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Influence of lncRNA MALAT1 on septic lung injury in mice through p38 MAPK/p65 NF-κB pathway

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Abstract. – OBJECTIVE: To investigate the influence of long non-coding ribonucleic acid (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) on septic lung injury in mice and its mechanism, so as to provide references for the clinical prevention and treatment of septic lung injury in the future.

MATERIALS AND METHODS: A total of 60 male C57 mice were randomly divided into Control group (n=20), lipopolysaccharide (LPS) group (n=20), and LPS+MALAT1 siRNA group (n=20) using a random number table. The mouse model of septic lung injury was established via intraperitoneal injection of LPS (10 mg/kg), and the MALAT1 knockdown model was established via tail intravenous injection of MALAT1 siRNA. After 12 h, the lung was taken to measure the wet weight/dry weight ratio. Also, the activity of myeloperoxidase (MPO) in lung tissues was detected. The number of neutrophils and macrophages in bronchoalveolar lavage fluid (BALF) was detected via bronchoalveolar lavage. Moreover, the messenger RNA (mRNA) expression levels of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, in lung tissues were detected via Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Finally, the expression level of p38 in lung tissues was detected via immunohistochemical staining, and the expressions of p38 mitogen-activated protein kinase (MAPK)/p65 nuclear factor-κB (NF-κB) signaling pathway-related proteins in lung tissues of mice were detected via Western blotting.

RESULTS: The expression of lncRNA MALAT1 in lung tissues of mice with septic lung injury was significantly increased ($p<0.05$). After knockdown of lncRNA MALAT1, the LPS-induced pathological injury of lungs could be improved, and the wet weight/dry weight ratio of lungs could be reduced ($p<0.05$). Compared with those in LPS group, the total number of inflammatory cells and the number of neutrophils and macrophages in BALF were significantly decreased in LPS+MALAT1 siRNA group ($p<0.05$), and the levels of inflammatory cytokines were also significantly inhibited ($p<0.05$). The immunohistochemical results manifested that the knockdown of lncRNA MALAT1 could inhibit the LPS-induced up-regulation of p38 in lung tissues in mice. According to the results of Western blotting, the p38 MAPK/p65 NF-κB signaling pathway was significantly activated in lung tissues in LPS group ($p<0.05$), while it was significantly suppressed after inhibition on lncRNA MALAT1 ($p<0.05$).

CONCLUSIONS: The knockdown of lncRNA MALAT1 can significantly improve the septic lung injury in mice, whose mechanism may be related to its inhibition on the p38 MAPK/p65 NF-κB signaling pathway.

Key Words:
LncRNA MALAT1, p38 MAPK/p65 NF-κB, Sepsis, Lung injury.

Introduction

Acute lung injury (ALI) is a common clinical symptom in multiple diseases, which is characterized by high morbidity and mortality rates[1]. ALI is usually caused by direct or indirect damage of alveolar epithelial cells and capillary endothelial cells, and the damage factors include pneumonia, entering of gastric contents, sepsis, severe shock, and drug damage[2]. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and a major pathogenic factor of ALI[3]. Studies[4,5] have demonstrated that the pro-inflammatory signaling pathway in the lungs

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of patients is activated and the inflammatory mediators are over-expressed after severe trauma or infection, thus leading to systemic inflammatory response and ultimately resulting in severe shock, multiple organ failure and even death. There are many studies on ALI currently, but the concrete molecular mechanism of occurrence and development of ALI has not been fully clarified yet.

Long non-coding ribonucleic acids (lncRNAs) are a kind of long-chain RNA molecules with more than 200 nucleotides in the transcription length[6]. Although lncRNAs cannot encode the corresponding proteins in cells, they can regulate the expression of the corresponding target genes at the transcriptional/post-transcriptional level and epigenetic modification, ultimately affecting the occurrence and development of disease[7,8]. LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), a member of the lncRNA family, plays an important role in a variety of diseases, including tumors, cardiovascular diseases, and endocrine diseases. For example, lncRNA MALAT1, an endogenous competitive RNA, regulates the expression of ZEB2 in clear cell renal carcinoma through sponging miR-200s, thereby facilitating the progression of renal carcinoma[9]. In lung adenocarcinoma, lncRNA MALAT1 can exert a cancer-promoting effect *via* the targeted inhibition on miR-204[10]. However, the role of lncRNA MALAT1 in septic lung injury has not been reported yet.

