

• BASIC RESEARCH •

# Role of interleukin 18 in acute lung inflammation induced by gut ischemia reperfusion

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## Abstract

**AIM:** To study the changes of endogenous interleukin 18 (IL-18) levels and evaluate the role of IL-18 on lung injury following gut ischemia/reperfusion.

**METHODS:** A superior mesenteric artery occlusion model was selected for this research. The mice were randomly divided into four groups: Sham operation (sham), ischemia (0.5 h) followed by different times of reperfusion (I/R), and I/R pretreated with exogenous IL-18 (I/R+IL-18) or IL-18 neutralizing antibody (I/R+IL-18Ab) 15 min before ischemia. Serum IL-18 levels were detected by Western blot and ELISA, and the levels of IL-18 in lung tissue were evaluated by immunohistochemical staining. For the study of pulmonary inflammation, the lung myeloperoxidase (MPO) contents and morphological changes were evaluated.

**RESULTS:** Gut ischemia/reperfusion induced rapid increase of serum IL-18 levels, peaked at 1 h after reperfusion and then declined. The levels of IL-18 in lung tissue were gradually enhanced as the progress of reperfusion. Compared with I/R group, exogenous administration of IL-18 (I/R+IL-18) further remarkably enhanced the pulmonary MPO activity and inflammatory cell infiltration, and in I/R+IL-18Ab group, the content of MPO were significantly reduced and lung inflammation was also decreased.

**CONCLUSION:** Gut ischemia/reperfusion induces the increase of IL-18 expression, which may make IL-18 act as an important proinflammatory cytokine and contribute to gut ischemia/reperfusion-induced lung inflammation.

**Key words:** IL-18; Ischemia; Reperfusion; Inflammation

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## INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is the leading cause of death in critically ill patients. Although systemic inflammation characteristic of MODS can result in damage to any organ, onset of the syndrome is usually heralded by the development of respiratory insufficiency<sup>[1,2]</sup>. Gut ischemia and reperfusion (I/R) is a prime mechanism that results in the pathogenesis of MODS partly dependent on neutrophils<sup>[2]</sup>. Neutrophils can be activated in response to reperfusion of ischemia tissue or exposure to endotoxin and pro-inflammatory cytokines<sup>[1]</sup>. Activated neutrophils adhere to endothelium through adhesion molecules, resulting in localized release of proteases, reactive oxygen species (ROS) and various cytokines and inflammatory mediators that contribute to tissue injury and failure<sup>[1,2]</sup>.

Interleukin 18 (IL-18), known initially as an IFN- $\gamma$ -inducing factor, is a pleiotropic proinflammatory cytokine and it has direct proinflammatory properties. In this respect, IL-18 induces production of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and chemokines such as IL-8, MIP-1 $\alpha$  and MCP-1<sup>[3,4]</sup>, and upregulates expression of adhesion molecules such as ICAM-1, VCAM-1 on endothelial cells<sup>[3,5]</sup>. Furthermore, IL-18 is found to activate neutrophils and promote neutrophil migration, adhesion and accumulation *in vivo*<sup>[6]</sup>. These data suggest that IL-18 may be involved in the inflammatory injuries associated with I/R<sup>[1,2,7,8]</sup>. Therefore, recent evidence showing that some local tissue ischemic injuries are associated with increased IL-18 levels<sup>[9,10]</sup>, and elevated IL-18 levels have been detected in response to hepatic I/R injury<sup>[11]</sup>. A direct correlation has been observed between IL-18 levels and liver neutrophils sequestration and liver injury, which suggests that IL-18 is required for facilitating neutrophil-dependent hepatic I/R injury<sup>[11]</sup>. Similarly, increased levels of IL-18 have been detected in isolated atrial trabeculae during I/R injury<sup>[12]</sup>. Inhibition of IL-18 or caspase-1 activity attenuated tissue and circulating levels of IL-18 and improved myocardial contractility, suggesting that endogenous IL-18 play a significant role in I/R-induced human myocardial injury<sup>[12]</sup>. Furthermore, Melnikov *et al*<sup>[13]</sup>, reported that caspase-1 and IL-18 play an important role in the ischemic acute renal failure; however,

there is also evidence suggesting that activated caspase-1 and its inflammatory products are not crucial to the induction of inflammation after renal I/R<sup>[14]</sup>. It is intriguing to consider the role of IL-18 in the I/R-induced local organ injury, and no evidence exists on the role of IL-18 in the MODS following I/R. Therefore, the major aim of present study was to determine the change of expression and role of IL-18 in the acute pulmonary injury after gut ischemia/reperfusion.

