

## Original Article

# Upregulation of miR-155 contributes to the suppression of inflammatory responses by targeting Socs1 in LPS-induced acute lung injury

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**Abstract:** In recent years, microRNAs (miRNAs) have been shown to have critical regulatory roles in inflammatory response. However, the contributions of miRNAs to inflammation induced acute lung injury (ALI) remains largely unknown. Differentially expressed miRNAs in the peripheral blood of patients with acute lung injury were identified by PCR arrays and validated using quantitative real-time polymerase chain reaction. Because miR-155 was one of the miRNAs that were significantly elevated, we investigated its function. The functions of microRNA-155 were assessed by silencing the miRNA in lipopolysaccharide (LPS)-induced ALI. Finally we using dual-luciferase reporter assay, qRT-PCR and western blot investigated the potential target of miR-155. Our study showed that miR-155 expression was significantly upregulated both in the peripheral blood of patients with ALI and in the lung tissues of LPS-challenged mice. We also found that knockdown of miR-155 decreased LPS-induced evident lung histopathological changes, lung wet-to-dry weight ratio, and oxygenation index. In addition, knockdown of miR-155 inhibited inflammatory cells and proinflammatory cytokines including interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) in BALF. Furthermore, Socs1, a negative regulator of IFN- $\gamma$  was identified as a direct target of miR-155 using a Luciferase reporter assay and western blot analysis. Our findings strongly demonstrated that reduce expression of miR-155 could effectively ameliorate the LPS-induced ALI by targeting Socs1, suggesting a potential application for miR-155-based therapy to treat clinical ALI.

**Keywords:** Acute lung injury, microRNA-155, IFN- $\gamma$ , Socs1

## Introduction

Acute Lung Injury/acute respiratory distress syndrome (ALI/ARDS) are acute noncardiogenic pulmonary inflammatory response, which is induced by direct and indirect injury to the lung that leads to disruption of the alveolar-capillary unit and a breakdown in the barrier and gas exchange functions of the lung [1, 2]. It is a clinically syndrome characterized by immune cell infiltration, excessive cytokine production, tissue damage, and pulmonary edema [3, 4]. It is the leading cause of death in critically ill patients. Although new therapies have been appeared, the mortality is as high as 30-50%. Therefore, new strategies are urgently required for achieving effective treatment of ALI, which might ultimately aid the clinical therapy for ARDS patients.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with 18-22 nt in length [5]. Mature miRNAs bind to the 3'-UTR of target mRNAs and repress translation of target mRNAs or induce degradation of target mRNAs [6, 7]. Recently, several studies have suggested that miRNAs played a role in modulating immune responses under various inflammatory conditions [8, 9]. And, kinds of miRNAs have been confirmed to participate in the progression of ALI [10, 11]. For example, in a mice ARDS model, induction of miR-146a, an anti-inflammatory microRNA targeting TLR4 signaling, could alternate macrophage activation and suppressed LPS-induced inducible NO synthase that resulted in amelioration of acid-induced lung injury [12]. Cai et al. revealed that the levels of miR-16 were reduced in lipopolysaccharide (LPS)-induced experimental ALI. In

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In addition, miR-16 treatment reduced the expression levels of the TNF- $\alpha$  and IL-6 proinflammatory cytokines following exposure of macrophages to LPS [13]. Recent study indicated miR-155 regulated the immune and inflammatory responses served as novel therapeutic targets and biomarkers for ALI/ARDS [14]. Furthermore, miR-155 has been identified as a component of macrophage and monocyte response to different types of inflammatory mediators, such as bacterial lipopolysaccharide (LPS), interferon- $\gamma$  (IFN- $\gamma$ ), and TNF- $\alpha$  [15, 16]. It implied that these microRNAs regulated lung injury and also represented a novel therapeutic strategy.

To define the LPS-induced miRNA pattern, we conducted a miRNA microarray screen that resulted in a set of differentially regulated miRNAs, including miRNA-155 (miR-155), which was upregulated in lung tissues of ALI mice. We found that down-regulation of miR-155 significantly reduced LPS-induced pulmonary inflammation and resulted in remarkable reversal of LPS-induced increases in lung permeability, accompanied by a significant reduction of histopathology changes of lung. Our findings strongly demonstrated that knockdown of miR-155 could effectively ameliorate the LPS-induced acute lung injury, suggesting the role of miR-155 in ALI and its potential application as a diagnostic and prognostic marker of the disease.

