IL-6/STAT3/miR-34a protects against neonatal lung injury patients

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Abstract. The present study aimed to investigate the protective role of the interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3)/microRNA (miR)-34a signaling pathway in patients with neonatal lung injury (NLI) and the underlying molecular mechanisms of these effects. It was demonstrated that miR-34a serum expression was significantly upregulated in patients and mice with NLI. Meanwhile, IL-6 and phosphorylated-STAT3 protein expression, and tumor necrosis factor (TNF)-α, IL-1β and IL-18 activity levels in NLI mice were significantly induced compared with the normal control group. The promotion of IL-6 protein expression resulted in significantly increased TNF-α, IL-1β and IL-18 activity levels, phosphorylated-STAT3 and p65 protein expression, and miR-34a expression in NLI mice compared with the corresponding normal control groups. In A549 cells treated with lipopolysaccharides, the promotion of miR-34a protein expression significantly increased TNF-α, IL-1β and IL-18 activity levels, and induced transcription factor p65 protein expression compared with the corresponding negative control groups. Collectively, the data of the present study indicate that IL-6R/STAT3/miR-34a possesses a protective role in patients with neonatal lung injury.

Introduction

Acute lung injury (ALI) refers to the acute inflammatory reaction that occurs in various causes of the body (1). The injuries in the acute diffuse alveolar epithelial cell and the alveolar capillary endothelial cell are the main pathologic changes. Dyspnea and hypoxemia are the clinical manifestations (2). To study ALI by different causes according to the clinical classification of endogenous and exogenous lung may be the future trend of ALI research (3). The most common causes of neonatal ALI include severe asphyxia, meconium aspiration, a large number of amniotic fluid inhalation, severe pulmonary infection, sepsis, and shock (4). At the same time, it is recognized that there are obvious differences in the treatment and prognosis for different causes of ALI in recent years, and it is recommended that ALI be divided into direct lung injury and indirect lung injury (5). There are differences for ALI of different causes in terms of pathophysiology, respiratory mechanics and the response to the treatment of ALI.

As a proinflammatory cytokine, IL-6 plays an important role in the inflammatory process (6). The biological function of IL-6 plays a role in signal transduction only through the formation of complex with its receptor IL-6/IL-6R/gp130 (7). With the deepening of its receptor signaling pathway, IL-6 receptor is expected to become an effective therapeutic target and play a role in the treatment of inflammatory diseases (8).

As the important member of signal transducers and activators of transcription (STATs), the activation of STAT3 can mediate the signals of various cytokines and growth factors into the nucleus, affecting the downstream transcription of many target genes, including c-fos, c-myc, cyclin D1, MMP2, VEGF and other key molecules related to cell proliferation and metastasis of tumor cells (9). There are epigenetic changes in the malignant transformation process of the cells regulated by the positive feedback loop, which are triggered by the initial inflammatory signal (10). The inflammatory signal activates NF-κB, and NF-κB directly activates IL6 transcription and inhibits let-7 indirectly by activating Lin28 to increase the expression of IL6 (11). The high expression of IL6 can activate STAT3 signaling pathway, which plays an important role in the process of cell malignant transformation (11).

MicroRNA (miRNA) is a class of non-coding small RNAs with a length of about 18-24 nucleotides. It is involved in the regulation of multiple life processes, including cell proliferation, differentiation and apoptosis (12). The recent researches show miRNA plays a significant role in inflammatory processes; for example, miR-155, miR-146a, miR-221 and mi R-192 take part in the development and progression of many inflammatory diseases (13). In this study, we investigated the IL-6/STAT3/miR-34a signaling pathway protects against neonatal lung injury patients and the underlying molecular mechanisms of these effects.