In this study, the mouse model of lncRNA MALAT1 knockdown was established *via* tail intravenous injection of lncRNA MALAT1 small-interfering RNA (siRNA), and the mouse model of septic lung injury was established using LPS, and then the role of lncRNA MALAT1 in the occurrence and development of septic lung injury and its mechanism were explored.

Materials and Methods

Grouping and Treatment of Laboratory Animals

A total of 60 male C57 mice aged 8-10 weeks old and weighing (25.31±2.44) g were randomly divided into Control group (n=20), septic lung injury group (LPS group, n=20), and lncRNA MALAT1 knockdown group (LPS+MALAT1 siRNA group, n=20) using a random number table. There were no statistically significant differences in the basic data, such as age and body weight, among the three groups. MALAT1 siRNA in a certain dose (2 mL/kg) was injected *via* caudal vein in MALAT1 siRNA group. After 3 d, LPS was intraperitoneally injected into mice in each group (10 mg/kg). After 12 d, the mice were executed, and the lungs were taken and stored at -80°C for later use. This study was approved by the Animal Ethics Committee of Henan University of Traditional Chinese Medicine Animal Center.

Inflammatory Cell Count in Bronchoalveolar Lavage Fluid (BALF)

The bronchoalveolar lavage was performed *via* tracheal intubation using phosphate-buffered saline (PBS) for 3 times, and the BALF was collected. The BALF samples were centrifuged at 3000 rpm and 4°C for 10 min. After the supernatant was discarded, the precipitated cells were resuspended in PBS, and the total cell count was detected using a hemocytometer. The number of neutrophils and macrophages was observed *via* Wright-Giemsa staining.

Detection of Expression of Related Genes Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA was extracted from cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA), the concentration and purity of RNA extracted were detected using an ultraviolet spectrophotometer, and the RNA with absorbance $(A)_{260}/A_{280}$ of 1.8-2.0 could be used. (2) The messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in the refrigerator at -80°C. (3) RT-PCR system: 2.5 μL 10 × Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L), and 19 μL ddH_2O. The amplification system of RT-PCR was the same as above. The primer sequences in RT-PCR are shown in Table I.

Hematoxylin-Eosin (HE) Staining

The lung tissues obtained in each group were placed in 10% formalin overnight, and then dehydrated and embedded in paraffin. Then, all lung tissues were sliced into 5 μm-thick sections, fixed on a glass slide and baked dry, followed by staining. According to the instructions, the sections were soaked in xylene, ethanol in gradient concentration and hematoxylin, respectively, and sealed with resin. After drying, the sections were observed and photographed under a light micro-

Table I. Primer sequences of each index in RT-PCR.

Target gene		Primer sequence
GAPDH	Forward	5'-GAGCTCAGCTCGCCTGGAGAAAC-3'
	Reverse	5'-TGCTGATCGTAGCCCTTTAGT-3'
LncRNA MALAT1	Forward	5'-TGCTGCCTTTTCTGTTCCTT-3'
	Reverse	5'-CTAGCTAGCTTTGGGTAGGGAAGT-3'
IL-1	Forward	5'-GTCCAACCCGTAAGGT-3'
	Reverse	5'-CGCTGCTGAGTCGATGCTAGCT-3'
IL-6	Forward	5'-ACGTAGCTAGCTAGTCGGTATG-3'
	Reverse	5'-TCGTAGCTTGGCTAGTCGATCG-3'
TNF-α	Forward	5'-TTGTGTTAGCTTAGCCCGATCGTA-3'
	Reverse	5'-CCGTCGTAAAAGCTAGTCGATC-3'

scope to detect the alveolar and alveolar interstitial structural changes in lung tissue sections.

