The RIPK3-IL-6 axis mediates kidney injury in cytokine storm syndrome

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted by the TRI Reagent method (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) and 1 μg was reverse transcribed with High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed in a Quant Studio 5 PCR System with the QuantStudio 3/5 Real-Time PCR Software using predeveloped primers (Applied Biosystems) and RNA expression of different genes was corrected for GAPDH expression [1].

Histological Assessment of Tubular Injury

Kidneys from mice were fixed in formalin for at least 48 hours and embedded in paraffin blocks. Sections 3 μm thick were stained with Hematoxylin and Eosin (H&E) and Periodic Acid-Schiff (PAS) using standard histological procedures. Tubular damage and inflammation were evaluated in four randomly chosen fields (two cortical and two from the outer stripe of the outer medulla, OSOM) at 400× magnification on H&E-stained sections. PAS staining was performed to enhance lesions visualization and confirm brush border loss and intratubular cast formation in renal tissue sections. Tubular injury was defined by the presence of lumen dilation and/or epithelial attenuation, cytoplasmic vacuolization or hydropic degeneration, cast formation, and epithelial regeneration (mitotic figures). Inflammation was assessed based on the presence of inflammatory cells infiltrating the renal interstitium. Each histological parameter was graded on a scale from 0 to 4: 0 = none, 1 = mild, 2 = moderate, 3 = marked, and 4 = severe, based on both the intensity and extent of the lesion (0%, ≤10%, 11–25%, 26–50%, >50%, respectively). The final renal injury score for each mouse was determined by summing the scores of all histological parameters within each individual field, followed by calculating the average of these sums across the four fields analyzed per animal. The scale for final score is 0-5=mild, 6-12= moderate, 13-18=marked.

Immunohistochemistry

Kidney tissue staining was performed in 3 μm thick sections of paraffin-embedded tissue using a PT-link device with a low pH solution. Sections were then washed with PBS for 5 minutes,

blocked by incubation with 4% PBS/BSA + 6% serum and incubated with primary antibody overnight. Primary antibodies were rabbit polyclonal anti-F4/80 (1:100, Ref. 70076, CST, Danvers, MA, USA) and anti-CD3 (ready to use, Ref. M7254, Agilent-DAKO, Santa Clara, CA, USA). Sections were counterstained with Carazzi‘s hematoxylin. Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody. The total number of positive cells was quantitated in 20 randomly chosen fields (20x) using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Samples were examined in a blind manner.

Western blot

Tissue samples were homogenized in lysis buffer T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) then separated by 10% SDS-PAGE under reducing conditions. After electrophoresis, samples were transferred to PVDF membranes (Millipore-Merck, Darmstadt, Germany), blocked with 5% skimmed milk in PBS/0.5% v/v Tween 20 for 1 hour, washed with PBS/Tween, and incubated with anti-RIPK3 (1:1000, Ref. 2283, ProSci, Poway, CA, USA), anti-cleaved-caspase 1 (1:500, Ref. AG20B-0042-C100, Adipogen, San Diego, CA), anti-NLRP3 (1:500, Ref. D4D8T, CST), anti-phospho-Stat3 (1:1000, CST) and Stat3 (1:1000, CST) antibodies diluted in 5% milk PBS/Tween. Blots were washed with PBS/Tween, incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:5000, GEHealthcare, Aylesbury, UK), developed with the chemiluminescence method (ECL) (Thermo Fisher Scientific) and probed with mouse monoclonal anti-α-tubulin (1:10000, Ref. T5168, Sigma-Aldrich) or anti-GAPDH (1:5000, Ref. mab374, Millipore-Merck) antibodies. Levels of expression were corrected for minor differences in loading. Uncropped gel scans merged with molecular weight images for all presented western blots are shown in **Fig. S7.**

Immunofluorescence

RIPK3 immunofluorescence in tissue sections was performed using anti-RIPK3 antibody (1:200, Ref. 2283, ProSci) and the Tyramide SuperBoost™ Kits with Alexa Fluor™ Tyramides, as recommended by the manufacturer (Invitrogen). For macrophage colocalization, RIPK3 staining was followed by incubation overnight at 4ºC with anti-F4/80 (1:50, Ref. MCA497,

BioRad, Hercules, CA). Then, slides were washed with PBS and incubated with anti-rat Alexa 633 conjugated secondary antibodies (1:200, Invitrogen). Nuclei were counterstained with DAPI (Sigma-Aldrich). Colocalization images were obtained using confocal fluorescence microscopy (LEICA TCS SP5 II) and imaging software ("LAS AF", Leica, Wetzlar, Germany).

