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LncRNA NEAT1 promotes inflammatory response in sepsis-induced liver injury via the Let-7a/TLR4 axis

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ABSTRACT

Background: Sepsis is a systemic inflammatory response that can lead to organ dysfunction and/or circulatory disorders in severe cases. The dysregulated inflammatory response plays a pivotal role in sepsis-induced liver injury. A variety of microRNAs and lncRNAs have been shown to be involved in the inflammatory response. However, their role in regulating sepsis-induced liver injury remains to be revealed.

Methods: Human hepatic tissue and healthy tissue were used for in vivo level detection. And Raw264.7 cells and Kupffer cells were used for in vitro modelling. The relative mRNA expression and the protein levels of TNF-α, IL-6 and IL-1β were detected by q-PCR or by enzyme-linked immunosorbent assay (ELISA), respectively. The binding of lncRNA NEAT1/Let-7a and Let-7a/TLR4 was detected by dual-luciferase reporter assay. RNA Immunoprecipitation (RIP) was used to detect the targeting relationship between lncRNA NEAT1 and Let-7a. Western blotting (WB) was used to detect TLR4 expression in different cell models.

Results: The overexpression of lncRNA NEAT1 accompanied by Let-7a inhibition and TLR4 activation was found in sepsis-induced liver injury patients. Similarly, LPS stimulation upregulated lncRNA NEAT1 expression, and lncRNA NEAT1 inhibition decreased the levels of inflammatory cytokines in vitro. Let-7a inhibitor treatment as well as TLR4 overexpression rescued the expression of inflammatory cytokines in lncRNA NEAT1-knockdown cells. Moreover, Let-7a interacted with both lncRNA NEAT1 and TLR4.

Conclusion: We demonstrate that lncRNA NEAT1 interacts with Let-7a, targeting TLR4 to contribute to the LPS-induced inflammatory response. Our assay can provide a potential therapeutic target for sepsis-induced liver injury.

1. Introduction

Sepsis is a deadly, worldwide and expensive disease caused by infection that induces systemic inflammatory response syndrome (SIRS). Sepsis happens when the body responds to an infection and then causes damage to its own tissues or organs [1,2]. The rate of sepsis mortality is approximately 25% to 30% [3,4]. Therefore, high sepsis incidence rates and hospital mortality make it a major public health concern. Sepsis-induced tissue damage is associated with multiple cellular and molecular events. In the case of sepsis, inflammatory cytokines are able to dysregulate the immune response and cause damage to various tissues or organs [5,6]. Tumour necrosis factor (TNF)-α and IL-1β regulate the immune response in the early stage, during which they are largely produced and released by immune cells through both transcriptional and posttranslational regulation [7]. TNF-α and IL-1β release can activate inflammatory cascades and generate reactive oxygen species (ROS), which are involved in organ injury [8–10]. The liver participates in the response to sepsis by releasing cytokines, acute-phase proteins, and coagulants and facilitating the clearance of infectious agents and products [11]. LPS is a type of prototypical endotoxin since it combines with the CD14/TLR4/MD2 receptor complex in various cell types, especially in dendritic cells, macrophages, monocytes and B cells, to promote the secretion of nitric oxide, proinflammatory cytokines and eicosanoids. During the progression of sepsis, the liver is a potential target of the dysregulated inflammatory response. At present, no specific and effective therapeutic interventions are available for sepsis-associated liver damage [12].

MicroRNAs (miRNAs) are a large class of small non-coding RNA molecules that target mRNA and regulate translational repression [13]. The main role of miRNAs is to post-transcriptionally mediate or silence gene expression [14,15]. miRNAs are involved in many biological processes, including developmental timing, cell proliferation, apoptosis,

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neuronal patterning, haematopoiesis, and organ development [16–20]. Dysregulation of several miRNAs has been found in the peripheral blood mononuclear cells (PBMCs) of sepsis patients [21]. The miR-143 level is also significantly increased in patients with sepsis compared with healthy controls [22]. A recent study demonstrated that miR-195 inhibition upregulates the levels of Bcl-2 and SIRT-1 and suppresses liver tissue injury in septic mice, indicating a possible role for miRNA in sepsis-induced liver injury [23]. It has been reported that Let-7a induces monocyte apoptosis by targeting Bcl-XL, leading to impaired monocyte-to-macrophage differentiation and thus ameliorating sepsis-induced liver dysfunction [24]. Moreover, the expression of Let-7a in serum was found to be decreased in sepsis patients compared with healthy controls. Let-7a regulates the anti-inflammatory process by inhibiting specific genes that target downstream signalling pathways [25]. However, it remains unclear whether Let-7a plays a critical role in sepsis-induced liver injury under inflammatory conditions.

LncRNAs are non-coding RNAs longer than 200 nt in length. As with miRNA, lncRNAs are also involved in multiple biological processes. Intriguingly, previous studies have revealed that lncRNAs and miRNAs can associate with each to further facilitate post-transcriptional regulation [26,27]. LncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) is functional in oncogenesis, and its aberrant overexpression is related to a poor prognosis in cancer patients [28]. Moreover, lncRNA NEAT1 is significantly upregulated in sepsis-induced acute kidney injury (AKI) patients, and the upregulation of lncRNA NEAT1 is associated with severity of AKI in sepsis patients [29]. However, its role in sepsis-induced liver injury remains unclear. TargetScan software predicted an interaction between lncRNA NEAT1 and Let-7a, suggesting its possible relation to the severity and poor prognosis of sepsis patients.

In the present study, we found that the expression of inflammatory factors, including TNF-α, IL-6, and IL-1β, along with lncRNA NEAT1 and Toll-like receptor 4 (TLR4) was increased in sepsis-induced liver injury patients. Conversely, the Let-7a level was decreased in this condition. We also found that lncRNA NEAT1 interacts with Let-7a, targeting TLR4 to regulate the LPS-induced inflammatory response. Our findings provide a potential target for sepsis-induced liver injury therapy.

2. Materials and methods

2.1. Patients and tissue specimens

Human hepatic tissue and healthy tissue were obtained from the Affiliated Hospital of Jining Medical University. Fifteen hepatic tissue samples and healthy tissue samples were collected to measure the mRNA expression of NEAT1, TLR4, Let-7a, TNF-α, IL-6 and IL-1β using q-PCR. Another fifteen paraffin sections of hepatic tissue and healthy tissue samples were used for ELISA to analyse the levels of TNF-α, IL-6 and IL-1β. The study protocol was approved by the institutional review board according to the Helsinki declaration. Written informed consent was obtained from each subject.

2.2. Extraction of Kupffer cells and cell culture

Kupffer cells were isolated from livers by collagenase perfusion. First, 20ml of 0.05% collagenase was added and incubated at 37 °C for 40 min. To remove hepatocytes, the cell suspension was centrifuged twofold for 5 min at 50 ×*g*. The supernatant containing Kupffer cells was pelleted by centrifugation at 300 ×*g* for 10 min. Cells were resuspended in serum-free RPMI 1640 medium supplemented with 0.1 mg/ml gentamycin and 2 mmol/l glutamine. After incubation for 30 min, Kupffer cells were separated from other nonparenchymal cells because they were adherent.

