Title: AKI and CKD Worsen Outcomes in Experimental Sepsis: Creating a Reverse-translational Model of Acute Peritoneal Infection and Recovery in an Immunocompromised Rodent.

Authors:

Floyd, Deana F.[1], Colbert, James T.[1,2], Furgeson, Seth B.[1], and Montford, John R[1,2]

Affiliations:

[1]Renal Section, Rocky Mountain Regional VA Medical Center, Aurora, CO

[2]Division of Nephrology and Hypertension, University of Colorado School of Medicine, Aurora, CO

[3]Division of Infectious Disease, University of Colorado School of Medicine, Aurora, CO

Corresponding Author:

John R. Montford MD

Associate Professor of Medicine and Nephrology

University of Colorado School of Medicine

Rocky Mountain Regional VA Medical Center

1700 N. Wheeling St.

Research B2-112

Aurora, CO 80045

John.Montford@CUAnschutz.edu

Abstract

Introduction: Infection is a leading cause of morbidity and mortality in individuals with kidney disease. Both acute kidney injury (AKI), and chronic kidney disease (CKD) are clinical states that have been associated with higher risk of incident infection, and poor outcomes once infection has been established. A variety of host- and pathogen-specific factors are implicated as potential causes for these disparate outcomes including an altered host microbiome, innate and adaptive immune defects, and poor renal clearance and cytokines. However, there remains significant difficulty in modeling both human kidney disease and infection into an animal host. Likewise, there remains a poor understanding of the mechanisms underlying the unique immunodeficiency imparted by AKI and CKD, and if either condition imparts disparate risk.

Methods: C57BL/6J mice were given vehicle or aristolochic acid (AA) to create AKI (control, AKI groups) or CKD (control, CKD groups). Donor mice from all four groups underwent sterile cecal dissection and creation of cecal slurry (CS) preparations, which was later injected into separate mice in a matched host-recipient manner, at either high or lower doses. Animals were clinically monitored for either 24- or 72-hours after inoculation, then euthanized. Animal survival, sepsis severity, temperature, weights, and transcutaneous glomerular filtration rate (tGFR) were tracked longitudinally throughout the study. Histology for kidney injury, peripheral blood flow cytometry for leukocyte counts, plasma cytokines, and typical markers for organ injury were determined.

Results: Compared to controls, animals with AKI experienced much more severe sepsis across virtually all tracked metrics, and no animals with AKI survived high-dose CS injection past 24-hours. AKI mice manifested with a peripheral defect in leukocytes early after sepsis, with severe and persistent cytopenias, and a dramatically heightened early pro-inflammatory cytokine response. Septic CKD mice also had worse outcomes than controls, though less severe, and occurring later than in animals with AKI. Interestingly, animals with AKI had worse clinical outcomes and evidence of organ injury than mice with CKD at any dose or time-point after inoculation, despite a higher mean baseline measured GFR.

Conclusions: Rodents with established AKI and CKD experience worse clinical outcomes and organ injury versus controls in a model intraperitoneal live-bacterial infection. Additionally, mice with AKI experienced earlier and more severe morbidity and mortality than animals with CKD.

Introduction

Infection is a leading cause of death and disability in individuals with both acute kidney injury (AKI) [1-3] and chronic kidney disease (CKD) [4-7]. AKI is thought to engender an acute inflammatory state [8], cause remote organ injury [9], and impart a state of immunoparalysis and poor function in innate immune cells[10-12]. In advanced CKD, the uremic state has long been recognized as immunomodulatory [10, 13], and infection remains a leading cause of mortality in patients with CKD, including in those with End Stage Kidney Disease (ESKD) receiving renal replacement therapies (RRT)[7].

We established a novel model of AKI using low dose aristolochic acid (AA), a phytotoxin responsible for outbreaks of kidney failure in the developing world [14, 15]. We crafted a model of kidney failure using AA that would mirror an indolent, "slow" form of AKI with depressed GFR for several weeks as opposed to a more rapid model of with high variability and mortality. Separately, we created a CKD model using the same approach, but continued to injure animals causing a slow, but persistent decline in GFR with uremic sequela, mimicking human CKD. We then collected cecal contents from donor mice in these groups (along with paired control animals), created a purified homogenous cecal slurry, and injected recipient mice with the solution to create a model of abdominal sepsis. *Our overall hypothesis was that mice with AKI and CKD would have worse clinical sepsis outcomes than control mice, and that mice with preexisting CKD, having a much lower baseline GFR and thus organ reserve, would have worse outcomes than mice with preexisting AKI.*

Results

Establishing separate rodent models of AKI and CKD using low-dose Aristolochic Acid administration

AKI Model C57BL6/J male mice were given either vehicle (N=10) or AA (N=10) at a dose of 2mg/kg i.p. three times weekly for three weeks to create the model of indolent AKI (**Figure 1A**). Compared to vehicle controls, mice with AKI experienced a gradual, but significant decline in measured transcutaneous glomerular filtration rate (tGFR) (**Figure 1B**) starting at 1 week after initial injection of AA; culminating with a mean reduction in tGFR by nearly half of controls by day 21 (537 vs 966 μL/min/100g BW, $p<0.0001$, AKI vs controls, respectively) (**Figure 1D**). AKI animals had also lost significant weight versus controls (24.6 vs 29.0 g body weight, $p=0.04$), experienced slight anemia (13.6 vs 15.6 g/dL hemoglobin, $p<0.0001$), and had gross evidence of nephrosarca (0.75 vs 0.62 % kidney weight/body weight, $p<0.0001$). Plasma BUN was unchanged, though creatinine was

significantly elevated in the AKI mice vs controls (0.35 vs 0.14 mg/dL, p<0.0001) (**Figure 1D**). Histologically, glomeruli were preserved, and moderate acute tubular injury (ATI) was observed in mice with AKI; characterized by dilated and simplified tubular lumens, an expanded, edematous cortex with evidence of mild acute interstitial nephritis, and a slight loss of volume in the outer medullary stripe (3.3 vs 0.1 acute tubular injury (ATI) score, p<0.0001, AKI vs controls, respectively) (**Figure 1C, 1F**). Interstitial fibrosis was significantly increased versus control mice, although a minor finding (2.2 vs 0.7 % cortical fibrosis, p=0.002).

CKD Model Separate groups of vehicle (N=9) or AA-injected (N=10) mice were given a similar initial AKI protocol but then underwent further injections and were euthanized on day 84, 12 weeks after the first injection (**Figure 1A**). CKD mice experienced a comparable indolent AKI phase with similar weight changes and loss of tGFR during the first 21 days of injury (**Figure 1I**). By the end of the study, CKD mice regained some weight but remained significantly smaller than age-matched controls (25.4 vs 31.6 g, p=0.0007). However, there were notable clinical, biochemical, and histologic differences in CKD- vs AKI-mice. tGFR continued to significantly decline in CKD mice over time, to a mean of 348 vs 847 μL/min/100g BW in control mice by day 84. By 12 weeks, both plasma BUN (55.0 vs 26.4 mg/dL, CKD vs vehicle mice, respectively, p<0.0001) and creatinine (0.54 vs 0.17 mg/dL, p<0.0001) were significantly elevated and hemoglobin concentration was significantly depressed (9.4 vs 14.4 g/dL, p<0.0001) (**Figure 1J**). Kidney atrophy, not edema, was appreciated in wet-kidney weights of CKD mice (0.63 vs 0.46 %kidney weight/body weight, p<0.0001) (**Figure 1K**). Histologic sections again revealed preserved glomeruli, profound tubular atrophy, nephron dropout, widespread tubulointerstital nephritis and fibrosis, proteinaceous cast formation, and “medullification” of the cortical regions with significant volume loss in the outer medullary stripe (**Figure 1H, 1K**).

Mice with pre-existing AKI experience heightened clinical sepsis severity versus controls

Figure 2A-2C outlines the approach used in sepsis studies. N=30 vehicle control (No-AKI) and N=30 AAN (AKI) mice were generated as before. Baseline weights (**Figure 3A**) and tGFRs (**Figure 3B**) are shown. Sham solution (PBS/10% glycerol), low dose (0.2mL, 20mg) or high dose (0.3mL, 30mg) host-recipient matched cecal slurry was administered to No-AKI and AKI animals as a single intraperitoneal injection and mice monitored for either 24- or 72-hours. Among No-AKI controls, the sham, low-dose, and high-dose sepsis animals all had

100% survival at their designated endpoint collections; whereas animals in the AKI group experienced high attrition: one animal in the AKI group receiving low dose CS (t=24h euthanized for moribund status), and all animals in high dose CS groups (three non-survival by 20h; and remaining 20-24h euthanized for moribund status) (**Figure 3C**). All animals had a graded response to CS dose as measured by clinical sepsis scores and degree of hypothermia (**Figure 3D-F**). However, compared to No-AKI controls, animals with AKI had significantly worse sepsis severity and hypothermia by as early as 4 hours after inoculation, even when receiving lower CS doses (8.7 vs 5.5 4-hour sepsis score, p=0.0038, respectively).

Mice without AKI demonstrated a reduction in 5-10% of baseline (pre-sepsis) body weight peaking at 24 hours after sepsis inoculation, with most regaining body weight by 72 hours. Interestingly, in mice with AKI there was a significantly less weight loss than in No-AKI mice at 24hrs after low dose CS (-4.6 vs -8.3% weight loss, p<0.007) and a similar trend after high dose CS (-6.1 vs -8.5% weight loss, p=0.09). Mice with AKI also had a comparative inability ability to regain body weight by 72 hours after low dose CS (-5.0 vs -0.8% weight loss, p=0.008). Only mice with AKI given sham-injections gained weight throughout the study, most likely resulting from the cessation of AA-injections and expected AKI recovery (**Figure 3D**). Additionally, all recovered high-dose AKI mice, and one low dose AKI mouse, had significant burdens of detectable bacteremia (**Supplemental Figure 1**).

Figure 3 shows changes in tGFR values in all No-AKI and AKI experiments. At baseline, No-AKI and AKI mice had significantly divergent tGFRs (**Figure 3B**), consistent with prior data. In control mice, kidney function was preserved after CS injections, with only animals receiving high doses showing a non-significant trend in reduced renal function 24-hours after inoculation vs baseline (747 vs 1044 µL/min/100g BW, p=0.11, respectively); which showed normalization vs baseline in the 72-hour group (1095 vs 1155 µL/min/100g BW, p=0.28) (**Figure 3F**). However, in mice with existing AKI, a trend towards renal failure was found even at lower CS doses (372 vs 586 µL/min/100g BW, p=0.34 24-hours vs baseline). After high dose sepsis, most animals with AKI had little detectable kidney function (58 vs 564 µL/min/100g BW, p=0.0008, 24-hours vs baseline) appreciated by a “flat” tGFR curve (**Supplemental Figure 2**). It is important to note that few AKI mice receiving high dose CS underwent tGFR monitoring given the degree of sepsis severity (4/7 surviving mice at 20-24h). Likewise, the mean reported tGFRs for this group may be overestimated as the most severely ill animals were excluded, and were immediately euthanized to minimize further distress.

