

Long non-coding RNA MALAT1 protects epithelial cells from LPS-induced acute lung injury by regulating miRNA-181a-3p /B

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Research

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Abstract

Background Long non-coding RNA metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) plays an important role in the pathophysiological process of inflammation. We aimed to investigate MALAT1 and its function in modulating miRNA-181a-3p and Bcl-2 in lipopolysaccharide (LPS)-induced acute lung injury (ALI).

Methods We analysed MALAT1 in ALI patients, as well as the alveolar epithelial cell models of LPS-induced injury. The expression of MALAT1 and miRNA-181a-3p were evaluated by qRT-PCR, and Bcl-2 was measured by western blot. Inflammatory factors tumour necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6 mRNA levels were also quantified by qRT-PCR. Luciferase reporter assay was used to verify direct interaction between MALAT1 and miRNA-181a-3p, or miRNA-181a-3p and Bcl-2. Transferase-mediated deoxyuridine triphosphate-biotin nick end labelling (TUNEL) assay was performed to detect alveolar epithelial cell apoptosis.

Results Serum MALAT1 and Bcl-2 levels decreased in ALI patients, whereas miRNA-181a-3p, TNF- α , IL-1 β and IL-6 levels increased ($P < 0.01$). MALAT1 was inversely correlated to miRNA-181a-3p ($R = -0.508$, $P = 0.0031$) in ALI patients. SiMALAT1 transfection upregulated miRNA-181a-3p level and downregulated Bcl-2 expression, aggravating alveolar epithelial cell apoptosis. MiRNA-181a-3p downregulated the Bcl-2 expression both in LPS-induced ALI rats and alveolar epithelial cells, as well as promoted apoptosis. TNF- α , IL-1 β and IL-6 levels increased after LPS stimulation and siMALAT1 transfection.

Conclusions The results demonstrate that LPS-induced ALI decreases lncRNA MALAT1, increases miRNA-181a-3p and inflammatory factor expression, downregulates the Bcl-2 level and promotes alveolar epithelial cell apoptosis. Down-regulation of MALAT1 may erase the protection of alveolar epithelial cells from LPS-induced ALI via up-regulating of miRNA-181a-3p.

Background

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), characterized by heterogeneous pathologic factors, are recognized as a severe respiratory dysfunction, and are associated with high morbidity and mortality[1]. Globally, ARDS affects approximately 3 million patients annually, accounting for 10% of intensive care unit (ICU) admissions, and 23% of patients receiving mechanical ventilation in the ICU[2]. ALI is characterized by a sustained and uncontrolled inflammatory process in the lung, and increased pulmonary alveolar capillary membrane permeability, leading to edema, hypoxemia, apoptosis and eventually respiratory failure[3, 4]. However, the potential mechanism of ALI and ARDS remains uncertain.

Recently, non-coding RNAs have been reported to play an important role in inflammatory mediator release and lung injury, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs)[5]. LncRNAs have recently been shown to play key roles in pathophysiological processes in respiratory diseases, such as cancer, chronic obstructive lung disease (COPD), asthma, pulmonary fibrosis and pulmonary

hypertension[6, 7]. They may function as “sponges” of miRNAs to downregulate the expression of specific target miRNAs[8]. It is also demonstrated that lncRNAs can regulate target genes by interacting with protein[9], DNA[10] and other RNAs[11].

Metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) is an lncRNA involved in many biologic processes. MALAT1 has been firstly identified as a prognostic marker of patient survival in stage I non-small-cell lung cancer[12]. The high expression levels of conserved MALAT1 are involved in the physiological progress of hypoxia[13], endothelial[14] and epithelial cell dysfunction[15]. A recent study also demonstrated that MALAT1 promoted inflammatory response and aggravated lipopolysaccharide (LPS)-induced ALI via downregulated miRNA-146a[16]. However, another study finds that MALAT1 decreases in the ALI rat model. Upregulation of MALAT1 alleviates the inflammation and cell apoptosis of pulmonary microvascular endothelial cells via the nuclear factor (NF)- κ B and p38 mitogen-activated protein kinase (MAPK) signalling pathways[17]. It is strongly suggested that MALAT1 plays an important role in ALI. Furthermore, because lncRNAs functions as competing endogenous RNAs (ceRNAs) or endogenous “sponge” RNAs in regulating the expression and biological functions of miRNAs, we have predicted miRNA-181a-3p as a target miRNA of MALAT1 by bioinformatics[8]. In addition, a study reports that inhibition of miRNA-181a, which could target the 3'UTR of B cell lymphoma/leukaemia-2 (Bcl-2) significantly aggravates LPS-induced ALI[6], but the mechanism underlying the genetic modulation of miRNA-181a is unclear.

We thus make the following hypothesis: Bcl-2 suppresses the development of LPS-induced ALI through inhibition of alveolar epithelial cell apoptosis. LPS leads to the development of ALI by suppressing MALAT1, which downregulates miRNA-181a-3p. In addition, miRNA-181a-3p downregulates the target gene Bcl-2.

Materials And Methods

Patient population

This was a prospective study conducted at Shanghai East Hospital, Tongji University School of Medicine in China between March 1, 2019 and December 20, 2019. The study followed the Ethical Principles for Medical Research Involving Human Subjects outlined in the Declaration of Helsinki. The ethics approval was obtained from the Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine. Written informed consent was obtained from all subjects. The inclusion criteria were as follows: 1) ages \geq 18 and \leq 80 years old; 2) ALI or ARDS. Patients were excluded if they were 1) premature; 2) had pulmonary fibrosis; 3) had abnormal liver or renal function; 4) showed pulmonary inflammation before the surgery; 5) had pulmonary edema due to cardiac dysfunction; 6) needed extracorporeal membrane oxygenation (ECMO) support after surgery. ALI was diagnosed according to the Berlin Definition of ARDS[18]. For each patient, 2 ml of fresh blood was drawn into a vacuum tube containing ethylenediaminetetraacetic acid (EDTA) within 30 minutes, 1 day and 3 days after ALI diagnosis, respectively. The serum was separated, and the tube was sent immediately to the molecular laboratory.

Data collection and definitions

During the ICU period, all patients underwent routine hemodynamic and blood gas surveillance. Mechanical ventilation (MV) if necessary in the ICU was performed by standard procedure and routines. Demographic and preoperative data were collected, including the patient's gender, age, weight, pulse oxygen saturation (SPO₂), duration of MV and the ratio of fraction of inspired oxygen to oxygen pressure (PaO₂/FiO₂). A bedside chest radiograph was taken every day. The left ventricular function was evaluated by the ejection fraction (EF) through echocardiography performed routinely. The Berlin Definition of ARDS was used to categorise the patients into the ALI and non-ALI groups[18].

LPS-induced ALI rat model

Forty-two male Sprague-Dawley (SD) rats (400–450 g) were divided randomly into six groups: control, sham, LPS, LPS plus Bcl-2, LPS plus anti-Bcl-2 and LPS plus siMALAT1 (n=6 per group). Firstly, rats were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.), then rats received intraperitoneal injection of 5 mg/kg LPS (Escherichia coli LPS serotype 0111:B4; Sigma-Aldrich, Saint Louis, USA) in LPS group. The rats in group without LPS received the same volume of 0.9% saline solution. Bcl-2, anti-Bcl-2 and siMALAT1 (Invitrogen, Carlsbad, CA, USA) were transfected with lentivirus for 48 h before the procedure and LPS injection, respectively. After LPS administration for 12 h, the rats were anaesthetized with 10% chloral hydrate and sacrificed. Then, the lungs of rats were harvested and stored in liquid nitrogen for later measurements[19]. All animals received humane care, and procedures were approved by the Animal Care and Use Committee of the Tongji University School of Medicine. Rats were kept at 22°C to 24°C and had no food and water restrictions. Rats were fed with a mixture of wheat, soybean oil, salt, ferric citrate and vitamins. The light cycle in the rat room was set at a circadian rhythm of 12h on and 12h off.

Arterial blood gas measurements

Arterial blood was obtained (0.3 ml) in heparinised syringes from the femoral artery of rats at 30 min before LPS treatment and 2 h and 4 h after LPS, respectively. To obtain PaO₂ values, the blood was harvested from the left femoral artery. The sample was measured with a GEM Premier 3000 gas analyser (Instrumentation Laboratory, Milan, Italy) immediately.