## MATERIALS AND METHODS

### Reagents and mice

C57BL/6J mice (SPF) were obtained from animal center, SIBS, Shanghai. For the experiments, 8-10-wk-old mice weighing 22-26 g were used. rmIL-18 was expressed and purified in our lab (purity >95% by SDS-PAGE) and the activity was confirmed by its ability to induce IFN- $\gamma$  production by mouse spleen cells. For immunohistochemical analysis, polyclonal rabbit anti-mouse IL-18 antibody was purchased from Boster (Wuhan, China). For immunoprecipitation and Western blot analysis, polyclonal rabbit anti-murine IL-18 antibody was provided by PeproTech (Rocky Hill, NJ, USA).

### Ischemia/reperfusion model

Mice were anesthetized by intraperitoneal injection of urethane (250 mg/mL, 1.25 mg/g wt of mouse, Sigma). The abdomen was rinsed with 75% ethanol, a midline laparotomy was performed and the superior mesenteric artery was occluded with an arterial clamp. Intestinal ischemia was confirmed by pulselessness of the mesenteric artery and paleness of the jejunum and ileum. Sham-operated mice underwent the same procedure, but without vascular occlusion. After 30 min, the clamp was removed, sterile saline was injected into the peritoneal cavity for resuscitation, and the mice were sutured.

### Experimental protocols

The mice were randomly divided into four groups ( $n = 5$  in each): sham, I/R, I/R+IL-18, and I/R+anti-IL-18. For exogenous IL-18 studies, animals were pretreated with either IL-18 (5  $\mu$ g/mouse) or sterile saline via the lateral tail vein 15 min before ischemia. For IL-18 neutralization studies, mice received monoclonal anti-IL-18 antibody (25  $\mu$ g/mouse, MBL, Nagoya, Japan) or sterile saline 15 min before ischemia. Animals underwent 30 min of superior mesenteric artery occlusion (or sham operation) followed by indicated periods of reperfusion were killed, lung tissues, and blood samples were taken for analysis. Excised lungs were frozen in liquid nitrogen and stored at -70 °C before determination of myeloperoxidase (MPO) activity. For histological or immunohistochemical studies, lungs were immersed in 10% neutral buffered formalin before sectioning. All blood samples were kept at room temperature for 2 h to clot, and serum was removed and stored at -70 °C until the time of assay.

### Serum IL-18 measurements

Animals underwent cardiac puncture to obtain blood at the indicated times in the reperfusion period. Systemic levels of IL-18 were evaluated by immunoprecipitation followed by Western blot. Serum was diluted to 1 mL with PBS, and

serum IL-18 was immunoprecipitated with rabbit anti-murine IL-18 antibody. Then, the immunoprecipitates were resolved by SDS-PAGE (12-15% acrylamide) under reducing conditions. Gels were transferred to NC membranes and incubated with primary antibodies (1:1 000) at 4 °C overnight. Then mouse anti-rabbit IgG peroxidase was added and developed by ECL. Serum levels of IL-18 were further measured by ELISA according to the manufacturer's instructions (Boster, Wuhan, China). The detection limit for IL-18 was <5 pg/mL.

### MPO assay

MPO activity is an indicator of neutrophil accumulation. The content of MPO in the tissues was measured as previously described with slight modification<sup>[7]</sup>. The lungs were homogenized for 30 s in 4 mL 50 mmol/L potassium phosphate buffer (pH 6.0) and then centrifuged for 30 min at 16 000 g at 4 °C. The pellet was resuspended in 1.5 mL 50 mmol/L potassium phosphate buffer (pH 6.0) containing 5 g/L cetrimonium bromide. The samples were sonicated for 90 s and then incubated in a 60 °C water bath for 2 h, and centrifuged for 30 min at 35 000 g at 4 °C. The supernatant 50  $\mu$ L was added to 950  $\mu$ L of 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL *o*-dianisidine (Sigma) and 5  $\mu$ L/L hydrogen peroxide. Absorbance of 460 nm was measured at 1 and 3 min. MPO content per gram of wet tissue was calculated as: MPO content ( $A/g$  wet tissue) =  $(A_{460}(3 \text{ min}) - A_{460}(1 \text{ min})) / \text{tissue weight (g)}$ .

