SiRNA directed against NF-κB inhibits mononuclear macrophage cells releasing proinflammatory cytokines *in vitro*

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Abstract. Acute lung injury (ALI) is a condition of acute respiratory failure, characterized by diffuse pulmonary infiltrates and severe hypoxemia. During ALI, the acute phase of inflammation induces the recruitment of activated inflammatory cells, including macrophages and lymphocytes, to the damaged lesions. Nuclear factor (NF)-κB is a key protein in many signal transduction pathways, over-activation of which is followed by an approach of inflammation cells and release of pre-inflammation cytokines. The aim of the present study was to explore the effect of NF-κB P65 siRNA retroviruses on the activation of NF-κB signaling pathway and release of pro-inflammatory cytokines in THP-1 cells. In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to detect the NF-κB p65 mRNA and protein expression at different times in THP-1 cells infected by p65 siRNA retroviruses. The results revealed that p65 siRNA retroviruses could significantly inhibit the expression levels of NF-κB p65 mRNA and protein at different times. In addition, to further investigate the effect of p65 siRNA retroviruses on the pro-inflammatory cytokines release stimulated by LPS, the expression of IL-1β in THP-1 cells and TNF-α in THP-1/M cells was also detected using RT-qPCR and ELISA. As a result, the level of released proinflammatory cytokine interleukin-1β and tumor necrosis factor-α stimulated was significantly inhibited at different times infected by p65 siRNA retroviruses, while increased at different times infected by siControl retroviruses in THP-1 and THP-1/M cells stimulated by LPS. In summary, the present study demonstrated that p65 siRNA retroviruses could suppress the activation of NF-κB signal pathway and release of pro-inflammatory cytokines in THP-1 cells which provided a clinically plausible method to inhibit the inflammation for ALI/ARDS utilizing RNA interference technology.

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Introduction

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), is a clinical syndrome of severe lung failure defined by acute onset, bilateral opacities on the chest radiograph, respiratory failure not fully explained by cardiac failure or fluid overload, and a ratio of arterial oxygen to inspired oxygen of <200 mmHg with a positive end-expiratory pressure of ≥5 cm H_2O. ALI/ARDS is characterized by a disruption of the endothelium and alveolar injury, resulting in an uncontrolled inflammatory response, including increasing release of reactive oxygen species (ROS), inflammatory cytokines, protein content and neutrophil accumulation. Despite numerous studies that have been performed in recent years, the underlying mechanisms of ALI/ARDS remain unclear, there are no effective therapies for the disease and the mortality rate of intensive care patients with ALI/ARDS is as high as 40-60%, which is a problem in respiratory medicine (1). Multiple factors may be involved in the increased vascular permeability, including endothelium injury, increased levels of pro-inflammatory cytokines TNF-α (tumor necrosis factor alpha), interleukin 1 (IL-1), or IL-6 and IL-8, and endovascular occlusion associated with the accumulation of erythrocytes with reduced deformability, leukocytes, and platelets (2).

Nuclear factor (NF)-κB is a key protein in numerous signal transduction pathways, the overactivation of which followed by activation and the response of inflammatory cells serves an important role in ALI/ARDS. The mammalian NF-κB family consists of p65 (or RelA), RelB, c-Rel, p50 (or NF-κB1), and p52 (or NF-κB2), which bind to the κB sites in the DNA of their target genes as homo- or heterodimers through the conserved Rel homology domain (RHD) (3). RNA interference is a specific and effective gene silencing technology, which is able to specifically inhibit target gene expression and reduce the corresponding protein level. The present study was primarily aimed at observing the NF-κB p65 silencing effect through small interfering RNA (siRNA) targeted to the NF-κB p65 gene, which prevents monocyte and phorbol myristate acetate (PMA)-induced THP-1 macrophages treated by lipopolysaccharide (LPS) from releasing IL-1β and TNF-α, providing a basis for novel treatments for ALI/ARDS.

