

# Intravenous Immunoglobulin Attenuates Cecum Ligation and Puncture-Induced Acute Lung Injury by Inhibiting Apoptosis of Alveolar Epithelial Cells

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**Purpose:** Intravenous immunoglobulin (IVIG) therapy has been used to treat sepsis, but its mechanisms of action remain unclear. Sepsis causes multiple organ failure, such as acute lung injury (ALI), which involves apoptosis of alveolar epithelial cells. In this study, we hypothesized that IVIG suppresses apoptosis in alveolar epithelial cells and evaluated mortality, cytokine levels, histological changes in the lung, and alveolar epithelial cell apoptosis after IVIG administration, in mice with experimentally induced sepsis.

**Methods:** Mice received an injection of vehicle (saline) or immunoglobulin (100 mg/kg or 400 mg/kg) into the tail vein, after which they underwent cecal ligation and puncture. A sham-operated group was used as the normal control. Survival was assessed in all groups after 72 hours. Plasma levels of TNF- $\alpha$  and IL-6, histopathological changes and wet-to-dry ratio in lung, and alveolar epithelial cell apoptosis were evaluated in all groups at 4 hours after surgery.

**Results:** In the vehicle group, histopathological injury of the lung was severe, and apoptosis of alveolar epithelial cells was significant. Survival and plasma cytokine levels were better in the IVIG treatment groups than in the vehicle group. IVIG 400 mg/kg suppressed apoptosis of alveolar epithelial cells and reduced ALI.

**Conclusion:** IVIG suppressed inflammatory cytokine levels and improved survival. Lung histopathology and alveolar epithelial cell apoptosis were improved by IVIG treatment, in a dose-dependent manner. Suppressing apoptosis in alveolar epithelial cells appears to be a mechanism by which IVIG improves survival. (J Nippon Med Sch 2020; 87: 129–137)

**Key words:** sepsis, intravenous immunoglobulin, animal, apoptosis of alveolar epithelial cells

## Introduction

In the Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3), sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection<sup>1</sup>. Despite significant advances in sepsis treatment in recent years, mortality from cardiogenic shock and multiple organ failure is still high<sup>2</sup>. The in-hospital mortality of sepsis is greater than 10%<sup>3,4</sup>. Septic shock, which causes marked circulatory, cellular, and metabolic abnormalities, is associated with greater mor-

tality risk than sepsis alone. In addition, sepsis can produce a general inflammatory response and systemic release of proinflammatory cytokines, resulting in acute organ dysfunction such as acute lung injury (ALI). ALI triggered by sepsis is particularly difficult to treat<sup>5</sup>. Recently, cell apoptosis has been reported to play a critical role in ALI progression<sup>6</sup>.

A recent study reported that intravenous immunoglobulin (IVIG) ameliorates acute brain dysfunction associated with sepsis by reducing apoptotic cell death in

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neuronal cells<sup>7,8</sup>. However, no study has examined the relationship between IVIG and apoptosis in alveolar epithelial cells. Immunoglobulin is a Y-shaped protein produced mainly by plasma cells and is used by the immune system to neutralize pathogens such as pathogenic bacteria and viruses. Immunoglobulin G is the most common type of human antibody. Recent studies indicate that IVIG is associated with anti-inflammatory responses to cytokine-related inflammation. Physicians use IVIG to treat several diseases, such as severe infection, agammaglobulinemia, Kawasaki disease, and idiopathic thrombocytopenic purpura (ITP)<sup>9</sup>. Kawasaki disease and ITP are treated with 2,000 mg/kg IVIG, and this high-dose IVIG regimen improved survival in animal model of sepsis<sup>10,11</sup>. A meta-analysis showed that high-dose IVIG was effective for clinical treatment of sepsis<sup>12</sup>. Low-dose IVIG (5,000 mg/day for 3 days; approximately total 200-300 mg/kg) is widely used as an adjunctive treatment for patients with sepsis in Asian countries, including Japan and Korea<sup>13</sup>.

Cecum ligation and puncture (CLP) is a common method used to create a sepsis model in animals. This method produces a septic state by causing polybacterial abdominal infection. The CLP animal model was used in several previous studies of IVIG<sup>14-16</sup>.

We hypothesized that the clinical dose of IVIG used in Asian countries would prevent mortality in a CLP-induced mouse model of sepsis. To understand the mechanism of action for IVIG we also measured plasma levels of cytokines and assessed lung tissue histopathology and apoptosis in alveolar epithelial cells by using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining.

