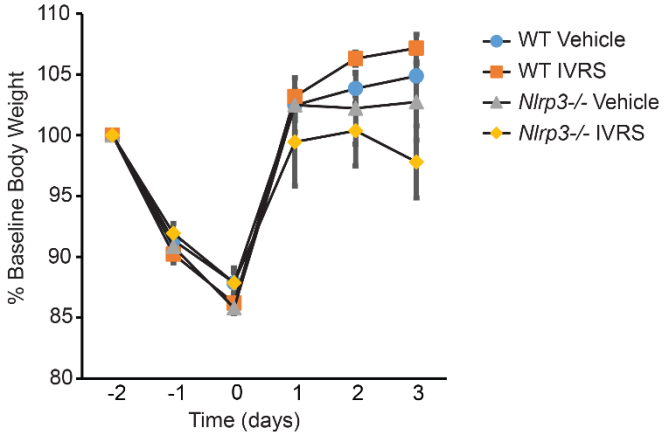
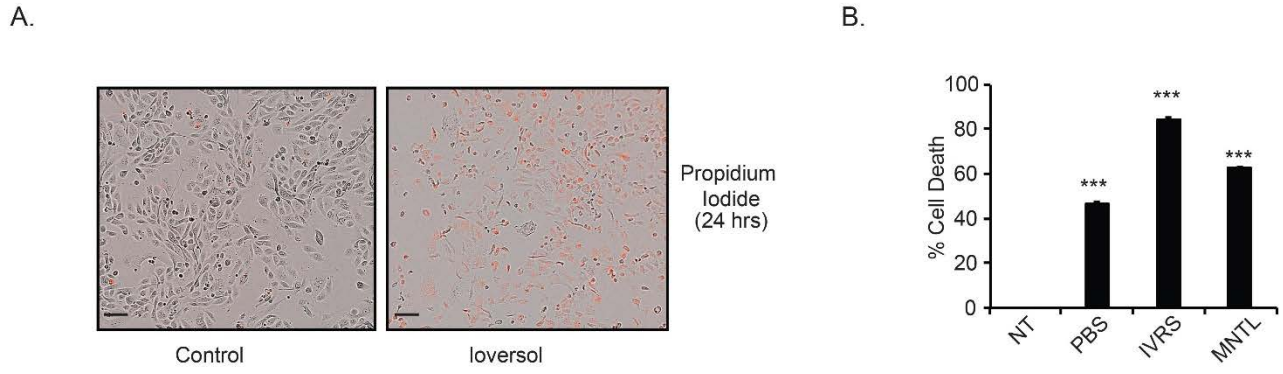
Lau et al 2018

Renal Immune Surveillance and Dipeptidase-1 Contribute to Contrast-Induced Acute Kidney Injury