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Materials and methods

NLI patients and quantitative RT-PCR analysis. We collected serums from neonatal lung injury patients (2-5 years old) and normal patients (2-5 years old), which gathered at Department of Pediatrics, Chengdu Military General Hospital (Chengdu, China). These studies were approved by Ethics Committee of Chengdu Military General Hospital. A total of 3-5 ml of whole blood was collected and centrifuged at 3,000 x g for 10 min, which serums were collected and saved at -80˚C.

Total RNA was prepared from serums using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized by using oligo-dT primers (Qiagen, Hilden, Germany) and M-MLV reverse transcriptase (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. RT-PCR was performed using Taq polymerase (Bioneer Corporation, Daejeon, Korea). ABI 7500 Fast Real-Time PCR System (Applied Biosystems Life Technologies, Foster City, CA, USA) was performed Real-Time PCR using SYBR-Green PCR Master Mix (Applied Biosystems Life Technologies).

Animals and cell culture. Male C57BL/6 wild-type mice were maintained in a specific pathogen-free facility. Animal studies were approved by Animal Care and Use Committee of Chengdu Military General Hospital.

A549 cell purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and cultured in DMEM medium (EuroClone S.p.A, Milan, Italy) with 10% fetal bovine serum (EuroClone S.p.A), 1% penicillin, and 1% streptomycinin at 5% CO_2 at 37˚C.

NLI model mice and A549 cell model. Mice were assigned at birth to Room air (RA) or hyperoxia (85-90% O2) from P2 to P21 and housed in a plexiglass chamber with O2 monitoring. Dams were rotated to standardize the nutrition provided to each litter at every 48 h. Oxygen exposure was continuous at brief intermittent interruptions (<10 min/day). A total of 30 mice were randomly assigned into three groups: Normal group, NLI model group and IL-6 group. In IL-6 group, NLI model mice were intraperitoneally injected with 1 μg/day of recombination IL-6 protein (14) for 1 week.

A549 cell was seeded at 6 well-plate (1-2x10^6) and miR-34a or negative control were transfected with Lipofectamine RNAiMAX Transfection Reagent (2 μl/ml) and Opti-MEM medium (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. After transfection for 48 h, A549 cell was treated by LPS (100 ng/ml) for 4 h.

ELISA kits. Cell or tissue samples were resuspended in lysis buffer in ice for 20-30 min and total proteins were measured using BCA assay. Total proteins (10 μg) used to analyze TNF-α, IL-1β and IL-18 contents using ELISA Kits.

Western blot analysis. Cell or tissue samples were resuspended in lysis buffer in ice for 20-30 min and total proteins were measured using BCA assay. Total proteins were resolved by 10-12% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Membrane was blocked with 5% skim milk powder in TBST and probed with primary antibodies against IL-6, phosphorylation-STAT3, p65 and GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C for 12 h. After washed with TBST for 3 times, protein blank was detected horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology, Inc.) and developed by a chemiluminescence-based detection system (ECL; Amersham Biosciences, Uppsala, Sweden). Protein blank were obtained by ChemiDoc-it 500 Imaging System and analyzed by Vision Works LS analysis software (UVP, Inc., Upland, CA, USA).

