Distinct pathophysiologic mechanisms of septic acute kidney injury: Role of immune suppression and renal tubular cell apoptosis in murine model of septic acute kidney injury

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Objective: **Sepsis is the most common cause of acute kidney injury in critically ill patients; however, the mechanisms leading to acute kidney injury in sepsis remain elusive. Although sepsis has been considered an excessive systemic inflammatory response, clinical trials that inhibit inflammation have been shown to have no effect. The purpose of this study was to examine the pathophysiology of septic acute kidney injury focusing on immune responses and renal tubular cell apoptosis by providing an on-site quantitative comparison between septic- and ischemia/reperfusion-induced acute kidney injury.**

Design: **Twenty-four hours after cecal ligation and puncture or ischemia/reperfusion injury, biochemical, histologic, and cytokine changes were compared in C57BL/6 mice. Apoptosis was assessed, and the effect of caspase 3 inhibition on renal function was also examined. The percentage of regulatory T cells and the effect of depletion were determined and compared with ischemia/reperfusion-induced acute kidney injury. The effect of interleukin-10 blocking was also compared.**

Measurements and Main Results: **Despite comparable renal dysfunction, acute tubular necrosis or inflammation was minimal in septic kidneys. However, tubular cell apoptosis was prominent, and caspase 3 activity was positively correlated with renal dysfunction. A decrease in apoptosis by caspase 3 inhibitor resulted in attenuation of renal dysfunction. In assessment of systemic immunity, septic acute kidney injury was associated with an increase in interleukin-10, and also showed massive immune cell apoptosis with increased regulatory T cells. In contrast to ischemia/reperfusion injury in which depletion of regulatory T cells aggravated renal injury, depletion of regulatory T cells before cecal ligation and puncture resulted in renoprotection. In addition, blocking interleukin-10 rescued septic mice from the development of acute kidney injury, whereas it had no effect in ischemia/reperfusion injury.**

Conclusions: **Pathogenesis of septic acute kidney injury is thought to be different from that of ischemia/reperfusion-induced acute kidney injury. Our data showed a link between apoptosis, immune suppression, and the development of acute kidney injury during sepsis and suggest that strategies targeting apoptosis or enhancing immunity might be a potential therapeutic strategy for septic acute kidney injury. (Crit Care Med 2012; 40:2997–3006)**

Key Words: acute kidney injury; apoptosis; immune suppression; ischemia/reperfusion injury; regulatory T cells; sepsis

Sepsis is the most common cause of acute kidney injury (AKI) in critically ill patients (1–3), and the presence of AKI in these patients is known to be an independent risk factor for a worse outcome (4). However, the mechanisms leading to AKI in septic patients remain elusive, but are thought to be similar to prerenal azotemia, which is mediated largely by intense renal vasoconstriction (5). Sepsis has traditionally been considered an excessive systemic inflammatory response to invading pathogens; however, several clinical trials have shown that suppression of inflammation has no effect on sepsis-induced AKI (6, 7). Previous study demonstrated the occurrence of compensated anti-inflammatory response syndrome follows systemic inflammatory response syndrome (8), and according to recent observations, mortality from sepsis or sepsis-induced organ dysfunction might be associated with paradoxical immune suppression, characterized by unregulated apoptosis of immune cells (9–12).

$CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) are important in suppression of immune responses or maintenance of immune tolerance. These cells were recently shown to have an important role in reducing innate immune responses and inflammation after ischemia/reperfusion (I/R) injury (13, 14). However, the role of these cells in septic AKI has never been examined.

Apoptosis plays an important role in health and diseases. It also contributes to kidney dysfunction in I/R or nephrotoxin-induced AKI (15–17). However, the role of renal cell apoptosis in the development of septic AKI has never been clearly defined.

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In this study, we used a cecal ligation and puncture (CLP) model to examine the pathophysiology of septic AKI, by focusing on the systemic immune response as well as kidney inflammation, apoptosis in septic mice. The role of immune and/or renal tubular cell apoptosis on septic AKI was tested by pretreating mice with a caspase 3 inhibitor. By comparing septic mice with I/R mice in which activation of innate immune responses is known to contribute to kidney injury (18, 19), we examined the effect of paradoxical immune suppression on the development of septic AKI. The role of immune suppression on septic AKI was also tested using a Treg depletion strategy or blocking the anti-inflammatory cytokine, interleukin (IL)-10.

MATERIALS AND METHODS

Animal Model

Approval for the study was obtained from the Korea University Institutional Animal Care and Use Committee. We used the CLP model to induce septic AKI in male C57BL/6 mice (6–7 wks old, weighing 20–25 g; Charles River Korea, Seoul, Korea) as previously described. To induce ischemic AKI, C57BL/6 mice were subjected to bilateral renal pedicle clamping for 32 mins, followed by reperfusion (I/R injury) and a sham operation was performed. Twenty-four hours after surgery, blood was collected, and the kidneys and spleens were removed for various molecular and flow cytometric analyses. Separately, the time course of plasma cytokines, creatinine, and hemodynamics in sepsis were measured.

Drug Administration

Anti-mouse CD25 monoclonal antibody (PC61) was obtained from ascitic fluid produced by a PC61 hybridoma (American Type Culture Collection, Rockville, MD) in *nu/nu* mice (Charles River Korea, Seoul, Korea). After purification by a Prosep G Ig purification kit (Millipore, Bedford, MA), antibody (Ab) (0.3 mg/mice) was administered intraperitoneally to C57BL/6 mice 4 days before surgery to deplete Tregs. As a negative control, rat isotype-control antibody was used. Depletion of $CD4^+$ $CD25^+$ cells was confirmed by flow cytometric examination of the spleen (supplemental data, Supplemental Digital Content 1, http://links.lww.com/CCM/A505; Supplemental Digital Content 2, http://links.lww.com/CCM/A506; and Supplemental Digital Content 3, http://links.lww.com/CCM/A507). For inhibition of apoptosis, caspase 3 inhibitor (25 mg; Calbiochem, Darmstadt, Germany) was administered intraperitoneally 2 hrs before CLP surgery. For determination of the effect of inhibition of IL-10, IL-10 blocking Ab (50 μg; Pierce-Endogen, Rockford, IL) or isotype control IgG was administered intraperitoneally 6 hrs after CLP or I/R.

Blood Chemistries

Blood urea nitrogen, lactate dehydrogenase, alanine aminotransferase, and Cr were measured using a Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan).

Histologic Examination

Tubular injury was assessed in PAS-stained kidney tissues. For assessing apoptosis, the number of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling -positive tubular cells per high power field was counted in the cortex and outer medulla in a blinded fashion. For immunohistochemical detection of neutrophils or macrophages, formalin-fixed and paraffin-embedded kidney sections were stained with Gr-1 Ab (Sigma-Aldrich, St. Louis, MO) or F4/80 Ab (Serotec, Oxford, UK).

Quantification of Chemokines and Cytokines by Cytometric Bead Array

Quantification of various chemokines and cytokines in kidney tissues and plasma was done by cytometric bead array. A mouse inflammation kit (BD Bioscience, San Diego, CA) was used according to the manufacturers instructions to simultaneously detect mouse IL-12p70, tumor necrosis factor-α, interferon-γ, monocyte chemoattractant protein-1, IL-10, and IL-6 as previously described (14).