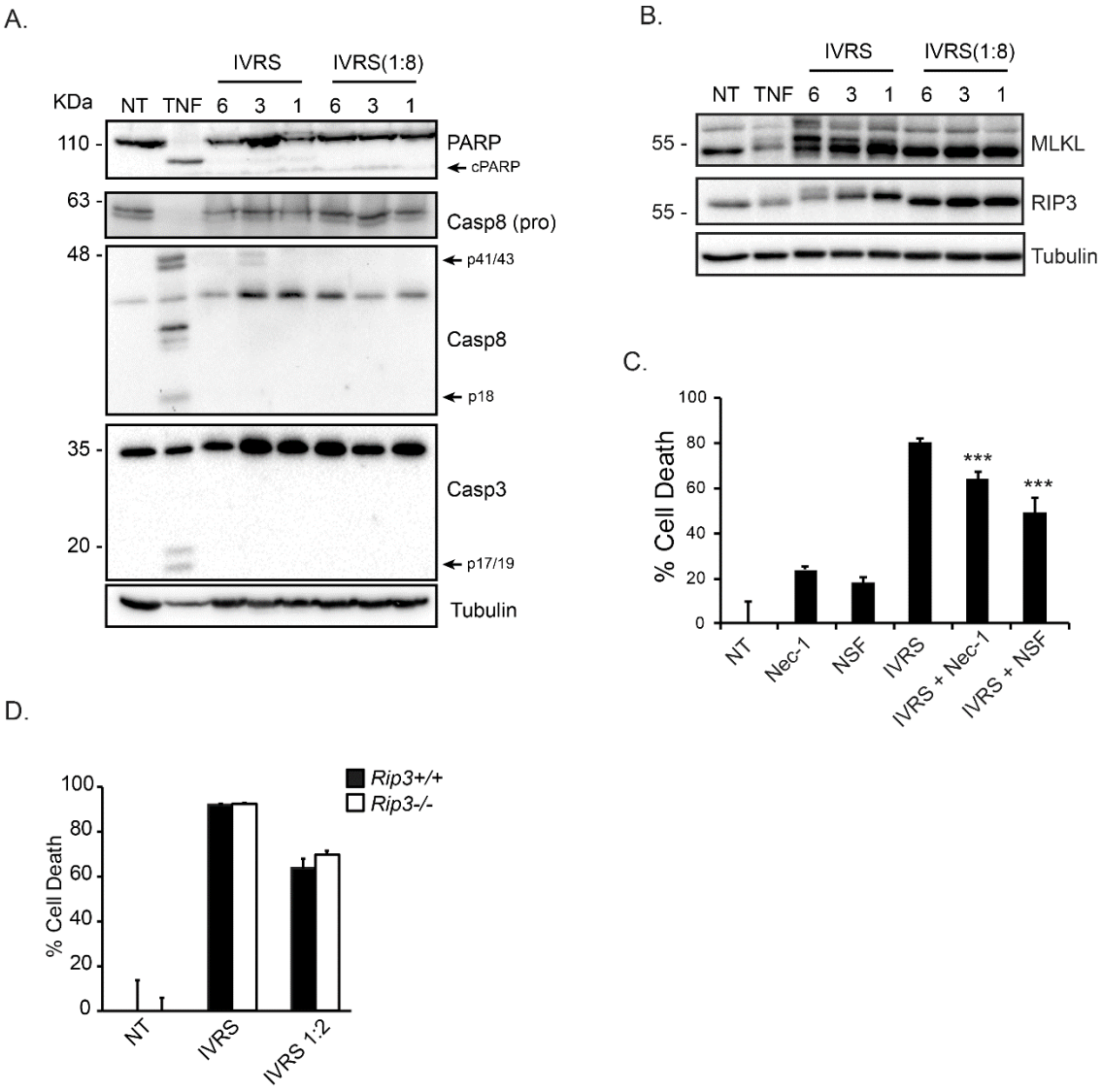
Supplementary Figures and Table



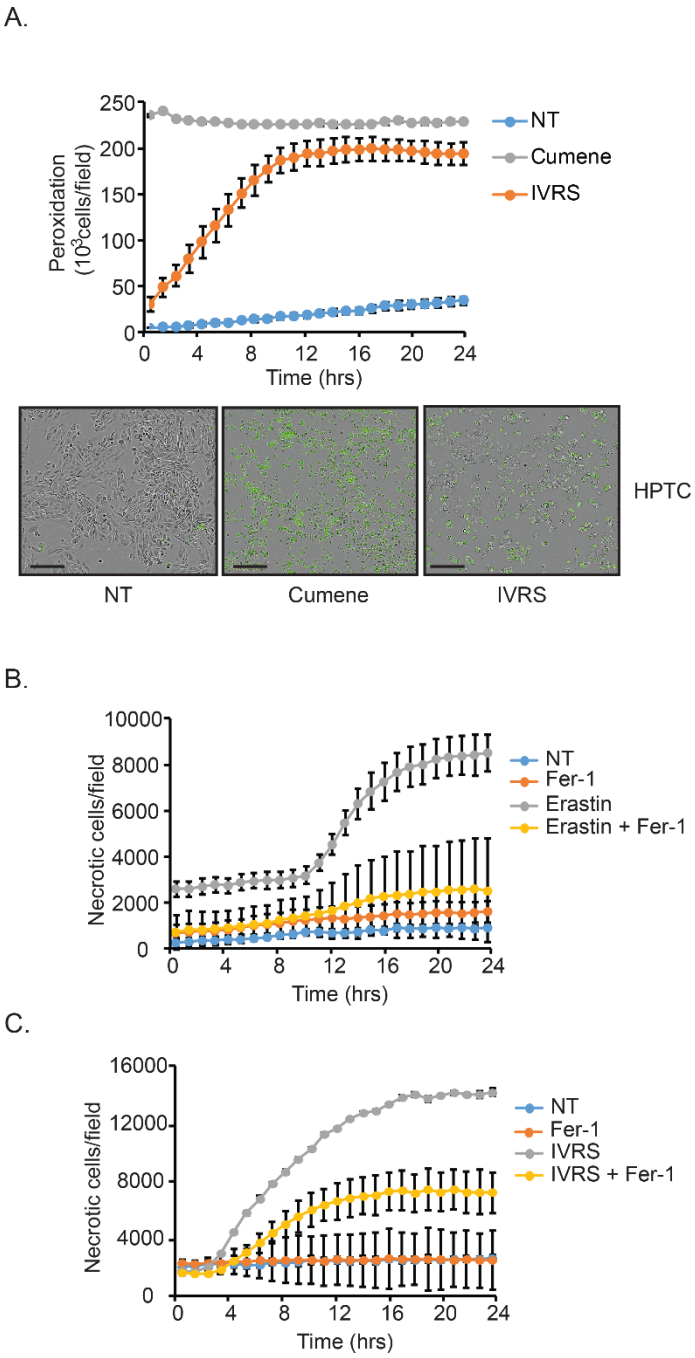
Supplementary Figure 1. Body weight changes in contrast-induced AKI model. Body weight change in *Nlrp3+/+* and *Nlrp3-/-* mice (n=4 for each group) following 48 hours of water deprivation and 3 days post ioversol (IVRS) or saline vehicle control with water *ad libitum*. Changes were not significant between groups (ANOVA).



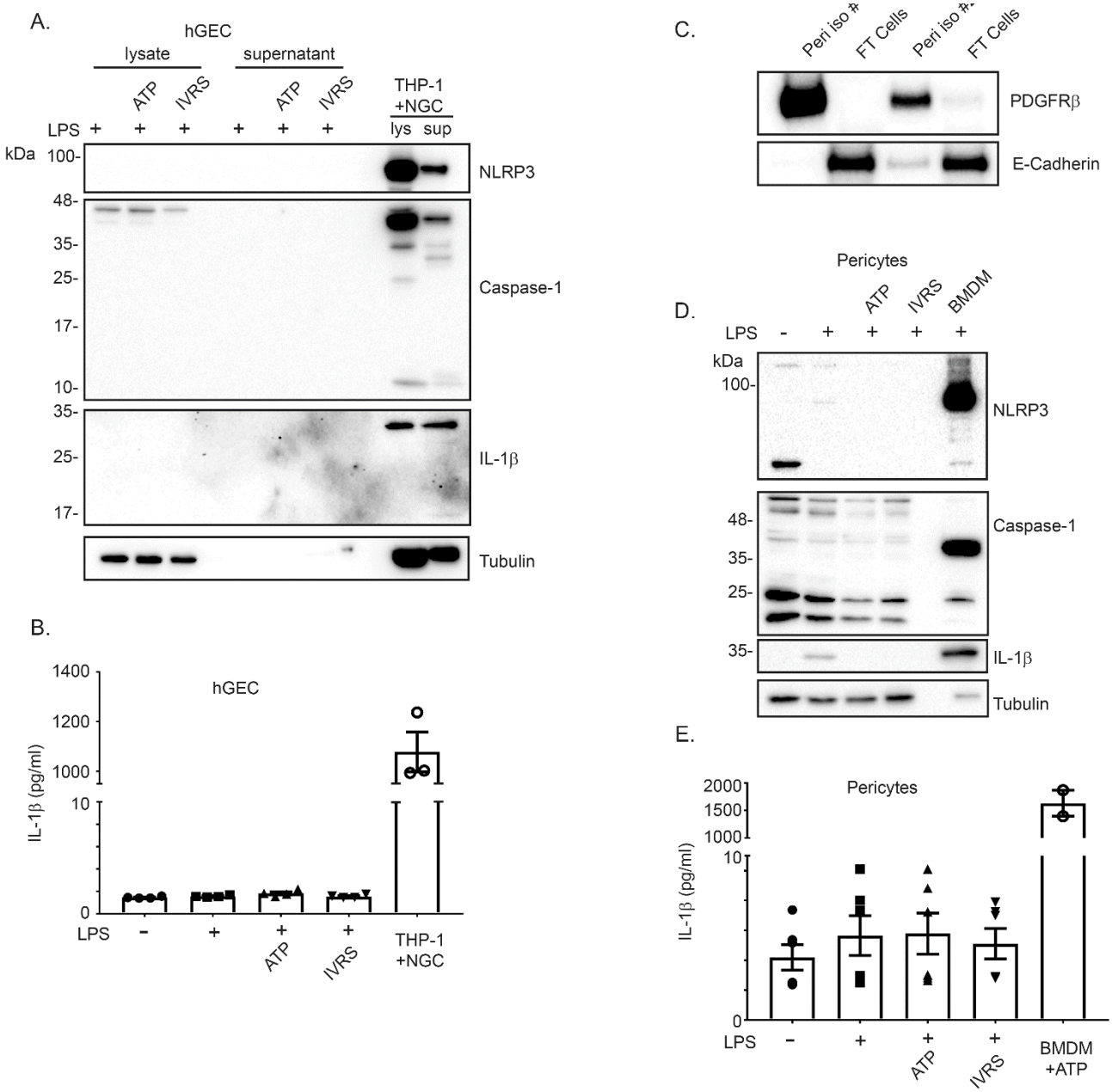
Supplementary Figure 2. Contrast mediates epithelial cell cytotoxicity in vitro. A) HPTC were treated with ioversol and labeled with propidium iodide (red) to identify necrotic cells for 24h. Images were captured with the Incucyte Live Cell Analysis System. (Scale bar: 150µm) B) HPTC were treated with ioversol (IVRS), an equivalent volume of PBS, or equi-osmolar mannitol buffer (MNTL) as controls and cell death was quantified by MTT assay. (n=6-12/group) (***: $p<0.001$ vs. NT; IVRS: $p<0.001$ vs. NT; MNTL: $p<0.001$ vs. NT, ANOVA).



