IκB kinase inhibitor attenuates sepsis-induced cardiac dysfunction in mice with chronic kidney disease

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Abstract

Patients with chronic kidney disease (CKD) requiring dialysis have a higher risk of sepsis and a 100-fold higher mortality. The severity of cardiac dysfunction predicts mortality in septic patients. Here we investigated (a) the roles of pre-existing CKD on cardiac function in mice with sepsis, and (b) whether inhibition of IκB kinase (IKK) reduces the cardiac dysfunction in CKD-sepsis. Male C57BL/6 mice underwent 5/6th nephrectomy (SNX), and were subjected 8 weeks later to either lipopolysaccharide (LPS; 2 mg/kg) or sepsis by cecum ligation and puncture (CLP). CKD mice with sepsis or endotoxemia received an IKK inhibitor (IKK 16, 1 mg/kg, 1 h-post CLP or LPS administration). SNX resulted in significant rises in urea and creatinine, and a small (P<0.05) reduction in ejection fraction (echocardiography), and increases in the cardiac phosphorylation of IκBα, nuclear translocation of the NF-κB subunit p65, iNOS expression, and phosphorylation of Akt and ERK1/2. When subjected to LPS or CLP, CKD mice exhibited exacerbation of (a) cardiac dysfunction, (b) lung inflammation and plasma cytokine levels (TNF-α, IL-1β, IL-6, IL-10) and (c) phosphorylation of IKKα/β, phosphorylation of IκBα, nuclear translocation of p65 and iNOS expression (heart). IKK 16 attenuated cardiac dysfunction, lung inflammation, cytokine formation and cardiac phosphorylation of IKKα/β and activation of NF-κB in CKD mice with sepsis or endotoxemia. Thus, pre-existing CKD aggravates the cardiac dysfunction caused by sepsis or endotoxemia in mice; this may be due to increased cardiac activation of NF-κB and iNOS expression.

Keywords: IKK 16; polymicrobial sepsis; endotoxemia; 5/6th nephrectomy; multiple organ dysfunction

Introduction

Sepsis is a systemic dysregulated inflammatory response to an infection, which, when excessive, may progress to multiple organ failure and death [1]. More than 40% cases of sepsis have cardiovascular impairment [2] and the overall mortality in septic patients who have myocardial dysfunction rises from 40% to 70% [3]. The lack of translatability of preclinical findings to patients with sepsis has many possible reasons including: interventions given relatively late, a great degree of heterogeneity in the patient population which often have co-morbidities including diabetes and chronic kidney disease (CKD), or both [4-7]. CKD is a growing public health burden with increasing number of patients receiving maintenance dialysis [8]. Cardiovascular disease is the leading cause of death in patients with CKD [9]. The cardiac injury caused by ischemia-reperfusion is greater in uremic rats compared to non-uremic controls [10]. Patients with CKD requiring dialysis have a higher risk of infection and sepsis [11] due to uremia-induced immune deficiency [12-14], significant co-morbidities and the dialysis procedure itself [15]. Once infected, dialysis patients with sepsis have an approximately 100-fold higher mortality rate compared with the general population with sepsis [16]. It is possible that alterations in cardiac function (at baseline, in response to sepsis or both) play a crucial role in the increased risk of death in CKD patients with sepsis.

Up-regulation of nuclear factor (NF)-κB has been linked to the development of cardiac dysfunction following the onset of sepsis [17, 18]. Physiologically, inhibitor of kappa B (IκB) α inactivates NF-κB by sequestering NF-κB as an inactive complex in the

cytoplasm [19, 20]. Phosphorylation of IκBα by IκB kinase (IKK) dissociates IκBα from NF-κB, which liberates NF-κB to enter the nucleus and activates the expression of NF-κB target genes [20]. Inhibition of IKK [21] attenuates sepsis-induced multiple organ dysfunction/injury in mice [22]. It is, however, unknown whether pre-existing CKD augments the cardiac dysfunction in sepsis, and whether excessive activation of NF-κB drives cardiac dysfunction in animals with CKD and sepsis.

Results

Characterization of organ dysfunctions and blood tests in mice that underwent subtotal (5/6th) nephrectomy (SNX).

When compared to a sham procedure, SNX resulted in significantly higher plasma urea and creatinine concentrations, this was paralleled by a mild cardiomyopathy indicated by slight, but significant, reductions in % ejection fraction (EF), fractional shortening (FS) and fractional area change (FAC). CKD mice exhibited a significantly higher mean arterial blood pressure (MABP), greater heart weights and heart weight to body weight ratio (a surrogate marker for myocardial hypertrophy [23]) (P<0.05; Table S1). Additionally, there was an increase in interventricular septum thickness in CKD mice (P<0.05), but no difference was observed in left ventricular dimensions (left ventricular internal-diastolic dimension or left ventricular end-diastolic volume) (P>0.05; Table S1), indicating the development of concentric hypertrophy of CKD hearts.

Additionally, full blood analysis indicated the development of anemia and an increase in neutrophil-to-lymphocyte ratio in CKDs (P<0.05; Table S1). Most notably, CKD mice had elevated plasma levels of the (mainly pro-inflammatory) cytokines interleukin (IL)-1β and keratinocyte-derived cytokine (KC) (Table S1, Figure S7), tumor necrosis factor (TNF)-α, IL-6 and IL-10 (Figure S7), indicating that CKD caused mild systemic inflammation.

Pre-existing CKD augmented the cardiac dysfunction caused by low dose lipopolysaccharide (LPS) administration.

In CKD sham animals, low dose LPS (2 mg/kg) had no effect on % EF, FAC and FS (P>0.05; Figure 1A - D, Figure S1A - C), however, in CKD mice, low dose LPS induced significant reductions in % EF, FAC and FS (P<0.05; Figure 1A - D, Figure S1A - C), indicating the development of a clear and significant cardiac dysfunction *in vivo.*

Pre-existing CKD augmented the cardiac dysfunction caused by cecal ligation and puncture (CLP).

The murine model of CLP with fluid resuscitation and antibiotics treatment offers a clinically relevant model of abdominal polymicrobial human sepsis. CLP induced-cardiac dysfunction was only observed in 8 month-old mice, but not in young mice [18]. As previously reported [18], CLP had no significant effect on cardiac parameters in young mice (P>0.05; Figure 1E - H, Figure S2A - C). However, in CKD mice, CLP caused significant reductions in % EF, FAC and FS (P<0.05; Figure 1E - H, Figure S2A - C), indicating the development of a pronounced cardiac dysfunction *in vivo.* The degree of systolic dysfunction in young CKD mice with CLP was similar to the cardiac dysfunction reported prevously in old (8 months) mice with CLP [18]. The cardiac dysfunction in CKD/CLP mice was paralleled with a reduced physical activity (P<0.05; Figure 2A). The drop in MABP was slightly greater in CKD/CLP mice compared to sham mice (P<0.05; Figure 2B), however, the drastic cardiac dysfunction

observed in CKD/CLP animals can not be attributed to such a small change in blood pressure, indicating that the cardiac dysfunction might not be primarily dependent on MABP.

Increases in the phosphorylation of IKKα/β, the phosphorylation of IκBα, the nuclear translocation of p65 NF-κB and the inducible nitric oxide synthase (iNOS) expression in hearts of mice with CKD subjected to low dose LPS administration or CLP.

To gain a better mechanical insight into the augmented sepsis-associated cardiac dysfunction in CKD mice, we investigated the effects of pre-existing CKD on signaling events in mouse hearts subjected to LPS or CLP. When compared to PBS-treated or sham-operated CKD sham mice, PBS-treated or sham-operated CKD mice exhibited significantly higher degrees of cardiac phosphorylation of IKKα/β on $Ser^{176/180}$, subsequent phosphorylation of IκBα on $Ser^{32/36}$, nuclear translocation of p65 NF-κB, and iNOS expression (P<0.05; Figure 3A - D, 4A - D). Exposure of CKD sham mice to low dose LPS or CLP had no significant effect on any of the above signaling pathways (P>0.05; Figure 3A - D, 4A - D). However, LPS or CLP further increased cardiac phosphorylation of IKKα/β and IκBα, nuclear translocation of p65, and iNOS expression (P<0.05; Figure 3A - D, 4A - D) to profound degrees in CKD mice.

Effects of low dose LPS administration or CLP on the phosphorylation of Akt and extracellular signal-regulated kinase (ERK) 1/2 in hearts of mice with CKD.