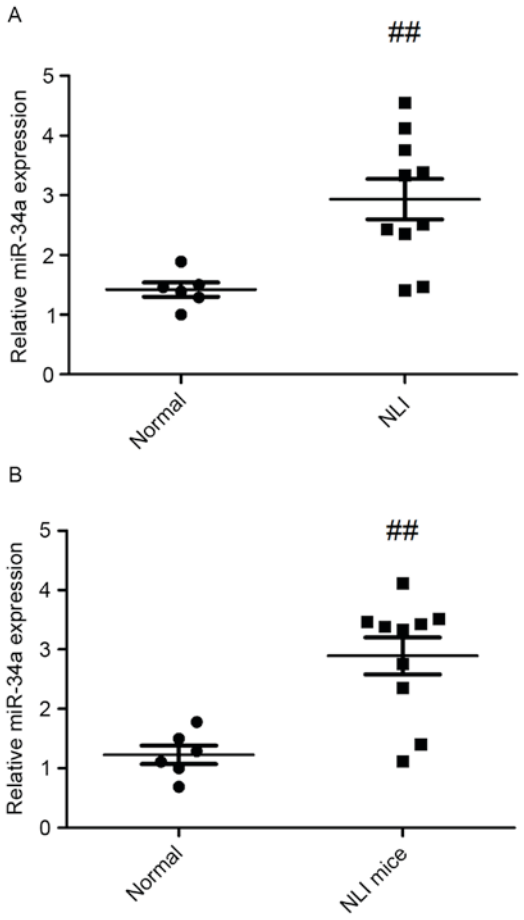

Figure 1. miR-34a serum expression of NLI patients and mice. miR-34a serum expression of (A) NLI patients and (B) mice. Normal, normal control group; NLI, neonatal lung injury patients; NLI mice, neonatal lung injury mice group. $^{##}$P<0.01 compared with normal control group.

Statistical analysis. Results are presented as mean ± SD. Significance was determined by Student's t-test and by ANOVA with Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-34a serum expression of NLI patients and mice. We asked miR-34a serum expression of NLI patients and mice. miR-34a serum expression of NLI patients and mice were very higher than those of normal control group (Fig. 1).

IL-6 and phosphorylation-STAT3 protein expression in NLI mice. To investigate verify IL-6 and phosphorylation-STAT3

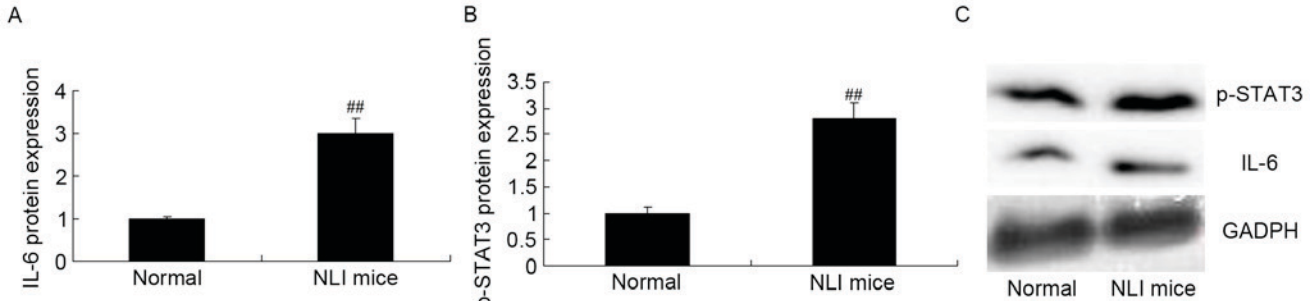

Figure 2. IL-6 and phosphorylation-STAT3 protein expression in NLI mice. (A and B) IL-6 and phosphorylation-STAT3 protein expression by statistical analysis and (C) IL-6 and phosphorylation-STAT3 protein expression by western blotting assays in NLI mice. Normal, normal control group; NLI mice, neonatal lung injury mice group. ##P<0.01 compared with normal control group.

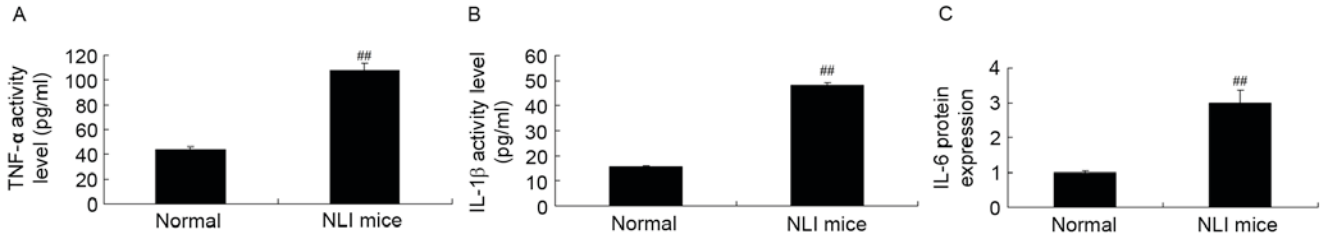

Figure 3. TNF-α, IL-1β and IL-18 contents in NLI mice. (A) TNF-α, (B) IL-1β and (C) IL-18 contents in NLI mice. Normal, normal control group; NLI mice, neonatal lung injury mice group. ##P<0.01 compared with normal control group.