Determination of Wet Weight/Dry Weight (W/D) Ratio of Lungs

At 12 h after LPS treatment, the lung tissues were collected, the residual connective tissues were removed, and the lung tissues were weighed immediately. Then the lung tissues were dried in an oven at 70°C for 48-72 h, and the dry weight of the lung was measured. The W/D ratio of lungs was calculated then.

Immunohistochemical Staining

The lung tissue sections were baked in the oven at 60°C for 30 min, deparaffinized with xylene (5 min ×3 times), and dehydrated with 100% ethanol, 95% ethanol, and 70% ethanol for 3 times. Then, the endogenous peroxidase activity was inhibited with hydrogen peroxide-methanol in a concentration of 3%. The tissues were sealed with goat serum for 1 h, incubated with the anti-p38 antibody (diluted at 1:100 with PBS) at 4°C overnight, and washed with PBS for 4 times on a shaker. Then, the secondary antibody was added, and the color was developed using diaminobenzidine. After that, 6 samples were randomly selected in each group, and 5 fields of view were randomly selected in each sample, followed by photography under the light microscope (200× and 400×).

Western Blotting

The lung tissues of mice in each group were fully ground in the lysis buffer, followed by ultrasonic lysis. Then, the lysis buffer was centrifuged, and the supernatant was taken and placed into the Eppendorf (EP) tube. The protein concentration was detected *via* ultraviolet spectrometry, and the protein samples were quantified to be the same concentration. The protein was sub-packaged and placed in the refrigerator at -80°C. After the total protein was extracted, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Then, the protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), incubated with the primary antibody at 4°C overnight, and then, incubated again with the goat anti-rabbit secondary antibody in a dark place for 1 h. The protein band was scanned and quantified using the Odyssey scanner, and the level of protein to be detected was corrected using glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the analysis of all data. Measurement data were expressed as mean ± standard deviation. The *t*-test was used for the comparison of data between two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of LncRNA MALAT1 in Lung Tissues of Rats With Septic Lung Injury

The expression level of lncRNA MALAT1 in lung tissues in Control group, LPS group, and LPS+MALAT1 siRNA group was detected. The results revealed that the expression level of lncRNA MALAT1 in lung tissues was significantly increased in LPS group ($p<0.05$), and the level of lncRNA MALAT1 in lung tissues was significantly inhibited after injection of MALAT1 siRNA *via* caudal vein ($p<0.05$) (Figure 1), indicating that the mouse model of MALAT1 knockdown was successfully induced.

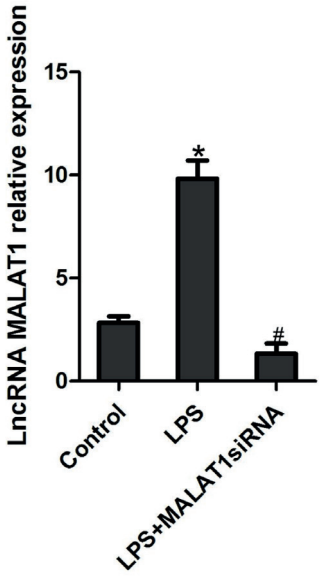

Figure 1. Expression of lncRNA MALAT1 in lung tissues of rats with septic lung injury. Control: Control group, LPS: septic lung injury group, LPS+MALAT1 siRNA: septic lung injury + MALAT1 knockdown group. There is a statistically significant difference *vs.* Control group (*p<0.05) and *vs.* LPS group (#p<0.05)

HE Staining Results of Lungs in Each Group of Mice

To evaluate the alveolar and alveolar interstitial microstructural changes in lungs in each group, HE staining was performed for lung tissues. The results showed that there were alveolar interstitial edema and wall thickening accompanied by infiltration of inflammatory cells in lung tissues in LPS group. After MALAT1 knockdown, the alveolar interstitial edema was significantly alleviated, the alveolar wall became thinner, and the infiltration of inflammatory cells was reduced (Figure 2), indicating that the knockdown of MALAT1 can alleviate the LPS-induced lung injury.