Measurement of Caspase-3 Activity

To determine the activity of caspase 3 kidney and spleen, we used the BD ApoAlert Caspase Colorimetric Assay Kit (BD Bioscience, Palo Alto, CA) according to manufacturers instructions.

Flow Cytometric Determination of Spleen Tregs and Kidney Leukocytes

To determine the effect of sepsis and kidney I/R injury on the systemic Treg population, mononuclear cells from the spleen were isolated, stained for 15 mins on ice with fluorochrome-labeled monoclonal antibodies (anti-mouse $CD4^+$-allophycocyanin and anti-mouse $CD25^+$-phycoerythrin; eBioscience, San Diego, CA) and analyzed using four-color flow cytometry (FACSCalibur; BD, San Jose, CA). For detection of kidney leukocytes, kidney single-cell suspensions were first incubated with anti-mouse CD45-fluorescein isothiocyanate Ab for 30 mins on ice. After washing, the cells were stained for 15 mins on ice with fluorochrome-labeled monoclonal antibodies against Gr-1-PE or F4/80-APC for detection of neutrophils or macrophages (anti-mouse CD45-fluorescein isothiocyanate, anti-mouse Gr-1-PE, anti-mouse F4/80-APC; eBioscience). Four-color fluorescence flow cytometric analyses were performed (FACSCalibur), and the data were analyzed with the FlowJo program (Treestar, Ashland, CA).

Statistical Analysis

Comparisons between groups were examined using Kruscal–Wallis test, Mann–Whitney test, and Spearman's correlation analysis by using SPSS 12.0 software (SPSS, Chicago, IL). Data are expressed as the means ± SE. Statistical significance was considered at a $p < .05$.

RESULTS

CLP Induced a Septic Shock and AKI

Our mouse model of CLP induced polymicrobial peritonitis with multiple organ dysfunction, including AKI and liver dysfunction (plasma creatinine 0.73 ± 0.065 mg/dL vs. 0.2 ± 0.05 mg/dL, alanine aminotransferase 1105 ± 882.6 IU/L vs. 24.66 ± 1.2 IU/L, CLP vs. sham). Table 1 summarizes the changes in hemodynamics. Hyperdynamic shock was evidenced by significant decrease in mean arterial pressure, increased heart rate, and fractional shortening of left ventricles. Creatinine level significantly increased at 24 hrs after CLP (Fig. 1*A*), and showed a positive correlation with plasma lactate dehydrogenase levels (Fig. 1*B*).

Direct Comparison Between Septic- and I/R-Induced AKI Showed That Septic AKI Is Associated With Paradoxical Immune Suppression

Septic Kidneys Are Characterized by Lack of Overt Tubular Damage and Sparse Inflammation. Despite a comparable level of functional impairment

Table 1. Hemodynamic changes in cecal ligation and puncture mice

Time	0 hrs	2 hrs	4 hrs	6 hrs	8 hrs	12 hrs	14 hrs	24 hrs
Mean arterial pressure (mm Hg)	89.1	75.6	57.5	54.3	56.8	55.7	55.6	50
Heart rate	348.1	392.5	411.2	408.5	419.2	392.8	404.3	369
Fractional shortening (%)	54.8	60.7	59.475					62.58

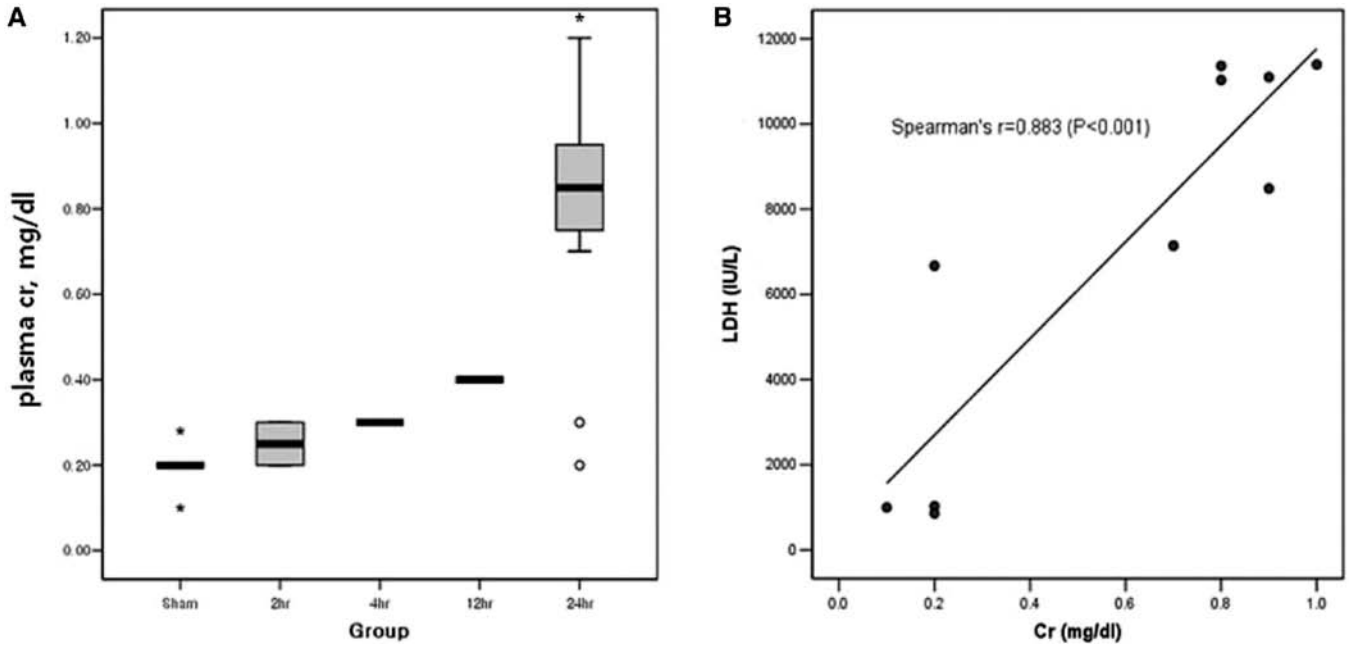

Figure 1. Cecal ligation and puncture (CLP)-induced acute kidney injury. *A*, Serial plasma creatinine was measured at 2 hrs (n = 2), 4 hrs (n = 2), 12 hrs (n = 2), and 24 hrs (n = 7) after CLP. Plasma creatinine reached to peak at 24 hrs, $*p < .05$ compared with sham. *B*, Lactate dehydrogenase (*LDH*) was measured 24 hrs after CLP. The plasma creatinine level had a significant positive correlation with the plasma LDH level. Values are expressed as the mean ± SEM (sham, n = 3; CLP, n = 7).

(0.73 ± 0.065 mg/dL vs. 0.78 ± 0.06 mg/dL, CLP vs. I/R), septic kidneys had a different morphology compared with I/R kidneys. Although typical acute tubular necrosis with tubular obstruction and brush border injury were evident in I/R-injured kidneys, septic kidneys were characterized by sparse vacuolar degeneration of tubular cells without overt tubular cell damage (data not shown) and were accompanied by sparse inflammatory cell infiltration. Gr-1 or F4/80-positive neutrophil or macrophage infiltration in septic kidneys were minimal, which is in contrast with I/R-injured kidneys (Fig. 2*A*). We confirmed the same findings using flow cytometric analysis of kidney leukocytes (Fig. 2*B*).