Raw264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Raw264.7 cells and Kupffer cells were cultured with DMEM (Thermo Fisher, 11995) supplemented with 10% FBS (GIBCO, Grand Island, USA) and 1% penicillin/streptomycin (GIBCO, 15140-122) in a 37 °C, 5% CO_2 humidified incubator.

2.3. Lentiviral transfection of sh-NEAT1 into Kupffer cells

The shNEAT1 gene with *Xho* I and EcoR I restriction sites was synthesized and inserted into the pMSCV-puro vector by Synbio Tech. The plasmid was then amplified and extracted with an endotoxin-free plasmid Maxiprep kit, stored at −20 °C or used for cell transfection. A total of 3 million GP2-293 cells were seeded in a 6-cm dish with transfection medium. After 24 h of culture, the cells were transfected with 2.5 μg pVSV-G and 2.5 μg pMSCV- shNEAT1 using Viafect (DNA: Viafect = 1:4) and incubated at 37 °C for 6 h. Subsequently, the medium was replaced with fresh growth medium and the cells cultured for 48 h. The supernatant was collected, centrifuged at 1000 rpm for 5 min, and then filtered through a 0.45 μM filter to remove cell pellets and debris. A total of 500,000 cells/well Kupffer cells were seeded in a 6-well plate with 3ml medium. Polybrene (Sigma, H9268) (stock concentration 8 mg/ml) was added to a final concentration of 4 μg/ml in the viral supernatant and mixed well. Then, 0.3, 0.5 and 1 ml of the mixture was added to the Kupffer cells and incubated at 37 °C for 48 h. After incubation, the medium was replaced with fresh selective Kupffer cell shNEAT1 medium and refreshed every 3 days. Seven days later, the cell density was checked, and the cells were diluted in a 96-well cell plate at 1 cell/well and incubated at 37 °C in 5% CO_2. The 96-well cell plates were observed every day, and the wells with single clones growing were labelled.

2.4. Transient transfection

HEK293 cells or Raw264.7 cells were transfected with sh-NEAT1 or other plasmids to investigate the interaction of the proteins. Cells were seeded in 10 cm dishes at 5×10^6 cells per well. The day after seeding, the plasmids were pre-incubated with Opti-MEM (Thermo Fisher, 51985901) and Fugene 6 (Promega, E2691) for 15 min. The mixture was then added in the 10 cm dishes and incubated for up to 48 h at 37 °C with 5% CO_2.

2.5. RNA extraction and q-PCR analysis

The cell culture dish was placed on ice, TRIzol added to lyse the cells for 5–10 min, and then the liquid was drained into an imported EP tube, Then, 1/5 volume of chloroform was added; the liquid was mixed up and down and left to stand for 10–15 min at 4 °C. Subsequently, the mixture was centrifuged for 15 min, 4 °C; the 400–500 μL supernatant was removed and placed in a new EP tube. An equal volume of isopropanol was added, incubated at 4 °C for 10 min, then centrifuged at 12,000 rpm for 10 min. The resulting pellet was washed with 75% alcohol, and 50 μL of DEPC water added after drying to fully dissolve the pellet, which was then stored at −80 °C. Total cellular RNA was extracted by TRIzol reagent (Thermo Fisher, San Jose, US) according to the manufacturer's protocol. The RNA integrity was confirmed by electrophoresis on ethidium bromide-stained 1% agarose gels. One microgram of total RNA was reverse transcribed using the PrimeScriptTM RT reagent kit (Takara, RR047A). The primer sequences used for amplification were as follows: NEAT1 forward primer: 5′-GTA ATT TTC GCT CGG CCT GG-3′, reverse primer: 5′-TAC CCG AGA CTA CTT CCC CA-3′. Let-7a forward primer: 5′-CAA CCT ACT ACC TCA TCC CA-3′, reverse primer: 5′-TAG GAA AGA CAG TAG ATT GT-3′. TLR4 forward primer: 5′-GGA GAC TTG GCC CTA AAC CA-3′, reverse primer: 5′-GAC ATG GAA ACA CAC CCA GG-3′. IL-6 forward primer: 5′-GCA AGG GTC TGG TTT CAG CC-3′, reverse primer: 5′-TGA GGT AAG CCT ACA CTT TCC AA-3′. IL-1β forward primer: 5′-ATT GCT CAA GTG TCT GAA GCA G-3′, reverse primer: 5′-AGA GAG CAC ACC AGT CCA A-3′. TNF-α forward primer: 5′-CCC TCT CTC CCC TGG AAA GG-3′, reverse primer: 5′-GCC ACT GAA TAG GGC GAT-3′. q-PCR was performed using