## Materials and Methods

### Animals and Ethics Statement

Male C57BL/6N mice (age 8-10 weeks, weight, 20-25 g; Sankyo Labo Service Corporation, Tokyo, Japan) were used for this study. Mice were housed in cages under specific pathogen-free conditions. The humane endpoint was defined as loss of ability to ambulate or labored respiration. The mice were checked every 6-8 hours, at least, and regarded as dead in survival analysis when they exhibited these symptoms, after which they were immediately euthanized. All procedures and animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals and the relevant committee for animal experiments. The protocols were approved by the Experimental Animal Ethics Review

Committee of Nippon Medical School, Tokyo, Japan (approval number 27-201). All researchers involved in this study were experienced and had received instruction on the ethics of animal experiments.

### Mouse Model of CLP

We developed a model of intra-abdominal infection and sepsis by using the CLP procedure. Mice were anesthetized with 2% isoflurane, and a 15-mm midline incision was made through the skin and peritoneum of the abdomen, to expose the cecum. After opening the abdomen, the cecum was ligated with a 3-0 silk ligature without obstructing intestinal continuity. The cecum was then punctured once by inserting an 18-gauge needle through both intestine walls. The cecum was returned to the abdominal cavity, and the peritoneal wall and skin incision were sutured. Sham-operated mice underwent a similar procedure, but the cecum was not ligated or punctured. All animals received 1 mL of normal saline in their abdominal cavity before closing the abdominal wall.

### Experimental Design

The animals were randomly assigned to one of four groups: 1) a Sham group that was given a bolus saline into the tail vein before operation without CLP, 2) a Vehicle group that received intravenous saline before CLP, 3) an IVIG 100 group that received a 100 mg/kg dose of human immunoglobulin before CLP, and 4) an IVIG 400 group that received a 400 mg/kg dose of human immunoglobulin before CLP. Each animal received the appropriate injection into the tail vein 30 minutes before operation. Human immunoglobulin (Venoglobulin IH 5%, polyethylene glycol-treated human normal immunoglobulin) was donated by the Japan Blood Products Organization (Tokyo, Japan). This preparation is produced from pooled human plasma obtained from multiple individuals.

### Survival Study

In the survival study, mice in each group were monitored for 72 hours (n = 10/group), and the surviving animals were euthanized on postoperative day 7.

### Cytokine Analysis

Previous studies reported that plasma levels of cytokines peak at 2 to 6 hours after CLP<sup>17,18</sup>. A previous study showed that ALI and acute kidney injury (AKI) could be established within 4 hours in an experimental sepsis model<sup>19</sup>. Therefore, after anesthesia, blood samples were collected by cardiac puncture with heparinized syringes 4 hours after operation. After centrifugation at 1,500 g for 17 min, plasma was stored at -80°C. Plasma levels of interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$  were

## Results

measured by enzyme-linked immunosorbent assay (ELISA) with Mouse IL-6 DuoSet ELISA DY406 and Mouse TNF-alpha DuoSet ELISA DY410 (R&D Systems, Minneapolis, MN, US) kits, respectively.

### Wet-to-Dry Weight Ratio

The wet-to-dry weight (W/D) ratio of the lungs was measured to evaluate lung tissue edema. Four hours after the operation, the animals were anesthetized, and their right ventricles were perfused with 10 mL of phosphate-buffered saline to clear pulmonary circulation. The right lung was then excised and weighed to obtain its wet weight. The lungs were then dried in an oven at 80°C for 72 hours and reweighed to establish a dry weight, to calculate the W/D ratio.

### Lung Histopathological Analysis

Histopathological changes in lungs were examined in each treatment group at 4 hours after surgery. Using the same procedure described above, we fixed the left lungs in 4% paraformaldehyde at 4°C. Lung sections were then stained with hematoxylin-eosin (H&E), and histological changes in lung tissue were observed under a light microscope. A blinded pathologist scored the tissues by using Murakami's criteria: 10 fields of lung parenchyma were graded on a scale of 0-4 (0, absent and appears normal; 1, light; 2, moderate; 3, strong; 4, intense) for congestion, edema, inflammation, and hemorrhage. The total lung injury score was calculated by adding the individual scores for each category<sup>20</sup>.

### TUNEL Assay

After removal of the left lung at 4 hours after surgery, lung sections were stained by the TUNEL method to detect fragmented DNA, which is indicative of apoptotic cells. Samples were then stained with an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, US). TUNEL-positive cells were counted at ×400 magnification. The number of apoptotic cells per lung section was then determined.

