

# Interleukin-6 RNA knockdown ameliorates acute lung injury induced by intestinal ischemia reperfusion in rats by upregulating interleukin-10 expression

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**Abstract.** Acute lung injury (ALI) is a common complication following intestinal ischemia/reperfusion (II/R) injury and contributes to the associated high mortality rate. However, the underlying mechanism is poorly understood and treatments are limited. RNA interference (RNAi) has been demonstrated to provide a promising disease treatment strategy both *in vitro* and *in vivo*. Therefore, the present study aimed to test whether blocking the proinflammatory cytokine IL-6 by RNAi may protect the lungs from remote organ injury following II/R, and to investigate the potential underlying mechanisms. A total of 176 adult healthy male Sprague-Dawley rats were randomly divided into sham, II/R, negative-control and IL-6-short hairpin (sh)RNA groups. The rats underwent II/R injury with occlusion of the superior mesenteric artery and coeliac artery to induce ischemia for 40 min, and were subsequently reperfused for 0-48 h. The negative-control group received a control lentiviral vector containing scrambled or non-specific sequences, and the IL-6-shRNA groups were administered with a vector containing an IL-6 shRNA sequence to affect RNAi-mediated knockdown of IL-6. ALI severity was determined by lung edema (lung wet/dry ratio) and histological

analysis (lung injury scores). IL-6 localization, and mRNA and protein expression levels, were detected by immunofluorescence, reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. IL-10 expression induced by IL-6 knockdown in lung tissues was additionally detected. IL-6 RNAi was revealed to significantly reduce the expression of IL-6, which was associated with upregulated IL-10 expression in lung tissues. Consequently, the severities of ALI and edema induced by II/R were substantially improved. In conclusion, the present study demonstrated that IL-6 RNAi may protect the lung from ALI induced by II/R, and that this protective role may be associated with upregulation of IL-10. These findings may contribute to the development of an IL-6-RNAi-based therapeutic strategy for the treatment of II/R-induced ALI.

## Introduction

Intestinal ischemia-reperfusion (II/R) is a common pathophysiological basis for numerous clinical diseases (1-3). It has been demonstrated that II/R is a multifactorial and complex pathophysiological process that contributes to multi organ failure and is associated with high mortality rates (4). In addition to local damage to the bowel, II/R leads to remote organ dysfunction (5,6), particularly in the lung, resulting in acute lung injury (ALI) (3,7), characterized by an excess elevation of pro-inflammatory cytokines and activated neutrophils (8-10).

Animal models and clinical data support the concept that interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), and IL-8 are typical cytokines involved in acute inflammation (7). Furthermore, it had been demonstrated that high levels of IL-6, a multifunctional cytokine, may be a marker of severity of inflammatory responses and are associated with inflammatory and immune diseases (11-14). IL-6 contributes to the inflammatory responses associated with II/R and serves important roles in the acute phase reaction and in the progression of ALI (15-17). It has been demonstrated that pretreatment with dexmedetomidine hydrochloride is useful for reducing lung damage caused by II/R, and this treatment was associated with decreased levels of TNF- $\beta$ , IL-6, toll-like receptor 4 (TLR4)

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and myeloid differentiation primary response gene 88 (18). Similarly, various studies have revealed that decreased levels of IL-6 are associated with attenuation of the inflammatory response in ALI (18-20). Additional evidence has suggested that IL-6 is directly involved in the mediation of inflammation via regulation of other inflammatory cytokine responses and neutrophil extravasation (17). In addition, a recent study demonstrated that IL-6 regulated intestinal epithelial tight junction permeability, and this effect was mediated by c-jun N-terminal kinase, activation of activator protein-1 (AP-1) and subsequent AP-1 activation of the claudin-2 gene (21). However, for the diseases caused by II/R, IL-6 instead was demonstrated to have potent anti-inflammatory roles in the liver following II/R injury, and may inhibit endotoxin-induced local acute inflammation of the trachea (22,23). Therefore, as IL-6 has been suggested to be involved in the development of almost all chronic inflammatory diseases (14), and may exert a potential protective effect on II/R-associated injuries, the exact role of IL-6 in acute inflammatory diseases requires further elucidation. Furthermore, whether IL-6 knockdown may serve as an effective strategy for the treatment of these diseases, and clarification of the potential underlying mechanisms requires additional further investigation. Previous studies have suggested that IL-10 may serve a protective role in II/R-induced ALI (24-26); thus, this requires further examination.

The RNA interference (RNAi) technique has become a reliable and powerful technique to inhibit the expression of targeted genes in both *in vitro* and *in vivo* (27-30). Accordingly, the present study aimed to test the hypothesis that blocking the proinflammatory cytokine IL-6 by RNAi may alleviate ALI following II/R, via upregulation of IL-10 expression.

## Materials and methods

**Animals and ethical statement.** The present study used 176 adult male Sprague Dawley rats (weight, 200-220 g), provided by the Experimental Animal Center of Sichuan University (Chengdu, China). Study protocols were followed according to the guidelines for laboratory animal care and safety as issued by the United States National Institutes of Health. All animals were raised in plastic cages (n=2/cage) with soft bedding and free access to food and water in a temperature (21-25°C) and humidity (45-50%)-controlled room. Animal care and all experimental protocols were approved by the Animal Care & Welfare Committee of Kunming Medical University (Kunming, China).

