Supplementary Material Online

IκB kinase inhibitor attenuates sepsis-induced cardiac dysfunction in CKD

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Methods and Materials

Animal models of subtotal (5/6th) nephrectomy (SNX)

Four to six week-old male C57BL/6 mice were subjected to a two-stage, SNX or sham surgery under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia. We followed the original SNX protocol introduced by Gagnon et al. [1] with slight modifications. Briefly, in the first stage of the SNX, the upper and lower poles of the left kidney were removed (2/6th NX), the mice were allowed to recover for 2 weeks, then the right kidney (3/6th NX) was removed. After the second stage of the surgery, the mice were kept for 8 weeks to develop chronic kidney disease (CKD). Mice subjected to sham operations were operated on without removing kidney.

Model of polymicrobial sepsis caused by cecal ligation and puncture (CLP)

We followed the original CLP protocol introduced by Wichterman and co-workers [2] with slight modifications including analgesia, antibiotic therapy and fluid resuscitation as described previously [3-5]. Based on previous evidence and preliminary data, an 18-G needle was used with the double puncture technique in order to generate reproducible cardiac dysfunction during the early phase of sepsis (24 hours). Briefly, mice were anesthetized i.p. with ketamine (100 mg/kg) and xylazine (10 mg/kg) prepared in the same solution by using 1.5ml/kg. Buprenorphine (0.05 mg/kg *i.p.*) was injected additionally to provide adequate analgesia. The rectal temperature of the animals was maintained at 37°C with a homeothermic blanket. The abdomen was opened via a 1.5 cm midline incision, and the cecum exposed. The cecum was ligated just below the ileocecal valve and punctured at both opposite ends. After a small amount of fecal matter was extruded from both ends, the cecum was placed back in its anatomical position and the abdomen was sutured. Ringer's solution was given *s.c.* for resuscitation directly after surgery (1 ml/mouse) and 6 hours and 18 hours after surgery (0.5 ml/mouse). Antibiotic (Imipenem/Cilastin; 20 mg/kg *s.c.*) and analgesia (buprenorphine; 0.05 mg/kg *i.p.*) was administered 6 hours and 18 hours after surgery.

Assessment of cardiac function *in vivo*

Cardiac function was assessed in mice by echocardiography *in vivo* as reported previously [3, 4]. At 18 hours after LPS administration or 24 hours after CLP, anesthesia was induced with 3 % isoflurane and maintained at 0.5 to 0.7 % for the duration of the procedure. Before assessment of cardiac function, mice were allowed to stabilize for at least 10 minutes. During echocardiography the heart rate was obtained from ECG tracing and the temperature was monitored with a rectal thermometer. Two-dimensional and M-mode echocardiography images were recorded using a Vevo-770 imaging system (VisualSonics, Toronto, Ontario, Canada). Percentage fractional area change (FAC) was assessed from a two-dimensional trace and percentage ejection fraction (EF) and fractional shortening (FS) were calculated from the M-mode measurements in the parasternal short axis view at the level of the papillary muscles.

Quantification of renal dysfunction and hepatocellular injury

Renal dysfunction and hepatocellular injury was assessed in mice subjected to LPS at 18 hours or CLP at 24 hours. Mice were anaesthetized with 1.5 ml/kg *i.p*. of a ketamine (100 mg/ml)/xylazine (20 mg/ml) solution in a 2:1 ratio before being sacrificed. Approximately 0.7 ml of blood was collected by cardiac puncture and immediately decanted into hirudin blood tubes (Roche, UK), after which the heart was removed to terminate the experiment. Approximately 0.1

ml of blood was used for analyses of haemoglobin, haematocrit and white blood cell counting by ProCyte Dx Haematology Analyser (IDEXX Laboratories, UK). The rest of the blood samples were centrifuged at 9900 *g* for 3 minutes to separate plasma, approximately 120 µl of the plasma was sent to an independent laboratory (IDEXX Laboratories, Buckinghamshire, UK) for analyses of plasma urea, creatinine and alanine aminotransferase (ALT), markers of renal dysfunction and hepatocellular injury, respectively. Additionally, heart samples were taken and stored at -80 °C for further analyses.

Western blot analysis

Semi-quantitative western blot analyses were carried out in mouse heart tissues as described previously [6]. Briefly, mouse heart samples were homogenized in 10 % homogenization buffer and centrifuged at 4000 RPM for 5 minutes at 4 °C. Supernatants were removed and centrifuged at 14 000 RPM at 4 °C for 40 minutes to obtain the cytosolic fraction. The pelleted nuclei were re-suspended in extraction buffer and centrifuged at 14 000 RPM for 20 minutes at 4 °C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined on both nuclear and cytosolic extracts using a bicinchoninic acid (BCA) protein assay following the manufacturer's directions (Therma Fisher Scientific, Rockford, IL). Proteins were separated by 8% sodium dodecyl sulphatepolyacrylamide gel

electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluoride (PVDF) membrane, which was then incubated with a primary antibody (rabbit anti-total IKKα/β, dilution 1:200; rabbit anti-pIKKα/β $Ser^{176/180}$, dilution 1:1000; mouse anti-total IκBα, dilution 1:1000; mouse anti-IκBα $pSer^{32/36}$, dilution 1:1000; rabbit anti-NF-κB p65, dilution 1:1000; rabbit anti-total iNOS, dilution 1:200; rabbit anti-total Akt, dilution 1:1000; mouse anti-Akt $pSer^{473}$, dilution 1:1000; rabbit anti-total ERK1/2, dilution 1:1000; mouse anti-pERK1/2, dilution 1:1000). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10000) for 30 minutes at room temperature and developed with the ECL detection system. The immunoreactive bands were visualized by autoradiography. Densitometric analysis of the bands was performed using the Gel Pro Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA). Each group was then adjusted against corresponding sham data to establish relative protein expression when compared with sham animals.

Measurement of cytokines

Concentrations of cytokines in culture supernatants and the plasma obtained from CKD + CLP + IKK 16 study were measured using a commercially available cytometric bead array (BD Bioscience Hatfield, UK) as described in the manufacturer's instructions. In this set of analysis, CKD mice exhibited significantly increased plasma levels of interleukin (IL)-1β and

keratinocyte-derived cytokine (KC) (P<0.05; Table S1), but increases in tumor necrosis factor (TNF)-α, IL-6 and IL-10 were not detected (possibly due to the relatively low sensitivity of the assay used). Therefore, for analyzing the concentrations of cytokines in the plasma obtained from CKD + LPS + IKK 16 study, we used a cytometric bead array with a higher sensitivity from Biolegend, UK, all of the above cytokines were detected in CKD mice, which is in line with previous studies[7, 8].

Bacteria counting

Accurate enumeration of bacteria in peritoneal lavages was performed by flow cytometry using the SYTO BC bacteria counting kit (Invitrogen, UK) according to the manufacturer's instructions.

Primary macrophage cultures

CKD control and CKD sham mice were injected with 1 mL of 2% Bio-Gel (Bio-Rad) i.p., and 4 days later, peritoneal lavages were harvested with 4 mL of EDTA (3 mM) in PBS. Cells (2 × 106) were plated in 24-well plates in RPMI medium 1640 containing 10% (vol/vol) FCS and 50 mg/mL of gentamicin. After 2 hours at 37 °C, nonadherent cells were washed and adherent cells (>90% macrophages) were incubated in RPMI 1640 1% FCS and treated with different concentrations of LPS (0.1 ng/ml, 1 ng/ml and 10 ng/ml) or vehicle (sterile PBS) for 6 hours at 37 °C. Supernatants were harvested and cytokine

production was determined by CBA (eBioscience, Hatfield, UK).

Materials

Reagents and compounds were purchased from Sigma Aldrich (Poole, Dorset, UK), unless otherwise stated. Antibodies for immunoblot analysis were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Results

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

When compared to PBS-treated CKD mice, CKD/LPS mice with vehicle treatment developed significant cardiac dysfunction (P<0.05; Figure S2A - C, Figure S5A - D)*;* this was significantly attenuated by delayed administration of IKK 16 one hour after LPS administration (P<0.05; Figure S2A - C, Figure S5A - D).

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

When compared to CKD/LPS mice with vehicle treatment, delayed administration of IKK 16 significantly attenuated the increases in cardiac phosphorylation of IKKα/β, phosphorylation of IκBα, nuclear translocation of p65 and iNOS expression (P<0.05; Figure S6A - D). Moreover, IKK 16 treatment significantly reduced cardiac phosphorylation of Akt and ERK1/2 (P<0.05; Figure S6E, S6F) in CKD/LPS mice.

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

Treatment of CKD/LPS mice with IKK 16 one hour after LPS administration significantly reduced the increases in lung MPO activity (P<0.05; Figure S1A)

and plasma inflammatory cytokine levels (P<0.05; Figure S1C - F).

To determine whether the observed higher levels of inflammatory cytokines in CLP challenged CKD control mice were due to increased cytokine production, macrophages isolated from either CKD sham or CKD control mice were incubated with different concentrations of LPS (0.1 ng/ml, 1 ng/ml and 10 ng/ml). Untreated CKD control-derived macrophages released significantly higher levels of IL-1β in the supernatant (P<0.05; Figure S7B). Yet significantly increased TNF-α released by CKD control-derived macrophages in comparison with CKD sham-derived macrophages was solely induced by a low dose LPS (0.1 ng/ml) stimulation (P<0.05; Figure S7A), no other significant difference was detected in the cytokine production levels in response to LPS stimulation (P>0.05; Figure S7).

References

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Figures and Tables

Table S1. Combined data sets from all groups studied prior to the intervention of endotoxemia/sepsis for the characterization of mice with chronic kidney disease (CKD) induced by subtotal (5/6th) nephrectomy (SNX).

