inhibitor could invert the promotion effect of silenced-NEAT1 on the viability of LPS-treated HL-1 cells (Figure 5B). Moreover, flow cytometry and WB analysis results suggested that the inhibitory effect of NEAT1 knockdown on LPL-induced HL-1 cells apoptosis could also be reversed by miR-144-3p inhibitor (Figure 5C-G). Furthermore, miR-144-3p inhibitor restored the contents of TNF-α, IL-1β, and IL-6 suppressed by NEAT1 silencing on LPL-treated HL-1 cells (Figure 5H-J). All data revealed that NEAT1 regulated LPS-induced myocardial cell injury by inhibiting miR-144-3p.

NEAT1 Sponged MiR-144-3p to Regulate LPS-Induced Myocardial Cell Injury Through the NF-κB Signaling Pathway

NF-κB signaling pathway is a classical signaling pathway associated with inflammatory response[27]. To confirm whether LPL-induced cellular inflammation response is achieved by regulating the NF-κB signaling pathway, we investigated the effects of NEAT1 and miR-144-3p on the NF-κB signaling pathway in LPS-treated HL-1 cells. Through the detection of p-IκBα and p-p65 protein levels, we discovered that LPS could induce the protein levels of p-IκBα and p-p65, while knockdown of NEAT1 could inhibit the protein levels of p-IκBα and p-p65. When treated with the miR-144-3p inhibitor, the sup-