**Experiments.** To investigate the function of IL-6 in the lungs of rats following II/R, human immunodeficiency virus (HIV) based lentiviral vectors were used to deliver double-stranded short hairpin RNA (shRNA) sequences to affect RNAi-mediated target gene knockdown in tissue. The recombinant IL-6 lentiviral vectors along with the specific shRNA sequences were designed and constructed by GeneCopeia™ (Guangzhou, China).

**Screening for effective RNAi sequences.** To screen for sequence segments that effectively mediate IL-6 knockdown, IL-6 gene sequences were obtained from the National Center

for Biotechnology Information database (Bethesda, MD, USA), and three potential shRNAs sequences targeting IL-6 mRNA and one nonsense shRNA as a negative control were designed and purchased from GeneCopeia. To test their knockdown efficacy *in vitro*, four 6-well plates of PC12 cells purchased from the Animal Research Institute of the Chinese Academy of Medical Sciences (Beijing, China) were seeded at  $8 \times 10^4$ /well and cultured at 37°C in 5% CO<sub>2</sub>. When the cells reached 50-60% confluency, the culture medium was replaced. Cells were then transfected with 1 µg shRNA fragment and 3 µl SuperFectin™ II DNA Transfection reagent (Shanghai Pufei Biological Technology Co., Ltd, Shanghai, China) according to the manufacturer's protocol. After an 18 h transfection, the medium was replaced with Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 50 U/ml penicillin-streptomycin (Hyclone; GE Healthcare Life Sciences). Subsequently, total RNA of PC12 cells was extracted 48 h post-transfection and the interference efficiency of IL-6 shRNA was examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) amplification as described in the following section. The most effective shRNA was selected to produce the recombinant lentivirus (31).

**Production of the recombinant lentivirus.** The procedure used to produce lentivirus was based on the Lenti-Pac™ HIV Expression Packaging kit user manual as previously described (31). In addition, mCherry fluorescent protein (mCherryFP) was fused to the plasmid vector. Briefly, 293Tα lentiviral packaging cells (GeneCopeia Co., Guangzhou, China) were cultured in DMEM supplemented with 10% heat-inactivated FBS and incubated at 37°C in 5% CO<sub>2</sub>. Following this, 1.25 µg lentiviral expression plasmid, 2.5 µl (0.5 µg/µl) Lenti-Pac HIV Expression Packaging mix and EndoFectin™ Lenti transfection reagent (all from GeneCopeia Co.) were diluted with Opti-Minimal Essential medium (Invitrogen; Thermo Fisher Scientific, Inc.) for 25 min at room temperature. When cell confluence reached 70-80%, the mixture was added to the culture medium of 293Tα cells. The culture medium was replaced 8 h after transfection with DMEM supplemented with 10% FBS. In addition, 10 µl TiterBoost reagent (GeneCopeia Co.) was added to improve the percentage of virus generation. A total of 72 h post-transfection, the culture medium was collected, centrifuged at 4°C, 3,000 x g for 30 min, and the supernatant was filtered. Lentiviral stocks were aliquoted and stored at -80°C for further use.

**II/R model and animal grouping.** Animals were randomly divided into the following groups as indicated in Table I: Sham, II/R (II/R without any injections), negative-control (NC; II/R+Lv-NC vector) and IL-6 shRNA (II/R+RSH048925-HIVmU6).

II/R was generated by blocking the superior mesenteric (SMA) and coeliac (CA) arteries as described previously (32,33). Rats were fasted with no restriction of water access for 24 h prior to surgery. Following this, the rats were anesthetized by intraperitoneal injection with ketamine-xylazine, which was provided by Kunming Medical University (Yunnan, China)

Table I. Animal grouping and samples used.

Group	Lung wet/ dry ratio	H&E/IF	WB/RT-qPCR
Sham	8	8	8
II/R	40	40	40
Negative-control	N	8	8
IL-6 shRNA	N	8	8

H&E, hematoxylin and eosin; IF, immunofluorescence; WB, western blotting; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; II/R, intestinal ischemia/reperfusion (reperfusion at 0, 8, 16, 24 or 48 h); N, none; shRNA, short hairpin RNA; IL-6, interleukin-6.

(100 and 20 mg/kg, respectively) and placed in a supine position. The intestines were separated gently until the SMA and CA were isolated, and traumatic microvascular clips were used to clamp the SMA and CA for 40 min. After 40 min ischemia, the artery clamps were removed and intestinal perfusion was induced for 0, 8, 16, 24 or 48 h. Sham control animals underwent the same surgical procedure with the exception of the SMA and CA clamping.

**Lentivirus injection.** For IL-6 RNAi, the respective prepared lentivirus constructs were injected into the right lung tissue of 16 subject rats over 3 min immediately following arterial clamping. After clamping arteries for 40 min, the SMA and CA clamps were loosened and the skin was sutured. Subsequently, 0.5 ml saline was injected into the enterocoelia. A total of 16 II/R NC rats were injected with the Lv-NC vector as a control.