### Histopathology and immunohistochemistry

Lungs were immersed in 10% neutral buffered formalin before sectioning. Sections (4  $\mu$ m) were obtained from paraffin-embedded tissue samples, stained with hematoxylin-eosin (HE), and examined for histological evaluation of tissue damage under a microscope and photographed. For immunohistochemical analyses, paraffin-embedded lung sections of 4  $\mu$ m were incubated overnight at 4 °C with polyclonal rabbit anti-mouse IL-18 antibody (dilution 1:100). Biotinylated IgG was added as second antibody. Horseradish peroxidase-labeled streptomycin-avidin complex was used to detect second antibody. Slides were stained with diaminobenzidine, counterstained with hematoxylin, and finally examined under light microscope. The brown or dark brown stain was considered as positive.

### Statistical analysis

All data are presented as mean  $\pm$  SD. Significant differences between groups were determined using Student's *t*-test.  $P < 0.05$  is considered as statistically significant.

## RESULTS

### Ischemia/reperfusion-induced upregulation of IL-18 levels

IL-18 has been shown to be upregulated after ischemia in the kidney, heart, and liver. Therefore, we investigated whether the levels of IL-18 changed in response to gut ischemia/reperfusion. The serum, obtained from sham and from gut I/R model at different times of reperfusion, was first evaluated by Western blot, and the change of IL-18

levels is shown in Figure 1A. Two bands were detected in all serum samples, which are consistent with the “pro” and “mature” form of IL-18. At time 0, the content of IL-18 from I/R group, for both pro and mature, was elevated as compared with that from sham-operated mice, and the levels of mature IL-18 (mIL-18) was further considerably elevated 1 h after reperfusion, and then decreased at 3 h (Figure 1A).

Mouse serum IL-18 was further detected by ELISA after gut ischemia/reperfusion or sham operation. As shown in Figure 1B, IL-18 levels from normal mouse were <200 pg/mL. Consistent with the previous results, there was a marked increase in serum IL-18 from I/R group, peak at 1 h (at 660 pg/mL), followed by a decline. In the sham group, there was little measurable change in IL-18 levels during all reperfusion time.