Parameter	CKD Sham	CKD
Urea (mmol/L)	8.18 ± 0.41 (n=12)	17.43 ± 0.61 (n=14)*
Creatinine (μmol/L)	29.72 ± 0.38 (n=12)	45.96 ± 1.76 (n=14)*
Ejection Fraction (%)	72.78 ± 0.56 (n=11)	65.25 ± 0.89 (n=23)*
Fractional Shortening (%)	41.25 ± 0.48 (n=11)	35.85 ± 0.67 (n=23)*
Fractional Area Change (%)	50.64 ± 0.64 (n=11)	44.34 ± 0.91 (n=23)*
Alanine Aminotransferase (U/L)	27.57 ± 1.78 (n=12)	37.30 ± 4.45 (n=14)
MABP (mmHg)	93.64 ± 3.17 (n= 3)	107.8 ± 2.79 (n=7)*
Body Weight (g)	31.11 ± 0.82 (n=5)	29.13 ± 0.41 (n=8)*
Heart Weight (g)	0.135 ± 0.006 (n=5)	0.154 ± 0.004 (n=8)*
Heart Weight Index[a]	4.34 ± 0.10 (n=5)	5.29 ± 0.19 (n=8)*
Interventricular Septum (mm)	0.86 ± 0.03 (n=7)	1.10 ± 0.04 (n=12)*
LVID (D) (mm)	3.56 ± 0.08 (n=7)	3.63 ± 0.05 (n=12)
LVEDV (μL)	52.69 ± 2.51 (n=7)	56.05 ± 1.98 (n=12)
Plasma IL-1β (pg/ml)	15.83 ± 4.85 (n=3)	78.08 ± 19.36 (n=6)*
Plasma KC (pg/ml)	25.55 ± 25.45 (n=3)	105.4 ± 12.78 (n=6)*

Haemoglobin (g/dL)	13.41 ± 0.37 (n=8)	10.97 ± 0.34 (n=6)*
Haematocrit (%)	42.83 ± 1.50 (n=8)	33.38 ± 1.14 (n=6)*
White Blood Cells (K/μL)	6.57 ± 0.76 (n=8)	7.75 ± 1.14 (n=5)
Neutrophils (K/μL)	0.64 ± 0.08 (n=8)	0.24 ± 0.07 (n=5)*
Lymphocytes (K/μL)	5.80 ± 0.68 (n=8)	5.20 ± 0.82 (n=5)
Monocytes (K/μL)	0.03 ± 0.01 (n=8)	0.13 ± 0.02 (n=5)*
Neutrophil-to-Lymphocyte Ratio[b]	0.11 ± 0.01 (n=8)	0.45 ± 0.09 (n=5)*
Neutrophils (%)	9.73 ± 0.53 (n=8)	30.44 ± 3.20 (n=5)*
Lymphocytes (%)	88.3 ± 0.57 (n=8)	66.44 ± 3.69 (n=5)*
Monocytes (%)	0.55 ± 0.19 (n=8)	1.68 ± 0.26 (n=5)*

Mice underwent a two-stage SNX were compared with mice which underwent sham surgery. All data are expressed as means ± SEM for *n* number of observations. Data were analyzed by unpaired Student's t-test, or Mann-Whitney *U* test when *n* number of the group equals 3. **P*<0.05 versus the CKD sham group. [a]Heart Weight Index was calculated by dividing the weight of the heart in grams by the weight of the animal in kilograms. [b]Neutrophil-to-lymphocyte ratio was calculated as the ratio of the neutrophils to lymphocytes. MABP, mean arterial blood pressure; LVID (D), left ventricular internal-diastolic dimension; LVEDV, left ventricular end-diastolic volume; IL, interleukin; KC, keratinocyte-derived cytokine.

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

Parameter	CKD		
	Sham-operated	CLP + Vehicle	CLP + IKK 16
Number	7	7	7
Urea (mmol/L)	17.61 ± 0.66*	34.01 ± 6.41	21.90 ± 1.85
Creatinine (μmol/L)	46.44 ± 2.75	62.17 ± 5.69	52.59 ± 4.78
ALT (U/L)	42.44 ± 8.10*	240.7 ± 36.78	626.0 ± 308.2[a]

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). Plasma urea, creatinine and alanine aminotransferase (ALT) levels were assessed at 24 hours after CLP. All data are represented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. *P<0.05 versus the CKD + CLP + Vehicle group. [a]Two extremely high ALT values were detected in CKD + CLP + IKK 16 group.