W/D Ratio of Lungs in Each Group of Mice

The W/D ratio of lungs in each group was detected, and it was found that the stimulation with LPS for 12 h could markedly increase the W/D ratio of mice (p<0.05), and it significantly declined after the expression of lncRNA MALAT1 was inhibited (p<0.05) (Figure 3), proving that the pulmonary edema in mice is significantly improved.

Influence of LncRNA MALAT1 Knockdown on Myeloperoxidase (MPO) Activity in Lung Tissues of Mice in Each Group

The activity of MPO in lung tissues of mice in each group was also detected. As shown in Figure 4, LPS stimulation could significantly increase the activity of MPO in lung tissues of mice (p<0.05), and it was reduced after lncRNA MALAT1 was inhibited in lung tissues of mice (p<0.05).

Influence of LncRNA MALAT1 Knockdown on Inflammatory Cells in BALF of Mice in Each Group

Moreover, the inflammatory cells in BALF of mice in each group were counted using the hemocytometer. As shown in Figure 5, the total number of inflammatory cells and the number of neutrophils and macrophages in BALF of mice were markedly increased after LPS stimulation (p<0.05), and they were significantly reduced after lncRNA MALAT1 knockdown (p<0.05).

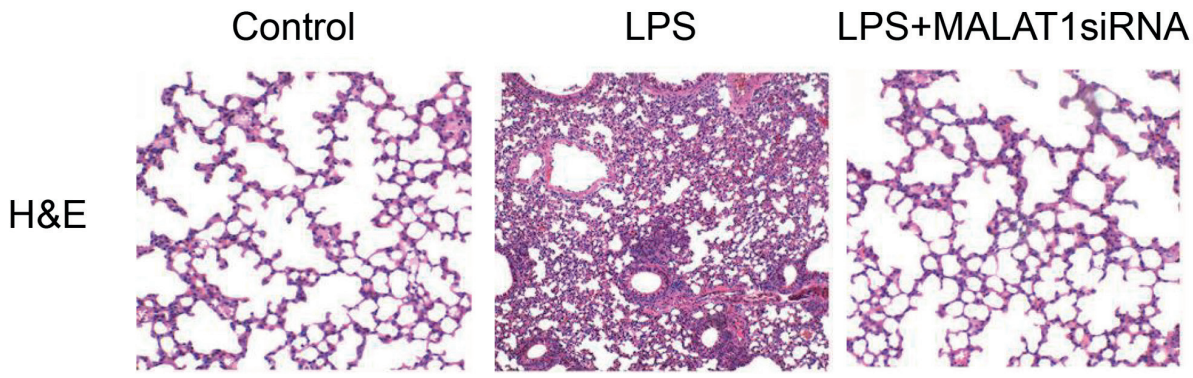

Figure 2. HE staining results of lungs in each group of mice (Magnification: 100×). Control: Control group, LPS: septic lung injury group, LPS+MALAT1 siRNA: septic lung injury + MALAT1 knockdown group.

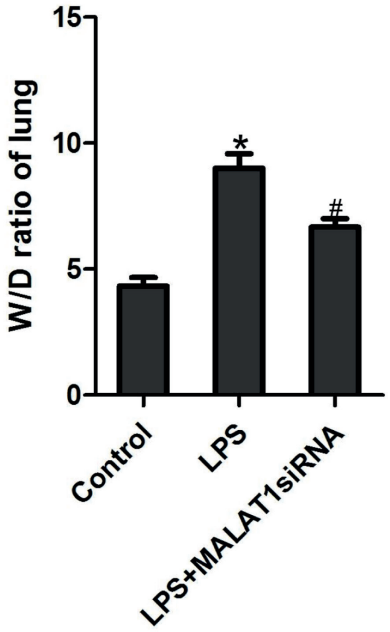

Figure 3. W/D ratio of lungs in each group of mice. Control: Control group, LPS: septic lung injury group, LPS+-MALAT1 siRNA: septic lung injury + MALAT1 knockdown group. There is a statistically significant difference *vs.* Control group (*p<0.05) and *vs.* LPS group (#p<0.05)