When compared to PBS-treated or sham-operated CKD sham mice, PBS-treated or sham-operated CKD mice demonstrated significantly higher degrees of cardiac phosphorylation of Akt on Ser^{473} and ERK1/2 on Tyr^{202} and Tyr^{204}, respectively (P<0.05; Figure 3E, 3F, 4E, 4F). CKD sham or CKD mice subjected to LPS or CLP demonstrated no significant change in the degree of phosphorylation of Akt or ERK1/2 (P>0.05; Figure 3E, 3F, 4E, 4F).

Pre-existing CKD increases severity of renal dysfunction and hepatocellular injury caused by low dose LPS administration or CLP.

In CKD sham animals, septic insults induced either by low dose LPS or CLP had no significant effect on plasma urea, creatinine or ALT level (P>0.05; Table 1), however, in CKD mice, low dose LPS further increased plasma urea, creatinine and ALT levels to profound degrees (P<0.05; Table 1); CLP resulted in significant increases in plasma urea and ALT levels (P<0.05; Table 1), indicating the augmentation of renal dysfunction and hepatocellular injury, respectively.

Pre-existing CKD increased lung inflammation and systemic inflammatory response caused by CLP.

In CKD sham animals, CLP had no significant effect on lung myeloperoxidase (MPO) activity or plasma inflammatory cytokine levels (TNF-α, IL-1β, IL-6, IL-10 or KC) (P>0.05; Figure 5A - F), however, in CKD mice, CLP resulted in significant increases in lung MPO activity and inflammatory cytokine levels (P<0.05; Figure 5A - E),

indicating an increased neutrophil infiltration in the lung and an enhanced systemic inflammatory response, respectively. No alteration was detected in peritoneal bacteria content between CKD and CKD sham mice following CLP (P>0.05; Figure S3).

Inhibition of IκB kinase attenuated CLP or LPS-induced cardiac dysfunction in mice with CKD.

When compared to sham-operated CKD mice, CKD mice that underwent CLP with vehicle treatment developed significant cardiac dysfunction (P<0.05; Figure 6A - D, Figure S2A - C)*;* this was significantly attenuated by delayed administration of IKK 16 one hour after CLP (P<0.05; Figure 6A - D, Figure S2A - C). CKD/CLP mice that received IKK 16 were significantly more active than CKD/CLP mice that received vehicle (P<0.05; Figure 2A). IKK 16 increased MABP in CKD/CLP mice (P<0.05; Figure 2B). However, IKK 16 did not affect MABP in anesthetized CKD mice (baseline: 84.26 ± 2.08 mmHg vs. IKK 16 administration: 82.52 ± 3.83 mmHg, n=3; P>0.05). Therefore, the higher MABP in IKK 16-treated CKD/CLP mice might be due to improved cardiac function or increased activity (secondary to an overall better health and cardiac performance). No significant change in plasma urea, creatinine or ALT level was seen with IKK 16 administration (P>0.05; Table S2). Similar protective effects of IKK 16 against cardiac dysfunction were found in CKD mice subjected to LPS administration (Figure S1A - C, Figure S5A - D).

Effects of IκB kinase inhibitor on signaling events induced by CLP or LPS in hearts of CKD mice.

When compared to CKD/CLP mice with vehicle treatment, delayed administration of IKK 16 significantly attenuated the increases in cardiac phosphorylation of IKKα/β and IκBα, nuclear translocation of p65 and iNOS expression (P<0.05; Figure 7A - D). Moreover, IKK 16 treatment significantly reduced cardiac phosphorylation of Akt and ERK1/2 (P<0.05; Figure 7E, 7F) in CKD/CLP mice. Similar signaling events were observed in CKD/LPS mice with delayed IKK 16 treatment (Figure S6A - F).

Inhibition of IκB kinase attenuated lung inflammation and systemic inflammatory response caused by CLP or LPS administration.

Treatment of CKD/CLP mice with IKK 16 one hour after CLP significantly reduced the increases in lung MPO activity and plasma inflammatory cytokine levels (P<0.05; Figure 8A - E). Similar protective effects of IKK 16 against lung inflammation and systemic inflammatory response were found in CKD/LPS mice (Figure S7A - F). However, IKK 16 treatment had no effect on peritoneal bacteria content in CKD mice following CLP (P>0.05; Figure S3).

Discussion

The presence of cardiac dysfunction in septic patients has been linked to a significantly raised mortality rate [3]. Patients with CKD also have a significantly higher risk of death followed by sepsis [15, 24], however, the reasons for this higher risk is unclear. The current study was designed to elucidate whether pre-existing CKD worsens cardiac performance in mice with sepsis, to identify (some of) the molecular mechanisms responsible in order to target/test new therapeutic interventions to reduce cardiac dysfunction in mice with CKD and sepsis.

In mice with SNX for 8 weeks (without sepsis), we found a small, but significant, impairment in systolic function (EF) and left ventricular hypertrophy (LVH), indicating the development of a cardiorenal syndrome (type IV as defined by Acute Dialysis Quality Initiative team) [25]. This result is consistent with a previous study revealing the presence of impaired cardiac function in SNX-induced mouse model of CKD [26]. Indeed, systolic dysfunction, cardiac hypertrophy and left ventricular dilation are present in patients with end-stage renal disease; only 16% of new dialysis patients show normal cardiac findings on echocardiography [27, 28]. The observed cardiac dysfunction and LVH in CKD mice are very likely caused by the significantly higher afterload (MABP increase of 14 mmHg). This is in line with a clinical study showing that dialysis patients have a 48% higher risk of LVH with each increase of 10 mmHg in blood pressure [29]. Hypertension is strongly related to the increased incidence of cardiovascular events in patients with 2-3 stage CKD [30].These structural and

functional alterations of heart associated with hypertension may contribute to the increased risk of cardiac death in patients with renal failure [28, 31]. Tight blood pressure control, thus, attenuating the hypertensive heart disease ("first hit") in patients with CKD, might be crucial to prevent the underlying predisposition to second insults such as sepsis.

Notably, we report here for the first time that the presence of CKD increases the severity of LPS-induced cardiac dysfunction, using a "two-hit" animal model that consists of pre-existing CKD followed by LPS injection. This is in agreement with the clinical findings that the pre-existing CKD worsens outcome in patients with infection or sepsis [16, 32]. We have recently reported that CLP-sepsis does not cause a significant cardiac (and indeed multiple organ) dysfunction in young mice, when these animals are treated with fluids and antibiotics, while older animals (8 month-old) do develop cardiac (multiple organ) dysfunction despite fluid resuscitation and antibiotics [18, 22]. We demonstrate here that young mice with CKD do develop a profound cardiac (systolic) dysfunction in response to CLP, which is similar to the cardiac dysfunction in aged mice with CLP. Like CKD, ageing is associated with a mild systemic inflammation, characterized by elevated plasma concentrations of IL-6, IL-1β and TNF [33]; this pro-inflammatory phenotype in ageing (or CKD) may be secondary to a) the observed activation of NF-κB, which is one of the signatures of ageing [33]; b) impaired excretion of cytokines by the kidneys due to decreased renal function (due to reduced number of functional glomeruli and lower glomerular filtration rate) [34]. Indeed,

24-month old mice exhibit systemic inflammation as well as an impairment in renal function (data not shown).

NF-κB is one of the most important pro-inflammatory transcription factors, consisting of heterodimer-subunits p50 and p65 [20]. CKD caused cardiac phosphorylation of $Ser^{176/180}$ on IKKα/β, indicating IKK activation, which in turn led to phosphorylation of IκBα and activation of NF-κB. Additionally, phosphorylation of IκBα can be induced by the exposure to pro-inflammatory cytokines, such as IL-1β and TNF-α [35]. Indeed, plasma pro-inflammatory cytokine levels were increased in CKD mice, paralleled by the increased cardiac phosphorylation of IκBα. The cardiac activation of NF-κB in CKD mice may also be attributable to the hypertensive state. NF-κB is significantly activated in rat cardiomyocytes subjected to cyclic mechanical stretch, which mimics some aspects of the pathophysiological changes associated with hypertension in cardiac myocytes [36]. It is possible that the activation of NF-κB has (at least in part) contributed to the cardiomyopathy through induction of expression of its target gene iNOS. Cardiac activation of NF-κB and the subsequent iNOS expression contribute to sepsis-related impaired left ventricular function [18, 37, 38]. Indeed, in the present study, nuclear translocation of p65 and iNOS expression were augmented in hearts of CKD/sepsis mice, and this was associated with a worsened cardiac dysfunction. As neither low dose LPS nor CLP significantly affected any of the above signaling pathways in mice without CKD, it is likely that the baseline cardiac activation of NF-κB during CKD acts as the prime driver of the observed excessive activation of NF-kB

(and expression of NF-kB dependent genes) and the associated cardiac dysfunction in CKD/sepsis.

