Pre-existing renal disease promotes sepsis-induced acute kidney injury and worsens outcome

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While it is known that risk of death from sepsis is higher in patients with pre-existing chronic kidney disease its mechanism is unknown. To study this we established a two-stage mouse model where renal disease was first induced by folic acid injection followed by sub-lethal cecal ligation and puncture to induce sepsis. Septic mice with pre-existing renal disease had significantly higher mortality, serum creatinine, vascular permeability, plasma vascular endothelial growth factor (VEGF) levels, bacteremia, serum IL-10, splenocyte apoptosis and more severe septic shock when compared to septic mice without pre-existing disease. To evaluate the contribution of vascular and immunological dysfunction, we treated the folate-septic mice with soluble Flt-1 to bind VEGF and chloroquine to reduce splenocyte apoptosis. These treatments together resulted in a significant improvement in kidney injury, hemodynamics and survival. Our study shows that the sequential mouse model mimics human sepsis frequently complicated by pre-existing renal disease and might be useful in evaluating preventive and therapeutic strategies.


KEYWORDS: acute-on-chronic; cecal ligation puncture; VEGF; SFlt-1; chloroquine

Sepsis is the leading cause of death in critically ill patients and the incidence of sepsis is increasing. 1, 2 Sepsis causes acute kidney injury (AKI) and patients with both sepsis and AKI show an especially high mortality rate. 3 Chronic kidney disease (CKD) is found in approximately 30% of AKI patients in ICU 4 and severe sepsis occurring in patients with underlying chronic diseases (comorbidities) including CKD, liver disease, and diabetes has an extremely high mortality rate. 1, 3 These findings suggest that clinical sepsis and sepsis-induced AKI are dramatically influenced by underlying diseases, which may explain why simple animal models of sepsis do not mimic human sepsis, and do not predict human response to therapeutics. 6 Developing a new animal model that allow us to investigate the mechanism by which preexisting CKD increases the mortality of sepsis might help discovery efforts to improve the mortality of sepsis, because the prevalence of CKD is increasing worldwide.

In the present study, we developed a new two-stage mouse model that mimics sepsis in patients with preexisting renal dysfunction. This ‘two-stage’ animal model consists of folic acid (FA)-induced renal injury followed by a sublethal cecal ligation and puncture (CLP) model of sepsis. FA injection induces renal injury, as documented by an increase of serum creatinine (Cr), about 60% decrease in glomerular filtration rate (GFR), and a remarkable interstitial fibrosis within 2 weeks. 7 Two weeks after FA injection, we induced sepsis by using a clinically relevant CLP sepsis model which we have established; 8 animals are treated with fluid resuscitation and antibiotics similar to septic patients in an ICU. We then used this new two-stage FA-CLP sepsis model to investigate the pathophysiological mechanisms through which CKD increases sepsis mortality. We used two agents that are known to modulate vascular (sFlt-1) and inflammatory (chloroquine; CQ) dysfunction in sepsis. As the two-stage model is sufficiently complex and models the propensity of preexisting disease to dramatically increase the risk of sepsis, we evaluated the effect of combination therapy in this model that better mimics human sepsis.

RESULTS

FA-CLP mice showed higher mortality after sepsis with amplified acute kidney injury

The protocol of the two-stage FA-CLP model is shown in Figure 1. Blood urea nitrogen (BUN) and serum Cr of FA
group at 48 h after injection were substantially higher than vehicle (Veh) group. Although BUN and serum Cr decreased at 2 weeks after injection, there was a significant and persistent elevation of both BUN and Cr in the FA group. GFR at 2 weeks after injection was 62% lower in the FA group than in the Veh group (Table 1).