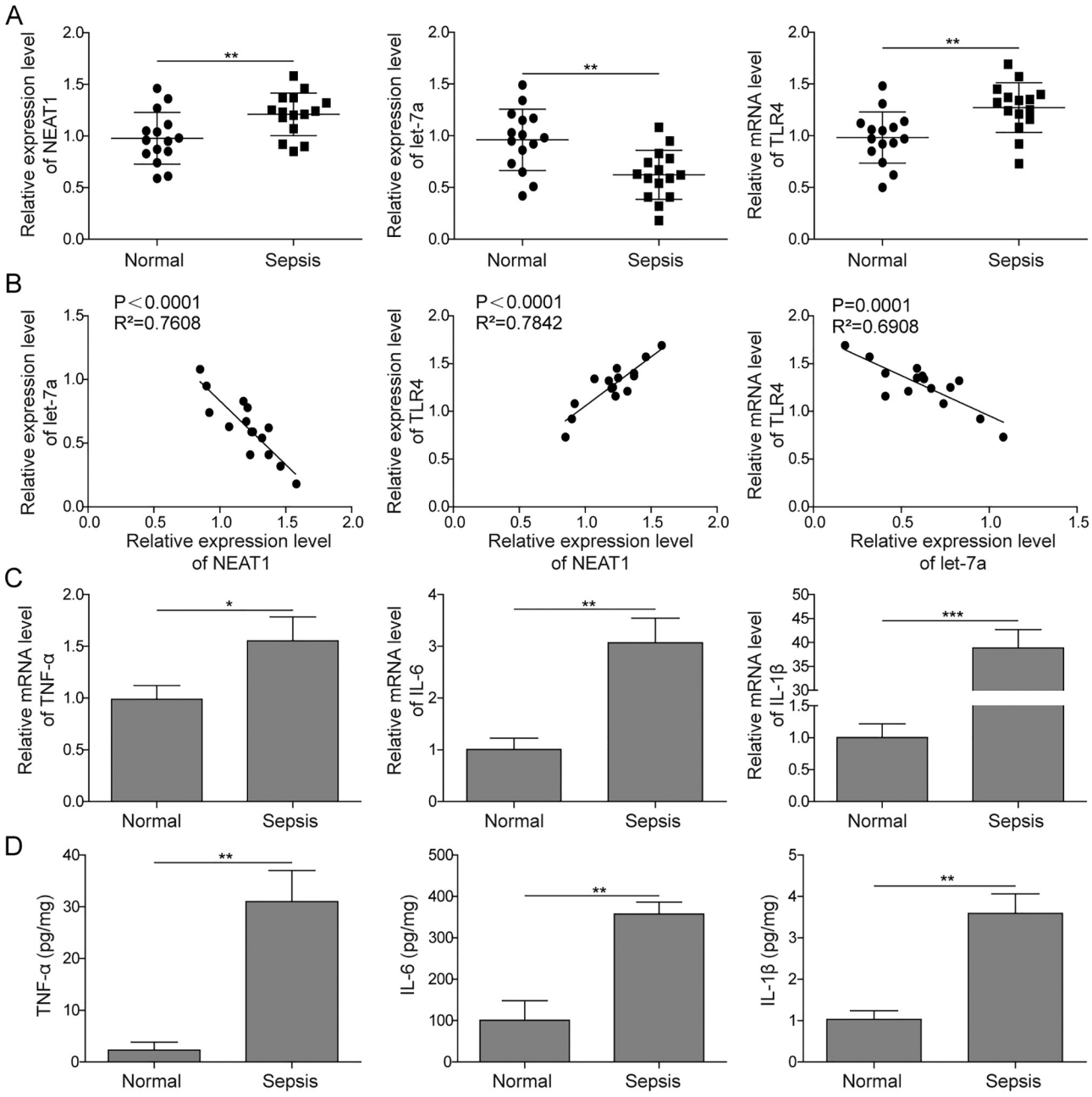

Fig. 1. Expression of lncRNA NEAT1, Let-7a, TLR4 and inflammatory factors in patients with sepsis and liver injury. A) lncRNA NEAT1, Let-7a and TLR4 expression in injured liver tissue and healthy tissue analysed by q-PCR. B) Correlation analysis of lncRNA NEAT1, Let-7a and TLR4. C) q-PCR to test the expression of TNF-α, IL-6, and IL-1β in injured liver and healthy tissue. D) The expression of TNF-α, IL-6, and IL-1β in liver tissue culture medium assessed through ELISA. The result is representative of three independent experiments, n = 3 in each group. Error bars represent the mean ± SD. P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P < 0.05.

SYBR real-time PCR kits in an Eppendorf Mastercycle EP. RNA expression was calculated based on the internal control β-actin.

2.6. Western blot

Cells were split and cultured overnight before exposure to the indicated treatments. Whole-cell lysates were prepared for lysis using RIPA lysis buffer, then separated on an SDS-PAGE gel and transferred to a PVDF membrane. Western blot assays were performed using the indicated primary antibodies: TLR4 (CST, 14358), GAPDH (CST, 5174S) and appropriate horseradish peroxidase-conjugated secondary antibodies (CST, 5571), followed by detection with an enhanced chemiluminescence reagent. Several X-ray films were analysed to verify the linear range of the chemiluminescence signals, and the quantifications were carried out using densitometry.

2.7. Enzyme-linked immunosorbent assay (ELISA)

For detecting TNF-α, IL-6, IL-1β, Human TNF-a Quantikine ELISA (R &D, DTA00D), Human IL-6 Quantikine ELISA (R&D, D6050) and Human IL-1 beta/IL-1F2 Quantikine ELISA kits (R&D, DLB50) were used. Kupffer cells were cultured with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a 96-well plate (Corning Costar #3599), with 5000 cells/well at 37 °C in a 5% CO_2 humidified incubator. Raw264.7 cells were also seeded in 96-well plates at 5000 cells/well. The day after plating, cells were treated under different

conditions, and the cell culture supernatants were collected and measured in accordance with the ELISA kit user manual.

2.8. RNA binding protein immunoprecipitation (RIP) assay

RIP assay. The RIP assay was performed to isolate target RNA-protein complexes using a Magna RIP RNA-binding Protein Immunoprecipitation kit according to the manufacturer's protocol (Millipore, Billerica, USA). Raw264.7 cells were lysed in RIP lysis buffer and then re-suspended in RIP lysis uffer. Then, anti-Argonaute 2 (Millipore, Billerica, USA) and normal mouse IgG (Millipore, Billerica, USA) were used for RIP assays and defined as the RIP-Ago2 group and RIP-IgG group (negative control). Next, the antibodies were incubated with protein A/G beads for 1 h at 4 °C with gentle rotation. After co-precipitated target RNAs were isolated from the protein A/G beads, lncRNA NEAT1 and Let-7a levels were measured by q-PCR.

2.9. Dual-luciferase reporter assay

A dual-luciferase reporter assay system was used to investigate the interaction of lncRNA NEAT1 and Let-7a, Let-7a and TLR4. LncRNA NEAT1/TLR4 gene fragments containing predicted Let-7a binding sites or corresponding mutant fragments were cloned into pGL3-basic vectors. Oligos of Let-7a mimics were used for Let-7a overexpression. The Let-7a mimics and respective negative controls were obtained from GenePharma (Shanghai, China). During transfection, pGL3, miRNA oligos and pRL plasmids were co-transfected into HEK293 cells with lipofectamine 3000 (Thermo Fisher Scientific, San Jose, CA, US) at a ratio of 1:3 (DNA: lipofectamine). After transfection for 48 h, firefly luciferase activity was measured and adjusted by Renilla luminescence using an assay kit according to the manufacturer's instructions (Promega, Madison, WI, US). Raw264.7 cells were seeded into a 96-well plate (5000 cells/well). Cells were incubated overnight at 37 °C with 5% CO_2. Then, the growth medium was removed from the cultured cells. The cells were rinsed with PBS once and lysed in 100 μl of 1 × PLB (included in the dual-luciferase reporter assay system kit) with gentle shaking for 15 min at room temperature. The detection procedure was guided by the dual-luciferase reporter assay system datasheet.

2.10. Statistical analysis

Statistical analysis was performed with SPSS statistics 22.0 (SPSS Inc., Chicago, USA). Normally distributed data are presented as the mean ± SD. Comparisons between groups were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The quantification of the chemiluminescence signal was carried out using densitometry. $P < 0.05$ was considered statistically significant in all cases.

3. Results

3.1. Expression of lncRNA NEAT1, Let-7a, TLR4 and inflammatory factors in sepsis patients with liver injury

We examined 30 samples, including 15 from sepsis patients with liver injury. The results of the q-PCR showed that, compared with that of the healthy controls, the expression of lncRNA NEAT1 and TLR4 in liver tissue of patients with sepsis-induced liver injury was clearly increased, whereas the level of Let-7a was significantly decreased (Fig. 1A). In order to understand the relationship of these three components in sepsis-induced liver injury, we conducted a correlation analysis of the expression of lncRNA NEAT1 and TLR4 in 15 healthy samples and 15 samples from patients with sepsis. The analysis results indicated a negative correlation of Let-7a with either lncRNA NEAT1 or TLR4 (Fig. 1B). As dysregulation of the inflammatory response may be related to sepsis-induced liver injury, we then detected the expression of TNF-α, IL-6 and IL-1β in sepsis-induced liver injury patients by q-PCR and ELISA (Fig. 1C and D). The q-PCR data showed that the expression of TNF-α, IL-6 and IL-1β in the livers of sepsis patients was 1.5-, 3-, 38-fold higher than that in the livers of healthy patients (Fig. 1C). Similar results were obtained using ELISA (Fig. 1D). Generally, these results show that sepsis-induced liver injury is associated with an intense inflammatory response along with the overexpression of lncRNA NEAT1 and TLR4 and a decrease in Let-7a levels.