In addition to inducing iNOS expression, NF-κB activation also leads to a pronounced increase in other pro-inflammatory cytokines [39]. Here we report a dramatic increase in plasma levels of TNF-α, IL-1β, IL-6 and IL-10, in CKD mice with CLP. More than 70% of inflammatory cytokines are excreted by the kidney [40]; and the half-lifes of TNF-α, IL-6 and IL-10 are 2-3-fold prolonged in CKD mice compared with normal mice [40]. Therefore, impaired renal function resulting in a prolonged half-life of cytokines in CKD mice may amplify systemic inflammation, which in turn may contribute to the excessive cardiac dysfunction and lung inflammation in CKD mice with sepsis [41, 42]. The augmented lung inflammation in CKD mice subjected to sepsis reported in this study is in line with a number of epidemiological studies showing that pre-existing CKD predisposes patients with pneumonia to higher mortality rates[43-45].

Having found the significant roles of phosphorylation of IKKα/β and the subsequent activation of NF-κB in the augmented cardiac dysfunction induced by sepsis/endotoxemia in CKD mice, we have then investigated the role of the selective inhibition of IKK complex *in vivo* in CKD mice that underwent CLP or LPS administration. The treatment protocol for IKK 16 used in the current study reduces systemic inflammation and organ injury in mice with sepsis without CKD [22]. We found for the first time that a single dose of IKK 16 started one hour after CLP or LPS

administration attenuated sepsis-induced cardiac dysfunction in CKD mice corresponded to significant attenuated cardiac activation of NF-κB and iNOS expression. Additionally, systemic inflammatory cytokine levels in CKD/CLP or CKD/LPS mice were reduced by IKK 16, presumably by inhibiting the production of inflammatory cytokines mediated by NF-κB activation and their release into plasma [21]. The attenuated lung inflammation with IKK 16 treatment in CKD/CLP or CKD/LPS mice was in line with previous studies, which showed therapeutic benefits of IKK 16 on sepsis-induced lung inflammation in normal mice [22] and on ventilation-induced lung injury [46].

Sustained high levels activation of the phosphoinositide 3-kinases/Akt and the ERK1/2 pathways have been involved in cardiomyocyte growth and the development of cardiac hypertrophy [47]. In the present study, the cardiac phosphorylation of Akt and ERK1/2 may contribute to the CKD-associated cardiac hypertrophy and cardiomyopathy. Similar to our results, the ERK1/2 pathway was also activated in rat hearts with adenine-induced CKD [48]. The activation of Akt and ERK1/2 was reduced by the administration of IKK 16 in septic CKD animals, presumably through the down-regulation of NF-κB activation and the decreased expression of inflammatory cytokines, such as TNF-α [49, 50]. In turn, down-regulated Akt and ERK1/2 phosphorylation may lead to less NF-κB activation, decreasing cytokine production, thus forming a feed-forward mechanism and further reducing the inflammatory reaction [49, 51].

Conclusions

We have discovered that pre-existing CKD augments the cardiac dysfunction caused by sepsis/endotoxemia. CKD alone resulted in moderate systemic inflammation and activation of NF-κB (and iNOS expression) in the heart, while sepsis/endotoxemia (second hit) in animals with pre-existing CKD resulted in a dramatic rise in a number of pro-inflammatory cytokines (in the plasma) as well as a dramatic increase in the activation of NF-κB (and iNOS expression) in the heart. Most notably, selective inhibition of IKK (by administration of IKK 16 after the onset of sepsis/endotoxemia) abolished the systemic inflammation and cardiac dysfunction caused by sepsis/endotoxemia in animals with CKD. Thus, inhibition of IKK may be useful to treat the excessive inflammation and systolic cardiac dysfunction associated with sepsis in patients with CKD.

Methods

Additional details on the methods are provided in the Supplementary Material online.

Animals

The local ‘Animal Use and Care Committee’ approved animal experiments in accordance with the derivatives of both, the ‘Home Office guidance on the Operation of Animals (Scientific Procedures) Act 1986’, and the ‘Guide for the Care and Use of Laboratory Animals’ of the National Research Council. This study was carried out on 117 four to six week-old male C57BL/6 mice (Charles River, Kent, UK), receiving a standard diet and water *ad libitum*.

Animal models of SNX

Mice were subjected to a two-stage SNX. We followed the original SNX protocol introduced by Gagnon et al. [52] with slight modifications, as described in the Supplementary Material online. Mice subjected to sham operations were operated on without removing kidney.

Model of LPS-induced organ dysfunctions

Mice with CKD and without CKD (CKD Sham) received *i.p.* injections of low dose LPS (2 mg/kg) or its vehicle (PBS). Sham-treated mice were not subjected to LPS, but were otherwise treated the same way.

Model of polymicrobial sepsis caused by CLP

Polymicrobial sepsis was induced by CLP (18-G needle, double puncture) in mice. Mice received volume resuscitation and antibiotic and analgesic therapy [53, 54]. The detailed CLP procedure is described in the Supplementary Material online. Sham-operated mice were not subjected to ligation or perforation of cecum but were otherwise treated the same way. One hour after CLP, CKD mice were treated either with IKK 16 (1 mg/kg *i.v.*) or vehicle (2 % DMSO).

Radiotelemetric recording of hemodynamics and activity *in vivo*

Blood pressure was recorded in conscious, freely moving mice using radiotelemetric transmitters (TA11PA-C10; Data Sciences International) implanted into the aortic arch. After 10 days recovery, the blood pressure and activity were recorded for 3 hours before, and for 20 hours after CLP surgery. Data were acquired for 2 minutes every 15 minutes, and the average values for MABP (mmHg) and activity (a.u) were calculated for every time point (Dataquest Art Acquisition System). ΔMABP and Δactivity were calculated at each time point by subtracting the reading from the average measurement during the 3 hour baseline recordings.

Blood pressure recording in anesthetized CKD mice

Mice were anesthetized with 2% isoflurane delivered in 0.4 ml/min oxygen. MABP was measured via carotid artery using a fluid filled catheter and a blood pressure transducer (MLT1199, AD Instruments, UK). A 10-minute baseline recording was

taken, IKK 16 (1 mg/kg) was given i.v via the jugular vein and MABP was monitored for 1 hour.

Quantification of organ dysfunction/injury

Cardiac function was assessed in mice subjected to LPS at 18 hours or CLP at 24 hours, respectively, by echocardiography using a Vevo-770 imaging system (Visual Sonics, Toronto, Canada) [54, 55]. Then, the experiment was terminated and organ and blood samples were collected for quantification of organ dysfunction/injury. Details are available in the Supplementary Material online.

Western blot analysis

We analyzed the degree of phosphorylation of IKKα/β on $Ser^{176/180}$, IκBα on $Ser^{32/36}$, Akt on Ser^{473} and ERK1/2, the nuclear translocation of the p65 subunit of NF-κB and the expression of iNOS. Semi-quantitative western blot analyses were carried out in mouse heart tissues as described previously [56] and outlined in the Supplementary Material online.

Determination of MPO activity in lung tissue

MPO was extracted from the tissue as described by Barone et al. [57] with slight modifications. MPO activity, used as a marker for neutrophil accumulation in tissues, was determined as previously described [58].

Measurement of cytokines

Concentrations of cytokines in culture supernatants and plasma were measured using a commercially available cytometric bead array (BD Bioscience Hatfield or Biolegend, UK) as described in the manufacturer's instructions. Details are available in the Supplementary Material online.

Statistics

Values are presented as mean ± standard error of the mean (SEM) of *n* observations. Data was assessed by a one-way ANOVA followed by Bonferroni's post hoc test (multiple comparison), a two-way ANOVA followed by Bonferroni's post hoc test (time course, multiple comparison), unpaired Student's t-test or Mann-Whitney *U* test. $P<0.05$ was considered to be statistically significant.