Sepsis induced by sublethal CLP surgery showed a significantly higher mortality in the FA-CLP group compared with the Veh-CLP group (FA-CLP 93%, Veh-CLP 18% at 96 h; \( P < 0.05 \); Figure 2a). In the Veh-CLP group, BUN but not serum Cr showed a modest increase following sublethal CLP surgery. In contrast, BUN and serum Cr in the FA-CLP group were both increased significantly at 18 h after CLP surgery compared to those at 0 h (Figure 2b and c). Renal morphologic evaluation was performed at 0 and 18 h after CLP surgery. In accordance with previous reports,7,9 FA induced patchy interstitial fibrotic lesions 2 weeks after injection, although the cortical tubules in nonfibrotic areas were grossly normal (Figure 2d and e). We found no histological evidence of damage to other organs (data not shown). We have reported previously that sepsis induced by CLP caused renal tubular damage mainly consisting of tubular vacuolization.8,10,11 In the present study, tubular vacuolization was found in the cortex of Veh-CLP group and nonfibrotic cortical area of FA-CLP (Figure 2f and g). The number of vacuolized tubules in these areas was higher in the FA-CLP group than in the Veh-CLP group (Figure 2h).

**Severe septic shock and hyperkalemia in FA-CLP mice**

Blood pressure (BP) and heart rate (HR) were measured in conscious animals by radiotelemetry. Presepsis BP was slightly, but not significantly, higher in FA-injected mice than vehicle-injected mice. Sepsis induced by sublethal CLP surgery caused mild decreases of BP and HR in Veh-CLP mice. On the other hand, severe hypotension and decreased HR were found in FA-CLP mice (Figure 3a and b).

![Diagram](https://example.com/diagram.png)

**Figure 1** | Two-stage mouse model of FA-induced renal injury and subsequent sepsis with CLP surgery. Schema of FA-CLP animal model protocol is shown.

Serum potassium levels of FA-CLP mice at 0 h (that is, before CLP) were similar to those of Veh-CLP mice. Sepsis induced hyperkalemia along with AKI in FA-CLP mice. Bilateral nephrectomy induced higher serum potassium levels than FA-CLP mice 18 h after surgery (Figure S1). However, bilateral nephrectomized mice started to die later than FA-CLP mice (time to death: FA-CLP, 41.5 ± 5.8 h (n = 10); bilateral nephrectomy, 51.3 ± 3.7 h (n = 11); \( P < 0.05 \)), although total survival rate were not significantly different between these two groups. This suggests that hyperkalemia is unlikely to be the primary cause of death in FA-CLP mice.

**FA-CLP mice showed higher vascular permeability, plasma VEGF**

Vascular permeability was examined with Evans blue dye, which binds to circulating albumin. Renal vascular permeability in FA-CLP mice at 0 h was increased compared with Veh-CLP (\( P < 0.05 \)). There was no difference in peritoneum or lung vascular permeability before CLP. FA-CLP mice showed a significantly higher vascular permeability compared with Veh-CLP mice in kidney (6 h), peritoneum (6, 18 h), and lung (6 h; Figure 4a–c). The plasma level of vascular endothelial growth factor (VEGF), a growth factor known to enhance vascular permeability,12 in FA-CLP mice was higher than that in Veh-CLP mice before CLP. Sepsis significantly increased plasma VEGF in FA-CLP compared with Veh-CLP mice (6, 18 h). Moreover, bilateral nephrectomy dramatically increased plasma VEGF 18 h after nephrectomy (Figure 4d). These results suggest that the kidney plays an important role in handling circulating VEGF. Recombinant sFlt-1 peptide can bind to circulating VEGF and has been reported to improve the survival of mouse sepsis models including CLP.13,14 We found that treatment with sFlt-1 peptide significantly reduced peritoneal, kidney, and lung vascular permeability in FA-CLP mice, best seen at 6 h after CLP (Figure 4a-c).