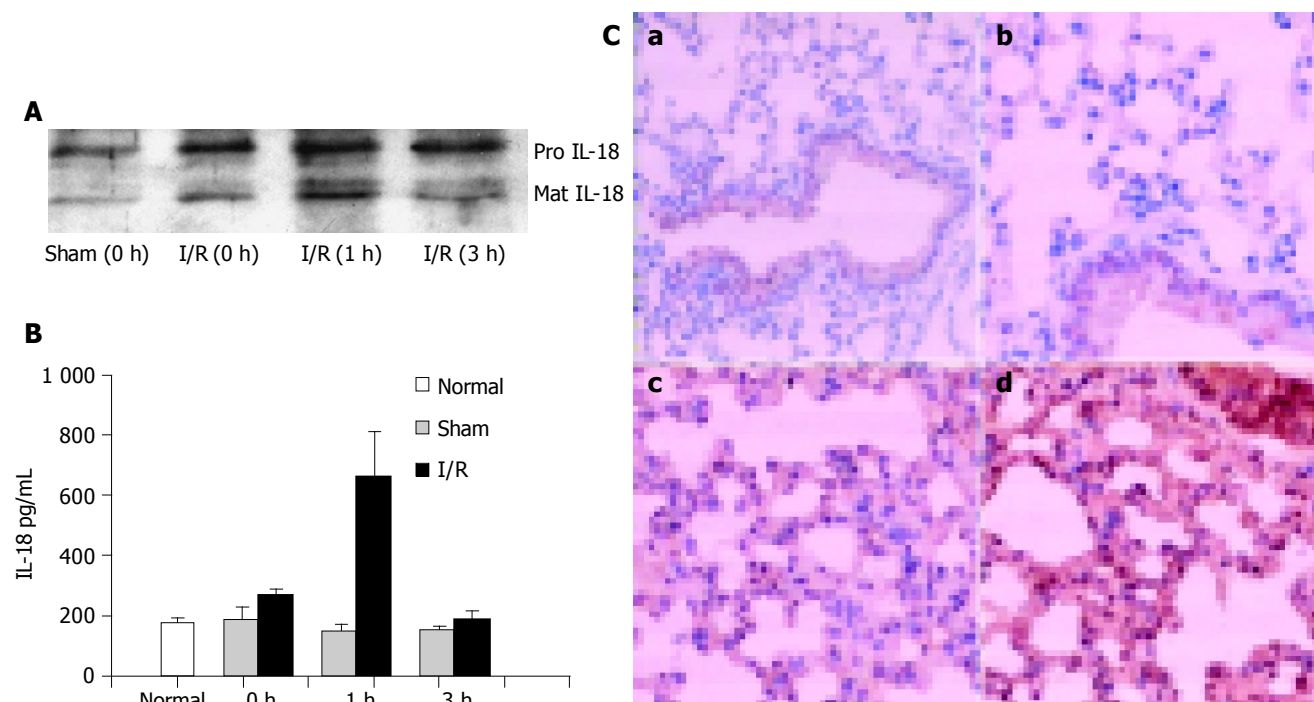
Furthermore, lung tissues obtained from normal mouse and I/R mice with 1 or 3 h reperfusion was examined by immunohistochemistry. Besides the constitutive expression of IL-18 in air way epithelium, normal lung tissue lacked immunoreactive IL-18 (Figure 1C, a and b); during 1-3 h of reperfusion, gradually increased levels of IL-18 were observed with prominent IL-18 staining (Figure 1C, c and d). These data indicated that IL-18 is induced at the early phase of ischemia/reperfusion and may participate in the pulmonary inflammation.

#### Effects of rmIL-18 on lung inflammation after gut I/R

Ischemia-induced expression of IL-18 increased remarkably at the early phase of reperfusion, suggesting that IL-18 may be an early inflammatory mediator and involved in the injurious processes in lung after gut I/R, which prompted