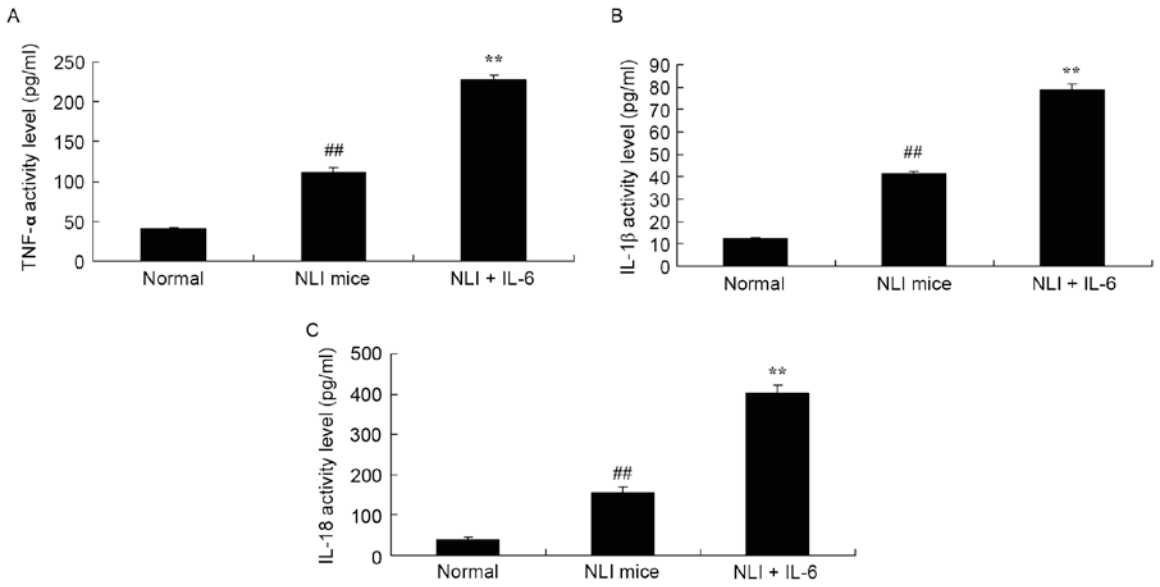

Figure 4. Promotion of IL-6 protein affects TNF-α, IL-1β and IL-18 contents in NLI mice. Promotion of IL-6 protein affects (A) TNF-α, (B) IL-1β and (C) IL-18 contents in NLI mice. Normal, normal control group; NLI mice, neonatal lung injury mice group; NLI + recombination IL-6 protein group. ##P<0.01 compared with normal control group, **P<0.01 compared NLI mice group.

protein expression in NLI mice, IL-6 and phosphorylation-STAT3 protein expression were measured using Western blot analysis. as showed in Fig. 2, IL-6 and phosphorylation-STAT3 protein expression were observably promoted in NLI mice, compared with normal group.

TNF-α, IL-1β and IL-18 contents in NLI mice. Then, we investigated TNF-α, IL-1β and IL-18 contents in NLI mice using ELISA kits. We found that TNF-α, IL-1β and IL-18 contents were signally increased in NLI mice, compared with normal group (Fig. 3).