ELISA

Plasma IL-6 (BD Pharmingen, San Diego, CA) was assessed by ELISA according to the manufacturer's instructions.

RNAseq

The study was performed at Unidad Genómica Cantoblanco, Fundación Parque Científico de Madrid, Madrid, Spain. Total RNA was extracted with PureLink RNA Mini Kit (Invitrogen, Thermo Fisher, Waltham, MA) from control, and CSS-AKI (3 animals per group) obtained 24h post induction of CSS-AKI. From 1 μg total kidney RNA, the PolyA+ fraction was purified and randomly fragmented, converted to double stranded cDNA and processed through subsequent enzymatic treatments of end-repair, dA-tailing, and ligation to adapters with NEBNext® Ultra™ II DNA Library Prep Kit (New England Biolabs, Ipswich, MA) following the manufacturer's recommendations. The adapter-ligated library was completed by PCR with Illumina PE primers (10 cycles). The resulting purified cDNA library was applied to an Illumina flow cell for cluster generation and sequenced for 50 bases in a single-read format (Illumina HiSeq 2000). Sequencing quality was first checked with FastQC. The FASTQ files were pre-processed to filter by size, quality and content of Ns using the Prinseq tool. Reads were then aligned to the mouse genome (GRCm38.p5. genome) with TopHat-2.0.1050. Transcripts assembly, estimation of their abundance and differential expression were calculated with Cufflinks-Cuffdiff in combination with CummeRbund 26. Transcripts with FPKM expression values lower than 0.05 in both conditions were considered not expressed and were excluded from further analysis. These data have been deposited in Gene Expression Omnibus (GEO) database (GSE243633). Upstream regulator analyses were performed using Ingenuity Pathway Analysis (IPA, Quiagen, Hilden, Alemania).

Olink Inflammation-Related Biomarker Analysis

Plasma and kidney samples from control (n=3), WT CSS-AKI (n=4) and Ripk3-KO CSS-AKI (n=4) mice were evaluated using the Olink mouse exploratory panel (Olink Proteomics AB, Uppsala, Sweden), which allows for simultaneous analysis of 92 biomarkers. In summary, the target protein binds specifically to the double-oligonucleotide-labeled antibody probe, and the oligonucleotide sequence is quantified via microfluidic real-time PCR amplification (Cobiomic Bioscience S.L, Córdoba, Spain).

Cells and reagents

MCT cells are a proximal tubular cell line harvested originally from the renal cortex of SJL mice and have been extensively characterized [2]. They were cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA), 10% decomplemented fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, in 5% CO_2 at 37ºC Haverty [2].

BM cells were collected from femur and tibia of WT and Ripk3-KO mice, sacrificed with CO_2. Isolated tibia and femur were flushed with RPMI 1640 (10% FBS). BM cells were cultured for 4 days in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin [3]. To this media, 5 ng/ml M-CSF (Biolegend, San Diego, CA, USA) was added for BM-derived macrophage (BMDM) cultures, while 5 ng/ml GM-CSF (Biolegend) and 5 ng/ml IL-4 (R&D Systems) were added for BM dendritic cell (BMDC) culture.