**Tissue collection.** At the end of each experimental time point, experimental and sham animals were sacrificed by cutting the abdominal aorta following intraperitoneal injection of ketamine-xylazine (100 and 20 mg/kg, respectively). Following this, the lung tissues were removed immediately for further analysis.

**Measurement of lung edema.** Lung edema was determined by the lung wet/dry weight ratio as described previously (34). The harvested lung tissue was immediately weighed to obtain the wet weight. Subsequently, the tissue was dried in an oven at 90°C for 24 h and reweighed to obtain the dry weight.

**Histological analysis.** Histological analysis of the lung tissue was performed by hematoxylin-eosin (H&E) staining. At the end point of the various reperfusion times, all rats were sacrificed by cutting the abdominal aorta following intraperitoneal injection of ketamine-xylazine (100 and 20 mg/kg, respectively), and harvested lung tissues were fixed in 4% paraformaldehyde in 0.1 M ice-cold phosphate buffer, pH 7.4, for >72 h at 4°C. Subsequently, fixed lung tissues were embedded in paraffin and sectioned at a thickness of 5 µm, transferred to glass slides, and stained with H&E. Finally, the stained sections were observed under a light microscope to detect morphological alterations. The degree of lung injury

was scored at 0 to 4 according to the severity of edema, neutrophil infiltration, hemorrhage and hyaline membrane formation as previously described (33). Furthermore, the number of red cells seeping into the alveolar interstitium and alveolar space from four fields of each section (3 sections/animal and 8 animals/group) were quantitatively analyzed. Each slide was evaluated by three investigators blinded to the experimental information.

**RT-qPCR.** At the end of the reperfusion period, the ischemic/reperfused lung tissues were frozen immediately and stored at -80°C until further required. The mRNA expression of IL-10 and IL-6 were assayed using RT-qPCR. Total RNA from the lung tissues was isolated using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and reverse transcribed to cDNA with the RevertAid<sup>™</sup> First Strand cDNA Synthesis kit (Takara Biotechnology, Co., Ltd., Dalian, China). Subsequently, RT-qPCR of cDNA was performed using the following primer sequences: Forward, 5'-AGAAGACCAGAGCAGATTTT-3' and reverse, 5'-GAGAAAAGAGTTGTGCAATG-3' for IL-6; annealing temperature, 52°C. Forward, 5'-CAGAAATCAAGGAGCATT TG-3' and reverse, 5'-CTGCTCCACTGCCTTGCTTT-3' for IL-10; annealing temperature, 50°C. Forward, 5'-GAAGAT CAAGATCATTGCTCCT-3' and reverse, 5'-TACTCCTGC TTGCTGATCCA-3' for β-actin; annealing temperature, 52°C. β-actin served as an internal control. PCR cycling conditions were as follows: Initial denaturation at 95°C for 2 min, denaturation at 95°C for 15 sec and amplification at 53°C for 20 sec, followed by extension at 60°C for 30 sec for a total of 40 cycles. The quantitation cycle (Cq) of each sample was recorded, and data were analyzed by normalization to β-actin values using the 2<sup>-ΔΔCq</sup> method (35). The experiment was replicated three times.

**Immunofluorescence staining.** To examine the location of IL-6 and IL-10 following II/R, a comparative analysis of immunofluorescence staining was performed in lung tissue sections. Following routine de-paraffinization and rehydration, the slices were permeated in PBS containing 3% goat serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min at 37°C and incubated overnight at 4°C with specific rabbit primary antibodies against IL-6 (Ab6672; 1:500; Abcam, Cambridge, UK) or IL-10 (Ab9969; 1:100; Abcam). Negative controls were performed by adding PBS instead of the primary antibody. The next day, sections were rinsed with PBS and incubated in the dark with an anti-rabbit cy3-labeled secondary antibody (111-165-003; 1:200; Jackson Laboratories, Bar Harbor, ME, USA), for 30 min at 37°C. The sections were subsequently washed three times with PBS, followed by incubation with DAPI (Beyotime Institute of Biotechnology, Shanghai, China) for 5 min to visualize the cell nuclei. Finally, sections were observed under a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**Western blot analysis.** Lung samples were frozen immediately and stored at -80°C until further required. To examine whether the protein expression levels of IL-6 and IL-10 altered following II/R and IL-6 shRNA transfection, protein was extracted from lung samples from each group and lysed