Materials and methods

Cell culture and plasmids. The pSUPER. retro. neo (VEC -PRT-0003 linear) plasmid DNA and the retrovirus packaging cell line 293A were kindly provided by Dr Yang Yizeng (NIH Center for Molecular Studies in Digestive and Liver Diseases, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA). The human monocyte THP-1 cell line was kindly provided by the Department of Pharmacology, Xuanwu Hospital of Capital Medical University (Beijing, China), and the NIH3T3 cell line was provided by Institute of Neurology, Basic Medical Sciences of Peking University Health Science Center (Beijing, China). THP-1 and NIH3T3 cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml) streptomycin (100 μg/ml). 293A cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and penicillin (100 U/ml) streptomycin (100 μg/ml). DMEM and RPMI-1640 medium were purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA), and FBS was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Two looped specific NF-κB P65 siRNA sequences and one scramble control sequence (list in Table I) were synthesized by Beijing Sunbiotech Co., Ltd. (Beijing, China) and ligated into pSUPER.retro.neo vector according to the protocol described in a previous study (4).

The NF-κB p65 siRNA vectors were transfected into 293A package cells using Lipofectamine2000^{TM} (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions, and the virus suspension was collected following 24, 48 or 72 h. Virus titration was performed with NIH3T3 cells. The viral titer was $3.72x10^6$ colony forming units (CFU)/ml for p65 siRNA1, $3.56x10^6$ CFU/ml for p65 siRNA2 and $3.66x10^6$ CFU/ml for p65 si-Control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs were exacted from the cell lines at 80-90% confluence by using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1 μg RNA was reverse transcribed using a RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.). The qPCR was performed using QuantiTect SYBR Green PCR kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. All the primers (listed in Table II) were obtained from Sanbio B.V. (Uden, The Netherlands). All the PCR reactions were initiated with incubation at 94˚C for 2 min, followed by 29 cycles of 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 2 min. Reactions were finished with a 72˚C 10 min extension. Data were normalized using the $2^{-\Delta\Delta Cq}$ method (5).

Western blotting. Total protein were isolated from cultured cells by 300 μl ice cold lysis buffer containing 1% NP-40, 50 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 0.1% SDS, 0.5% deoxycholate, 200 μg/ml phenylmethanesulfonyl fluoride and 50 μg/ml aprotinin. Insoluble materials were removed by ultracentrifugation at 15,000 x g for 30 min for 4˚C. The concentration of the extracted protein was measured spectrophotometrically with Coomassie G-250. Clarified protein lysates (50 μg) were electrophoretically resolved on denaturing SDS-PAGE (8-12%). The proteins were transferred onto polyvinylidene fluoride membranes using a wet transfer method following PAGE, and subsequently blocked with 3% bovine serum albumin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature and washed with Tris-buffered saline and Tween-20 three times. Antibodies specific for NF-κB p65 (mouse anti-human IgG; 1:500; cat. no. sc-8008) and GAPDH (mouse anti-human IgG; 1:10,000; cat. no. sc-47724) were used to probe membranes for 2 h at room temperature, followed by peroxidase-conjugated secondary antibodies (goat anti-mouse IgG; 1:5,000; cat. no. sc-3697) for 1 h at room temperature and enhanced chemiluminescence detection (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). All primary and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. For quantification of band intensity, appropriate films were scanned and band densities were determined using Quantity One software (version 4.6.6; Bio-Rad Laboratories, Inc., Hercules, CA, USA), normalized against GAPDH, and presented as a ratio of control.

ELISA. Secretory levels of IL-1β and TNF-α in culture supernatants were determined by the Quantikine ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA, cat. nos. DLB50 and DTA00C). The color generated was determined by measuring the OD at 450 nm using a spectrophotometric microplate reader. A standard curve was run on each assay plate using serial dilutions of recombinant IL-1β and TNF-α. The experiment was repeated three times and results were presented as the mean value.

Statistical analysis. Data are presented as the mean ± standard deviation. All data analyses were performed using SPSS software version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and independent-samples t-tests were conducted. Tukey's test was used for post-hoc testing following the ANOVA. All experiments were performed at least three times with similar results. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of mRNA expression by p65 siRNA retroviruses. In a previous study, the authors successfully constructed the p65 siRNA retroviruses using gene recombination technology. In the present study, the effect of p65 siRNA retroviruses on the expression of NF-κB p65 mRNA in THP-1 cells was investigated. Fig. 1A revealed the levels of p65 mRNA at 0, 4, 8, 12 and 24 h following infection with p65 siRNA retroviruses, as measured by RT-qPCR. Statistical analysis demonstrated that the p65 mRNA expression levels were significant reduced at 4, 8, 12 and 24 h following infection with p65 siRNA1 and p65 siRNA2 retroviruses, while no significant difference in THP-1 cells infected with siControl retroviruses (Fig. 1B).