Primary tubular epithelial cells (TECs) were obtained from WT and Ripk3-KO mice [3]. After sacrificing with CO_2, the kidney cortex was decapsulated, dissected from the medulla, sliced, minced, and digested with 1.25 mg/mL collagenase Type I (GIBCO) at 37°C for 20 minutes. The rubber end of a syringe plunger was used to filter the pellet through a 100-µm cell strainer (Falcon-Corning, Corning, NY, USA) over a 50-ml conical tube by washing with Medium 199 1X (GIBCO). After depleting red blood cells with erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.5M EDTA), the pellet was passed through a 40-µm cell strainer (Falcon), and resuspended in RPMI 1640 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM

glutamine and ITS (Thermo Fisher, Waltham, MA, USA). Cells were seeded in gelatin type B-coated plates. After 7 days, cells were reseeded in the corresponding plates for experiments.

Subconfluent cells were rested in serum-free medium overnight and 100 ng/ml LPS was added for 6 hours.

To test the impact of the BMDM secretome on MCT cells, WT and *Ripk3*-KO BMDM were stimulated with 100 ng/mL LPS for 6 h. Then, the media was replaced by fresh media and cells were further incubated for 24 h. Then, MCT cells were cultured in BMDM-conditioned media for 24 h [3].

Data mining

To explore the clinical relevance of the murine findings in CSS-AKI, we chose AKI associated to COVID-19, a recognized cause of CSS-AKI[4] in which targeting IL-6 pathway activation with tocilizumab decreased the risk of early death (rate ratio 0.85; 95% CI 0.76-0.94) and of severe AKI requiring kidney replacement therapy (rate ratio 0.72, 0.58–0.90)[5]. Additionally, the literature on COVID-19 CSS included publicly available human data on kidney tissue transcriptomics representing COVID-19 AKI and control kidneys[6]. We searched in Gene Expression Omnibus and PubMed using key words IL-6 AND RIPK3 AND COVID-19. GSE202182 data were downloaded from https://www.ncbi.nlm.nih.gov/geo/ and processed using the tool GEO2R. Differences between healthy and COVID-19 AKI subjects were assessed by Mann-Whitney test. An adjusted p value <0.05 was considered statistically significant. IL-6 and RIPK3 were considered genes of interest.