Figure S1

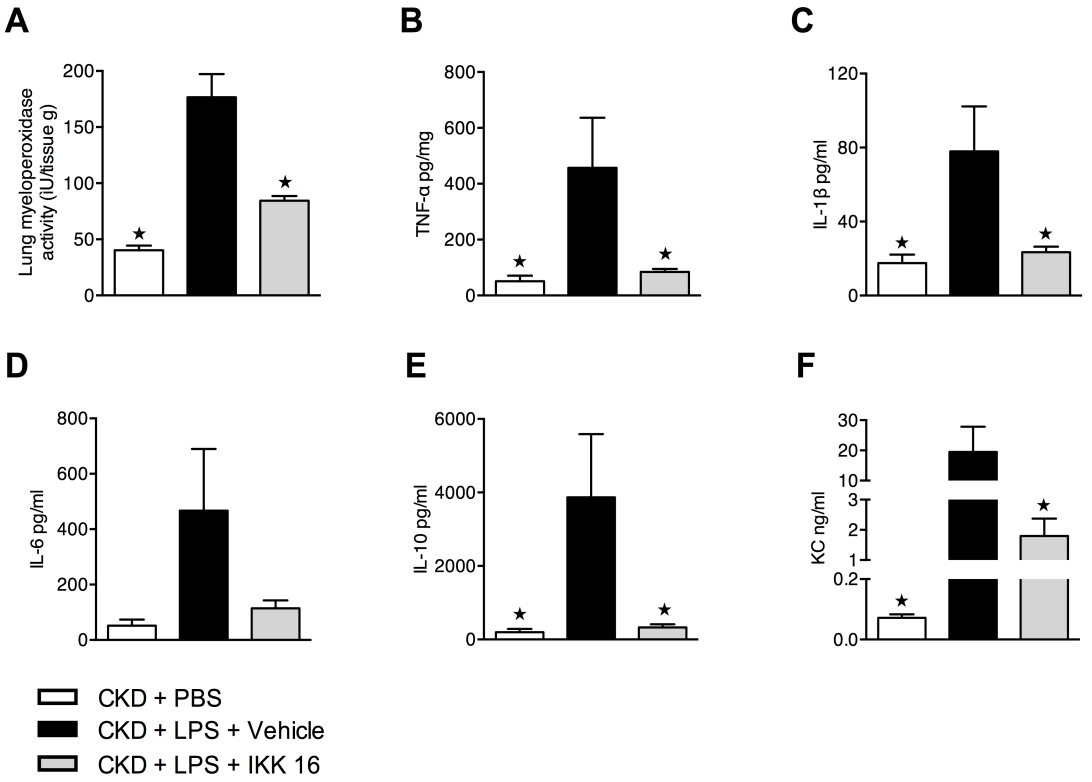

Figure S1. Effects of IκB kinase inhibitor on lung inflammation and systemic response in mice with chronic kidney disease (CKD) subjected to LPS administration. CKD mice received LPS (2 mg/kg) or PBS (5 ml/kg) intraperitoneally. One hour after LPS administration, CKD mice were treated with either IKK 16 (1 mg/kg i.v.) or vehicle (2% DMSO). Markers of lung inflammation and systemic response were assessed at 18 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=4-5 per group. All data are represented as mean ± SEM. Data

were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★P <0.05 versus the CKD + LPS + Vehicle group.

Figure S2

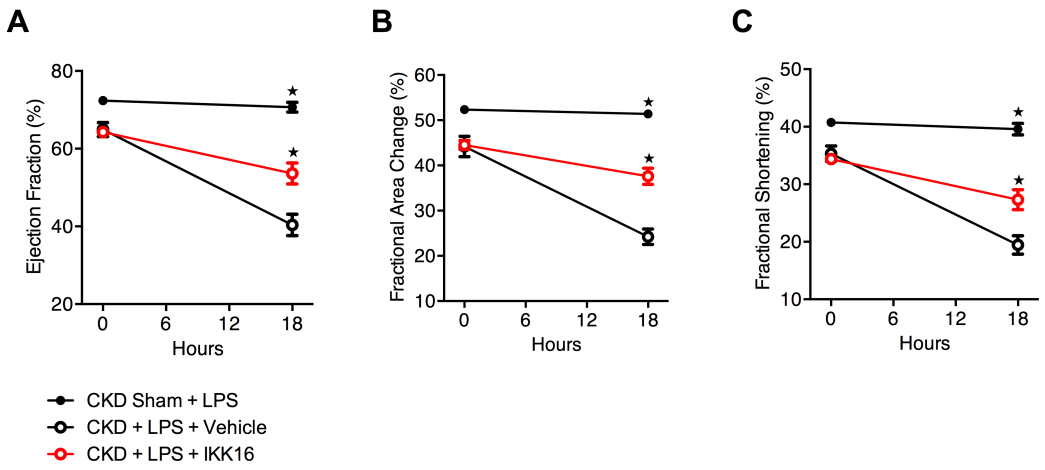

Figure S2. Effects of low dose of LPS (2 mg/kg) administration and IκB kinase inhibitor on cardiac function in mice with chronic kidney disease (CKD). CKD sham or CKD mice received LPS (2 mg/kg) intraperitoneally. One hour after LPS administration, CKD mice were treated with either IKK 16 (1 mg/kg i.v.) or vehicle (2% DMSO). Cardiac function was assessed befroe LPS administration and at 18 hours after LPS injection. Percentage (%) (**A**) ejection fraction (EF); (**B**) fractional area change (FAC); and (**C**) fractional shortening (FS). The following groups were studied: CKD sham + LPS (n = 7); CKD + LPS + Vehicle (n = 7); CKD + LPS + IKK 16 (n = 7). All data are represented as mean ± SEM. Data were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. ★P<0.05 versus the CKD + LPS + Vehicle group.