Western blot analysis

The superior lobe of left lung was harvested from the rats and stored in -80 °C liquid nitrogen. Total proteins were extracted, and their concentrations were measured by protein assay. Cell lysates (20µg protein/lane) were separated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were subsequently blocked with 5% skim milk in a phosphate-buffered saline with Tween (PBST) solution (100mM NaCl, 50mM Tris, 0.1% Tween-20, PH 7.5) for 1h at room temperature and probed overnight at 4 °C with appropriate primary antibodies (anti-Bcl-2, Santa Cruz, CA, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit immunoglobulin G (IgG) and anti-rat IgG (Sigma-Aldrich, Saint Louis, USA) were used as secondary antibodies according to

the primary antibodies. Immunoreactive bands were visualised by enhanced chemiluminescence. The antibody for β -actin (1:1000 dilution; Beyotime, China) was used as the endogenous control.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was isolated from tissues using TRIzol reagent (Invitrogen, USA). All samples were centrifuged at 4,000 Xg for 10 min at 4°C in a refrigerated microfuge. The total RNA was extracted from 350 μ l of plasma/serum using a tRNA extraction kit (Ambion, mirVana PARIS, USA). The RNA was then eluted with 50 μ l of Ambion elution solution. The samples were measured within 30 min before ALI diagnosis and 1 and 3 days after ALI occurred. Gene expression in each sample was normalised to glyceraldehyde 3-phosphate dehydrogenase (GADPH) expression. The primer sequences used were as follows: for GAPDH-forward, 5'-GCAAGTTCAACGGCACAG-3' and GAPDH-reverse, 5'-CAGTAGACTCCACGACAT-3'; MALAT1-forward, 5'-GCTCTGTGGTGTGGGATTGA-3' and MALAT1-reverse, 5'-GTGGCAAATGGCGGACTTT-3'; miRNA -181a-3p -forward, 5'-ACACTCCAGCTGG.GAACATTCAACGCTGTCGG-3', and miRNA-181a-3p -reverse 5'-TGGTGTCTGGAGTCG-3'; Bcl-2-forward, 5'-ACTGAGTACCTGAACCGGCATC-3' and Bcl-2-reverse, 5'-GGAGAAATCAAACAGAGGTCGC-3'. TNF- α -forward, 5'-GCTGCACTTTGGAGTGATCG-3' and TNF- α -reverse, 5'-TCACTCGGGGTTTCGAGAAGA-3'. IL-1 β -forward, 5'-CTGAGCTCGCCAGTGAAATG-3' and IL-1 β -reverse, 5'-TGTCCATGGCCACAACAAC-3'. IL-6-forward, 5'-TTCTACAGACTACGGTTTGAG-3' and IL-6-reverse, 5'-GGATGACACAGTGATGCT-3'. Real-time PCR reactions were performed using the ABI7500 system (Applied Biosystems, Foster City, CA, USA). The relative expression fold change of mRNAs was calculated by the $2^{-\Delta\Delta C_t}$ method.

Histopathological examination

The superior lobe of the left lung was harvested when rats were sacrificed, fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Tissues were cut into a series of microsections (4 μ m), and then stained with haematoxylin and eosin (HE) using standard protocols. Immunohistochemistry staining with Bcl-2 antibody (anti-Bcl-2, Santa Cruz, CA, USA) was performed appropriately. In brief, immunostaining for Bcl-2 was analyzed under a light microscope (Olympus CH30, Olympus, Tokyo, Japan) at a final magnification of 400x. Positive staining was defined as brown staining of epithelial cells and negative staining was the complete absence of staining[20]. Pathological changes of lung tissues were observed under a light microscope (BXFM; Olympus, Tokyo, Japan). The severity of lung injury was evaluated by a semi-quantitative histological index of quantitative assessment (IQA). This assessment was divided into four grades from 0–3 means: minimal, mild, moderate, and severe, respectively. The items were alveolar edema, infiltration of neutrophils and hyaline membrane formation[21]. The standard lung injury score was measured by a blinded pathologist to objectively quantify the lung injury.

Measurement of alveolar epithelial permeability

To evaluate the alveoli-capillary barrier function, ^{99m}Tc-labeled albumin was prepared via a commercial kit (Vasculocis; Gif sur Yvette, France). Paper chromatography was performed to verify the amount of free ^{99m}Tc. The solution was instilled in one lung to compare pulmonary microvascular permeability between the instilled and the noninstilled lung[22].

Lung wet/dry weight ratio

As an indication of lung edema, the lung wet/dry ratio was calculated by dividing the wet weight by the dry weight in LPS-induced rats. The lower lobe of the left lung was excised and weighed to obtain the wet weight. Drying was carried out until the weight of the sample becomes consistent at least twice. The lobe was weighed again to obtain the dry weight.

MALAT1 transfection for rats

The siRNA (sense: 5'-GGAGUACCCUGAAGCUAUUU-3'; antisense: 5'-UUAUAGCUUCAGGGUACUCCUU-3') targeting MALAT1 was synthesised by GenePharma (Shanghai, China). The scrambled siRNA (sense: 5'-UUCUCCGAACGUGUCACGUUU-3'; antisense: 5'-ACGUGACACGUUCGGAGAAUU-3') was used as the control. All siRNAs were dissolved by diethylpyrocarbonate (DEPC)-treated water to a final concentration of 40 µg/ml. Lentivirus vectors overexpressing MALAT1 (200 nM) were injected through tail vein 48 h before experiments. In brief, The lentiviral pcDNA3.1-YFP-puro-MALAT1 expression vector ("LV-MALAT1") was designed and synthesized by GenePharm Co. (Shanghai, China). The LV-MALAT1 sequence was sub-cloned into the plasmid to generate pCDH-CMV-MALAT1-EF1-coGFP-puro, which was transfected into rats via tail vein with the lentiviral packaging vectors, psPAX2 and pMD2.G, to produce the LV-MALAT1.

MiRNA-181a-3p modulation

MiRNA-181a-3p mimics and inhibitors (Dharmacon, 50 nM) were used to upregulate or downregulate the miRNA-181a-3p expression. The sequences of miRNA-181a-3p mimics of 5'-UAACACUGUCUGGUAACGAUGU-3' and inhibitor as 5'-ACAUCGUUACCAGACAGUGUUA-3' were obtained from GenePharma (Shanghai, China). Oligo transfection was performed according to the manufacturer's instructions. Briefly, 1 × 10⁵ cells per well were transfected with 1 OD miRNA-181a-3p or AMOs using Lipofectamine2000 Reagent (Invitrogen, USA) for 24 h before animal experiments via tail vein and for 18 hours after 24-hour serum starvation for alveolar epithelial cells.

Cell culture and transfection

Rat alveolar epithelial and HEK 293T (human embryonic kidney) cell lines were purchased from the Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin in an atmosphere of 5% CO₂ at 37°C.

siRNA against MALAT1 (si-MALAT1), siRNA control (si-control), pcDNA-MALAT1 and pcDNA empty vector were purchased from Ambion (Foster City, CA, USA). MiRNA control (vector), miRNA-181a-3p mimic and

miRNA-181a-3p inhibitor (anti-miRNA-181a-3p) were purchased from GenePharma (Shanghai, China). Cell transfection with oligonucleotides or vectors into epithelial cells was transfected by Lipofectamine 2000 (Invitrogen, USA) for miRNA transfection for 24 h before LPS treatment. MALAT1 and siMALAT1 transfection were conducted for 48 h before LPS treatment.

LPS treatment of alveolar epithelial cells

We employed the LPS-treated alveolar epithelial cell model. Alveolar epithelial cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM high-glucose medium (HyClone, Beijing, China) and supplemented with 10% foetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. LPS was incubated with cells in glucose-free DMEM medium.

Measurement of alveolar epithelial apoptosis induced by LPS

Rat alveolar epithelial cells cultured in 0.1% FBS were treated with LPS, miRNA-181a-3p or MALAT1. Afterwards, cell apoptosis was measured by TUNEL staining. TUNEL staining was done using the in situ cell death detection kit (Roche, Indianapolis, USA) according to the manufacturer's protocol. The number of TUNEL-positive cells were counted under a fluorescence microscope. Results were shown from the data of five independent experiments ($n = 6$) with 20 random microscopic fields from each sample.

Identification of target genes of MALAT1 and miRNA-181a-3p

Previous studies have shown that lncRNAs function as ceRNAs or “sponges” to modulate miRNAs[8]. We used the Starbase V2.0 (<http://starbase.sysu.edu.cn/>) to determine that miRNA-181 is the potential target RNA of MALAT1. Starbase V2.0 also predicted that Bcl-2 is the target gene of miRNA-181a-3p.

Dual-luciferase reporter assay

We used dual-luciferase reporter assay to further investigate the interaction of miRNA-181a-3p with MALAT1 and Bcl-2. The fragments of the 3'-UTR of Bcl-2 mRNA containing the wild-type (WT) or mutant (Mut) were amplified and used in the dual-luciferase reporter assay system (Promega, WI, USA). siRNA-Bcl-2 (si-Bcl-2) was obtained using the sequence 5'-GCAAUGACUCAGAUGCAUATT-3', which was synthesised by GenePharma (Shanghai, China). To generate the over-expressing vector of Bcl-2, the PCR products containing the Bcl-2 were similarly digested and inserted into pcDNA3.1, and then plasmid pcDNA3.1-Bcl-2 was obtained. The primer used in Bcl-2 PCR was sequenced as follows: sense: 5'-ATGTCCATAATTATATGGAACATTTA-3', and antisense: 5'-TAAGTTGATGACCCATTATTAACCA-3'. Transient transfection was performed using the Lipofectamine 2000 reagent in antibiotic-free Opti-MEM medium following the manufacturer's protocol. All experiments were performed in duplicate and repeated at least 3 times.

