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Inhibition of miR-21 ameliorates LPS-induced acute lung injury through increasing B cell lymphoma-2 expression

Junke Ge, Yanfen Yao, Haiyan Jia, Pibao Li and Wei Sun

Abstract

The aberrant expression of microRNAs (miRNAs) is associated with the pathogenesis of inflammation-related diseases. However, the biological functions of miR-21 in acute lung injury (ALI) remain largely unknown. In this study, the level of miR-21 was obviously increased, but B cell lymphoma-2 (Bcl-2) expression was markedly decreased in LPS-treated human pulmonary alveolar epithelial cells (HPAEpiC). Suppression of miR-21 attenuated LPS-induced apoptosis and inflammation in HPAEpiC and promoted the survival of mice with ALI by decreasing the inflammatory cell count, release of cytokines and permeability in lung tissues. Importantly, Bcl-2 was a direct target of miR-21, and its expression was significantly inhibited by miR-21 mimics at a post-transcriptional level. Besides, Bcl-2 over-expression reversed miR-21-induced apoptosis and inflammation status and showed synergic effects with miR-21 inhibitor in LPS-treated HPAEpiC. In conclusion, inhibition of miR-21 could ameliorate apoptosis and inflammation by restoring the expression of Bcl-2 in LPS-induced HPAEpiC and mice, which might provide therapeutic strategies for the treatment of ALI.

Keywords

miR-21, Bcl-2, ALI, HPAEpiC, mice

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Introduction

Acute lung injury (ALI) is one leading cause of death from inflammation, characterised by excessive inflammation in lung tissues, which results in a loss of alveolar–capillary membrane integrity and lung dysfunction.[1,2] Although significant advances in the treatment of ALI have been achieved, the annual morbidity and mortality of patients with ALI or acute respiratory distress syndrome (ARDS) remain high.[3,4] Consequently, it is imperative to investigate the molecular mechanisms of ALI and novel therapeutic methods.

MicroRNAs (miRNAs), a type of small endogenous non-coding single-stranded RNA, play a key role in the regulation of gene expression. Generally, their pairing with the 3′-untranslated region (3′-UTR) of target mRNAs results in mRNA degradation or translational inhibition.[5] In previous studies, miRNAs have been found to be down-regulated and involved in certain inflammatory conditions.[6–8] For example, miR-297 is reduced in LPS-induced inflammatory human umbilical vein endothelial cells,[6] and miR-146b and miR-27a overexpression ameliorate LPS-induced ALI through inhibiting inflammation and apoptosis.[7,8] In addition, miRNAs have been identified to be up-regulated in inflammatory conditions, such as miR-155[9] and miR-21.[10] Jansing et al. demonstrated that inflammatory reactions were impaired in miR-21 knock-out mice with reduced platelet and neutrophil activation compared to wild type (WT) mice that showed inflammatory and pathological changes in the presence of LPS.[10] However, more *in vitro* assays and *in vivo* experiments should be explored to elucidate the mechanisms underlying miR-21-involved ALI.

Department of Intensive Care Unit, Shandong Provincial Third Hospital, Cheeloo College of Medicine, Shandong University, PR China

Corresponding author:
Wei Sun, Department of Intensive Care Unit, Shandong Provincial Third Hospital, Cheeloo College of Medicine, Shandong University, No. 11, Central Wuying Hill Road, Jinan 250031, Shandong, PR China.
Email: qdqd1999@126.com

In this work, we investigated the expression of miR-21 in ALI model using LPS-induced human pulmonary alveolar epithelial cells (HPAEpiC) and mice. Then, miR-21 inhibitor transfection and antagonist injection were conducted to inhibit miR-21 level in HPAEpiC and mice with ALI, respectively. Further, the target gene was predicted by Targetscan and verified through a dual luciferase reporter gene assay. This study will provide a theoretical basis for the treatment of ALI.

Methods

Cell culture and transfection

HPAEpiC from ScienCell Research Laboratories (Carlsbad, CA) were cultured in alveolar epithelial cell medium (ScienCell Research Laboratories) with poly-L-lysine-coated culture vessels (Corning, Inc., Tewksbury, MA). HEK293T was originally obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% FBS, 10 IU/ml penicillin and 10 mg/ml streptomycin. Cells were grown in a humidified atmosphere at 37°C at gas tensions of 20% O_2/5% CO_2 for normoxic incubation. MiR-21 inhibitor, inhibitor-negative control (NC) and Bcl-2 plasmids were purchased from RiboBio (Guangzhou, PR China), nd transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Cell viability assay

Cell proliferation was determined with a Cell Counting Kit-8 (CCK-8) assay. Briefly, cells were placed on a 96-well plate at a density of 2×10^3 cells/well and incubated at 37°C in a 5% CO_2 humidified environment. CCK-8 reagent was added and incubated for 2 h. Light absorbance at 450 nm was measured daily with a microplate reader. Experiments with were performed independently in triplicate.