Promotion of IL-6 protein affects TNF-α, IL-1β and IL-18 contents in NLI mice. To determine whether the promotion of IL-6 protein affects TNF-α, IL-1β and IL-18 contents in NLI mice, TNF-α, IL-1β and IL-18 contents were measured using ELISA kits. As showed in Fig. 4, the promotion of IL-6 protein significantly increased s TNF-α, IL-1β and

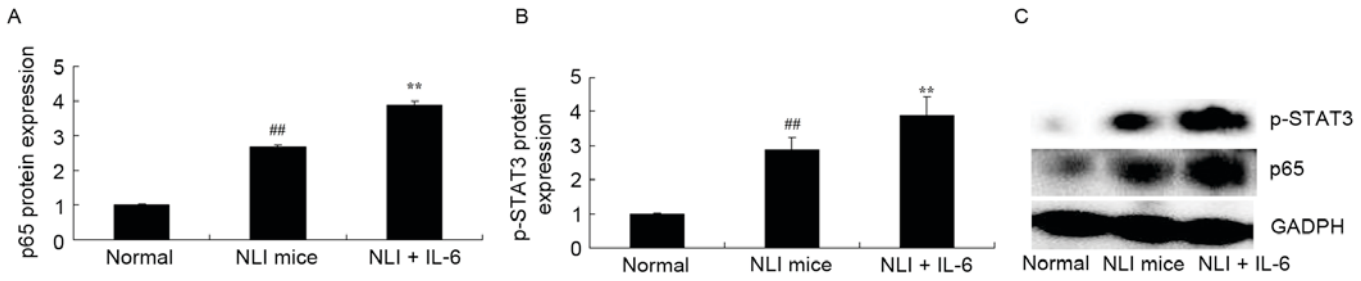

Figure 5. Promotion of IL-6 protein affects phosphorylation-STAT3 and p65 protein expression in NLI mice. Promotion of IL-6 protein affects phosphorylation-STAT3 and p65 protein expression by statistical analysis (A and B) and phosphorylation-STAT3 and p65 protein expression by western blotting assays (C) in NLI mice. Normal, normal control group; NLI mice, neonatal lung injury (NLI) mice group; NLI+ recombination IL-6 protein group. $^{\#\#}$P<0.01 compared with normal control group, **P<0.01 compared NLI mice group.

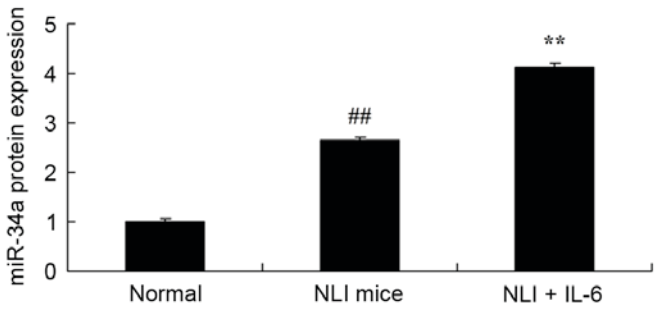

Figure 6. Promotion of IL-6 protein affects increased miR-34a expression in NLI mice. Normal, normal control group; NLI mice, neonatal lung injury (NLI) mice group; NLI+ recombination IL-6 protein group. $^{\#\#}$P<0.01 compared with normal control group, **P<0.01 compared NLI mice group.

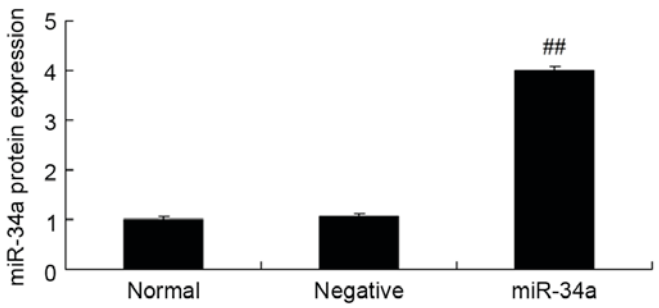

Figure 7. In A549 cell treated by LPS, promotion of miR-34a protein. Normal, normal control group; NLI mice, neonatal lung injury (NLI) mice group; NLI+ recombination IL-6 protein group. $^{\#\#}$P<0.01 compared with normal control group.