Mice with AKI experience worse organ injury with both low- and high-sepsis doses

Plasma BUN and creatinine largely mirrored the tGFR findings, with BUN showing the better early sensitivity reflecting functional decline in mice with AKI (**Figure 4A**). Compared with sham-injected No-AKI animals after 24-hours, only the No-AKI high dose sepsis groups had significantly increased BUN (64 vs 23 mg/dL, p=0.0001, respectively), but no changes in creatinine consistent with measured GFR. Animals with AKI showed more severe azotemia at both low- and high-CS doses having significantly increased BUN (55 and 101 vs 24 mg/dL, p=0.002 and <0.0001, respectively). Creatinine showed a delayed increase in AKI animals vs tGFR changes, peaking at 72h in the low-dose CS group, but was significantly elevated acutely in the surviving high CS dose groups at 20-24 hours. We also compared global sepsis biomarkers such as AST and plasma NGAL between the two groups. AST results paralleled those of BUN, and plasma NGAL performed the best as a sepsis biomarker, being significantly elevated with both low- and high-CS doses vs shams in the No-AKI group. Even so, mice with AKI had significantly higher plasma NGAL than control mice across the study at 24-hours (34852 vs 11635 ng/mL, p=0.02 (low dose) ; 66466 vs 29325 ng/mL, p<0.0001 (high dose), respectively). Plasma NGAL in AKI might come from many sources, including both the kidney and liver in an IL-6 dependent manner as we and others have previously published [16]. Therefore, we analyzed frozen kidney tissue for NGAL message by qPCR which showed a similar pattern of: upregulation only in high-sepsis dosed No-AKI animals, and significantly higher upregulation in both low- and higher-sepsis dosed AKI animals. Finally, frozen lung tissues were analyzed for MPO-enzymatic activity to determine if any remote lung injury from sepsis was present. Interestingly, lung MPO activity appeared to increase the most after 72-hours post-inoculation in a delayed fashion in recovering animals.

PAS-stained kidney sections from AKI mice and controls showed subtle changes in tubular injury assessed by a combination score of tubular simplification/dilation, presence of proteinaceous casts, and degree of interstitial nephritis. Control mice showed significant increases in tubular simplification and slight interstitial nephritis peaking at 72-hours (ATI scores 1.2 and 2.4 vs 0, p=0.001 and p<0.0001, low- and high-dose sepsis vs sham, respectively) though overall degree of histologic injury was minor. Determining differences in histologic injury in mice with preexisting AKI and sepsis was much more difficult; and while higher-dose CS injection trended towards worse tubular injury, driven by objectively more dilated tubules and interstitial nephritis (**Figure**

4C), these results were not statistically different versus baseline degree of tubular injury in sham-injected animals (**Figure 4B**).

The presence of AKI creates peripheral immune defects and aggravated acute immune response after sepsis inoculation

Flow cytometry was performed on peripheral blood specimens from affected animals. CS injection induced a dose- and time-dependent leukopenia in No-AKI mice, and this was worsened in mice with AKI (**Figure 5A**). Specifically, populations of lymphocytes, granulocytes, and monocytes all acutely dropped then partially rebounded by 72 hours after CS injection in No-AKI mice. Among mice with AKI no, or very little, rebound was appreciated among surviving animals. In lymphoid cells in AKI mice (**Figure 5A, middle panel**), CD19+ B-cells acutely fell 24-hours after CS injection and failed to rebound by 72 hours in low dose mice. However, CD4+, CD8+, and γΔ T-cells all increased as a percentage of leukocytes in AKI mice vs non-AKI mice.

Interestingly, neutrophils increased in no-AKI mice at 24-hours after CS inoculation, then fell by 72-hours. Opposite dynamics were appreciated AKI mice (**Figure 5A, left panel**). Eosinophilopenia was appreciated in a dose-dependent fashion similar to dynamics in B-cells. In mice without AKI, evidence of an acute increase in “M1-like” Ly6C-Hi expressing monocytes predominated early, followed by increasing “M2-like” Ly6C-Low monocytes later in sepsis (**Figure 5A, right panel**). Mice with AKI had impaired peripheral monocyte differentiation and, as with other leukocytes, trended towards cytopenias more than Non-AKI counterparts.

In order to examine if mice with AKI undergoing lower dose sepsis, who had more subtle clinical and biochemical worsening of sepsis severity vs No-AKI mice, had evidence of immune dysregulation- a multiplex cytokine array was performed on peripheral plasma samples (**Figure 5B**). No-AKI animals demonstrated modest, but significant increases in cytokines by 24-hours after CS injection vs sham controls (IL-1β: 1.3 vs 0.2 pg/mL p=0.004, IL-2: 1.3 vs 0.8 pg/mL p=0.02, IL-4: 0.45 vs 0.008 pg/mL, IL-6: 456 vs 40 pg/mL, p=0.002, CXCL1: 795 vs 58 pg/mL, p=0.0005, IL-10: 168 vs 30 pg/mL, p=0.001, IL-12p70: 1.69 vs 0 pg/mL, p=0.01, TNF-α: 39.4 vs 6.8 pg/mL, p=0.0004, respectively). In comparison, AKI animals with low dose sepsis were observed to have massively higher increases in all cytokines profiled 24-hours after CS inoculation vs AKI sham animals, and vs corresponding No-AKI animals, which largely returned to near normal values in mice collected at 72 hours.

Body weight CS dose adjustment does not correct differences in sepsis severity between AKI mice and controls

Mice with AKI have lower body weights than controls (**Figures 1D & 3A**), therefore at fixed volume doses of cecal slurry, mice with AKI are receiving a higher dose-to-body weight than control mice, potentially skewing sepsis severity worse in the AKI mice. Therefore, a separate group of No-AKI (N=10) and AKI (N=10) mice were generated and received a dosing strategy of 10ml cecal slurry per kg body weight (1mg stool / 1g body weight). Mean weights were 27.8g in No-AKI mice and 22.8g in AKI mice resulting in dosing that was slightly below the 0.3mL high dose strategy in No-AKI mice, and a dose slightly above the 0.2mL low-dose strategy in AKI mice (**Supplemental Figure 3A**). Body weight correction did not result in narrowing of comparative sepsis severity in No-AKI and AKI mice. And despite only slightly higher delivered dose to AKI mice vs low-flat dose strategy, these animals experienced significantly more severe sepsis and rapid animal attrition, while No-AKI mice experienced minimal distress (**Supplemental Figure 3B, 4C**).

CKD Studies

Generation of No-CKD (N=30) and CKD (N=30) mice proceeded as previously outlined (**Figure 1A**), and the CKD donor stool collection and experimental details was performed similar to AKI studies (**Figure 2A-C**). Baseline measurements are displayed in **Figure 6A, 6B**. As with No-AKI controls, the No-CKD control group had no mortality at either low or high sepsis doses, with similar dynamic trends of temperatures and sepsis scores (**Figure 6C-F**). In contrast to No-AKI controls, No-CKD controls lost considerably more weight across either low or higher CS doses at 24h (-8.8% and -11.1% weight loss, respectively) and failed to regain weight by 72 hours (-11.1% and -13.4% weight loss, respectively). This may be a consequence of the older age of mice, or higher baseline body weight vs the No-AKI controls before sepsis studies. Mice with CKD tolerated sepsis better than mice with AKI at either flat-dosing CS strategy, although two animals were euthanized (one at 36 hours and another at 48-hours, according to criteria) in the 72-hour high-dose studies. Surviving mice with CKD had similar weight changes after CS inoculation compared with No-CKD mice, with a minor (statistically insignificant) trend towards less overall weight loss.

tGFR measurements in sham animals from either group showed no significant changes across the study (**Figure 6D**). With low- or higher-dosed sepsis No-CKD and CKD mice showed similar patterns of functional decline, with only high-CS doses inducing significant decline in tGFR in 24 hour groups, though the magnitude of decline from baseline was not significantly different between the two groups (**Figure 6F**). CKD mice showed a minor, statistically insignificant decline in tGFR by 24 hours with low CS doses (**Figure 6E**). As in AKI studies, surviving mice at 72 hours in either group mostly had return of baseline kidney function. Bacteremia was again assessed, and only the two CKD mice with severe sepsis requiring euthanasia at 48 hours had evidence of detectable bacteremia (**Supplemental Figure 1A**).

Plasma measurements of BUN, AST, and NGAL showed similar overall dynamics in the CKD studies as appreciated in AKI studies, with some notable exceptions (**Figure 7A**). Baseline levels of BUN were higher in CKD mice than No-CKD mice (49 vs 24 mg/dL, $p=0.005$, respectively). As an overall sepsis biomarker, AST again performed reasonably well, peaking at 24-hours after high-dose sepsis in No-CKD mice (71 vs 26 U/L, $p<0.0001$), and in CKD mice (90.8 vs 24 U/L, $p<0.0001$); with CKD mice having significantly higher levels than No CKD mice. Plasma NGAL again performed well as a sensitive and specific sepsis biomarker, mirroring the clinical data in the CKD group the strongest. Acute tubular injury was noted in a dose-dependent manner in No-CKD control mice (**Figure 7C**), peaking again at 72-hours after injury. As with AKI specimens, kidneys from CKD mice had severe baseline tubular injury, and determining any new changes in new tubular injury vs sham-animals was extraordinarily difficult by histological methods. Objectively more proteinaceous cast formation was appreciated in kidneys from septic CKD mice, though this finding was not statistically significant (**Figure 7B**).

CKD mice have isolated, modest peripheral immune defects and cytokine elevation after CS inoculation vs No-CKD controls

Unlike AKI studies, mice with CKD had less severe differences in flow cytometry-sorted populations of leukocytes vs No CKD controls. Patterns of absolute leukopenia after CS injection were similar in both groups as compared to AKI studies, with CKD mice showing non-significantly lower total leukocyte blood numbers at 72 hours in both low and high dose CS studies (**Figure 8A**). CKD mice demonstrated significantly lower neutrophils, eosinophils, and significantly higher T-cell populations vs No-CKD mice, but these differences were overall modest. No differences were determined in peripheral blood mononuclear cell (PBMC) differentiation. Plasma

cytokines profiled from animal groups receiving low dose CS were again measured (**Figure 8B**), and CKD mice showed significant increases in IL-2, IL-6, CXCL1, IL-10, IL-12p70, and TNF-α vs No CKD control mice at 24-hours, though absolute increases at this time point and with this dose were comparatively lower than in AKI mice. Interestingly, IL-1β and IL-5 levels were significantly higher in septic controls vs CKD mice at 72 hours. The relative increase in IL-5 levels in control septic mice was accompanied by higher eosinophil levels profiled by flow cytometry at this time point (**Figure 8A, left panel**).

Body weight CS dose adjustment partially corrects sepsis severity between CKD mice and controls

As with AKI mice, CKD mice and controls have divergent weights (**Figures 1I, 6A**), potentially leading to inappropriate skewing of sepsis severity in CKD mice if using a flat-dosing strategy. Body weight adjustment experiments were again performed at 10ml/kg CS to body weight. Mean body weights at time of inoculation were 34.4 and 27.7g, resulting in an administration of cecal slurry dose that was higher in No-CKD mice than the 0.3 mL flat-high dose experiments, and dosing slightly below the 0.3mL flat-high dose experiments in CKD mice (**Supplemental Figure 3D**). Body weight adjustment partially corrected sepsis severity between No-CKD and CKD mice. CKD mice continued to experience moderate later attrition of numbers, though importantly the No-CKD control mice did experience low levels of mortality as well (**Supplemental Figures 4E, 4F**). These were the only control animals with preexisting normal kidney function in all the studies to suffer mortality from CS injection.

Of note, these two animals in the control group, which had high clinical sepsis scores requiring euthanasia by 36- and 48-hours were also the animals receiving the highest CS doses given their baseline weights, which were comparatively heavier than littermates (40.1g and 37.9g vs mean of 33.3g in the other littermates). The heaviest dosed mouse in the CKD group (31.0g) was also one that experienced the highest sepsis severity. Among other CKD mice there was no relationship between dose or sepsis severity, though there was little variability in baseline weights, and therefore doses (standard deviation of 1.39g body weight in other 9 CKD animals). If the two heaviest control mice were excluded from analysis, sepsis severity would appear similar to the flat-high dose No-CKD control group. These data indicate that a weight adjustment dosing strategy might cause *more* variability in sepsis response, especially if: 1) there is a large standard deviation of weights between animals in the same experimental group, and 2) adjustments are made at relatively higher CS doses, since

effects of CS appear to work in a linear dose-response manner, until a certain threshold is reached then sepsis severity appears to progress in a non-linear manner.