Development of septic AKI is associated with anti-inflammatory condition. To assess systemic and kidney inflammation, we measured multiple chemokines and cytokines in plasma and kidneys in septic- and I/R-injured mice at 2 hrs, 4 hrs, and 24 hrs following surgery. Proinflammatory chemokines and cytokines, including MCP-1 and IL-6, showed a curve forming parabola, increased at 2–4 hrs, and then decreased at 24 hrs. However, the anti-inflammatory cytokine, IL-10 increased relatively late after CLP (Fig. 3*A*). The time course of cytokines indicates that the cytokine balance inclined to anti-inflamamtory condition at 24 hrs after CLP, and this late increase in IL-10 was associated with development of AKI (Fig. 1*A* and Fig. 3*A*) in sepsis. However, in ischemic mice, systemic inflammatory/anti-inflammatory cytokines concentrations were significantly lower than those of septic mice, and delayed IL-10 peak observed in septic mice was not demonstrated (Fig. 3*A*). Similar findings were also observed in septic kidneys; the ratio of the increase in proinflammatory cytokines to anti-inflammatory IL-10 was much lower in septic AKI compared with I/R-injured kidneys in which IL-10 was not detectable at 24 hrs after insult (Fig. 3*B*).

Septic AKI is associated with a relative increase in the percentage of Tregs. We also measured the percentage of spleen Tregs and observed that the relative frequency of $CD4^+$ $CD25^+$ Tregs increased with near significance ($p = .056$) in septic mice compared with I/R-injured mice (Fig. 4).

Role of Renal Tubular Cell/Immune Cell Apoptosis in Septic AKI

We observed a substantial number of TUNEL-positive apoptotic cells in septic kidneys and spleens. Caspase 3 activity also increased in kidney and spleen tissue, and it had a significant positive correlation with the plasma Cr level (Fig. 5), suggesting that renal cell/immune cell apoptosis might contribute to kidney dysfunction during sepsis.

To confirm the role of apoptosis on kidney dysfunction in septic AKI, caspase 3 inhibitor was administered 2 hrs before CLP surgery. Caspase 3 inhibitor pretreatment resulted in a significant reduction in renal cell and/or immune cell apoptosis (Fig. 6*A*), and it was associated with a significant attenuation of functional kidney impairment (Fig. 6*B*).

Effect of Blocking Immune Suppression on Kidney Dysfunction in Sepsis

To determine the functional significance of increased Tregs, we used a Treg depletion strategy. Depletion of Tregs before CLP surgery resulted in marked attenuation of kidney dysfunction, whereas depletion of these cells before I/R injury worsened kidney dysfunction (Fig. 7*A*). In addition, to achieve better insight about the direct causal relationship between late increase of IL-10 and the development of AKI in sepsis, we administered an IL-10 blocking Ab and compared kidney function in I/R and septic mice. Although IL-10 blocking did not affect renal function in I/R-injured mice, it was protective in septic mice, suggesting that increased IL-10 in the compensated anti-inflammatory response syndrome period might

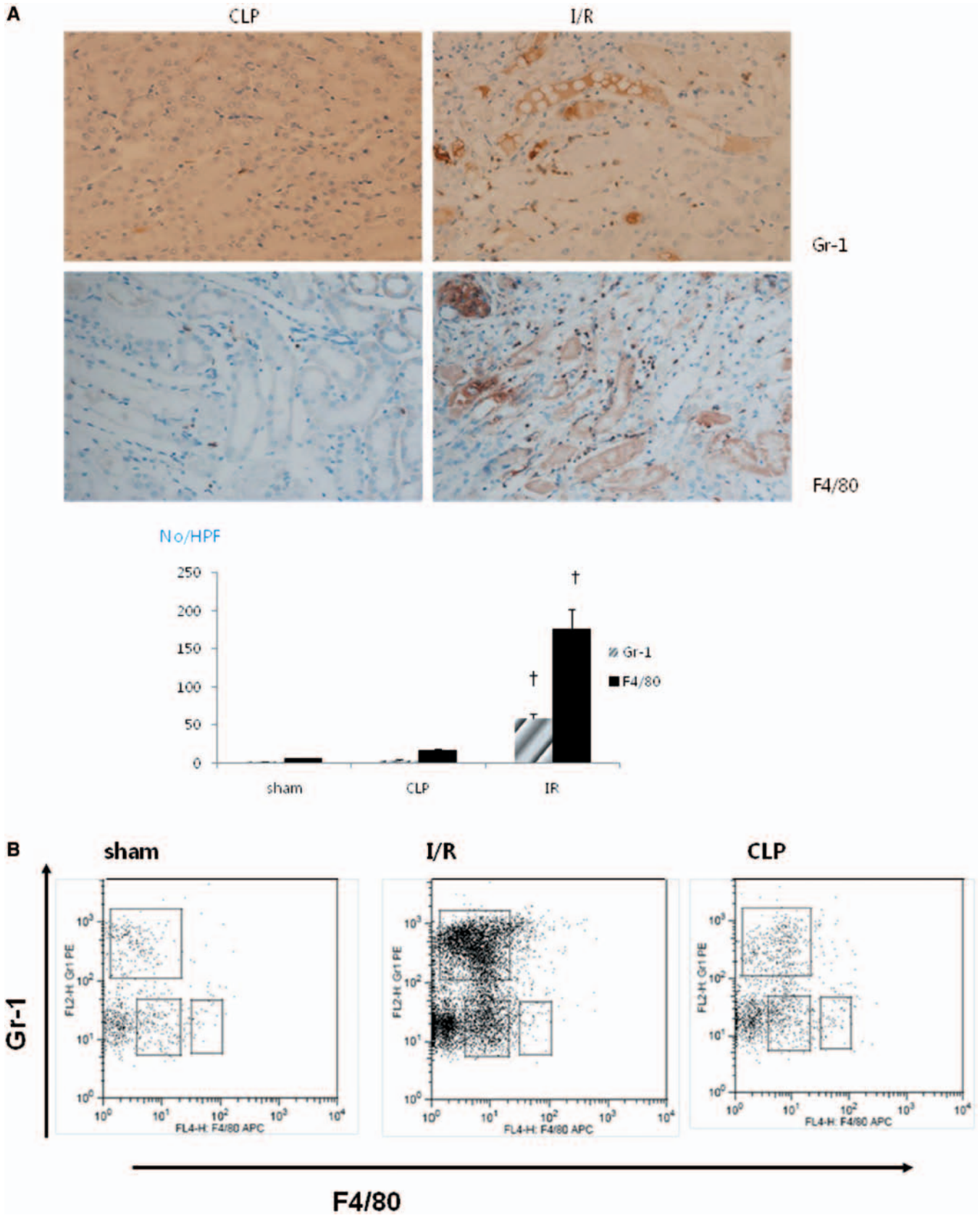

Figure 2. Tissue inflammation was not evident in septic acute kidney injury. For assessment of tissue inflammation, immunohistochemical detection of Gr-1-positive neutrophils (*upper panel*) or F4/80-positive macrophages (*lower panel*) in kidneys was performed. *A*, In contrast to ischemic kidneys (*right panel*), septic kidneys were characterized by lack of neutrophil or macrophages infiltration (*left panel*). (Gr-1 or F4/80, ×200, †$p < .05$, n = 4 mice/group). *B*, Flow cytometric analysis of kidney leukocytes also demonstrated that neither F4/80 positive macrophages nor Gr-1 positive neutrophils were detected in septic kidneys compared with a massive infiltration of these cells in ischemic kidneys (n = 4 mice/group). *CLP*, cecal ligation and puncture; *I/R*, ischemia/reperfusion; *HPF*, high power field.