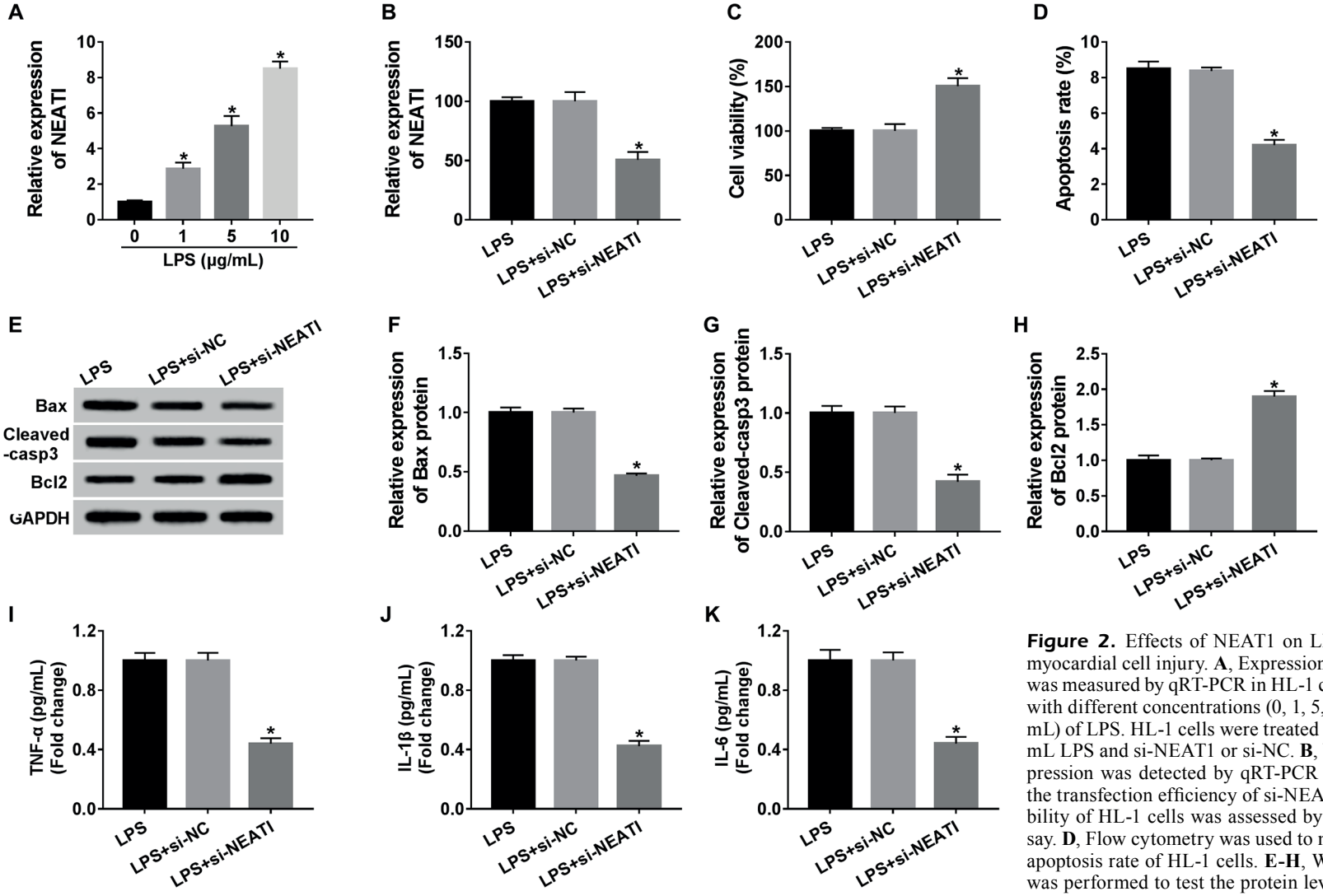

Figure 2. Effects of NEAT1 on LPS-induced myocardial cell injury. **A**, Expression of NEAT1 was measured by qRT-PCR in HL-1 cells treated with different concentrations (0, 1, 5, and 10 μg/mL) of LPS. HL-1 cells were treated with 10 μg/mL LPS and si-NEAT1 or si-NC. **B**, NEAT1 expression was detected by qRT-PCR to evaluate the transfection efficiency of si-NEAT1. **C**, Viability of HL-1 cells was assessed by CCK-8 assay. **D**, Flow cytometry was used to measure the apoptosis rate of HL-1 cells. **E-H**, WB analysis was performed to test the protein levels of Bax, Cleaved-casp3, and Bcl2. **I-K**, ELISA assay was used to determine the contents of TNF-α, IL-1β, and IL-6. *$p < 0.05$.

