Intravenous immunoglobulin attenuates cecum ligation and puncture-induced acute lung injury by inhibiting apoptosis of alveolar epithelial cells

IVIG improves survival of septic mice.

Jun Hagiwara¹, Marina Yamada², Norio Motoda³, Hiroyuki Yokota¹

¹Department of Emergency and Critical Care Medicine, Graduate School of Medicine, Nippon Medical School, Bunkyo-ku, Tokyo, Japan
²Faculty of Medical Science, Nippon Sport Science University, Yokohama, Kanagawa, Japan
³Department of Pathology, Nippon Medical School Musashi-Kosugi Hospital, Kawasaki, Kanagawa, Japan

Correspondence to Jun Hagiwara, MD, Department of Emergency and Critical Care Medicine, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

E-mail: jun-nms@pop21.odn.ne.jp

FAX: +81-3-3821-5102
Abstract

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

Methods: Mice were administrated either 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 group after 72 hours. Plasma levels of TNF-α and IL-6, histopathological changes and wet-to-dry ratio of lung, and alveolar epithelial cell apoptosis were evaluated in all groups 4 hours after surgery.

Results: In the vehicle group, the histopathological injury of the lung was severe, and significant apoptosis of alveolar epithelial cells was observed. Both survival and plasma cytokine levels were improved in each of the IVIG treatment groups compared to the vehicle group. IVIG at 400 mg/kg suppressed apoptosis of alveolar epithelial cells and
reduced ALI.

Conclusion: IVIG can suppress inflammatory cytokine levels and improve 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 one of the mechanisms by which IVIG improves survival.

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 was defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. Despite significant advances in the treatment of sepsis in recent years, there is still high mortality due to cardiogenic shock and multiple organ failure. The in-hospital mortality of sepsis is greater than 10%. Septic shock, which causes particularly profound circulatory, cellular, and metabolic abnormalities, is associated with a greater risk of mortality than sepsis alone. In addition, sepsis can produce a general inflammatory response and the systemic release of pro-inflammatory cytokines, resulting in acute organ dysfunction, such as acute lung injury (ALI). ALI triggered by sepsis is particularly difficult to treat. Recently, cell apoptosis has been reported to play a critical role in the progression of ALI.

Interestingly, a recent study has shown that IVIG ameliorates the acute brain dysfunction associated with sepsis by reducing apoptotic cell death in neuronal cells. However, no study has examined the relationship between IVIG and apoptosis in alveolar epithelial cells.

Immunoglobulin is a Y-shaped protein that is produced mainly by plasma cells that 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 have indicated that IVIG is associated with anti-inflammatory responses to cytokine-related inflammation. Physicians use intravenous immunoglobulin (IVIG) to treat several disease, such as severe infection, agammaglobulinemia, Kawasaki disease and idiopathic thrombocytopenic purpura (ITP). Kawasaki disease and ITP are treated with 2000 mg/kg IVIG, and such a high-dose of IVIG has been shown to improve survival in animal model of sepsis. Also in the clinic, Turgeon et al. have reported a meta-analysis showing high-dose IVIG is effective for treating sepsis.

The administration of low-dose IVIG (5000 mg/day for 3 days; approximately total 200-300 mg/kg) is widely used as an adjunctive treatment for patients with sepsis in Asian counties, including Japan and Korea.

Cecum ligation and puncture (CLP) is a common method used to create a model of sepsis in animals. This method produces a septic state by causing a polybacterial abdominal infection. The CLP animal model has been used frequently in several previous IVIG studies.

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 of IVIG we also measured the plasma levels of cytokines, assessed
lung tissue histopathology, and assessed apoptosis in alveolar epithelial cells by terminal
deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining.