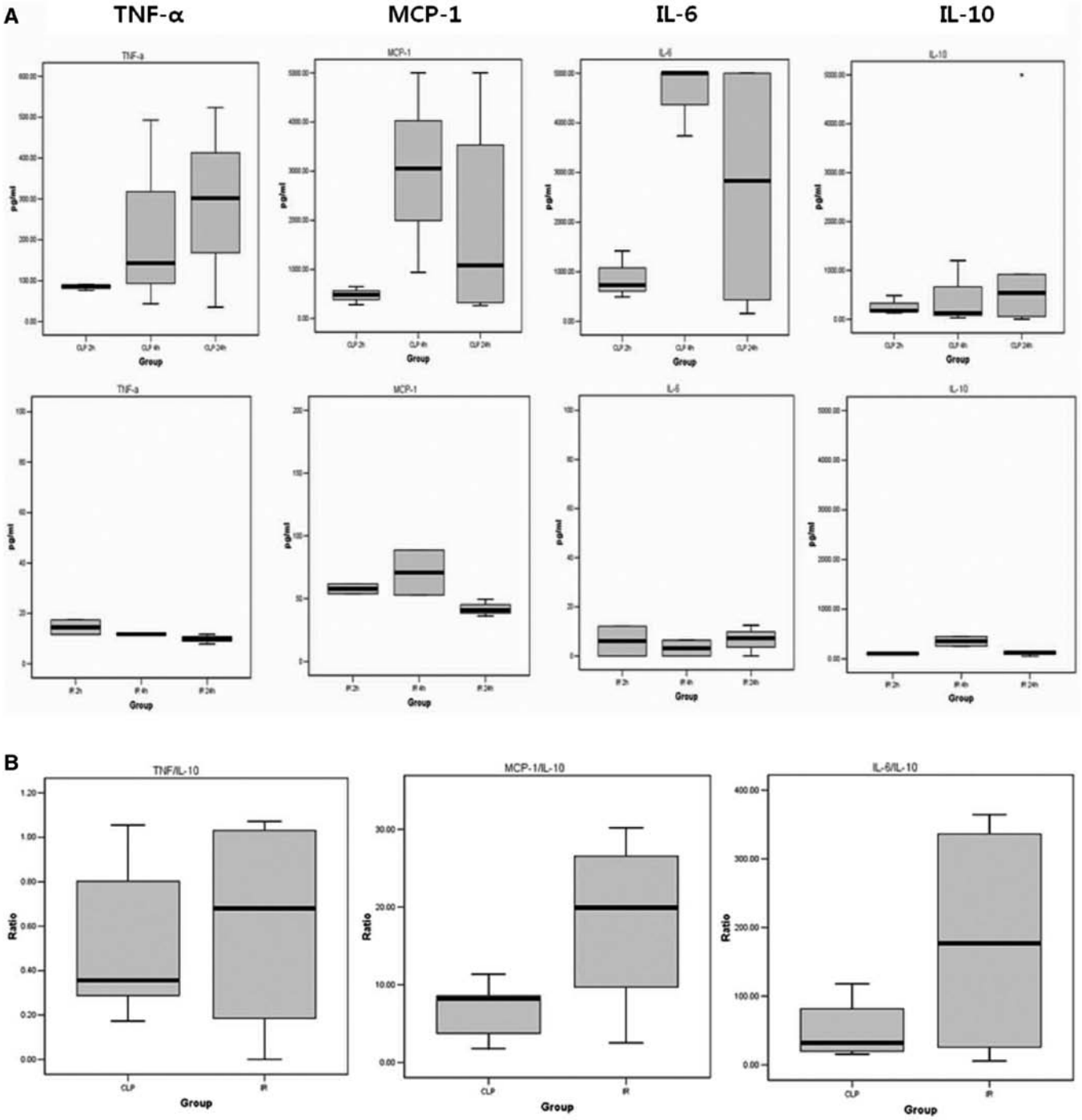

Figure 3. Chemokine and cytokines in septic and ischemic acute kidney injury. *A*, Mice subjected to cecal ligation and puncture (CLP) and ischemia/reperfusion (IR) injury were sacrificed at 2 hrs, 4 hrs, and 24 hrs after surgery, and the plasma levels of chemokine/cytokines were measured using cytometric bead array. Although in CLP mice, proinflamamtory monocyte chemoattractant protein-1 (*MCP-1*), interleukin (*IL*)-6 increased 2–4 hrs after CLP, IL-10 significantly increased late at 24hrs (n = 3 at 2 hrs, n = 3 at 4 hrs, n = 6 at 24 hrs; *upper panel*). However, in I/R mice, systemic cytokine levels were much lower and delayed increase in anti-inflammatory IL-10 was not observed (n = 3 at 2 hrs, n = 3 at 4 hrs, n = 4 at 24 hrs; *lower panel*). *B*, The levels of chemokine and cytokines in kidney tissue from septic or ischemic acute kidney injury at 24 hrs after insults were also measured, and the ratio of the level of each proinflammatory cytokine to IL-10 was compared (CLP, n = 6; I/R, n = 4).

directly contribute to the development of AKI in sepsis (Fig. 7*B*). IL-10 blocking Ab and Treg depletion treatment decreased renal tubular cell apoptosis and caspase-3 activity (Fig. 8), and Treg depletion also resulted in better survival (Fig. 9).

DISCUSSION

Severe sepsis and septic shock are the most common cause of AKI in critical illness, and the development of AKI in these patients is known to increase patients' morbidity and mortality (1–4). Generalized arterial dilatation and the paradoxical intense renal vasoconstriction have been considered to be the main mechanisms of loss of glomerular filtration rate in septic AKI (5). This "hemodynamically-mediated ischemic paradigm" has recently been challenged by several studies that