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Disclosure

All authors declared no conflicts of interest.

Supplementary Material

Table S1. Combined data sets from all groups studied prior to the intervention of endotoxemia/sepsis for the characterization of mice with CKD induced by SNX.

Table S2. Effects of IκB kinase inhibitor on renal dysfunction and hepatocellular injury induced by polymicrobial sepsis in mice with CKD.

Figure S1. Effects of low dose of LPS (2 mg/kg) administration and IκB kinase inhibitor on cardiac function in mice with CKD.

Figure S2. Effects of polymicrobial sepsis induced by CLP and IκB kinase inhibitor on cardiac function in mice with CKD.

Figure S3. Peritoneal bacterial loads following CLP and IKK 16 treatment in CKD mice.

Figure S4. Cytokine production by macrophages derived from CKD sham and CKD mice following LPS incubation.

Figure S5. Effects of IκB kinase inhibitor on cardiac dysfunction induced by LPS in mice with CKD.

Figure S6. Effects of IκB kinase inhibitor on signaling pathways in hearts of mice with CKD subjected to LPS administration

Figure S7. Effects of IκB kinase inhibitor on lung inflammation and systemic response in mice with CKD subjected to LPS administration.

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Figures and Tables

Figure 1

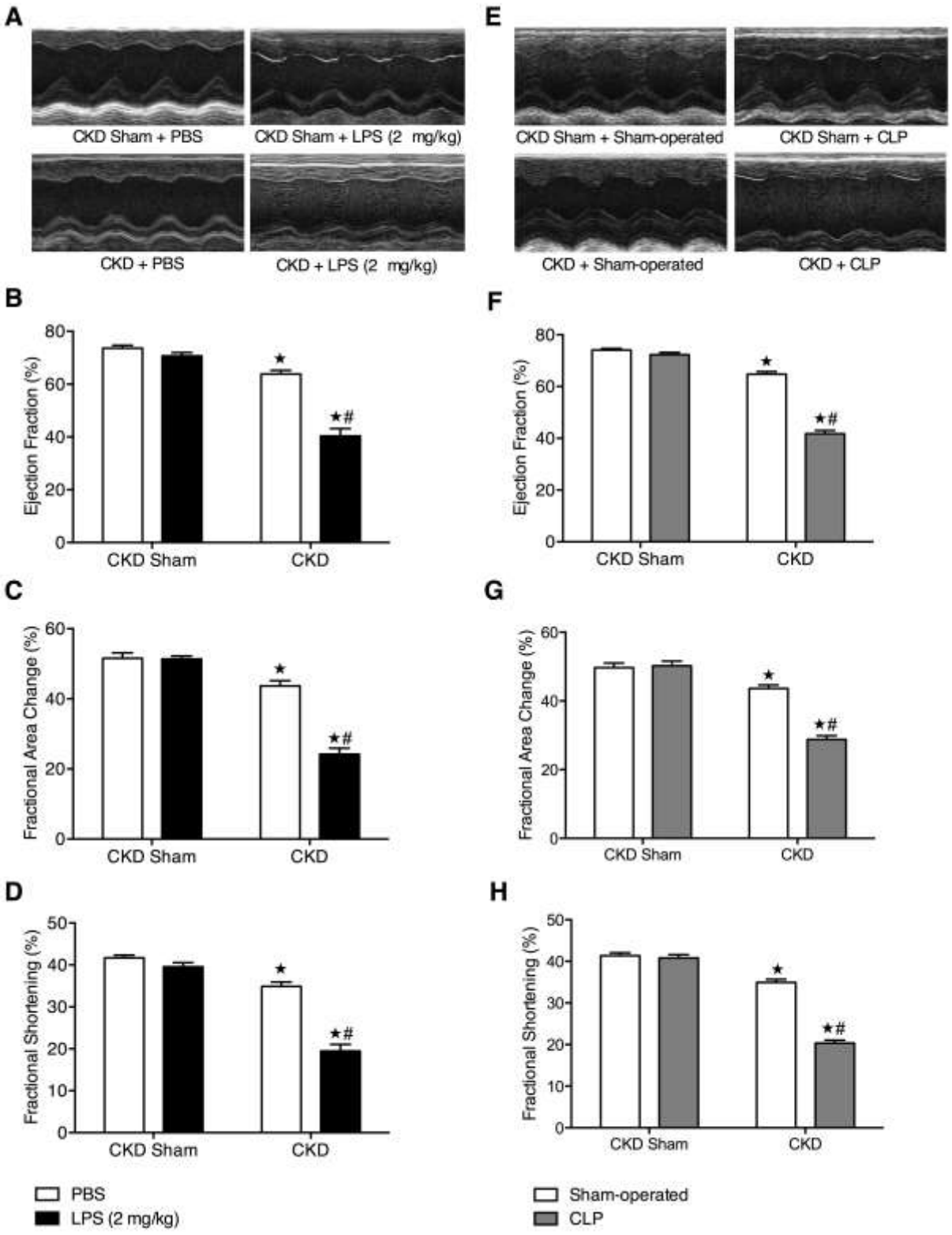

Figure 1. Effects of low dose of LPS (2 mg/kg) administration or polymicrobial sepsis induced by cecal ligation and puncture (CLP) on cardiac function in mice

with chronic kidney disease (CKD). Panel **A** - **D**: CKD sham or CKD mice received either LPS (2 mg/kg) or PBS (5 ml/kg) intraperitoneally. Cardiac function was assessed at 18 hours. (**A**) Representative M-mode echocardiograms; percentage (%) (**B**) ejection fraction (EF); (**C**) fractional area change (FAC); and (**D**) fractional shortening (FS). The following groups were studied: CKD sham + PBS (n = 6); CKD + PBS (n = 7); CKD sham + LPS (2 mg/kg) (n = 7); CKD + LPS (2 mg/kg) (n = 7). Panel **E – H:** CKD sham or CKD mice were subjected to CLP or sham-operated surgery. Cardiac function was assessed at 24 hours. (**E**) Representative M-mode echocardiograms; percentage (%) (**F**) EF; (**G**) FAC; and (**H**) FS. The following groups were studied: CKD sham + sham-operated (n = 6); CKD + sham-operated (n = 7); CKD sham + CLP (n = 7); CKD + CLP (n = 7). Panel **A – H**: all data is represented as mean ± SEM. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★P<0.05 versus the CKD sham group with respective treatment, #P<0.05 versus the respective PBS or sham-operated group.