Materials and Methods

Animals and ethics statement

Male C57BL/6N mice, 8-10-weeks old and weighing 20–25 g (Sankyo Labo Service Corporation, Tokyo, Japan) were used for this study. Mice were housed in cages under specific pathogen-free conditions. Humane endpoint was defined as the loss of ability to ambulate and/or as the labored respiration. The mice were checked every 6 - 8 hours at least and regarded to be dead in the analysis for survival study when they exhibited these symptoms, and euthanized immediately. All procedures and animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals and the local 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 the researchers involved in this study were skilled who received lectures on animal experiments ethics.
We created a model of intra-abdominal infection and sepsis using the CLP procedure. Mice were anesthetized with 2% isoflurane, and a 15 mm midline incision was then 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. Following this, the cecum was punctured one single time crossing both intestine walls with an 18 gauge needle. 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**

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) the IVIG 100 group that received a 100 mg/kg dose of human immunoglobulin before CLP and 4) the IVIG 400 group that received a 400 mg/kg dose of human immunoglobulin before CLP. Each animal
received their 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 the pooled human plasma obtained from multiple individuals.

**Survival study**

For 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 have shown that the plasma level of cytokines peak at 2 to 6 hours after CLP\(^ {17,18}\). A previous study also showed that ALI and acute kidney injury (AKI) could be established within 4 hours in an experimental sepsis model\(^ {19}\). Accordingly, following anesthesia, blood samples were collected by cardiac puncture with heparinized syringes 4 hours after operation. After centrifugation 1500 \(g\) for 17 min, the plasma was stored at -80°C. Plasma levels of Interleukin (IL)-6 and tumor necrosis factor (TNF)-\(\alpha\) were measured by enzyme-linked immunosorbent assay (ELISA) with a Mouse IL-6 DuoSet
ELISA DY406 and a Mouse TNF-alpha DuoSet ELISA DY410 (R&D Systems, Minneapolis, MN, US), respectively.

**Wet-to-dry weight (W/D) ratio**

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

**Lung histopathological analysis**

Histopathological changes in the lung were examined in each of the treatment groups 4 hours after surgery. Following the same procedure described above, left lungs were fixed in 4% paraformaldehyde at 4°C. The lung sections were then stained with hematoxylin and eosin (H&E) and histological changes in the lung tissue were observed under a light microscope. A blinded pathologist scored the tissues on the following criteria (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.

**TUNEL assay**

Following removal of the left lung 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 a Kaplan-Meier analysis. A log-rank test was used to analyze the survival rate. All data were expressed as mean ± standard error. Differences were evaluated using an unpaired Student’s t test between two group and one-way ANOVA and Tukey’s Post Hoc tests for multiple comparisons. For the histopathological study, a nonparametric Kruskal-Wallis test was performed. A $p < 0.05$ was considered statistically significant.
Results

Mortality

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

Effect of IVIG on the plasma levels of IL-6 and TNF-α

The plasma levels of IL-6 were increased in all mice in the CLP groups ($n = 6-8/group$) at 4 hours after the CLP operation. The plasma levels of IL-6 were very low in the Sham group (310.9 ± 9.4 pg/mL). In both the IVIG 100 and IVIG 400 groups, the plasma levels of IL-6 were significantly lower compared to the Vehicle group (both $p < 0.01$). The IL-6 levels in the IVIG 100 group, the IVIG 400 group, and the Vehicle group were
3692.2 ± 484.8, 2730.9 ± 556.3, and 10530.0 ± 1600.4 pg/mL, respectively (Fig 2A).

The plasma levels of TNF-α were also increased in all mice in the CLP groups. In both the IVIG 100 and IVIG 400 groups, the plasma levels of TNF-α were significantly lower compared with the Vehicle group (p < 0.01). There was no clear significant difference between the IVIG 100 group and the IVIG 400 group. The TNF-α levels in the Sham group, the IVIG 100 group, the IVIG 400 group, and the Vehicle group 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 the lungs in the CLP groups (n = 7-8/group) were significantly higher than the Sham group. The W/D ratios of the IVIG groups (both 100 and 400) were however statistically significantly lower than the Vehicle group (Fig 3).