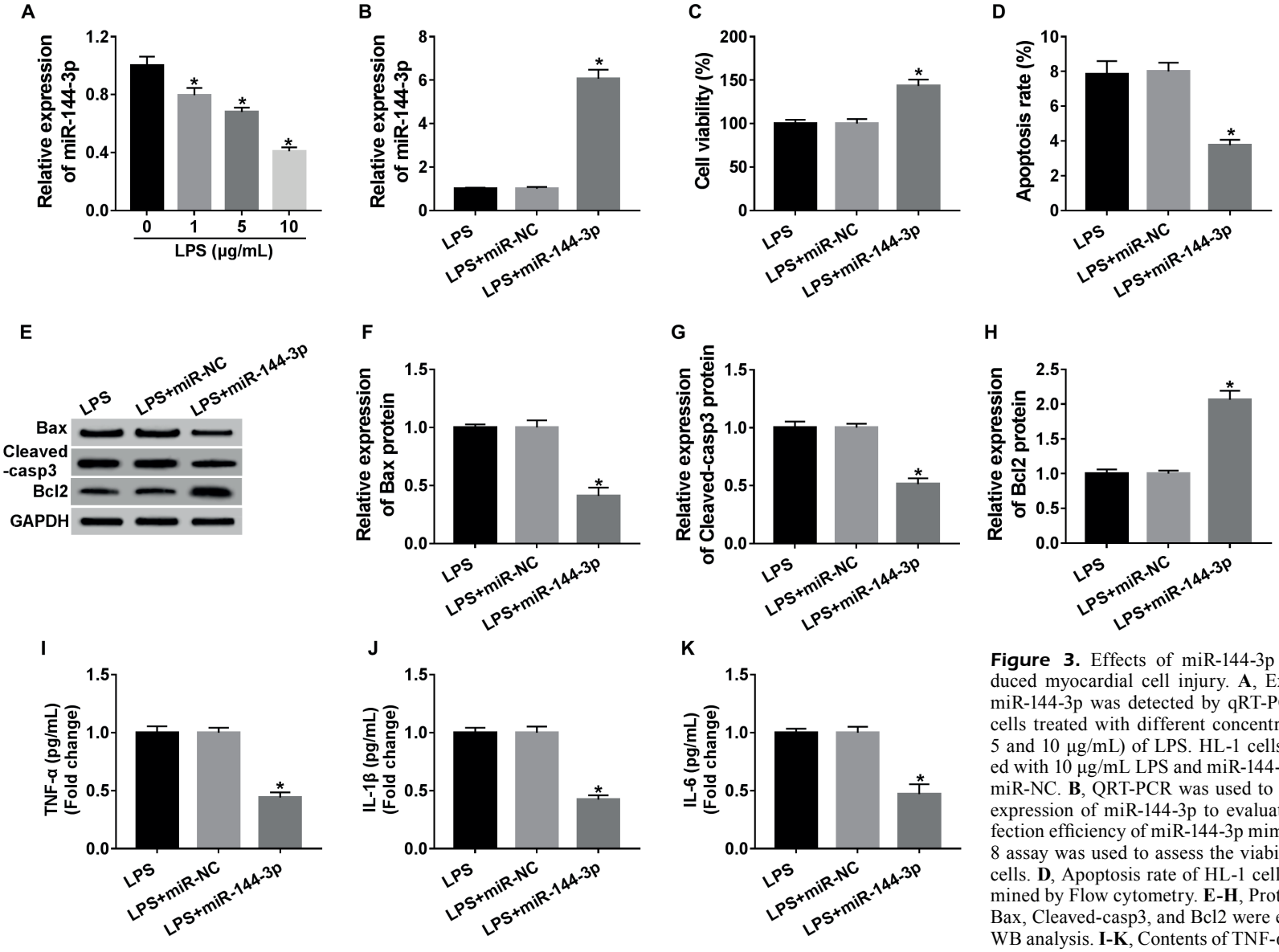

Figure 3. Effects of miR-144-3p on LPS-induced myocardial cell injury. **A**, Expression of miR-144-3p was detected by qRT-PCR in HL-1 cells treated with different concentrations (0, 1, 5 and 10 μg/mL) of LPS. HL-1 cells were treated with 10 μg/mL LPS and miR-144-3p mimic or miR-NC. **B**, QRT-PCR was used to measure the expression of miR-144-3p to evaluate the transfection efficiency of miR-144-3p mimic. **C**, CCK-8 assay was used to assess the viability of HL-1 cells. **D**, Apoptosis rate of HL-1 cells was determined by Flow cytometry. **E-H**, Protein levels of Bax, Cleaved-casp3, and Bcl2 were employed by WB analysis. **I-K**, Contents of TNF-α, IL-1β, and IL-6 were tested by ELISA assay. *$p < 0.05$.