Expression of Inflammatory Cytokines in Lung Tissues of Mice in Each Group

The mRNA expression levels of IL-1, IL-6, and TNF-α in lung tissues of mice in each group were detected *via* RT-PCR. It was found that LPS stimulation could up-regulate the transcriptional levels of IL-1, IL-6, and TNF-α (p<0.05), which could be significantly reversed by the inhibition on lncRNA MALAT1 (p<0.05) (Figure 6).

Immunohistochemical Staining Results of p38 in Lung Tissues of Mice in Each Group

Considering the important role of a p38 MPAK signaling pathway in LPS-induced ALI, the expression of p38 MPAK in lung tissues of mice in each group was detected *via* immunohistochemical staining. It was found that the protein expression level of p38 MAPK in alveolar epithelial cells was markedly increased in LPS group, but it was significantly inhibited in LPS+MALAT1 siRNA group compared with that in LPS group (Figure 7).

Influence of LncRNA MALAT1 Knockdown on p38 MAPK/p65 NF-κB Signaling Pathway

Furthermore, the total protein (T) and phosphorylated protein (P) levels of p38 MAPK and p65 NF-κB in lung tissues of mice in each group were detected *via* Western blotting. As shown in Figure 8, the P-p38 MAPK/T-p38 MAPK ratio and P-p65 NF-κB/T-p65 NF-κB ratio in lung tissues of mice in LPS group were remarkably increased compared with those in Control group (p<0.05), but the lncRNA MA-

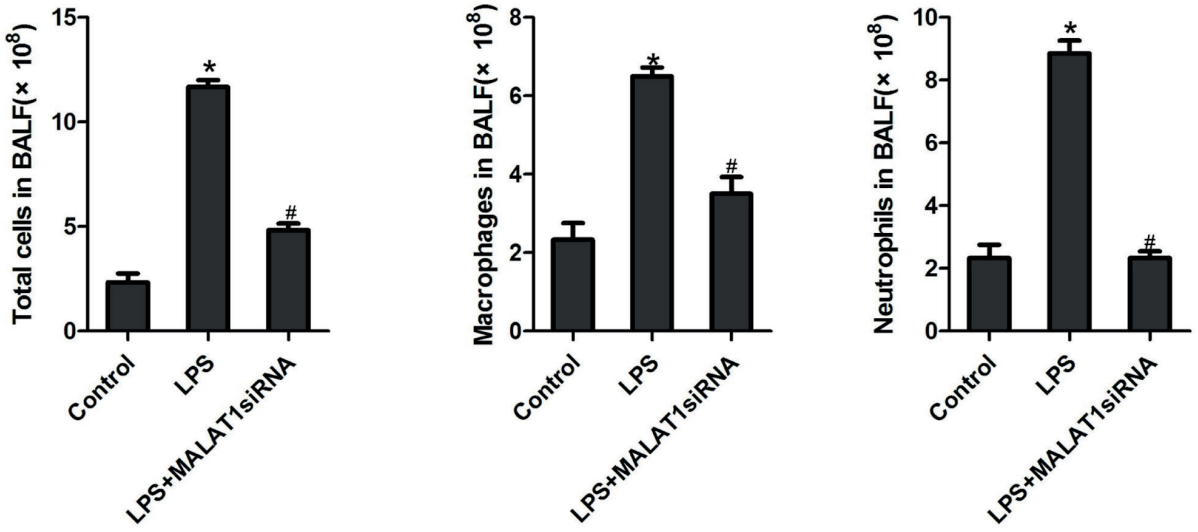

Figure 4. Influence of lncRNA MALAT1 knockdown on MPO activity in lung tissues of mice in each group. Control: Control group, LPS: septic lung injury group, LPS+MALAT1 siRNA: septic lung injury + MALAT1 knockdown group. There is a statistically significant difference *vs.* Control group (*p<0.05) and *vs.* LPS group (#p<0.05)