Supplementary references

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Fig. S1

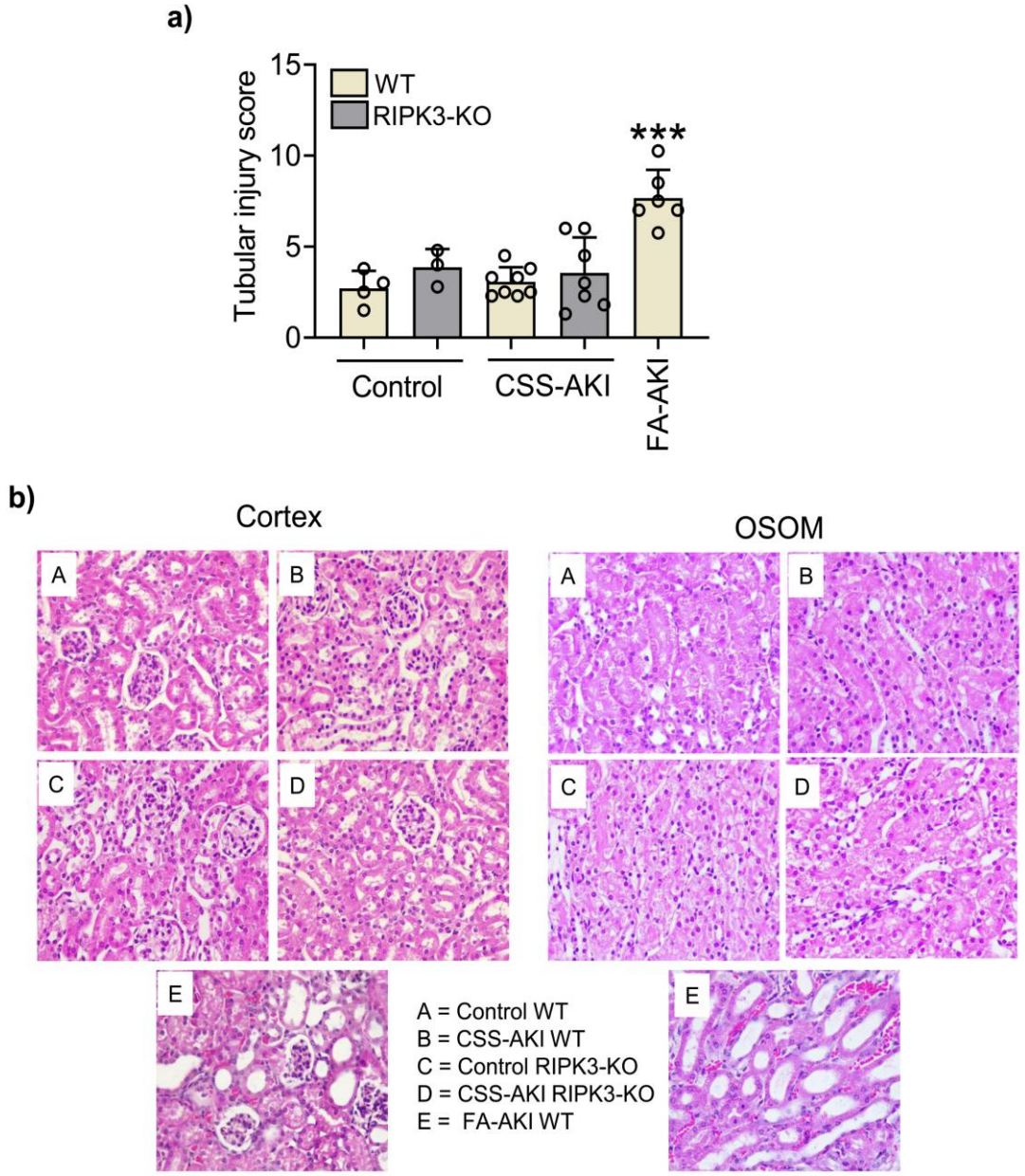

Fig. S1. CSS-AKI at 24 hours develops minor tubular injury in kidneys compared with FA-AKI at 48h. Quantification and representative image of H&E staining. Magnification 400x. Mean ± SD of 4-8 mice per group. ***p<0.001 vs Control WT. OSOM: outer stripe of the outer medulla.