### Statistical Analysis

The mice survival curves were obtained by Kaplan-Meier analysis, and the log-rank test was used to analyze survival rate. All data were expressed as mean ± standard error. Differences between two groups were evaluated with the unpaired *t* test, and one-way ANOVA and the Tukey Post Hoc test were used for multiple comparisons. For the histopathological study, the nonparametric Kruskal-Wallis test was performed. A *p* value of <0.05 was considered to indicate statistical significance.

### Mortality

The survival rates of mice were assessed at 72 hours after the surgical operation. Moribund mice were all euthanized at humane endpoints. In the Sham group, all animals survived. In the Vehicle group, however, all mice died within 72 hours. In the IVIG 100 and IVIG 400 groups, 30% and 50% of the animals survived, respectively. These survival rates were significantly higher than that of the Vehicle group (*p* = 0.018 and <0.01, respectively) (Fig. 1). Therefore, IVIG 100 mg/kg and IVIG 400 mg/kg improved survival in this mouse CLP model.

### Effect of IVIG on Plasma Levels of IL-6 and TNF-α

Plasma levels of IL-6 were higher in all mice in the CLP groups (*n* = 6-8/group) at 4 hours after the CLP operation. The plasma IL-6 level was very low in the Sham group (310.9 ± 9.4 pg/mL). In the IVIG 100 and IVIG 400 groups, plasma levels of IL-6 were significantly lower than in the Vehicle group (both *p* < 0.01). IL-6 levels in the IVIG 100, IVIG 400, and Vehicle groups were 3,692.2 ± 484.8, 2,730.9 ± 556.3, and 10,530.0 ± 1,600.4 pg/mL, respectively (Fig. 2A).

Plasma levels of TNF-α were also higher in all mice in the CLP groups. In the IVIG 100 and IVIG 400 groups, plasma TNF-α levels were significantly lower than in the Vehicle group (*p* < 0.01). There was no significant difference between the IVIG 100 and IVIG 400 groups. TNF-α levels in the Sham, IVIG 100, IVIG 400, and Vehicle groups were 26.7 ± 13.7, 265.4 ± 10.5, 302.6 ± 33.2, and 482.5 ± 44.8 pg/mL, respectively (Fig. 2B).

### Effect of IVIG on the Lung

#### W/D ratio of lungs

The W/D ratios of lungs in the CLP groups (*n* = 7-8/group) were significantly higher than in the Sham group. However, the W/D ratios of the IVIG groups (100 and 400) were significantly lower than in the Vehicle group (Fig. 3).

#### Histological evaluation of the lungs

Four hours after the operation, a clear inflammatory reaction was seen in the lungs, as indicated by the presence of cellular infiltrate in the interstitium and air spaces of the lung (Fig. 4). Inflammatory cell infiltration, interstitial edema, vascular congestion, and hemorrhage were more obvious in the Vehicle group than in the Sham group. For the IVIG 400 group, the lung injury scores for four parameters—congestion, edema, inflammation, and hemorrhage—were significantly lower than in the Vehicle group. In contrast, there were no significant differences in these lung injury scores between the IVIG