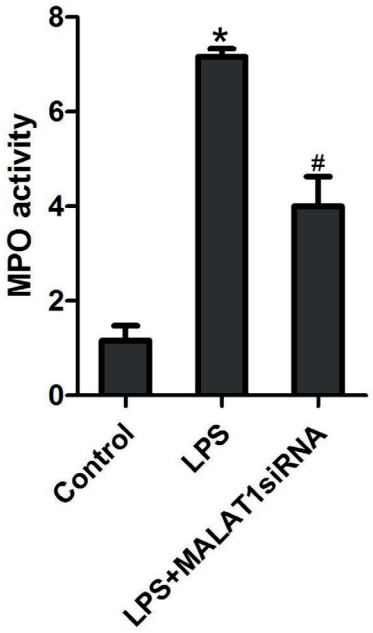

Figure 5. Influence of lncRNA MALAT1 knockdown on inflammatory cells in BALF of mice in each group. Control: Control group, LPS: septic lung injury group, LPS+MALAT1 siRNA: septic lung injury + MALAT1 knockdown group. There is a statistically significant difference *vs.* Control group (*p<0.05) and *vs.* LPS group (#p<0.05)

LAT1 knockdown could remarkably reverse the phosphorylation levels of p38 MAPK and p65 NF-κB.

Discussion

Sepsis is a highly fatal disease, but there are no effective treatment means currently. The lung is one of the most susceptible organs to sepsis[11]. According to previous studies[12,13], about 45% of patients with severe sepsis suffer from ALI, and the lung function level directly determines the survival rate of patients. Current studies have revealed that the pathogenesis of septic lung injury mainly includes: 1) microcirculation and microvascular abnormalities, 2) autonomic nervous imbalance, 3) metabolic abnormality, 4) mitochondrial dysfunction, 5) cell death, 6) activation of inflammatory cells and factors, 7) damage of alveolar epithelial cells. The inflammatory response is the most common and the most studied mechanism currently[14,15]. In the serum and lung tissues of patients with LPS-induced ALI and laboratory mouse models, the levels of IL-1, IL-6, and TNF-α are significantly increased[16]. Therefore, inhibiting the production of these inflammatory factors can significantly alleviate LPS-induced lung injury.

NF-κB, one of the important inflammatory gene transcription factors, is involved in the transcriptional regulation of expression of various inflammatory factors, such as IL-1, IL-6, and TNF-α[17]. Previous studies showed that lncRNA MALAT1 can aggravate the sepsis-induced cardiac insufficiency and inflammation through the competitive binding to miR-125b and the acti-