3.2. LPS induces an inflammatory response by upregulating the expression of lncRNA NEAT1

Lipopolysaccharide (LPS) is one of the most powerful bacterial virulence factors in terms of proinflammatory properties. The LPS-induced inflammatory response exacerbates sepsis pathogenesis. In our study, LPS treatment was used for in vitro modelling, and the concentration and time of LPS treatment was 1 μg/ml for 24 h [30]. Q-PCR was used to detect the expression of lncRNA NEAT1, Let-7a, TLR4 and inflammatory factors. LPS increased the expression of lncRNA NEAT1 and TLR4, decreased Let-7a expression in both Kupffer and Raw264.7 cell lines. However, the abrogation of lncRNA NEAT1 significantly reversed the effects induced by LPS

(Fig. 2A and B). In addition, inflammatory factors, including TNF-α, IL-6 and IL-1β, were increased by LPS treatment, as revealed by q-PCR in the Kupffer cell line (Fig. 2C). The same result was found in Raw264.7 cell lines (Fig. 2D). The abrogation of lncRNA NEAT1 obviously decreased the expression of these inflammatory factors induced by LPS (Fig. 2C and D). To confirm these results, an ELISA was conducted to detect the levels of these inflammatory factors in Kupffer cell line and Raw264.7 cell lines, and the consistent results are shown in Fig. 2E and F. These data indicate that LPS induces an inflammatory response through upregulating the expression of lncRNA NEAT1.

3.3. LncRNA NEAT1 functions as a molecular sponge for Let-7a

TargetScan software was used to predict the binding domain between lncRNA NEAT1 and Let-7a (Fig. 3A). A luciferase assay showed that the Let-7a mimic significantly decreased the wild -type fluorescence intensity but did not affect the expression of the binding domain-mutated NEAT1 (Fig. 3B). To confirm these results, an RNA binding RIP assay was performed to detect the interaction between Let-7a and lncRNA NEAT1. The results showed that lncRNA NEAT1 and Let-7a co-precipitated with pulled-down ago2, which is indicative of the targeted binding of lncRNA NEAT1 to Let-7a (Fig. 3C). Moreover, a q-PCR assay revealed that shRNA-targeted lncRNA NEAT1 silencing increased Let-7a an expression (Fig. 3D). Therefore, lncRNA NEAT1 and Let-7a interact, and the expression of the two is inversely proportional.

3.4. LncRNA NEAT1 interacts with Let-7a to regulate the LPS-induced inflammatory response

LPS is a major component of gram-negative bacterial cell walls. LPS interacts with specific receptors, including TLR4, and induces an NF-κB-dependent inflammatory cascade, consequently resulting in the overexpression of a group of proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β. Our q-PCR assay results showed that shRNA-targeted lncRNA NEAT1 silencing significantly decreased the levels of TNF-α, IL-6 and IL-1β induced by LPS stimulation in Raw264.7 cells (Fig. 4A). As expected, the Let-7a inhibitor rescued the expression of TNF-α, IL-6 and IL-1β in lncRNA NEAT1-knockdown cells (Fig. 4A). Similar results were obtained using ELISA (Fig. 4B). These results indicate that lncRNA NEAT1 antagonises Let-7a to regulate the LPS-induced inflammatory response.