### Materials and methods

#### *Sample collection*

All subjects contributed to the study with a single blood donation. All the peripheral blood samples (approximately 5 ml) were drawn from each subject into heparinized tubes. Samples were coded and processed within 2 h.

#### *Induction of LPS-induced acute lung injury (ALI)*

Studies were performed in male Sprague Dawley mice (8-10 weeks old) that were purchased from Nanchang University (Nanchang, China). All animal procedures were conducted after approval from the Animal Committee of Nanchang University. ALI model was induced in mice as described previously [17, 18]. Male mice were randomly divided into three groups with 10 mice in each group: (1) control group

(saline), (2) LPS group, (3) LPS + miR-155 antagomir (8 mg/kg). Mice were anesthetized by an intraperitoneal injection with chloralhydrate (3%) and kept in a supine position while spontaneous breathing was monitored. Mice were sacrificed at the indicated time after injury. ALI induction was verified by pathological examination of the lung.

#### *Harvest of lungs and bronchoalveolar lavage*

Lungs were harvested 24 h after LPS administration. As described previously, bronchoalveolar lavage (BAL) was obtained by cannulating the trachea with a blunt 20-gauge needle and then lavaging the lungs three times with 1 ml of ice-cold PBS. Total cell counts were measured in the BAL fluid with a hemocytometer (Hausser Scientific).

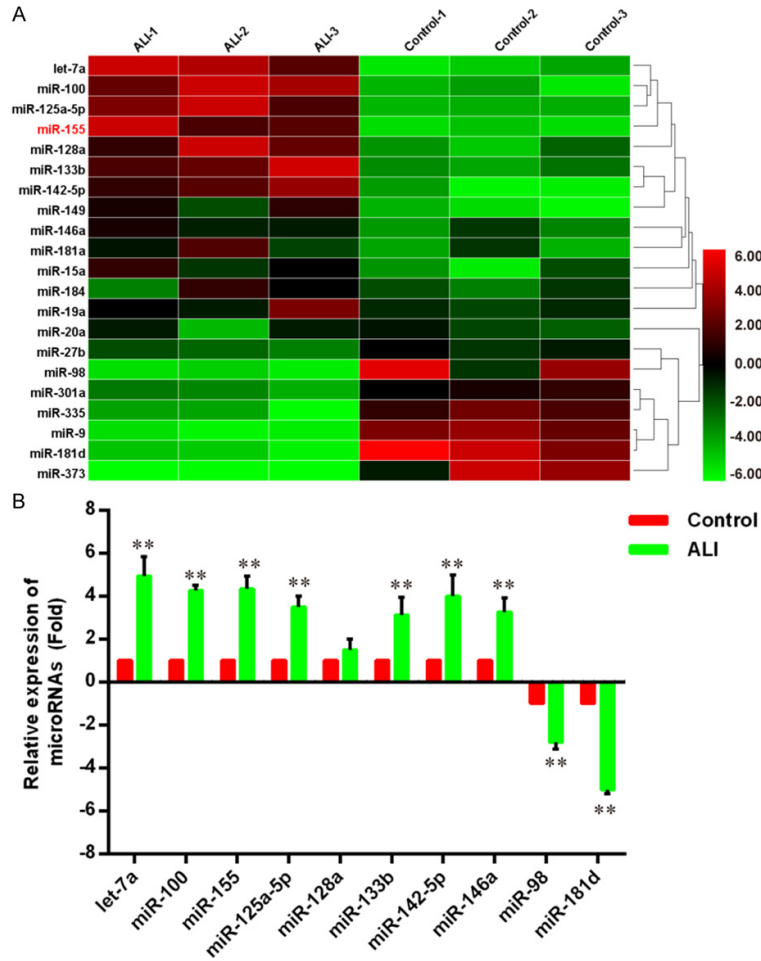
#### *RNA extraction*

Total RNA was extracted from the peripheral blood from patients with ALI using an RNeasy® mini kit (QIAGEN GmbH, Hilden, Germany). Potential genomic DNA contamination was removed from the samples by treatment with RNase-free DNase (QIAGEN) for 15 min at room temperature. Concentration and purity were determined using a NanoDrop 1000™ spectrophotometer (Thermo Fisher, Dubuque, IA, USA), while the integrity of miRNA was further assessed using an Agilent 2100 Bioanalyzer (Agilent Tech, Palo Alto, CA).