Inhibition of protein expression by p65 siRNA retroviruses. To further investigate the inhibitory effects of p65 siRNA retroviruses on the NF-κB signaling pathway, the expression of NF-κB p65 protein was detected using western blot

Table I. siRNA targeting human nuclear factor-κB p65 and the siControl sequence.

Group	Sequence
siRNA1	5'-GATCCCCAGCATCCCAGGCGAGAGGATTCAAGAGATCGTAGGGTCCGCTCTCCTTTTTTA-3'
	3'-GGGTCGTAGGGTCCGCTCTCCTAAGTTCTCTAGCATCCCAGGCGAGAGGAAAAAATTCGA-5'
siRNA1	5'-GATCCCCGGACATATGAGACCTTCAATTCAAGAGATTGAAGGTCTCATATGTCCTTTTTA-3'
	3'-GGGCCTGTATACTCTGGAAGTTAAGTTCTCTACTTCCAGAGTATACAGGAAAAATTCGA-5'
siControl	5'-GATCCCCAACGAGTGTGCCTACATCCTTCAAGAGAGGATGTAGGCACACTCGTTTTTTTA-3'
	3'-GGGTTGCTCACACGGATGTAGGAAGTTCTCTCCTACATCCGTGTGAGCAAAAAAATTCGA-5'

siRNA, small interfering RNA.

Table II. Specific primers for NF-κB, IL-1β, TNF-α and β-actin.

Gene	Direction	Primer sequence (5'-3')	Size (bp)
NF-κB p65	Forward	GTGTTCACAGACCTGGCATCC	230 bp
NF-κB p65	Reverse	TCCGCAATGGAGGAGAAGTCT	
IL-1β	Forward	TGTACCTGTCCTGCGTGTTG	316 bp
IL-1β	Reverse	GCCCTAGGGATTGAGTCCAC	
TNF-α	Forward	CCCATCCCCAATAACAATCCA	262 bp
TNF-α	Reverse	GAGCTCTGCAGTTGGGACAGT	
β-actin	Forward	GGGAAATCGTGCGTGACAT	385 bp
β-actin	Reverse	TCAGGAGGAGCAATGATCTTG	

NF-κB, nuclear factor-κB; IL, interleukin; TNF-α, tumor necrosis factor-α.