Figure 2

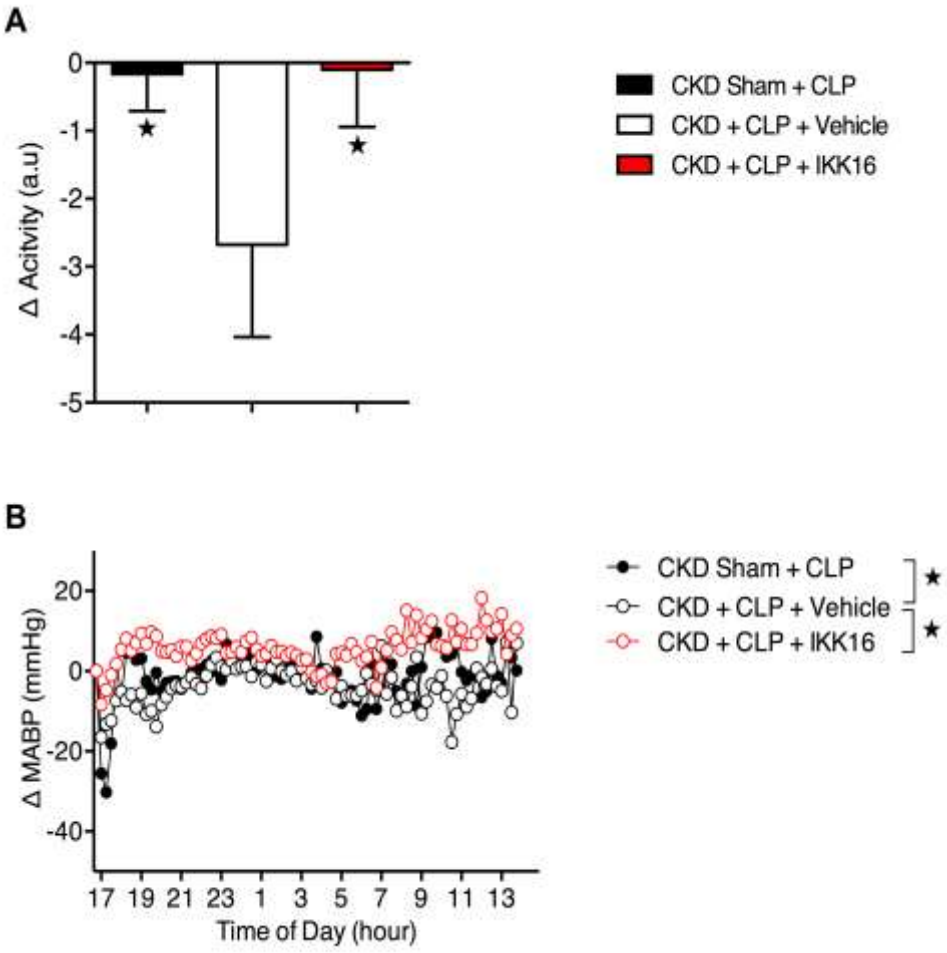

Figure 2. Effects of cecal ligation and puncture (CLP) and/or IKK 16 treatment on the change in activity (Δactivity) and the change in mean arterial blood pressure (ΔMABP) in CKD sham and CKD mice. Radiotelemetric recording of Δactivity (**A**) and conscious ΔMABP (**B**) of CKD sham (black) or CKD mice (white, red) subjected to CLP. After one hour (at 15:00) of CLP, CKD mice were injected with vehicle (black) or IKK 16 (red). N=3-4 per group. All data is represented as mean ± SEM. Panel **A**: Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. Panel **B**: Data was analyzed by two-way ANOVA followed by Bonferroni's post hoc test. ★P<0.05 versus the CKD + CLP + Vehicle group.

Figure 3

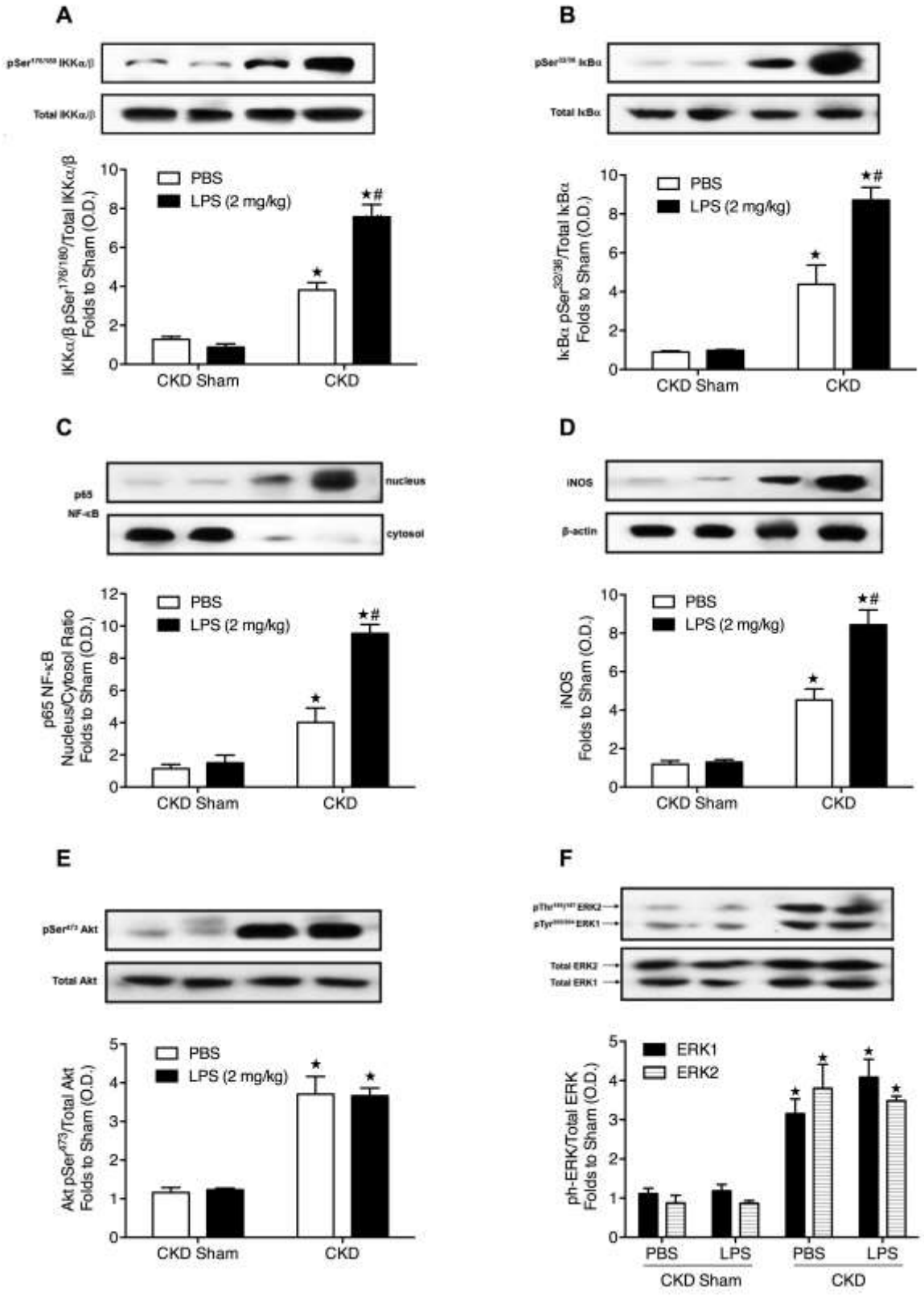

Figure 3. Effects of pre-existing chronic kidney disease (CKD) on signaling

pathways in hearts of mice subjected to low dose of LPS (2 mg/kg) administration. CKD sham or CKD mice received either LPS (2 mg/kg) or PBS (5 ml/kg) intraperitoneally. Signaling events in heart tissue were assessed at 18 hours. Densitometric analysis of the bands is expressed as relative optical density (O.D.) of (**A**) phosphorylated inhibitor of kappa B (IκB) kinase (IKK) α/β ($pSer^{176/180}$) corrected for the corresponding total IKKα/β content and normalized using the related sham band; (**B**) phosphorylated IκBα ($pSer^{32/36}$) corrected for the corresponding total IκBα content and normalized using the related sham band; (**C**) nuclear factor (NF)-κB p65 subunit levels in both, cytosolic and nuclear fractions expressed as a nucleus/cytosol ratio normalized using the related sham bands; (**D**) inducible nitric oxide synthase (iNOS) expression corrected for the corresponding tubulin band; (**E**) phosphorylated Akt ($pSer^{473}$) corrected for the corresponding total Akt content and normalized using the related sham band; (**F**) extracellular signal-regulated kinase (ERK)1/2 phosphorylation, corrected for the corresponding total ERK1/2 content and normalized using the related sham band. Each analysis (**A - F**) is from a single experiment and is representative of three separate experiments. Data is expressed as mean ± SEM for *n* number of observations. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★$P<0.05$ versus the CKD sham group with respective treatment, #$P<0.05$ versus the respective PBS group.