Fig. S2

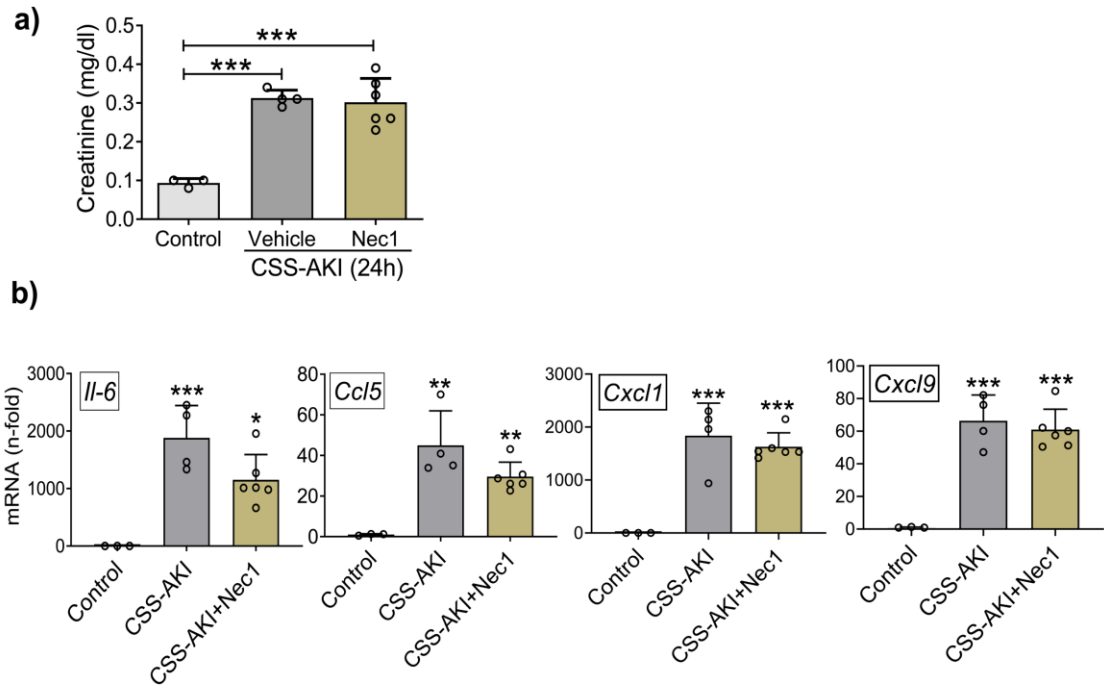

Fig. S2. Necroptosis inhibition does not protect from CSS-AKI kidney failure. WT mice were treated with the necroptosis inhibitor Necrostatin-1 (1.65 mg/kg) 30 minutes before CSS-AKI induction. **A)** Renal function assessed by plasma creatinine levels at 24 hours after CSS-AKI. **B)** Kidney mRNA levels of proinflammatory cytokines measured by RT-PCR 24 hours after induction of CSS-AKI. Mean ± SD of 3-6 mice per group. *p <0.05; **p <0.01; ***p <0.001.