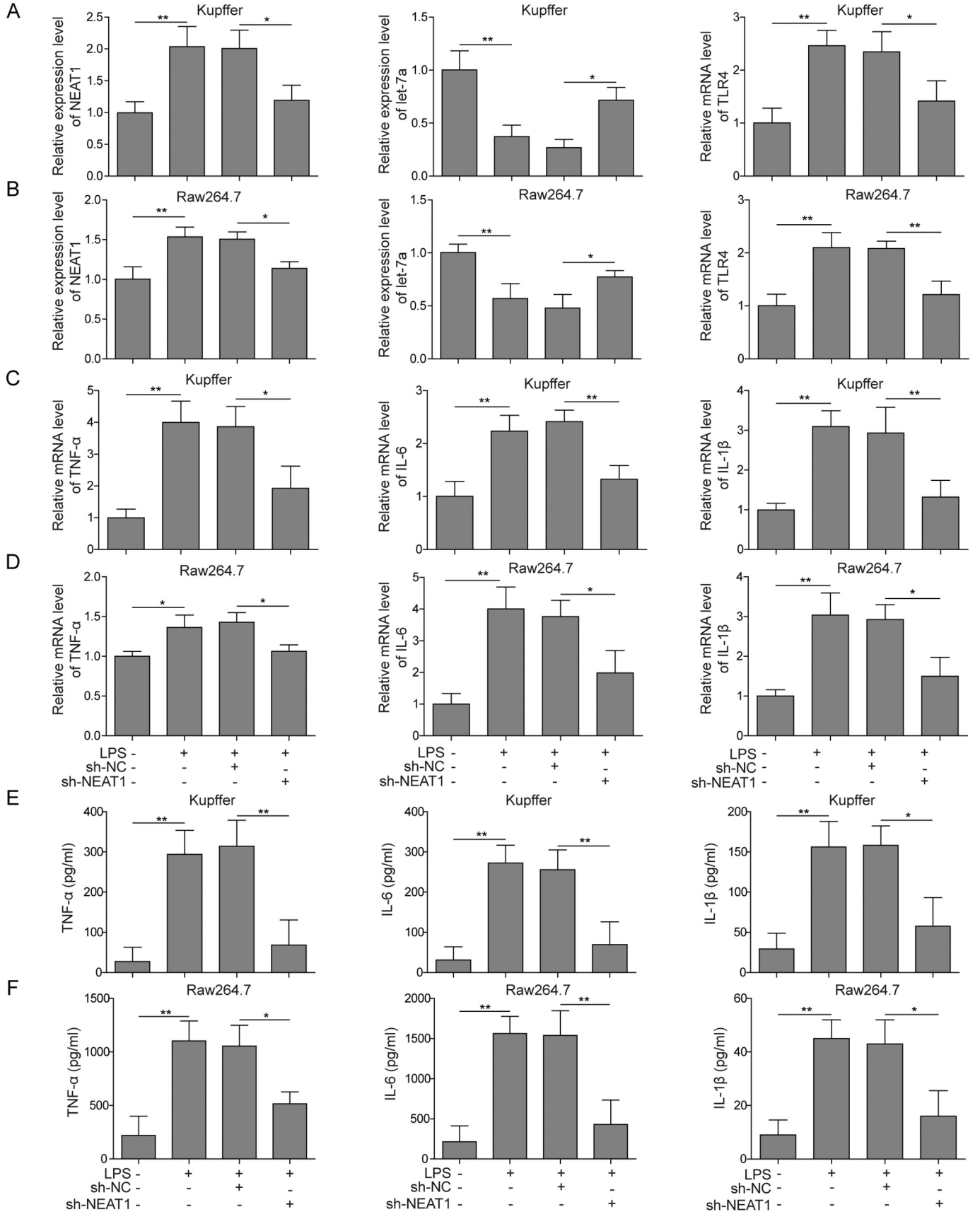

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Fig. 2. LPS induces an inflammatory response by upregulating the expression of lncRNA NEAT1. A) The expression of NEAT1, Let-7a and TLR4 in sh-NC and sh-NEAT1 Kupffer cell models assessed by q-PCR. B) The expression of NEAT1, Let-7a and TLR4 in sh-NC and sh-NEAT1 Raw264.7 cell models assessed by q-PCR. C) The expression of TNF-α, IL-6, and IL-1β in sh-NC and sh-NEAT1 Kupffer cell models assessed by q-PCR. D) The expression of TNF-α, IL-6, and IL-1β in sh-NC and sh-NEAT1 Raw264.7 cell models assessed by q-PCR. E) The expression of TNF-α, IL-6, and IL-1β in sh-NC and sh-NEAT1 Kupffer cell models assessed through ELISA. F) The expression of TNF-α, IL-6, and IL-1β in sh-NC and sh-NEAT1 Raw264.7 cell models assessed through ELISA. The results are representative of three independent experiments, n = 3 in each group. Error bars represent the mean ± SD. P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P < 0.05.

3.5. Let-7a directly interacts with TLR4

Previously, we verified that lncRNA NEAT1 binds to Let-7a to participate in the LPS-induced inflammatory response. Additionally, we predicted the binding sites of Let-7a and TLR4 through bioinformatics software Targetscan (http://www.targetscan.org) (Fig. 5A). The results of the dual-luciferase reporter system analysis indicated that there is a targeting relationship between Let-7a and TLR4. The Let-7a mimic significantly reduced the expression of fluorescence intensity in wild-type cells, whereas it did not affect the expression of binding domain-mutated TLR4 (Fig. 5B). To verify this result, we treated cells with the Let-7a mimic or inhibitor. The Let-7a mimic downregulated TLR4 expression. In contrast to the Let-7a mimic, the Let-7a inhibitor upregulated TLR4 expression detected, as detected by q-PCR (Fig. 5C and D). Consistent results were obtained by Western blot (Fig. 5E). Therefore, TLR4 targets Let-7a, and there is a negative correlation between the two proteins.

3.6. Let-7a regulates LPS-induced inflammatory responses by targeting TLR4

In order to study the role of Let-7a in regulating the LPS-induced inflammatory response by targeting TLR4, we used LPS to induce the inflammatory response in Raw264.7 cells and detected the expression of TNF-α, IL-6 and IL-1β. As the q-PCR results showed, the Let-7a mimic inhibited the expression of all three indicated inflammatory factors induced by LPS, while the overexpression of TLR4 (OE-TLR4) reversed this effect (Fig. 6A). To confirm these results, all of the experiments were analysed by ELISA, and the results were similar to those of the q-PCR (Fig. 6B). Therefore, TLR4 functions as a downstream effector of Let-7 regulating the LPS-induced inflammatory response. Let-7 negatively regulates the LPS-induced inflammatory response by targeting TLR4.

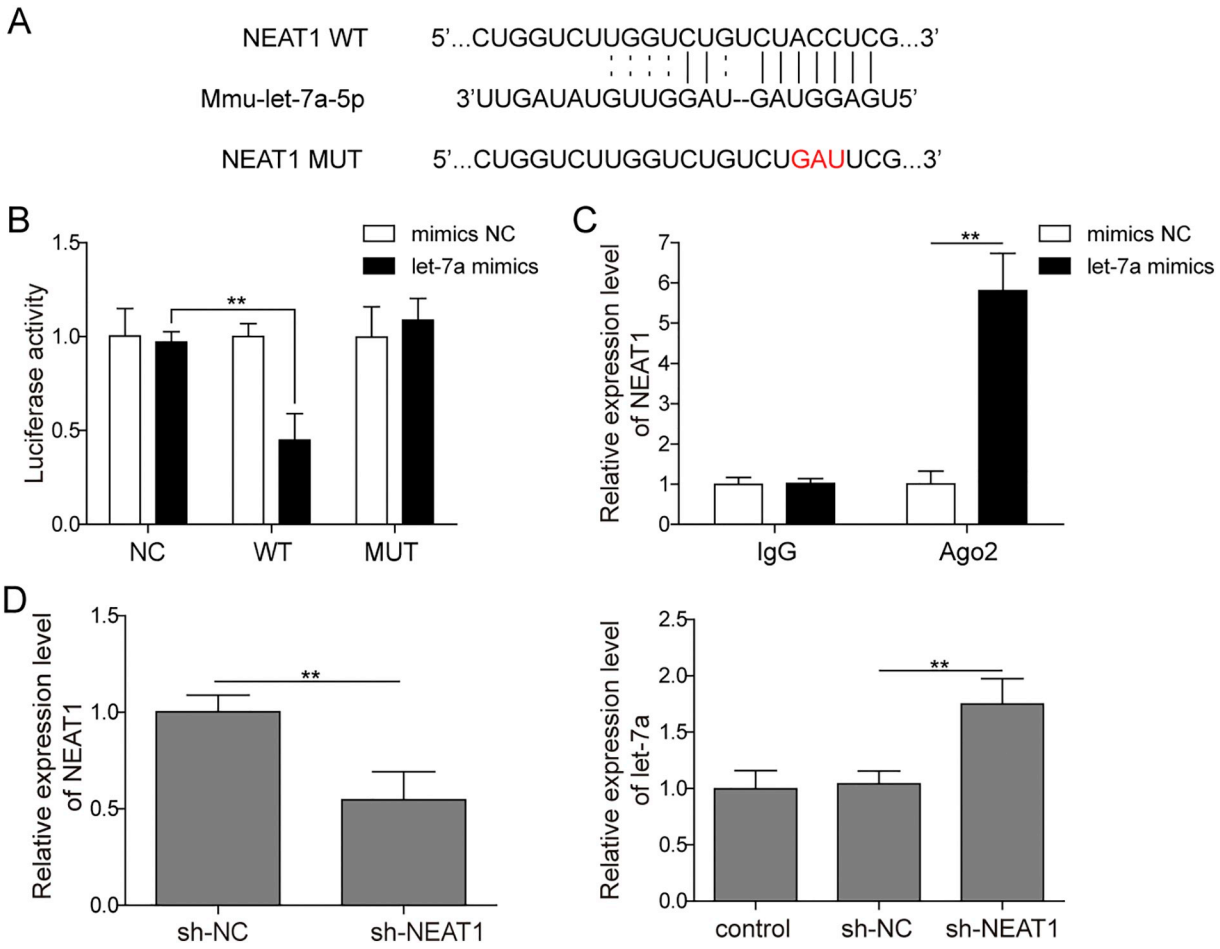

Fig. 3. LncRNA NEAT1 functions as a molecular sponge for Let-7a. A) Bioinformatics prediction of binding sites between lncRNA NEAT1 and Let-7a; B) Targeting relationship between lncRNA NEAT1 and Let-7a by fluorescein analysis; C) Targeting relationship between lncRNA NEAT1 and Let-7a detected by RIP; D) Detection of lncRNA NEAT1 and Let-7a expression by q-PCR in sh-NC and sh-NEAT1 Raw264.7 cell models. The results are representative of three independent experiments, n = 3 in each group. Error bars represent the mean ± SD. P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P < 0.05 in B), ***P < 0.001 in C).