**Bacterial count, splenocyte apoptosis, and serum IL-10 increased in FA-CLP mice**

Sepsis induces a state of altered host defenses, which was assessed via measurement of bacterial counts from blood and peritoneal cavity, splenocyte apoptosis, and serum interleukin-10 (IL-10) levels. FA-CLP mice showed higher bacterial counts both in blood and peritoneal cavity compared with Veh-CLP (Figure 5a and b). Splenocyte apoptosis evaluated by activated caspase-3 immunohisto-
chemistry was not increased by FA injection alone. After CLP surgery, the number of activated caspase-3 cells was higher in FA-CLP compared with Veh-CLP mice at 18 h after CLP surgery (Figure 5c). Serum IL-10 levels were not different between FA-CLP and Veh-CLP mice 2 weeks after FA injection. After induction of sepsis, FA-CLP mice showed significantly higher serum IL-10 levels compared with Veh-CLP mice (Figure 5d). CQ is reported to improve mortality of several CLP models via improving splenocyte function,15 splenocyte apoptosis, and serum IL-10.16 In the current FA-CLP model, we found that CQ treatment also significantly attenuated the bacterial count in blood, splenocyte apoptosis, and serum IL-10 levels. In contrast, CQ did not significantly decrease peritoneal fluid bacterial counts (Figure 5a–d).

Liver damage and lung pathological damage in FA-CLP mice
There was no statistically significant difference of aspartate aminotransferase and alanine aminotransferase between FA-CLP and Veh-CLP mice, although both groups showed increases of liver enzymes after CLP surgery (Figure 6a and b). FA injection did not cause any lung damage (data not shown). Although lung vascular leakage in FA-CLP mice at 6 h was significantly increased, there was no detectable histological change (that is, increase of interstitial cellularity and/or extension of cellular infiltrates into the alveolar space) in either FA-CLP or Veh-CLP at 6 and 18 h after CLP (Figure 6c and d).

Combination treatment with soluble Flt-1 and chloroquine improved survival of FA-CLP mice
As sFlt-1 peptide and CQ appear to affect sepsis by different mechanisms, we treated FA-CLP mice with sFlt-1 and CQ, alone or in combination. Each treatment alone tended to decrease BUN and serum Cr levels 18 h after CLP surgery, but only the combination treatment showed a statistically significant protective effect (Figure 7a and b). Hypotension and bradycardia after sepsis induction were improved only in sFlt-1 and combination treatment groups (Figure 7c and d), whereas CQ partially improved BP without improving HR at all. In the survival analysis, sFlt-1 and CQ treatment decreased mortality compared with control group individually (sFlt-1 40%, CQ 29%, control 12% at 96 h), however
only the combination treatment group showed a statistically significant improvement of survival compared with the control group (survival rate: 60% at 96 h, \( P < 0.05 \) versus FA-CLP; Figure 7c).

**DISCUSSION**

We have developed a new two-stage mouse model of FA-induced renal injury and subsequent sepsis with CLP surgery. This model replicated the clinical finding that preexisting CKD amplifies sepsis-induced AKI, induces severe septic shock, and worsens outcome in sepsis.\(^1,4\) We demonstrated the participation of several different pathophysiological mechanisms (that is, increased capillary permeability, decreased bacteria clearance, and splenocyte apoptosis), and the benefit of combination treatment with soluble Flt-1 and CQ, compared to treatment with the individual agents.