#### *PCR array*

The human RT<sup>2</sup> profiler PCR autophagy array (QIAGEN) was used to study the expression of 84 inflammation-associated genes in the peripheral blood from patients with ALI and controls. Briefly, using an RT<sup>2</sup> first Strand kit (QIAGEN), 1  $\mu$ g total RNA obtained from the peripheral blood was incubated with the kit's genomic DNA elimination mixture at 42°C for 5 min and then transferred to ice for no less than 1 min to remove any residual DNA contamination. The kit's reverse transcription mixture was added to the purified RNA sample. The mixture was incubated at 42°C for 15 min and then 95°C for 5 min to convert total RNA back into cDNA. After cDNA synthesis, real-time RT-PCR was performed using RT2 SYBR® Green master mix (QIAGEN), according to the manufacturer's instructions. The amplification data (fold chang-

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**Figure 1.** MiRNA expression in peripheral blood of ALI patients. A. Heat map of microRNA (miRNA) profiles represented the significantly regulated miRNAs in peripheral blood from ALI patients and controls. B. Listing of miRNAs in heat map with fold change. Among those, miR-155 was one of the highest expressed miRNA in peripheral blood from ALI patients and controls. \*\*P < 0.01 vs Controls.

es in the threshold cycle [Ct] values of all the genes) were analyzed by the  $\Delta\Delta C_t$  method.

### Real-time PCR

For miRNAs analysis, total RNA was isolated from lungs using the MicroRNA Extraction and Purification Kit (Novland, Shanghai, China). Real-time PCR was performed using two-step Stemaim-it miRqRT-PCR Quantitation Kit (SYBR Green) (Novland, Shanghai, China) on BIO-RAD IQ5 real-time PCR instrument. Specific primers and probes for mature microRNAs and snRNA RNU6B were obtained from Genepharma, Shanghai, China. All reactions were conducted in triplicate. Quantitative normalization was performed on U6 and  $\beta$ -actin for miRNA and mRNA detection, respectively.

### Cytokine assay

Levels of IFN- $\gamma$ , IL-1 $\beta$ , and IL-6 in BALF were determined by ELISA kits according to the instructions recommended by the manufacturers. The optical density of each well was read at 450 nm.

### Measurement of wet-to-dry ratio of the lungs

At 6 h after treatment with LPS, mice were euthanized, the right lung was then removed and the wet weight was determined. Subsequently, the lungs were incubated at 60°C for 3 to 4 days to remove all moisture, then the dry weight was measured and the ratio of wet-to-dry weight calculated.

### Oxygenation index ( $PaO_2/FiO_2$ ) analysis

At 24 h after ALI (or control), animals were anesthetized and given endotracheal intubation with a 20-gauge catheter. The animals were mechanically ventilated with pure oxygen at 7 mL/kg (120 breaths/min). After 20 min-ventilation, the arterial blood was obtained from carotid

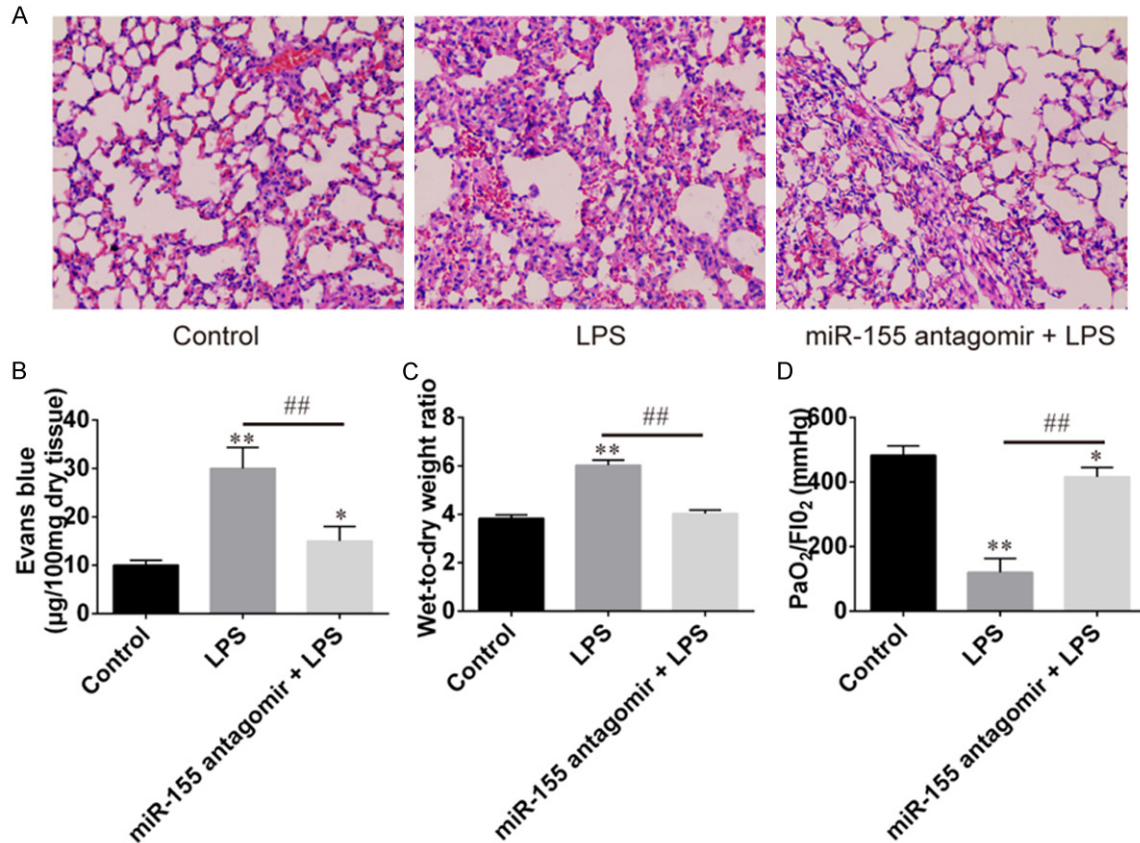
artery and measured using a commercial blood gas analyzer (model ABL8000; Radiometer Copenhagen, Westlake, Ohio).