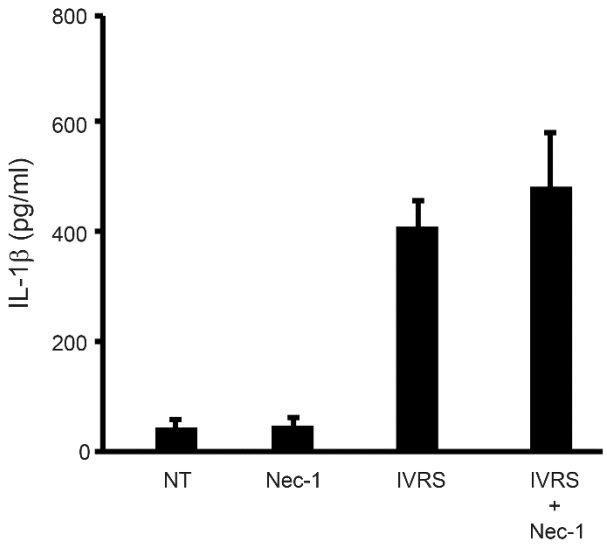
Supplementary Figure 3. Contrast-induced activation of cell death pathways in epithelial cells. A, B) Human proximal tubular epithelial cells (HPTC) were analyzed for markers of apoptosis (Caspase-8, Caspase-3, PARP) and markers of necroptosis (MLKL, RIP3) using high dose (1:1) and low dose (1:8) ioversol (IVRS) diluted in culture medium. Tumor necrosis factor-alpha (TNF) was used as a positive control for apoptosis. Immunoblots are representative of 3 independent experiments. C) HPTC were pretreated with the RIP-1 inhibitor necrostatin-1 (Nec-1) or the MLKL inhibitor necrosulfonamide (NSF) prior to stimulation with IVRS. Cell death was analyzed by MTT assay (vs. IVRS alone, IVRS+Nec-1: ***p=0.001, IVRS+NSF: ***p=0.0009, 6-12/group, ANOVA). D) MTT assay of IVRS-treated mouse tubular epithelial cells (TEC) isolated from wild type or *Rip3-/-*mice (p=NS, n=4/group, ANOVA).



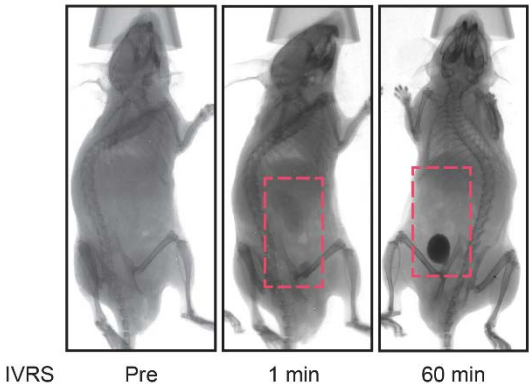
Supplementary Figure 4. Contrast induces ferroptosis in tubular epithelial cells. A) Lipid peroxidation (green) was visualized and quantified in human proximal tubular cells (HPTC) after ioversol (IVRS) treatment using the Incucyte Live Cell Analysis System. Cumene was used as a control for lipid peroxidation. (n=3/group) (Scale bar: 300µm) B) HPTC were treated with a erastin (50 µM) as a positive control for ferroptosis while a ferroptosis inhibitor (Fer-1) was used to inhibit cell death. Necrosis was measured via propidium iodide labeling and quantified using the Incucyte Live Cell Analysis System. (n=3/group) C) HPTC were treated with IVRS and a ferroptosis inhibitor (Fer-1). Necrosis was measured via propidium iodide labeling and quantified using the Incucyte Live Cell Analysis System. (n=3/group)



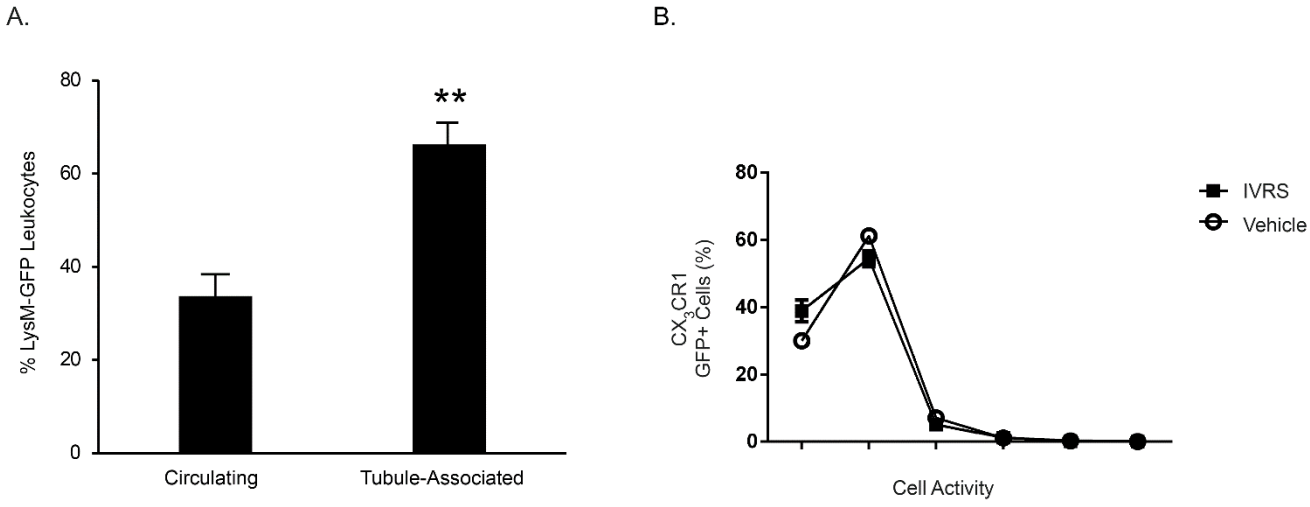
Supplementary Figure 5. Glomerular endothelial cells and renal pericytes do not activate inflammasomes in response to contrast. A) Human glomerular endothelial cells (hGEC) were primed with LPS and treated with either ATP or IVRS for 6 hours. THP-1 cells treated with nigericin was used as positive control. Inflammasome markers NLRP3, caspase-1, and IL-1β were analyzed by immunoblot and tubulin was used as loading control. B) IL-1β ELISA was used to test for inflammasome activation in hGEC after treatment with ATP or IVRS for 6 hours. THP-1 treated with nigericin was used as positive control. C) Murine renal pericytes were isolated by magnetic bead isolation and characterized by PDGFRβ expression as positive marker and E-cadherin as negative marker. Separated flow-through cells (FT) were also characterized as negative control for pericyte markers. D) Isolated murine renal pericytes were primed with LPS and treated with ATP or IVRS for 6 hours. Murine bone marrow derived macrophages (BMDM) were used as positive control. Inflammasome markers NLRP3, caspase-1, and IL-1β were analyzed by immunoblotting. Tubulin was used as loading control. E) IL-1β ELISA was used to test for inflammasome activation in murine renal pericytes after treatment with ATP or IVRS for 6 hours. BMDM treated with ATP was used as positive control.