Apoptosis analysis

Cell apoptosis analyses were performed using a Phycoerythrin-Annexin V Apoptosis Detection kit I (BD Pharmingen; BD Biosciences, San Jose, CA). For cell apoptosis analysis, 8×10^5 cells were seeded in each well of six-well plates. Then, 48 h after transfection, cells were harvested and labelled with Annexin V for 15 min. Subsequently, 50 μg/ml propidium iodide was added for 1 h at 37°C to each sample prior to flow cytometry using the BD LSR II (BD Biosciences).

Quantitative RT-PCR

Bulge-Loop™ miRNA quantitative RT-PCR (qRT-PCR) primer sets for hsa-miR-21 were purchased from Ribobio (Guangzhou, PR China). Total RNA (1 μg) was reverse transcribed with either the miRNA-specific primers or an Oligo dT primer using a reverse transcription system kit (Promega, Fitchburg, WI). Extraction of serum miRNA was performed using the miRcute Serum/Plasma miRNA Isolation Kit (Tiangen, Beijing, PR China) following the manufacturer's instructions. Subsequently, qRT-PCR was performed with a GoTaq qPCR Master Mix Kit (Promega) to quantify the miRNA and mRNA levels, using U6 and GAPDH, respectively, as the internal controls. The fold changes of the miRNA and mRNA levels were calculated using the $2-\Delta Ct$ method.

Western blotting

Equal amounts of protein lysates were subjected to 12% SDS-PAGE gel and then transferred to polyvinylidene fluoride membrane (Millipore, Danvers, MA) and probed with primary Abs at 4°C overnight and secondary Abs at room temperature for 2 h. Bound Abs were detected by the ECL Plus Western blotting substrate (Thermo Fisher, Waltham, MA), and the results were recorded using the Biorad ChemiDoc MP Gel Imaging System. The band density of specific proteins was quantified after normalisation with GAPDH.

Animal model

Experimental protocols involving animals were approved by the Animal Care and Use Committee of Shandong Provincial Third Hospital (SLSY-A-201801). BALB/c mice (aged 6–8 wk old and weighing 18–22 g) were obtained from the animal experimental centre of Shandong University. The mice were kept under specific pathogen-free conditions: 22 ± 2°C, 40% humidity, 12:12 h light/dark cycle and free access to food and water. Thirty mice were instilled intra-tracheally with 5 mg/kg of LPS (Sigma–Aldrich, St Louis, MO). For the treatment group, mice ($n = 10$) were injected with antagomir-21 via the tail vein. Antagomir-NC was used as control ($n = 10$). Cold PBS was instilled and aspirated into the lungs for the collection of bronchoalveolar lavage fluid (BALF). After centrifugation, the concentration of total proteins in the supernatant was determined by the BCA method (Beyotime Biotechnology, Shanghai, PR China). Then, pro-inflammation cytokines in the BALF from ALI mice and cells lysis were detected using ELISA kits (Beyotime) for the quantification of TNF-α, IL-1β and IL-6 according to the manufacturer's protocol. Optical density value was measured at 450 nm on an ELISA plate reader (Synergy HT; BioTek Instruments, Inc., Winooski, VT).

Luciferase reporter assay

The Bcl-2 3′-UTR luciferase reporter gene plasmids were constructed by GenePharma, Inc. (Shanghai, PR China). 3′-UTR of Bcl-2 was cloned and inserted downstream of the firefly luciferase of plasmid pmirGLO to generate pmirGLO-Bcl-3′-UTR-WT and pmirGLO-Bcl-2-3′-UTR-mutant (MUT) reporters. The HEK-293T cells were co-transfected with Bcl-2-3′-UTR-WT and miR-21 mimics or Bcl-2-3′-UTR-MUT and miR-21 mimics using Lipofectamine 2000. After incubation, we used a dual luciferase reporter gene assay kit (Beyotime) to detect the luciferase activity according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5 (GraphPad Software, Inc., La Jolla, CA). The results are shown as the mean ± SEM of triplicate experiments. Data were analysed by one-way ANOVA with Bonferroni test or Student's *t*-test. *P* values < 0.05 were considered statistically significant.

Results

MiR-21 level increases in inflammatory HPAEpiC

To identify whether the expression of miR-21 is deregulated in LPS-induced inflammatory conditions, we detected miR-21 expression in HPAEpiC using a qRT-PCR assay. As shown in Figure 1a, the abundance of miR-21 was remarkably increased in LPS-induced HPAEpiC compared to DMSO control ($P < 0.01$). Besides, the protein level of Bcl-2 was apparently reduced in LPS-treated HPAEpiC (Figure 1b). These findings indicate that miR-21 and Bcl-2 might play important roles in the development of ALI. In addition, LPS obviously increased miR-21 expression or inhibited Bcl-2 level in a dose- and time-dependent manner ($P < 0.01$). Both expressions reached the highest or lowest level, respectively, at 48 h after 1000 ng/ml of LPS treatment. Further, the proliferation of HPAEpiC was significantly suppressed (Figure 1c), while its apoptosis was obviously increased by 1000 ng/ml of LPS compared to DMSO control ($P < 0.01$; Figure 1d). qRT-PCR was employed to detect the mRNA expression. ELISA was used to determine the levels of pro-inflammatory cytokines. The mRNAs and concentrations of IL-1β, IL-6 and TNF-α in HPAEpiC and supernatant, respectively, were markedly increased after stimulation of LPS ($P < 0.01$; Figure 1e and f). Based on the previously mentioned results, we inferred that miR-21 level was increased by LPS in inflammatory HPAEpiC.