### Histopathological analysis

Lung tissue was fixed in 4% (v/v) paraformaldehyde and embedded in paraffin, sectioned at 4- $\mu$ m thickness and stained with H&E solution (Sigma-Aldrich) to estimate inflammation.

### Luciferase reporter assay

To determine whether Socs1 is a direct target of miR-155, dual luciferase assays were conducted in a 24 well plate format. pGL3-Socs1 3'UTR report/pGL3-Socs1 3'UTR Mutant report + TK100 Renilla report were transfected into



**Figure 2.** miR-155 plays a critical role in LPS-induced ALI in mice. MiR-155 antagonist was injected into the tail veins after LPS challenge and mice were euthanized 48 h later. Histopathological changes, Evans blue (EB), Wet-to-dry (W/D) values, Oxygenation index (PaO<sub>2</sub>/F<sub>i</sub>O<sub>2</sub>) were detected. A. Representative H&E images (×20) of sections of lung indicating immune cell infiltration. B. Evans blue (EB) content in lungs. C. Wet-to-dry (W/D) lung weight ratio. D. Oxygenation index (PaO<sub>2</sub>/F<sub>i</sub>O<sub>2</sub>). Data are presented as mean ± SD (n = 10 in each group). \*P < 0.05, \*\*P < 0.01 vs control group; ##P < 0.05 vs. LPS group.

70% confluent HEK293 cells, along with miR-155 mimic, miR-155 inhibitor or each control. After 48-h transfection, firefly and renilla luciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, USA) following the manufacturer's recommendations.

#### Western blot analysis

Total cell protein extracts (20 µg) were separated by 10% SDS polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked for 1 h in PBST with 5% non-fat milk at 4°C. Then, the blots were incubated with primary antibodies against Socs1 and β-actin (Santa Cruz, USA) followed by horseradish peroxidase-conjugated secondary antibody and detected by chemiluminescence detection kit (Millipore, Billerica, MA, USA). The intensity of protein fragments was quantified using Image-Pro Plus software.

#### Statistical analysis

All parameters were recorded for individuals within all groups. The statistical comparisons of data were implemented using ANOVA and t-test in the SPSS 13.0 system. P value < 0.05 was considered significant.

#### Results

##### MiRNA expression profiles and qPCR validation in the peripheral blood from patients with ALI

Hierarchical clustering showed systematic variations in the expression of miRNAs in the peripheral blood from patients with ALI. After normalization, 14 upregulated and 7 downregulated miRNAs were found in the peripheral blood compared with those in the normal control group (Figure 1A). To validate the miRNA PCR array analysis findings, we randomly

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selected 10 miRNAs among the differential miRNAs and analyzed their expression using quantitative real-time polymerase chain reaction (qRT-PCR) in the peripheral blood from patients with ALI. These data confirmed that let-7a, miR-100, miR-155, miR-125b-5p, miR-128a, miR-133b, miR-142-5p and miR-146a were over-expressed in the peripheral blood, whereas the expression of miR-98 and miR-181d was decreased ( $P < 0.05$  for all), which corroborated the expression patterns seen using the PCR array (**Figure 1B**). Thus, our data indicate that a set of miRNAs is frequently aberrantly expressed in the peripheral blood from patients with ALI. It is also interesting that the expression of miR-155 is important for SEB-mediated inflammation during ALI [19]. Based on this, we hypothesized that miR-155 may be involved in the development of LPS-induced ALI.