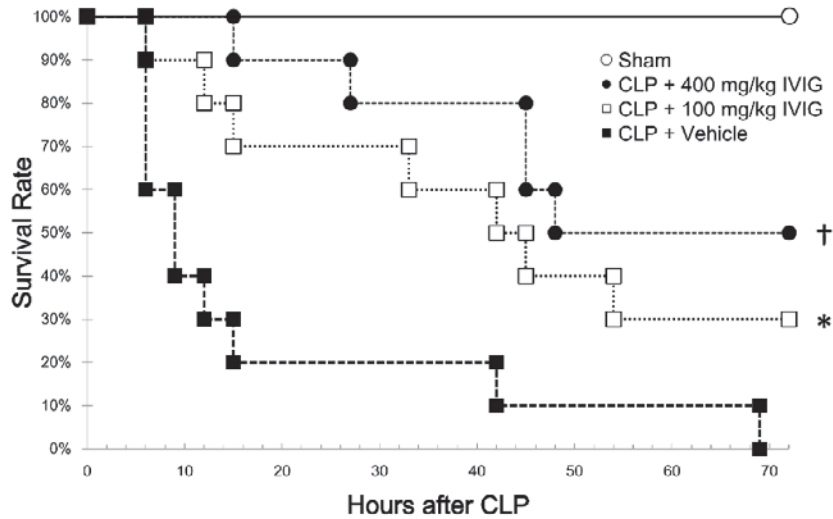


Fig. 1 Survival curves

Survival was significantly better for mice in the IVIG treated groups than in the Vehicle group (n = 10/group). The mortality rate was significantly lower in the IVIG 100 group than in the Vehicle group (p = 0.018) and significantly lower in the IVIG 400 group than in the Vehicle group (p = 0.005). In the Sham group (laparotomy only, no CLP), all mice survived. There was no significant difference between the IVIG 100 and IVIG 400 groups (p = 0.279).

\* p < 0.05 vs Vehicle; † p < 0.01 vs Vehicle

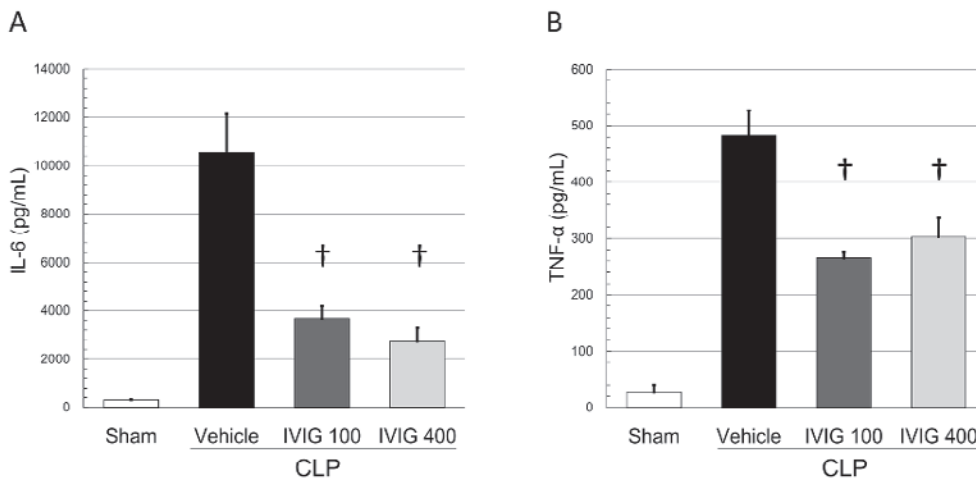


Fig. 2 Plasma cytokine levels 4 hours after CLP

(A) Plasma levels of IL-6 (n = 7 in the Sham, Vehicle, and IVIG 100 groups; n = 8 in the IVIG 400 group). Plasma IL-6 levels were significantly lower in the IVIG groups than in the Vehicle group. There was no significant difference between the IVIG 100 and 400 groups. IL-6 levels were very low in the Sham group.

(B) Plasma level of TNF-α (n = 7 in the Sham and IVIG 100 groups; n = 6 in the Vehicle group; and n = 8 in the IVIG 400 group). TNF-α levels were significantly lower in both IVIG treatment groups than in the Vehicle group. TNF-α levels in the Sham group were very low. Data are expressed as the mean ± SE.

† p < 0.01 vs Vehicle

100 and Vehicle groups (Fig. 5). The total lung injury score was also significantly attenuated by IVIG 400 (Fig. 6).

TUNEL assay

Alveolar epithelial cell apoptosis was evaluated by TUNEL staining at 4 hours after surgery. Numerous TUNEL-positive cells were present in the Vehicle group

( $21.7 \pm 2.7$ /section). In contrast, there were few TUNEL-positive cells in the Sham group ( $4.8 \pm 0.9$ /section). In the IVIG 400 group ( $10.3 \pm 1.5$ /section), the number of TUNEL-positive cells was significantly lower than in the Vehicle group. In contrast, the number of TUNEL-

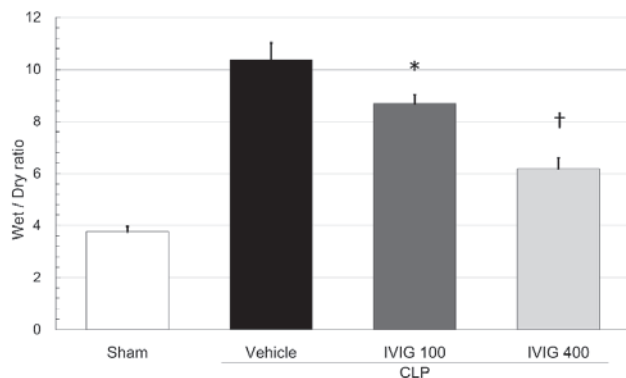


Fig. 3 Effect of IVIG on W/D ratio of sepsis-induced mice.