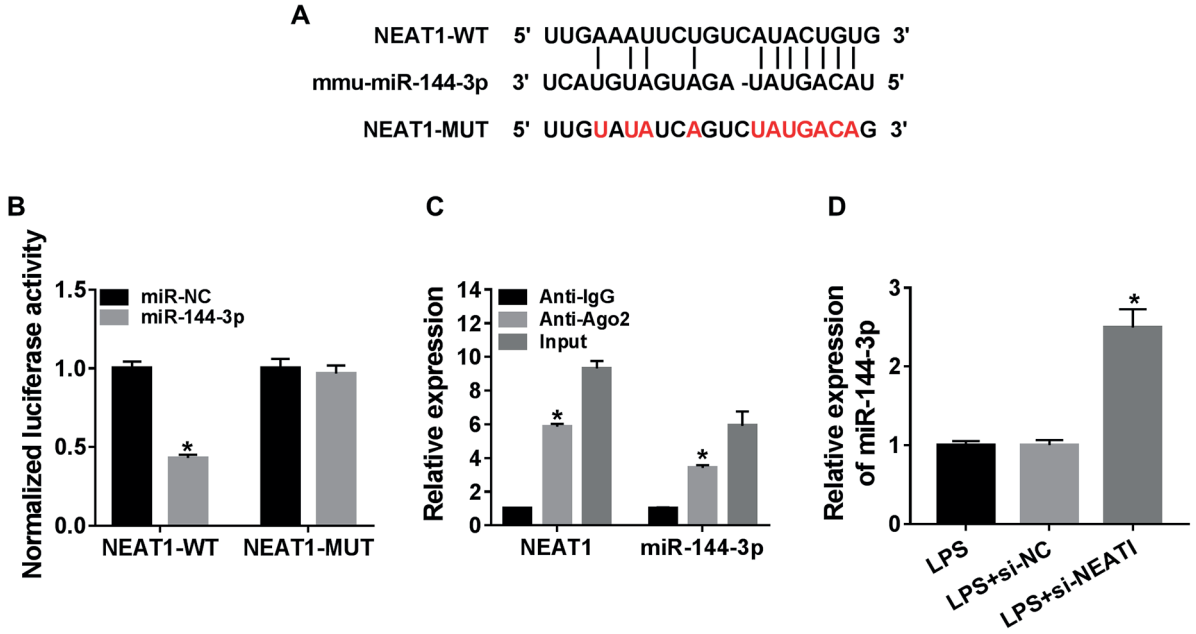

Figure 4. NEAT1 directly sponged miR-144-3p. **A**, Binding sites and mutant binding sites between NEAT1 and miR-144-3p were shown. **B**, Dual-Luciferase reporter assay was used to detect the luciferase activity of NEAT1-WT/MUT. **C**, RIP assay was performed to assess the enrichment of NEAT1 and miR-144-3p in Anti-Ago2 or Anti-IgG. **D**, Expression of miR-144-3p was measured by qRT-PCR in HL-1 cells treated with LPS and si-NEAT1 or si-NC. *$p< 0.05$.

pression effects of NEAT1 on p-IκBα and p-p65 protein levels could be reversed (Figure 6). Hence, these results suggested that the roles of NEAT1 and miR-144-3p in LPS-induced inflammation response were achieved by activating the NF-κB signaling pathway.

Discussion

LPS is an endotoxin that exhibits a variety of biological activities when it acts on other biological cells, including mediating the development of myocardial injury caused by sepsis[28]. Previous studies[29,30] have shown that LPS can induce the activity of the NF-κB signaling pathway to promote the contents of inflammatory cytokines. Here, we probed the effect of LPS on myocardial cells and found that LPS could inhibit viability and promote apoptosis in myocardial cells. Besides, the contents of pro-inflammatory cytokines and the protein levels of p-IκBα and p-p65 were improved in LPS-treated myocardial cells, which was consistent with previous studies[16,30].

Many researches[14-16] have confirmed that NEAT1 is upregulated in sepsis patients. Similar to previous studies, we uncovered that NEAT1 expression was highly expressed in LPS-treated myocardial cells. Wang et al[31] revealed that knockdown of NEAT1 inhibited apoptosis, reduced oxidative stress, and alleviated inflammation in LPS-induced myocardial tissues. In our study, we also discovered that NEAT1 silencing promoted viability, suppressed apoptosis, and relieved inflammation in LPS-induced myocardial cells, which was in agreement with the results of Wang et al[31] and Zhang et al[16]. Besides, the decreased expression levels of p-IκBα and p-p65 confirmed that NEAT1 alleviated the inflammatory response of LPS-induced myocardial cells mainly by inhibiting the NF-κB signaling pathway. This suggested that the presence of NEAT1 was essential to the maintenance of the sepsis models.