Discussion

We have described a new parallel method of creating AKI and CKD with repeated, low-dose injections of aristolochic acid; which was then utilized to study the dynamics of acute intrabdominal infection in an at-risk host. We believe these findings are important for several key reasons. 1) The AAN model, while having some variability, nevertheless appears to be well suited to reliably reproduce distinct clinical states of AKI and CKD. 2) The CS live-bacterial infection approach allows for a study of acute sepsis dynamics that may not be possible with more blunt methods like CLP given the fragility of the underlying host. 3) We have shown that AKI predisposes to worse sepsis outcomes than CKD in this AAN model; and this observation occurred despite higher baseline measured GFR in mice with AKI, and at a slightly younger animal age. 4) Finally, we have demonstrated that “AKI-on-AKI” and “AKI-on-CKD” can be effectively modeled. Resultingly, our findings may have major translational implications beyond the immediate scope of this study.

Our studies add to the literature regarding the dysregulated nature of immunity in hosts with AKI and CKD. One group of investigators was able to successfully model cecal ligation and puncture (CLP) in animals suffering AKI from folic acid nephropathy[17] and in mice with accelerated CKD from remnant nephrectomy[18]. Animals receiving CLP with preexisting AKI or CKD in these studies suffered high mortality. We believe the models we have crafted, with more indolent form of AKI and CKD using AA is an ideal method to study sepsis in these unique immunocompromised states as it has low periprocedural mortality, doesn’t rely on prior surgical manipulation, is a model of live-bacterial infection, and allows for the study of different sepsis dynamics in a dose-response manner.

Our data raise interesting new questions regarding the early acute inflammatory response and its apparent dysregulation in the presence of kidney disease. Animals with AKI experienced evidence of a hyperacute inflammatory response, yet had early attrition high degrees of bacteremia, and severe cytopenias. We hypothesize that early exhaustion in innate immune cells (possibly in neutrophils and activated macrophages) results in poor pathogen clearance in the infected peritoneum. The presence of persistent cytopenias in AKI mice are a particularly interesting finding and warrant further study. In CKD mice there was

delayed mortality, and we are unable to determine if these animals have a unique counterregulatory innate or adaptive immune defect; or a similar, though less severely dysregulated, acute aggravated response as in AKI mice.

Of note, there is an appreciation that acute tubular injury can accompany high loads (30-50%) glycerol administration [19] . We found no appreciable increase in tubular injury in control, AKI, or CKD mice receiving sham PBS-glycerol injections assessed by histology, biochemically with BUN, Cr, as well as directly measured GFR. We administered sham animals the higher dose of PBS-glycerol (0.3mL) to mirror the highest possible dose of PBS-glycerol the infected animals were given in anticipation of this potential confounder. Sham injected AKI mice actually had improvement in renal function, which is similar to an unpublished observation we previously made when generating the original AAN-CKD model, leading to the addition of the 4-6week period of repeated AA-injections as necessary to maintain sustained GFR decline (**Figure 1A**). We presume this transient improvement in renal function and rebound in weights represents partial AKI recovery after cessation of AA injections.

Our study has some limitations. Some physiological data of sepsis severity is lacking; and tGFR measurements were performed at baseline and at terminal collection only. Given the degree of frailty in the animals after CS inoculation, we chose to manipulate them as infrequently as possible, so as not to influence their clinical outcome. Importantly, we chose not to perform serial blood draws on individual animals given that this intervention might worsen sepsis severity. This concern was especially relevant among animals with existing kidney injury, whom have evidence of anemia at baseline (**Figures 1E, 1J**). Separate sham-operated animals were used as alternative controls in this regard. We did not administer prophylactic fluids (except in a reactive manner with severe sepsis as outlined in methods), nor antibiotics so cannot determine if the mice with high morbidity and mortality would be rescued with these interventions.

We chose 24- and 72-hours as medium-term clinical endpoints for the model based on published data and our own observations of sepsis evolution after this method of cecal slurry injection. We acknowledge that our data on plasma cytokines and flow cytometry is not a complete picture- that understanding much earlier events, such as occurring at 1-4-hours after inoculation when evidence of clinical divergence in animal groups was first observed, is necessary to draw more firm conclusions about our observations. Additionally, clinical mortality should be assessed beyond 72-hours in surviving mice, particularly given our observations that persistent immune dysregulation in some animals is apparent at this later time point.

The AA-model itself might influence the sepsis phenotype, independent of degree of kidney injury and measured GFR. However, AA is not known to induce other immune cell defects; and we did not appreciate that AKI or CKD mice had significantly different peripheral immune cell populations versus controls at baseline. Finally, we acknowledge that our findings might be different with alterations in animal strains, species, sex, and different models of kidney injury and sepsis. There is an enormous number of potential variables which might result in different outcomes with altered acute host-pathogen interactions in early sepsis. Nevertheless, our results offer intriguing observations of the dysregulated nature of the early immune response against bacterial pathogens in hosts encumbered by different types of pre-existing kidney disease.

In conclusion, we have demonstrated that stable, clinically relevant AKI and CKD states can be modeled in a rodent with slow aristolochic acid injections; and that by applying a novel method of live-bacterial sepsis to these established models, many different dynamics of sepsis initiation and recovery can be studied. Furthermore, our data indicate that sepsis severity is worse in AKI than CKD, possibly because of a comparatively more dysregulated early innate immune response.

Methods

Rodent model of Acute and Chronic Aristolochic Acid Nephropathy (AAN)

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Colorado Anschutz Medical Campus and the Rocky Mountain Regional VA Medical Center. C57BL/6J male mice were procured and underwent experimentation starting at 8-10 weeks of age. Vehicle control (phosphate buffered saline, PBS) or AA (AA-1 sodium salt, Sigma) was administered at a dose of 2 mg/kg intraperitoneal (i.p.) thrice weekly x 3 week for the AKI model, and thrice weekly x 3 weeks plus weekly x 3 weeks, then allowed to recover for an additional six weeks for the CKD model (**Figure 1A**). Animals used for the initial model description underwent serial assessments of weight and transcutaneous glomerular filtration rate (tGFR, see below) at baseline, and weekly until euthanasia in the AKI model, and baseline followed by assessment at 3, 6, and 12 weeks in the CKD model. These animals were euthanized on day 21 (AKI model) or day 84 (CKD model). Separate animals used for the cecal slurry (CS) sepsis studies underwent weight and tGFR assessment only immediately prior to inoculation, after kidney disease had been established.

Cecal Slurry Preparation

Cecal slurry creation was performed as described originally [20], with important recent modifications [21, 22]. Sham-sepsis animals (see procedure below) were identified as suitable cecal slurry donors and, at the time of terminal collection, underwent isolation of cecum, sterile dissection of contents, suspension of contents into 10% glycerol in sterile PBS at 100mg stool/mL (**Figure 2B**). Contents were press-filtered serially through sterile 860, 320, 180µM filters (Bellco Glass, Inc., Vineland, NJ) and a 100µM sterile cell strainer (ThermoFisher Scientific, Waltham, MA). A small aliquot (50-75µL) of the final solution from each animal was separated for later 16s DNA analysis. The final solution from each animal was combined with similar grouped littermates to create a homogenous host-matched cecal slurry. Slurry was then aliquoted into separate cryovials under conditions of continuous stirring using a sterile magnetic bar on stir plate, frozen on dry ice immediately and stored at -80degC. Cecal slurry stocks were used within 6 weeks of generation, though published data indicate bacterial loads are completely stable up to 6 months and beyond using this method[21]. On day of experimentation, slurry vials were rapidly thawed in a 37degC shaking water bath, transported to the vivarium and kept warm on a 37degC heat block for a short period until injection. Shortly before injection, vials were agitated by hand, sample drawn up with a sterile 0.5mL tuberculin syringe and injected into the right lower ventral abdomen into the peritoneal space at the indicated volume.

Cecal Slurry Peritonitis

Separately generated AKI- and CKD-mice underwent cecal slurry sepsis experimentation starting at the two previously established AAN time points (**Figure 2A**). Each AKI- and CKD-cohort was accompanied by separate vehicle injected animals as before, designated hereafter as “No-AKI” and “No-CKD” controls. No-AKI, AKI, No-CKD, and CKD mice received either a single intraperitoneal injection of sham-sepsis vehicle solution (10% glycerol in PBS at 0.3mL), low dose (0.2mL, 20mg), or high dose (0.3mL, 30mg) host-matched cecal slurry. Additional groups were generated for the supplemental body weight dose-adjustment experiments (10ml slurry/kg body weight, or 1mg/g, **Supplemental Figure 3**). With each dosing strategy, separate paired groups were generated for planned analysis at either 24- or 72-hours after inoculation. Sepsis scores (Adapted by Shrum B et al.[23]) with slight modifications (**Supplemental Figure 4**) and temperatures obtained by rectal probe (PhysioSuite, Kent Scientific, Torrington, CT) were procured at baseline (immediately before inoculation), 1 hour,

4 hours, and 24 hours in all animals; and at 48 and 72 hours in the 72-hour designated group. tGFRs were only assessed at baseline and time of euthanasia (24 or 72 hours). Daily weights were also assessed until collection (**Figure 2C**). Mice were euthanized after final tGFR performed via terminal isoflurane inhalation using a drop method, dissected with bilateral thoracotomy and laparotomy, EDTA-whole blood obtained by cardiac puncture and separated for analysis of frozen plasma or flow cytometry; kidneys and lungs dissected and placed in 4% paraformaldehyde for histology and separate tissues frozen on dry ice.

Transcutaneous GFR

tGFRs were assessed for animals in **Figure 1** as previously mentioned. For sepsis studies, all measurements were taken on days 21 or 84: (1) as a baseline measurement and (2) 24- or 72-hours after sham or cecal slurry inoculation. Mice were shaved 1-2 days prior to initial baseline measurement to reduce skin aggravation on the day of device readings. Animals were anesthetized with 1.5-2.5% isoflurane, placed on a heating platform (Kent Scientific), where the shaved area on the right dorsal thorax was cleaned with 70% ethanol and allowed to dry. A charged battery was attached to the tGFR-mini device (Medibeacon, Mannheim Germany), which was then attached to the animals with a proprietary transdermal sticky pad. Surgical tape was used to snugly secure the device in place. FITC-Sinistrin (10mg/100g body weight, Medibeacon) was then administered via a retro-orbital injection and mice monitored for a minimum of 70 minutes. Measurements were read with proprietary software and GFR derived from FITC-Sinistrin half-life as per the manufacturers recommendations[24] and as detailed in our previous publications.[25-27]

Cytokine analysis, organ injury, bacteremia

Plasma was isolated from EDTA-anticoagulated whole blood, centrifuged at 2,000 xg for 10 minutes at 4degC, and frozen at -80degC for future measurements. Blood urea nitrogen (BUN), Creatinine, and Aspartate transaminase (AST) were assessed using commercially available kits (BioAssay Systems, Hayward CA). Lipocalin-2 (NGAL) was assessed with a Quantikine ELISA Kit (Biotechne R&D Systems, Minneapolis MN). Additionally, a multiplex inflammatory cytokine panel (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO (CXCL1), and TNF-α, MesoScale Discovery, Mouse V-Plex Proinflammatory Panel 1) was performed following manufacturer instructions and analyzed using the MESO Quickplex SQ120 instrument and Discovery

Workbench software. Lung myeloperoxidase (MPO) activity was measured with frozen harvested tissue. Lung samples (30-50mg) were suspended with 1mL of HTAB buffer (2g hexadecyltrimethylammonium bromide in 400mL MPO Buffer- 3.4g of potassium dihydrogen phosphate and 4.35g of dipotassium hydrogen phosphate in 450mL deionized water, pH 6.0) and homogenized using a sterile 5mm stainless steel bead in a Tissue Lyser 2 (Qiagen, Hilden, Germany) for 90 seconds at 1/60Hz. The homogenate was transferred to a 2mL tube and sonicated for 10 seconds, then centrifuged at 14,000 xg for 30 minutes at 4degC. MPO activity was measured by combining supernatant with 3% hydrogen peroxide in o-dianisidine MPO solution (16.7mg o-dianisidine in 90mL DI water with 10mL MPO buffer, heated to 37degC). A plate reader was used to measure absorbance at 450nm. Total protein quantity was measured using a Bradford Assay Kit (ThermoFisher Scientific). Bacteremia was assessed by plating 10µL of EDTA-whole blood onto trypsic soy agar plates, serial diluted 1:2, then by a factor of 10 to 1:1000, incubated for 24 hours at 37degC, and detection was determined. Of note in several of the high grade bacteremia samples we were unable to quantify number of colonies as they were not diluted enough, therefore results were reported as positive/negative rather than determination of CFU counts due to incomplete data.