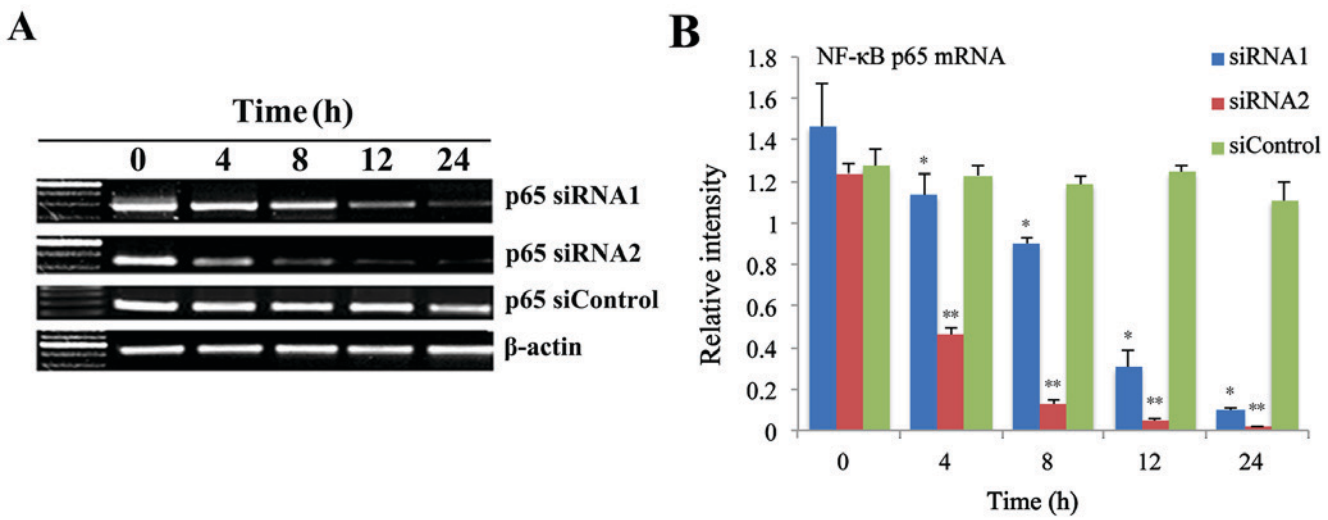

Figure 1. Inhibitory effects of p65 siRNA retroviruses on p65 mRNA expression levels. (A) The expression levels of p65 mRNA at different time in THP-1 cells transfected with p65 siRNA retroviruses were measured by reverse transcription-quantitative polymerase chain reaction. (B) The mRNA expression levels of p65 were significantly reduced at 4, 8, 12 and 24 h in THP-1 cells transfected with p65 siRNA1 and p65 siRNA2, while no significantly difference transfected with siControl retroviruses. Data are expressed as the mean ± standard deviation (n=5). *P<0.05, **P<0.01 vs. 0 h. siRNA, small interfering RNA; NF-κB, nuclear factor-κB.

analysis. The level of p65 protein in THP-1 cells infected with p65 siRNA retroviruses at 0, 4, 8, 12, 24, 48 and 72 h was presented in Fig. 2A. Statistical analysis revealed that the protein expression levels of p65 in THP-1 cells was significantly decreased from 12 h to 72 h following infection with p65 siRNA1 retroviruses, while decreased from 8 h to 72 h following infection with p65 siRNA2 retroviruses. There was no significant difference of p65 protein expression at different time in THP-1 cells following infection with siControl retroviruses (Fig. 2B).

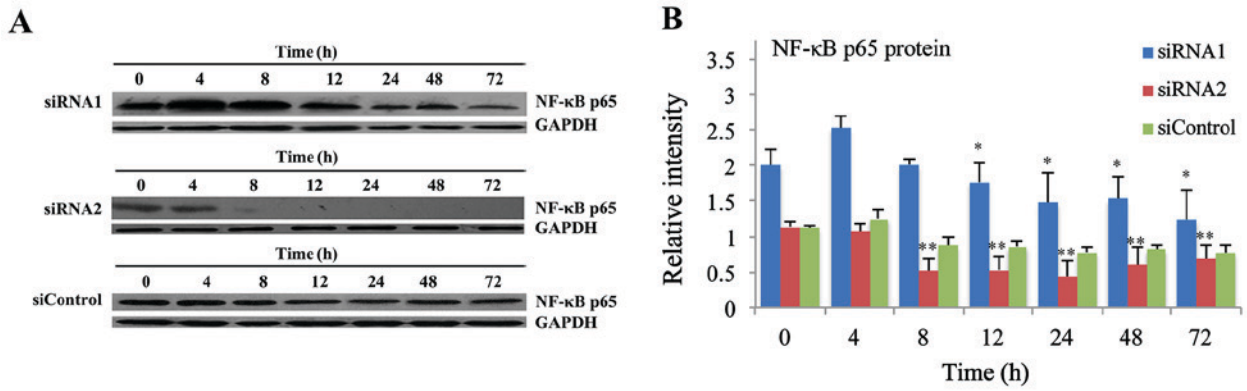

Figure 2. Inhibitory effects of p65 siRNA retroviruses on p65 protein expression levels. (A) The expression levels of p65 protein at 0, 4, 8, 12, 24, 48 and 72 h in THP-1 cells transfected with p65 siRNA retroviruses were measured by western blot analysis. (B) The protein expression levels of p65 were significantly reduced at 12, 24, 48 and 72 h in THP-1 cells following transfection with p65 siRNA1 retroviruses, while decreased at 8, 12, 24, 48 and 72 h following transfection with p65 siRNA2 retroviruses. There was no significant difference of protein expression at different times in THP-1 cells infected with siControl retroviruses. Data are expressed as the mean ± standard deviation (n=5). *P<0.05, **P<0.01 vs. 0 h. siRNA, small interfering RNA; NF-κB, nuclear factor-κB.