### *Knockdown of miR-155 ameliorates LPS-induced ALI in mice*

Given the increased expression of miR-155 in patients with ALI, we sought to detect whether knockdown of miR-155 could ameliorate the LPS-induced ALI. Thus, we injected miR-155 antagomir into the tail veins every other day after LPS challenge. Compared with the control group, the lungs of LPS-challenged mice in model control group showed marked inflammatory alterations characterized by the presence of interstitial edema, hemorrhage, and thickening of the alveolar wall. In contrast, histological damage was improved in the ALI mice treated with miR-203 antagomir (**Figure 2A**).

Meanwhile, pretreatment with miR-155 antagomir significantly attenuated the increase of Evans blue (EB) content in lungs (**Figure 2B**) and the lung wet-to-dry (W/D) ratios (**Figure 2C**) levels compared with that in the LPS group, respectively. Furthermore, the ratio of  $\text{PaO}_2/\text{FiO}_2$  was remarkably reduced in LPS + miR-155 antagomir compared with that in the LPS group (**Figure 2D**). This finding demonstrates that miR-155 negatively regulates lung injury induced by LPS.

### *Effects of miR-155 on cytokine in BALF*

Mice treated with LPS demonstrated a significant increase in the inflammatory cell content of the BALF compared with the normal control

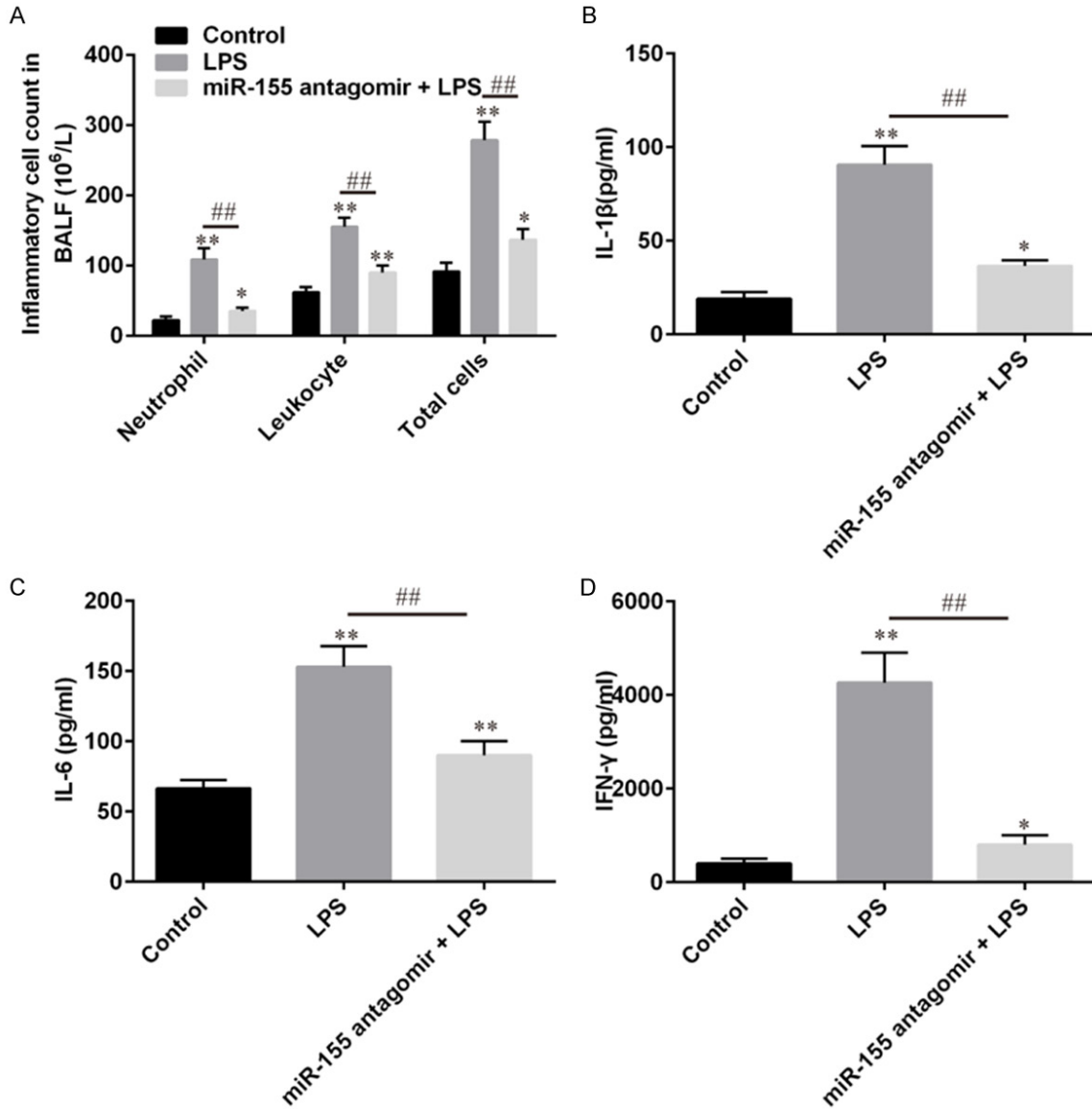
mice. In particular, the number of neutrophil and leukocyte in BALF are markedly increased in LPS group compared with the control group; however, miR-155 antagomir pretreatment significantly reduced the number of neutrophil and leukocyte in BALF compared with the LPS group (**Figure 3A**).