IL-18 contents in NLI mice, compared with normal control group.

Promotion of IL-6 protein affects phosphorylation-STAT3 and p65 protein expression in NLI mice. Next, we determined the promotion of IL-6 protein affects phosphorylation-STAT3 and p65 protein expression in NLI mice. The results of Western blot analysis showed that the promotion of IL-6 protein markedly induced hosphorylation-STAT3 and p65 protein expression in NLI mice, compared with normal control group (Fig. 5).

Promotion of IL-6 protein affects miR-34a expression in NLI mice. To investigate whether IL-6 affects miR-34a expression in NLI mice, miR-34a expression was measured using Quantitative RT-PCR analysis. Fig. 6 indicated that the promotion of IL-6 protein significantly enhanced miR-34a miRNA expression in NLI mice, compared with normal control group.

In A549 cell treated by LPS, promotion of miR-34a protein. To further investigate whether miR-34a plasmid increased miR-34a expression in A549 cell treated by LPS, miR-34a expression was measured using Quantitative RT-PCR analysis. miR-34a plasmid observably promoted miR-34a expression in A549 cell treated by LPS, compared with negative group (Fig. 7).

In A549 cell treated by LPS, promotion of miR-34a protein expression affects TNF-α, IL-1β and IL-18 contents. To evaluate the role of IL-6 in miR-34a-induced inflammation in A549 cell treated by LPS, TNF-α, IL-1β and IL-18 contents were measured using ELISA Kits. Overexpression of miR-34a significantly increased TNF-α, IL-1β and IL-18 contents in A549 cell treated by LPS, compared with negative group (Fig. 8).

In A549 cell treated by LPS, promotion of miR-34a protein expression affects p65 protein expression, not affects IL-6 and phosphorylation-STAT3 protein expression. To further evaluate the role of IL-6 in miR-34a-induced p65 and STAT3 protein expression, p65 and STAT3 protein expression were measured using western blot analysis. We found that overexpression of miR-34a significantly induced p65 protein expression, not affects IL-6 and phosphorylation-STAT3 protein expression in A549 cell treated by LPS, compared with negative group (Fig. 9).

Discussion

ALI is an alveolar inflammation initiated by perinatal asphyxia or infection and under the other internal and external factors (15). The activation and increase of neutrophil as well as the release of a variety of inflammatory mediators and the impairment of epithelial cell and vascular endothelial cell functions, compose the main pathogenesis of ALI (16). In the current study, miRNA-34a serum expression of NLI patients and mice were very higher than those of normal control group.