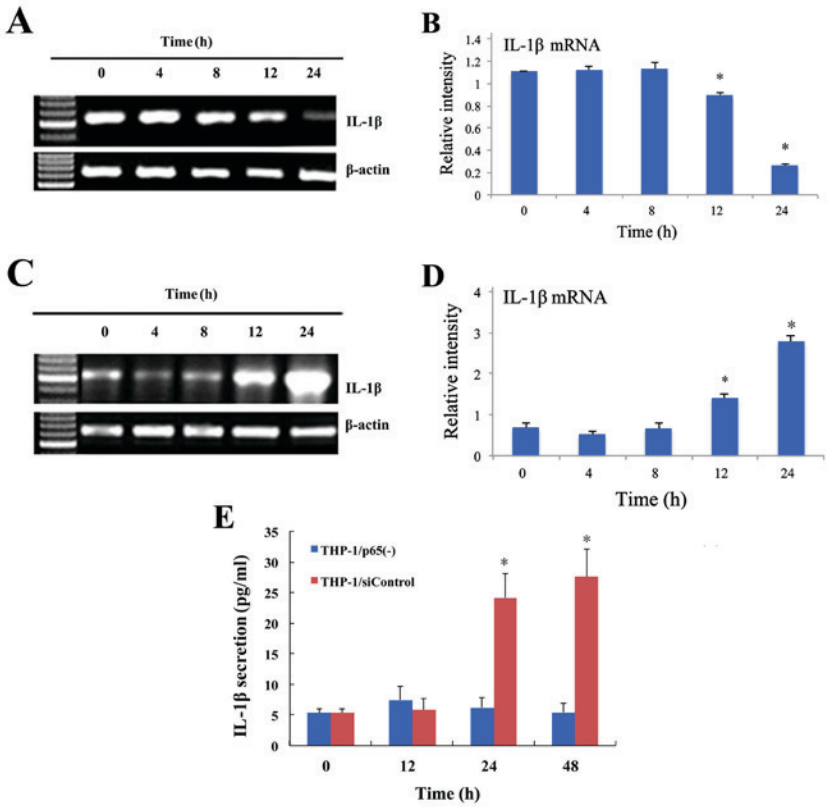

Figure 3. Inhibitory effects of p65 siRNA retroviruses on IL-1β expression, as assessed by RT-qPCR and ELISA in THP-1/p65(-) and THP-1/siControl cells stimulated by LPS. (A) The THP-1/p65(-) cells were treated with LPS (0.1 g/ml) and mRNA levels of IL-1β at 0, 4, 8, 12 and 24 h were measured by RT-qPCR. (B) The mRNA expression levels of IL-1β were significantly reduced at 12 h and 24 h compared with at 0 h. Data are expressed as the mean ± standard deviation (n=5). (C) The THP-1/siControl cells were treated with LPS (0.1 g/ml) and mRNA levels of IL-1β at 0, 4, 8, 12 and 24 h were measured by RT-qPCR. (D) The mRNA expression levels of IL-1β were significant increased at 12 h and 24 h compared with at 0 h. Data are expressed as the mean ± standard deviation (n=5). (E) Inhibitory effect of p65 siRNA retroviruses on IL-1β secretion assessed by ELISA in THP-1 cells stimulated by LPS. TheTHP-1/p65(-) and THP-1/siControl cells were treated with LPS (0.1 g/ml) and levels of IL-1β at 0, 12, 24 and 48 h was measured by ELISA. Treatment with LPS resulted in a significant increase in the secretion of IL-1β at 24 h and 48 h in THP-1/siControl cells. However, the secretion of IL-1β sustained a lower level and there was no significant difference at 0, 12, 24 and 48 h in THP-1/p65(-) cells stimulated by LPS. Data are expressed as the mean ± standard deviation (n=5). *P<0.05 vs. 0 h. THP-1/p65(-) cells, THP-1 cells infected with p65 siRNA2 retrovirus. THP-1/siControl cells, THP-1 cells infected with siControl retroviruses. siRNA, small interfering RNA, IL, interleukin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LPS, lipopolysaccharide.