Histological evaluation of the lungs

Four hours after the operation, a clear inflammatory reaction could be seen in the lungs as characterized by the presence of a cellular infiltrate in the interstitium and air spaces.
of the lung (Fig 4). Inflammatory cell infiltration, interstitial edema, vascular congestion, and hemorrhages were very evident in the Vehicle group compared with the Sham group. For the IVIG 400 group, the lung injury scores for four different parameters, namely congestion, edema, inflammation, and hemorrhage, were significantly lower than the Vehicle group. In contrast, there were no clear significant differences in these same lung injury scores between the IVIG 100 group and the Vehicle group (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 4 hours after surgery. Numerous TUNEL-positive cells were found in the Vehicle group (21.7 ± 2.7/section). On the other hand, there were very few TUNEL-positive cells in the Sham group (4.8 ± 0.9/section). In the IVIG 400 group (10.3 ± 1.5/section), the number of TUNEL-positive cells was significantly reduced compared with the Vehicle group. In contrast, the number of TUNEL-positive cells in the IVIG 100 group (19.6 ± 3.2/section) was not significantly different compared from that in the Vehicle group (Fig 7 and Fig 8).
Discussion

These data clearly show that both 100 mg/kg IVIG and 400 mg/kg IVIG significantly improve survival and reduce the plasma levels of IL-6 and TNF-α. On the other hand, ALI and apoptosis of alveolar epithelial cells were found to be improved only with 400 mg/kg IVIG.

IVIG has been used to treat sepsis, but previous clinical studies have indicated that there are differences in the efficacy of immunoglobulin preparations in sepsis patients. Thus, its benefit remains unclear \(^{21-25}\). In the study by Werdan et al., IVIG therapy was ineffective in treating septic shock patients \(^{26}\), whereas a meta-analysis study by Laupland et al. suggested that IVIG might reduce the mortality rate of adults with septic shock \(^{22}\).

IVIG has several theoretical advantages in the treatment of patients with sepsis. These advantages include pathogen recognition, pathogen clearance, and toxin scavenging. IVIG preparations may also have beneficial effects on the host response to infections \(^{21,27}\). Other studies have demonstrated its mechanism of action includes the stimulation of Fc receptor-mediated antibiotic-dependent cellular cytotoxicity,
neutralization of viruses and toxins, suppression of inflammatory cytokine activity,

promotion of complement-mediated bacteriolysis, and opsonization of targets to

promote phagocytosis. The Fab-dependent effects include neutralization of bacteria,

neutralization of bacterial toxins, neutralization of bacterial superantigens, suppression

of cytokines produced by immune cells, and autoantibodies. Previous studies have

also shown IVIG has an anti-inflammatory effect. Thus, immunoglobulins

could theoretically improve survival in our mouse sepsis model not only through direct

antibacterial effects but also by exerting anti-inflammatory effects.

Hagiwara et al. and Yoshikawa et al. have suggested that immunoglobulins

improve survival in animal sepsis models but only at high doses (over 1000mg). However, in our study, we observed that both 100 mg/kg and 400 mg/kg IVIG improved

the survival rate. Furthermore, there were no significant differences between the 100

mg/kg and 400 mg/kg doses. Therefore, IVIG, even in the absence of antibiotics, can

improve the outcome in a mouse model of sepsis.

The data in the present study also demonstrated that IVIG treatment reduces

the levels of two pro-inflammatory cytokines (TNF-α, and IL-6). This findings is
consistent with previous studies\textsuperscript{10,11,34}. In our study, the degree of cytokine reduction was not necessarily dependent on the IVIG dose. In the previous studies, the plasma levels of the high-mobility group box chromosomal protein 1 (HMGB1), as a late mediator of lethal systemic inflammation, were also reduced by IVIG treatment\textsuperscript{10,11,34}.