MiR-21 inhibitor ameliorates inflammatory injury of HPAEpiC

To analyse the impact of miR-21 on apoptosis and inflammatory response in LPS-induced HPAEpiC, miR-21 inhibitor was transfected into HPAEpiC to decrease the intracellular level of miR-21 prior to LPS treatment. First, apoptosis-related proteins were analysed by Western blotting. The results suggested that LPS prominently increased the protein levels of Bax and cleaved caspase 3/9 but reduced the protein expression of Bcl-2, which were significantly reversed by miR-21 inhibitor ($P < 0.01$; Figure 2a and b). As expected, the reduced cell viability and increased apoptotic rate in LPS-treated HPAEpiC were also reversed by miR-21 inhibitor ($P < 0.01$; Figure 2c and d). Besides, elevated levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in HPAEpiC induced by LPS were significantly attenuated after pretreatment with miR-21 inhibitor ($P < 0.01$; Figure 2e and f). Altogether, these findings indicated that miR-21 inhibitor reversed LPS-induced apoptosis and inflammatory response in HPAEpiC.

MiR-21 inhibition promotes survival and alleviates the lung injury of mice with ALI

We established a LPS-induced ALI model in mice and examined the pathological alterations at a molecular and cellular level. Then, we inhibited miR-21 level in mice with ALI using transfection of antagomir-21. The level of miR-21 was significantly dropped by antagomir-21 in the BALF and blood 24 h post treatment ($P < 0.01$; Figure 3a). After 72 h, mice with antagomir-21 had a significantly longer overall survival time than those with antagomir-NC ($P < 0.01$; Figure 3b). As shown in Figure 3c, it was found that there were reduced total leucocytes and neutrophils in the BALF of antagomir-21-treated ALI mice ($P < 0.01$; Figure 3c). Further, we measured the concentrations of IL-1β, IL-6 and TNF-α in the BALF using ELISA. Twenty-four h after LPS administration, the concentration levels of IL-1β, IL-6 and TNF-α in the LPS+antagomir-21 group were significantly reduced compared to those in the LPS+antagomir-NC group ($P < 0.01$; Figure 3d). Total protein, albumin and IgM concentrations were higher in the BALF of mice with ALI, which was consistent with exudation of plasma proteins into the alveolar cavity. Inhibition of miR-21 ameliorated LPS-induced exudation of plasma proteins, as characterised by decreased total protein, albumin and IgM levels ($P < 0.01$; Figure 3e and f). Our results suggested that inhibition of miR-21 promoted survival and alleviates the lung injury of mice with ALI.