Statistical analysis

Statistical analyses were performed using SPSS statistics, version 17.0 (IBM Inc., Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). The results were presented as mean±SD if normal distribution or medians and interquartile ranges if non normal distribution for continuous variables, and as percentage for categorical variables. Continuous data were tested for normal distribution with the one-sample Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was conducted in multiple group comparison. Fisher's exact test was used to compare categorical data as appropriate. A Pearson correlation test was performed to determine the correlation between biomarker data and clinical parameters. The multivariate variables included age, body mass index (BMI), sex, ICU duration and the concentrations of MALAT1, miRNA-181a-3p and Bcl-2, as well as inflammatory factors. A P value < 0.05 was considered statistically significant.

Results

Correlation of MALAT1 with miRNA-181a-3p, Bcl-2 and inflammatory factors in ALI patients

Three hundred and sixty-eight patients were recruited in the present study. ALI occurred in 38 patients (10.3% of total). Characteristics of patients were comparable between groups with and without ALI (Table. 1). MALAT1 and Bcl-2 expression in serum were inversely associated with occurrence of ALI (both $P < 0.01$), whereas miRNA-181a-3p in serum was positively associated with ALI ($P < 0.01$). Nevertheless, the mRNA levels of TNF- α , IL-1 β and IL-6 were increased in the serum of ALI patients 1 and 3 days after ALI, respectively (both $P < 0.01$, Figure. 1). Meanwhile, to determine the expression changes of mRNA translation, we used western blot to measure protein levels. As Figure. 2 A and B show, Bcl-2 protein expression decreased in serum of ALI patients.

Moreover, the expression level of MALAT1 was inversely correlated with miRNA-181a-3p expression in serum in ALI patients (Figure. 3, Pearson's correlation between MALAT1 and miRNA-181a-3p, $R = -0.508$, $P = 0.0031$).

Rat model of LPS-induced ALI

To investigate the effect of LPS on ALI, we established a LPS-induced ALI rat model. In the sham group, HE staining showed that the alveolar wall was intact and very thin. In the LPS-treated group, the performance of acute inflammatory response, such as the alveolar wall not being intact, and inflammatory cells increased (Figure. 4).

We analysed artery blood gas and W/D ratio of lungs after ALI and found that there was no significant difference between the ALI group and the sham group in terms of PO_2 and PCO_2 , whereas the W/D ratio was increased in the ALI group (Table. 2).

Bcl-2 decreased inflammatory-related factors in rats

To explore the effect of Bcl-2 on ALI, we injected LPS-induced ALI rats with Bcl-2 or anti-Bcl-2. Compared to control, anti-Bcl-2 increased inflammatory infiltration (Figure. 5 A and B). Bcl-2 decreased the expression of TNF- α , IL-1 β and IL-6, whereas anti-Bcl-2 increased their expression (Figure. 5 C).

Effect of miRNA-181a-3p on Bcl-2 and inflammatory factor expression in lungs of rats undergoing ALI

After miRNA-181a-3p mimic was transfected to rat for 48 h, Bcl-2 mRNA expression was significantly decreased, whereas miRNA-181a-3p inhibitor increased the Bcl-2 mRNA expression (Figure. 6 A). As Figures. 6 B and C show, miRNA-181a-3p mimic downregulated, whereas miRNA-181a-3p inhibitor upregulated the protein expression of Bcl-2. In addition, levels of TNF- α , IL-1 β and IL-6 expression increased after miRNA-181a-3p mimic transfection, whereas levels of inflammatory factors decreased after miRNA-181a-3p inhibitor transfection (Figure. 6 D). As Figure. 6 E shows, the protein expression of TNF- α , IL-1 β and IL-6 was significantly increased after miRNA-181a-3p mimic stimulation and decreased after miRNA-181a-3p inhibitor administration. Furthermore, miRNA-181a-3p mimic aggravated alveolar epithelial apoptosis, whereas miRNA-181a-3p inhibitor alleviated it (Figure. 6 F).

Effects of MALAT1 on the expression of miRNA-181a-3p, Bcl-2, inflammatory factors and apoptosis in ALI rats

We found that MALAT1 transfection decreased miRNA-181a-3p expression and increased Bcl-2 expression in LPS-induced ALI in rats. After siMALAT1 was transfected to rats, miRNA-181a-3p expression levels increased and as a result, Bcl-2 expression decreased accordingly (Figure. 7 A). As Figure. 7 B shows, MALAT1 transfection downregulated the inflammatory factors TNF- α , IL-1 β and IL-6, whereas siMALAT1 reversed the effect of MALAT1 (Figure. 7 C). Furthermore, MALAT1 transfection alleviated alveolar epithelial apoptosis, whereas siMALAT1 aggravated it (Figure. 7 D).

MiRNA-181 modulated the expression of Bcl-2 and inflammatory factors in LPS-treated alveolar epithelial cells

After transfection with miRNA-181a-3p mimic or inhibitor for 24 h, alveolar epithelial cells showed LPS insult. As Figure. 8 A shows, miRNA-181a-3p mimic decreased the mRNA level of Bcl-2, and miRNA-181a-3p inhibitor increased it. Accordingly, miRNA-181a-3p mimic also downregulated the protein level of Bcl-2 and miRNA-181a-3p inhibitor reversed it (Figures. 8 B and C). However, no dramatic decrease of endogenous MALAT1 was detected after transfection of miRNA-181a-3p mimics. Indeed, increased or decreased miRNA-181a-3p expression with miRNA-181a-3p mimic or inhibitor did not affect the expression of MALAT1 (Figure. 8 A). When miRNA-181a-3p mimic was transfected to alveolar epithelial cells, the level of inflammatory factors TNF- α , IL-1 β and IL-6 increased after LPS treatment (Figure. 8 D and E).

MALAT1 inversely regulated miRNA-181 and Bcl-2 expression in LPS-treated alveolar epithelial cells

To explore the interaction between MALAT1 and miRNA-181a-3p, we conducted a bioinformatics analysis of the lncRNA sequence and miRNA for recognition sequences using Starbase V2.0

(<http://starbase.sysu.edu.cn>) and found that miRNA-181a-3p has a binding site for MALAT1 (Figure. 9). We next assessed whether miRNA-181a-3p could be negatively regulated by MALAT1. Alveolar epithelial cells were transfected with MALAT1 or siMALAT1 for 48 h via lentivirus. After 24 h of exposure to LPS, miRNA-181a-3p was examined by qRT-PCR, and we found MALAT1 knockdown increased miRNA-181a-3p and MALAT1 stimulation decreased the expression of miRNA-181a-3p. In contrast, the mRNA level of Bcl-2 was upregulated after transfection of MALAT1 and downregulated after transfection of siMALAT1 (Figure. 10 A). Furthermore, transfection of MALAT1 decreased epithelial cell apoptosis, whereas siMALAT1 increased apoptosis (Figure. 10 C).

Effects of MALAT1 on inflammatory factors and apoptosis in LPS-treated alveolar epithelial cells

We found that MALAT1 decreased the protein levels of TNF- α , IL-1 β and IL-6 in alveolar epithelial cells treated with LPS, and siMALAT1 reversed the effect, which was verified by western blot measurement (Figure. 10 B).

MALAT1 inversely regulated miRNA-181 expression in HEK293 cells

A recent study suggested that lncRNAs could serve as a competing endogenous RNAs (ceRNAs) to modulate miRNA expression[8]. Thus, we predicted the potential miRNA binding sequence for MALAT1 using Starbase V2.0. As displayed in Figure 8, there were putative binding sites of miRNA-181a-3p in MALAT1 transcripts. To further verify the interaction between MALAT1 and miRNA-181a-3p, we established luciferase reporter plasmids containing the miRNA-181a-3p-binding site wild- or mutant-type MALAT1 to co-transfect with miRNA-181a-3p mimic or miRNA-control into HEK293 cells. The luciferase activities of pGL3-MALAT1-WT were significantly inhibited in miRNA-181a-3p-overexpressing HEK293 cells, but miRNA-181a-3p transfection exhibited no inhibitory effect on luciferase activities of pGL3-MALAT1-MUT (Figure. 11 A). Furthermore, qRT-PCR was carried out to detect the expression of miRNA-181a-3p in HEK293 cells transfected with si-MALAT1 or pcDNA-MALAT1. The results showed that MALAT1 knockdown markedly improved miRNA-181a-3p expression, while MALAT1 overexpression distinctly suppressed miRNA-181a-3p expression in HEK293 cells. The result of dual-luciferase assay showed that miRNA-181 significantly reduced luciferase activity for the wild-type reporter vectors in the HEK293 cells, but mutagenesis of the predicted miRNA-181a-3p target sites abolished that effect (Figure. 11 B). These results verified the predicted miRNA-181a-3p target site on MALAT 1 in vitro.

Discussion

The present study demonstrated that lncRNA MALAT1 expression decreased during the LPS treatment period, upregulating target miRNA-181a-3p, which, in turn, downregulated target anti-apoptotic Bcl-2 both in the LPS-induced ALI rat and alveolar epithelial cells, and as a result, deteriorating epithelial cell apoptosis and LPS-induced ALI.