Fig. S3

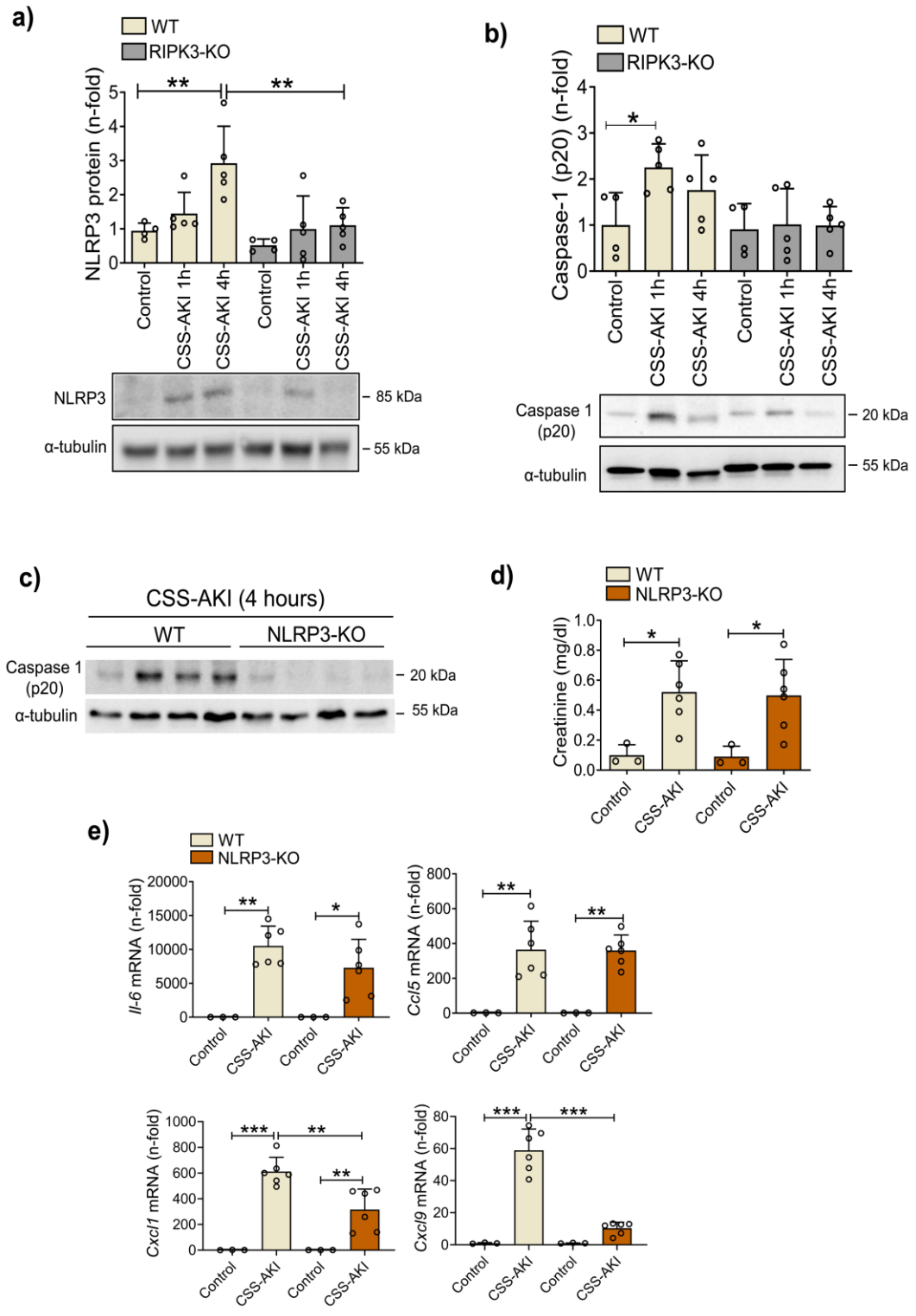

Fig. S3. NLRP3 targeting does not protect from CSS-AKI kidney failure. A, B) Representative western blot and quantification of kidney NLRP3 (A) and cleaved caspase 1 (B) in CSS-AKI at 1 and 4 hours. Mean ± SD of 4-5 animals per group. *p<0.05; **p<0.01. **C)** Western blot of cleaved caspase 1. Cleaved caspase 1 is not observed in Nlrp3-KO mice with

CSS-AKI, supporting the NLRP3-dependence of caspase 1 cleavage in this model. Representative western blot. **D)** Kidney function was assessed by plasma creatinine at 24 hours after CSS-AKI induction in WT and Nlrp3-KO mice. **E)** Kidney Il-6, Ccl5, Cxcl1 and Cxcl9 mRNA level expression assessed by RT-PCR at 24 hours of CSS-AKI. D, E) Mean ± SD of 3-6 mice per group. *p<0.05; **p<0.01; ***p<0.001.