The lung W/D ratio was examined after 4 h in each group ( $n = 8$  in the IVIG groups;  $n = 7$  in the Sham and Vehicle groups). Data are expressed as mean  $\pm$  SE. The W/D ratio in the IVIG 100 and 400 groups was significantly lower than in the Vehicle group ( $p = 0.046$  and  $<0.001$ , respectively).

\*  $p < 0.05$  vs Vehicle; †  $p < 0.01$  vs Vehicle

positive cells in the IVIG 100 ( $19.6 \pm 3.2$ /section) and Vehicle groups did not significantly differ (Fig. 7, 8).

### Discussion

Both 100 mg/kg and 400 mg/kg IVIG significantly improved survival and reduced plasma levels of IL-6 and TNF- $\alpha$ ; however, ALI and apoptosis of alveolar epithelial cells improved only with IVIG 400 mg/kg.

IVIG has been used to treat sepsis, but previous clinical studies reported differences in the effectiveness of immunoglobulin preparations in sepsis patients; thus, its benefit remains unclear<sup>21-25</sup>. Werdan et al. found that IVIG therapy was ineffective in treating septic shock patients<sup>26</sup>, whereas a meta-analysis suggested that IVIG might reduce the mortality rate among adults with septic shock<sup>22</sup>.

IVIG has several theoretical advantages in the treatment of sepsis, including pathogen recognition, pathogen clearance, and toxin scavenging. IVIG preparations may also have beneficial effects on host response to infection<sup>21,27</sup>. Other studies reported that its mechanism of action includes stimulation of Fc receptor-mediated antibiotic-dependent cellular cytotoxicity, neutralization of viruses and toxins, suppression of inflammatory cytokine activity, promotion of complement-mediated bac-

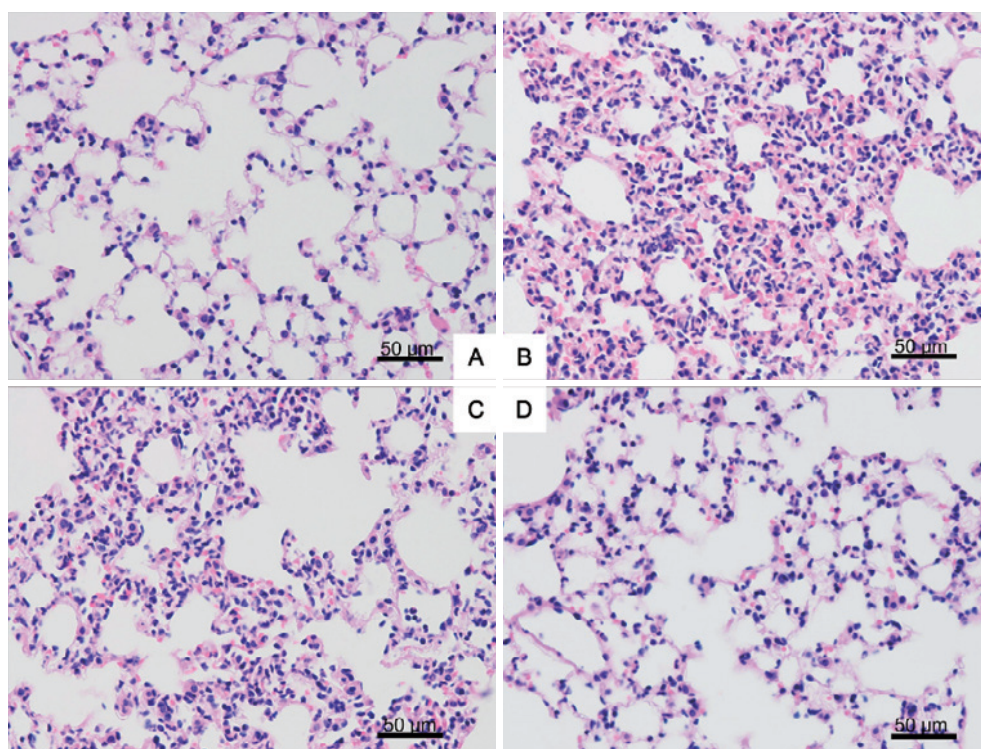


Fig. 4 Representative histopathological findings in the lung.