The RT-qPCR and western blot analysis revealed that the siRNA2 was the most effective in decreasing p65 expression, so p65 siRNA2 was used for further research.

Inhibition of pre-inflammation cytokine IL-1β release by p65 siRNA retroviruses. In order to investigate the effect of p65 siRNA retroviruses on the pro-inflammatory cytokines

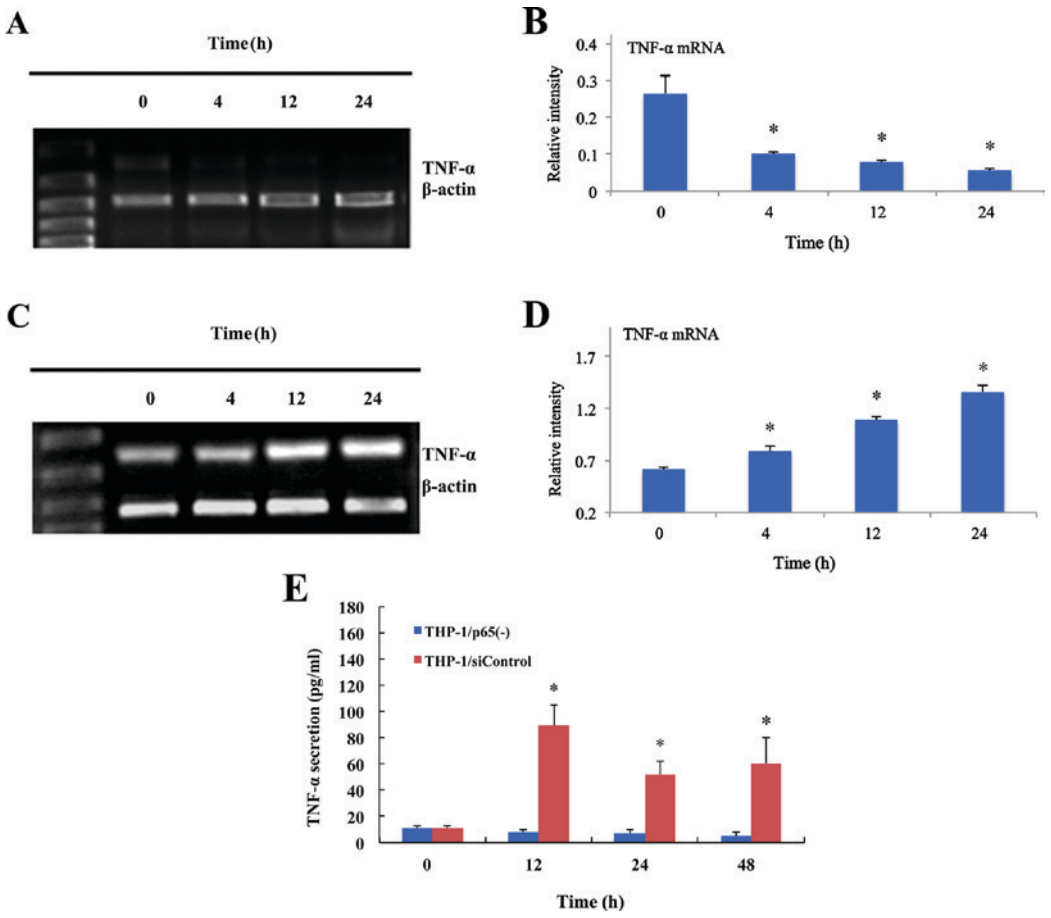

Figure 4. Inhibitory effect of p65 siRNA retroviruses on TNF-α expression assessed by RT-qPCR and ELISA in THP-1 cells stimulated by LPS. (A) The THP-1/siControl cells were treated with LPS (0.1 g/ml) and mRNA levels of TNF-α at 0, 4, 12 and 24 h were measured by RT-qPCR. (B) The mRNA expression levels of TNF-α were significant reduced at 4, 12 and 24 h compared with at 0 h (n=4). (C) The THP-1/siControl cells were treated with LPS (0.1 g/ml) and mRNA levels of TNF-α at 0, 4, 12 and 24 h were measured by RT-qPCR. (D) The mRNA expression levels of TNF-α were significantly increased at 4, 12 and 24 h compared with at 0 h (n=4). (E) Inhibiting effect of p65 siRNA retroviruses on TNF-α secretion assessed by ELISA in THP-1 cells stimulated by LPS. The THP-1/p65(-) and THP-1/siControl cells were treated with LPS (0.1 g/ml) and secretory levels of TNF-α at 0, 12, 24 and 48 h were measured by ELISA. Treatment with LPS resulted in a significant increase in the secretion of TNF-α at 12, 24 and 48 h in THP-1/M/siControl cells. However, the secretion of TNF-α sustained a lower level and there was no significant difference at 0, 12, 24 and 48 h in THP-1/M/p65(-) cells (n=5). Data are expressed as the mean ± standard deviation. *P<0.05 vs. 0 h. THP-1/p65(-) cells, THP-1 cells infected with p65 siRNA2 retroviruses; THP-1/siControl cells, THP-1 cells infected with siControl retroviruses. siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide.

release stimulated by LPS, RT-qPCR was used to detect the expression of IL-1β in THP-1 cells. The level of IL-1β mRNA in THP-1/p65(-) and THP-1/siControl cells stimulated by LPS (0.1 μg/ml) at 0, 4, 8, 12 and 24 h is presented in Fig. 3. Statistical analysis revealed that the mRNA expression levels of p65 was significantly decreased at 12 h and 24 h in THP-1/p65(-) cells following stimulation with LPS, while increased at 12 h and 24 h in THP-1/siControl cells (Fig. 3B and D).