In the present study, only 10 percent patients manifested ALI, consistent with epidemic analysis and data reports[2]. There were more COPD cases in the ALI group compared with the non-ALI, but the difference

was not significant. The occurrence of pulmonary infection in the ALI group was higher than in no-ALI group. For MALAT1 and Bcl-2 concentration, the relative expression was lower in ALI group than in no-ALI group. However, the level of miRNA-181a-3p and inflammatory factors were higher in ALI patients, demonstrating that these non-coding RNA participate the pathological process of ALI and play an important role.

Until now, the cellular mechanisms of ALI that modulate inflammatory signalling during ALI are incompletely understood. Recent studies have reported the role of epigenetic factors in the development of hypoxia[13] and apoptosis[23,24]. In the hypoxia-induced mice model, MALAT1 is most strongly produced in lung tissue, and hypoxia-inducible factor (HIF)-2 seems to be the preferred transcriptional activator of MALAT1, demonstrating the strong induction of MALAT1 in organs of mice exposed to inspiratory hypoxia[13]. MALAT1 was firstly discovered a symbol of lung cancer transfer[12], but also participated in the physiological progress of hypoxia[13], endothelial[14] and epithelial cell dysfunction[15]

However, in the present study, the MALAT1 level decreased 3 days after ALI diagnosis, which limited the protective profile of MALAT1. The inconsistency between the above study and our results might lie in unknown mechanisms that remain to be unveiled. Nevertheless, the lncRNA MALAT1 definitely participates in the process of LPS-induced ALI.

Another characteristic of MALAT1 is its role in apoptosis. In the cerebral ischaemia/reperfusion injury, MALAT1 appears to protect human brain vascular endothelial cells from oxygen glucose deprivation (OGD)-induced apoptosis via increasing phosphatidylinositol 3-kinase (PI3K) activities, activating protein kinase B (Akt) phosphorylation and decreasing cell apoptosis and caspase-3 activity[23,24]. In human glioma, MALAT1 promotes proliferation and suppresses apoptosis of glioma cells through derepressing Rap1B by sponging miRNA-101[25]. In the present study, MALAT1 manifestes anti-apoptotic characteristics through degradation of miRNA-181a-3p by acting as a “sponge” and downregulates it by RNA-RNA interaction. These results are displayed via decrease of MALAT1 and Bcl-2 in ALI patients, anti-apoptosis experiments in rat model, effects of miRNA-181a-3p and MALAT1 modulation on apoptosis. Our results demonstrate that MALAT1 protects alveolar epithelial cells from apoptosis due to LPS-induced ALI.

MicroRNAs are a kind of non-coding RNA with 22 to 29 bases involved in early development, cell proliferation, differentiation, apoptosis, energy metabolism and immune regulation[26]. MiRNA-181a-3p belongs to the miRNA-181 family, and the gene sequence is highly conserved[27]. It has been demonstrated that in an LPS-induced ALI model, downregulation of miRNA-181a protected mice from apoptosis by targeting the gene Bcl-2[6]. In accordance with previous studies, our results show that miRNA-181a-3p downregulates its target gene Bcl-2 without affecting the expression level of MALAT1. In general, lncRNAs act as “molecular sponges” that compete with mRNAs for the binding of miRNAs and thus dampen the mRNA-destabilising potential of miRNAs. Although in the present study, the luciferase

assay in HEK293 cells confirm that miRNA-181a-3p, as a target of MALAT1, directly repressed Bcl-2 expression, there is little evidence to support the influence of miRNAs on lncRNAs.

One of the central concepts in ALI is that an unbalanced quantity or quality of the inflammatory response aggravates epithelial or endothelial injury. This includes a dysregulated recruitment of leukocytes as well as inappropriate expression of cytokines which involves TNF- α , IL-1 β and IL-6[28]. In cardiomyocytes, IL-6 induces MALAT1 overexpression in HL-1 cell response to LPS[29]. Furthermore, MALAT1 can enhance TNF- α expression at least partly via serum amyloid A-3 (SAA3) in LPS-treated cardiomyocytes[29]. However, in our results, the expression of MALAT1 is decreased, and the TNF- α level is increased. The different expression of MALAT1 from the present study may be attributed to the cell phenotype and organ condition. In LPS-induced ALI, infiltrating epithelial cells are largely responsible for creating a proinflammatory environment. Furthermore, IL-6 induces MALAT1 upregulation in cardiomyocytes, which is in accordance with our finding that the IL-6 level is increased after LPS stimulation. IL-6 is a pro-inflammatory mediator that has been well discussed[30]. In accordance with our study, IL-6 manifests a pro-inflammatory profile. The present study demonstrates that TNF- α , IL-1 β and IL-6 are increased in ALI patients and have been regulated by MALAT1 and miRNA-181a-3p. These results manifest that TNF- α , IL-1 β and IL-6 are target inflammatory factors of lncRNA and miRNAs.

The above-mentioned mediators not only mediate inflammatory response, but also participate in epithelial cell apoptosis. According to some studies, TNF- α is associated with apoptosis[31]. It is a cytokine with multiple biological activities produced by epithelial cells. Its mechanism of apoptosis induction involves activation of the cascade reaction of the apoptotic kinase family[32]. IL-1 β is also another pro-apoptosis cytokine that can induce inflammation and apoptosis in chondrocytes[33], which is in accordance with the present study. In addition, siMALAT1 downregulation decreased the TNF- α , IL-1 β and IL-6 levels and reversed the alveolar epithelial apoptosis, demonstrating the anti-apoptosis characteristics of lncRNA MALAT1.

Conclusions

The present study demonstrates that LPS-induced ALI inhibits the expression of lncRNA MALAT1, which downregulates target RNA miRNA-181. The downregulation of miRNA-181a-3p inhibits the target gene, anti-apoptosis Bcl-2, resulting in alveolar epithelial apoptosis and CPB-induced ALI. We can foresee real-time clinical interventions performed at the time of bypass that take advantage of the protective effects of MALAT1.

Limitations

The first limitation of the present study is the lack of gene knockout animals. We used antibodies, anti-oligonucleotides and mimics or inhibitors to elucidate the mechanism of LPS-induced ALI in rats. However, all results in patients, animals or cells drew the same conclusions. The second limitation is that the extrapolation of data obtained from different animal or cell models to humans.

Declarations

Ethics approval and consent to participate

The study follows the Ethical Principles for Medical Research Involving Human Subjects outlined in the Declaration of Helsinki. The ethics approval was obtained from the Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine.

Consent for Publication

Consent for publication has been obtained from each individual.

Availability of Data and Materials

All data and materials generated or analysed during this study are included in this published article.

Competing Interests

Author Yaling Liu has received research grants from the National Natural Science Foundation of China. Author Xiaodong Wang has received research grants from the Science and Technology Commission of Shanghai Municipality, the Key Disciplines Group Construction Project of Pudong Health Bureau of Shanghai and Beijing United Heart Foundation.

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Authors' contributions

Yaling Liu wrote the draft. Xiaodong Wang conducted animal experiments and revised the manuscript. Liqun Yang performed cell experiments. Hong Xie devised the concept and conducted molecular biology experiments.

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Figures

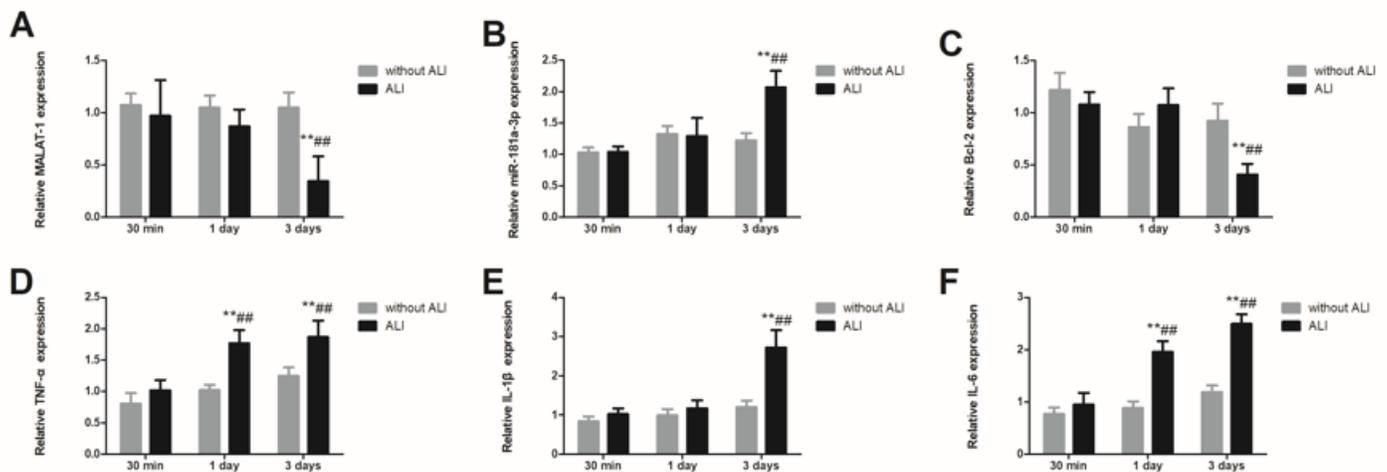


Figure 1

The expression of MALAT1, miRNA-181a-3p, Bcl-2 and inflammatory factors in 368 patients. Patient serum was separated 30 min, 1 day and 3 days after occurrence of ALI or not. The RNA was measured by qRT-PCR. The normalization was U6. Control group means baseline status before ALI diagnosis. MALAT1 and Bcl-2 expression were both inversely associated with ALI (both $P < 0.01$), whereas miRNA-181a-3p was positively associated with ALI ($P < 0.01$). In addition, mRNA levels of TNF- α , IL-1 β and IL-6 increased in ALI patients after 1 or 3 days, respectively (**compared to patients without ALI, $P < 0.01$, ##compared to 30 min and 1 day, $P < 0.01$).