Lung tissue sections were stained with hematoxylin-eosin. (A) Sham, (B) CLP + Vehicle, (C) CLP + IVIG 100, (D) CLP + IVIG 400.

Scale bar = 50  $\mu$ m

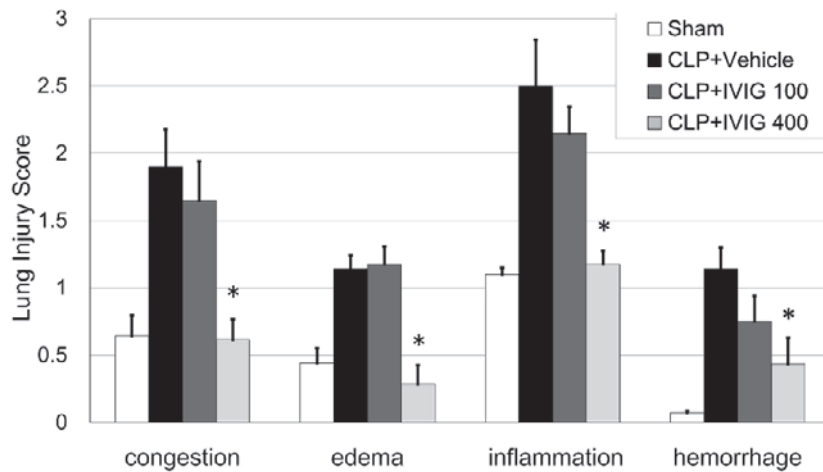


Fig. 5 Lung injury scores for four parameters of lung injury.

Lung injury scores were examined in each group at 4 h after CLP (n = 8 in IVIG groups; n = 7 in the Sham and Vehicle groups). Data are expressed as mean ± SE. In the IVIG 400 group, all four individual lung injury parameters assessed were significantly lower than in the Vehicle group:  $p = 0.033$  (congestion),  $p = 0.018$  (edema),  $p = 0.024$  (inflammation), and  $p = 0.038$  (hemorrhage).

\*  $p < 0.05$  vs Vehicle

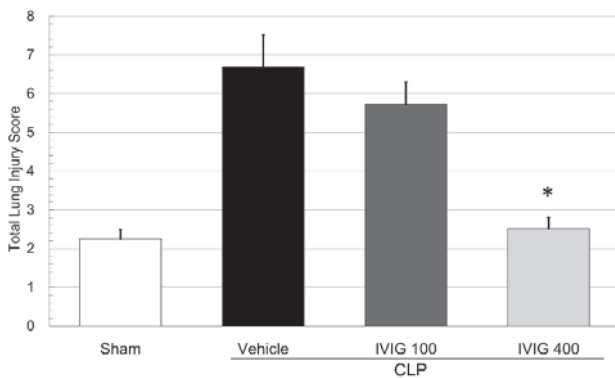


Fig. 6 Total lung injury score.

The total lung injury score for each group was calculated after examining lung injury parameters at 4 h after CLP (n = 8 in the IVIG groups; n = 7 in the Sham and Vehicle groups). Data are expressed as mean ± SE. Similar to the results for the individual parameters (Fig. 5), total lung injury score was significantly lower in the IVIG 400 group than in the Vehicle group ( $p = 0.014$ ).

\*  $p < 0.05$  vs Vehicle

teriolysis, and opsonization of targets to promote phagocytosis<sup>28</sup>. Fab-dependent effects include neutralization of bacteria, bacterial toxins, and bacterial superantigens, suppression of cytokines produced by immune cells, and autoantibodies<sup>29</sup>. Previous studies have shown that IVIG has anti-inflammatory effects<sup>10,11,30-33</sup>. Thus, immunoglobulins could theoretically improve survival in our mouse sepsis model not only through direct antibacterial effects but also by exerting anti-inflammatory effects.

Previous studies reported that immunoglobulins im-

proved survival in animal sepsis models, but only at high doses (>1,000 mg)<sup>10,11</sup>. However, we observed that 100 mg/kg and 400 mg/kg IVIG improved the survival rate. Furthermore, there was no significant difference between these doses. Therefore, even in the absence of antibiotics, IVIG improved outcomes in a mouse model of sepsis.