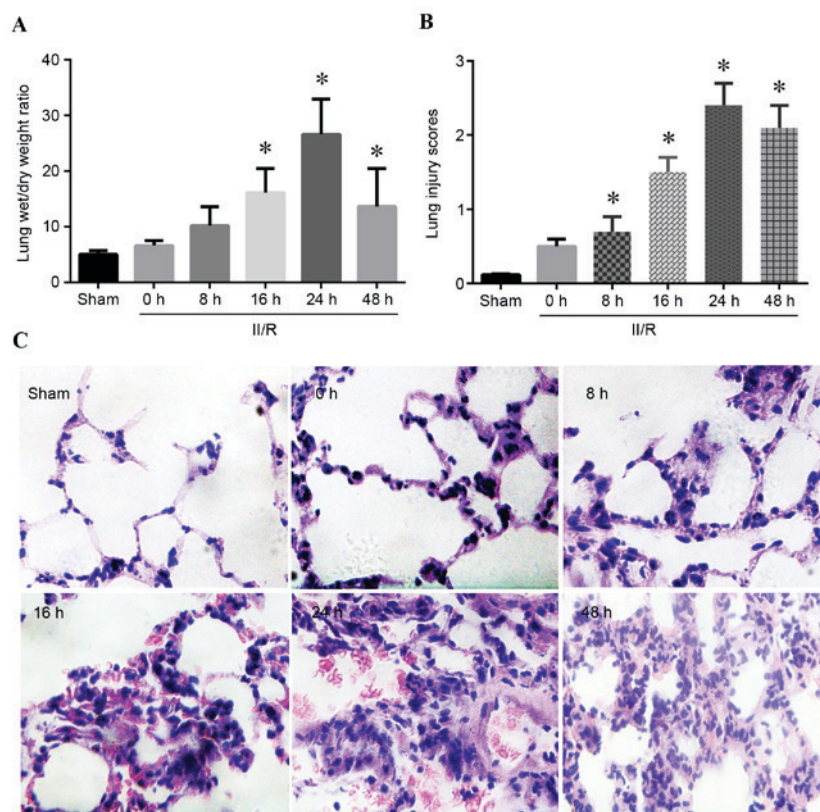


Figure 1. Assessment of lung injury following II/R. (A) Lung wet/dry weight ratio in sham and II/R groups at the indicated times following reperfusion. (B) Lung injury scores of the sham and II/R groups at the indicated times following reperfusion. Data are presented as the mean  $\pm$  standard deviation ( $n=8$ ). \* $P<0.05$  vs. sham group. (C) Representative photomicrographs of hematoxylin and eosin stained lung sections in sham and II/R groups following reperfusion (original magnification,  $\times 400$ ). II/R, intestinal ischemia/reperfusion.

with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing 2% inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Samples were centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  and the supernatant was collected. A Bicinchoninic Acid protein assay kit (Beyotime Institute of Biotechnology) was used to detect the protein concentration. Protein ( $100 \mu\text{g}$ ) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%) at 60 V for 30 min and 100 V for 1.5 h, following which proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) for 4 h at 350 mA. The membranes were subsequently blocked in 5% skimmed milk in TBS containing Tween-20 (TBST) for 1 h at room temperature and incubated with rabbit anti-rat primary antibodies against IL-6 (1:800) or IL-10 (1:2,500) overnight at  $4^{\circ}\text{C}$ .  $\beta$ -actin (ABM40028; 1:1,000; Abbkine Scientific Co., Ltd., Wuhan, China) served as an internal control. Subsequently, the membranes were rinsed four times with TBST and incubated with a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (ab6721; 1:5,000; Abcam) for 1.5 h. Finally, membranes were rinsed four times with TBST and the immune complexes were detected using ChemiDoc XRS System with Image Lab Software version 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with enhanced chemiluminescence reagent (BL520A; Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Statistical analysis was performed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA).

Data are expressed as the mean  $\pm$  standard deviation and were analyzed using a Student's *t*-test with a two-tailed distribution. For multiple group comparisons, one-way analysis of variance followed by Tukey's post hoc test was used.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Lung edema and damaged morphology induced by II/R.** Lung edema and morphological alterations were detected by lung wet/dry weight ratio and H&E staining following II/R. As presented in Fig. 1A, compared with the sham group, rats subjected to II/R injury demonstrated significantly increased lung wet/dry weight ratio, reaching the highest level 24 h post-reperfusion ( $P<0.05$ ). The lung injury scores of the II/R rats were significantly higher compared with the sham group at 8, 16, 24 and 48 h post-reperfusion ( $P<0.05$ ; Fig. 1B). H&E staining revealed that II/R injured rats exhibited histological evidence of ALI based on a grading system that assessed congestion, intra-alveolar cellular infiltration and hemorrhage (Fig. 1C).

**Expression of IL-6 in lung tissues following II/R.** To determine whether IL-6 may be associated with the ALI following II/R, the protein expression levels of IL-6 were measured in the lungs by western blot analysis 8, 16, 24 and 48 h after reperfusion. The results revealed that IL-6 protein expression levels significantly increased in rats subjected to II/R compared