Animal studies typically examine sepsis and related organ failure in otherwise healthy animals, despite the inability of simple sepsis models to predict human drug response\(^6\) and numerous epidemiological studies of human sepsis that show the importance of preexisting comorbidity.\(^1,5\) Angus et al.\(^1\) reported that 55% of 200,000 patients with severe sepsis had underlying comorbidity. As CKD patients have been recently recognized as being at high risk for cardiovascular death and mortality from all causes,\(^17,18\) patients with CKD also have an increased risk of morbidity and mortality of sepsis.\(^1,19-22\) In one large multicenter study involving 30,000 critically ill patients, 50% of AKI was associated with septic shock and 30% of AKI had preexisting renal dysfunction.\(^4\) A new animal model that mimics the complexity of human sepsis is required. To simulate CKD, we employed a mouse FA injection model. This model causes acute tubular damage with increases of BUN and serum Cr peaking 2 days after injection, and mild renal dysfunction with remarkable interstitial fibrosis were subsequently found after 2 weeks. FA injection did not cause other organ damage (that is, liver and lung), possibly because the rodent folate receptors are highly expressed in the kidney\(^23\) and/or the kidney damage is induced by chemical precipitation of FA in the renal tubules. Although the FA-induced renal injury model does not show any glomerular lesions nor does it progress to end-stage renal disease, it does transiently mimic human CKD in terms of decreased GFR (Table 1) and histologic evidence of kidney

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**Figure 3** Blood pressure and heart rate. (a, b) Telemetric recordings of mean arterial pressure (MAP) and heart rate (HR) in Veh-CLP (open circle) and FA-CLP (closed circle; \( n = 5 \) per group). \( *P < 0.05 \) versus Veh-CLP.

**Figure 4** Vascular permeability and plasma VEGF levels. Evans blue dye leakage in (a) peritoneum, (b) kidney, and (c) lung was measured at 0, 6, and 18 h after CLP (\( n = 4-8 \) per group). (d) Plasma VEGF levels were measured at 0, 6, and 18 h after CLP by ELISA (\( n = 5-6 \) per group). \( #P < 0.05 \) versus Veh-CLP. \( *P < 0.05 \) versus FA-CLP.
Serum IL-10 levels were measured at 0 and 18 h after CLP by ELISA (Figure 5). Splenic apoptosis was evaluated by activated caspase-3 immunohistochemistry (n = 4 per group). Serum IL-10 levels were measured at 0 and 18 h after CLP by ELISA (n = 5–6 per group). *P < 0.05 versus Veh-CLP, **P < 0.05 versus FA-CLP.

Figure 5 | Bacterial counts in blood and peritoneal fluid, splenic apoptosis, and serum IL-10 levels. Bacterial counts in (a) blood and (b) peritoneal cavity were evaluated at 18 h after CLP (n = 8 per group). (c) Splenic apoptosis was evaluated by activated caspase-3 immunohistochemistry (n = 4 per group). (d) Serum IL-10 levels were measured at 0 and 18 h after CLP by ELISA (n = 5–6 per group). *P < 0.05 versus Veh-CLP, **P < 0.05 versus FA-CLP.

Figure 6 | Liver damage and lung pathology. (a, b) Serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured at 0, 6, and 18 h after CLP (n = 5–6 per group). (c, d) Lung histology in Veh-CLP and FA-CLP at 18 h with HE staining. Original magnification: ×200.

To verify these findings, we set up a second CKD model in CD-1 mice utilizing a modified 5/6 nephrectomy ‘remnant kidney’ model. CKD was confirmed by robust proteinuria, increases in serum Cr and BUN, and histological evidence of chronic glomerular changes and interstitial fibrosis at 4 weeks. Subsequent sublethal CLP produced significant increases in BUN, aspartate aminotransferase, alanine aminotransferase at 18 h in the CKD animals compared to non-CKD animals; Cr was increased but not significantly (as in the FA/sublethal model). There was also a trend toward higher mortality within 18 h (Leelahavanichkul et al., unpublished data). Therefore the worsening of sepsis by preexisting kidney disease is not limited to the FA model.