To further evaluate whether p65 siRNA retroviruses could suppress IL-1β production, ELISA was used to measure the secretion of IL-1β in THP-1/p65(-) and THP-1/siControl cells stimulated by LPS. As presented in Fig. 3E, treatment with LPS resulted in a significant increase in the secretion of IL-1β at 24 h and 48 h in THP-1/siControl cells. However, the secretion of IL-1β sustained a lower level and there was no significant difference at 0 h, 12 h, 24 h and 48 h in THP-1/p65(-) cells following stimulation with LPS (Fig. 3E).

Inhibition of proinflammatory cytokine TNF-α release by p65 siRNA retroviruses. To explore the effect of p65 siRNA retroviruses on the production of TNF-α, THP-1 differentiated macrophage-like (THP-1/M) cells were used. When the THP-1 cells were pretreated with PMA (100 nM/ml) for 48 h, the THP-1 cells were almost completely induced to differentiate into macrophage-like cells (Fig. 4).

The level of TNF-α mRNA in THP-1/M/p65(-) and THP-1/M/siControl cells stimulated by LPS (0.1 μg/ml) at 0, 4, 12 and 24 h was presented in Fig. 4A and C, as measured by RT-qPCR. Statistical analysis revealed that the mRNA expression levels of TNF-α was significantly decreased in THP-1/M/p65(-) cells at 4, 12 and 24 h stimulated by LPS, while increased in THP-1/M/siControl cells at 4, 12 and 24 h stimulated by LPS (Fig. 4B and D).

In addition, to confirm whether the inhibition of TNF-α mRNA correspond to a decrease in TNF-α protein, the secretion of TNF-α in THP-1/M/p65(-) and THP-1/M/siControl cells stimulated by LPS (0.1 μg/ml) was measured by ELISA. As demonstrated in Fig. 4E, treatment with LPS resulted in a significant increase in the secretion of TNF-α at 12, 24 and 48 h in THP-1/M/siControl cells. However, the secretion of

TNF-α sustained a lower level and there was no significant difference at 0, 12, 24 and 48 h in THP-1/M/p65(-) cells stimulated by LPS (Fig. 4E).

Discussion

ALI is a condition of acute respiratory failure, characterized by diffuse pulmonary infiltrates and severe hypoxemia (6). During ALI, the acute phase of inflammation induces the recruitment of activated inflammatory cells, including macrophages and lymphocytes, to the damaged lesions (7). Macrophages and lymphocytes are circulating immune cells that serve a crucial role in secreting proinflammatory cytokines and inactivating inflammatory mediators in the ischemic region (8).