Harvested mouse organs (kidney, lung) were preserved in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 24 hours before being processed and embedded in paraffin-blocks. Blocks were cut to 5µM sections and stained for collagen using Picrosirius Red Staining; and fibrosis was quantified using an analysis of polarized PSR-stained cortical tissues as we have previously outlined[28]. Separate sections were stained using a commercially available periodic acid Schiff (PAS) staining kit (Newcomer Supply, Middleton, WI). Acute tubular injury was scored on PAS sections using a modification of published methods[29, 30] as follows: Injury including evidence of brush border loss, tubular dilation or atrophy, cell necrosis, presence of proteinaceous casts, and degree of tubulitis and interstitial nephritis was graded from 0-5 with none (0), < 10% (1), 11-25% (2), 26-45% (3), 46-75% (4), and > 76% (5) of affected corticomedullary kidney regions. If corticomedullary boundary could not be determined (as in CKD samples), random mid-cortical regions were analyzed. Approximately 3 separate sections of transverse cut kidney tissues were examined with 4-6 20x light microscopic fields examined per section and scored in blinded fashion, with sample identification masked. Samples were randomly scored a second time and an average of the two scores were obtained.

Peripheral Blood Flow Cytometry

EDTA-treated whole blood obtained by cardiac puncture (150µL) was treated with RBC lysis buffer (eBioscience, ThermoFisher Scientific) for 3 minutes, washed with FA3 buffer (PBS, 10mM HEPES, 2mM EDTA, 1% FBS), centrifuged at 300 xg x 5 minutes (underwent an additional RBC lysis for 1 minute and wash/centrifuge again if needed to clear sample), passed through a 70µM filter, and resuspended in FA3 buffer before being plated on 96-well plate for staining. Cells were counted using an automated cell counter (BioRad, Hercules, CA). After block with anti-mouse CD16/CD32 (Invitrogen, ThermoFisher Scientific) and stain with a viability dye (GhostDye Violet 510, Tonobo, Cytek Biosciences, Fremont, CA), cells were incubated at 4degC with the following fluorochrome conjugated primary anti-mouse antibodies for 45 minutes (all Biolegend, San Diego, CA): anti-CD45 PE-CF594 (clone 30-F11), anti-Ly6G PECy7 (clone 1A8), anti-CD11b PE (clone M1/70), anti-Ly6C BV785 (clone HK1.4), anti-TCRβ APC (clone H57-597), anti-CD4 BV605 (clone RM4-5), anti-CD8 APC-Cy7 (clone 53-6.7), and anti-CD19 PacBlue (clone 6D5). Cells were then centrifuged, washed and resuspended in FA3 buffer, and finally fixed in 2% PFA. Cells were processed through a Cytoflex LX flow cytometer (Beckman Coulter, Indianapolis, IN), and data analyzed using FlowJo single-cell analysis software (FlowJo, Ashland, OR). Gating proceeded as follows: Live → singlets → CD45+ → (CD19+ B-cells) CD19- → (TCRβ+ T-cells → CD4 vs CD8 T-cells (γΔ-Tcells as double negative population)) TCRβ- → (Ly6G+SSC-Hi Neutrophils) Ly6G-SSC-Low → CD11b+ → Ly6C+SSC-low Macrophages, Ly6C-SSC-low Macrophages, Ly6C-intSSC-high Eosinophils.

Statistics

A student's T-test was performed for 2 variable comparison, 1-way ANOVA for more than 2 variables, with Tukey's multiple comparison test. For grouped analyses, a 2-way ANOVA was performed. For comparisons within groups, statistical significance was only determined between time- and dose-endpoints versus the corresponding sham condition (i.e. sham vs low-dose 24hr, sham vs low dose 72hr), whereas comparisons between groups included analysis of only parallel condition and timed endpoints (i.e. Control low-dose sepsis 24hr vs AKI low-dose sepsis 24hr). Aggregated clinical data was used in the case of weights, sepsis scores, and temperatures, where data from N=10 experimental animals was used at: baseline, 1 hour, 4 hours, and 24 hours after inoculation. Thereafter (48h, 72h) data included only the N=5 remaining animals in the 72-hour designated

group. If the comparisons between groups contained missing data because animals did not survive to predetermined endpoint, these data were excluded from analysis. Samples collected prior to predetermined endpoint due to moribund status were included in either 24- or 72-hour analyses on an individual basis. The affected animals included: all AKI animals receiving high dose sepsis designated for collection at 72 hours (all euthanized at, or shortly before, 24 hours as outlined and were considered as additional 24-hour high-dose sepsis animals). Additionally, two animals in the high dose CKD 72-hour studies needed euthanasia before predetermined endpoint; one (moribund by 36 hours) was included with animals in the 24-hour group, and the other (moribund by 48 hours) was included with the 72-hour group in analysis of biochemical and histologic measurements.

Acknowledgments

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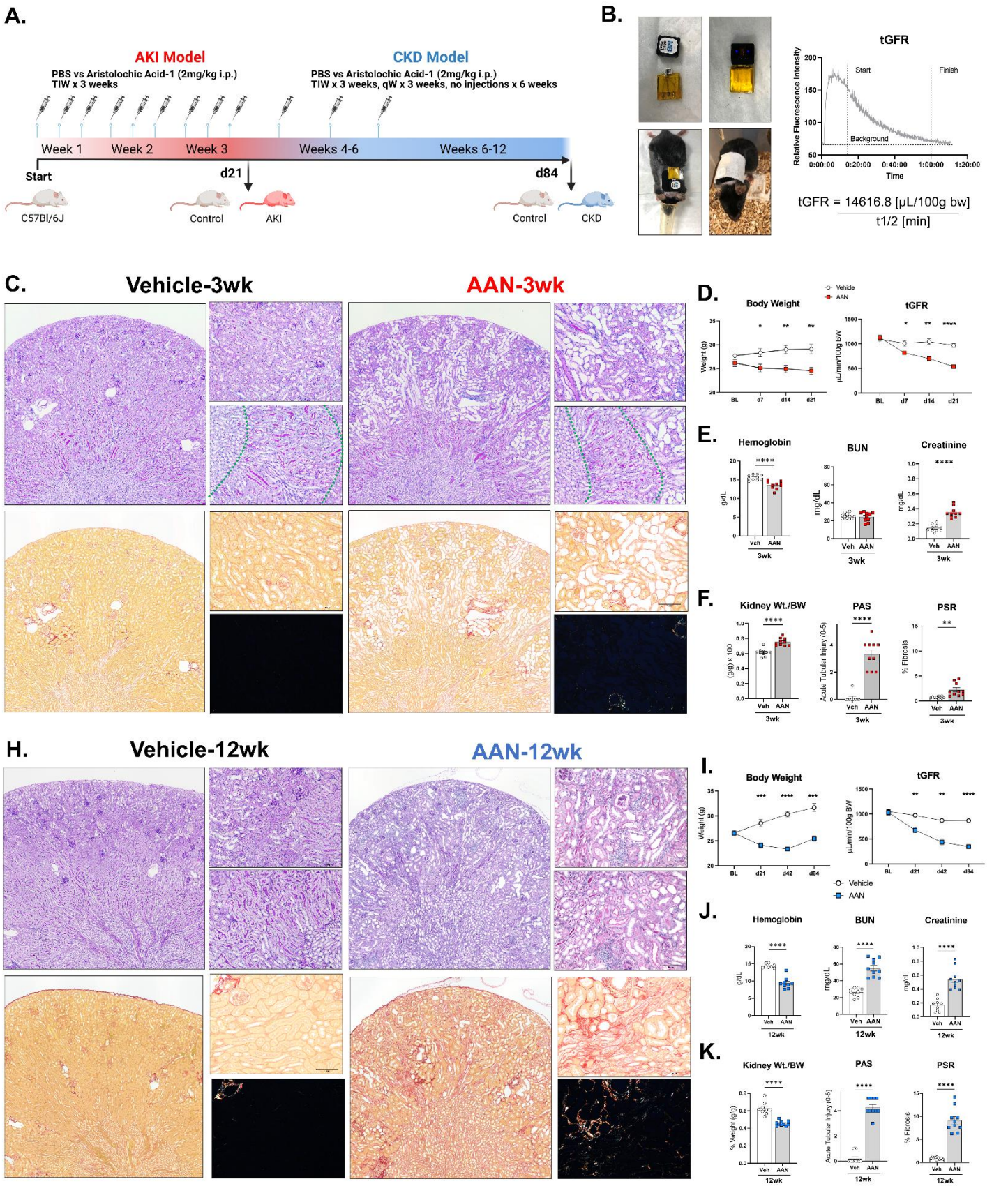


Figure 1. Creation of AAN-AKI and -CKD Models in C57BL/6 Mice (A) AKI and CKD was induced using AA. (B) An example of tGFR procedure and derivation of tGFR in µL/min/100g body weight. Shown are (C, H) 4x and 20x(2) histologic images of PAS-stained kidneys, along with 4x and 20x (with corresponding polarized

image) PSR stained sections. (F) Quantification of PAS and PSR sections are shown. During the studies, (D,I) body weights and tGFR trends were determined. Upon terminal collection, (E,J) whole blood hemoglobin, plasma BUN and creatinine concentrations, and (F) kidney weight/body weights were determined. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