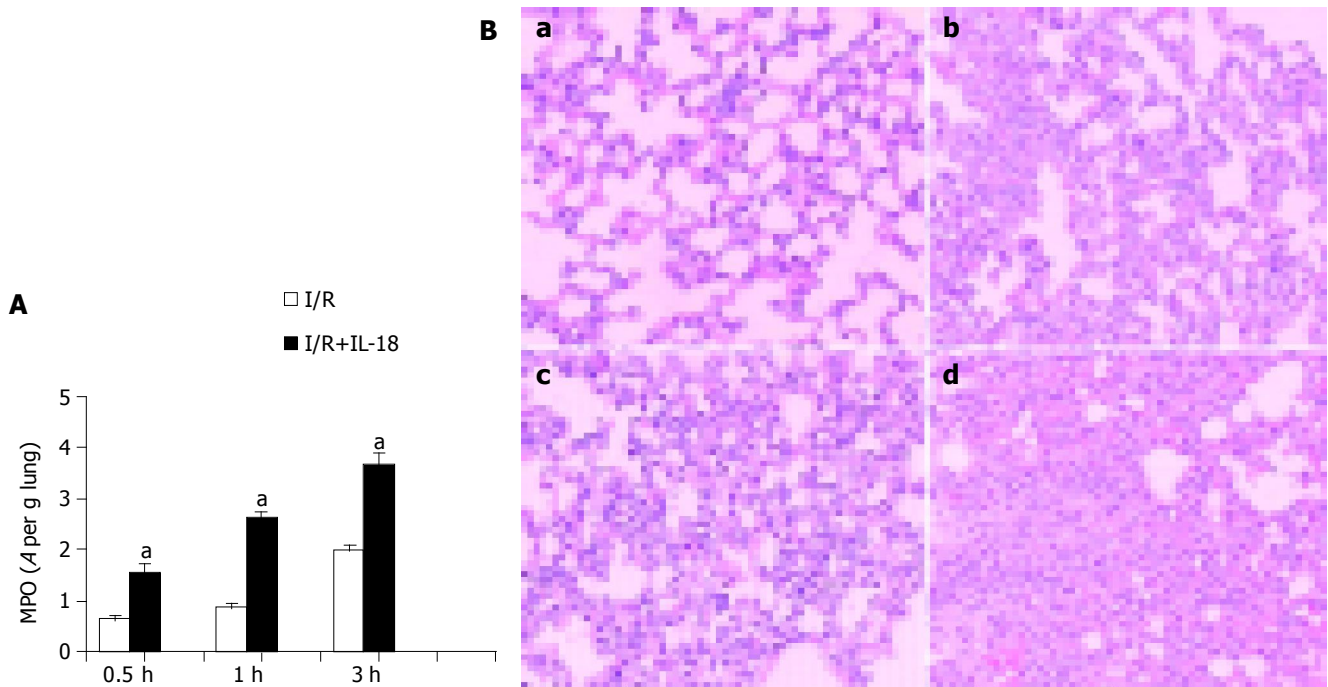
us to suppose whether administration of exogenous IL-18 could exert early effects on neutrophil activation and accelerate lung injury. As shown in Figure 2, in the IL-18-nontreated group, gut ischemia/reperfusion induced gradually increased MPO activity in the lungs at 0.5, 1, or 3 h after reperfusion ( $0.65\pm0.08$ ,  $0.86\pm0.19$ , or  $1.99\pm0.17$  respectively), and IL-18 injection further dramatically enhanced the MPO activity in the lung tissues at each indicated times after reperfusion: the MPO value in IL-18-treated mice was further increased to  $1.54\pm0.38$  (a 237% increase), and  $2.63\pm0.20$  (a 306% increase) at 0.5 or 1 h after ischemia respectively, as compared with IL-18-nontreated group ( $P<0.05$ ); and IL-18 administration induced much more neutrophils infiltration in lung within 1 h of reperfusion than that of 3 h of reperfusion in IL-18-nontreated group (Figure 2A). Furthermore, after 3 h of reperfusion, the MPO activity was further increased to higher level ( $3.68\pm0.45$ , a 184% increase) compared with that from IL-18-nontreated mice ( $P<0.05$ ). These data suggested that IL-18 injection enhanced pulmonary MPO activity and augmented the neutrophil accumulation in lung.

Lung inflammation in this model was evident from histological analysis (Figure 2B). As previously reported, massive neutrophil infiltration was observed in the lung tissues from gut I/R model within 3 h of reperfusion (Figure 2B, b). Although the infiltration in IL-18-nontreated mice at 1 h after ischemia was not obvious (Figure 2B, a), IL-18 injection significantly enhanced the pulmonary infiltration after 1 h of reperfusion (Figure 2B, c). Furthermore, treatment of IL-18 induced more serious inflammatory cell infiltration compared with that of IL-18-nontreated mice as the reperfusion



**Figure 1** Gut ischemia/reperfusion results in increased levels of IL-18 in serum and lung. (A and B) The circulating levels of IL-18 after gut ischemia/reperfusion. Mice were subjected to gut ischemia/reperfusion or sham. Serum IL-18 at the indicated times after reperfusion was collected, part of which was immunoprecipitated with polyclonal rabbit anti-murine IL-18 antibody and analyzed by immunoblotting

(A), and another part was assayed with ELISA (B) ( $n = 5$ ). (C) Gut ischemia/reperfusion results in elevated levels of IL-18 in lung tissue. Immunohistochemistry of lung sections obtained from normal (a, ×100, and b, ×200), 1 h (c) or 3 h (d) I/R model. The positive staining for IL-18 shows as dark brown. Magnification (c and d) ×200.