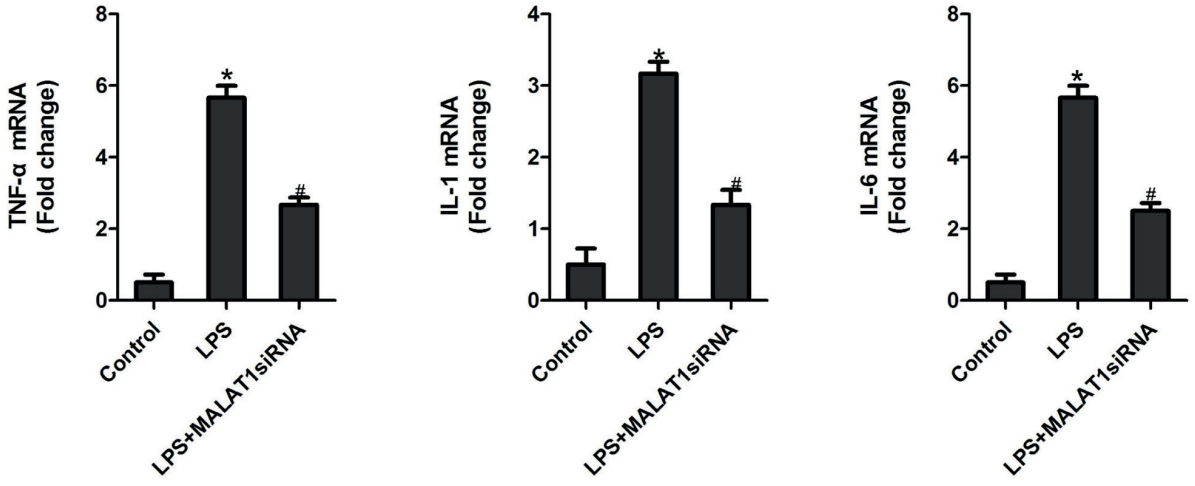

Figure 6. Influence of lncRNA MALAT1 knockdown on mRNA expression of pro-inflammatory cytokines in lung tissues of mice in each group. Control: Control group, LPS: septic lung injury group, LPS+MALAT1 siRNA: septic lung injury + MALAT1 knockdown group. There is a statistically significant difference *vs.* Control group (*p<0.05) and *vs.* LPS group (#p<0.05).

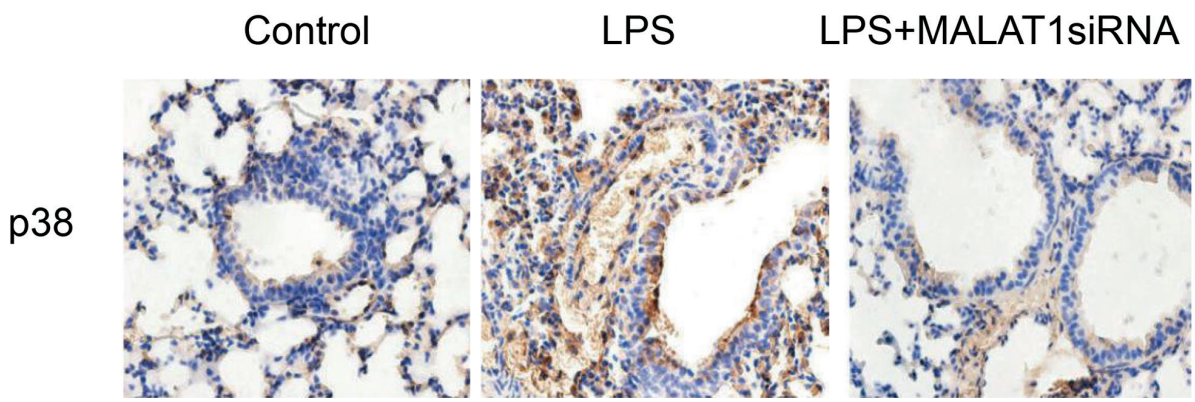

Figure 7. Influence of lncRNA MALAT1 knockdown on p38 expression in lung tissues of mice in each group (Magnification: 100×). Control: Control group, LPS: septic lung injury group, LPS+MALAT1 siRNA: septic lung injury + MALAT1 knockdown group. There is a statistically significant difference *vs.* Control group (*p<0.05) and *vs.* LPS group (#p<0.05).