In a study by Yang et al., treatment with anti-HMGB1 antibodies beginning 24 h after CLP surgery significantly increased the survival rate of CLP-induced septic mice. This study concludes that HMGB1 was indeed an important mediator under septic conditions, but less so, in the case of septic shock, where TNF-\(\alpha\) plays a major role\textsuperscript{35}. Thus, it appears that a reduction in pro-inflammatory cytokines through the use of IVIG contributes to an improvement in the prognosis of 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 the onset of ALI\textsuperscript{36}. The pathological processes in ALI are mainly caused by neutrophil- and platelet-dependent damage to the endothelial and epithelial barriers of the lung. This causes a fibrin-rich edema fluid, red blood cells and neutrophils to enter the alveoli, and this influx leads to the inactivation of surfactant. These changes disrupt lung tissue thereby reducing lung function\textsuperscript{37,38}. Inflammation plays an important role during injury
and repair in ALI \(^{39}\). Pro-inflammatory cytokines, such as IL-6 and TNF-\(\alpha\), are involved in this complex systemic inflammatory response \(^{40,41}\). In particular, TNF-\(\alpha\) induces the production of other inflammatory cytokines, promotes migration and adhesion of neutrophils to endothelial cells \(^{42}\), and causes apoptosis of alveolar epithelial cells \(^{6,43}\).

The W/D ratio was also higher in the CLP group than in the IVIG groups. IVIG treatment significantly reduces the lung W/D weight ratio and the amount of excess lung fluid in experimental ALI. IVIG treatment therefore improves the lung edema seen in septic mice. On the other hand, using a histopathological assessment, we demonstrated that only treatment with 400 mg/kg IVIG reduced ALI. The reasons for the differences between the W/D ratio data and histopathological data are not known but will require further study.

Both clinically and experimentally, in addition to acute lung injury, severe sepsis is known to cause the failure of other organs, especially the kidney dysfunction. Currently, there is growing interest in the potential cross talk that may exist between injured organs, particularly in regard to the relationship between acute kidney injury (AKI) and ALI \(^{44,45}\). In this experiment, IVIG was found to contribute to improved
survival even in the 100 mg/kg IVIG group. On the other hand, from the experiments examining lung lesions, in particular the histopathological study, it appeared that ALI is improved in a dose-dependent manner by IVIG treatment, suggesting that IVIG also acts on organs other than the lung.

This study is the first assess the effects of IVIG on the apoptosis of alveolar epithelial cells in an animal sepsis model. A previous study demonstrated that sepsis could cause the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is involved in the process of apoptosis in ALI. In addition, Murakami et al. showed that IVIG suppressed activation of NF-κB. By these mechanisms, we seem that IVIG suppresses apoptosis of alveolar epithelial cells.

Compared to the Vehicle group, the number of apoptotic cells was not significantly different in the IVIG 100 group but was significantly reduced in the IVIG 400 group. This result suggests that IVIG suppresses apoptosis in alveolar epithelial cells in a dose-dependent manner.

In this study, IVIG, at either 100 mg/kg or 400 mg/kg, was also shown to
improve survival and the plasma levels of the pro-inflammatory cytokines IL-6 and TNF-α. On the other hand, ALI caused by sepsis was shown to be improved histologically, but only at 400 mg/kg IVIG. Most likely this is due to the suppression of apoptosis by 400 mg/kg IVIG.

It is interesting to note that 100 mg kg IVIG improved survival despite not suppressing ALI. In a study by Bhargava et al., TNF-α was shown to play a key role in pro-inflammatory cytokine production, ALI and AKI occur together early in the course of sepsis in an animal sepsis model. When an anti-TNF-α antibody was administered to these mice with induced sepsis, the serum levels and functional activities of IL-6 and TNF-α decreased, and renal function was improved. This suggests that a reduction in pro-inflammatory cytokines can play an important role in improving renal function. As mentioned above, IVIG has been shown to suppress the acute brain dysfunction associated with sepsis by reducing apoptotic cell death in neuronal cell.