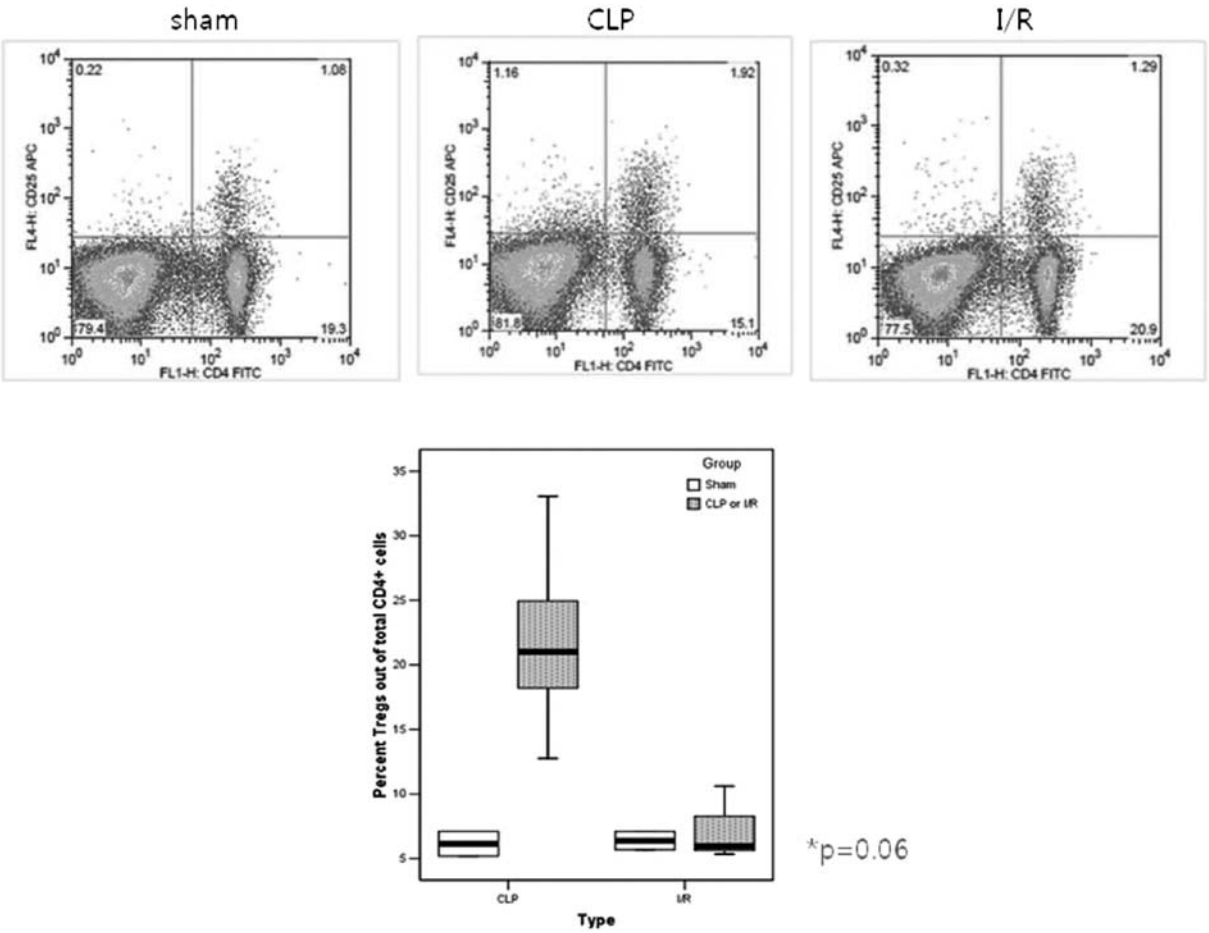

Figure 4. Comparison of splenic Treg frequency between septic and ischemic acute kidney injury (AKI). Twenty-four hours after cecal ligation and puncture (*CLP*), flow cytometric analysis of splenic Tregs was performed using mononuclear cells. Relative frequency of immune suppressive $CD4^+$ $CD25^+$ Tregs to total $CD4^+$ cells showed increase in mice with septic AKI compared with sham or ischemic AKI with near significance (sham, n = 3; ischemia/reperfusion [*I/R*], n = 3; CLP, n = 7; *p = .06).

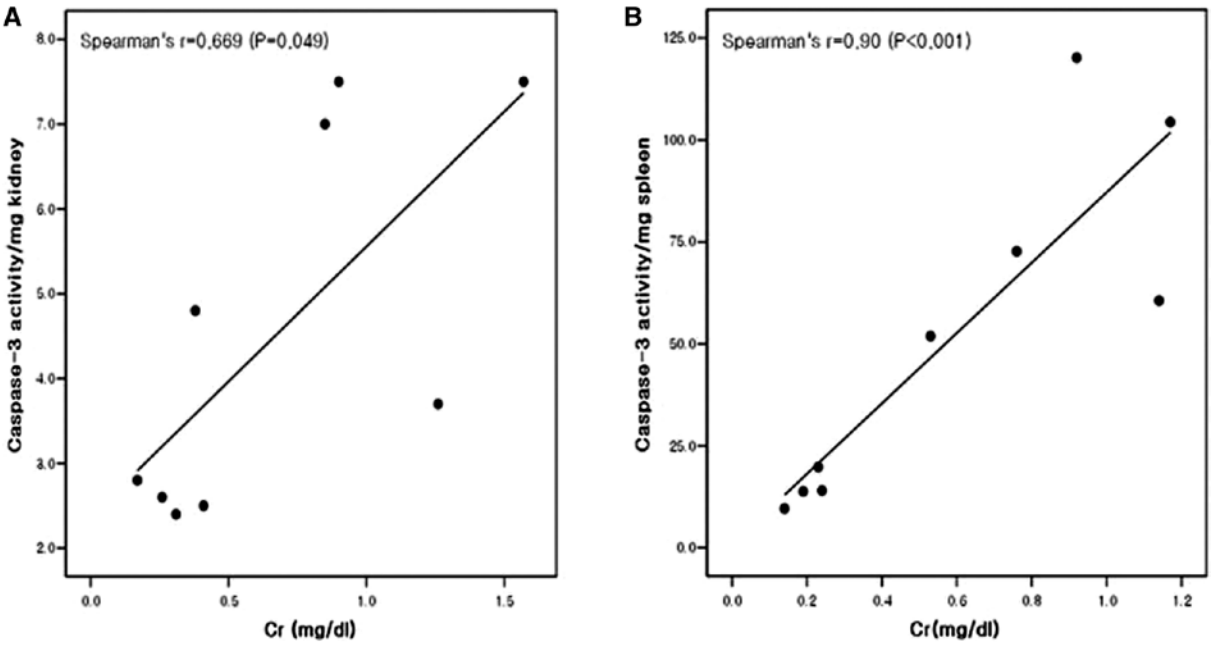

Figure 5. Renal tubular cell/immune cell apoptosis is related to kidney dysfunction in septic acute kidney injury. Caspase 3 activity in kidney and spleen tissue was measured. (*A*) kidney (*B*) spleen. Caspase 3 activity in spleen or kidney showed a significant positive correlation with the plasma creatinine level determined at 24 hrs in septic mice (sham, n = 3; cecal ligation and puncture, n = 7).