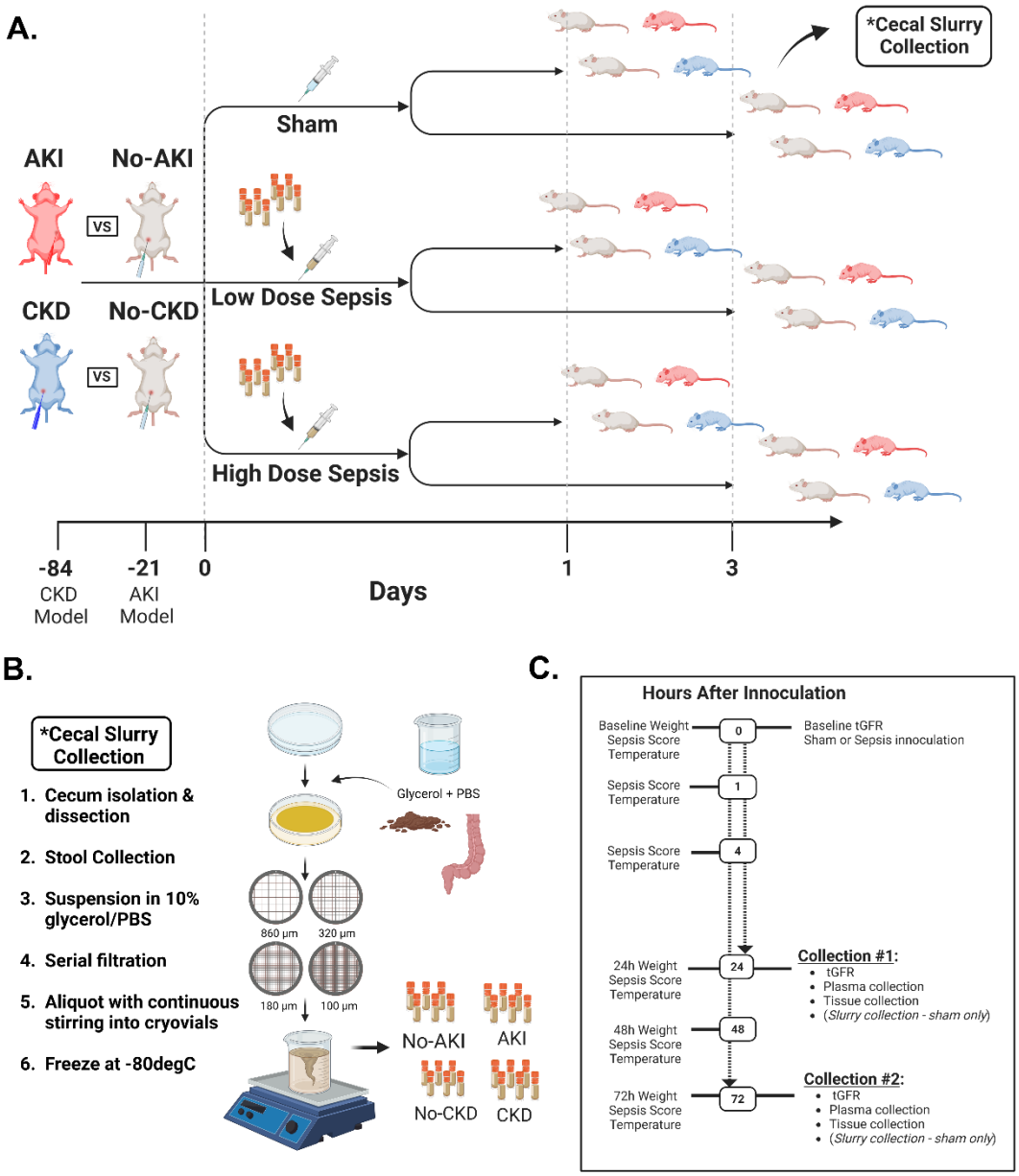


Figure 2. Design of CS Studies (A) Outline of study design. AKI or CKD groups generated, animals receiving sham injections also served as CS donors. (B) Creation of CS host-matched stock solutions. (C) Diagram of post-CS inoculation clinical monitoring and endpoint analyses.

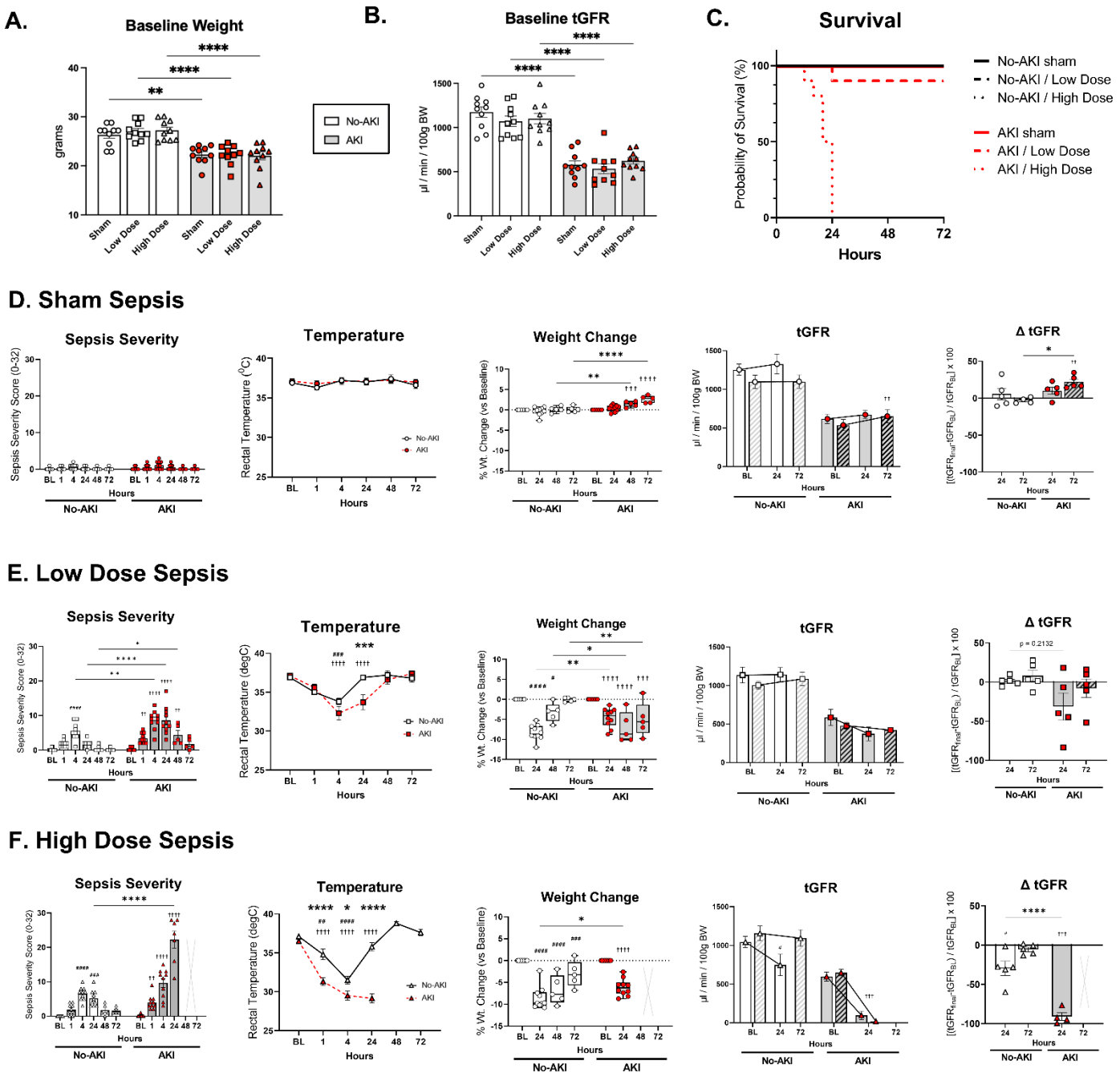


Figure 3. AKI CS Sepsis Studies. (A) Baseline weights and (B) tGFRs are shown. (C) AKI animals suffered early, severe mortality in high dose groups, No-AKI animals had 100% survival in all groups. (D) Sham, (E) Low-dose, and (F) High-dose sepsis studies are shown with AKI and control animals. Sepsis severity and rectal temperatures revealed worse sepsis severity starting by 1-4 hours after CS inoculation in AKI animals. AKI mice lost modest weight, and both No-AKI and AKI animals had significant lowering of tGFR by 24-hours in high-sepsis doses, with AKI mice suffering the most decline. (Within No-AKI group: # p<0.05, ##p<0.01, ###p<0.001,

####p<0.0001; Within AKI group: †p<0.05, ††p<0.01, †††p<0.001, ††††p<0.0001; No-AKI vs AKI group: * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

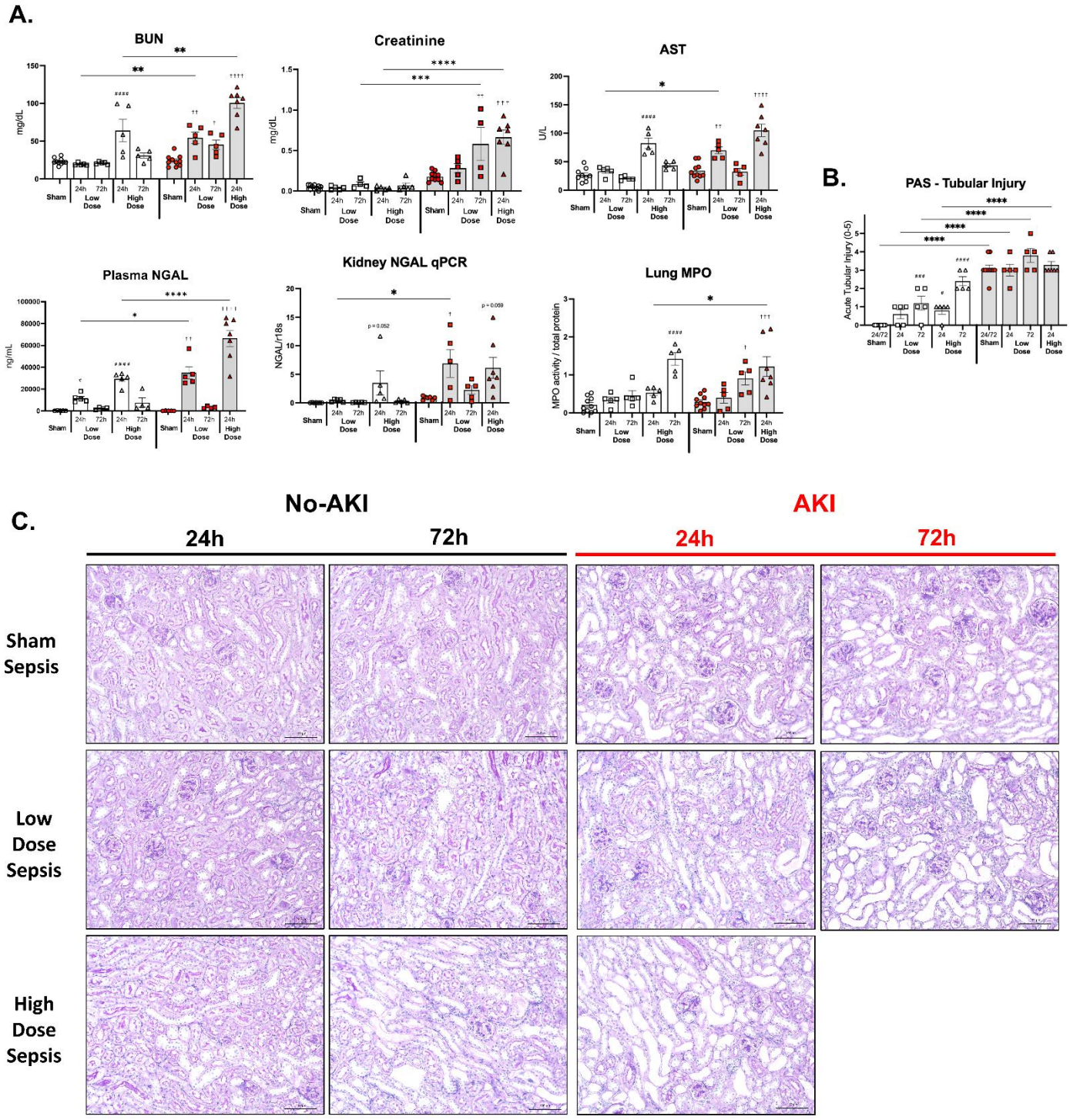


Figure 4. AKI mice show evidence of early and late organ dysfunction during sepsis. (A) Plasma BUN, Creatinine, AST, NGAL levels and kidney NGAL qPCR message levels demonstrated significantly elevated levels in both low- and high CS dosed AKI animals vs No-AKI controls. (B,C) PAS images (20x) from cortical regions of affected animals demonstrate a delayed increase in acute tubular injury, peaking at 72 hours after CS inoculation. ATI in mice with preexisting AKI was difficult to discern vs in No-AKI control mice. (Within No-AKI

group: # $p<0.05$, ##$p<0.01$, ###$p<0.001$, ####$p<0.0001$; Within AKI group: †$p<0.05$, ††$p<0.01$, †††$p<0.001$, ††††$p<0.0001$; No-AKI vs AKI group: * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