VEGF plays a critical role in promoting endothelial survival and maintaining the microvasculature. On the other hand, high levels of VEGF can cause vascular leakage by destruction of vascular barrier function and are associated with heightened severity of human sepsis. Interestingly, plasma VEGF levels were already increased in FA-CLP mice just before CLP and bilateral nephrectomy caused a large increase of plasma VEGF. Increased plasma VEGF levels have been reported in predialysis or hemodialyzed patients and subtot al nephrectomized rats. VEGF production in fibrotic kidney in mouse FA-induced renal injury model was decreased. These data suggest that the kidney plays an important role in removing VEGF from the systemic circulation. Other factors induced by renal dysfunction might enhance systemic VEGF production and/or suppress VEGF degradation. Impaired handling of VEGF in FA-CLP mice and CKD patients could contribute to the high mortality from sepsis. In the present study, we found a protective effect of sFlt-1 in our FA-CLP model that was associated with a decrease in vascular permeability and prevention of severe hypotension. Recently, two different groups reported that VEGF plays an important pathophysiologic role in sepsis. Injection of recombinant human VEGF worsened the survival of lipopolysaccharide-injected mice. Soluble Flt-1 (VEGF receptor-1) peptide injection improved the survival in a lipopolysaccharide injection model and a simple CLP model. Soluble Flt-1 treatment had multiple procedures, acute hemorrhagic shock or burns followed in rapid succession by sepsis induced by CLP, endotoxin, or bacteria injections. These two-hit models generate substantially more severe sepsis than induced by sepsis alone. This heightened susceptibility was also found in our current two-stage acute-on-chronic disease animal model that showed a higher mortality in animals with reduced renal function (FA-CLP) compared with normal renal function (Veh-CLP). FA-CLP mice had substantially increased AKI and severity of septic shock, but not liver enzyme elevations or lung inflammatory changes compared to Veh-CLP mice. It is well known that severe shock and sepsis-induced AKI are the most important predicting factors for sepsis outcomes. Our data indicate that preexisting renal dysfunction worsens sepsis by amplifying additional renal damage and promoting septic shock.

Our study is the first ‘two-stage’ sepsis animal model that consists of prior kidney injury followed by subsequent sepsis. Previous ‘two-hit’ animal models of sepsis generally include two closely adjacent ‘hits’ that mimic prior surgical damage (Figure 2e and f) as defined in the K/DOQI guideline.

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effects including attenuation of increased vascular permeability, depression of cardiac function, and enhanced proinflammatory cytokine productions. Soluble Flt-1 modulates sepsis by several different mechanisms and further investigations are required to clarify the precise mechanisms of action of sFlt-1 in this FA-CLP model.

Infectious complications in CKD patients are important causes of their morbidity and mortality. CKD patients are at significant risk of hospitalization for sepsis. It is reported that mortality associated with systemic bacteremia is significantly higher in patients with preexisting CKD (serum Cr above 3 mg per 100 ml). Uremia is associated with alterations in host defense systems and increases the risk of bacterial infections through a number of possible mechanisms such as impaired neutrophil activation and deficient cell immunity. Numerous basic research studies have investigated the multiple pathophysiological mechanisms of sepsis. However, clinical trials targeting specific pathways have not been successful, with the possible exception of activated protein C, although this is controversial. There are several possible reasons. First, some of these drug targets (for example, tumor necrosis factor-α) were selected on the basis of results from animal models that do not replicate human sepsis. We established our CLP model to replicate human sepsis and sFlt-1 and chloroquine.

Figure 7 | Combination treatment of sFlt-1 and chloroquine. (a, b) Renal function of combination treatment. BUN and serum Cr were measured 18 h after CLP in control group (n = 18), sFlt-1 treatment group, CQ treatment group, and combination treatment group (n = 6–7 per treatment group). (c, d) Telemetric recordings of mean arterial pressure (MAP) and heart rate (HR) (n = 5–6 per group) and (e) Survival analysis of combination treatment (n = 15–17 per group). *P < 0.05 versus control.