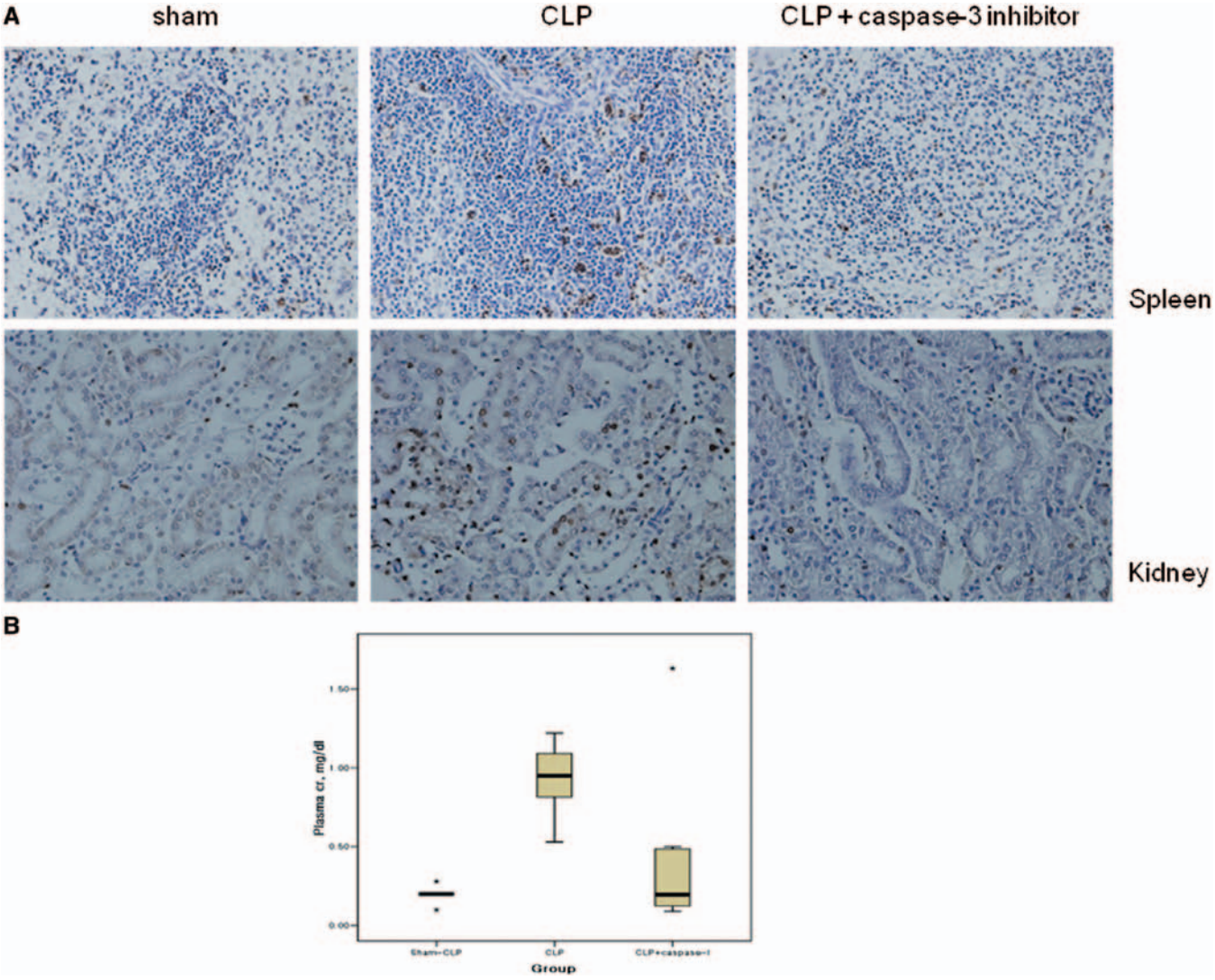

Figure 6. Caspase 3 inhibitor pretreatment induced inhibition of immune cell and/or renal tubular cell apoptosis and attenuated kidney dysfunction in septic acute kidney injury. Caspase 3 inhibitor was administered 2 hrs before cecal ligation and puncture (*CLP*) surgery, and mice were sacrificed at 24 hrs. *A*, Pretreatment of mice with caspase 3 inhibitor resulted in marked decrease in immune cell and/or renal tubular cell apoptosis (*upper panel*: spleen, *lower panel*: kidney, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling stain, ×200). *B*, Caspase 3 inhibitor pretreatment was associated with a marked renoprotective effect in septic acute kidney injury. Values are expressed as the mean ± sem, sham (n = 4) vs. CLP (n = 7) vs. CLP+caspase 3 inhibitor (n = 8), *$p < .05$ compared with CLP.

demonstrated the loss of glomerular filtration rate was accompanied by normal or increased renal blood flow in an early sepsis model established in awakened sheeps by live gram-negative bacterial infusion (20). This implies that the long-held belief that an experimental model of ischemic injury could tell what happens in septic kidneys might be wrong and suggests that mechanisms other than hemodynamic alteration might contribute to the pathophysiology of septic AKI.

In this study, we demonstrated that septic AKI is mediated by a distinct pathophysiologic mechanism that is completely different from I/R injury by providing an on-site quantitative comparison between septic and I/R injury. Morphologically, septic kidneys were characterized by only mild vacuolization of tubular cells without overt acute tubular necrosis. The lack of overt tubular cell injury in septic kidneys has also been reported (21, 22). Langenberg et al (23) analyzed several human studies and found that ATN was observed in only 11%–22% of patients, suggesting that the view that ischemia and consequent cell necrosis are responsible for the loss of GFR in sepsis might be wrong. Instead, we observed that a substantial number of tubular cells underwent apoptosis. In addition to increased numbers of TUNEL-positive cells, caspase 3 activity also increased in septic kidneys compared with sham kidneys, and more importantly, kidney caspase 3 activity showed a significant positive correlation with plasma creatinine level. These data might suggest the possible contribution of renal tubular cell apoptosis in development of septic AKI. The possible contribution of various types of proinflammatory cytokines or endotoxin or lipopolysaccharide in sepsis on tubular cell apoptosis has been previously demonstrated (24). Using human proximal tubular cells in culture, Jo et al (25) reported that tumor necrosis factor-α, IL-1β, or lipopolysaccharide treatment induced Fas-dependent apoptosis in tubular cells. The presence of tubular cell apoptosis in human sepsis has also been recently demonstrated by Lerolle et al who examined immediate postmortem kidney biopsies from 19 septic patients (26). They showed increased numbers of TUNEL-positive cells,