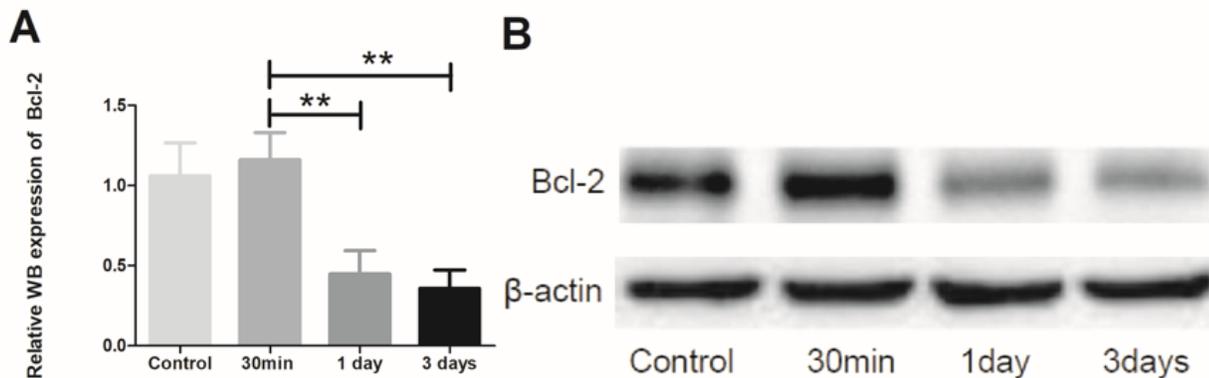


Figure 2

The protein expression of Bcl-2 in ALI patients. After diagnosis of ALI for 1 and 3 days, respectively, the Bcl-2 protein level decreased compared to levels before ALI (** $P < 0.01$).

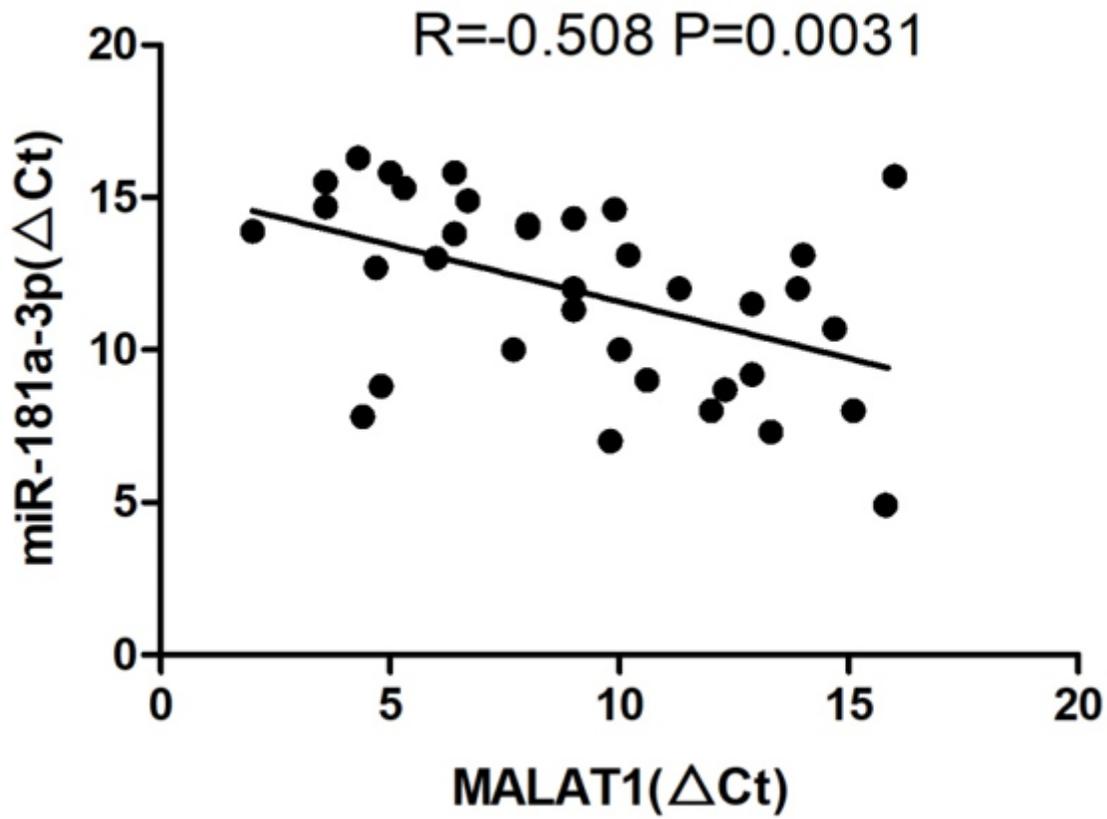


Figure 3

Relationship between MALAT1 and miRNA-181 in ALI patients. MALAT1 level was inversely correlated to miRNA-181 expression in Pearson's correlation ($R = -0.508$, $P = 0.0031$).

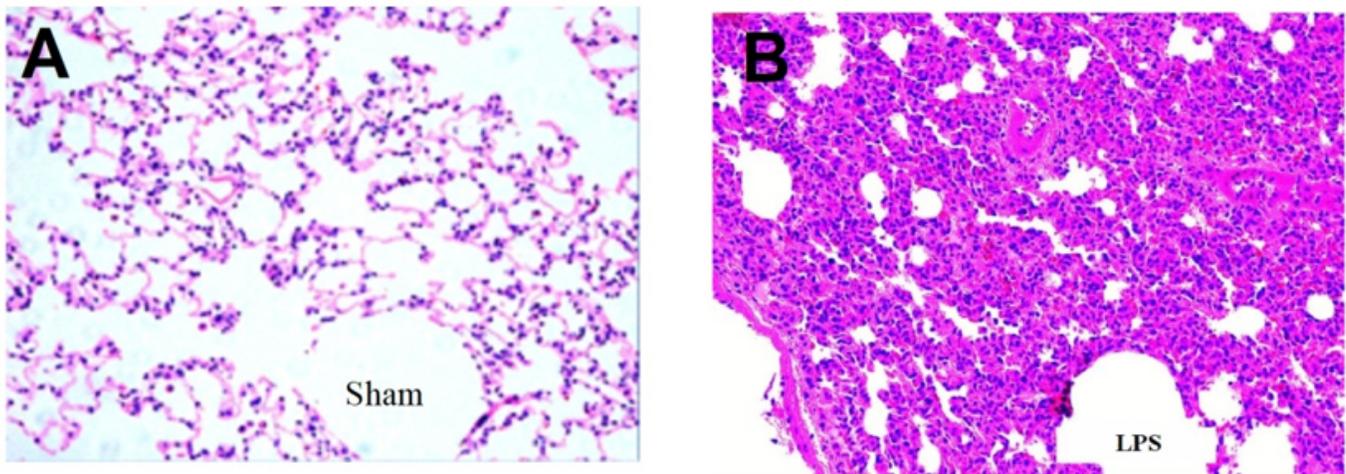


Figure 4

The change of lung tissue before and after LPS stimulation in rats. The superior lobes of the left lungs of rats were harvested. HE staining showed that the alveolar wall was intact and very thin in the sham group, whereas in LPS-induced ALI group, the alveolar wall was destroyed, and neutrophil and lymphocyte infiltration was observed.