Fig. S4

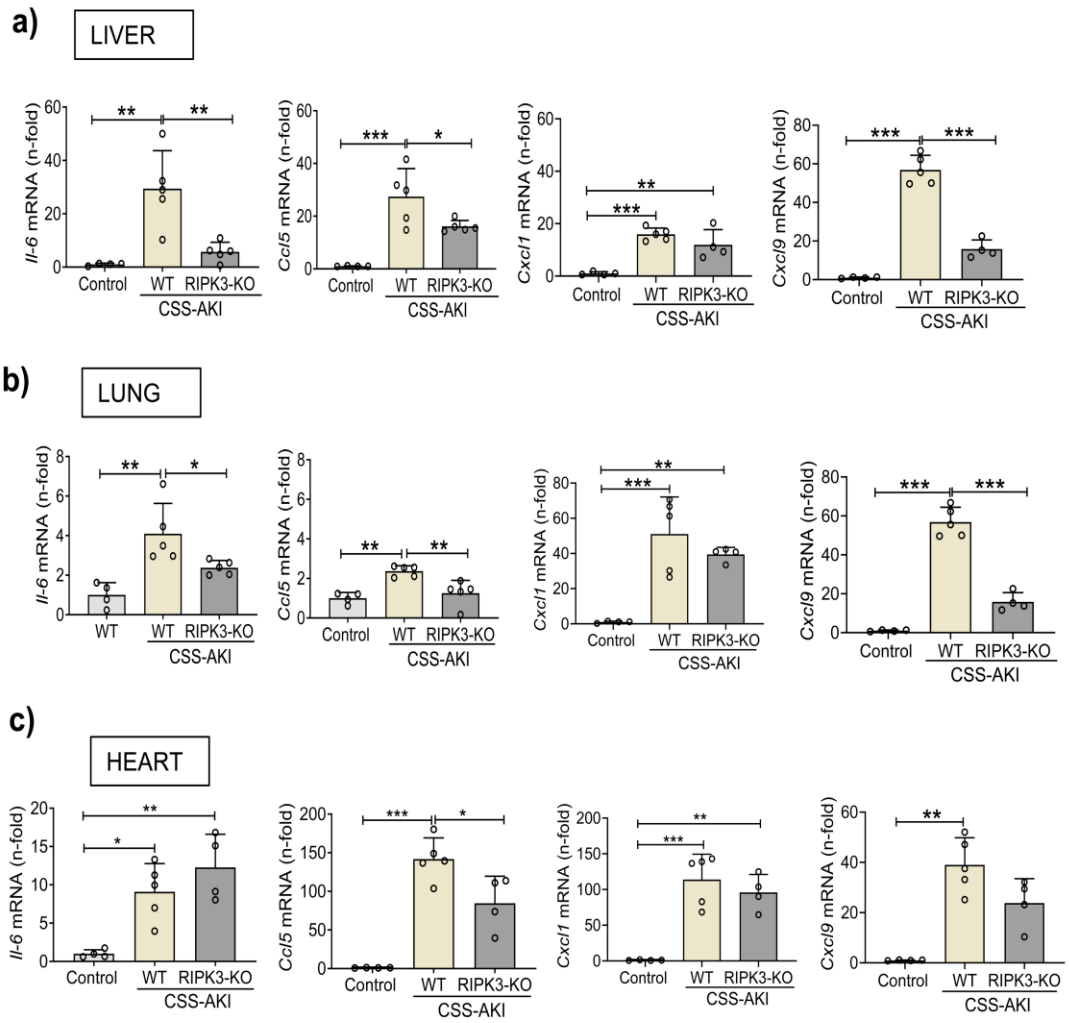

Fig. S4. ***Ripk3*** **deficiency reduces systemic inflammation in CSS. A-C)** *Il-6, Ccl5, Cxcl1* and *Cxcl9,* mRNA levels, measured by RT-PCR, in liver (A), lung (B) and heart (C) of mice with CSS at 24 hours. Mean ± SD of 4-5 animals per group. *p<0.05; **p<0.01; ***p<0.001.

Fig. S5.

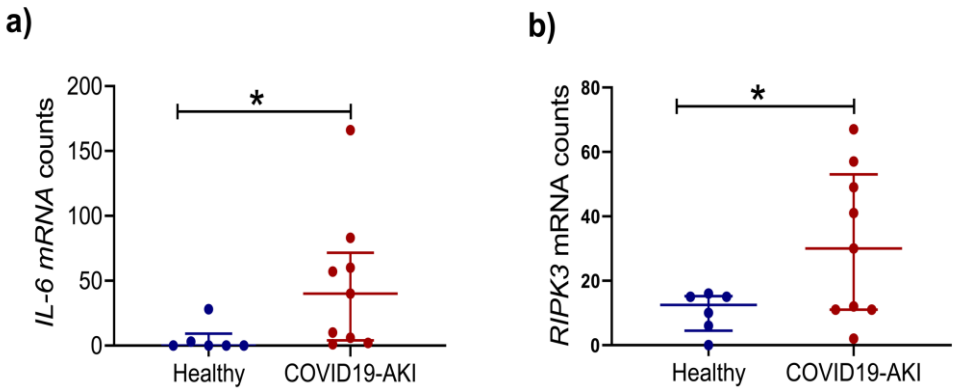

Fig. S5. Human kidney transcriptomics in CSS-AKI. Datamining of a human kidney transcriptomics dataset representing AKI associated to COVID-19 CSS and controls (GSE202182[6]) identified upregulation of *IL-6* (A) and *RIPK3* (B) mRNA in AKI associated to COVID-19 CSS. Data expressed as median with interquartile range of 6-9 subjects per group. *P <0.05

Fig. S6.