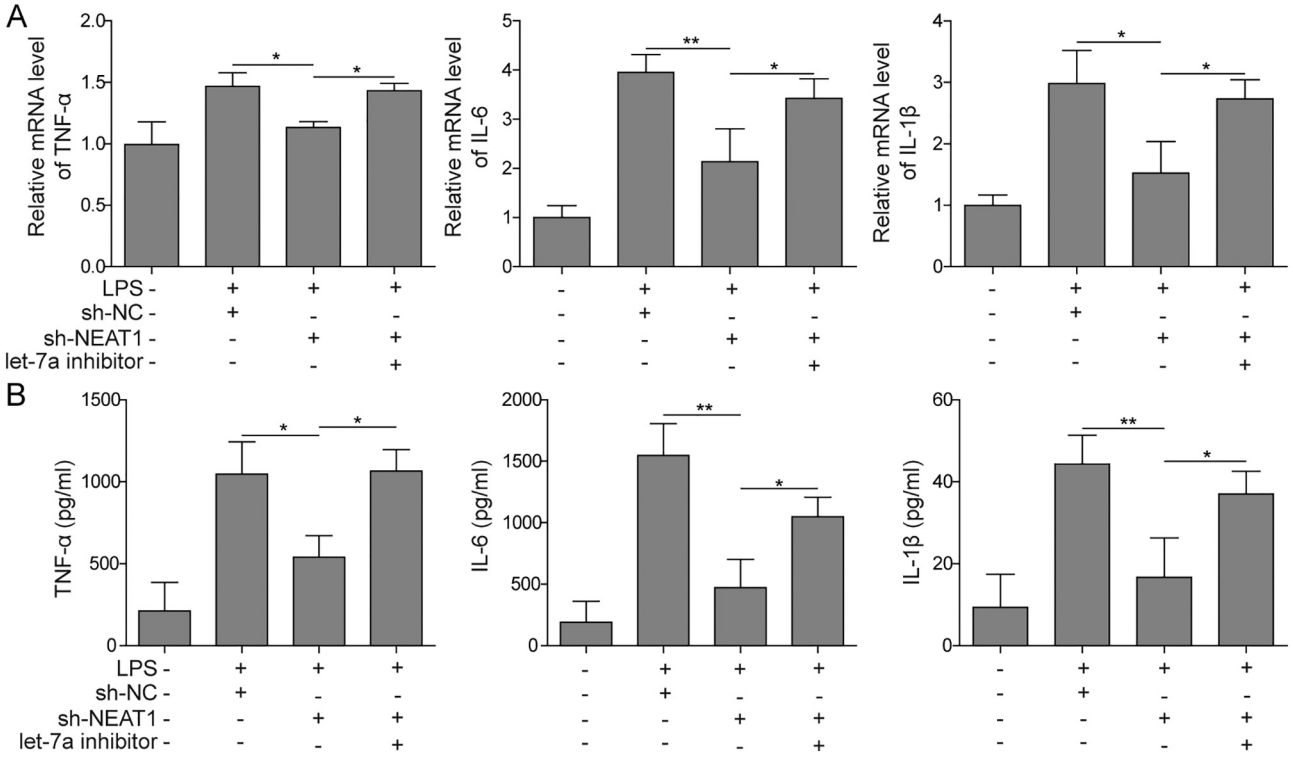

Fig. 4. lncRNA NEAT1 binds to Let-7a and participates in the LPS-induced inflammatory response. A) The expression of TNF-α, IL-6, and IL-1β in sh-NC, sh-NEAT1 and sh-NEAT1 + Let-7a inhibitor Raw264.7 cell models assessed by q-PCR. B) ELISA was used to test the expression of TNF-α, IL-6, and IL-1β in the same cell models as Fig. 4A. The results are representative of three independent experiments, n = 3 in each group. Error bars represent the mean ± SD. P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P < 0.05.

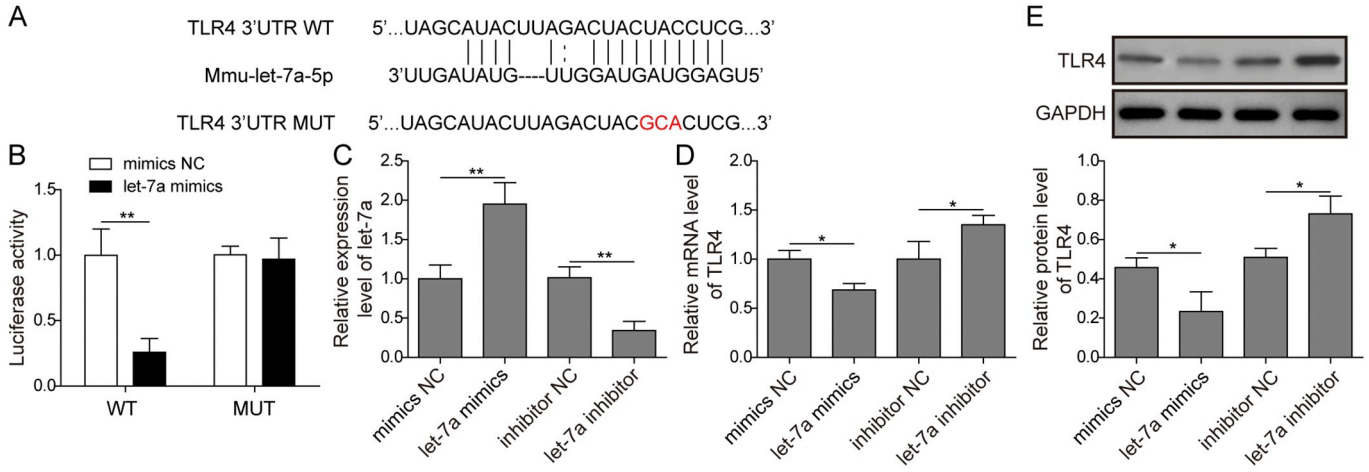

Fig. 5. Let-7a directly interacts with TLR4. A) Bioinformatics prediction of binding sites between Let-7a and TLR4 using Targetscan; B) Targeting relationship between Let-7a and TLR4 in the dual-luciferase reporter system analysis; C) The effect of the Let-7a inhibitor and mimic detected by q-PCR; D) The expression of TLR4 in Let-7a inhibitor- and mimic-treated cells assessed by q-PCR; E) TLR4 expression in the Let-7a inhibitor and mimic WB assay. The results are representative of three independent experiments, n = 3 in each group. Error bars represent the mean ± SD. P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P < 0.05.