The secretion of inflammatory cytokines is a pivotal cellular response to ALI [20, 21]. Thus, IFN- $\gamma$ , IL-6 and IL-1 $\beta$  in BALF were measured by ELISA. IL-1 $\beta$ , and IL-6 levels were found to be significantly increased in the LPS group compared with the control group. Conversely, miR-155 antagomir pretreatment efficiently decreased the levels of IL-1 $\beta$ , and IL-6 (**Figure 3B, 3C**). Additionally, cytokine analysis of the bronchoalveolar lavage fluid (BALF) in the lungs from LPS-exposed mice demonstrated high concentrations of proinflammatory cytokine IFN- $\gamma$ . In contrast, IFN- $\gamma$  levels were significantly diminished in the mice injected with miR-155 antagomir after LPS challenge (**Figure 3D**). These results provided clear evidence that knockdown of miR-155 attenuated the LPS induced inflammation.

### *MiR-155 targets Socs1, a negative regulator of IFN- $\gamma$*

Several studies demonstrated that miR-155 could control the inflammatory process by its ability to target genes such as FADD and Ripk1 for translational repression [22, 23]. Among the predicted miR-155 targets, Socs1 is an important negative regulator of IFN- $\gamma$ . To examine whether miR-155 directly targets Socs1, we conducted a luciferase assay (**Figure 4A**). The luciferase assay used in the present study is to evaluate the effect of miRNA-dependent post-transcriptional regulation of target genes. We transfected HEK293 cells with miR-155 mimic and miR-155 inhibitor in which miR-155 expression was significantly increased or decreased. The results showed that overexpression of miR-155 significantly decreased the luciferase activity in pGL3-Socs1 3'-UTR transfected cells, whereas it had no effect on pGL3-mut Socs1 3'-UTR (**Figure 4B**).

To evaluate whether miR-155 regulated Socs1 expression, we detected the protein expression level of Socs1 in miR-155 mimic or miR-155 inhibitor infected cells. Western blot analysis showed that miR-155 overexpression markedly



**Figure 3.** Knockdown of miR-155 inhibits inflammatory cell content and pro-inflammatory cytokine levels in BALF. When miR-155 was inhibited by antagomir-155, The circulating levels of inflammatory mediators of IFN- $\gamma$ , IL-1 $\beta$ , and IL-6 were elevated. A. Cells were isolated by centrifugation and stained with Diff-Quik stain reagent. B. IL-1 $\beta$  level in BALF. C. IL-6 level in BALF. D. INF- $\gamma$  level in BALF. Data are presented as mean  $\pm$  SD (n = 10 in each group). \*P < 0.05, \*\*P < 0.01 vs control group; ##P < 0.05 vs. LPS group.

decreased the protein level of Socs1, whereas miR-155 inhibition increased the protein expression of Socs1 (Figure 4C). These data indicated that miR-155 functions by directly targeting Socs1 expression.