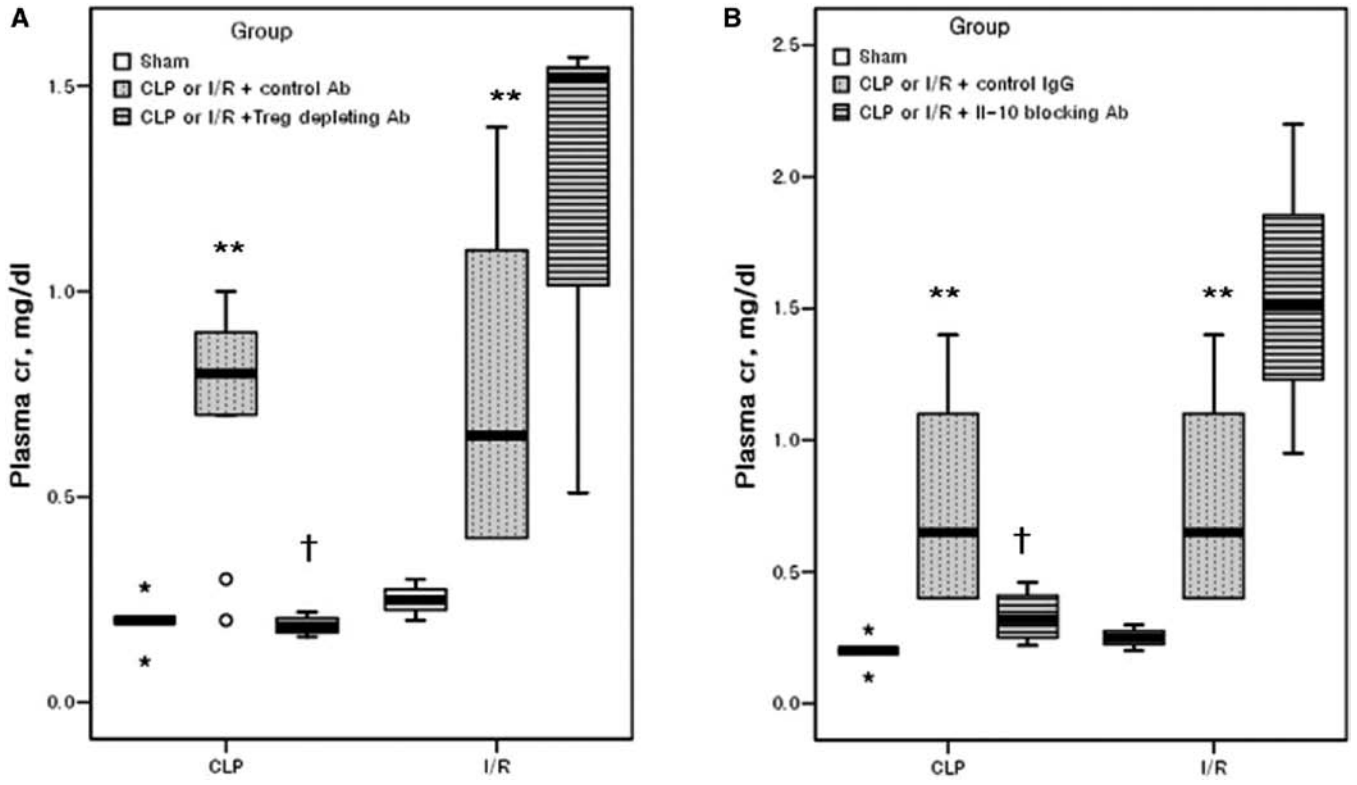

Figure 7. The effect of Treg depletion and interleukin (*IL*)-10 blocking on kidney dysfunction in septic acute kidney injury (AKI) and ischemic AKI. PC61 was administered intraperitoneally 96 hrs before cecal ligation and puncture (*CLP*) and ischemia/reperfusion (*I/R*) injury. IL-10 blocking monoclonal Ab was administered intraperitoneally 6 hrs after surgery, and mice were sacrificed at 24 hrs. *A*, The effect of Treg depletion on kidney function in septic and ischemic AKI. Compared with worsened renal function in I/R, depletion of Tregs significantly attenuated functional impairment in septic AKI. *B*, Blocking IL-10 attenuated kidney dysfunction in septic mice, whereas same treatment did not affect kidney function in I/R mice. Values are expressed as the mean ± SEM, sham (n = 3), CLP (n = 7), CLP+PC61 (n = 4), CLP+IL-10 Ab (n = 7), I/R (n = 6), I/R+PC61 (n = 4), I/R+IL10Ab (n = 4), $^{**}p < .05$ compared with sham, $^{\dagger}p < .05$ compared with control Ab group.

as well as activated caspase 3-positive cells, in kidneys of patients who died from septic shock in contrast to patients in the intensive care unit or post-trauma. Although whether or not the occurrence or extent of apoptosis is specific to sepsis or correlates with renal dysfunction needs to be further verified, the role of renal tubular cell apoptosis following I/R injury is well known. To achieve better insight regarding the contribution of renal tubular cell apoptosis to kidney dysfunction, we pretreated mice with caspase 3 inhibitor and observed that inhibition of apoptosis was renoprotective in sepsis. However, because caspase 3 inhibition was also associated with marked inhibition of immune cell apoptosis, we could not make a firm conclusion about the direct contribution of renal cell apoptosis in septic AKI and needs to be further studied.

The marked difference in histopathology in septic kidneys compared with I/R kidneys was also evident in that either neutrophil or macrophage infiltration, known to be important in I/R injury, was minimal in septic kidneys. Neither immunohistochemical detection nor flow cytometry with a kidney single-cell suspension revealed a substantial amount of inflammation. This is an unexpected finding, considering that sepsis is defined by the body's severe inflammatory response to invading pathogens. The absence of interstitial inflammation in septic kidneys was also recently demonstrated by Lerolle et al (26); however, in contrast to a lack of interstitial inflammation, Lerolle et al observed intense monocytic infiltration in glomeruli or interstitial capillaries, and currently the reason for this discrepancy is not clear.

We measured various cytokine and chemokine levels in the circulation and kidneys from septic or I/R mice and compared the time course. The time course of systemic cytokine levels showed that the cytokine balance inclined to anti-inflammatory condition at 24 hrs after CLP transition from systemic inflammatory response syndrome 2–4 hrs after CLP to compensated anti-inflammatory response syndrome at 24 hrs, when deterioration of kidney function was evident in sepsis. Although proinflammatory mediators, such as monocyte chemoattractant protein-1 and IL-6, peaked early and started to decrease, a classical anti-inflammatory cytokine (IL-10) showed a significant increase 24 hrs after CLP. This anti-inflammatory cytokine balance in sepsis has been previously reported (8). In addition to systemic cytokine level, we also measured kidney cytokines and compared with those of I/R injury. Although absolute level of proinflamamtory cytokine levels in septic kidneys were significantly higher than those in I/R kidneys, the relative ratio of proinflammatory mediators against IL-10 was higher in I/R kidneys compared with septic kidneys suggesting that kidney cytokine balance also has a trend toward anti-inflammation or immune suppression. Therefore, there is possibility that this paradoxical anti-inflammatory milieu in kidney might be responsible for the absence of kidney inflammation in sepsis.

In addition to the cytokine balance, paradoxical immune suppression 24 hrs after CLP was also evident by massive immune cell apoptosis determined by TUNEL staining and measurement of caspase 3 activity. Apoptosis of immune effector cells might