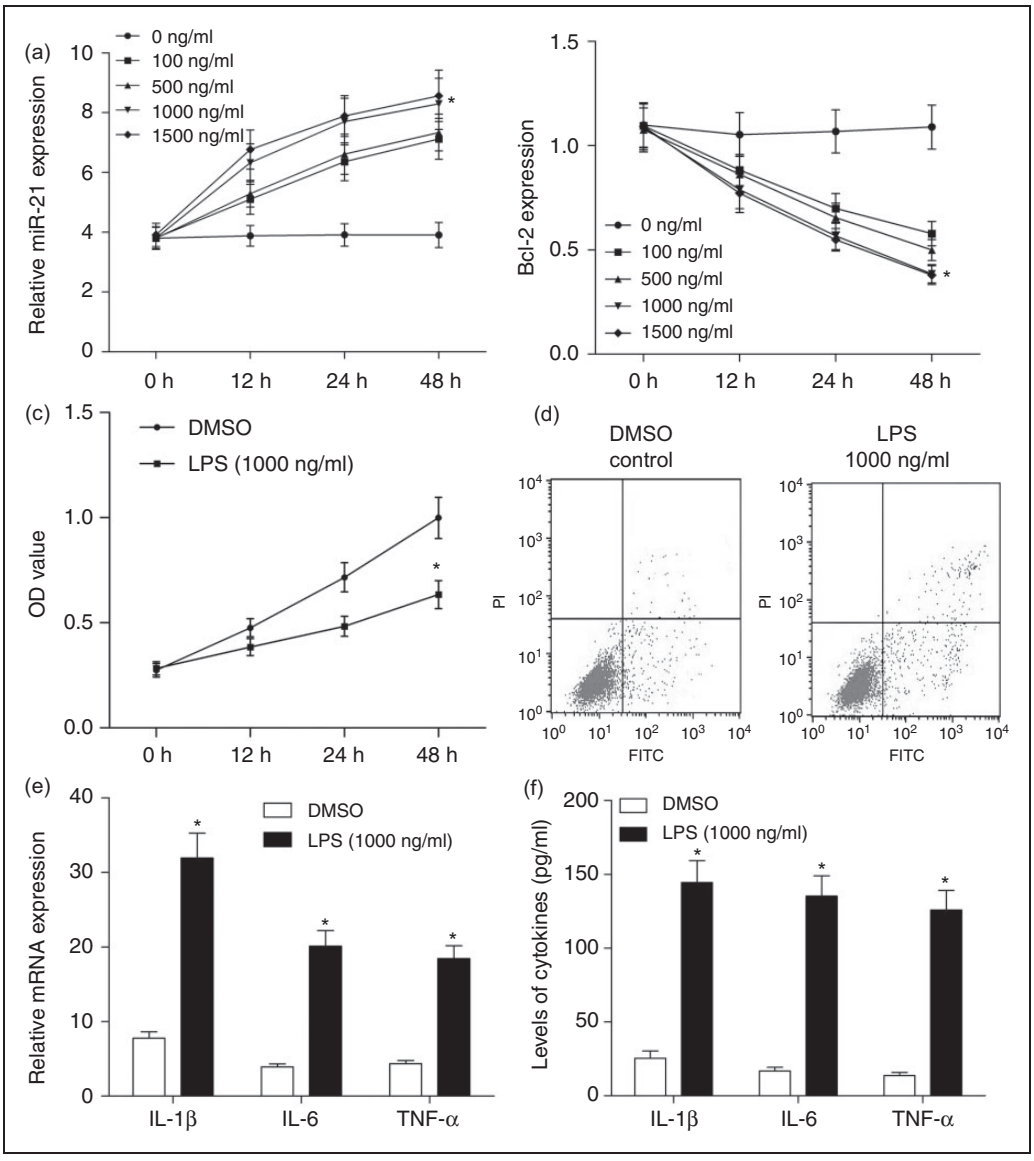

Figure 1. microRNA-21 (miR-21) level is increased by LPS in inflammatory human pulmonary alveolar epithelial cells (HPAEpiC). HPAEpiC were exposed to LPS (0, 100, 500, 1000 and 1500 ng/ml) at different time points (0, 12, 24 and 48 h). (a) miR-21 expression was evaluated by quantitative RT-PCR (qRT-PCR) at different time points (0, 12, 24 and 48 h). (b) B-cell lymphoma-2 (Bcl-2) expression was evaluated by Western blotting at different time points (0, 12, 24 and 48 h). HPAEpiC were exposed to 1000 ng/ml LPS. Then, cell viability (c) was measured with a Cell Counting Kit-8 (CCK-8) assay at different time points (0, 12, 24 and 48 h). Cell apoptosis (d) was detected by flow cytometry 48 h post treatment. The mRNAs (e) and concentrations (f) of inflammatory cytokines were assessed by qRT-PCR and ELISA, respectively. *$P < 0.01$ versus DMSO control.

Bcl-2 is a direct target of miR-21

We screened the candidate target genes through the Targetscan website. Then, we found that the 3′-UTR of Bcl-2 contains the targeting site of miR-21 (Figure 4a). Further, Western blotting was carried out to detect the expression of Bcl-2 in HEK-293T cells. Bcl-2 protein level was decreased in HEK-293T cells with miR-21 mimics plus Bcl-2 plasmids with its 3′-UTR-WT compared to NC mimics plus Bcl-2 plasmids with its 3′-UTR-WT, whereas there was no significance between miR-21 mimics+Bcl-2 plasmids with 3′-UTR-MUT and NC mimics+Bcl-2 plasmids with 3′-UTR-MUT groups ($P < 0.01$; Figure 4b). Next, we designed a WT Bcl-2 3′-UTR sequence and a MUT vector and inserted them into luciferase reporter plasmids. We verified the binding relationship between miR-21 and Bcl-2 using the dual luciferase reporter assay. MiR-21 mimics attenuated luciferase activity of pmirGLO-Bcl-2-3′-UTR-WT reporter ($P < 0.01$) but had no significant effects on