NF-κB is a critical signal transcription factor for regulating immune and inflammatory responses (9). Importantly, the activity of NF-κB is regulated by its subcellular localization, and NF-κB is retained in the cytosol when bound to its inhibitor, IκB. Activation of the IκB proteins, which can be induced by a variety of stimuli, such as pro-inflammatory cytokines, allows NF-κB to be released from IκB and translocate to the nucleus where it can initiate transcription by binding to numerous specific gene promoter elements. The activation of the nuclear transcription factor NF-κB is closely associated with the excessive release of pro-inflammatory and inflammatory factors, such as TNF-α and IL-1β (10).

IL-1β is a member of inflammatory factors that is implicated in the pathogenesis of acute respiratory distress syndrome. It has been reported that IL-1β is one of the most biologically active cytokines in early phase of ALI, which is elevated in plasma, and that IL-1β is a potent inducer of lung which causes release of a variety of pro-inflammatory cytokines, such as monocyte chemotactic protein-1, macrophage inflammatory protein-1, IL-6 and IL-8 with subsequent recruitment of inflammatory cells into the airspaces as well as being able to alter endothelial-epithelial barrier permeability and fluid transport leading to edema (11). TNF-α, a pro-inflammatory cytokine, is secreted in the pulmonary tissue by activated immune cells, which control the inflammatory process and accelerate secondary inflammatory processes by inducing inflammatory molecules, such as intercellular adhesion molecules, vascular cell adhesion molecules-1 and selectin (12,13). TNF-α is also responsible for the accumulation of inflammatory cells in the peripheral nucleus of pulmonary tissue, and it induces a second inflammatory response.

Several studies have proven that the inhibition of NF-κB activity leads to the reduction of the excessive release of inflammatory factors and may be a potential method for the clinical treatment of ALI (14,15). However, an effective drug target to inhibit NF-κB has not been reported so far. RNA interference, which was first identified in *Caenorhabditis elegans* in 1998, is a process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by siRNA homologous in sequence to the silenced gene. RNA interference technique is now extensively employed in genetic engineering as a simple and effective gene knockdown tool (16,17). The results demonstrated that p65 siRNA could significantly suppress the expression of NF-κB p65 mRNA and protein level, which confirmed that p65 siRNA could significantly inhibit the activity of NF-κB.

As the NF-κB signaling pathway is activated during ALI, the authors also investigated the effect of siRNA retroviruses on the activity of the NF-κB signaling pathway. The results showed that the expression level of NF-κB p65 mRNA and protein significant decreased in THP-1 cells infected by p65 siRNA retroviruses, which indicated that p65 siRNA may inhibit the expression of NF-κB p65.

Furthermore, the authors detected the effect of siRNA retroviruses on the release of inflammatory cytokines in THP-1 and THP-1/M cells stimulated by LPS. LPS, a component of the outer membrane of Gram-negative bacteria, is considered to be the most potent activator of monocytes and macrophages. Monocytes and macrophages serve an essential role in inflammation and mobilization of the host defense against bacterial infection (18).

As a result, the expression levels of IL-1β and TNF-α mRNA and secreted protein were significantly increased in THP-1 and THP-1/M cells, which suggested that LPS may promoted the release of inflammation cytokines, such as IL-1β and TNF-α. For the siRNA group, the results indicated that IL-1β and TNF-α mRNA expression levels was decreased, while the secreted protein expression levels exhibited no significant difference at different times in THP-1/p65(-) cells stimulated by LPS. The authors speculated that the protein secretion levels of IL-1β and TNF-α were very low without LPS stimulation, so the p65 siRNA may only inhibit the release of IL-1β and TNF-α protein secretion stimulated by LPS, rather than reduce the expression levels of secreted protein in THP-1 cells.

The results proved that NF-κB serves an essential role in regulating the release of inflammatory factors. The inhibition of the excessive release of inflammatory factors through NF-κB signal pathway may be an important method for the clinical treatment of ALI.

In summary, the results demonstrated that suppressing the expression of the NF-κB signaling pathway through NF-κB p65 siRNA causes an inhibition to release of inflammatory factors induced by LPS. The results suggested that disruption of the NF-κB signaling pathway represents an opportunity for rational drug design for ALI prevention or treatment. These insights may help develop NF-κB activity based pharmacological strategies to regulate inflammation but further study is required *in vivo*.

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