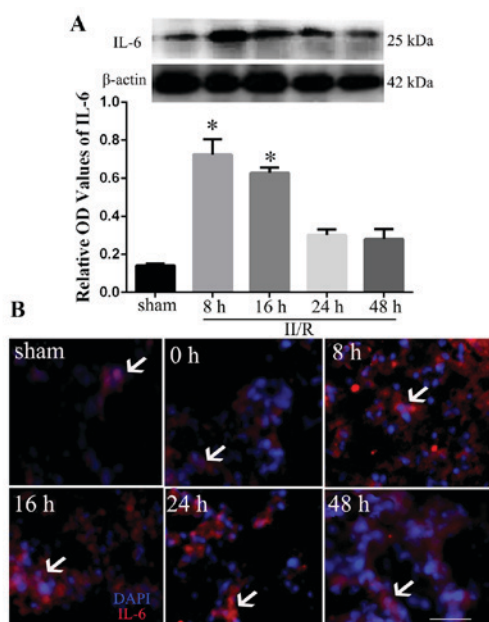


Figure 2. Alterations in lung IL-6 expression levels following II/R. (A) Representative western blot images and quantification of IL-6 protein expression levels in sham and II/R groups. Data are presented as the mean  $\pm$  standard deviation (n=8). \*P<0.05 vs. sham group. (B) Representative photomicrographs of IL-6 revealed increased immunofluorescence intensity in the II/R groups compared with the sham group, with the highest intensities observed 8 and 16 h post reperfusion. IL-6-positive cells stained in red are indicated by white arrows. Nuclei were counterstained with DAPI. Scale bar=50  $\mu$ m. II/R, intestinal ischemia/reperfusion; OD, optical density; IL-6, interleukin-6.

with sham operated rats, with the highest level observed 8 h post-reperfusion (P<0.05; Fig. 2A). Furthermore, immunofluorescence staining identified that II/R induced significantly increased intensities of IL-6 immunofluorescence in invading neutrophils, with the strongest intensity observed at 8 and 16 h after reperfusion (Fig. 2B).

**Identification of lentiviral recombinants.** Lentivirus-introduced IL-6 RNAi was used to regulate the expression of IL-6. Details of the lentivirus constructs with the inserted shRNA sequence are presented in Fig. 3A. Compared with the transfection (transfection reagent+random sequence) or negative control groups, IL-6 mRNA expression levels were reduced in the IL-6 shRNA plasmid transfection groups, and the shRNA-F2 sequence exhibited the highest RNAi efficiency (P<0.01; Fig. 3B). Therefore, the shRNA-F2 segment was used to construct a plasmid and generate a lentiviral recombinant, following which a recombinant containing the targeted gene with mCherryFP (IL-6-shRNA) was packaged into 293Ta cells. Immunofluorescence detection revealed that 293Ta cells emitted red fluorescence, confirming successful transfection (Fig. 3C). These results confirmed the efficacy of the lentivirus-mediated IL-6 RNAi in these experimental conditions.

**Protective role of IL-6 knockdown on lung injury.** As presented in Fig. 4A, the addition of the IL-6 shRNA lentivirus significantly decreased the expression of IL-6. The distribution of red cells (biconcave red in the alveolar interstitium

and alveolar space) in the two groups was identified by H&E staining. A total of 16 h after reperfusion, the number of red cells in the pulmonary alveoli in the IL-6 shRNA group was dramatically decreased compared with the negative control group, which had a large number of red cells (P<0.05; Fig. 4B). As a result, the lung injury scores of rats were improved in the IL-6 shRNA group compared with the negative control group (P<0.05; Fig. 4C). These results supported the hypothesis that transfection of a shRNA lentivirus may downregulate the expression of IL-6 and alleviate the effects of the inflammatory response.

**Role of IL-6 knockdown on the expression of IL-10.** To investigate the effect of IL-6 on the expression of IL-10, the mRNA (Fig. 5A) and protein (Fig. 5B) expression levels of IL-10 were analyzed by RT-qPCR and western blot analysis, respectively. The results revealed that 16 h after reperfusion, mRNA and protein expression levels of IL-10 were increased compared with the negative control group (P<0.05). Furthermore, immunofluorescence analysis demonstrated that following IL-6 shRNA transfection, the density of IL-10 immunoreactivity in lung tissue was significantly increased compared with the control Lv-NC-transfected group (Fig. 5C).

## Discussion

An II/R model was used in the present study to induce ALI, including lung edema and elevated lung injury scores, and IL-6 expression levels were demonstrated to be elevated. To understand the physiological role of IL-6 in mediating II/R-associated lung injuries, an IL-6 shRNA lentiviral construct was used to specifically suppress IL-6 expression in the lungs of experimental animals. RT-qPCR and western blot analysis revealed that IL-6 silencing contributed to a progressive and parallel reduction in IL-6 mRNA and protein expression levels, which was followed by improved lung injury scores and increased IL-10 expression. Therefore, these results provided experimental support for the clinical application of IL-6 knockdown in the treatment of ALI caused by II/R.

The results of the histological analyses performed in this study closely associate with those identified in previous studies (36-38), thus confirming the presence of ALI caused by II/R and the success of this rat model. In the present study, subsequent analyses were performed at various time intervals to determine the period at which the observed phenomena were most significant. From this, it was determined that the most severe lung injury occurred 24 h post-reperfusion, as indicated by lung edema and increased lung injury scores. Furthermore, it was observed that the mRNA and protein expression levels of IL-6 increased in lung tissues following II/R, suggesting that elevated IL-6 may be associated with lung injury caused by II/R (18). In support of this, previous studies have reported that intra-alveolar cell infiltrates, alveolar hemorrhages and excessive elevation of proinflammatory cytokines including IL-1, IL-6, TNF and IL-8 are important factors in the occurrence and development of II/R-mediated ALI (9,10,39). In addition, TLR4 has been demonstrated to serve an important role in the pathogenesis of II/R-induced ALI and inflammation, and p38 kinase and nuclear factor- $\kappa$ B may be involved