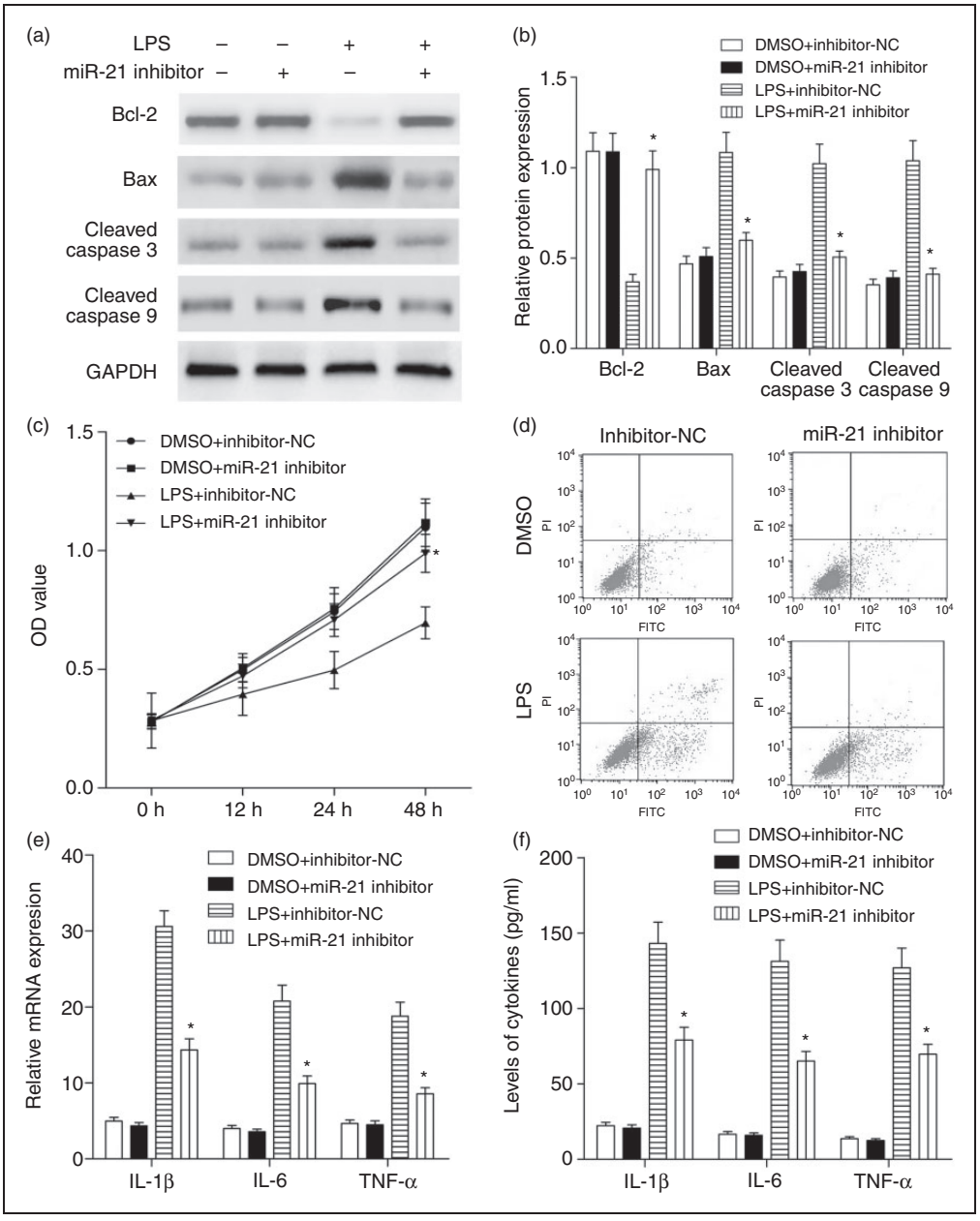

Figure 2. MiR-21 inhibitor attenuates LPS-induced cell injury. HPAEpiC were subjected to 1000 ng/ml LPS or/and transfected with 20 nM (final concentration) miR-21 inhibitor or inhibitor-NC. (a) Western blotting analysis was performed to detect apoptosis-related proteins (Bcl-2, Bax and cleaved caspase 3/9) 48 h post treatment. (b) Protein expression was quantified by densitometric analysis and normalised to GAPDH. (c) Cell viability was measured by CCK-8 assay 48 h post treatment. (d) Cell apoptosis was detected by flow cytometry 48 h post treatment. The mRNAs (e) and concentrations (f) of inflammatory cytokines were assessed by qRT-PCR and ELISA, respectively, 48 h post treatment. *$P < 0.01$ versus DMSO + inhibitor-NC.

that of pmirGLO-Bcl-2-3′-UTR-MUT reporter ($P > 0.05$; Figure 4c).

Over-expression of Bcl-2 enhances the effect of miR-21 inhibitor in LPS-induced HPAEpiC

To explore whether Bcl-2 was involved in miR-21-mediated inhibition of progression of ALI, miR-21 inhibitor/inhibitor NC and Bcl-2 plasmids/empty vector were transfected into HPAEpiC treated with LPS. As displayed in Figure 5a, miR-21 prominently reduced the protein level of Bcl-2 in LPS-induced HPAEpiC, which was further reversed by Bcl-2 plasmids ($P < 0.01$). Moreover, up-regulation of Bcl-2 enhanced the protective effects of miR-21 on cell viability (Figure 5c), and inhibited apoptosis by further