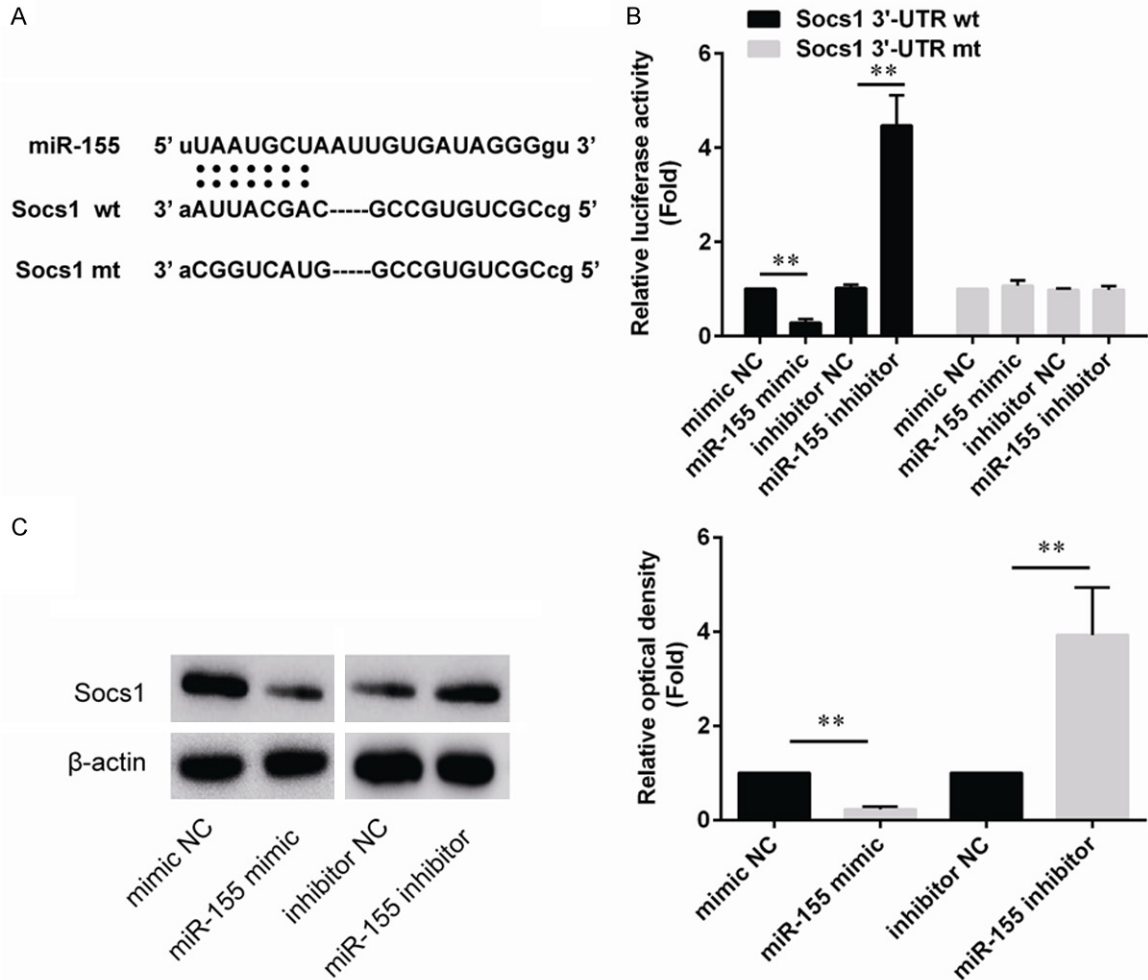
**Discussion**

In the present study, we demonstrated miR-155 expression was upregulated in the peripheral blood from patients with ALI. More impor-

tantly, we found that knock down of miR-155 could partially reversed LPS-induced inflammatory responses. In addition, the expression level of Socs1, a negative regulator of IFN- $\gamma$  that could always be activated, was also inhibited by overexpression of miR-155.

A growing body of scientific evidence has suggested a regulatory role and the potential impact of microRNAs in treatment for ALI. WANG et al. found that miR-181b may be

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**Figure 4.** miR-155 directly binds and downregulates SOCS1. A. Schema of the firefly luciferase reporter constructs for the SOCS1, indicating the interaction sites between miR-155 and the 3'-UTRs of the SOCS1. B. Luciferase activities. HEK-293 cells were co-transfected with firefly luciferase constructs containing the SOCS1 wild-type or mutated 3'-UTRs and miR-155 mimic, mimic NC, miR-155 inhibitor or inhibitor NC, as indicated (n = 6). C. Protein expression of SOCS1 after treatment with miR-155 mimic or miR-155 inhibitor (n = 6). All data represent the mean  $\pm$  SD results of three independent experiments. \*\*P < 0.01.