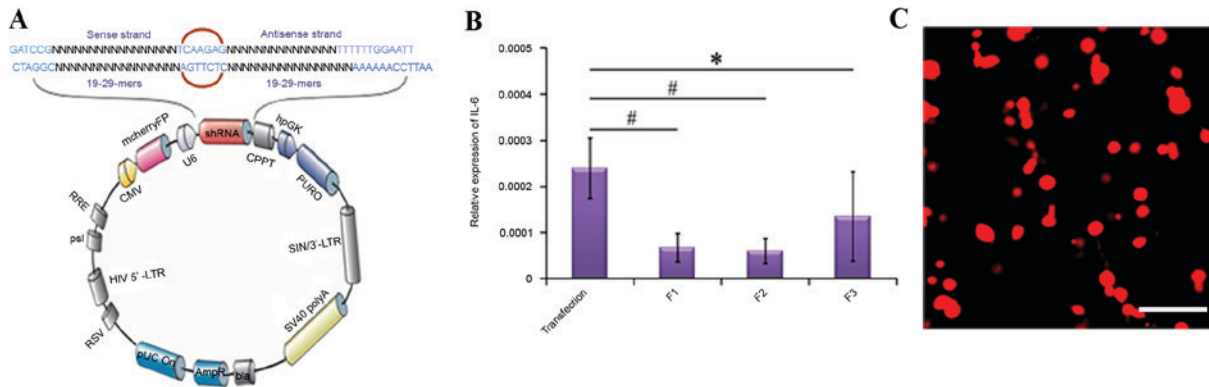


Figure 3. Packaging and RNAi efficacy of recombinant lentivirus. (A) Vector schematic for the IL-6 shRNA construct RSH048925-HIVmU6. (B) The effectiveness of the shRNA IL-6 fragments was assessed by reverse transcription-quantitative polymerase chain reaction in the PC12 cell line. Data are presented as the mean  $\pm$  standard deviation ( $n=8$ ). F1, F2 and F3 represent fragments 1, 2 and 3 respectively. \* $P<0.05$ ; # $P<0.01$ . (C) The successfully recombined IL-6-shRNA mCherryFP (IL-6-shRNA) lentivirus packaged in 293To cells emitted red fluorescence as visualized under a fluorescence microscope, indicating successful transfection. Scale bar=50  $\mu\text{m}$ . mCherryFP, mCherry fluorescent protein. shRNA, short hairpin RNA; IL-6, interleukin-6.

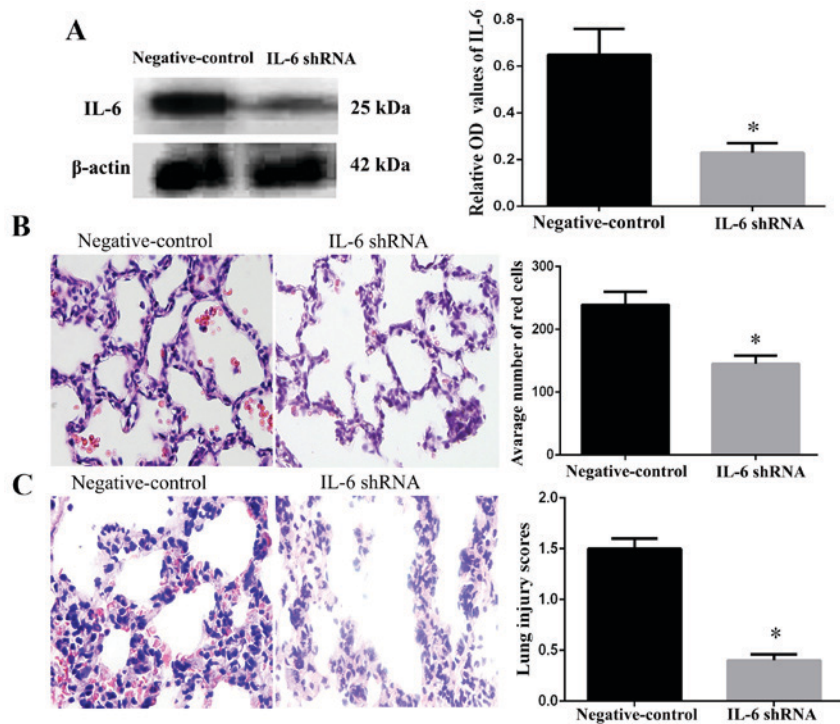


Figure 4. Alterations in IL-6 expression and lung injury following shRNA-mediated IL-6 knockdown. (A) Representative western blot images and quantification of IL-6 protein expression levels in the lung following 16 h II/R. (B) H&E staining of the distribution of red cells in the alveolar interstitium and alveolar space (original magnification,  $\times 200$ ) and quantitative analysis of the number of red blood cells in the negative control and IL-6 shRNA groups. (C) Lung injury scores evaluated by H&E staining of the negative control and IL-6 shRNA groups. Data are presented as the mean  $\pm$  standard deviation ( $n=8$ ). \* $P<0.05$ . II/R, intestinal ischemia/reperfusion; H&E, hematoxylin and eosin; IL-6, interleukin-6; OD, optical density; shRNA, short hairpin RNA.

in TLR4 signaling-mediated lung inflammatory processes during II/R (3,40), illustrating the complexity of this physiological response.