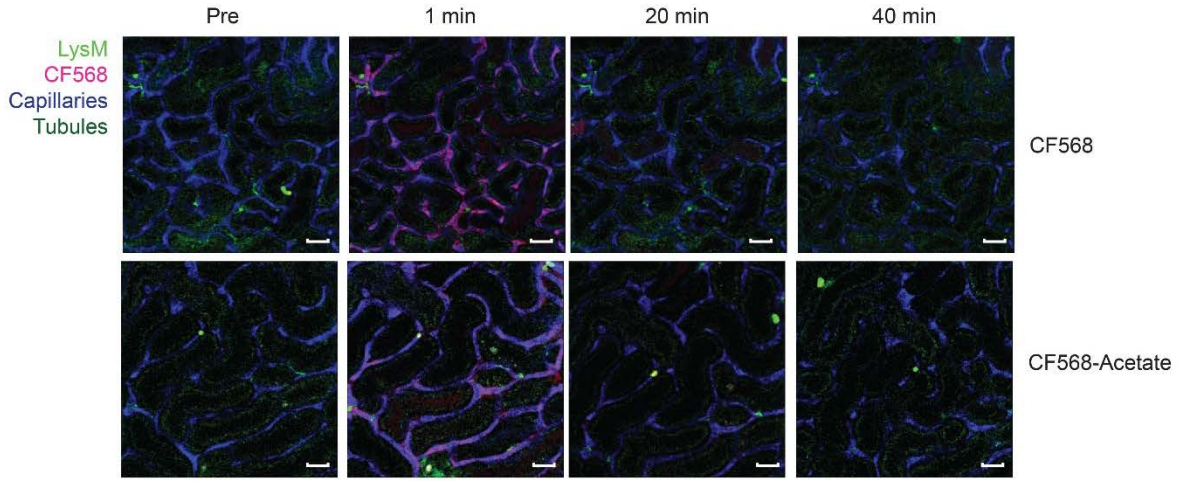
Supplementary Figure 6. Necrostatin-1 does not inhibit contrast-induced inflammasome activation. THP-1 cells were treated with ioversol with or without the RIPK1 inhibitor Necrostatin-1 (Nec-1) in the presence of ioversol (IVRS). IL-1β production was quantified by ELISA. (IVRS vs. IVRS + Nec-1, p=NS, n=3/group, ANOVA).



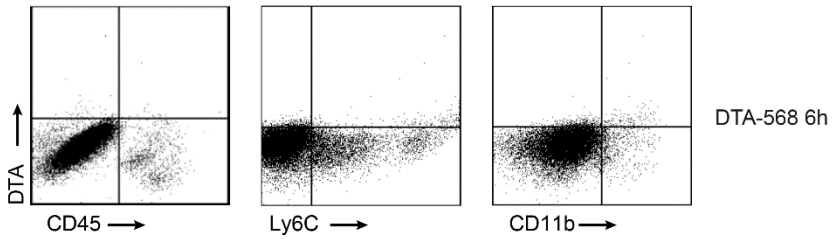
Supplementary Figure 7. Contrast is largely excreted after intravenous administration. Ioversol (IVRS) was administered intravenously and localization of contrast was visualized by X-ray imaging over 60 minutes in volume depleted mice.



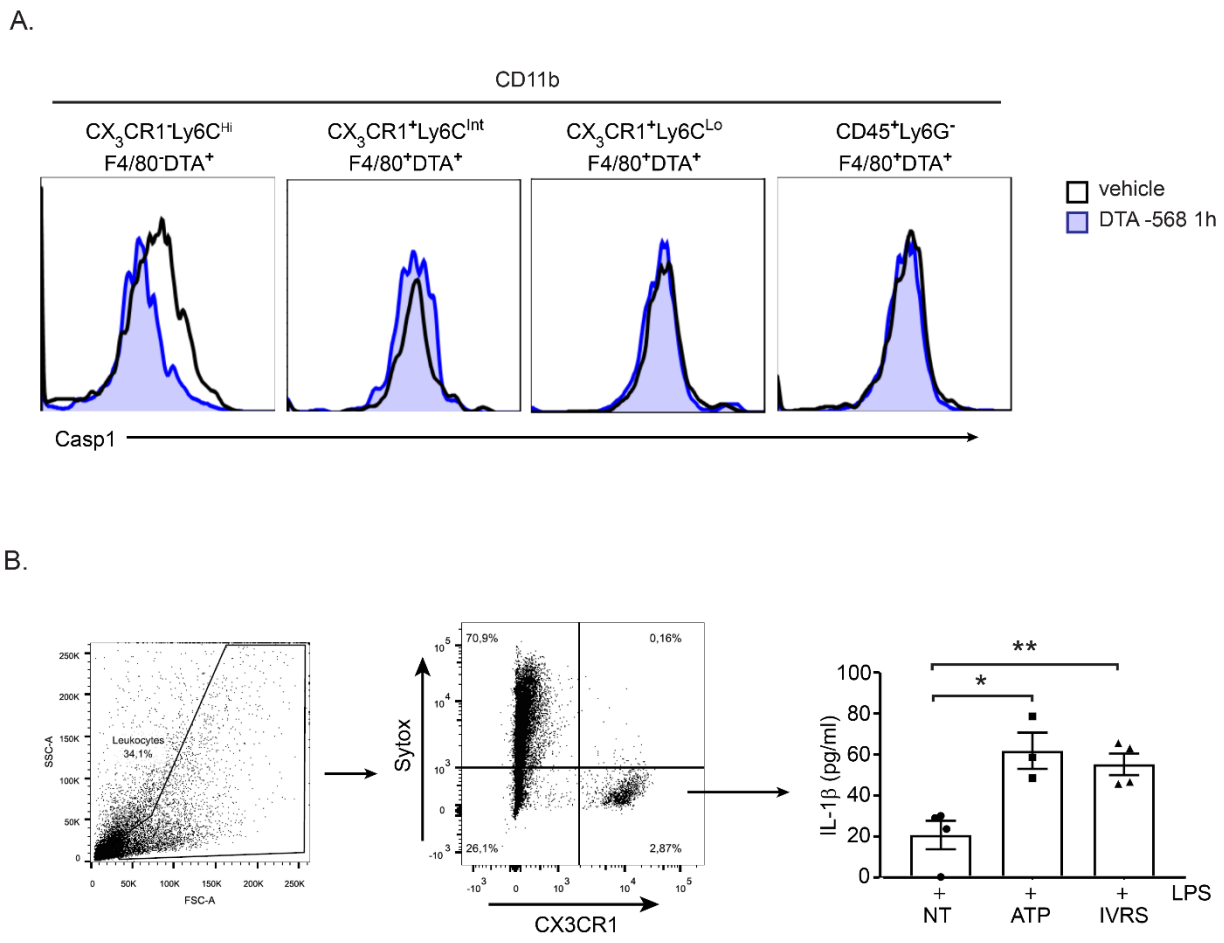
Supplementary Figure 8. Behaviour of recruited monocyte/macrophages and resident renal phagocytes in response to intravenous contrast. A) *LysM*$^{(gfp/gfp)}$ mice were volume depleted and treated with CF-568 labeled diatrizoate and kidneys imaged using multiphoton intravital microscopy over 1 hour. LysM-GFP cells containing contrast in the circulation (capillaries) or associated with contrast-laden tubules were quantified (**p=0.002, n=4/group, Student's t-test). B) *CX$_3$CR1*$^{(gfp/+)}$ mice were treated with either saline vehicle or ioversol (IVRS) and imaged by multiphoton intravital microscopy over 1 hour. Z-stacks of single fields were taken and analyzed by Imaris software to determine changes in CX$_3$CR1$^+$ cell extension and retraction as a measure of cell activity. IVRS did not induce a change in resident phagocyte activity compared to controls.