Our experimental results suggest that IVIG reduces blood cytokines levels and reduces mortality arising from organ dysfunction in organs other than lung, perhaps the kidney or central nerve system.
Further studies are needed to understand the mechanisms underlying the action of IVIG and appropriate dose of immunoglobulin to treat sepsis.

Conclusion

In the present study, we show that IVIG can improve the survival of mice with induced sepsis. We also demonstrated that IVIG reduces the level of inflammatory cytokines in the plasma and also improves ALI and apoptosis in alveolar epithelial cells. Inhibition of apoptosis in alveolar epithelial cells appears to be one mechanism by which IVIG improves survival.

Acknowledgments

The authors thank Dr. Masatoku Arai, Professor Hisashi Matsumoto and Dr. Tomohiko Masuno for thoughtful comments on this research.

Conflict of Interest: The authors declare that they have no conflicts of interest.

References

1. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus
Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA*. 2016;315(8):801-810.

2. Matsuda A, Jacob A, Wu R, et al. Novel therapeutic targets for sepsis: regulation of exaggerated inflammatory responses. *J Nippon Med Sch*. 2012;79(1):4-18.

3. Hagel S, Ludewig K, Frosinski J, et al. Effectiveness of a hospital-wide educational programme for infection control to reduce the rate of health-care associated infections and related sepsis (ALERTS)—methods and interim results. *Dtsch Med Wochenschr*. 2013;138(34-35):1717-1722.

4. Seymour CW, Rea TD, Kahn JM, Walkey AJ, Yealy DM, Angus DC. Severe sepsis in pre-hospital emergency care: analysis of incidence, care, and outcome. *Am J Respir Crit Care Med*. 2012;186(12):1264-1271.

5. Andrews P, Azoulay E, Antonelli M, et al. Year in review in intensive care medicine, 2004. I. Respiratory failure, infection, and sepsis. *Intensive Care Med*. 2005;31(1):28-40.

6. Beasley MB. The pathologist's approach to acute lung injury. *Arch Pathol Lab Med*. 2010;134(5):719-727.

7. Esen F, Ozcan PE, Tuzun E, Boone MD. Mechanisms of action of intravenous immunoglobulin in septic encephalopathy. *Rev Neurosci*. 2018;29(4):417-423.

8. Esen F, Orhun G, Ozcan PE, et al. Neuroprotective effects of intravenous immunoglobulin are mediated through inhibition of complement activation and apoptosis in a rat model of sepsis. *Intensive care medicine experimental*. 2017;5(1):1.

9. Abe Y. Therapeutic application of intravenous human natural immunoglobulin preparation. *Front Biosci*. 1996;1:e26-33.

10. Hagiwara S, Iwasaka H, Hasegawa A, Asai N, Noguchi T. High-dose intravenous immunoglobulin G improves systemic inflammation in a rat model of CLP-induced sepsis. *Intensive Care Med*. 2008;34(10):1812-1819.

11. Yoshikawa T, Takeuchi H, Suda K, et al. High-dose immunoglobulin preparations improve survival in a CLP-induced rat model of sepsis. *Langenbeck's archives of surgery*. 2012;397(3):457-465.

12. Turgeon AF, Hutton B, Fergusson DA, et al. Meta-analysis: intravenous immunoglobulin in critically ill adult patients with sepsis. *Ann Intern Med*. 2007;146(3):193-203.

13. Nishida O, Ogura H, Egí M, et al. The Japanese Clinical Practice Guidelines for Management of Sepsis and Septic Shock 2016 (J-SSCG 2016). *Acute Med Surg*. 2018;5(1):3-89.

14. McCuskey RS, Nishida J, McDonnell D, Baker GL, Urbaschek R, Urbaschek B. Effect of immunoglobulin G on the hepatic microvascular inflammatory response during sepsis. *Shock (Augusta, Ga)*. 1996;5(1):28-33.
15. Nishida J, Ekataksin W, McDonnell D, Urbaschek R, Urbaschek B, McCuskey RS. Ethanol exacerbates hepatic microvascular dysfunction, endotoxemia, and lethality in septic mice. *Shock (Augusta, Ga).* 1994;1(6):413-418.

16. Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock—a review of laboratory models and a proposal. *J Surg Res.* 1980;29(2):189-201.

17. Eyenga P, Roussel D, Morel J, et al. Time course of liver mitochondrial function and intrinsic changes in oxidative phosphorylation in a rat model of sepsis. *Intensive care medicine experimental.* 2018;6(1):31.

18. Seemann S, Zohles F, Lupp A. Comprehensive comparison of three different animal models for systemic inflammation. *J Biomed Sci.* 2017;24(1):60.

19. Bhargava R, Altmann CJ, Andres-Hernando A, et al. Acute lung injury and acute kidney injury are established by four hours in experimental sepsis and are improved with pre, but not post, sepsis administration of TNF-alpha antibodies. *PLoS One.* 2013;8(11):e79037.

20. Murakami K, McGuire R, Cox RA, et al. Heparin nebulization attenuates acute lung injury in sepsis following smoke inhalation in sheep. *Shock (Augusta, Ga).* 2002;18(3):236-241.

21. Di Rosa R, Pietrosanti M, Luzi G, Salemi S, D’Amelio R. Polyclonal intravenous immunoglobulin: an important additional strategy in sepsis? *Eur J Intern Med.* 2014;25(6):511-516.

22. Laupland KB, Kirkpatrick AW, Delaney A. Polyclonal intravenous immunoglobulin for the treatment of severe sepsis and septic shock in critically ill adults: a systematic review and meta-analysis. *Crit Care Med.* 2007;35(12):2686-2692.

23. Neilson AR, Burchardi H, Schneider H. Cost-effectiveness of immunoglobulin M-enriched immunoglobulin (Pentaglobin) in the treatment of severe sepsis and septic shock. *J Crit Care.* 2005;20(3):239-249.

24. Ohlsson A, Lacy JB. Intravenous immunoglobulin for suspected or proven infection in neonates. *Cochrane Database Syst Rev.* 2013(7):CD001239.

25. Pildal J, Gotzsche PC. Polyclonal immunoglobulin for treatment of bacterial sepsis: a systematic review. *Clin Infect Dis.* 2004;39(1):38-46.

26. Werdan K, Pilz G, Bujdosó O, et al. Score-based immunoglobulin therapy of patients with sepsis: the SBI TS study. *Crit Care Med.* 2007;35(12):2693-2701.

27. Shankar-Hari M, Spencer J, Sewell WA, Rowan KM, Singer M. Bench-to-bedside review: Immunoglobulin therapy for sepsis: biological plausibility from a critical care perspective. *Critical care (London, England).* 2012;16(2):206.

28. Negi VS, Elluru S, Siberil S, et al. Intravenous immunoglobulin: an update on the clinical use and mechanisms of action. *J Clin Immunol.* 2007;27(3):233-245.
29. Bruhns P, Samuelsson A, Pollard JW, Ravetch JV. Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. *Immunity*. 2003;18(4):573-581.

30. Fabrizio K, Groner A, Boes M, Pirofski LA. A human monoclonal immunoglobulin M reduces bacteremia and inflammation in a mouse model of systemic pneumococcal infection. *Clin Vaccine Immunol.* 2007;14(4):382-390.

31. Hoffman JN, Fertmann JM, Vollmar B, Laschke MW, Jauch KW, Menger MD. Immunoglobulin M-enriched human intravenous immunoglobulins reduce leukocyte-endothelial cell interactions and attenuate microvascular perfusion failure in normotensive endotoxemia. *Shock (Augusta, Ga).* 2008;29(1):133-139.

32. Galeotti C, Hegde P, Das M, et al. Heme oxygenase-1 is dispensable for the anti-inflammatory activity of intravenous immunoglobulin. *Sci Rep.* 2016;6:19592.