IL-6 is a pleiotropic acute reactant cytokine involved in inflammatory responses (17) that may be promptly and transiently produced in response to infections and tissue injuries, contributing to host defenses via stimulation of acute phase responses, hematopoiesis, and immune reactions (40). Furthermore, IL-6 exerts various effects in addition to those on hepatocytes and lymphocytes that are frequently detected in chronic inflammatory diseases (14,41,42).

The present study primarily investigated acute inflammatory disease following II/R treatment, which differs from previous studies that focused on the chronic inflammatory process (14).

Conversely, IL-6 may have an anti-inflammatory effect *in vitro* and *in vivo*, as IL-6 administration has been demonstrated to inhibit TNF- $\alpha$  and IL-1 $\beta$  signaling, reduce aggregation of polymorphonuclear cells (PMNs), and protect against the damage caused by toxins *in vivo* (43). Together, these disparate findings indicated that the function of IL-6 on the process of ALI is complex; therefore, further confirmation

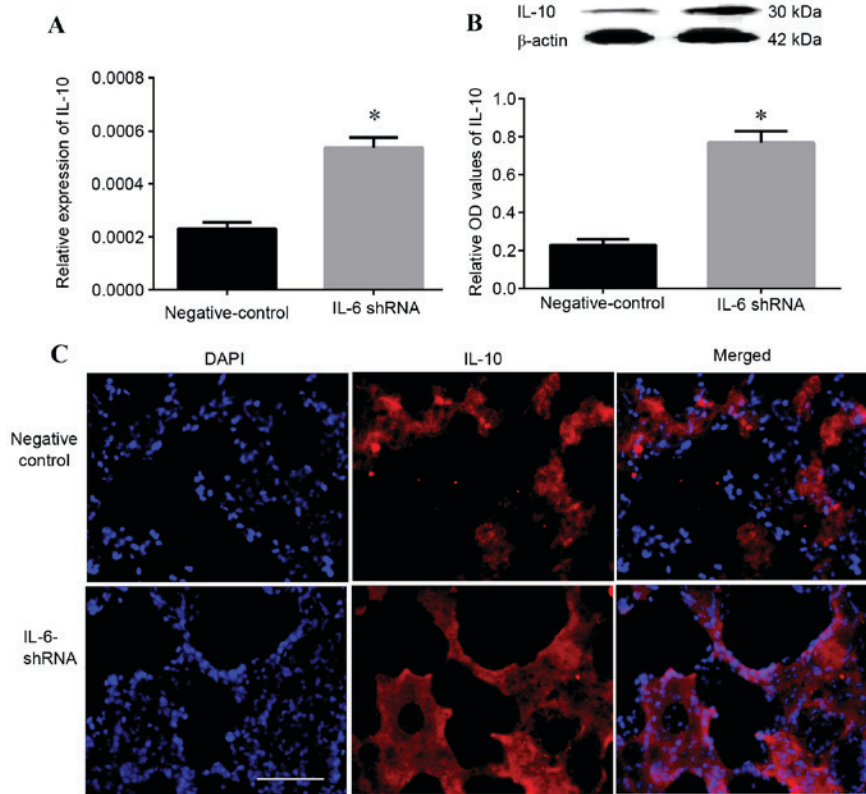


Figure 5. Alterations in IL-10 expression following IL-6 knockdown. IL-10 (A) mRNA and (B) protein expression levels 16 h after reperfusion as assessed by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The results were quantified and normalized to  $\beta$ -actin values. Data are presented as the mean  $\pm$  standard deviation (n=8). \*P<0.05. (C) IL-10 immunoreactivity in the lung tissues from negative controls and IL-6 shRNA injected rats 16 h post-reperfusion. Scale bar=100  $\mu$ m. IL, interleukin; shRNA, short hairpin RNA; OD, optical density.