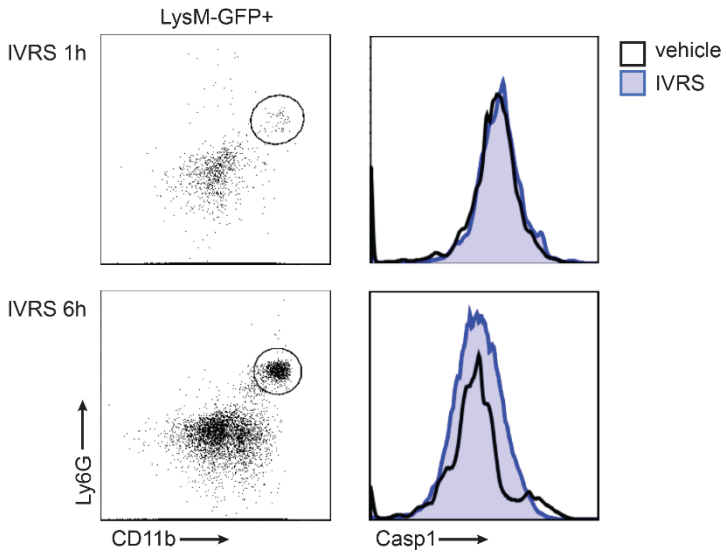
Supplementary Figure 9. Administration of free CF568 or CF568 conjugated to acetic acid does not result in tubular accumulation and uptake in the kidney. Volume-depleted *LysM*$^{(gfp/gfp)}$ mice were treated with free dye alone (CF568) or acetic acid conjugated to dye (CF568-Acetate) as vehicle controls. Multiphoton intravital microscopy was used to confirm the lack of uptake in the tubules over time. Labels: capillaries, Q-tracker (blue); LysM-GFP+ leukocytes (green); CF568-label (red); and tubules, autofluorescence (dark green). (Scale bar: 100µm)



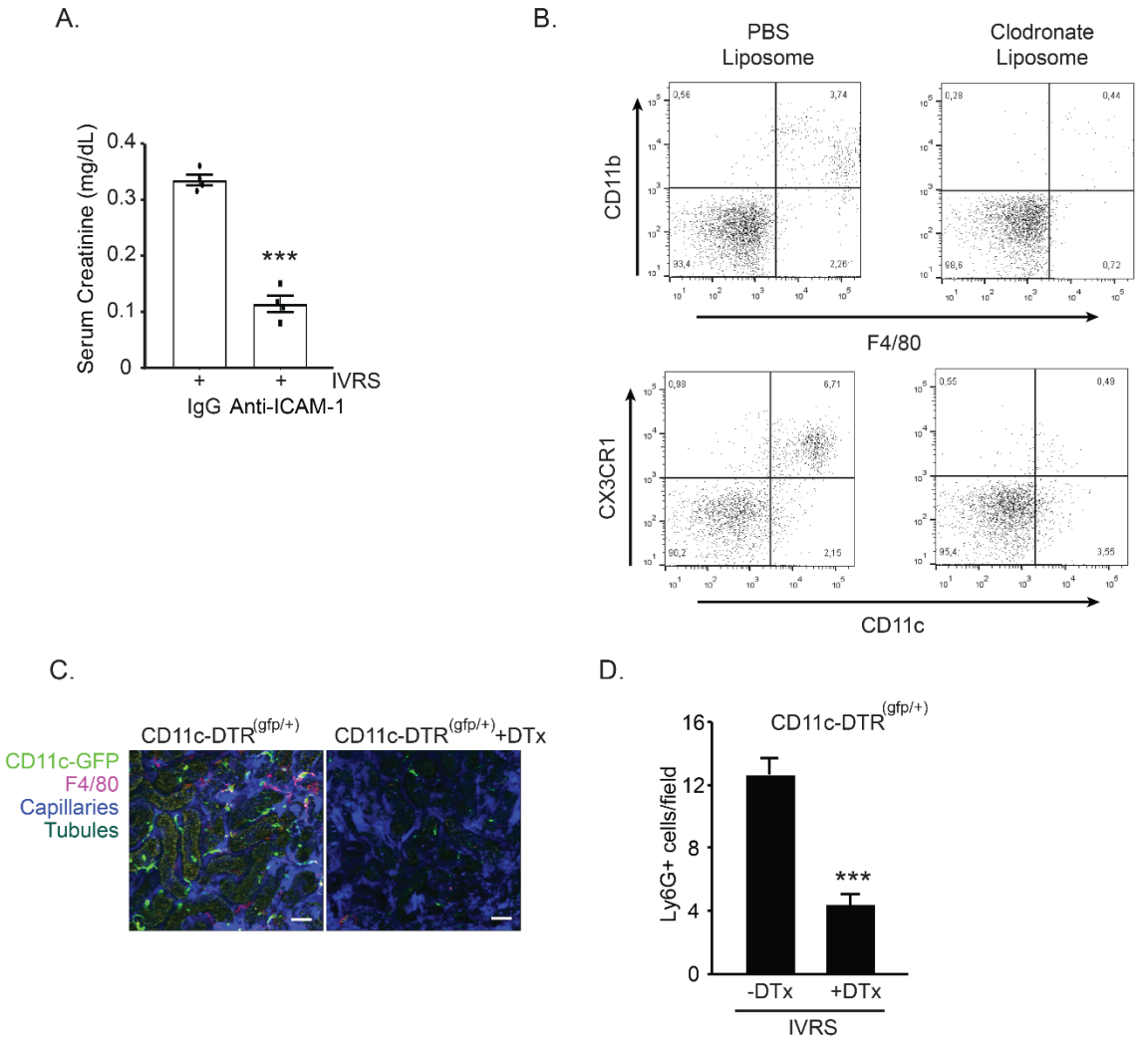
Supplementary Figure 10. Contrast uptake is not detected in leukocytes at 6 hours after treatment. Mice were volume depleted and treated with CF-568 labeled diatrizoate (DTA-568). Renal leukocytes were isolated at 6 hours and sorted for CD45, Ly6C and CD11b by flow cytometry followed by analysis for DTA uptake. CF568-DTA was not detectable at the 6 hour timepoint.