Figure S3

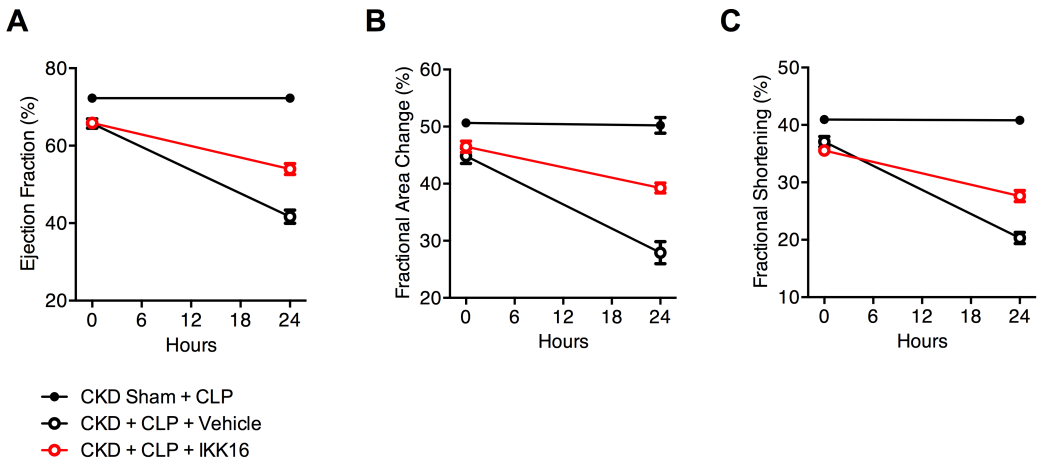

Figure S3. Effects of polymicrobial sepsis induced by cecal ligation and puncture (CLP) and IκB kinase inhibitor on cardiac function in mice with chronic kidney disease (CKD). CKD sham or CKD mice were subjected to CLP surgery. One hour after CLP, CKD mice were treated with either IKK 16 (1 mg/kg i.v.) or vehicle (2% DMSO). Cardiac function was assessed before CLP surgery and at 24 hours after CLP. Percentage (%) (**A**) ejection fraction (EF); (**B**) fractional area change (FAC); and (**C**) fractional shortening (FS). The following groups were studied: CKD sham + CLP (n = 7); CKD + CLP + Vehicle (n = 7); CKD + CLP + IKK 16 (n = 7). All data are represented as mean ± SEM. Data were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. ★P<0.05 versus the CKD + CLP + Vehicle group.