33. Watanabe M, Uchida K, Nakagaki K, et al. High avidity cytokine autoantibodies in health and disease: pathogenesis and mechanisms. *Cytokine Growth Factor Rev.* 2010;21(4):263-273.

34. Murakami K, Suzuki C, Kobayashi F, et al. Intravenous immunoglobulin preparation attenuates LPS-induced production of pro-inflammatory cytokines in human monocytic cells by modulating TLR4-mediated signaling pathways. *Naunyn Schmiedebergs Arch Pharmacol.* 2012;385(9):891-898.

35. Yang H, Ochani M, Li J, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A.* 2004;101(1):296-301.

36. Ware LB, Matthay MA. The Acute Respiratory Distress Syndrome. *New England Journal of Medicine.* 2000;342(18):1334-1349.

37. Matthay MA, Zemans RL. The acute respiratory distress syndrome: pathogenesis and treatment. *Annu Rev Pathol.* 2011;6:147-163.

38. Wheeler AP, Bernard GR. Acute lung injury and the acute respiratory distress syndrome: a clinical review. *Lancet.* 2007;369(9572):1553-1564.

39. Curley G, Hayes M, Laffey JG. Can 'permissive' hypercapnia modulate the severity of sepsis-induced ALI/ARDS? *Critical care (London, England).* 2011;15(2):212.

40. Riedemann NC, Neff TA, Guo RF, et al. Protective effects of IL-6 blockade in sepsis are linked to reduced C5a receptor expression. *J Immunol.* 2003;170(1):503-507.

41. Simpson SQ, Casey LC. Role of tumor necrosis factor in sepsis and acute lung injury. *Crit Care Clin.* 1989;5(1):27-47.

42. Galani V, Tatsaki E, Bai M, et al. The role of apoptosis in the pathophysiology of Acute Respiratory Distress Syndrome (ARDS): an up-to-date cell-specific review. *Pathol Res Pract.* 2010;206(3):145-150.
43. Ji Q, Sun Z, Yang Z, et al. Protective effect of ginsenoside Rg1 on LPS-induced apoptosis of lung epithelial cells. *Mol Immunol.* 2018.

44. Faubel S. Pulmonary complications after acute kidney injury. *Adv Chronic Kidney Dis.* 2008;15(3):284-296.

45. Singbartl K, Bishop JV, Wen X, et al. Differential effects of kidney-lung cross-talk during acute kidney injury and bacterial pneumonia. *Kidney Int.* 2011;80(6):633-644.

46. Lin WC, Chen CW, Huang YW, et al. Kallistatin protects against sepsis-related acute lung injury via inhibiting inflammation and apoptosis. *Sci Rep.* 2015;5:12463.
Mice in the IVIG treated groups had significantly improved survival compared to the Vehicle group (n= 10/group). The mortality rate was significantly lower in the IVIG 100 group compared with the Vehicle group (p = 0.018). Similarly, the mortality rate was significantly lower in the IVIG 400 group compared with the Vehicle group (p = 0.005). In the Sham group (laparotomy only, no CLP), all the mice survived. There were no significant differences between the IVIG 100 and the IVIG 400 (p = 0.279). * p < 0.05 vs Vehicle; † p < 0.01 vs Vehicle.

(A) Plasma levels of IL-6. n = 7 in the Sham, the Vehicle and the IVIG 100 groups, and n= 8 in the IVIG 400 group. Sham and Vehicle groups In both the IVIG 100 and 400 groups, IVIG significantly reduced the plasma levels of IL-6 compared with the Vehicle group. There was no statistically significant difference between the IVIG 100 and 400 groups. The levels of IL-6 were very low in the Sham group. (B) Plasma level of TNF-α. n = 7 in the Sham and the IVIG 100 groups, n= 6 in the Vehicle group and n = 8 in the IVIG 400 group. The TNF-α levels were significantly lower in both the IVIG treatment groups compared with the Vehicle group. The TNF-α levels in the Sham group were...
very low. The data are expressed as the mean ± SE. † p < 0.01 vs Vehicle.