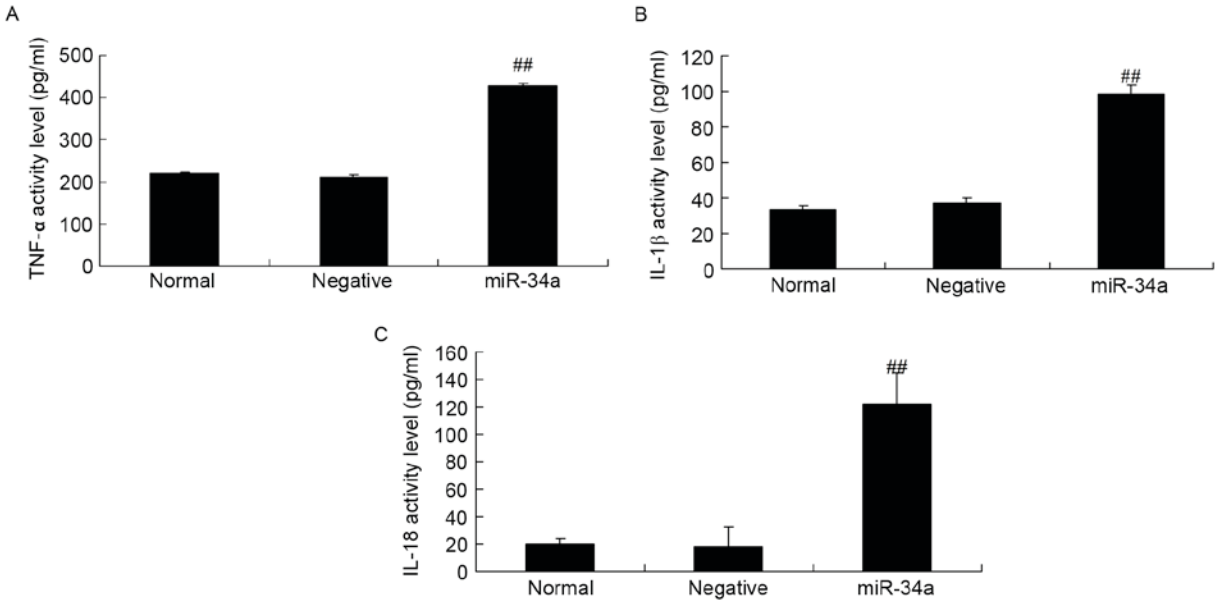

Figure 8. In A549 cell treated by LPS, promotion of miR-34a protein expression affects TNF-α, IL-1β and IL-18 contents. In A549 cell treated by LPS, promotion of miR-34a protein expression affects TNF-α (A), IL-1β (B) and IL-18 (C) contents. Normal, normal control group; NLI mice, neonatal lung injury (NLI) mice group; NLI+ recombination IL-6 protein group. ##P<0.01 compared with normal control group.

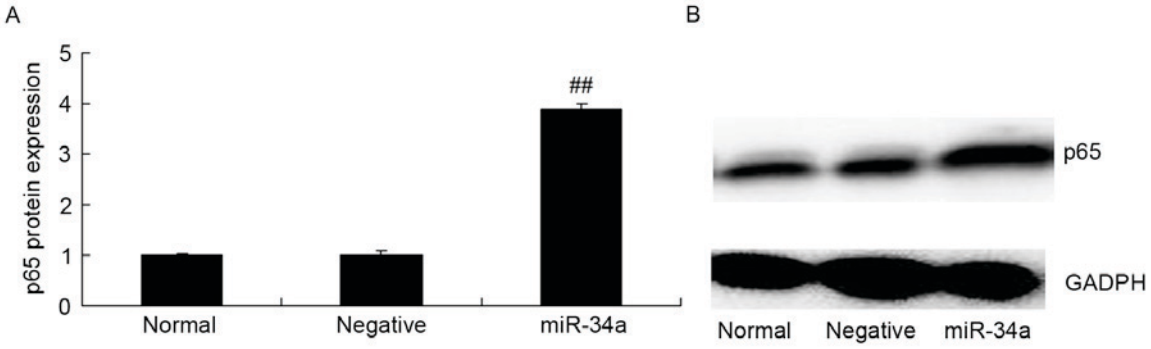

Figure 9. In A549 cell treated by LPS, promotion of miR-34a protein expression affects p65 protein expression, not affects IL-6 and phosphorylation-STAT3 protein expression. In A549 cell treated by LPS, promotion of miR-34a protein expression affects p65 protein expression by statistical analysis (A) and western blotting assays (B), not affects IL-6 and phosphorylation-STAT3 protein expression and phosphorylation-STAT3 and p65 protein expression by western blotting assays (B). Normal, normal control group; NLI mice, neonatal lung injury (NLI) mice group; NLI+ recombination IL-6 protein group. ##P<0.01 compared with normal control group.