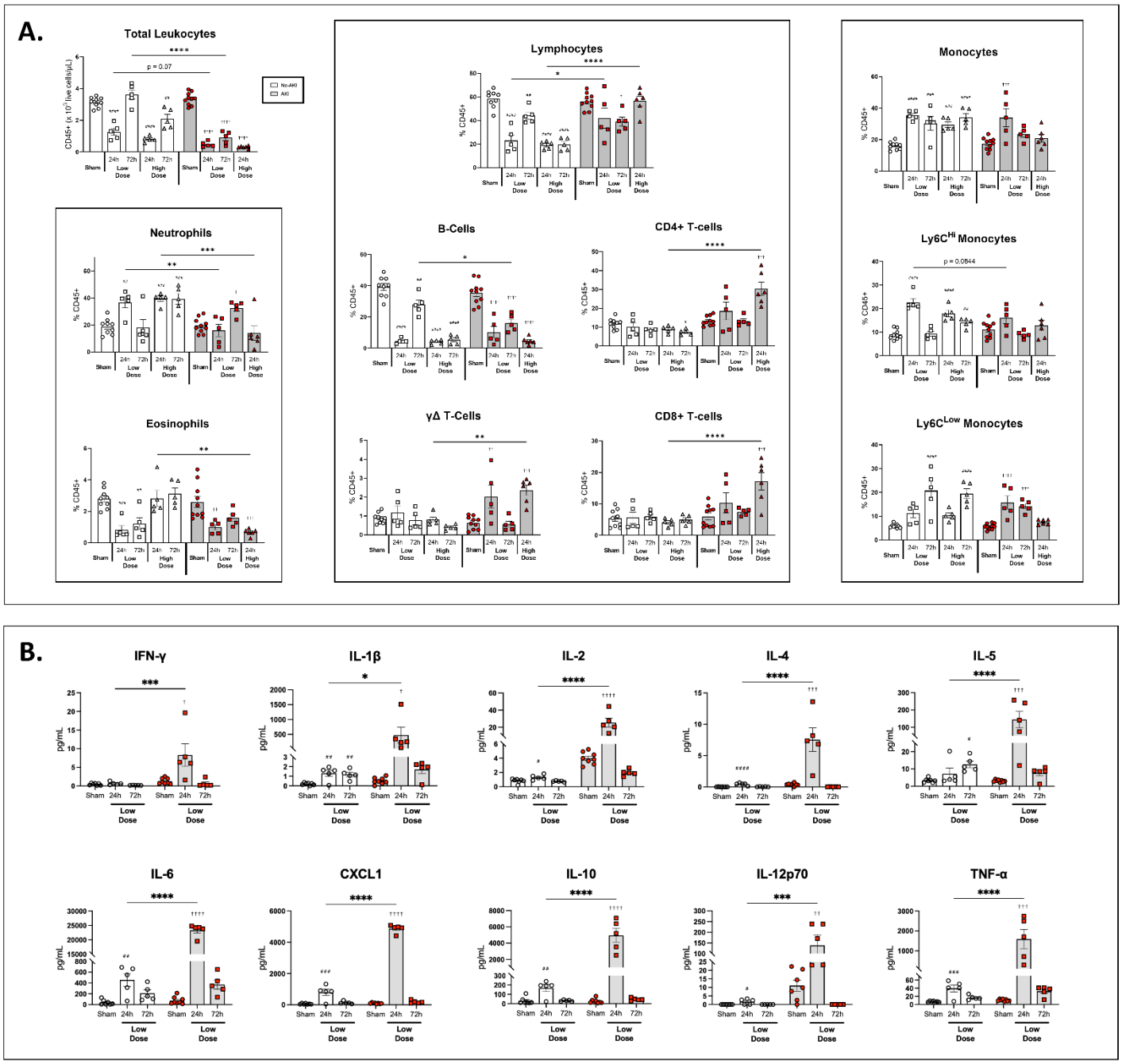


Figure 5. Mice with AKI experience leukopenia, multiple peripheral leukocyte abnormalities, and altered pro-inflammatory cytokine production during sepsis. A) Flow cytometry of peripheral blood leukocytes was performed on mice in the No-AKI and AKI groups at 24- and 72-hours after inoculation (24/72-hour samples are pooled in sham animals). Leukocytes dropped acutely after sepsis then rebound in total numbers in mice without AKI. Conversely, mice with AKI experienced significant leukopenia with an inability to reconstitute total numbers among surviving mice at 72-hours. Among CD45+ leukocytes, individual populations of immune cells were profiled including: granulocytes (A, left panel), lymphocytes (A, middle panel), and monocytes (A, right panel). Compared to No-AKI animals, animals with AKI experienced significant neutropenia and eosinophilopenia, an

increase in T-cells, and unchanged to slightly diminished monocyte differentiation in a dose dependent manner. B) Plasma cytokine analysis using a Mesoscale Vplex assay was performed in sham, low-dose 24h, and low-dose 72h samples from No-AKI controls and AKI animals. All profiled cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5,, IL-6, CXCL1, IL-10, IL-12p70, and TNF-α were significantly increased in mice with AKI at 24-hours after low-dose sepsis, indicating an exaggerated acute cytokine response, which returned to levels similar to controls by 72-hours after sepsis. (Within No-AKI group: # $p<0.05$, ##$p<0.01$, ###$p<0.001$, ####$p<0.0001$; Within AKI group: †$p<0.05$, ††$p<0.01$, †††$p<0.001$, ††††$p<0.0001$; No-AKI vs AKI group: * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

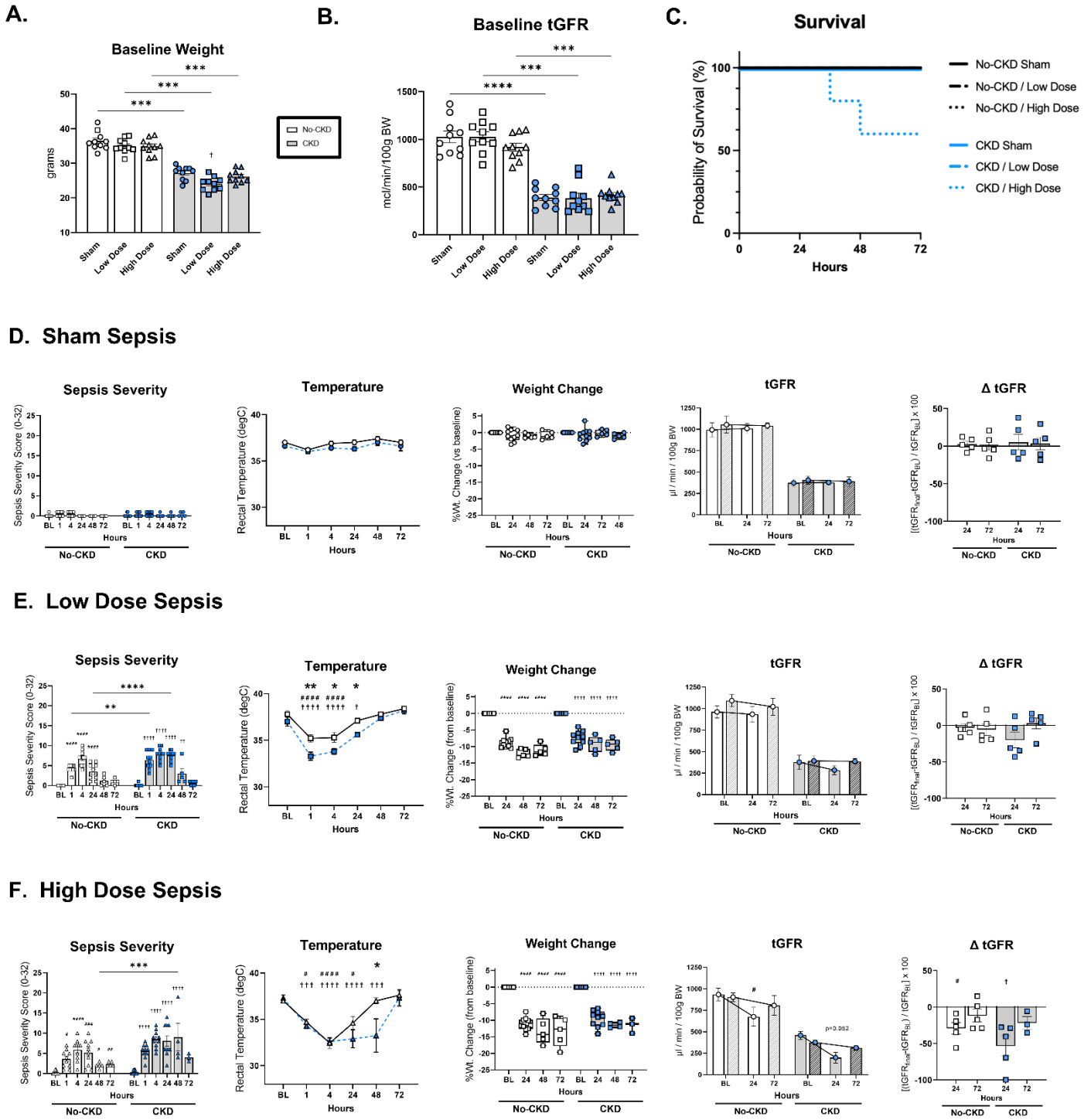


Figure 6. CKD CS Sepsis Studies. (A) Baseline weights and (B) tGFRs are again shown in CKD mice. (C) CKD mice showed delayed mortality in only two animals occurring at 36- and 48-hours after CS inoculation. As in Aki studies, No-CKD animals had 100% survival in all groups. (D) Sham, (E) Low-dose, and (F) High-dose sepsis studies are shown with CKD and control animals. Sepsis severity and rectal temperatures revealed slightly worse sepsis severity after CS inoculation in CKD animals starting at 1-4 hours after CS inoculation. CKD, like AKI mice, lost modest weight, however despite divergent baseline tGFRs, the change in tGFR from baseline to

collection was not significantly different in CKD mice vs Controls. (Within No-CKD group: # $p<0.05$, ##$p<0.01$, ###$p<0.001$, ####$p<0.0001$; Within CKD group: †$p<0.05$, ††$p<0.01$, †††$p<0.001$, ††††$p<0.0001$; No-CKD vs CKD group: * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