Figure 4

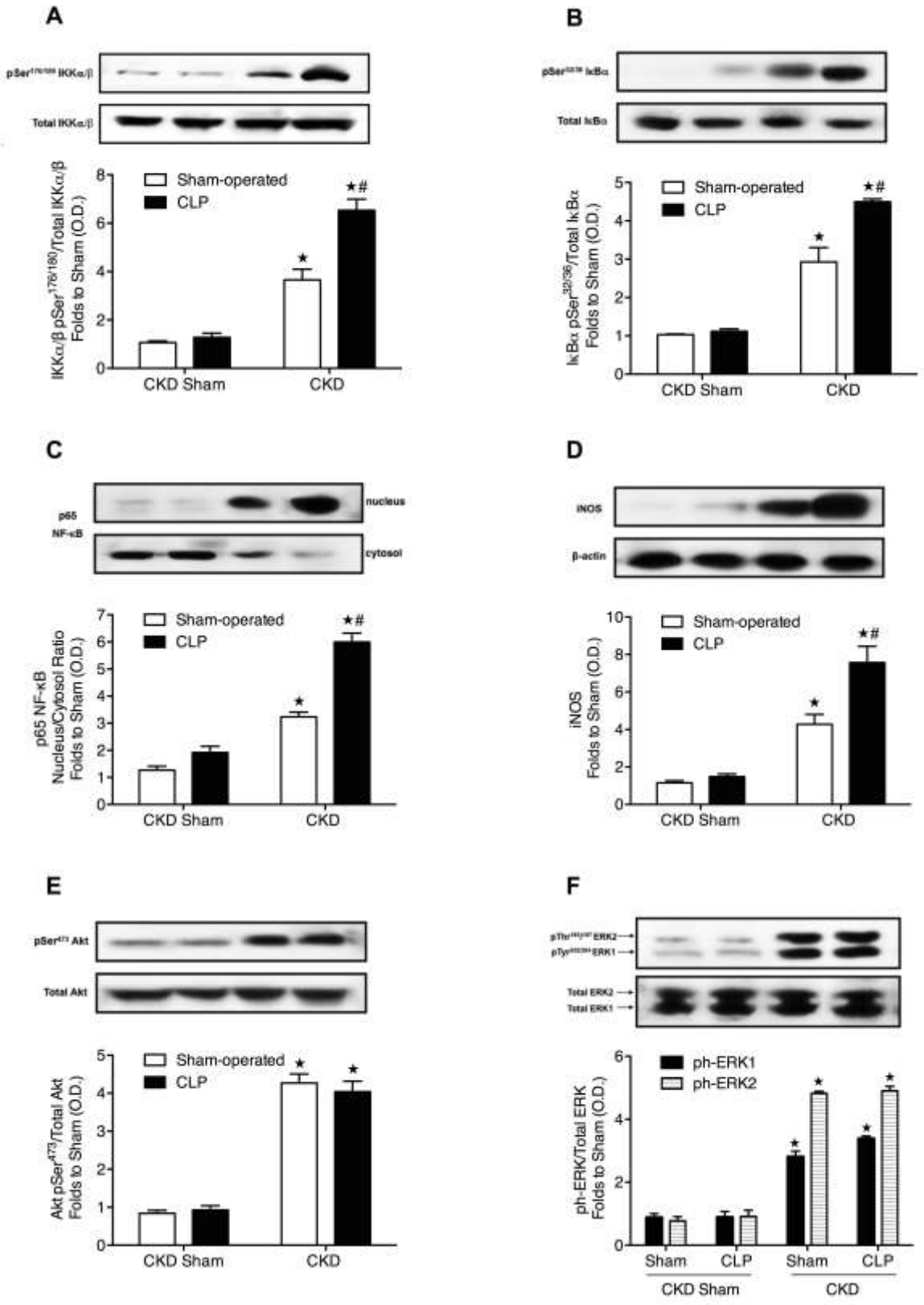

Figure 4. Effects of pre-existing chronic kidney disease (CKD) on signaling

pathways in hearts of mice subjected to polymicrobial sepsis induced by cecal ligation and puncture (CLP). CKD sham or CKD mice were subjected to CLP or sham-operated surgery. Signaling events in heart tissue were assessed at 24 hours. Densitometric analysis of the bands is expressed as relative optical density (O.D.) of (**A**) phosphorylated inhibitor of kappa B (IκB) kinase (IKK) α/β ($pSer^{176/180}$) corrected for the corresponding total IKKα/β content and normalized using the related sham band; (**B**) phosphorylated IκBα ($pSer^{32/36}$) corrected for the corresponding total IκBα content and normalized using the related sham band; (**C**) nuclear factor (NF)-κB p65 subunit levels in both, cytosolic and nuclear fractions expressed as a nucleus/cytosol ratio normalized using the related sham bands; (**D**) inducible nitric oxide synthase (iNOS) expression corrected for the corresponding tubulin band; (**E**) phosphorylated Akt ($pSer^{473}$) corrected for the corresponding total Akt content and normalized using the related sham band; (**F**) extracellular signal-regulated kinase (ERK)1/2 phosphorylation, corrected for the corresponding total ERK1/2 content and normalized using the related sham band. Each analysis (**A - F**) is from a single experiment and is representative of three separate experiments. Data is expressed as mean ± SEM for *n* number of observations. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★$P<0.05$ versus the CKD sham group with respective treatment, #$P<0.05$ versus the respective sham-operated group.

Figure 5

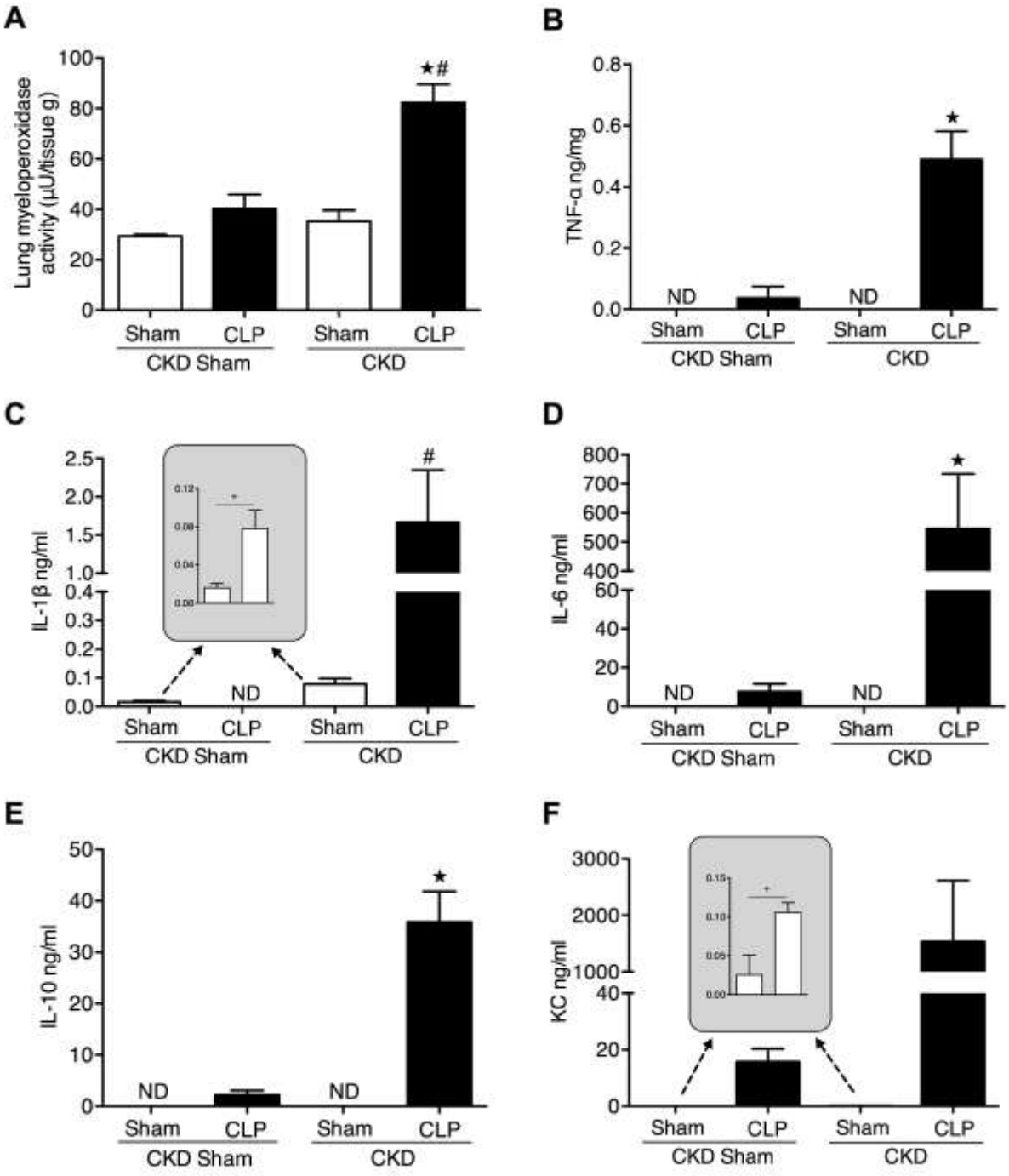

Figure 5. Effects of polymicrobial sepsis induced by cecal ligation and puncture (CLP) on lung inflammation and systemic response in mice with chronic kidney disease (CKD). Markers of lung inflammation and systemic response were assessed at 24 hours in mice that underwent CLP. (**A**) Myeloperoxidase (MPO) activity in lung

tissue; (**B**) plasma tumor necrosis factor (TNF)-α concentration; (**C**) plasma interleukin (IL)-1β concentration; (**D**) plasma IL-6 concentration; (**E**) plasma IL-10 concentration; and (**F**) plasma keratinocyte-derived cytokine (KC) concentration. Panel **A**: n=3 per group; Panel **B – F**: n=3 for CKD Sham + Sham-operated group, n=5-6 for other groups. All data is represented as mean ± SEM. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons or by Student's t-test for comparisons between two groups. ★P<0.05 versus the CKD sham group with respective treatment, #P <0.05 versus the respective sham-operated group, +P<0.05 versus the CKD sham group with sham operation. ND, not detected.