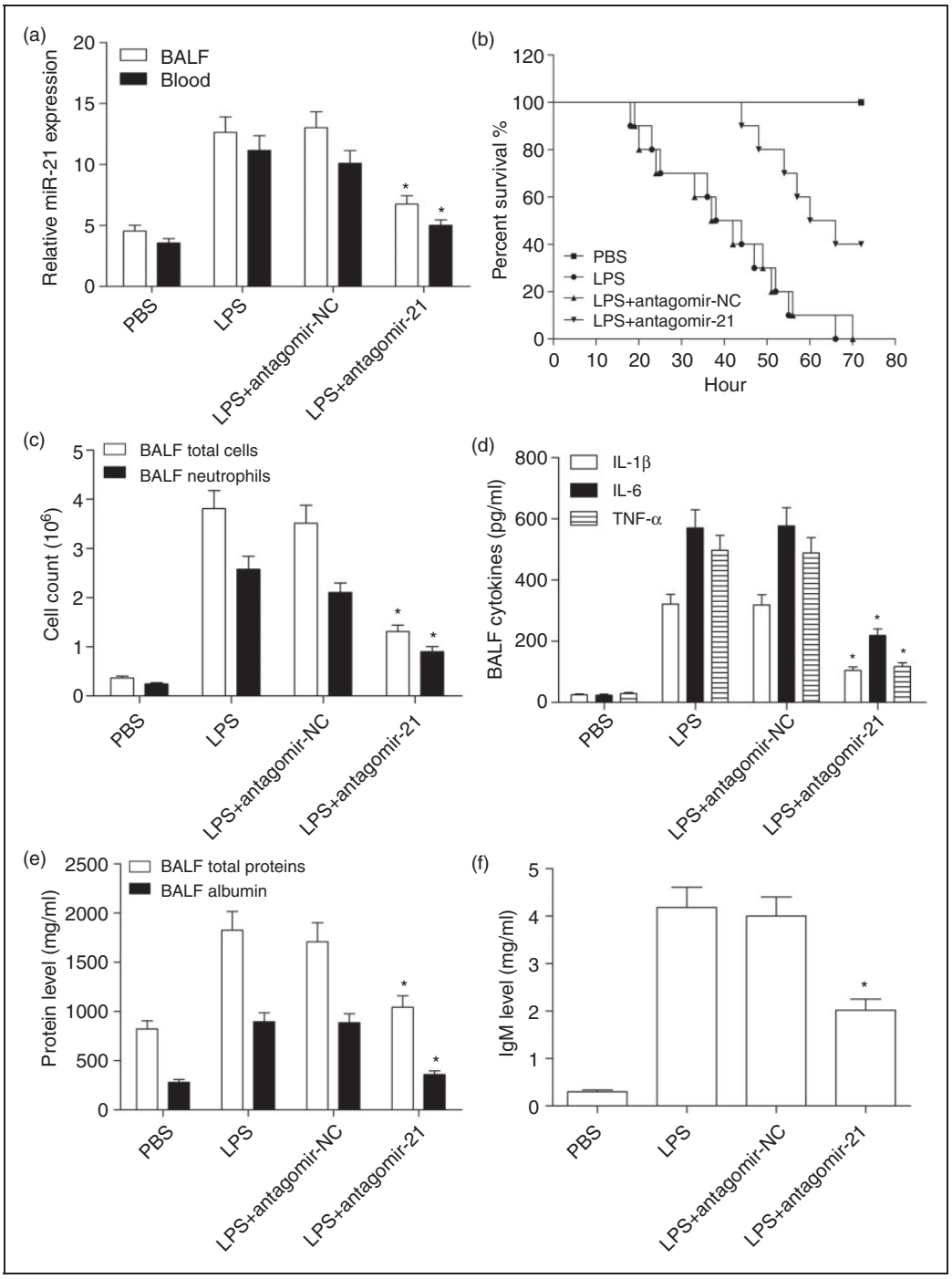

Figure 3. MiR-21 inhibition promotes survival and attenuates the lung injury of mice with acute lung injury (ALI). Mice were instilled intra-tracheally with 5 mg/kg of LPS. Then, mice were injected with antagomir-21 (25 nM of antagomir-21 diluted in 100 μl PBS; $n = 10$) or 25 nM antagomir-NC ($n = 10$) via the tail vein. (a) MiR-21 expression in the bronchoalveolar lavage fluid (BALF) and blood was measured by qRT-PCR 24 h post treatment. (b) A 72 h survival rate of mice was observed and recorded ($n = 10$ mice/group). (c) Total cell numbers in the BALF were counted with a haemocytometer. The number of neutrophils was determined by the ratio of cell population in cytospin at 24 h post treatment. (d) The cytokine levels in the BALF were detected by ELISA 24 h post treatment. (e) The protein content was measured by Bio-Rad DC protein assay reagent 24 h post treatment. (f) The IgM level was detected by ELISA. *$P < 0.01$ versus LPS+antagomir-NC or LPS alone.