In human sepsis, apoptosis was detected dominantly in lymphocytes and several strategies to decrease immune cell apoptosis has been reported to improve the survival from sepsis by CLP. FA-CLP mice showed more splenocyte apoptosis after sepsis compared with Veh-CLP mice. It is reported that CQ improved survival of CLP-induced sepsis following hemorrhagic shock while increasing splenocyte proliferation and IL-2 production. We also found that CQ improved sepsis via decreasing splenocyte apoptosis and serum IL-10 levels. In the present study, CQ treatment improved bacteremia, decreased IL-10 levels, and splenocyte apoptosis in FA-CLP mice.

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treatment group suggests that multiple therapeutic interventions may be required for the treatment of sepsis complicated with comorbidity. We did not measure the therapeutic window for the combination therapy; this needs to be tested to determine whether this strategy might be considered to preempt or treat patients with established sepsis and/or sepsis-AKI.

In conclusion, we developed a new clinically relevant murine two-stage model of sepsis in the setting of preexisting renal dysfunction. This model replicated the clinical findings of higher mortality of sepsis in CKD patients. We also found that combination therapy of soluble Flt-1 and CQ, which block vascular and immunological dysfunction, respectively showed the best survival rate. Our results strongly suggest that combination of complementary therapeutic approaches might be needed to treat human sepsis.

MATERIALS AND METHODS

Folic acid injection and subsequent cecal ligation and puncture model

All animal experiments were conducted in accordance with an animal study protocol approved by the NIDDK animal care and use committee. Eight-week-old male CD-1 mice (Charles River Laboratories, Wilmington, MA, USA) were used. Mice were administered FA (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally at a dose of 250 mg/kg in vehicle (0.2 ml of 0.3 mM NaHCO3) or given vehicle alone. Two days later, 60–70% of the mice developed AKI (defined as BUN >100 mg per 100 ml) as previously described. In all experiments, we used animals only with sufficient acute renal damage (BUN >100 mg per 100 ml) at 48 h after FA injection.

CLP surgery was performed on FA-treated (FA-CLP) and vehicle-treated mice (Veh-CLP) at 2 weeks after injection. Under isoflurane anesthesia, a 4-0 silk ligature was placed 8 mm from the cecal tip. The length of 8 mm was shown to cause sublethal sepsis in normal CD-1 mouse. The ceccum was excised twice with a 21-gauge needle and gently squeezed to express a 1 ml column of fecal material. Prewarmed normal saline (NS; 1 ml) was injected intraperitoneally. Treatment with fluid and antibiotic was started at 6 h after surgery with subcutaneous injection of imipenem/cilastatin (14 mg/kg) in 1 ml of NS. Animals were killed 6 and 18 h after surgery for collecting specimens. In the survival study, treatment was continued every 12 h with imipenem/cilastatin (7 mg/kg) in 1 ml of NS.

Bilateral nephrectomy

Under isoflurane anesthesia, the kidneys were exposed from flank, dissected, and removed after the pedicles were ligated using 4-0 silk sutures. The wounds were closed in two layers using 6-0 nylon sutures and surgical staples. Prewarmed NS (1 ml) was injected intraperitoneally. Animals received antibiotics and fluid treatment at 6 h as described above and were killed 18 h after surgery or observed for survival analysis.

Measurement of GFR in conscious mice

GFR was measured by fluorescein isothiocyanate labeled inulin clearance. Blood samples were collected from the tail vein at 3, 7, 10, 15, 35, 55, and 75 min after single fluorescein isothiocyanate labeled inulin (3.7 μl/g body weight) injection. Fluorescence was determined by a Nanodrop-ND-3300 fluorescence spectrometer (Nanodrop Technologies, Wilmington, DE, USA). GFR was calculated using a two-compartment model of two-phase exponential decay.

Treatment of soluble Flt-1 and chloroquine

Recombinant human soluble Flt-1 domain D1-3 (Cell Sciences, Canton, MA, USA) at the dose of 1 μg per mouse or an equal volume of NS was injected intravenously every 3 h (four doses), beginning 1 h after CLP. CQ (Sigma-Aldrich) at the dose of 50 mg/kg or an equal volume of saline was administered by oral gavage at 3 h before CLP surgery. When testing combination treatment, mice were randomly assigned to the following groups: sFlt-1 and CQ, sFlt-1 and vehicle (peritoneally), CQ and vehicle (intravenously), or vehicle only (peritoneally and intravenously).