Figure 6

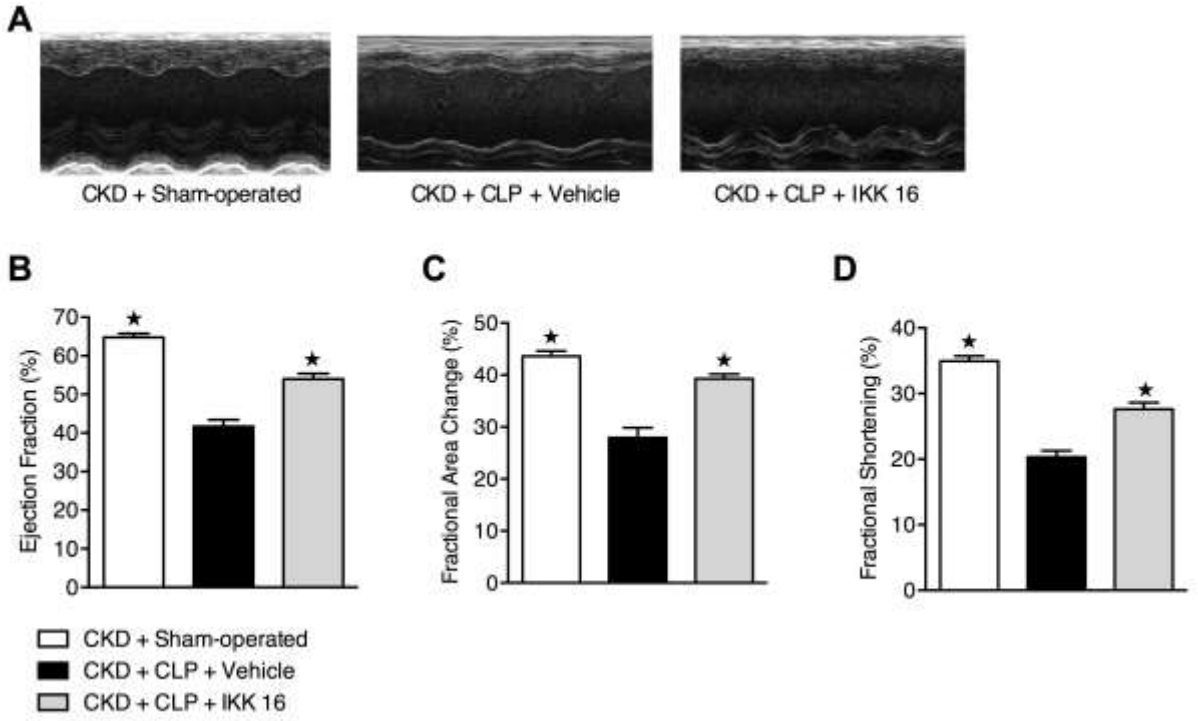

Figure 6. Effects of IκB kinase inhibitor on cardiac dysfunction induced by polymicrobial sepsis in mice with chronic kidney disease (CKD). CKD mice underwent sham-operated surgery or cecal ligation and puncture (CLP). One hour after CLP, mice were treated with either IKK 16 (1 mg/kg i.v.) or vehicle (2% DMSO). Cardiac function was assessed at 24 hours. (**A**) Representative M-mode echocardiograms; percentage (%) (**B**) ejection fraction; (**C**) fractional area change; and (**D**) fractional shortening. The following groups were studied: CKD + sham-operated (n = 7); CKD + CLP + Vehicle (n = 7); CKD + CLP + IKK 16 (n = 7). All data is represented as mean ± SEM. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★P<0.05 versus the CKD + CLP + Vehicle group.

Figure 7

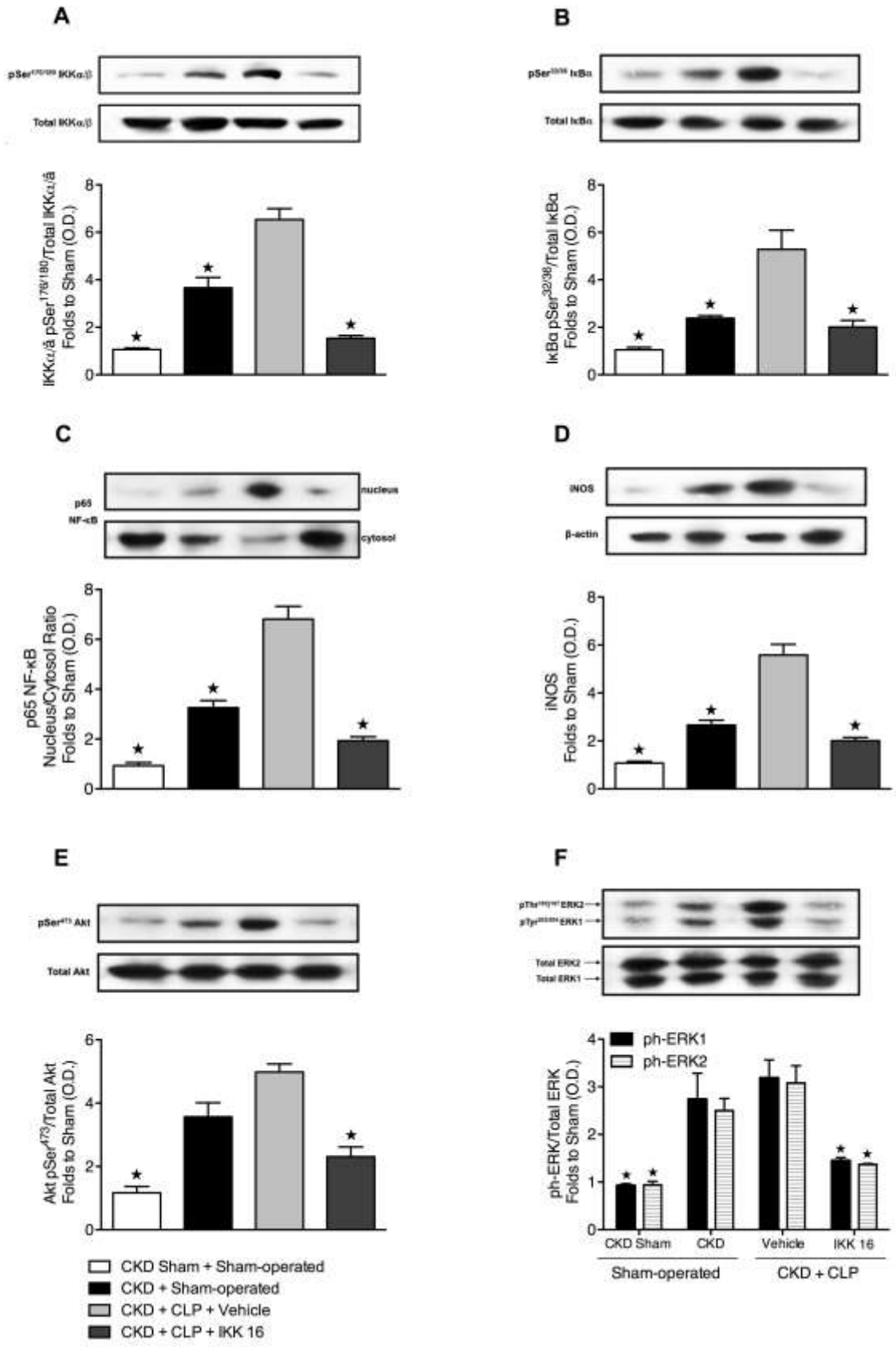
A
Total IKKα,β
IKKα,β pSer176/180/Total IKKα,β
Folds to Sham (O.D.)
B
Total IκBα
IκBα pSer32/36/Total IκBα
Folds to Sham (O.D.)
C
p65
NF-κB
nucleus
cytosol
p65 NF-κB
Nucleus/Cytosol Ratio
Folds to Sham (O.D.)
D
iNOS
β-actin
iNOS
Folds to Sham (O.D.)
E
Total Akt
Akt pSer473/Total Akt
Folds to Sham (O.D.)
F
Total ERK2
Total ERK1
ph-ERK1
ph-ERK2
ph-ERK/Total ERK
Folds to Sham (O.D.)
CKD Sham
CKD
Vehicle
IKK 16
Sham-operated
CKD + CLP
CKD Sham + Sham-operated
CKD + Sham-operated
CKD + CLP + Vehicle
CKD + CLP + IKK 16