Figure S4

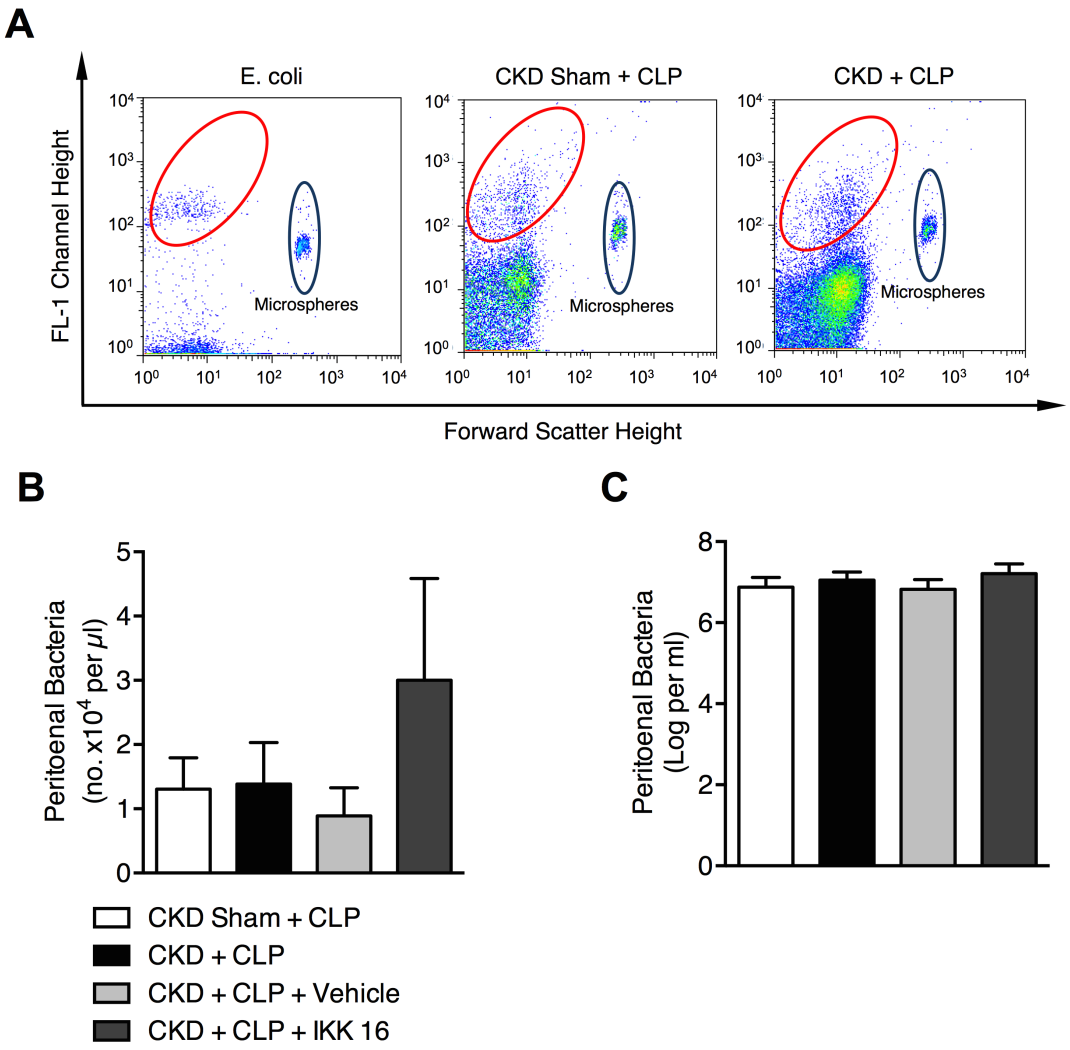

Figure S4. Peritoneal bacterial loads following cecal ligation and puncture (CLP) and IKK 16 treatment in chronic kidney disease (CKD) mice. Mice underwent CLP surgery. One hour after CLP, two groups of CKD control mice were treated with either IKK 16 (1 mg/kg *i.v.*) or vehicle (2% DMSO). (**A**) Representative flow cytometry scattergrams illustrating bacteria (SYTO BC bacteria dye) positive events in E. coli suspension (Left) as well as 24 hours post-CLP peritoneal exudates from CKD sham and CKD control mice. The density of bacteria in the experimental samples was determined from the

ratio of bacterial to microsphere signals. (**B - C**) Bacteria levels in peritoneal lavages from CKD sham and CKD control mice. N=3-6 per group. All data are represented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test.

Figure S5

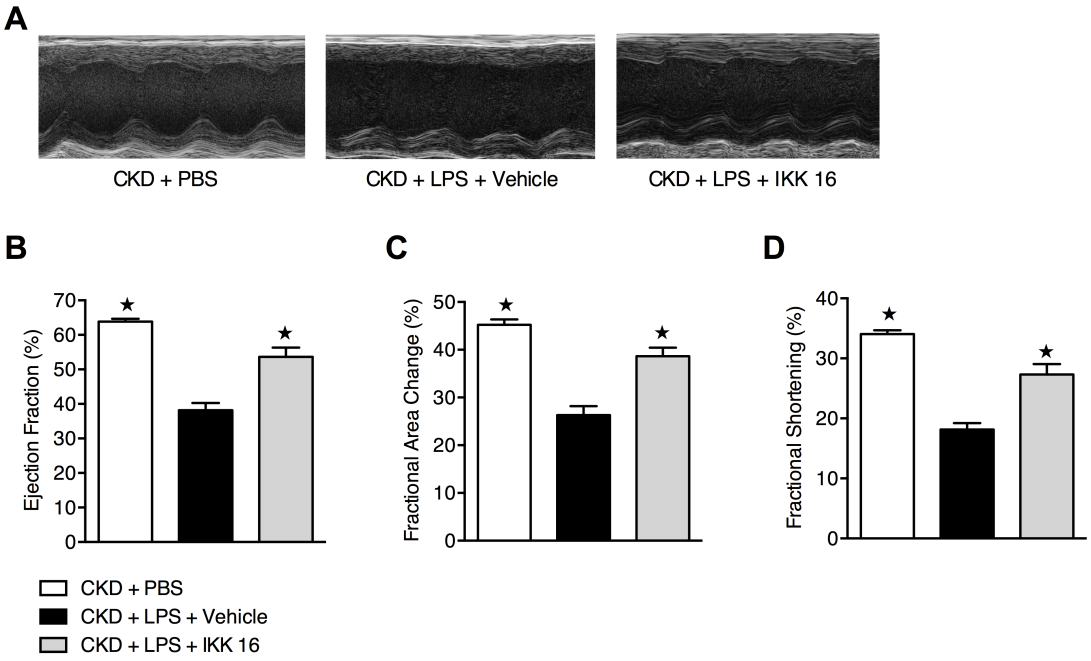

Figure S5. Effects of IκB kinase inhibitor on cardiac dysfunction induced by LPS in mice with chronic kidney disease (CKD). CKD mice received LPS (2 mg/kg) intraperitoneally. One hour after LPS administration, mice were treated with either IKK 16 (1 mg/kg i.v.) or vehicle (2% DMSO). Cardiac function was assessed at 18 hours. (**A**) Representative M-mode echocardiograms; percentage (%) (**B**) ejection fraction; (**C**) fractional area change; and (**D**) fractional shortening. The following groups were studied: CKD + PBS (n = 7); CKD + LPS + Vehicle (n = 7); CKD + LPS + IKK 16 (n = 7). All data are represented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★P<0.05 versus the CKD + LPS + Vehicle group.