Measurement of blood pressure and heart rate

The mean BP and HR were measured by radiotelemetry. A telemeter catheter was implanted in the left carotid artery and advanced to the aortic arch. The attached telemetry transmitter (model TA11PA-C10, Data Sciences International, St Paul, MN, USA) was placed in a subcutaneous pocket on the left flank 3–5 days before CLP surgery. BP and HR data were analyzed from 9 h before CLP surgery for 27 h.

Measurement of blood chemistry, vascular endothelial growth factor, and IL-10

BUN and serum Cr was measured by a modified method of the Berthelot reaction with Urea Nitrogen Colorimetric Reagent (Teco Diagnostics, Anaheim, CA, USA) and HPLC method respectively. Serum potassium, aspartate aminotransferase, and alanine aminotransferase were measured using an autoanalyzer (Hitachi 917, Boehringer Mannheim, Indianapolis, IN, USA). Plasma VEGF and IL-10 were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

Vascular permeability assay with Evans blue dye

Mice were injected intravenously with 20 mg/kg Evans blue dye (Sigma-Aldrich). Thirty minutes after injection, peritoneal fluid was collected with 1.5 ml NS lavage. Mice were perfused with phosphate-buffered saline through the right ventricle until blood was visibly eliminated. The kidneys and lungs were weighed, snap frozen in liquid nitrogen, and stored at −80°C. Peritoneal fluid was centrifuged for 10 min at 3000 g. The kidneys and lungs were homogenized in 1 ml formamide and incubated 55°C for 18 h and centrifuged at 10,000 g for 30 min. The amount of Evans blue dye in the supernatants was analyzed by measuring absorbance at 620 and 740 nm as described previously. Results were expressed as concentration of Evans blue dye in peritoneal fluid lavage and micrograms of Evans blue dye per gm of kidney or lung (wet weight).

Bacterial count in blood and peritoneal cavity

The peritoneal cavity was lavaged with 1.5 ml sterile saline, and blood was collected by cardiac puncture 18 h after CLP surgery. Serial dilutions of blood or peritoneal fluid were plated onto tryptic soy agar (Remel, Lenexa, KS, USA) and colony counting after 24 h incubation at 37°C. Bacterial counts were log normalized; samples that did not have detectable bacteria were assigned a value of 0.5 colony forming unit.
Morphologic evaluation of kidney and lung
Kidney and lung specimens (4 μm), fixed in 10% formalin and embedded in paraffin, were stained with Masson’s trichrome and hematoxylin and eosin staining, respectively. Renal tubular damage caused by CLP-induced sepsis was assessed by counting vacuolated tubules at ×400 magnification using >100 randomly selected tubules from each animal.10,11

Immunohistochemical analysis of activated caspase-3 in spleen
Immunohistochemical staining of 4 μm paraffin sections was performed with anti-activated caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA) as described previously.12 The number of positive stained cells was determined from the mean of five randomly selected nonoverlapping 200 × fields in each section.

Statistical analysis
Results are expressed as mean ± s.e.m. Differences among groups were analyzed by Student’s t-test or Mann–Whitney rank sum test. Differences among groups in the combination treatment experiments were confirmed by one-way ANOVA followed by Dunnett’s test for individual comparison. Survival analyses were compared by a log-rank test with a multiple comparison correction. These calculations were done using SigmaStat version 3.10 (Systat Software Inc., Richmond, CA, USA). The null hypothesis was rejected when P < 0.05.

DISCLOSURE
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SUPPLEMENTARY MATERIAL
Figure S1. Hyperkalemia in FA-CLP and bilateral nephrectomized mice.

REFERENCES