**Fig 3. Effect of IVIG on W/D ratio of sepsis-induced mice.**
The lung W/D ratio was examined after 4 h for each group (n = 8 in the IVIG groups, n = 7 in the Sham and Vehicle groups). The data are expressed as the mean ± SE. The W/D ratio in the IVIG 100 and 400 groups were significantly lower than the Vehicle group (p = 0.046 and < 0.001, respectively). * p < 0.05 vs Vehicle; † p < 0.01 vs Vehicle.

**Fig 4. Representative histopathological findings in the lung.**
Lung tissue sections were stained with hematoxylin and eosin. (A) Sham, (B) CLP + Vehicle, (C) CLP + IVIG 100, (D) CLP + IVIG 400; Scale bar = 50 μm

**Fig 5. Lung injury scores for four different parameters of lung injury.**
Lung injury scores were examined in each group 4 h after CLP (n = 8 in the IVIG groups, n = 7 in the Sham and Vehicle groups). The data are expressed as the mean ± SE. In the IVIG 400 group, all four individual lung injury parameters assessed were significantly lower compared with 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 4 h after CLP (n = 8 in the IVIG groups, n = 7 in the Sham and Vehicle groups). The data are expressed as the mean ± SE. Similar to the results seen for each individual parameter (Fig 5), the total lung injury score was significantly lower in the IVIG 400 group compared to the Vehicle group (\( p = 0.014 \)). * \( p < 0.05 \) vs Vehicle.

**Fig 7. Representative TUNEL stain findings in the lung.**

Lung tissue sections stained with TUNEL to identify apoptotic cells. Brown stained cells were called as 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 the TUNEL staining performed 4 h after surgery in each group (n = 8 in the IVIG groups, n = 7 in the Vehicle group, and n = 4 in the Sham group). The data are expressed as the mean ± SE.
The number of cells undergoing apoptosis was significantly decreased in the IVIG 400 group compared with the Vehicle group ($p = 0.014$). There was no significant difference between the IVIG 100 group and the Vehicle group in the number of cells undergoing apoptosis. *$p < 0.05$ vs Vehicle.
Fig. 1

The figure shows a survival rate over time after CLP (Cecal Ligation and Perforation). The survival rate is plotted against hours after CLP. Different treatments are represented by different symbols and line styles:

- **Sham**: Open circle (○)
- **CLP + 400 mg/kg IVIG**: Black circle (●)
- **CLP + 100 mg/kg IVIG**: White square (□)
- **CLP + Vehicle**: Black square (■)

The survival rate decreases over time, with some groups showing higher survival rates than others. The figure includes symbols indicating significant differences (*) and trends marked with a dagger (†).
Fig. 2
Fig. 3
Fig. 4
Fig. 5

The bar chart shows the lung injury score for different conditions and treatments:

- **Sham**
- **CLP+Vehicle**
- **CLP+IVIG 100**
- **CLP+IVIG 400**

The conditions are categorized as:

- **congestion**
- **edema**
- **inflammation**
- **hemorrhage**

Significance is indicated by asterisks (*) on the bars.
Fig. 6

Bar graph showing the total lung injury score in different groups: Sham, Vehicle, IVIG 100, and IVIG 400. The Vehicle group has the highest total lung injury score, followed by IVIG 100, and Sham. IVIG 400 shows a lower score compared to the other groups.
Fig. 7
Fig. 8

The bar chart illustrates the number of TUNEL-positive cells across different treatment groups: Sham, Vehicle, IVIG 100, and IVIG 400. The Vehicle group shows the highest number of TUNEL-positive cells, followed by IVIG 100 and Sham, with IVIG 400 showing the least. The asterisk (*) indicates a statistically significant difference in the IVIG 400 group compared to the other groups.