**Figure 2** The effect of exogenous IL-18 on the ischemia/reperfusion-induced lung injury. **A:** IL-18 injection further remarkably enhanced the neutrophil sequestration in lung. After indicated times of reperfusion, the MPO activity was determined in lung tissue ( $n = 5$ ), <sup>a</sup> $P < 0.05$  vs I/R. **B:** Comparison of pulmonary

histopathology. Lungs from IL-18-nontreated (a, for 1 h of reperfusion and b, for 3 h) or IL-18-treated (c, for 1 h and d, for 3 h of reperfusion) mice subjected to gut I/R and stained with HE. Magnification  $\times 100$ .

time extended to 3 h (Figure 2B, b and d), which in accordance with the higher MPO activity in IL-18-treated group. Both indices of lung inflammation, including histological changes and MPO activity, suggest that IL-18 injection may accelerate and augment the lung inflammatory injury in response to gut I/R, and IL-18 may be involved in the lung pathogenesis in this I/R model as an early inflammatory mediator.

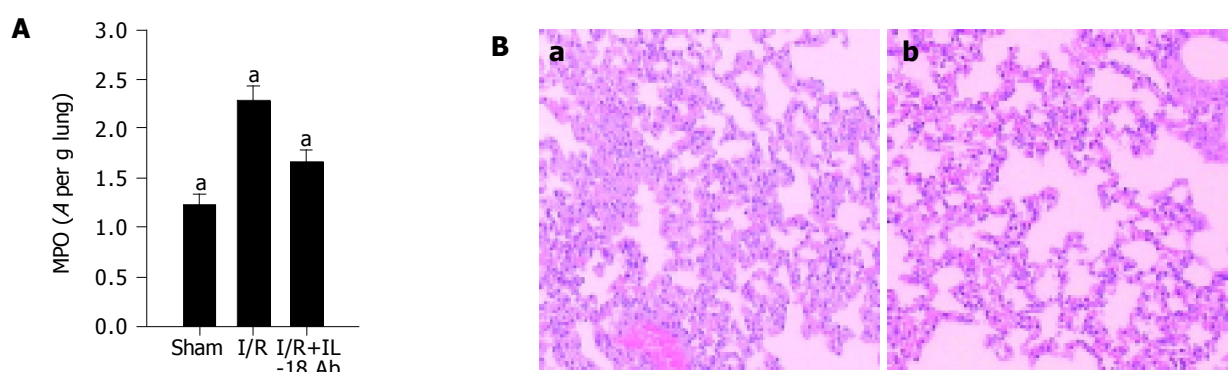
#### Effects of anti-IL-18 on lung inflammatory induced by gut ischemia/reperfusion

To further determine the role of induced IL-18 in the lung pathogenesis, we investigated whether *in vivo* neutralization of IL-18 could modify neutrophil-mediated acute inflammatory response induced by gut I/R. Anti-IL-18Ab (25  $\mu$ g/mouse) was administered 15 min before ischemia insult, and after 3 h of reperfusion, the MPO levels were determined and

the lung inflammation was evaluated histopathologically. As shown in Figure 3, anti-IL-18 recipient mice exhibited significantly reduced tissue MPO activity (more than 50% decrease in the lung MPO levels) as compared with the positive control ( $P < 0.05$ , Figure 3A). Consistent with this, the inflammatory infiltration was significantly reduced in anti-IL-18-treated mice (Figure 3B). These data suggested the IL-18Ab attenuated lung inflammation induced by gut I/R and supported the concept that endogenous IL-18 functioned as an important proinflammatory factor, which may be involved in the neutrophil sequestration and lung injury in this gut I/R model.

#### DISCUSSION

To date, IL-18 has been known for its role in infectious



**Figure 3** The effect of anti-IL-18 antibody on the ischemia-induced lung injury. **A:** Anti-IL-18 antibody injection remarkably inhibited the lung MPO activity. After 3 h of reperfusion, the MPO activity was determined in lung tissues ( $n = 5$ ),

<sup>a</sup> $P < 0.05$  vs others. **B:** Comparison of pulmonary histopathology. Lungs from ischemic mice (a, for 3 h of reperfusion), and ischemic mice treated with IL-18Ab (b, for 3 h of reperfusion) and stained with HE. Magnification  $\times 100$ .



inflammatory injury. However, few investigations have been accomplished regarding the role of IL-18 during non-infectious acute inflammatory reactions such as those that occur in ischemia/reperfusion. Previous reports have shown that IL-18 have important regulatory role during organ injury induced by local ischemia/reperfusion<sup>[11-13]</sup>. In the current study, we used a model of acute lung inflammation induced by gut ischemia/reperfusion, which results in damage of capillary endothelial cells and neutrophil influx, and oxidants, proteases released from inflammatory cells damage lung cells and tissue matrix<sup>[1-3]</sup>. The role of IL-18 in this model of lung injury has not been explored. The current study provides evidence that endogenous IL-18 may act as a proinflammatory cytokine and contribute to gut ischemia/reperfusion-induced lung inflammation.