3.7. lncRNA NEAT1 regulates the LPS-induced inflammatory response via Let-7a targeting of TLR4

We have indicated that lncRNA NEAT1 binds to Let-7a and participates in the LPS-induced inflammatory response. Given that Let-7 negatively regulates the LPS-induced inflammatory response by targeting TLR4, a lncRNA NEAT1/Let-7/TLR4 axis might exist to mediate LPS-induced inflammation. To verify our hypothesis, lncRNA NEAT1 was depleted using sh-NEAT1. q-PCR and Western blot results showed that knockdown of lncRNA NEAT1 reduced the LPS-induced expression of TLR4 (Fig. 7A and B). The expression of inflammatory factors, including TNF-α, IL-6 and IL-1β, induced by LPS was significantly reduced in lncRNA NEAT1-silenced cells, whereas this effect was reversed by the overexpression of TLR4, as detected by q-PCR and ELISA (Fig. C and D).Taken together, these results show that lncRNA NEAT1 interacts with Let-7a, targeting TLR4 to promote the LPS-induced inflammatory response.

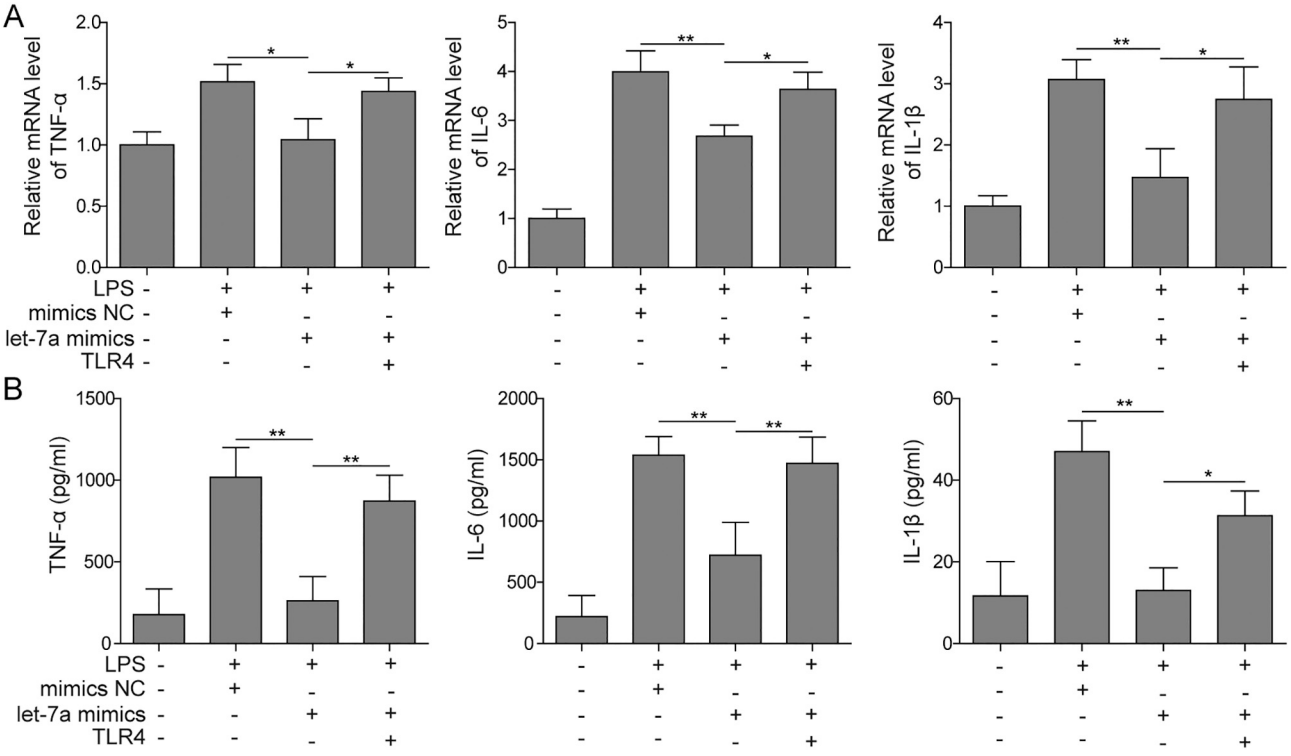

Fig. 6. Let-7a regulates LPS-induced inflammatory responses by targeting TLR4. A) The expression of TNF-α, IL-6, and IL-1β in miR-NC, Let-7a mimic and Let-7a mimic + OE-TLR4 Raw264.7 cell models assessed by q-PCR; B) The expression of TNF-α, IL-6, and IL-1β in the LPS-induced inflammatory response of miR-NC, Let-7a mimic and Let-7a mimic + OE-TLR4 Raw264.7 cell models assessed by ELISA. The results are representative of three independent experiments, n = 3 in each group. Error bars represent the mean ± SD. P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P < 0.05.

4. Discussion

Sepsis is a complex syndrome accompanied by dysregulation of the immune, endocrine, and metabolic responses to infection. This excessive response can result in multi-organ failure, shock, and death. Sepsis pathogenesis is exacerbated by the inflammatory response to the pathogen-associated molecular pattern ligand LPS [28]. Recent studies have targeted the inflammatory response as a potential treatment for sepsis. Our present study reveals that lncRNA NEAT1 and TLR4 are upregulated, while the level of Let-7a is decreased in sepsis-induced liver injury patients compared with the healthy controls. Moreover, lncRNA NEAT1 interacts with Let-7a, targeting TLR4 to regulate the LPS-induced inflammatory response. These findings uncover a new pathway for sepsis-induced liver injury therapy.

The upregulation of lncRNA NEAT1 expression in patients with sepsis has been shown in a previous study [30]. It has also been demonstrated that overexpressed lncRNA NEAT1 plays an important role in sepsis-induced acute kidney injury [29]. Similar to previous studies, we found the overexpression of lncRNA NEAT1 in sepsis-induced liver injury patients accompanied by Let-7a inhibition and TLR4 activation. LncRNA NEAT1 has been reported to be upregulated by activated TLR1 upon LPS stimulation [31]. In addition, a recent study revealed that lncRNA NEAT1 contributes to non-small cell lung cancer proliferation and metastasis through its direct binding to Let-7a [32]. Thus, we hypothesised that there is a correlation among lncRNA NEAT1, Let-7a and TLR in mediating sepsis-induced liver injury.