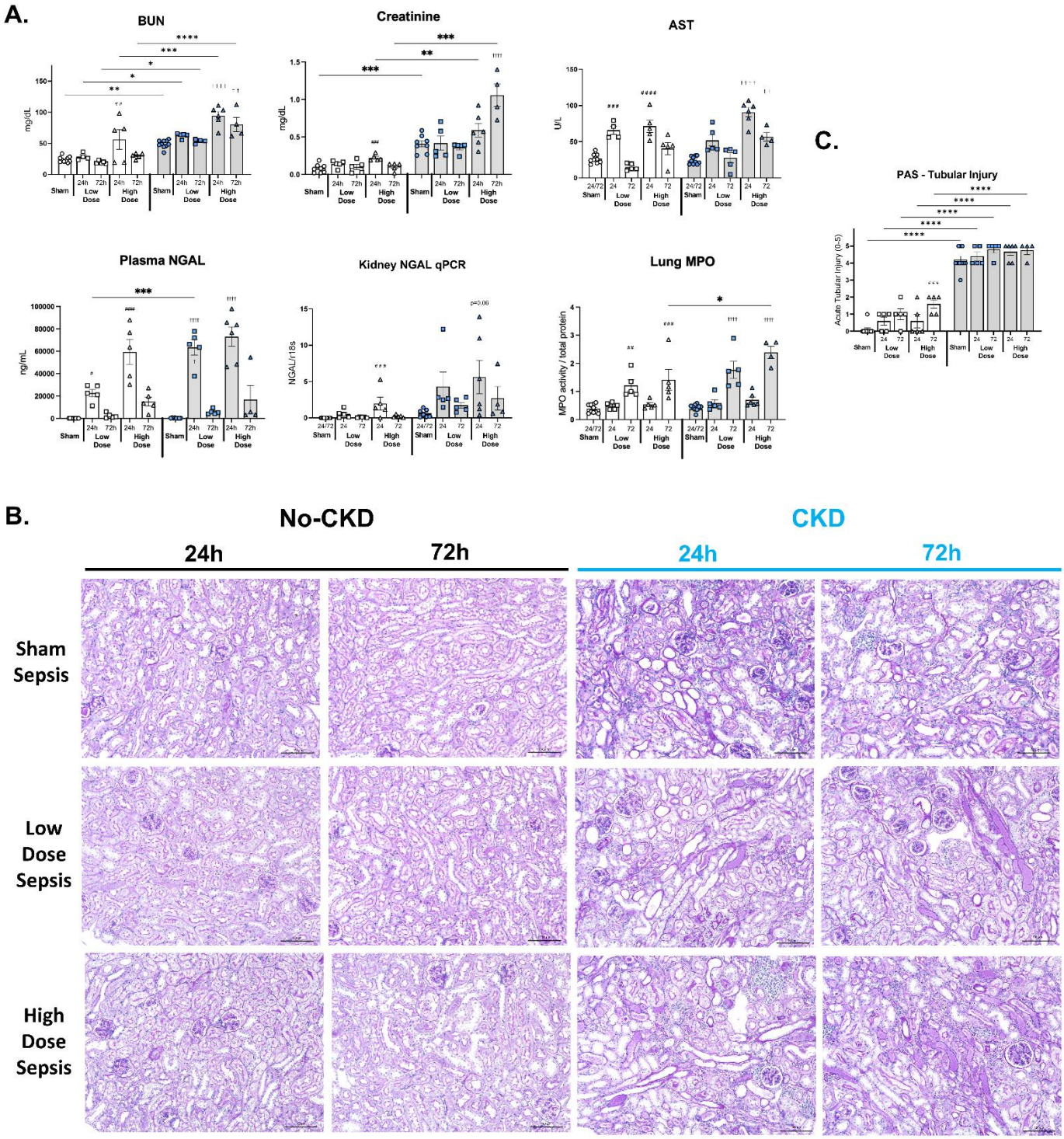


Figure 7. Mice with CKD have worse organ injury than No-CKD controls after sepsis. (A) At baseline, mice with CKD had significantly higher plasma BUN and creatinine than control mice. A significant increase in azotemia was only appreciated after high-dose sepsis in both control and CKD mice. As before in AKI studies, both plasma AST and NGAL performed better as sepsis biomarkers, being significantly elevated after even low dose sepsis, though only NGAL was significantly higher in CKD mice at 24-hours after low dose sepsis. Lung

MPO had a similar delayed increase reaching maximum values at 72 hours after sepsis in CKD and controls. (B,C) PAS-stained cortical tissues from no-CKD controls showed significantly increased tubular injury by 72 hours in both low and high-dose sepsis. However, due to the extreme baseline tubular injury in CKD mice, only a minor, non-significant trend towards increased tubular injury was appreciated in higher dose sepsis studies, driven primarily by the appearance of more proteinaceous tubular casts vs. controls. (Within No-CKD group: # $p<0.05$, ##$p<0.01$, ###$p<0.001$, ####$p<0.0001$; Within CKD group: †$p<0.05$, ††$p<0.01$, †††$p<0.001$, ††††$p<0.0001$; No-CKD vs CKD group: * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

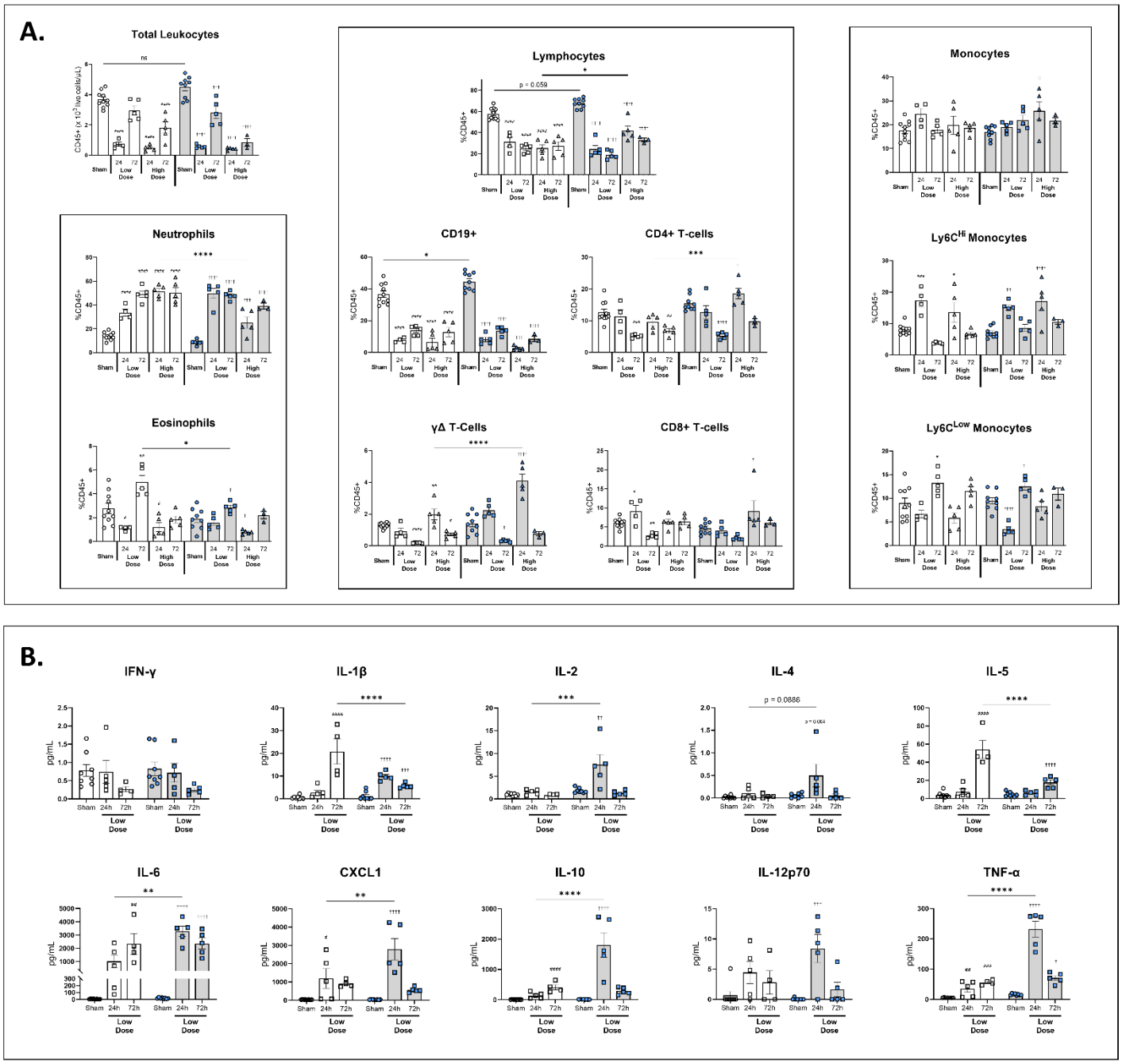
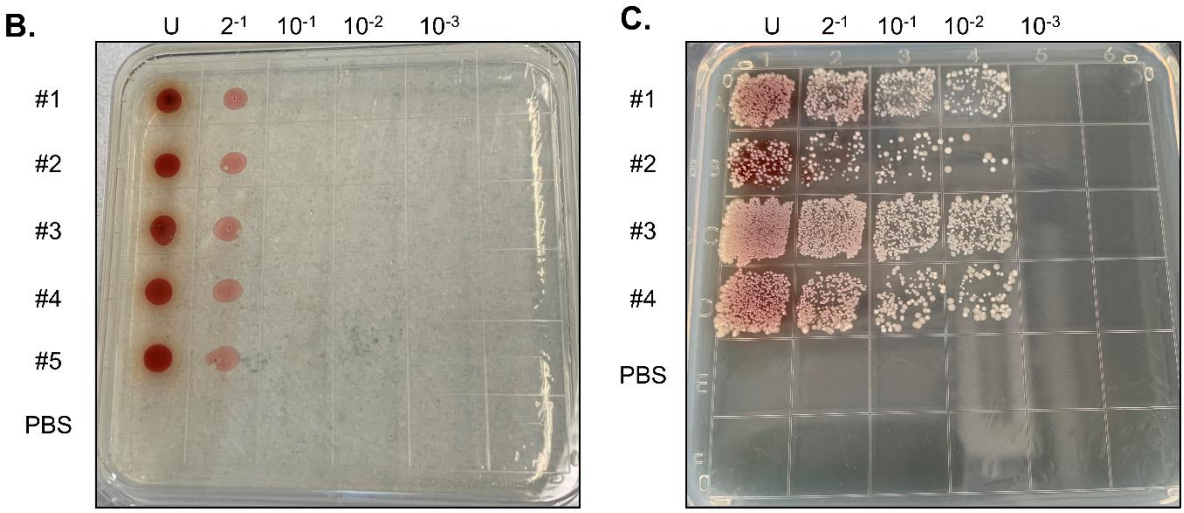


Figure 8. Mice with CKD have a similar peripheral blood immune response to sepsis vs control mice. (A) Flow cytometry of peripheral blood leukocytes was performed on mice in the No-CKD and CKD groups. 24- and 72-hour sham samples are shown pooled as before. Populations are expressed as percent CD45+ cells. CKD mice had similar degrees of leukopenia after sepsis compared to control animals (A, upper left panel). Aside from a significant increase in CD4 and γΔ T-cells, and significant decrease in neutrophils in high-dose sepsis 24h studies, populations of profiled immune cells from CKD mice largely mirrored no-CKD controls. (B) Plasma cytokine analysis using Mesoscale Vplex assay. Mice with CKD were observed to have significantly higher IL-2, IL-6, CXCL1, IL-10, and TNF-α levels vs controls at 24-hours after low-dose sepsis. Interestingly, mice with CKD