The present findings indicate that IVIG treatment reduces levels of two proinflammatory cytokines (TNF- $\alpha$  and IL-6), which is consistent with previous results<sup>10,11,34</sup>. In our study, the degree of cytokine reduction did not depend on IVIG dose. In previous studies, plasma levels of high-mobility group box chromosomal protein 1 (HMGB1), a late mediator of lethal systemic inflammation, were reduced by IVIG treatment<sup>10,11,34</sup>. In a study by Yang et al., treatment with anti-HMGB1 antibodies, beginning at 24 h after CLP surgery, significantly increased the survival rate of CLP-induced septic mice. The authors concluded that HMGB1 was an important mediator under septic conditions, but less so in the case of septic shock, in which TNF- $\alpha$  has a major role<sup>35</sup>. Thus, it appears that a reduction in proinflammatory cytokines through the use of IVIG helps improve outcomes in septic mice.

ALI can be caused by several pathological processes that affect the lungs. In particular, sepsis is a common cause of indirect lung injury and is associated with onset of ALI<sup>36</sup>. The pathological processes in ALI are mainly caused by neutrophil- and platelet-dependent damage to

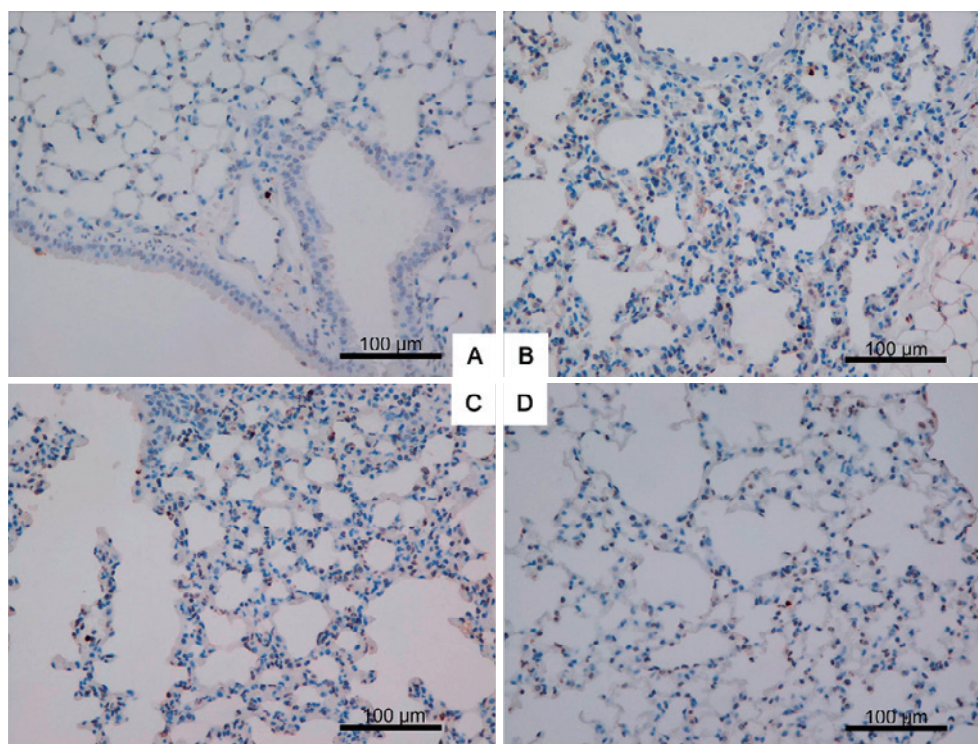


Fig. 7 Representative TUNEL stain findings in the lung.

Lung tissue sections stained with TUNEL to identify apoptotic cells. Brown-stained cells were considered TUNEL-positive cells. (A) Sham, (B) CLP + Vehicle, (C) CLP + IVIG 100, (D) CLP + IVIG 400.

Scale bar = 100 µm.

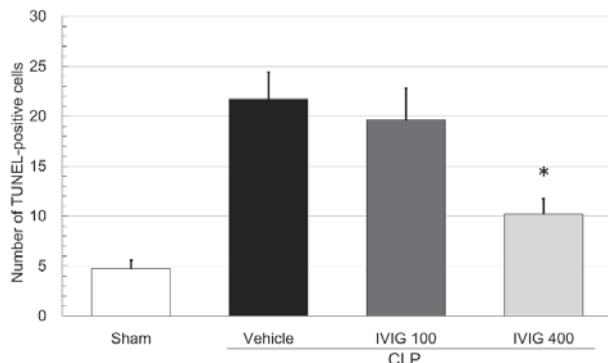


Fig. 8 Number of TUNEL-positive cells in each group. Quantification of TUNEL staining performed at 4 h after surgery in each group (n = 8 in the IVIG groups; n = 7 in the Vehicle group; n = 4 in the Sham group). Data are expressed as mean ± SE. The number of cells undergoing apoptosis was significantly lower in the IVIG 400 group than in the Vehicle group (p = 0.014). There was no significant difference between the IVIG 100 group and Vehicle group in the number of cells undergoing apoptosis.