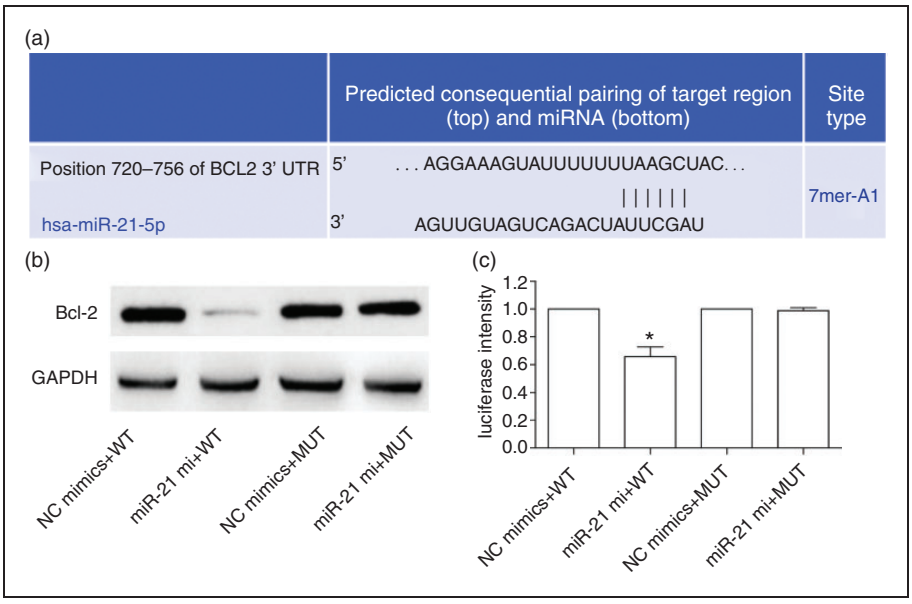

Figure 4. Bcl-2 is a direct target of miR-21. (a) Schematic diagram of miR-21-binding site in Bcl-2 3′-UTR region by bioinformatics analyses. (b) HEK-293T cells were co-transfected with 2 μg of Bcl-2 plasmids containing WT or MUT 3′-UTR in the presence of miR-21 or NC mimics (50 nM); the protein level of Bcl-2 was measured by Western blotting analysis 48 h later. (c) Luciferase activity was measured in HEK-293T cells co-transfected with pmirGLO-Bcl-3′-UTR-WT/MUT (2 μg) and 50 nM miR-21/NC mimics by dual-luciferase reporter assay 48 h post transfection. *$P<0.01$.

down-regulating miR-21 inhibitor-decreased Bax and caspase 3/9 in LPS-induced HPAEpiC (Figure 5a and d). Besides, up-regulation of Bcl-2 aggravated the inhibitory effects of miR-21 inhibitor on levels of inflammatory cytokines IL-1β, IL-6 and TNF-α ($P < 0.01$; Figure 5e and f). Overall, these findings indicated that over-expression of Bcl-2 enhanced the effect of miR-21 inhibitor in LPS-induced HPAEpiC.

Discussion

Recent reports have demonstrated the potential role of miRNAs in the pathogenesis of ALI. These studies showed that certain miRNAs are significantly up-regulated or down-regulated in inflammation or apoptosis-related diseases, which are concomitant with increased levels of inflammatory cytokines, including IL-1β, IL-6 and TNF-α.[11] Using a LPS-induced mice lung injury model, Cai et al. found that miR-214 and miR-451 were significantly up-regulated, while miR-16, miR-23a, miR-24 and miR-199a were obviously down-regulated. In addition, they found miR-16 was involved in regulation of post-transcription of IL-6 and TNF-α in LPS-stimulated A549 cells.[12] A substantial body of evidence suggests that certain miRNAs are indeed related to the pathophysiology of ALI and may be interesting diagnostic biomarkers and therapeutic targets. In the present study, we found that the level of miR-21 was obviously increased in the BALF of mice with ALI and LPS-treated HPAEpiC. These preliminary findings indicated that miR-21 may be involved in the progression of ALI.

MiR-21 has been suggested to affect some cellular behaviour in many inflammation and cancer-related diseases, including proliferation, apoptosis and epithelial–mesenchymal transition.[13] The present study identified that suppression of miR-21 expression attenuated LPS-induced apoptosis and inflammation in HPAEpiC, and promoted survival of mice with ALI by decreasing inflammatory cell count, release of cytokines and permeability in lung tissues. These findings suggested that miR-21 aggravated the progression of ALI, and its inhibition indeed protects lung epithelial cells against ALI. Some previous research found that miR-21 negatively regulated LPS-induced ALI. For example, miR-21 up-regulated by Liang-Ge-San (a classic traditional Chinese medicine formula) attenuates LPS-induced ALI in murine RAW264.7 cells.[14] MiR-21 represses LPS-induced ALI by targeting NF-κB in murine NR8383 cells.[15] It should be noted that the authors of both studies applied murine monocyte macrophages,[14,15] whereas we used human pulmonary alveolar epithelial cells. In addition, in humans, some studies indicated that miR-21 inhibition protects against liver injury and attenuates inflammatory