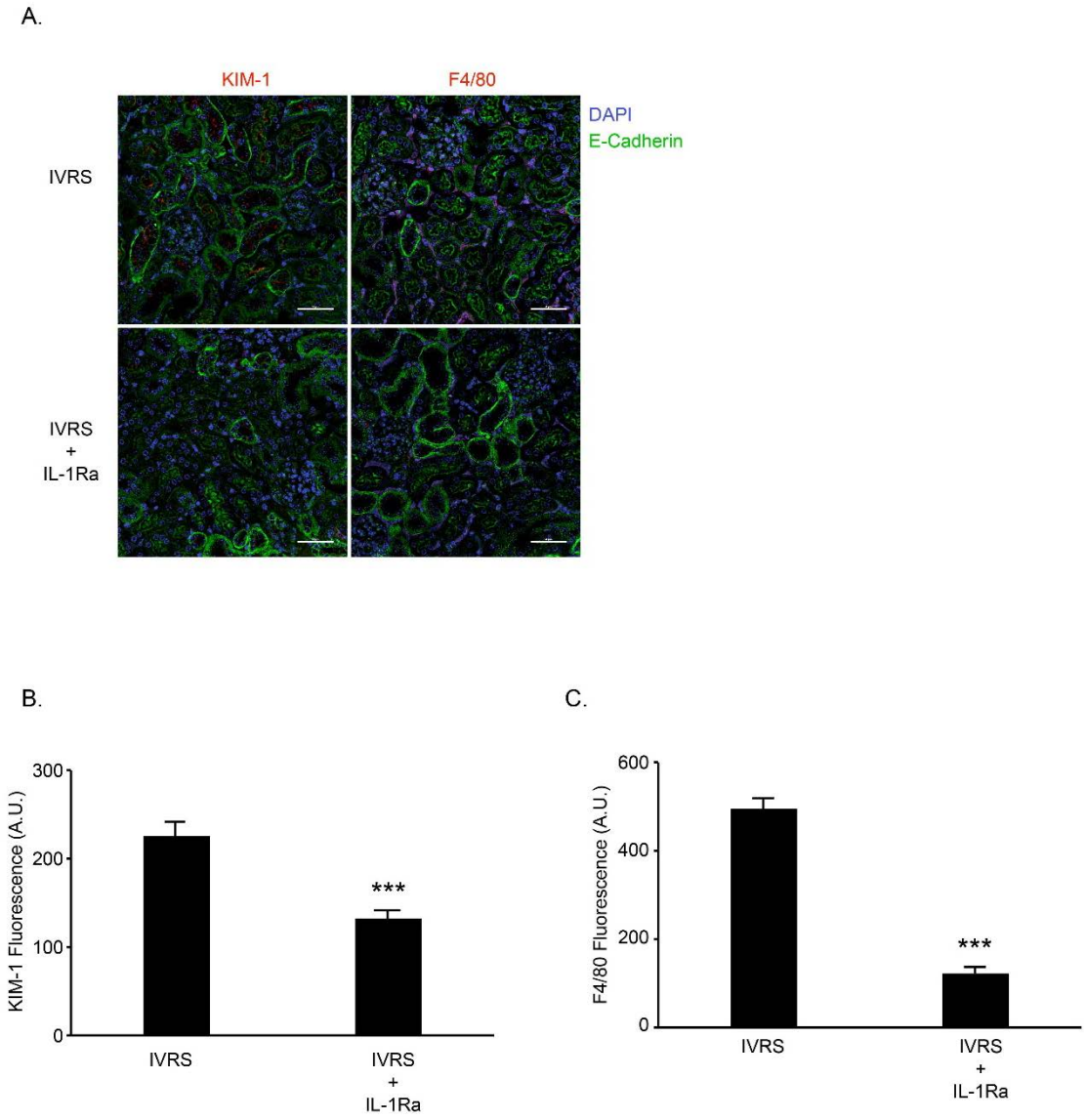
Supplementary Figure 11. Caspase-1 and Inflammasome activation in recruited monocyte/macrophages and resident renal phagocytes. A) Mice were volume depleted and treated with CF-568 labeled diatrizoate (DTA-568) and renal leukocytes were sorted by CD11b and analyzed by flow cytometry at 1h. DTA-568+ monocyte/macrophage populations were further separated by Cx_3CR1, Ly6C, Ly6G, and F4/80 markers and analyzed for caspase-1 activation. Active caspase-1 is not detectable in macrophages at 1 hour in vivo. Leukocytes isolated from the kidneys of vehicle treated mice are used as the reference control. Representative of data obtained from three different mice. B) Resident renal phagocytes were isolated from wild type $CX_3CR1^{(gfp/+)}$ mice using fluorescent cell sorting. Viable resident renal phagocytes comprised of approximately 3% of total renal leukocytes. Cells were cultured and primed with LPS before treatment with ioversol (IVRS) for 6 hours or nigericin as positive control. Supernatants were analyzed for IL-1β by ELISA (untreated, NT vs. ATP: *p=0.001, NT vs. IVRS: **p=0.008, n=3-4/group, ANOVA).



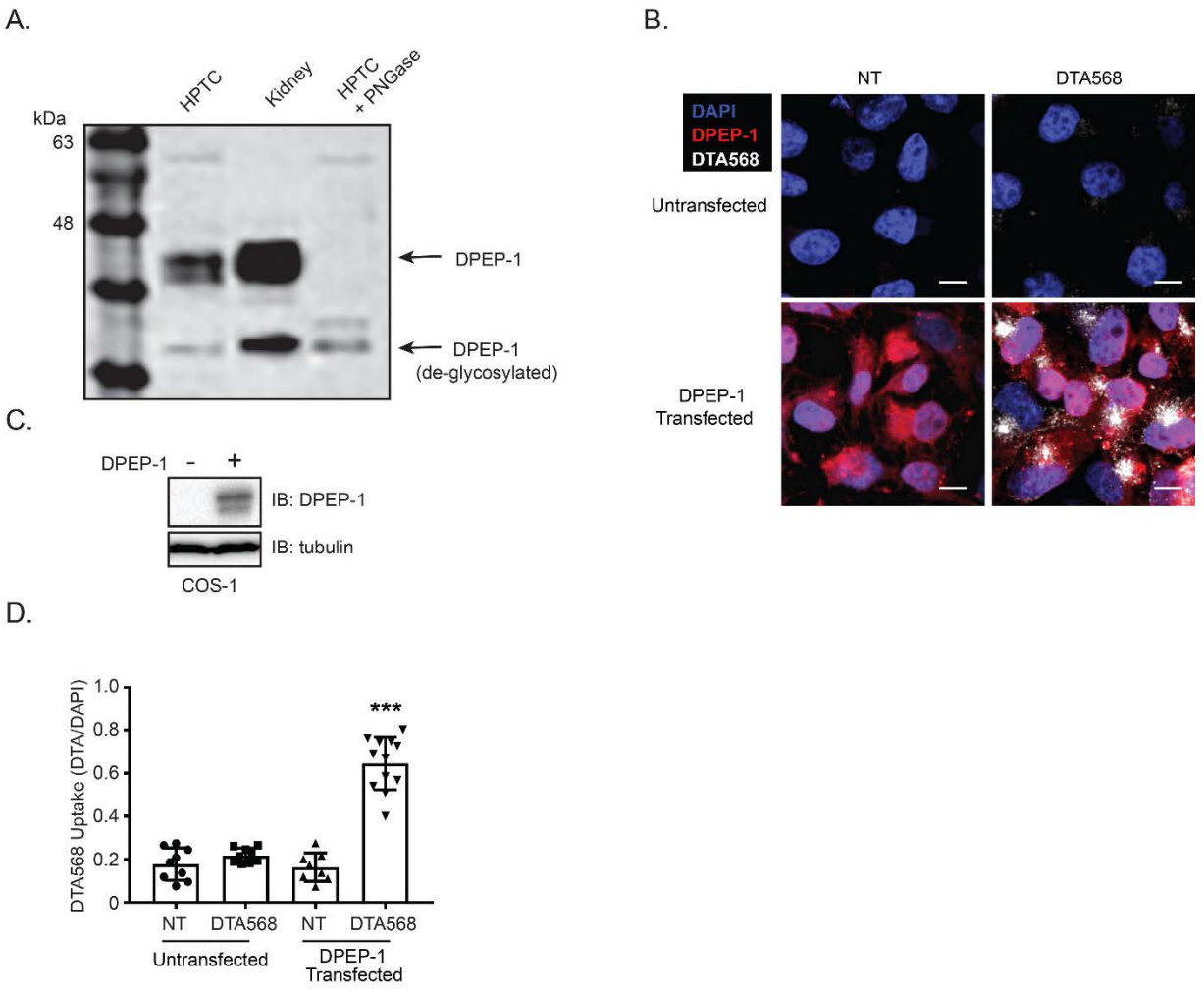
Supplementary Figure 12. Inflammasome activation in neutrophils. *LysM*$^{(gfp/gfp)}$ mice were volume depleted and treated with IVRS for 1 or 6 hours. Renal leukocytes were sorted by LysM-GFP. Neutrophils were identified by CD11b and Ly6G and analyzed for caspase-1 activation. Active caspase-1 is not detected in neutrophils recruited to the kidney. Neutrophils from vehicle treated mice are used as the reference control. Representative of data obtained from three different mice.