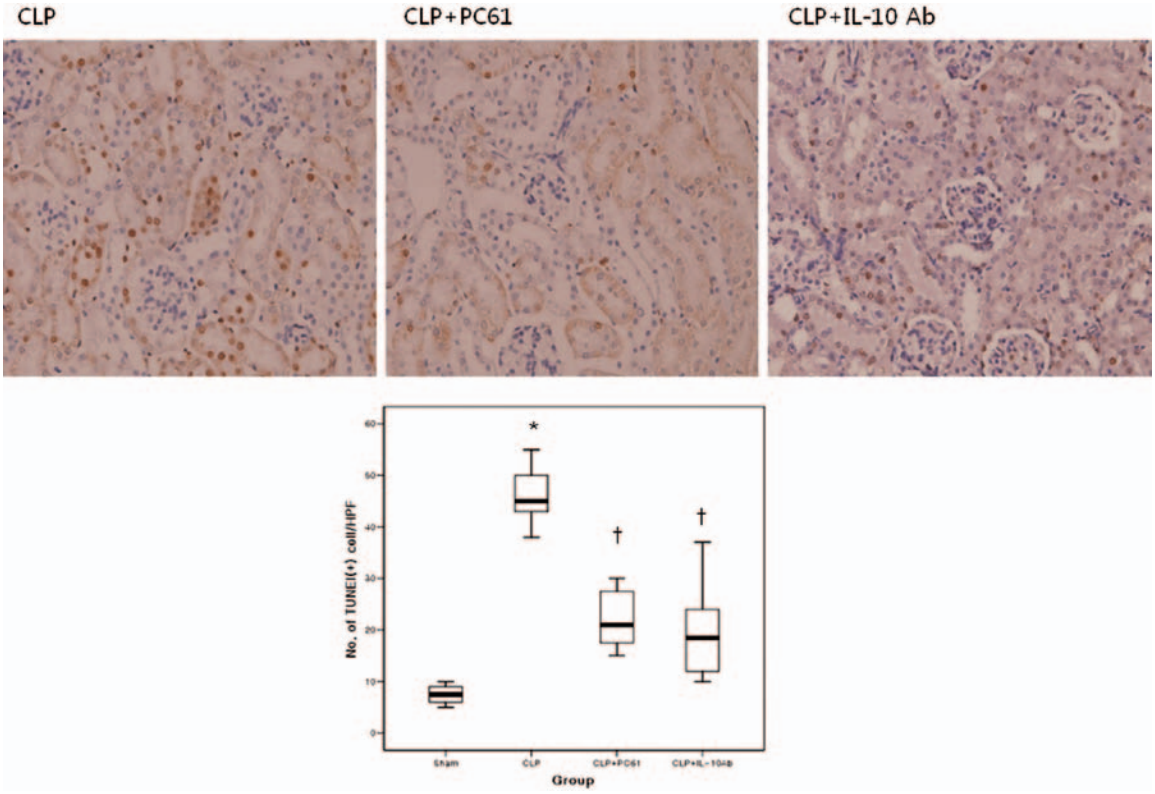

Figure 8. The effect of Treg depletion and interleukin (*IL*)-10 blocking on renal tubular cell apoptosis in septic acute kidney injury. Pretreatment of mice with PC61 and IL-10 blocking Ab resulted in marked decrease in renal tubular cell apoptosis. Number of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (*TUNEL*)-positive tubular cells per high power field (*HPF*) was counted in the cortex and outer medulla in a blinded fashion. (*upper panel*: kidney TUNEL stain, ×200). *Lower panel*, number of TUNEL(+) cells/HPF, 10 fields, sham (n = 3) vs. cecal ligation and puncture (*CLP*) (n = 7) vs. CLP+PC61 (n = 4), CLP+IL-10 Ab (n = 7), *$p < .05$ compared with sham, †$p < .05$ compared with CLP group.

lead to immune paralysis, and previous studies have demonstrated that strategies that inhibit or suppress immune cell apoptosis ultimately improve survival or organ dysfunction (27, 28). We also observed that the caspase 3 inhibitor induced renoprotective effect in sepsis was associated with significant inhibition of apoptosis, not only in renal tubular cells but also in immune cells. Recently, $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in various types of immunologic diseases are getting much attention due to their function in suppressing the immune response or maintaining immune tolerance. Depletion of these cells before I/R or cisplatin has been demonstrated to enhance the innate immune response, to worsen renal function, and adoptive transfer of these cells back into Treg-depleted mice partially restored the injury (29, 30). Flow cytometry of splenocytes revealed that the relative percentage of Tregs increased in sepsis compared with sham or I/R mice. To determine the functional significance of increased Tregs in septic AKI, we depleted these cells and observed that depletion of these cells

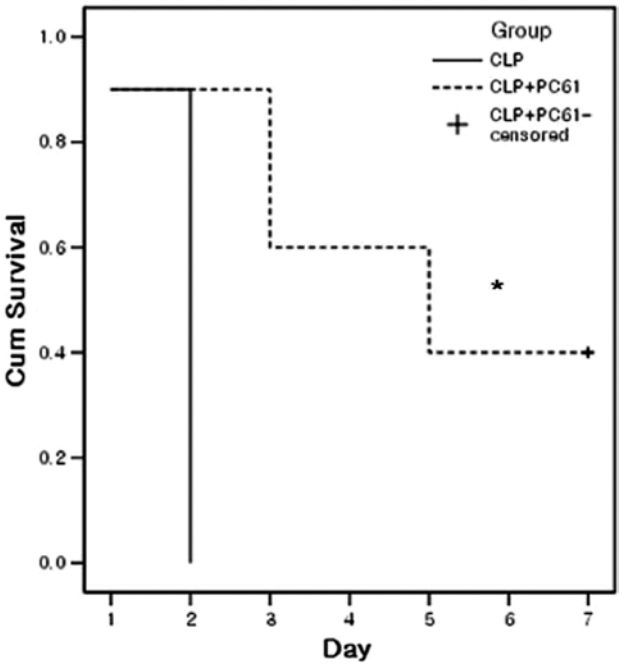

Figure 9. The effect of Treg depletion on survival in septic acute kidney injury (AKI). Kaplan–Meier survival curve in mice with septic AKI (n = 5) vs. septic AKI+PC61 treatment group (n = 5). PC61 was administered 96 hrs before cecal ligation and puncture (*CLP*). Depletion of Tregs resulted in better survival. *$p < .05$ compared with CLP.

before CLP resulted in reduced number of apoptosis and marked improvement of kidney function. This is a completely opposite finding from I/R mice because predepletion of these cells resulted in worsening of kidney function following I/R. These data might suggest that mechanisms involved in induction of renal tubular cell apoptosis in septic vs. I/R-induced AKI are also different. In addition to its renoprotective effect, depleting Tregs also resulted in much better survival.

The role of immune suppression in the development of septic AKI was also tested by using an IL-10 blocking strategy. Although blocking IL-10 in I/R injury did not affect kidney dysfunction, blocking IL-10 in CLP was renoprotective, suggesting that immune suppression characterized by late increase of IL-10 might also be causally linked to the development of septic AKI. Recently, Hiraki et al (31) demonstrated that neutralization of IL-10 reduced the relative expansion of Tregs and subsequently improved survival in sepsis. These results are in accordance with our finding that excessive immune suppression is associated with poor outcome. But it also brings about the issue that blocking IL-10 exerts its effect directly or through Tregs that cannot be determined in our study.

All these results could suggest that immune suppression characterized by immune cell apoptosis, relative expansion of immune suppressive Tregs, and relative anti-inflammatory cytokine balance might be causally linked to the development of AKI in sepsis. Although decreased renal tubular cell apoptosis in Treg depleted or IL-10 blocking Ab-treated CLP mice has been demonstrated, the exact downstream mechanisms leading to improved survival or renoprotective effect is currently unknown. In addition, the results that the same intervention that depletes Tregs or blocking IL-10 evoked completely opposite results in renal function in septic or I/R mice might indicate that the pathophysiology of septic AKI is completely different and not thought to result from a classical "ischemic paradigm."

CONCLUSIONS

Although overzealous activation of immune system to invading pathogen is clearly a prerequisite for sepsis or sepsis-induced organ dysfunction, subsequent immune suppression seems to play some important roles in septic AKI. This study can also suggest that ischemic renal injury resulting from intense renal vasoconstriction is not likely to be pathophysiologic mechanism of septic AKI. Further studies identifying new targets for prevention or treatment of sepsis or sepsis-induced organ dysfunction are needed.

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