Figure S6

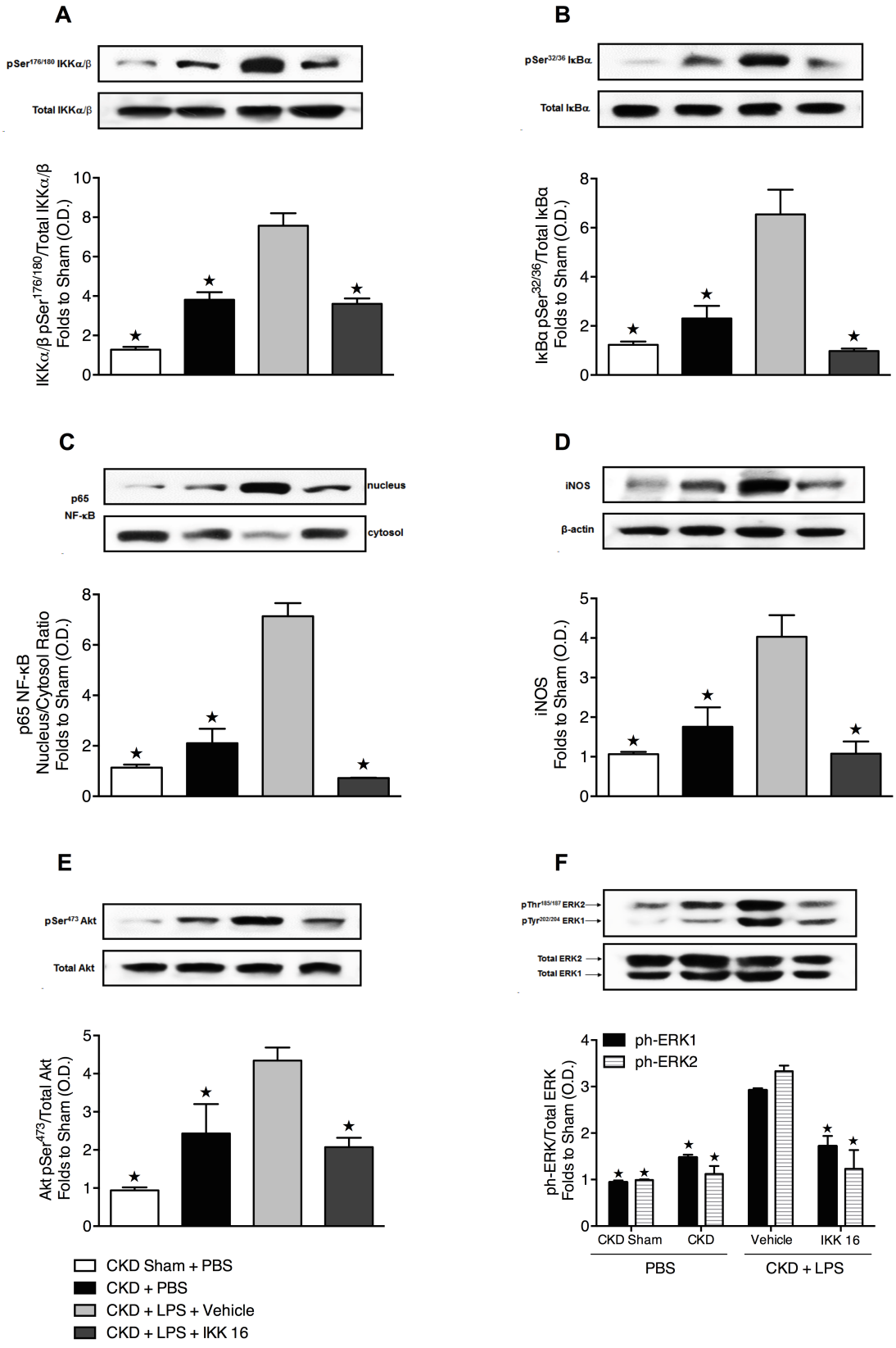

Figure S6. Effects of IκB kinase inhibitor on signaling pathways in hearts

of mice with chronic kidney disease (CKD) subjected to LPS administration. CKD sham received PBS (5 ml/kg), CKD mice received LPS (2 mg/kg) or PBS intraperitoneally. One hour after LPS administration, 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 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 are expressed as mean ± SEM for *n* number of observations. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. ★P <0.05 versus the CKD + LPS + Vehicle group.