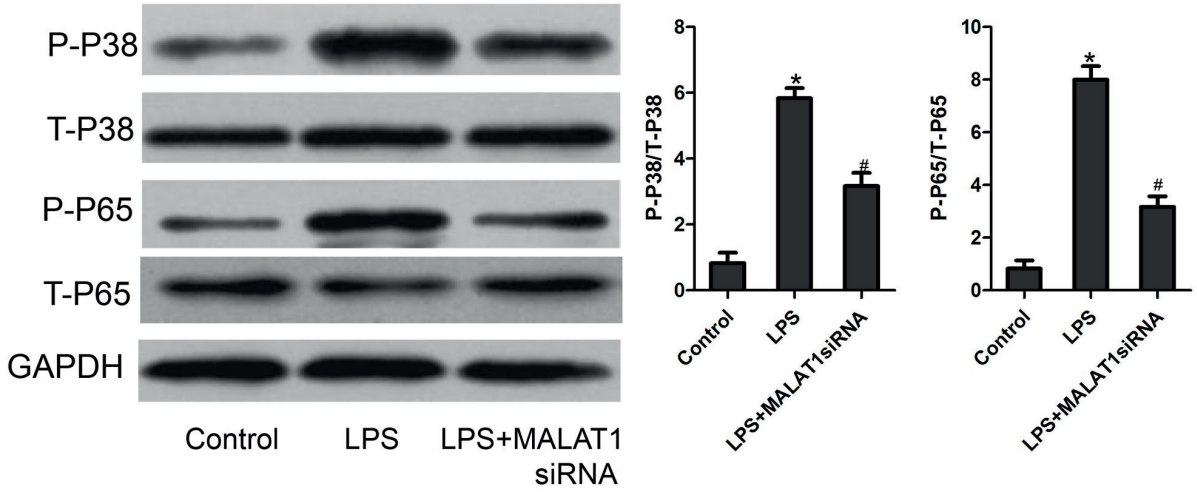

Figure 8. Influence of lncRNA MALAT1 knockdown on p38 MAPK/p65 NF-κB signaling pathway. Control: Control group, LPS: septic lung injury group, LPS+MALAT1 siRNA: septic lung injury + MALAT1 knockdown group. There is a statistically significant difference *vs.* Control group (*p<0.05) and *vs.* LPS group (#p<0.05).

vation of the p38 MAPK/p65 NF-κB signaling pathway[18]. It was reported previously that LPS stimulation can induce the expression of lncRNA MALAT1 in macrophages *in vitro*, thereby activating NF-κB and transcribing various downstream inflammatory cytokines[19]. In sepsis-induced acute kidney injury, lncRNA MALAT1 can lead to the massive release of various inflammatory cytokines in the kidney through competitively adsorbing miR-125b and inducing phosphorylation of NF-κB[20]. The above studies indicate that lncRNA MALAT1, as an important lncRNA, can competitively adsorb multiple miRNAs to regulate the inflammatory response in the body.

In this study, lncRNA MALAT1 in mice was knocked down for the first time *via* tail intravenous injection of MALAT1 siRNA, and LPS in a certain dose was intraperitoneally injected to establish the model of septic lung injury. The experimental results showed that the knockdown of MALAT1 could significantly relieve the LPS-induced lung injury, which was mainly manifested as the inhibition of increased W/D ratio of lungs at 12 h after LPS injection. Moreover, the HE staining results also revealed that the knockdown of MALAT1 significantly inhibited LPS-induced lung tissue damage, which was mainly manifested as the changes in alveolar wall thickness and the reduced number of inflammatory cells in the alveolar interstitium. Furthermore, the number of inflammatory cells (neutrophils and macrophages) in BALF of mice in each group was

evaluated using the hemocytometer, and it was found that the total number of inflammatory cells and the number of neutrophils and macrophages in BALF of mice significantly declined after knockdown of lncRNA MALAT1 compared with those in LPS group. Besides, the knockdown of lncRNA MALAT1 could also affect the MPO activity in lung tissues of sepsis mice. At the same time, the levels of pro-inflammatory factors in lung tissues of mice in each group were further detected *via* RT-PCR. The results manifested that inhibiting lncRNA MALAT1 could markedly reverse the LPS-induced transcriptional activation of inflammatory factors in lung tissues of mice. Finally, the mechanism of lncRNA MALAT1 knockdown in affecting septic lung injury was explored, and it was found that the abnormal increase in phosphorylation of p38 MAPK and p65 NF-κB in LPS+MALAT1 siRNA group was reversed. However, there were still some limitations in this study: 1) The cell experiment was not designed for verification, 2) the lncRNA MALAT1 level in serum of mice (or people) was not detected in Control group and LPS group, and 3) the direct target of lncRNA MALAT1 was not found.

Conclusions

We revealed the role of lncRNA MALAT1 in LPS-induced lung injury in mice for the first time, so lncRNA MALAT1 is expected to be an important target for the prevention and treatment of septic lung injury in the future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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