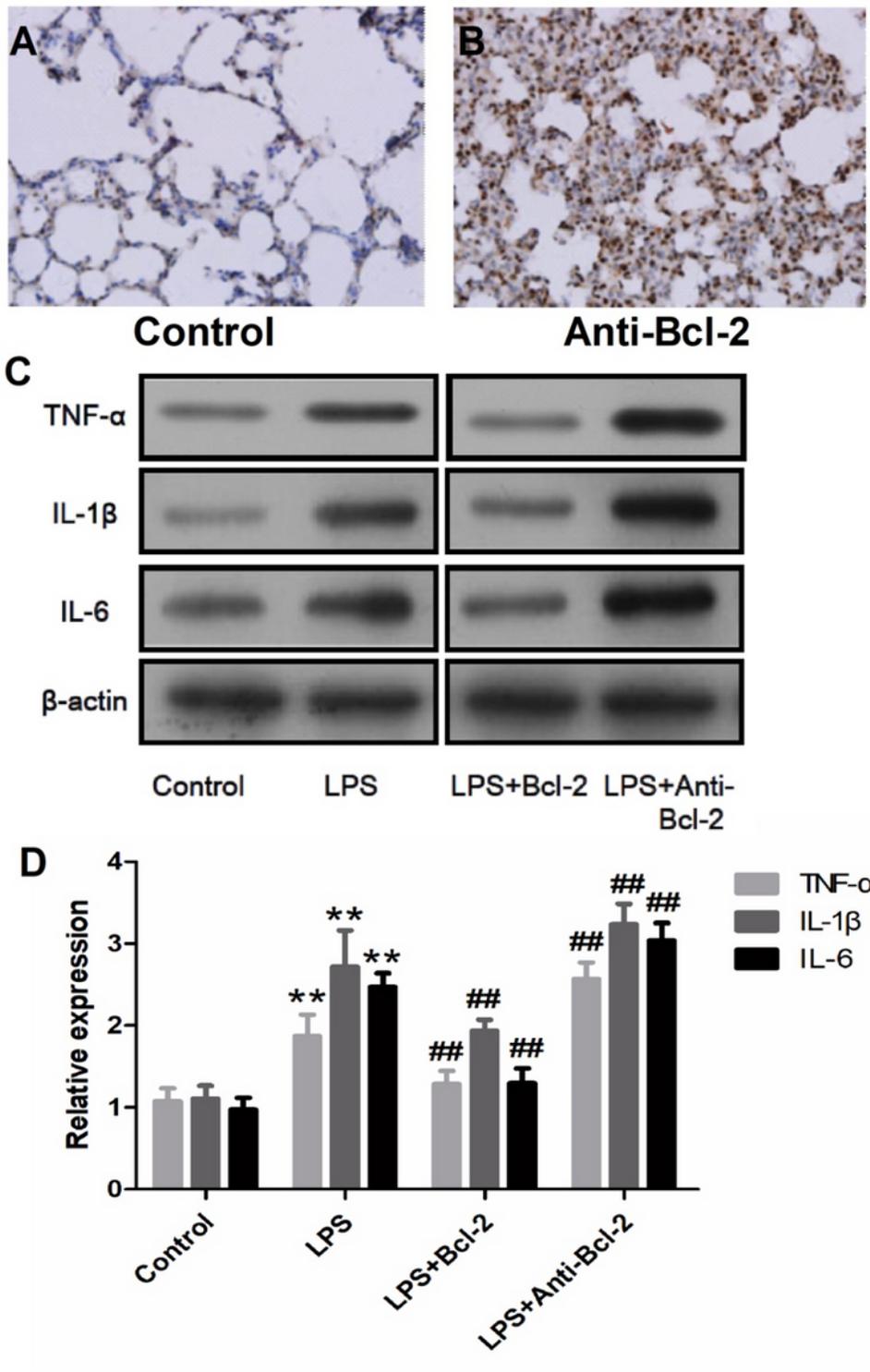


Figure 5

The effect of Bcl-2 on inflammatory factor expression in LPS-induced ALI in rats. Compared to control (A), anti-Bcl-2 increased inflammation infiltration (B). Bcl-2 increased, whereas anti-Bcl-2 decreased, protein levels of TNF- α , IL-1 β and IL-6 (C and D) (compared to control, ** $P \leq 0.01$; compared to LPS, ## $P \leq 0.01$).

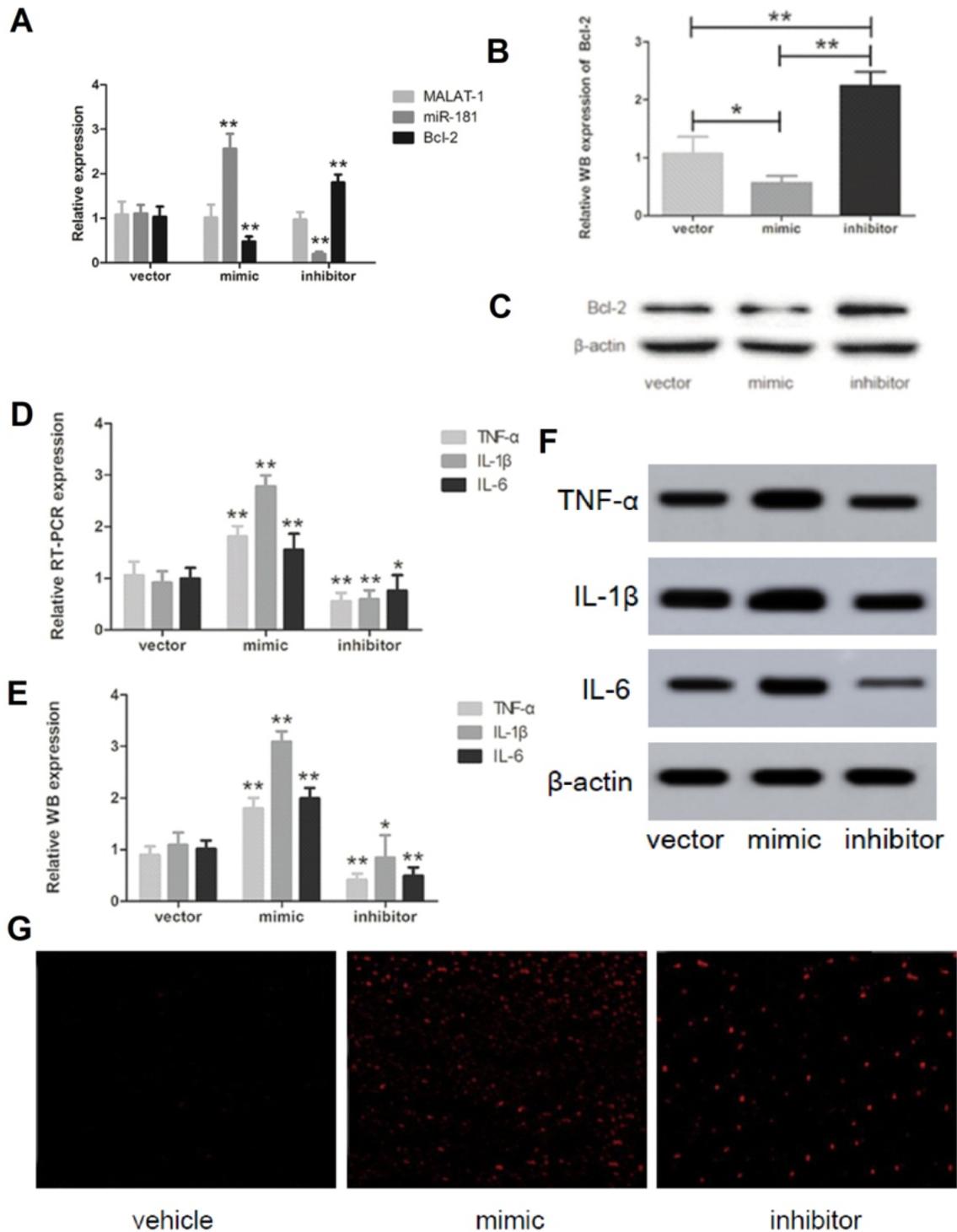


Figure 6

The effect of miRNA-181a-3p on the expression of Bcl-2 and inflammatory factors and apoptosis in LPS-induced ALI in rats. MiRNA-181a-3p mimic and inhibitor were transfected to rats for 24 h. MiRNA-181a-3p

mimic decreased Bcl-2 mRNA expression, whereas miRNA-181a-3p inhibitor increased Bcl-2 mRNA expression (A). MiRNA-181a-3p mimic downregulated protein expression of Bcl-2, whereas miRNA-181a-3p inhibitor upregulated it (B and C). MiRNA-181a-3p mimic increased the mRNA level of TNF- α , IL-1 β and IL-6, whereas miRNA-181a-3p inhibitor reversed the effect (D). The protein expression of TNF- α , IL-1 β and IL-6 increased significantly after miRNA-181a-3p mimic stimulation and decreased after miRNA-181a-3p inhibitor administration (E and F) (* $P \leq 0.05$, ** $P \leq 0.01$). Apoptosis was measured by TUNEL staining. TUNEL staining was done using the in situ cell death detection kit according to the manufacturer's protocol. The number of TUNEL-positive cells were counted under a fluorescence microscope. Compared to vehicle, miRNA-181a-3p mimic promoted alveolar epithelial cell apoptosis, whereas the miRNA-181a-3p inhibitor alleviated it (G).