Inflammation is a defensive reaction for the living tissue with the vascular system to the damaged factors, which can remove the harmful substances out of the body, but sometimes it causes damage to the body through reaction (17). In recent years, many studies have proved that this view, especially TNF-α, IL-6 and other cytokines and inflammatory mediators are involved directly (18). TNF-α is a multi-dominant cytokine generated by monocyte-macrophages (18). It is an inflammatory mediator with important biological activity. It can induce chemotaxis and local infiltration of neutrophils, to phagocytose and kill pathogens, thus starting the inflammatory response (19). IL-6 is generated by monocyte-macrophages and lymphocytes spontaneously or under the stimulation of various factors. IL-6 is an important cytokine in inflammatory reaction, which is an important mediator for acute phase synthesis (20). Intriguingly, in our study, IL-6 and phosphorylation-STAT3 protein expression were observably promoted, and TNF-α, IL-1β and IL-18 contents were signally increased in NLI mice, compared with normal group.

MiRNA is a non-coding RNA that plays a role in post-transcriptional regulation in gene transcription (21). It plays an important role in the development, reproduction, apoptosis, and pathogenesis of target genes by regulating transcriptional expression of target genes (22). Now it is known a variety of diseases are related to the inflammatory response, so the mechanism of inflammation is particularly important (22). Recent studies have shown that miRNA can regulate a variety of life activities of the body, including the regulation on the inflammatory response. Many miRNAs have been found involved in the regulation of inflammatory reactions and inflammatory diseases (13). This suggests that the promotion of miR-34a protein expression increased TNF-α, IL-1β and IL-18 contents, induced and p65 protein expression, not affects IL-6 and phosphorylation-STAT3 protein expression.

IL-6 is a multifunctional inflammatory cytokine, which is a key component of the inflammatory mediator network, and plays an important role in the inflammatory response (7). As anti-inflammatory cytokine or long-term cytokine, it can balance the damage effect of proinflammatory cytokine or early cytokine, to play a protective effect. IL-6 can act as a cytoprotective and anti-inflammatory agent in bacterial endotoxin-induced experimental lung injury by inhibiting the generation of IL-1 by macrophage and tumor necrosis factor (6). As it has dual function of inflammation and anti-inflammation, its role is related to the contentin tissue; the normal level is beneficial for the body, and excessive generation can cause a series of inflammatory damage (23). Our data indicated that The promotion of IL-6 protein increased TNF-α, IL-1β and IL-18 contents in neonatal lung injury mice.

NF-κB and STAT3 are two important signaling pathways for the connection of inflammation and malignancy, which are often in abnormal activation state in lung cancer. They can promote the development of lung cancer through the regulation of downstream hyperplasia, apoptosis and inflammation-related genes (24). NF-κB is one of the most important nuclear transcription factors in the intracellular signal transduction mediated by inflammatory response (24). It is usually activated by proinflammatory cytokines such as TNF-α and IL-1β (15). The studies from Takahashi have shown that the continuous abnormal activation of NF-κB signal, can lead to the overexpression of TNF-α, IL-6 and other cytokinesin the downstream of NF-κB, to promote lung epithelial cell hyperplasia. In addition, the dysfunction of p53 gene in lung cancer cells and the activation of KRAS can activate NF-κB, and the inhibition on NF-κB pathway can inhibit the further development of lung cancer (25).

As important transcriptional factors in inflammation pathway, NF-κB and STAT3 have interactions with each other by multiple ways (26). NF-κB can transcribe and activate IL-6, and IL-6 can further activate JAK2/STAT3 pathway in the downstream; it is proved that the constitutively activated STAT3 maintains the activity of NF-κB in tumor by inhibiting the output from nuclear of NF-κB (16). In this view, the promotion of IL-6 protein induced phosphorylation-STAT3 and p65 protein expression, and increased miR-34a expression in neonatal lung injury mice.

Our study however clearly demonstrates IL-6R/STAT3/miR-34a protects against NLI patients and NLI-inflammation (TNF-α, IL-1β and IL-18). Finally, clinical implications of miR-34a as biomarker of inflammation in neonatal lung injury, and its release could be also linked to lung injury.

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