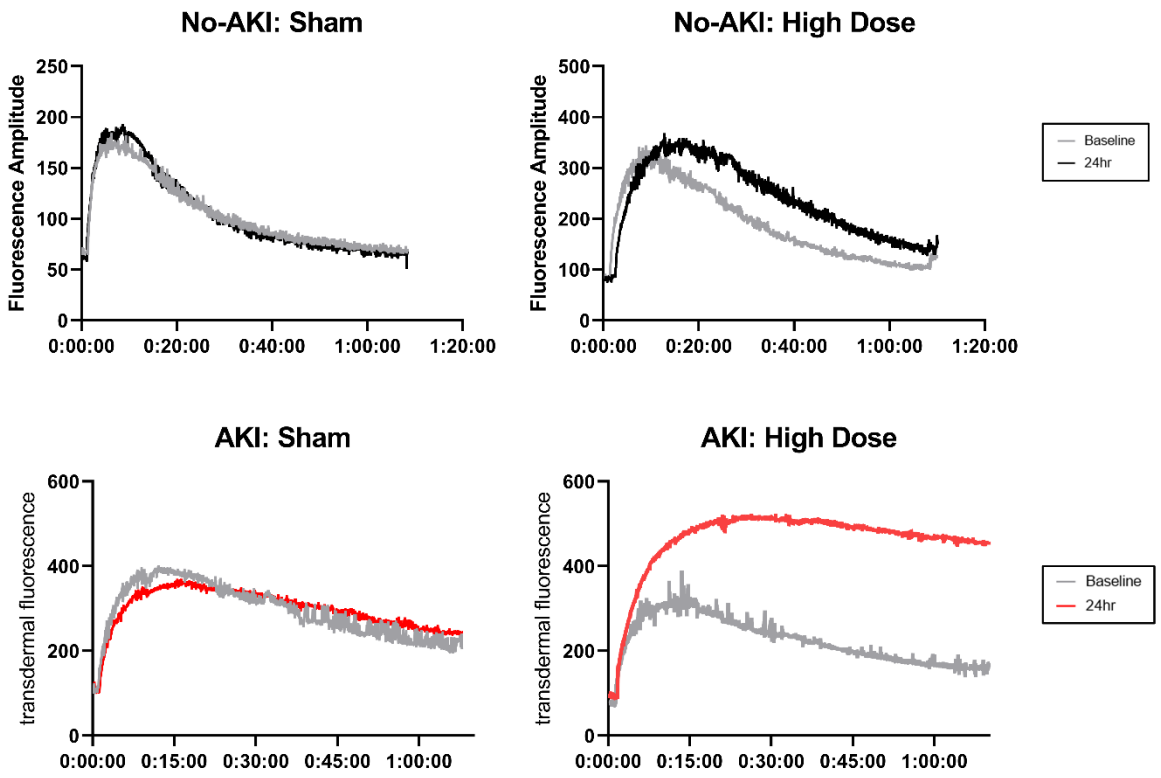
had blunted IL-1β and IL-5 levels by 72-hours after low-dose sepsis vs controls. (Within No-CKD group: # $p<0.05$, ##$p<0.01$, ###$p<0.001$, ####$p<0.0001$; Within CKD group: †$p<0.05$, ††$p<0.01$, †††$p<0.001$, ††††$p<0.0001$; No-CKD vs CKD group: * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

A.

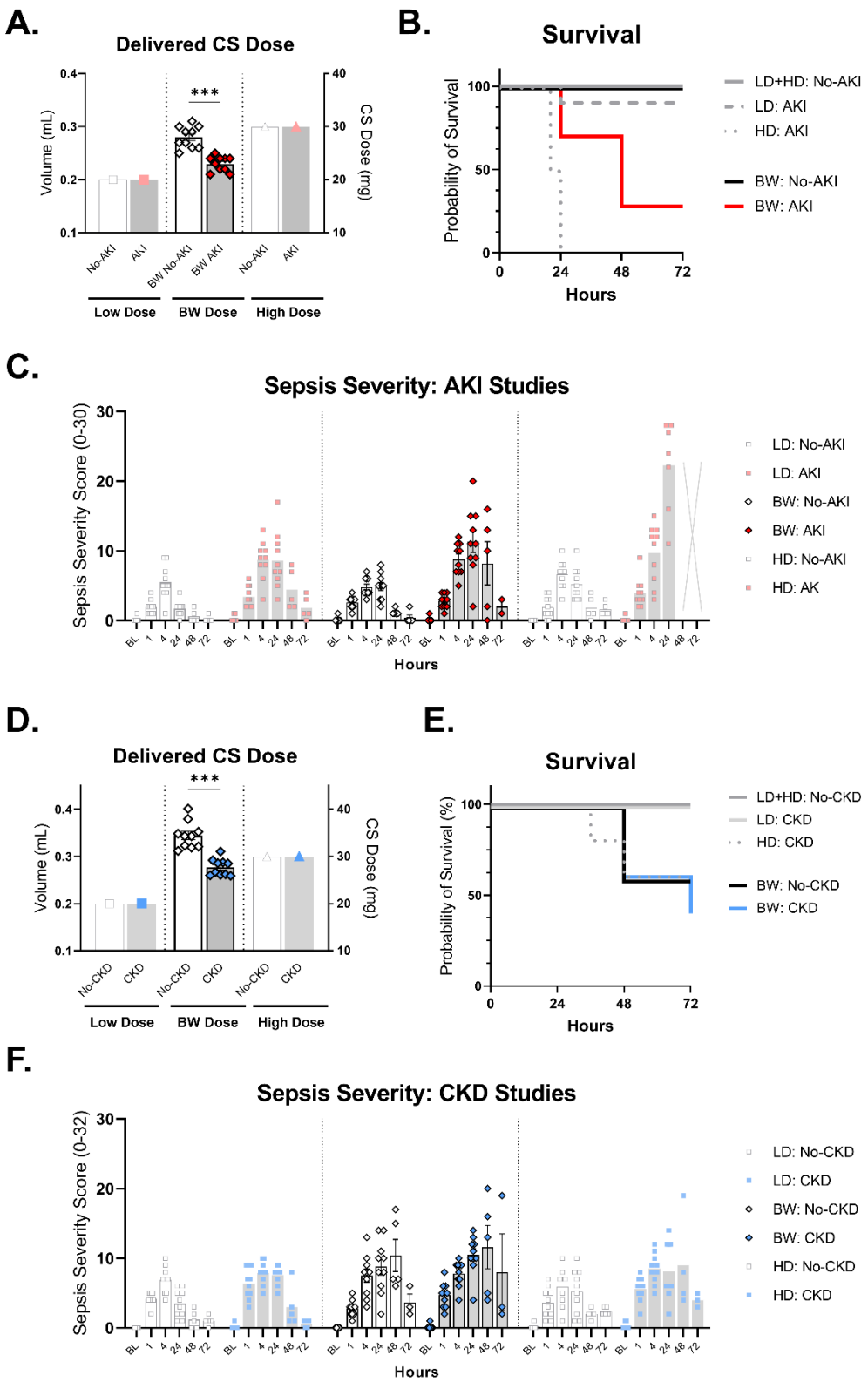
	Sham		Low Dose		High Dose	
	24h	72h	24h	72h	24h	72h
No-AKI	-	-	-	-	-	-
AKI	-	-	1/5	-	5/10*	n/a
No-CKD	-	-	-	-	-	-
CKD	-	-	-	-	-	2/5**



Supplemental Figure 1. Burden of Bacteremia in Septic Mice. Blood samples were plated on trypsic-soy agar plates to determine bacterial growth. (A) Table demonstrating the numbers of animals with positive blood culture plates. (B) Pictures of plates from a group of No-AKI and (C) AKI animals at 24-hour time collection. Undiluted (U) whole blood specimens were plated followed by a 1:2, 1:10, 1:100, and 1:1000 dilution of whole blood in sterile PBS. Control diluent (PBS) samples were plated alongside specimens in each case. None of the AKI- or CKD-control mice had evidence of bacteremia. In AKI mice, 1/5 (20%) of the low-dose CS animals were bacteremic. *Among the N=10 AKI mice receiving high dose CS, all 10 suffered mortality (N=3 before collection, among the N=7 remaining, only 5 underwent plating for bacterial culture and all were positive. N=2 of the remaining collected mice were severely ill and blood specimens were prioritized for other biological measurements, thus 5/10 represents 5/5 of the actual plated samples). **Among CKD mice, 2/5 72h-designated animals were euthanized before 72h (one at 36h, one at 48h), and were the identified bacteremic animals.



Supplemental Figure 2. tGFR curves from AKI studies. Pictured are No-AKI (upper graphs) and AKI (lower graphs) 24-hour animal tGFR curves. Examples of graphs from both sham and high-dose CS animal are shown in comparison. In No-AKI: sham animals, a typical pattern of FITC-sinistrin high amplitude peak after retroorbital injection was appreciated, followed by a decline near background fluorescence skin levels after 1:05h. In the AKI: sham mouse there is a slower decline from peak amplitude and it does not return to background levels by the end of the measurement period. 24-hour follow up tGFR curves are overlayed on the same animal, demonstrating little change in tGFR from baseline to 24 hours in either group receiving sham injections. Conversely, there is significant flattening out of 24-hour follow up tGFR curves in animals receiving high CS doses. In AKI mice, severe renal failure was observed with almost complete plateau of FITC-sinistrin signal, indicating little functional renal clearance. Note the relatively muted signal in AKI-high dose 24-hour curve vs all other curves, attributed to moribund state and little observed animal activity during tGFR measurement period.



Supplemental Figure 3. Body-weight adjustment CS AKI and CKD Experiments. (A) Body weight (BW) dose adjustments in AKI groups did not reverse differences in sepsis severity between controls and AKI mice as measured by mortality (B) and clinical sepsis severity (C). In CKD groups, the delivered CS dose was highest in the No-CKD group (D), and two animals suffered severe sepsis and needed euthanasia in this group (E). BW

adjustment partially reversed sepsis severity in CKD mice (F), though higher attrition than No-CKD mice was appreciated, again in a delayed fashion. Flat low dose and high dose CS studies are included in AKI and CKD BW data in background for comparison.

Date/Cage:			N					R					L					B					R2x				
Experiment:			1	4	24	48	72	1	4	24	48	72	1	4	24	48	72	1	4	24	48	72	1	4	24	48	72
Coat	0	Smooth coat																									
	1	Patches of hair piloerected																									
	2	Majority of back piloerected																									
	3	"Puffy mouse"																									
	4	Emaciated																									
LOC	0	Alert																									
	1	Alert, avoids upright stance																									
	2	Alertness slow, ambulates																									
	3	Slow, barely moves																									
	4	Stationary																									
Activity	0	Normal activity																									
	1	Slight suppressed activity																									
	2	Sig supressed activity																									
	3	No activity, stationary																									
	4	No activity + tremors																									
Stimuli	0	Resp. to audio/tactile stim.																									
	1	Slow resp. to stimuli																									
	2	No resp. to aud, few touch																									
	3	No resp. to aud, min touch																									
	4	No resp., cannot right self																									
Eyes	0	Open, no secretions																									
	1	Not fuly open +/-min secr.																									
	2	Half closed +/-mod. secr.																									
	3	Nearly closed +/-mod. secr.																									
	4	Closed or milky																									
Resp rate	0	Normal, rapid resp.																									
	1	Slight decreased resp.																									
	2	Moderately reduced resp.																									
	3	Severely reduced resp.																									
	4	Extremely red. resp.																									
Resp quality	0	Normal																									
	1	Brief Labored breathing																									
	2	Labored, no gasping																									
	3	Labored with int. gasps																									
	4	Gasping																									
		Time (__0h__) Total Score																									
		Time (__1h__) Total Score																									
		Time (__4h__) Total Score																									
		Time (__24h__) Total Score																									
		Time (__48h__) Total Score																									
		Time (__72h__) Total Score																									
		Temp @ Time (__0h__)																									
		Temp @ Time (__1h__)																									
		Temp @ Time (__4h__)																									
		Temp @ Time (__24h__)																									
		Temp @ Time (__48h__)																									
		Temp @ Time (__72h__)																									

Supplemental Figure 4. Modified Clinical Sepsis Score. Adopted by Shrum et. al [23]. Animals were graded on a scale of 0 – 4 using individual metrics listed at t=0h (baseline), 4h, 24h, (and 48h, 72h in those designated groups). Total score from all metrics was added for composite score. Rectal temperatures were also recorded alongside sepsis scores.