Figure S7

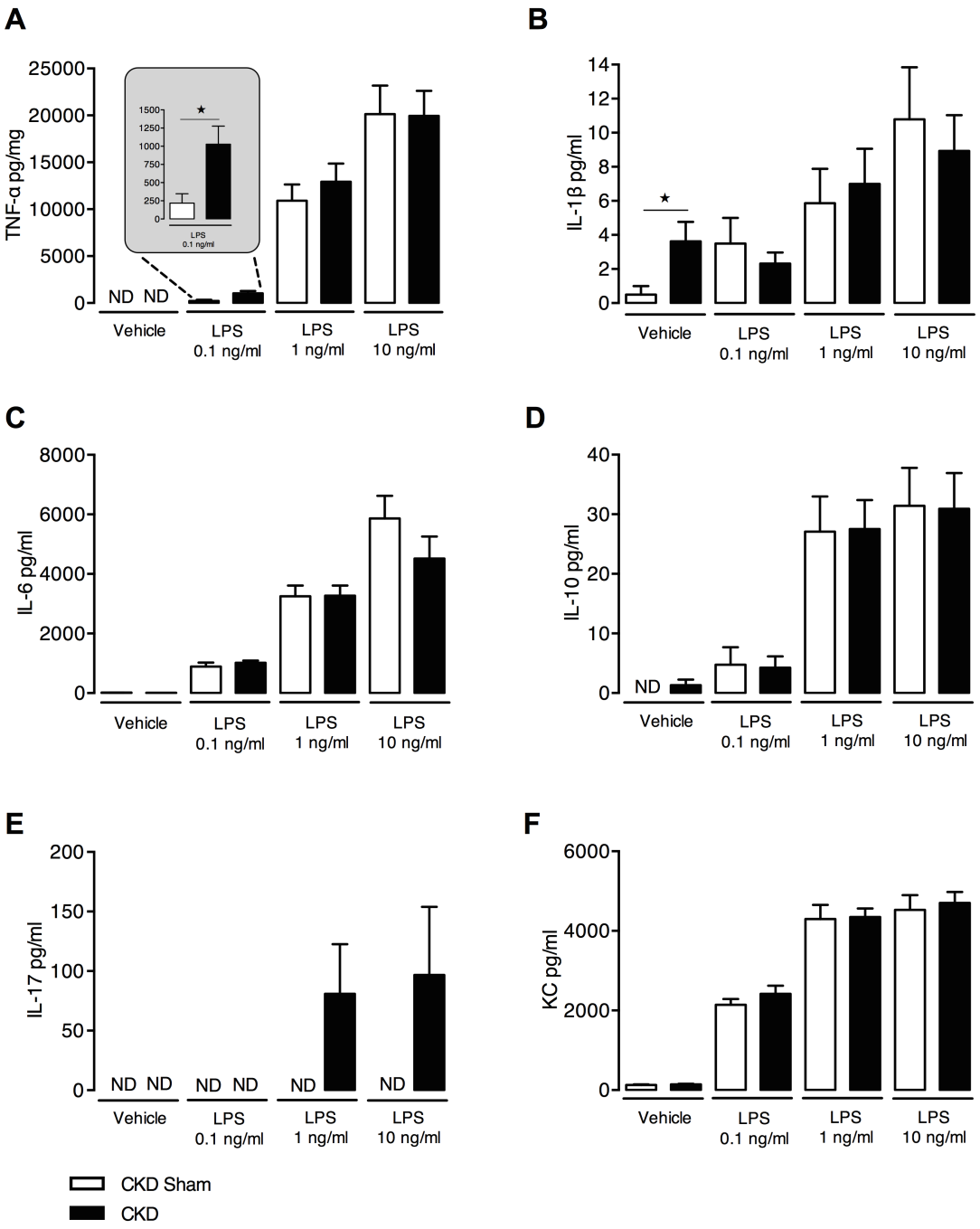

Figure S7. Cytokine production by macrophages derived from chronic kidney disease (CKD) sham and CKD control mice following LPS incubation. Biogel-elicited macrophages from CKD sham and CKD control mice were incubated with the indicated concentrations of LPS or vehicle (sterile PBS) for 6 hours at 37 °C before assessment of cytokine production in

supernatants by CBA. (**A**) Supernatant tumor necrosis factor (TNF)-α concentration; (**B**) supernatant interleukin (IL)-1β concentration; (**C**) supernatant IL-6 concentration; (**D**) supernatant IL-10 concentration; (**E**) supernatant IL-17 concentration; and (**F**) supernatant keratinocyte-derived cytokine (KC) concentration. N=4-7 per group. All data are represented as mean ± SEM. Data were analyzed by unpaired Student's t-test for comparisons between two groups with the same PBS or LPS treatment. ★P<0.05 versus corresponding CKD sham group. ND, not detected.