In view of the differential expression of miR-144-3p in sepsis[26], we first investigated its expression in LPS-treated myocardial cells and found that it was markedly decreased, which was similar to the expression trend of miR-144-3p in other diseases[24,25]. Furthermore, miR-144-3p mimic enhanced viability and inhibited apoptosis in LPS-treated myocardial cells. Also, miR-144-3p mimic reduced the contents of inflammatory cytokines. These anti-apoptotic and anti-inflammatory functions of miR-144-3p were similar to that

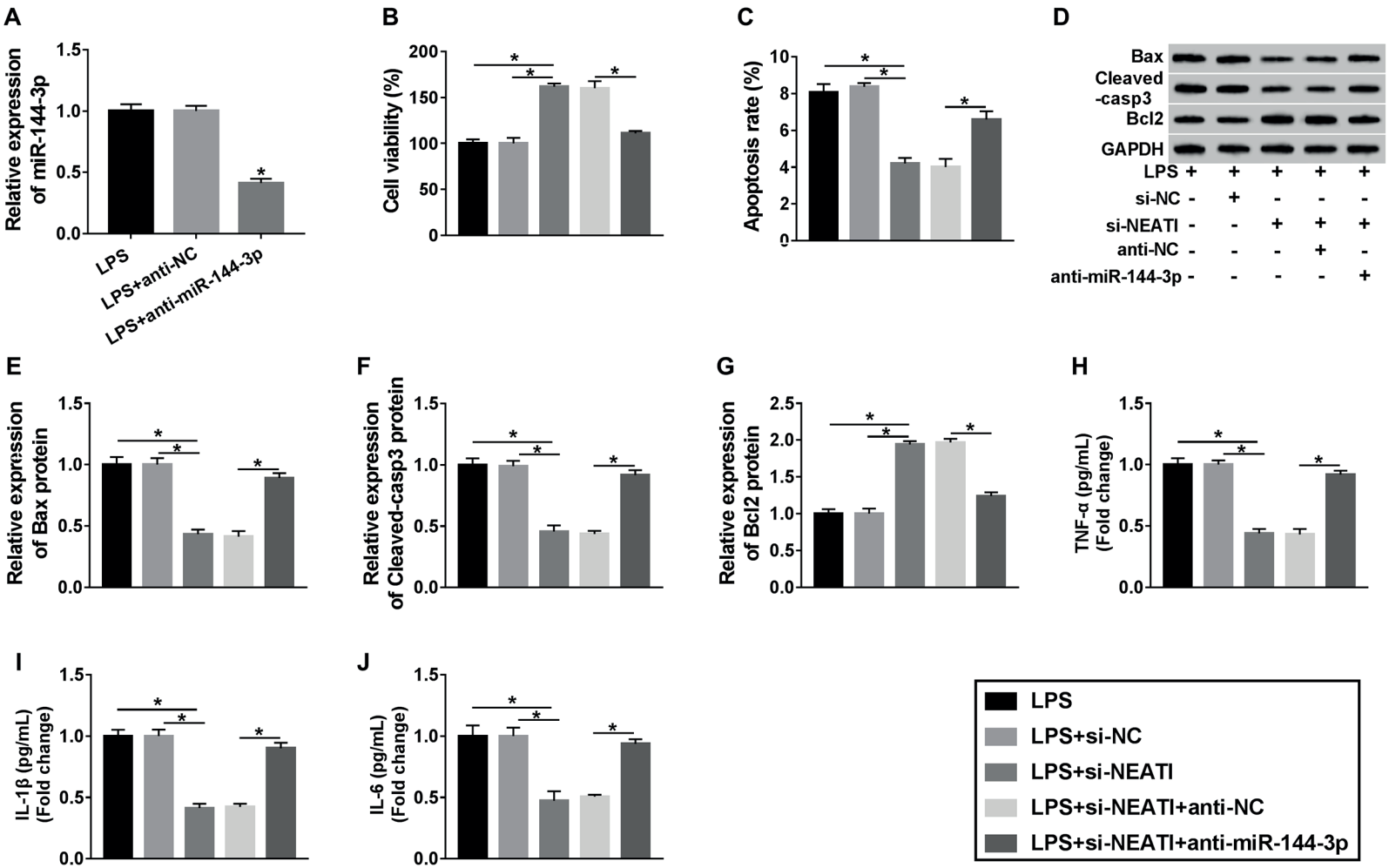

Figure 5. Effects of NEAT1 and miR-144-3p on LPS-induced myocardial cell injury. HL-1 cells were treated with 10 μg/mL LPS and si-NEAT1 or anti-miR-144-3p or their negative controls (si-NC or anti-NC). **A**, Expression of miR-144-3p was detected by qRT-PCR to evaluate the transfection efficiency of anti-miR-144-3p. **B**, Viability of HL-1 cells was assessed by CCK-8 assay. **C**, Apoptosis rate of HL-1 cells was measured by Flow cytometry. **D-G**, Protein levels of Bax, Cleaved-casp3, and Bcl2 were determined by WB analysis. **H-J**, Contents of TNF-α, IL-1β, and IL-6 were tested by ELISA assay. *$p < 0.05$.

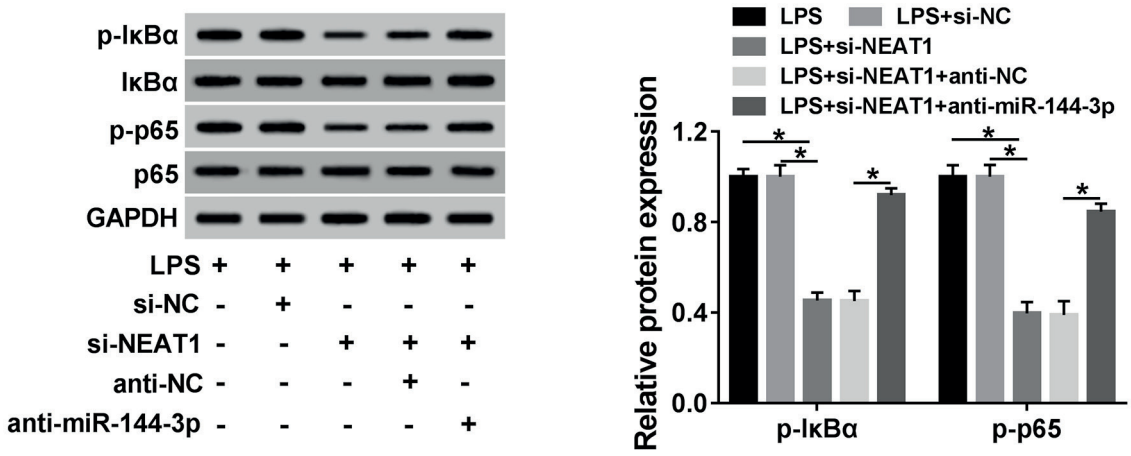

Figure 6. Effects of NEAT1 and miR-144-3p on the activity of NF-**κB** signaling pathway. HL-1 cells were treated with 10 μg/mL LPS and si-NEAT1 or anti-miR-144-3p or their negative controls (si-NC or anti-NC). The protein levels of p-IκBα and p-p65 were detected by WB analysis. *$p< 0.05$.

of NEAT1 silencing in LPS-induced myocardial cells, so we speculated that both of them might be correlated. Through bioinformatics prediction and experimental verification, we uncovered that NEAT1 could sponge miR-144-3p. Meanwhile, miR-144-3p inhibitor reversed the inhibitory effects of NEAT1 knockdown on LPS-induced myocardial cell injury and inverted its suppression effect on the NF-κB signaling pathway, indicating that miR-144-3p acted with an essential role in the regulation of NEAT1 on sepsis.

Conclusions

In summary, we demonstrated that lncRNA NEAT1 sponged miR-144-3p to promote the progression of sepsis-induced myocardial injury by regulating the NF-κB signaling pathway. This study reveales the role of NEAT1 on sepsis and provides a potential therapeutic target for sepsis-induced myocardial injury.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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