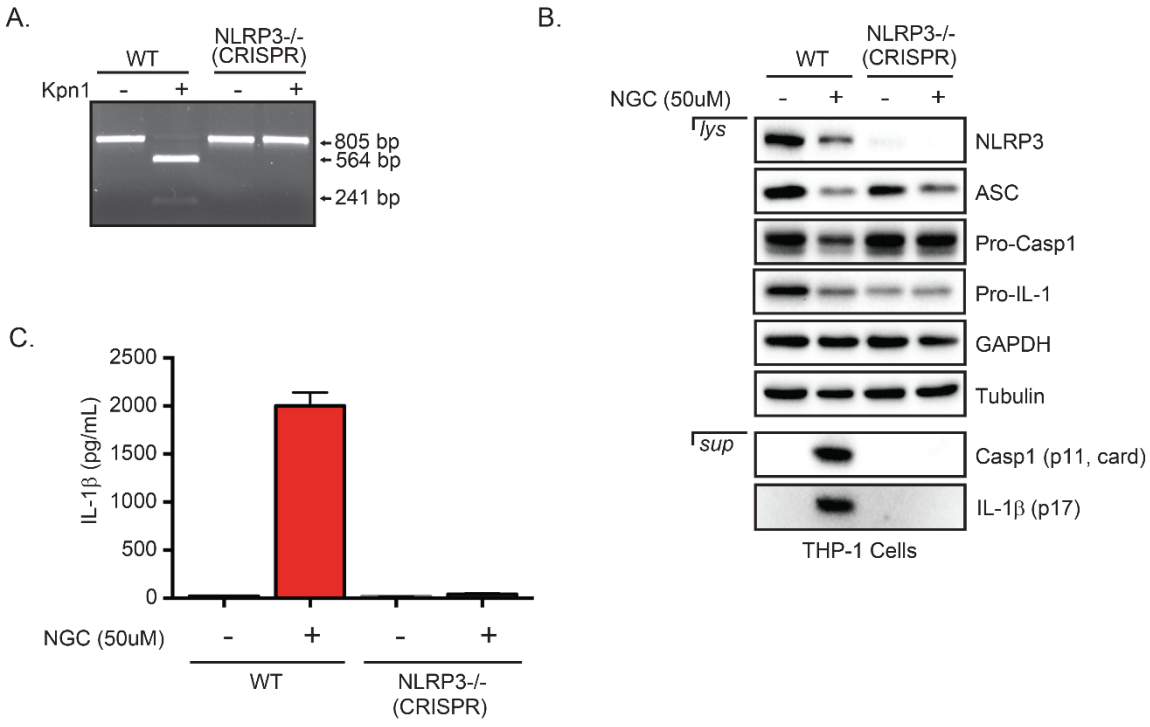
Supplementary Figure 13. Depletion of phagocytic monocytes by clodronate liposome and CD11c⁺ monocytes in *CD11c-DTR(gfp/+)* mice. A) Mice were volume depleted and treated with either IgG or anti-ICAM-1 before ioversol (IVRS) treatment. Renal function was measured by serum creatinine at day 3 (ref. values 0.04-0.08 mg/dL) (***p=0.0001, n=4/group, two-tailed Student's t-test). B) *LysM(gfp/gfp)* mice were treated with either clodronate liposomes or PBS liposomes as a control. Renal phagocytes were isolated and analyzed by flow cytometry for CD11b, F4/80, CD11c, CX_3CR1 to confirm efficacy of depletion procedure. C) *CD11c-DTR(gfp/+)* were depleted of CD11c⁺ macrophages via diphtheria toxin (DTx) or treated with PBS control. GFP expression and F4/80 immunofluorescence confirms depletion of kidney resident phagocytes. Labels: capillaries, Q-tracker (blue); neutrophil, Ly6G (pink); CD11c, GFP (green); tubules, autofluorescence (dark green). (Scale bar: 100µm) D) Neutrophil recruitment to the kidney in *CD11c-DTR(gfp/+)* mice at 6 hours following IVRS administration (intravital microscopy). Neutrophils were quantified by manual counting (***: p<0.001 vs. -DTx, n=3/group, two-tailed Student's t-test).



Supplementary Figure 14. IL-1R antagonist ameliorates contrast-induced AKI. A, B, C) Mice were volume depleted and pre-treated with IL-1Ra prior to ioversol (IVRS) administration. Kidneys were imaged and quantified for tissue injury and inflammation by immunofluorescence using KIM-1 and F4/80 at 3 days. (IVRS vs. IVRS+IL-1Ra, KIM-1: ***p=0.0003, F4/80: ***p=0.0001, n=9/group, two-tailed Student's t-test) (Scale bar: 50µm)



Supplementary Figure 15. Contrast uptake is mediated by dipeptidase-1 (DPEP1). A) Expression of DPEP1 was analyzed by immunoblot in total human kidney and human renal proximal tubular epithelial cells (HPTC). HPTC were treated with PNGaseF to de-glycosylate DPEP1. B) Monkey kidney cells (COS-1) were transfected to overexpress DPEP-1. Untransfected and DPEP-1 transfected cells were incubated with CF568-diatrizoate (DTA568) for 30 minutes, washed and imaged using live-cell confocal microscopy. The live-cell dye MitoTracker green was used to label cells (Scale bar: 10µm). C) Immunoblot confirming DPEP-1 expression in transfected COS-1 cells. D) Contrast uptake in DPEP-1 transfected COS-1 cells was quantified. (DTA-568, untransfected vs. transfected, ***p=0.0001, ANOVA)



Supplementary Figure 16. Validation and characterization of CRISPR NLRP3-/- THP-1 cells. NLRP3 was targeted by CRISPR-Cas9 in THP-1 cells. A) Genomic DNA was isolated from wild type and NLRP3-/- THP-1 cells and the targeted region of interest in the NLRP3 gene was amplified by PCR. PCR products were digested by Kpn1 and DNA fragments were visualized by gel electrophoresis. The effect of CRISPR-Cas9 mutation was validated by removal of endogenous Kpn1 site. B) Total protein was isolated from wild type and NLRP3-/- THP-1 cells treated with and without nigericin (NGC). Expression of inflammasome proteins were analyzed by immunoblot including NLRP3, ASC, Caspase-1, and IL-1β. GAPDH and tubulin was used as loading control. C) IL-1β ELISA from wild type and NLRP3-/- THP-1 cell supernatant after treatment with and without nigericin (NGC).