involved in the process of LPS-induced inflammation in BEAS-2B cells by activating the NF- $\kappa$ B signaling pathway [24]. A study performed by Cai et al revealed that miR-16 treatment reduced the expression levels of the TNF- $\alpha$  and IL-6 proinflammatory cytokines following exposure of macrophages to LPS [13]. Similarly, miR-127 treatment was also demonstrated to reduce the IL-1 $\beta$ , TNF- $\alpha$  and IL-6 production in macrophages that had been exposed to LPS, as well as to reduce the lesion degree in an experimental ALI model in vivo [25]. In order to identify potential miRNAs involved in ALI, the miRNAs expression profile was analyzed in the peripheral blood from

patients with ALI. Notably, 21 miRNAs displayed significantly differential expression levels. Of these potential candidates, the focus was laid on miR-155, since it was one of the most clearly altered miRNAs and is known to be deregulated in inflammation, although its function remains unclear.

A variety of studies have demonstrated that miR-155 participated in the mediation of ALI. For example, a study from Roshni Rao et al. found that miR-155 played an important role in SEB-induced acute inflammatory lung injury [19]. Dr. Baltimore and his group showed the up-regulation of the miR-155 level in response

to LPS and to virally relevant stimuli in human monocytes [15, 26], whereas two recent reports showed an impaired response of B cells from miR-155<sup>-/-</sup> knockout mice toward LPS [27, 28]. Recently, miR-155 has been confirmed to effectively alleviate the lung inflammation in mice with acute lung injury induced by LPS [29]. Our data showed that knockdown of miR-155 suppressed increasing of important pro-inflammation cytokines in of ALI mice, including IL-1 $\beta$  and IL-6. These results suggested that miR-155 might play an anti-inflammation role in the development of LPS-induced ALI.

Usually, an appropriate regulation of IFN- $\gamma$  is necessary for mediating Th1 responses and blunting infection [30]. LPS exposure, however, causes an excessive release of IFN- $\gamma$ . T cells exposed to IFN- $\gamma$  proliferate further, thus perpetuating a cycle of inflammation [31, 32]. Studies carried out with LPS activation of macrophages have demonstrated that early cytokines released include IL-1 $\beta$  and TNF- $\alpha$ , followed by the massive production of IFN- $\gamma$  [15, 16]. Our data indicated substantial release of LPS-induced IFN- $\gamma$  in the BALF, suggesting that this particular cytokine may significantly contribute to LPS-induced inflammation. In addition, we found that while transfection with a synthetic miR-155 antagomir leads to the reduction of IFN- $\gamma$  induced by LPS. Our results clearly indicate that the LPS-mediated induction of IFN- $\gamma$  can be explained, at least in part, by the induction of miR-155.

SOCS1 is a critical feedback inhibitor of both IFN- $\gamma$ /STAT. In particular, SOCS1 is induced by IFN- $\gamma$  for autoregulation of the IFN- $\gamma$  pro-inflammatory response by inhibiting the JAK/STAT1 signaling pathway [33]. Recent experiments in numerous cell types have revealed that miR-155 targets SOCS1 [31, 32, 34]. For example, miR-155 could enhance Th1 cell differentiation by targeting SOCS1 and strengthen the signal transduction of IFN- $\gamma$  [35]. Wang P et al. demonstrate that inducible miR-155 feedback positively regulates host antiviral innate immune response by promoting type I IFN signaling via targeting SOCS1 [36]. Accordingly, we hypothesized that miR-155 showed both anti- and pro-inflammatory effects by regulating Socs-1. In the current study, gain and loss-of-function studies with miR-155 mimic and inhibitor clearly demonstrated that miR-155-mediated targeting of Socs1.

Because miRNAs target multiple mRNAs to regulate gene expression, a single miRNA might regulate the protein synthesis of thousands of genes either directly or indirectly [37]. It is likely that miR-155 may also function through different mechanisms by targeting different molecules during the process. Consequently, further studies to reveal additional targets of miR-155 are warranted.

In conclusion, we found miR-155 was highly expressed in the peripheral blood of ALI patients and we found that knockdown of miR-155 could protect from LPS-mediated inflammation and acute lung injury. Furthermore, the present study demonstrated that the high levels of IFN- $\gamma$  production associated with LPS exposure can be attributed to the miR-155-mediated repression of Socs1, a critical regulator of IFN- $\gamma$ . Thus, miR-155 may be a potential apoptotic biomarker and potent therapeutic target for LPS-induced ALI.

### Disclosure of conflict of interest

None.

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