The experimental LPS-induced sepsis model is commonly used for the study of the inflammatory response processes during sepsis [33,34]. Consistent with the results in sepsis-induced liver injury patients, LPS stimulation upregulated lncRNA NEAT1 expression, and lncRNA NEAT1 knockdown decreased the levels of inflammatory cytokines, including TNF-α, IL-6 and IL-1β, induced by LPS. Additionally, Let-7a inhibitor treatment as well as overexpression of TLR4 rescued the expression of inflammatory cytokines in lncRNA NEAT1 knockdown cells, suggesting that Let-7a and TLR4 function downstream of lncRNA NEAT1, which is in contrast to a previous study [31]. Moreover, the overexpression of TLR4 reversed the inflammatory response inhibited by the Let-7a mimic, indicating that TLR4 is a downstream effector of Let-7a.

It has been reported that lncRNA NEAT1 competes against Let-7a for IGF-2 to regulate lung cancer cell progression [32]. We also found direct binding of lncRNA NEAT1/Let-7a and Let-7a/TLR4, and these complexes might function through a similar mechanism revealed in a former study [32]. In the case of LPS stimulation, the expression of lncRNA NEAT1 was upregulated. Increased lncRNA NEAT1 subsequently competitively bound to Let-7a and released TLR4 from Let-7a. Then, the released TLR4 was activated and stimulated downstream signalling, causing a severe inflammatory response and consequent liver injury.

5. Conclusion

Our data clearly show the biological function of lncRNA NEAT1 in sepsis-induced liver injury. We demonstrated that lncRNA NEAT1 competes with Let-7a and regulates TLR4 to promote sepsis-induced liver injury. The identified lncRNA NEAT1/Let-7a/TLR4 axis uncovers a novel insight into the function of lncRNA NEAT1 in sepsis-induced liver injury, providing a potential therapeutic target for sepsis-induced liver injury.

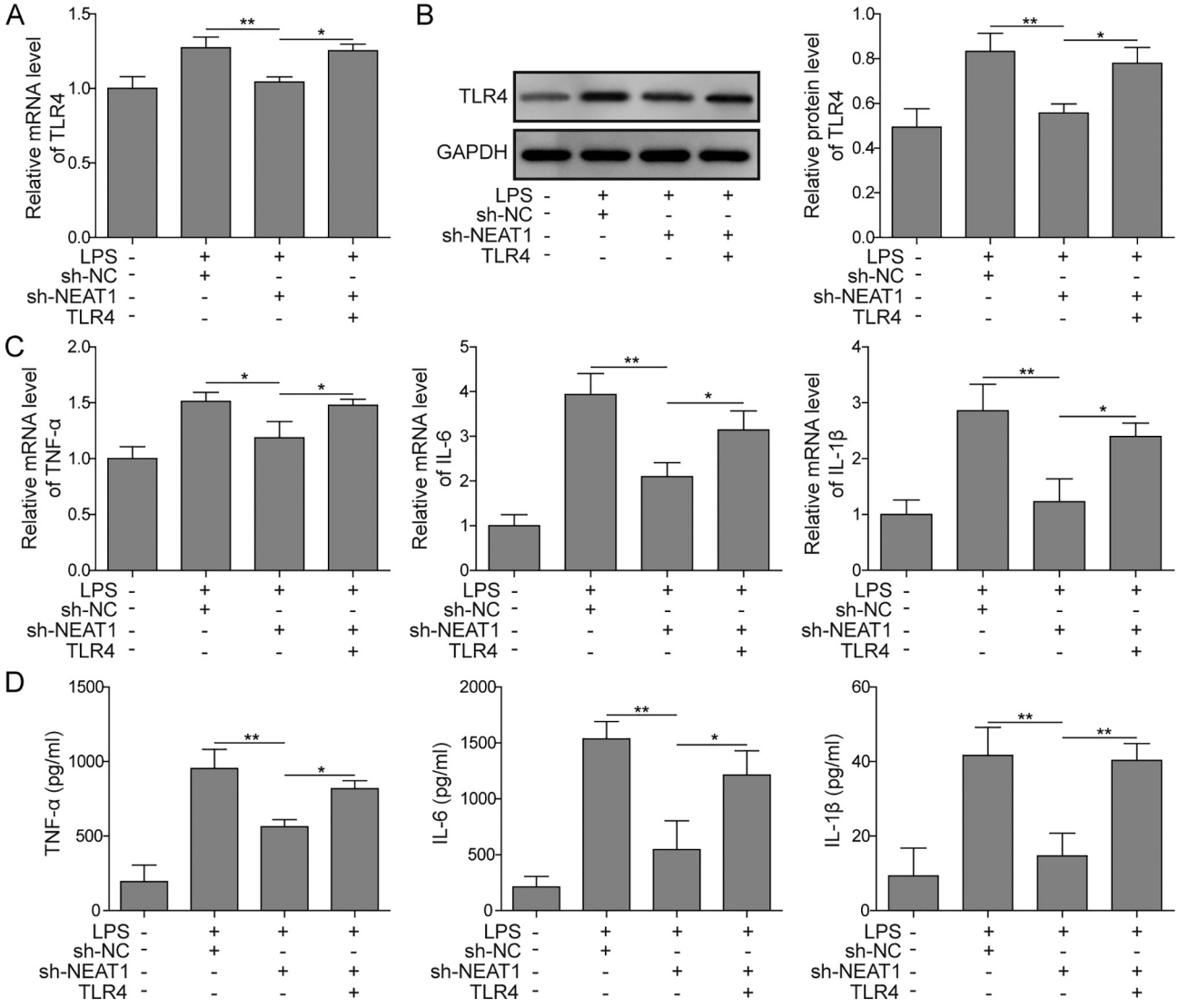

Fig. 7. lncRNA NEAT1 regulates the LPS-induced inflammatory response via Let-7a targeting of TLR4. A) TLR4 expression in LPS-induced inflammatory responses of sh-NC, sh-NEAT1 and sh-NEAT1 + OE-TLR4 Raw264.7 cell models assessed by q-PCR assay. B) TLR4 expression in LPS-induced inflammatory responses of sh-NC, sh-NEAT1 and sh-NEAT1 + OE-TLR4 Raw264.7 cell models assessed by WB assay. C) The expression of TNF-α, IL-6, and IL-1β in LPS-induced inflammatory responses of sh-NC, sh-NEAT1 and sh-NEAT1 + OE-TLR4 Raw264.7 cell models assessed by q-PCR. D) ELISA was used to test the expression of TNF-α, IL-6, and IL-1β in LPS-induced inflammation. The results are representative of three independent experiments, n = 3 in each group. Error bars represent the mean ± SD. P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P < 0.05.

List of abbreviations

AKI	acute kidney injury
ELISA	enzyme-linked immunosorbent assay
LncRNA	non-coding RNAs longer than 200 nt in length
LPS	lipopolysaccharide
MiRNAs	microRNAs
NEAT1	nuclear paraspeckle assembly transcript 1
OE-TLR4	overexpression of TLR4
PBMCs	peripheral blood mononuclear cells
RIP	RNA-binding protein immunoprecipitation
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
TLR4	Toll-like receptor 4
TNF	tumour necrosis factor

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Declaration of Competing of Interest

The authors declare that there are no conflicts of interest.

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