	Pre-Angiogram n=42	Post-Angiogram n=42
Age (years)	71 ± 10	
Male sex (%)	31 (70%)	
Caucasian ethnicity (%)	39 (89%)	
Serum creatinine (µmol/L)	128 ± 36	126 ± 15
Urinalysis		
Specific gravity	1.02 ± 0.03	1.01 ± 0.005
pH	5.59 ± 0.73	5.72 ± 0.65
Leukocyte (scale from 0 – 3)	0.17 ± 0.53	0.27 ± 0.58
Protein (scale from 0 – 3)	0.60 ± 0.65	0.62 ± 0.78
Glucose (scale from 0 – 4)	0.11 ± 0.53	0.16 ± 0.64
Blood concentration (scale from 0 – 4)	0.19 ± 0.50	0.61 ± 1.19

Supplementary Table 1. Patient Characteristics. Urines were collected pre-coronary angiogram and 12-24 hours post, before any detectable clinical kidney injury. Urinalysis was unchanged pre- and post angiogram, although hematuria was higher post-angiogram (p=0.004). All values presented as mean ± SD

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Supplementary Movie Legend

Supplementary Movie 1. Intravital microscopy of the kidney in control mice. Intravital microscopy in vehicle treated *LysM$^{(gfp/gfp)}$* and *Nlrp3-/- LysM$^{(gfp/gfp)}$* mice. Labels: capillaries, Q-tracker (Blue); leukocytes, LysM-GFP+ (green); injured/necrotic cells, Sytox orange (red); tubules, autofluorescence, (dark green).

Supplementary Movie 2. Contrast-induced leukocyte recruitment and tubular cell injury at 6 hours. Dehydrated *LysM$^{(gfp/gfp)}$* and *Nlrp3-/- LysM$^{(gfp/gfp)}$* mice were treated with ioversol and imaged by multiphoton intravital microscopy of the kidney at 6 hours (time-lapse over 2-5 minutes). Contrast induces significant recruitment of LysM-GFP$^+$ leukocytes in *LysM$^{(gfp/gfp)}$* mice that is significantly reduced or absent in *Nlrp3-/- LysM$^{(gfp/gfp)}$* mice. Little to no tubular cell injury/necrosis is observed at this time point. Labels: capillaries, Q-tracker (Blue); leukocytes, LysM-GFP$^+$ (green); injured/necrotic cells, Sytox orange (red); tubules, autofluorescence, (dark green).

Supplementary Movie 3. Contrast-induced leukocyte recruitment and tubular cell injury at 72 hours. Dehydrated *LysM$^{(gfp/gfp)}$* and *Nlrp3-/- LysM$^{(gfp/gfp)}$* mice were treated with ioversol and imaged by multiphoton intravital microscopy of the kidney at 72 hours (time-lapse over 2-5 minutes). Recruitment of LysM-GFP$^+$ leukocytes is less prominent in both mouse strains compared to 6 hours. Tubular epithelial cell injury/necrosis however is observed in *LysM$^{(gfp/gfp)}$* but not *Nlrp3-/- LysM$^{(gfp/gfp)}$* mice at this time point. Labels: capillaries, Q-tracker (Blue); leukocytes, LysM-GFP$^+$ (green); injured/necrotic cells, Sytox orange (red); tubules, autofluorescence, (dark green).

Supplementary Movie 4. Renal contrast handling in hydrated mice. Hydrated *LysM$^{(gfp/gfp)}$* mice were administered CF568-labeled diatrizoate and imaged by multiphoton intravital microscopy of the kidney at 0-20 minutes after injection (time lapse). Hydration prevents contrast accumulation in renal tubules as seen by the rapid appearance and disappearance CF568-diatrizoate from the tubular lumen. Labels: capillaries, Q-tracker (Blue); leukocytes, LysM-GFP$^+$ (green); diatrizoate, CF568 (pink); tubules, autofluorescence, (dark green).

Supplementary Movie 5. Renal contrast handling in dehydrated mice. (A) Volume-depleted *CX$_3$CR1$^{(gfp/+)}$* mice were administered CF568-diatrizoate and imaged by multiphoton intravital microscopy of the kidney at 0-30 minutes after injection (time lapse). (B) High power fields (red box) were used to demonstrate tubular uptake of contrast at 0-30 minutes after contrast injection in a dehydrated mouse. Compared to hydrated mice, volume depletion results in the reabsorption and accumulation of contrast in the tubules. Labels: capillaries, Q-tracker (Blue); resident renal phagocytes, CX$_3$CR1-GFP$^+$ (green); diatrizoate, CF568 (pink); tubules, autofluorescence, (dark green).

Supplemental Movie 6. Contrast uptake by resident renal CX$_3$CR1$^+$ phagocytes. Volume depleted *CX$_3$CR1$^{(gfp/+)}$* mice received CF568-DTA and imaged by multiphoton intravital microscopy of the kidney at 30-40 minutes (time lapse). High power field to visualize co-localization of contrast in CX$_3$CR1$^+$ phagocytes. Labels: resident renal phagocytes, CX$_3$CR1-GFP$^+$ (blue); diatrizoate, CF568 (white).

Supplemental Movie 7. Contrast is taken up intracellularly by infiltrating LysM$^+$ monocytes. Volume-depleted *LysM$^{(gfp/gfp)}$* mice received CF568-diatrizoate and imaged by multiphoton intravital microscopy of the kidney at 30-40 minutes (time lapse). High power and magnified fields to visualize leukocyte crawling

along and interacting directly with tubules to ingest contrast material reabsorbed from urine. Labels: leukocytes, LysM-GFP^{+} (blue); diatrizoate, CF568 (white).

Supplemental Movie 8. Cilastatin prevents tubular uptake of contrast. Volume – depleted C57/B6 mice were pretreated with cilastatin (25 mg/kg), administered CF568-diatrizoate and imaged by multiphoton intravital microscopy of the kidney at 1-30 minutes (time lapse). Inhibition of DPEP-1 by cilastatin prevents tubular accumulation of contrast. Clearance of contrast is slower than in hydrated mice (Movie 3) due to the volume-depleted state. Labels: capillaries, Q-tracker (Blue); diatrizoate, CF568 (pink); tubules, autofluorescence, (dark green).