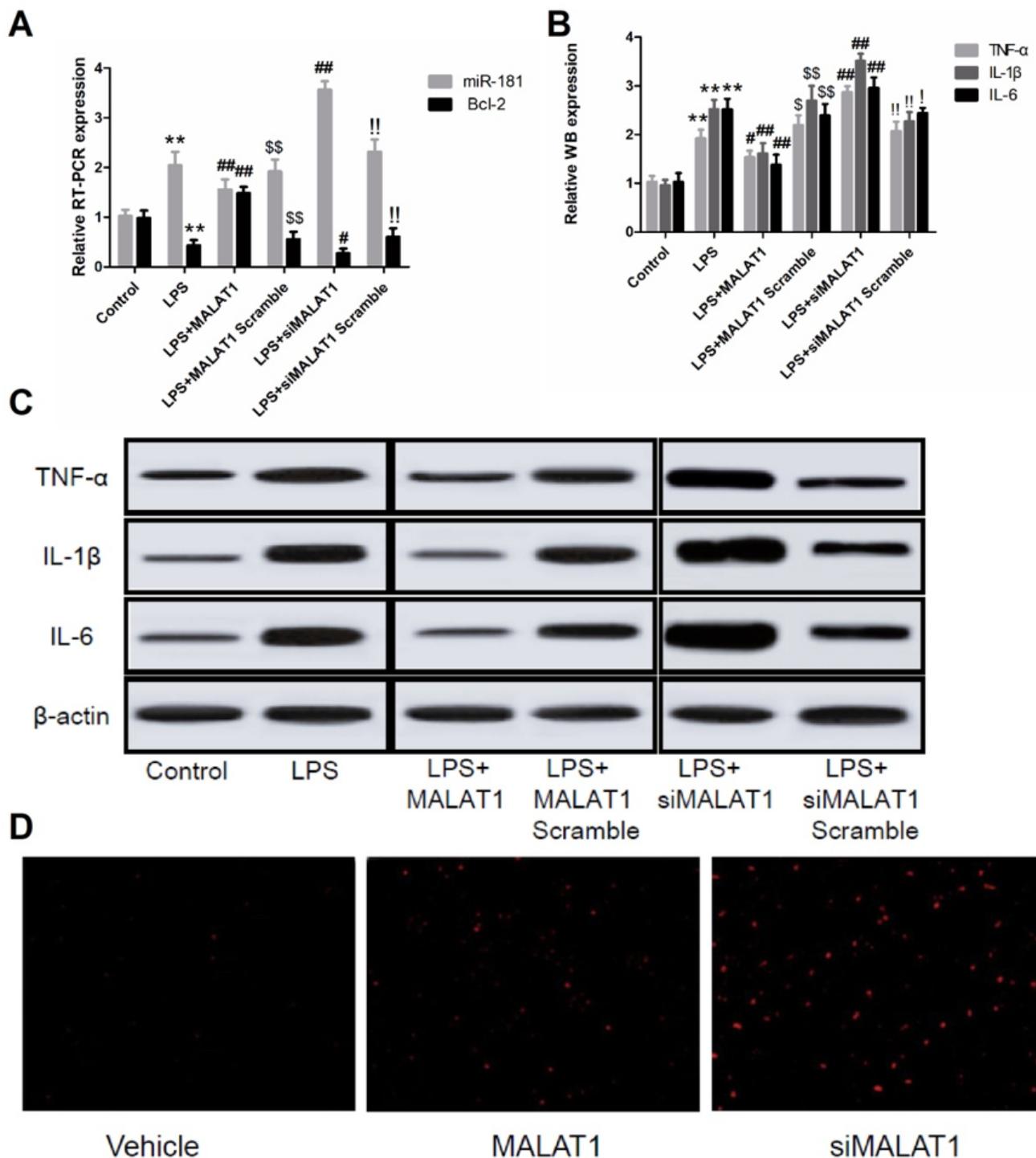


Figure 7

The effect of MALAT1 on the expression of miRNA-181a-3p, Bcl-2, inflammatory factors and apoptosis in LPS-induced rats. LPS increased miRNA-181a-3p expression and decreased Bcl-2 expression (A). After transfection of MALAT1 for 48 h, the miRNA-181a-3p expression decreased and Bcl-2 level increased, whereas transfection of siMALAT1 increased miRNA-181a-3p expression and decreased Bcl-2 levels (A). In addition, LPS induced TNF- α , IL-1 β and IL-6 expression in rats. MALAT1 transfection decreased protein

levels of TNF- α , IL-1 β and IL-6, whereas siMALAT1 reversed the effect (B and C). Apoptosis was measured by TUNEL staining. Compared to vehicle, MALAT1 alleviated apoptosis, whereas siMALAT1 aggravated apoptosis of alveolar epithelial cells (D). (compared to control, **P \leq 0.01; compared to LPS, #P \leq 0.05, ##P \leq 0.01; compared to LPS+MALAT1, \$P \leq 0.05, \$\$ P \leq 0.01; compared to LPS+siMALAT1, !P \leq 0.05, !! P \leq 0.01).

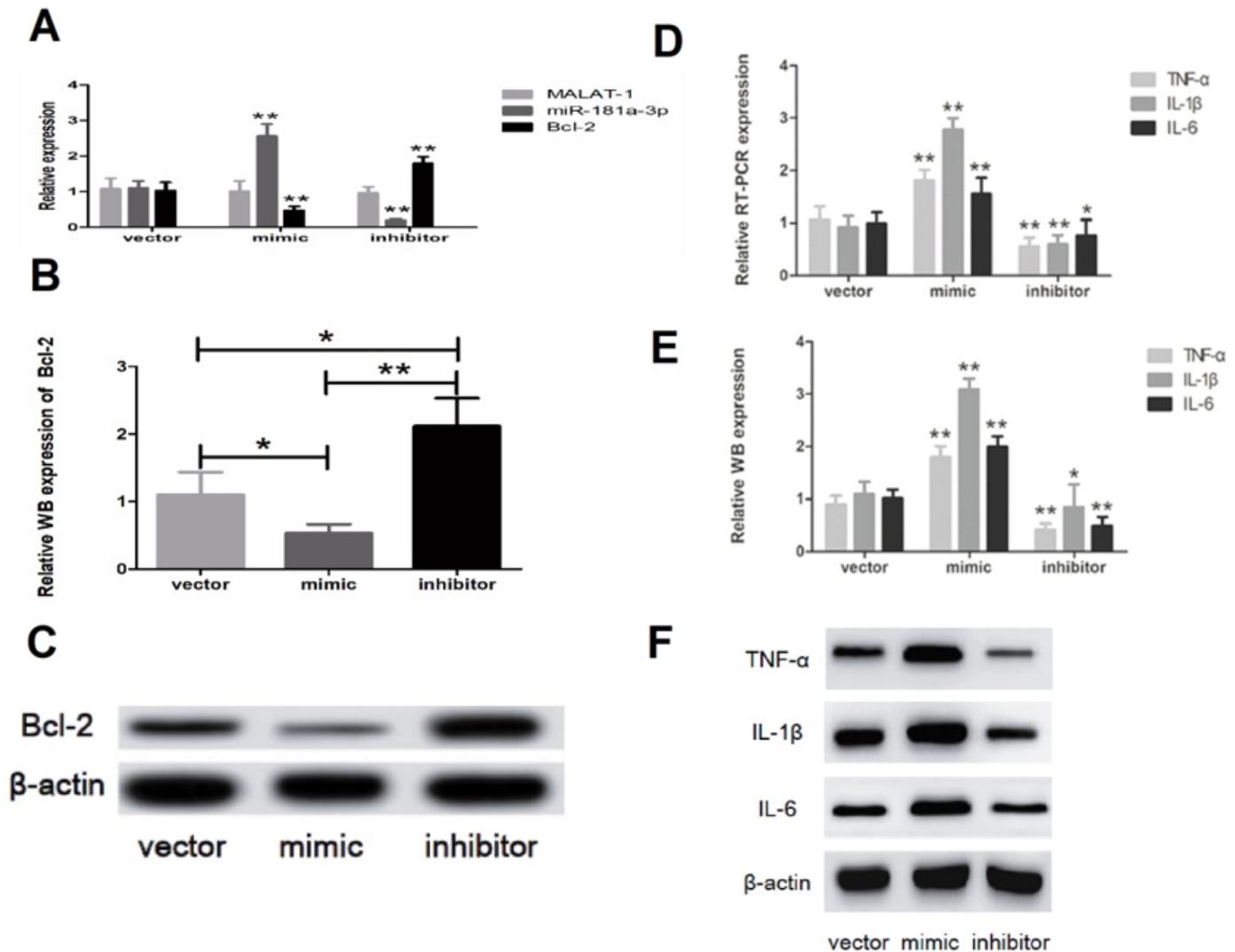


Figure 8

The effect of miRNA-181a-3p on the expression of Bcl-2 and inflammatory factors in LPS-induced alveolar epithelial cells injury. MiRNA-181a-3p mimic and inhibitor were transfected to alveolar epithelial cells for 24 h, and miRNA-181a-3p mimic decreased Bcl-2 mRNA expression significantly, whereas miRNA-181a-3p inhibitor decreased the it (A). In addition, the miRNA-181a-3p mimic decreased and miRNA-181a-3p inhibitor increased the Bcl-2 protein level (B, C). MiRNA-181a-3p mimic increased the mRNA and protein levels of TNF- α , IL-1 β and IL-6, whereas miRNA-181 inhibitor reversed the effect (D, E, F) (compared to vector, *P \leq 0.05, **P \leq 0.01).