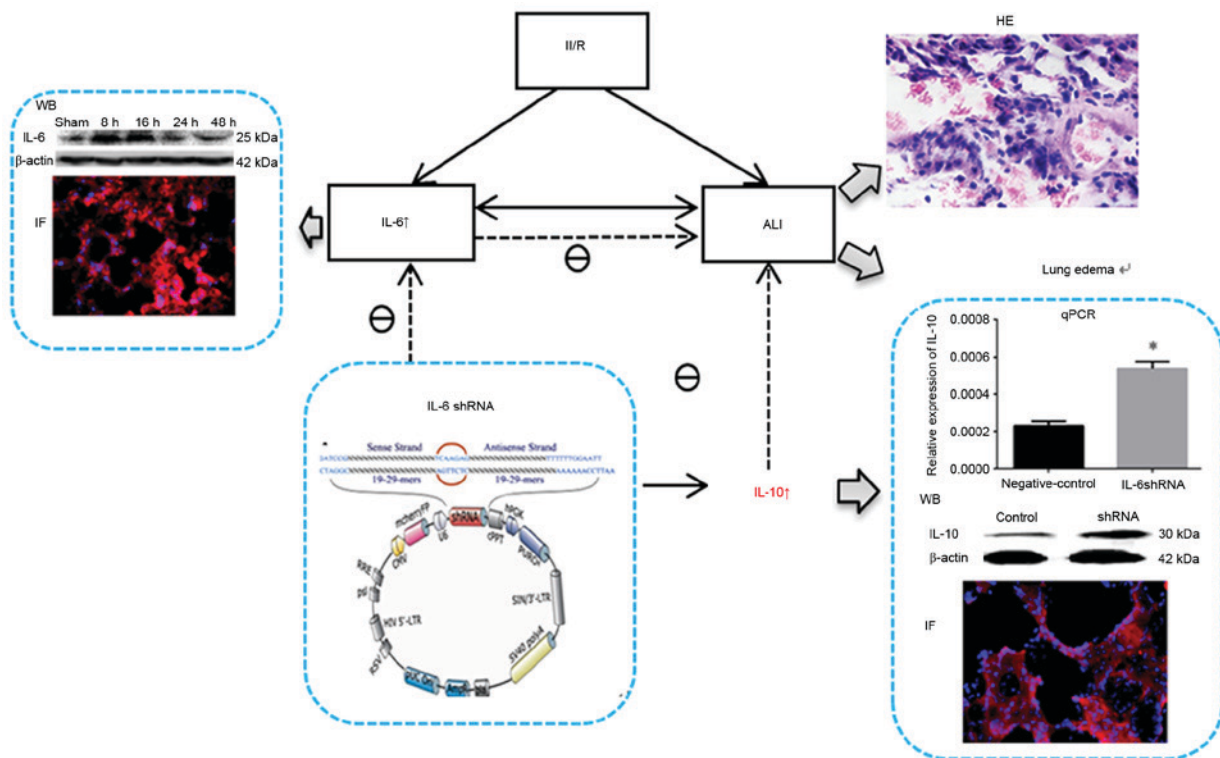


Figure 6. Potential mechanisms for the protective role of IL-6 shRNA on II/R-mediated ALI. At various time points following II/R, ALI was confirmed as indicated by lung edema and H&E staining. Together with these injuries, IL-6 expression levels were increased significantly in lung tissues. Subsequently, it was demonstrated that IL-6 shRNA-mediated improvement of ALI was associated with IL-10 upregulation. II/R, intestinal ischemia/reperfusion; ALI, acute lung injury; IL, interleukin; shRNA, short hairpin RNA; H&E, hematoxylin and eosin; qPCR, quantitative polymerase chain reaction; WB, western blot; IF, immunofluorescence.

of the exact role of IL-6 in acute inflammatory diseases associated with II/R is required.

The present study demonstrated that IL-6 knockdown led to significantly increased expression of IL-10, and markedly reduced lung edema scores and leakage of red blood cells to the pulmonary alveoli in II/R model rats compared with the control group. These results are particularly relevant as no specific therapeutic treatments are currently available for ALI caused by II/R. Based on the observation that oxidative stress and mast cells interact together and promote II/R-induced ALI (44), previous studies have revealed that osthonol, valproic acid, protocatechuic acid and cyclic arginine-glycine-aspartate peptide are able to affect survival and the development of ALI in a rat model of II/R. These factors may impact recovery following lung injury via anti-oxidant and anti-inflammatory effects (33,45-48). Support for the potential use of IL-6 knockdown in II/R-associated ALI, as studied here, is provided by numerous reports. Goodman *et al* (49) injected IL-6 into the healthy endotrachea of mice, and demonstrated that this led to PMN aggregation and pulmonary edema in murine lung tissue, suggesting that IL-6 may cause lung injury. Conversely, Bhatia *et al* (50) confirmed that injection of an IL-6 antibody may decrease the level of C-reactive protein (CRP) in patients with sepsis to healthy levels, suggesting that IL-6 inhibits CRP. Furthermore, another study reported that IL-6 serves a protective role in inflammation, as increased IL-6 was negatively correlated with sepsis mortality (17). However, few studies have placed emphasis on the protective effect of IL-6 knockdown and its regulation of inflammatory cytokines in ALI induced by II/R. Therefore, to the best of our knowledge, this is the first study to demonstrate a protective effect of IL-6 RNA knockdown on ALI after II/R, and further, to demonstrate the potential underlying mechanism of IL-10 upregulation. These data provide the foundation for the potential application of IL-6 RNAi for the treatment of II/R-associated ALI.

In conclusion, the present study demonstrated that II/R may induce ALI and enhance the expression of IL-6 in an II/R rat model. In addition, IL-6 inhibition in lungs by an IL-6-shRNA-lentivirus alleviated the inflammatory response. This protective role was associated with upregulated IL-10 expression in lung tissues. Therefore, the results of the present study may serve as a basis for the development of novel treatment strategies for ALI and associated clinical diseases including systemic inflammatory response syndrome and multiple organ dysfunction syndrome evoked by II/R.

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