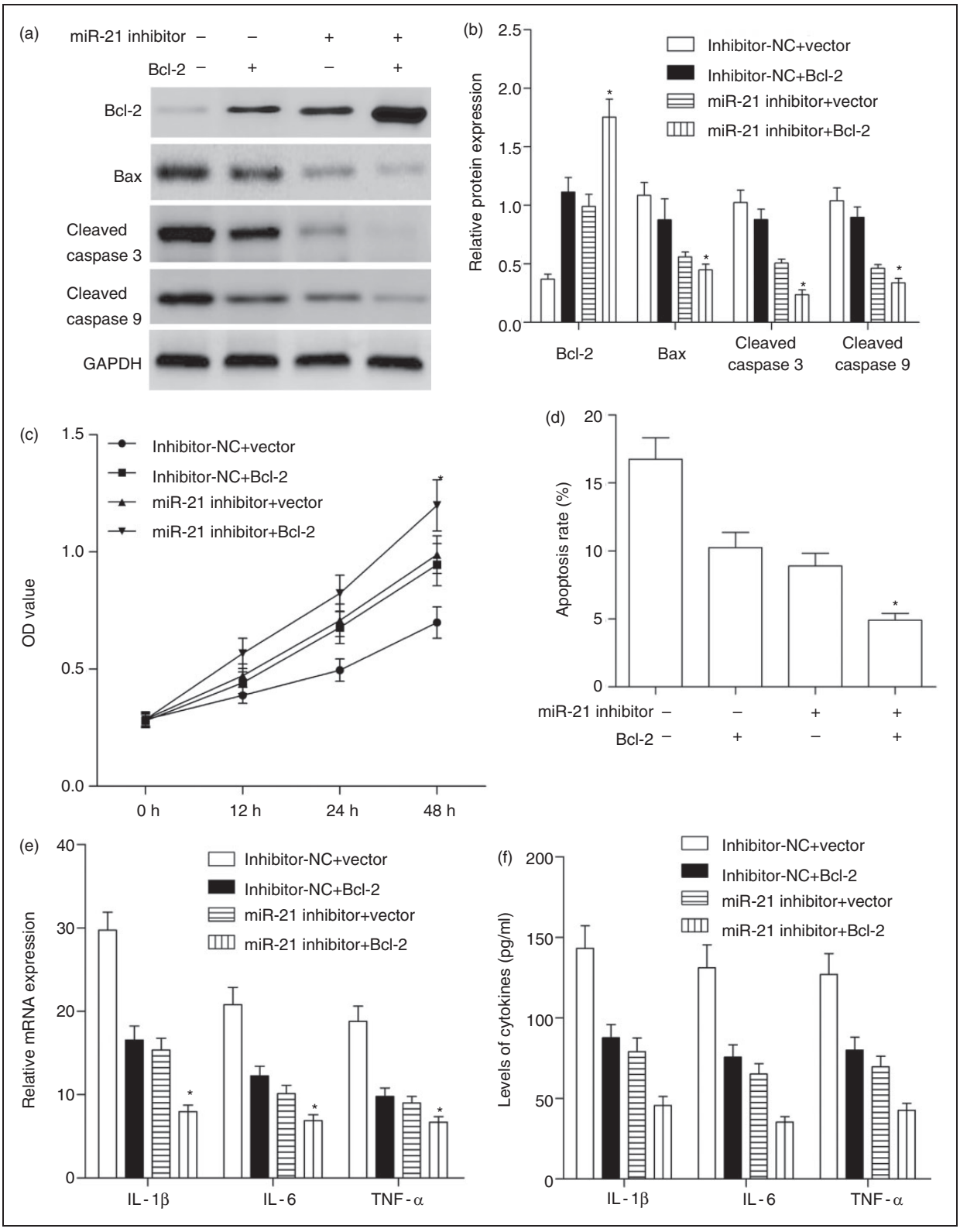

Figure 5. Over-expression of Bcl-2 enhances the effect of miR-21 inhibitor in LPS-induced HPAEpiC. Twenty-five nM of miR-21 inhibitor, inhibitor-NC, 2 μg of Bcl-2 plasmids or empty vector were transfected into HPAEpiC prior to 1000 ng/ml LPS treatment. (a) The protein levels of Bcl-2, Bax and cleaved caspase 3/9 were detected by Western blotting analysis 48 h post treatment. (b) Protein expression was quantified by densitometric analysis and normalised to GAPDH. (c) Cell viability was measured by CCK-8 assay 48 h post treatment. (d) Cell apoptosis was measured by flow cytometry 48 h post treatment. (e) and (f) Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) were detected by ELISA 48 h post treatment. $^*P < 0.01$.

responses and apoptosis in LPS-induced retinal pigment epithelial cells.[16,17] Inhibition of miR-21 contributes to the recovery of spinal cord injury by inhibiting inflammation.[18] Consistent with our results, Jansing et al. demonstrated that miR-21 knock-out alleviated alveolar structural remodelling and inflammatory signalling in ALI.[10] Therefore, miR-21 may exert its roles depending on different cell types and species.

MiRNAs exert their effects on cells through directly binding to target mRNAs and inhibiting mRNA translation.[5–8] In our study, we found that Bcl-2 was a direct target of miR-21, and the expression of Bcl-2 was negatively regulated by miR-21. Bcl-2 family members not only play an integral role in apoptosis, but also contribute to other cellular functions. Bcl-2 is involved in many miRNAs-related pathways to regulate diverse biological and pathological processes, which has been recognised as a target for improving or developing new therapies.[19] Zhao et al. demonstrated that miR-21 regulated mycobacterial survival and inflammatory responses by targeting Bcl-2.[11] Zhuang et al. showed that miR-202 suppressed hepatocellular carcinoma progression via down-regulating Bcl-2 expression.[20] Here, we found that Bcl-2 over-expression reversed miR-21-induced apoptosis and inflammation, and showed synergic effects with miR-21 inhibitor in LPS-induced HPAEpiC. Further research on miRNAs/Bcl-2-related pathways may provide a new insight into the pathogenesis of numerous diseases and contribute to the exploration of future treatment strategies.

In conclusion, our study suggested that inhibition of miR-21 suppressed apoptosis and inflammation by restoring Bcl-2 expression in LPS-induced HPAEpiC and mice with ALI, thereby providing new therapeutic approaches for the treatment of ALI.

Declaration of conflicting interests

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ORCID iD

Wei Sun https://orcid.org/0000-0002-8970-9037

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