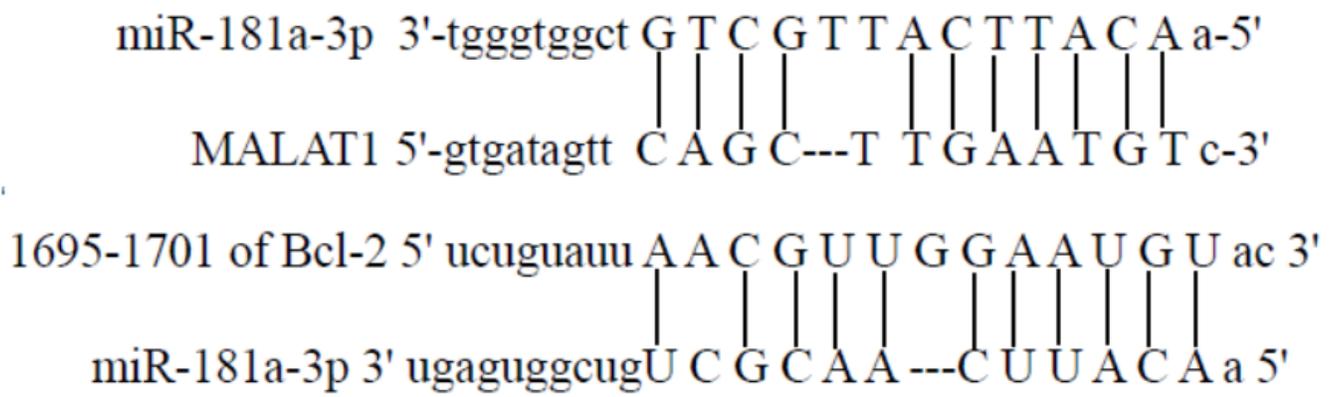


Figure 9

The predicted base pairs of MALAT1, miRNA-181a-3p and Bcl-2 by TargetScan.

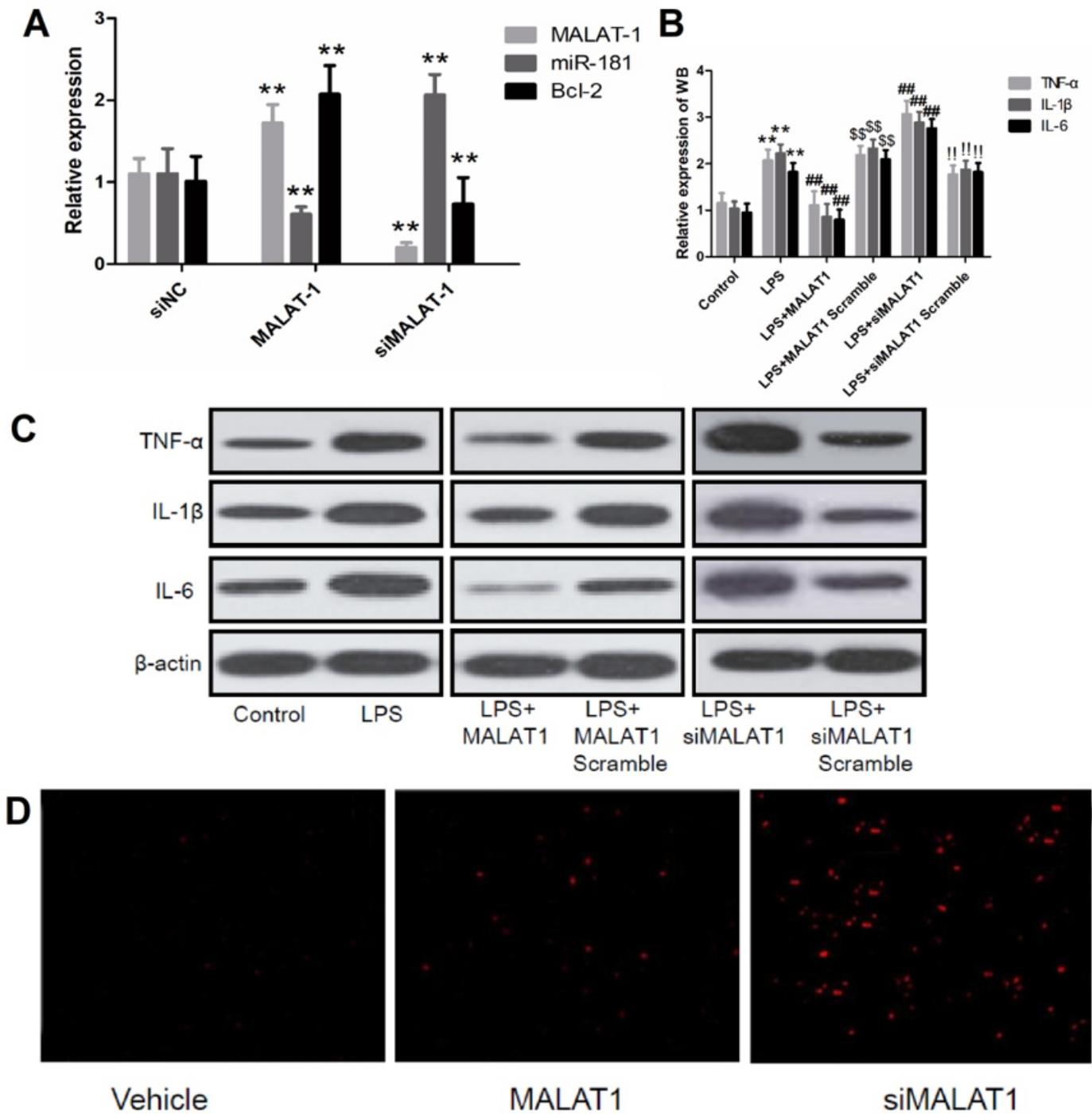


Figure 10

The effect of MALAT1 on the expression of miRNA-181a-3p and Bcl-2 mRNA, inflammatory factor proteins and apoptosis in LPS-induced alveolar epithelial cells injury. MALAT1 and siMALAT1 were transfected to alveolar epithelial cells via lentivirus for 48 h. MALAT1 decreased the expression of miRNA-181a-3p, and increased the level of Bcl-2, whereas siMALAT1 had the opposite effect of MALAT1 (A). MALAT1 increased, whereas siMALAT1 decreased, the protein levels of TNF-α, IL-1β and IL-6 (B and C).

Apoptosis was measured by TUNEL staining. Compared to vehicle, MALAT1 alleviated apoptosis, whereas siMALAT1 aggravated apoptosis in alveolar epithelial cells (D). (compared to control, $**P \leq 0.01$; compared to LPS, $##P \leq 0.01$; compared to LPS+MALAT1, $$$$P \leq 0.01$; compared to LPS+siMALAT1, $!!P \leq 0.01$).

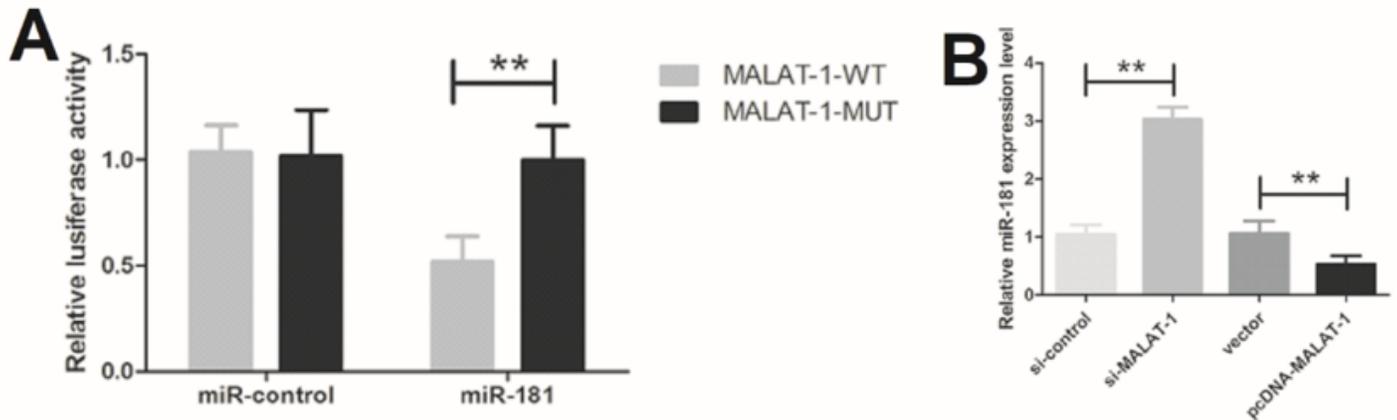


Figure 11

The luciferase reporter analysis in HEK293 cells of MALAT1 and miRNA-181a-3p. The luciferase activities of pGL3-MALAT1-WT were significantly inhibited in miRNA-181a-3p-overexpressing HEK293 cells, but miRNA-181a-3p transfection exhibited no inhibitory effect on luciferase activities of pGL3-MALAT1-MUT (A). MALAT1 knockdown markedly improved miRNA-181 expression, while MALAT1 overexpression distinctly suppressed miRNA-181 expression in HEK293 cells (B). ($**P \leq 0.01$).

Supplementary Files

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