Figure 7. Effects of IκB kinase inhibitor on signaling pathways in hearts of mice with chronic kidney disease (CKD) subjected to polymicrobial sepsis induced by cecal ligation and puncture (CLP). CKD sham underwent sham-operated surgery, CKD mice were subjected to CLP or sham-operated surgery. One hour after CLP, CKD mice were treated with either IKK 16 (1 mg/kg i.v.) or vehicle (2% DMSO). Signaling events in heart tissue were assessed at 24 hours. Densitometric analysis of the bands is expressed as relative optical density (O.D.) of (**A**) phosphorylated inhibitor of kappa B (IκB) kinase (IKK) α/β ($pSer^{176/180}$) corrected for the corresponding total IKKα/β content and normalized using the related sham band; (**B**) phosphorylated IκBα ($pSer^{32/36}$) corrected for the corresponding total IκBα content and normalized using the related sham band; (**C**) nuclear factor (NF)-κB p65 subunit levels in both, cytosolic and nuclear fractions expressed as a nucleus/cytosol ratio normalized using the related sham bands; (**D**) inducible nitric oxide synthase (iNOS) expression corrected for the corresponding tubulin band; (**E**) phosphorylated Akt ($pSer^{473}$) corrected for the corresponding total Akt content and normalized using the related sham band; (**F**) extracellular signal-regulated kinase (ERK)1/2 phosphorylation, corrected for the corresponding total ERK1/2 content and normalized using the related sham band. Each analysis (**A - F**) is from a single experiment and is representative of three separate experiments. Data is expressed as mean ± SEM for *n* number of observations. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★P <0.05 versus the CKD + CLP + Vehicle group.

Figure 8

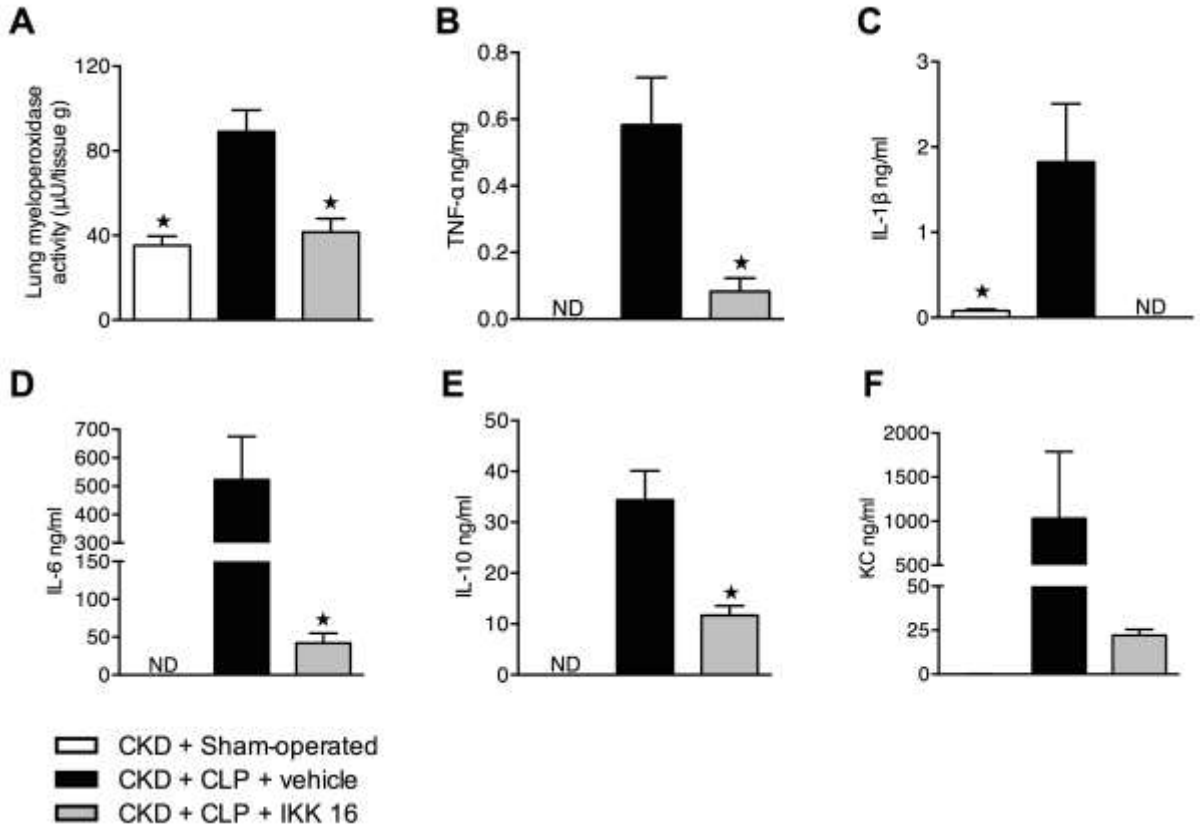

Figure 8. Effects of IκB kinase inhibitor on lung inflammation and systemic response in mice with chronic kidney disease (CKD) subjected to polymicrobial sepsis induced by cecal ligation and puncture (CLP). CKD mice underwent CLP or sham-operated surgery. One hour after CLP, CKD mice were treated with either IKK 16 (1 mg/kg i.v.) or vehicle (2% DMSO). Markers of lung inflammation and systemic response were assessed at 24 hours. (**A**) Myeloperoxidase (MPO) activity in lung tissue; (**B**) plasma tumor necrosis factor (TNF)-α concentration n; (**C**) plasma interleukin (IL)-1β concentration; (**D**) plasma IL-6 concentration; (**E**) plasma IL-10 concentration; and (**F**) plasma keratinocyte-derived cytokine (KC) concentration. Panel **A**: n=3 per group; Panel **B – F**: n=5-6 per group. All data is represented as mean ± SEM. Data was analyzed by one-way ANOVA followed by Bonferroni's post

hoc test. ★*P* <0.05 versus the CKD + CLP + Vehicle group. ND, not detected.

Table 1. Effects of low dose of LPS (2 mg/kg) administration or polymicrobial sepsis induced by cecal ligation and puncture (CLP) on renal dysfunction and hepatocellular injury in mice with chronic kidney disease (CKD).

Parameter	CKD Sham		CKD	
	PBS	LPS (2mg/kg)	PBS	LPS (2mg/kg)
Number	6	7	7	7
Urea (mmol/L)	8.26 ± 0.47	16.13 ± 3.88	17.24 ± 1.09*	38.56 ± 2.11*†
Creatinine (umol/L)	30.22 ± 0.55	30.23 ± 2.35	45.47 ± 2.42*	58.43 ± 2.55*†
ALT (U/L)	27.23 ± 3.01	52.06 ± 2.11	32.16 ± 3.34	83.35 ± 14.11*†
	Sham-operated	**CLP**	**Sham-operated**	**CLP**
Number	6	6	7	7
Urea (mmol/L)	8.08 ± 0.72	13.08 ± 087	17.61 ± 0.66	37.60 ± 6.91*†
Creatinine (umol/L)	29.22 ± 0.50	27.30 ± 0.93	46.44 ± 2.75	67.43 ± 12.92*
ALT (U/L)	23.62 ± 2.90	103.52 ± 15.31	42.44 ± 8.10	287.10 ± 49.86*†

Plasma urea, creatinine and alanine aminotransferase (ALT) levels were assessed at 18 hours in mice subjected to LPS administration and at 24 hours in mice that underwent CLP. All data is represented as mean ± SEM. Data was analyzed by one-way ANOVA followed by Bonferroni's post hoc test. *P<0.05 versus the CKD sham group with respective treatment, †P <0.05 versus the respective PBS or sham-operated group.