\* p < 0.05 vs Vehicle

endothelial and epithelial barriers of the lung. This causes fibrin-rich edema fluid, red blood cells, and neutrophils to enter alveoli, which leads to inactivation of surfactant. These changes disrupt lung tissue, thereby re-

ducing lung function<sup>37,38</sup>. Inflammation is important during injury and repair in ALI<sup>39</sup>. Proinflammatory cytokines such as IL-6 and TNF-α are involved in this complex systemic inflammatory response<sup>40,41</sup>. In particular, TNF-α induces production of other inflammatory cytokines, promotes migration and adhesion of neutrophils to endothelial cells<sup>42</sup>, and causes apoptosis of alveolar epithelial cells<sup>6,43</sup>.

The W/D ratio was higher in the CLP group than in the IVIG groups. IVIG treatment significantly reduced lung W/D weight ratio and the amount of excess lung fluid in experimental ALI. IVIG treatment therefore improved the lung edema seen in septic mice. In contrast, using a histopathological assessment, we found that only treatment with 400 mg/kg IVIG reduced ALI. The reasons for the differences between the W/D ratio and histopathological data are not known.

In addition to acute lung injury, severe sepsis caused clinical and experimental failure of other organs, especially kidney dysfunction. Currently, there is growing interest in potential crosstalk between injured organs, particularly in regard to the relationship between AKI and ALI<sup>44,45</sup>. In this experiment, IVIG improved survival even at a dose of 100 mg/kg. On the other hand, analysis of

lung lesions, particularly histopathological analysis, indicated that ALI was improved in a dose-dependent manner by IVIG treatment, which suggests that IVIG acts on organs other than the lung.

This study is the first assess the effects of IVIG on apoptosis of alveolar epithelial cells in an animal model of sepsis. A previous study reported that sepsis could cause activation of nuclear factor kappa-light-chain-enhancer of activated B (NF- $\kappa$ B) cells, which is involved in apoptosis in ALI<sup>46</sup>. In addition, Murakami et al. showed that IVIG suppressed activation of NF- $\kappa$ B<sup>34</sup>. IVIG appears to suppress apoptosis of alveolar epithelial cells by these mechanisms.

The number of apoptotic cells was not significantly different in the IVIG 100 and Vehicle groups but was significantly lower in the IVIG 400 group. This suggests that IVIG suppresses apoptosis in alveolar epithelial cells in a dose-dependent manner. In this study, both 100 mg/kg and 400 mg/kg IVIG improved survival and plasma levels of proinflammatory cytokines IL-6 and TNF- $\alpha$ . However, ALI caused by sepsis improved histologically but only at an IVIG dose of 400 mg/kg, probably because apoptosis was suppressed at this dose. Interestingly, 100 mg/kg IVIG improved survival but did not suppress ALI. TNF- $\alpha$  has a key role in proinflammatory cytokine production, and ALI and AKI occurred together early in the course of sepsis in an animal sepsis model. When an anti-TNF- $\alpha$  antibody was administered to mice with induced sepsis, serum levels and functional activities of IL-6 and TNF- $\alpha$  decreased and renal function improved, which suggests that a reduction in proinflammatory cytokines is important in improving renal function<sup>19</sup>.

As mentioned above, IVIG suppressed acute brain dysfunction associated with sepsis, by reducing apoptotic cell death in neuronal cells<sup>7,8</sup>. Our experimental results suggest that IVIG reduces blood cytokines levels and mortality arising from organ dysfunction in organs other than lung, eg, the kidney and central nervous system. Future studies should examine the mechanisms underlying the action of IVIG and the optimal immunoglobulin dose for sepsis treatment.

### Conclusion

In mice with induced sepsis, IVIG improved survival, reduced levels of inflammatory cytokines in plasma, and improved ALI and apoptosis in alveolar epithelial cells. Inhibition of apoptosis in alveolar epithelial cells appears to be one mechanism by which IVIG improves survival.

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**Conflict of Interest:** The authors declare no conflicts of interest.

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