Our results, consistent with the previous reports, have shown that local ischemia may induce the elevation of IL-18 level<sup>[11-13]</sup>. It promptly reached peak at 1 h and then declined to baseline. However, the levels of IL-18 in lung tissue were not the same. Constitutively, expression of IL-18 in airway epithelium has been observed by using immunohistochemistry, and as the reperfusion time extended to 3 h, the level of IL-18 in air way epithelium was greatly enhanced and immunoreactive IL-18 dispersed all over the lung tissue, which suggests that pulmonary expression of IL-18 may be enhanced during reperfusion as the inflammatory agents secreted from intestine reached the lung tissue and further, the IL-18 receptor mediated mechanism may also account for the pulmonary accumulation of IL-18, which in accordance with the rapid decline of serum IL-18 level during reperfusion.

IL-18 has been known to be produced as a proform and stored in cytoplasm. After the cleavage of caspase-1, it turns to a functional, mature protein. By Western blot analysis, the IL-18 mature protein was quickly increased after 1 h of reperfusion (Figure 1A), which suggests that gut ischemia may result in the activation of caspase-1 and enhance the maturation and secretion of IL-18. Furthermore, previous reports have shown that ischemia also induces IL-1 $\beta$  expression. Therefore, it is possible that intestinal caspase-1 may play a key role in expression of both the mature IL-18 and IL-1 $\beta$ , and ischemic gut may serve as a major source for circulating IL-18 in this model.

Experimental studies have suggested that IL-18 protects against bacterial and viral agents<sup>[3]</sup>, which is thought to be attributable in part to the infiltration of inflammatory cells after treatment with IL-18. There is also evidence suggesting that IL-18 may activate neutrophils and promote neutrophil migration and accumulation *in vivo*<sup>[6]</sup>. In the current studies, we showed that the *in vivo* exogenous administration of IL-18 before gut ischemia greatly enhanced neutrophil recruitment as well as lung inflammation (Figure 2). Conversely, IL-18 blockade reduced the evidence of lung inflammation by neutralizing Ab (Figure 3). These results suggest that IL-18 may, like IL-1 $\beta$ , be an important early pro-inflammatory cytokine involved in the pathogenesis of acute lung inflammation induced by gut ischemia/reperfusion. Furthermore, these data also suggest that the preoperative levels of serum IL-18 may represent a valuable parameter to aid the postoperative prognostic determinant in clinical

patients with ischemia-induced MODS.

But how IL-18 is activated, and how it enhances the pulmonary inflammation during ischemia/reperfusion is still unknown. There is evidence indicating that NO suppresses IL-18 processing by inhibiting caspase-1 activity<sup>[15,16]</sup>. It is likely that ischemia-induced decrease of NO levels may result in the activation of caspase-1 and the further secretion of mature IL-18. Furthermore, it is clear that IL-18, a pleiotropic proinflammatory cytokine, has an endogenous role in enhancing production of several early response cytokines during lung inflammation, such as TNF- $\alpha$ , IFN- $\gamma$ , and IL-1 $\beta$ . Previous reports have shown that IL-18 may activate neutrophil and promote neutrophil migration and accumulation *in vivo* via a TNF- $\alpha$ -dependent pathway<sup>[6,17]</sup>. However, the mechanism of pulmonary neutrophils sequestration is possibly independent of TNF- $\alpha$  in this lung injury model<sup>[18]</sup>, which suggests that the function of IL-18 may not be totally dependent on the role of TNF- $\alpha$ . These works are now in progress in our group.

The present data expand our knowledge of the immunoregulatory properties of IL-18. Our findings are clinically applicable to ischemia/reperfusion injury during surgical operation such as small intestinal transplantation, and modulation of IL-18 expression may enhance endogenous protective effects, leading to a reduction in organ injury.

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