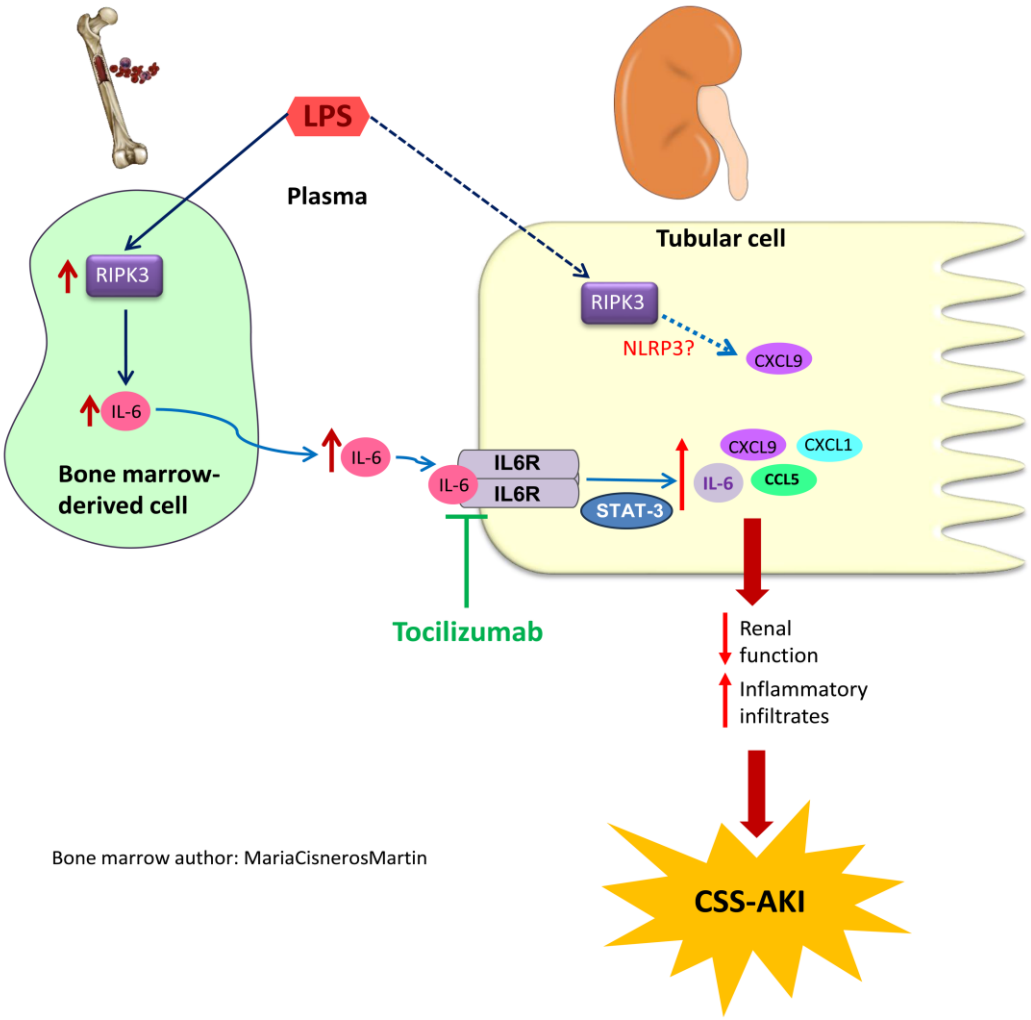

Fig. S6. Conceptual representation of RIPK3 role in CSS-AKI. Cytokine storm syndrome (CSS) is defined as a massive immune response that can lead to multi-organ dysfunction, including AKI, and even death. *In vivo* and *in vitro* studies have shown that RIPK3 in bone marrow cells promotes the expression of IL-6, thereby increasing plasma levels of this cytokine. Plasmatic IL-6 then reaches the kidney, where it activates the STAT3 pathway and triggers the expression of renal IL-6, CCL5, CXCL1 and CXCL9 leading to inflammatory infiltrates and renal injury. Targeting IL-6R inhibits IL-6 signaling in the kidney, preventing cytokine expression, inflammatory infiltrate and kidney dysfunction. In parallel, RIPK3 also induced the kidney expression of CXCL9, maybe through NLRP3 inflammasome, but our results do not support a major contribution of NLRP3, at least at the time points analyzed

Fig. S7.

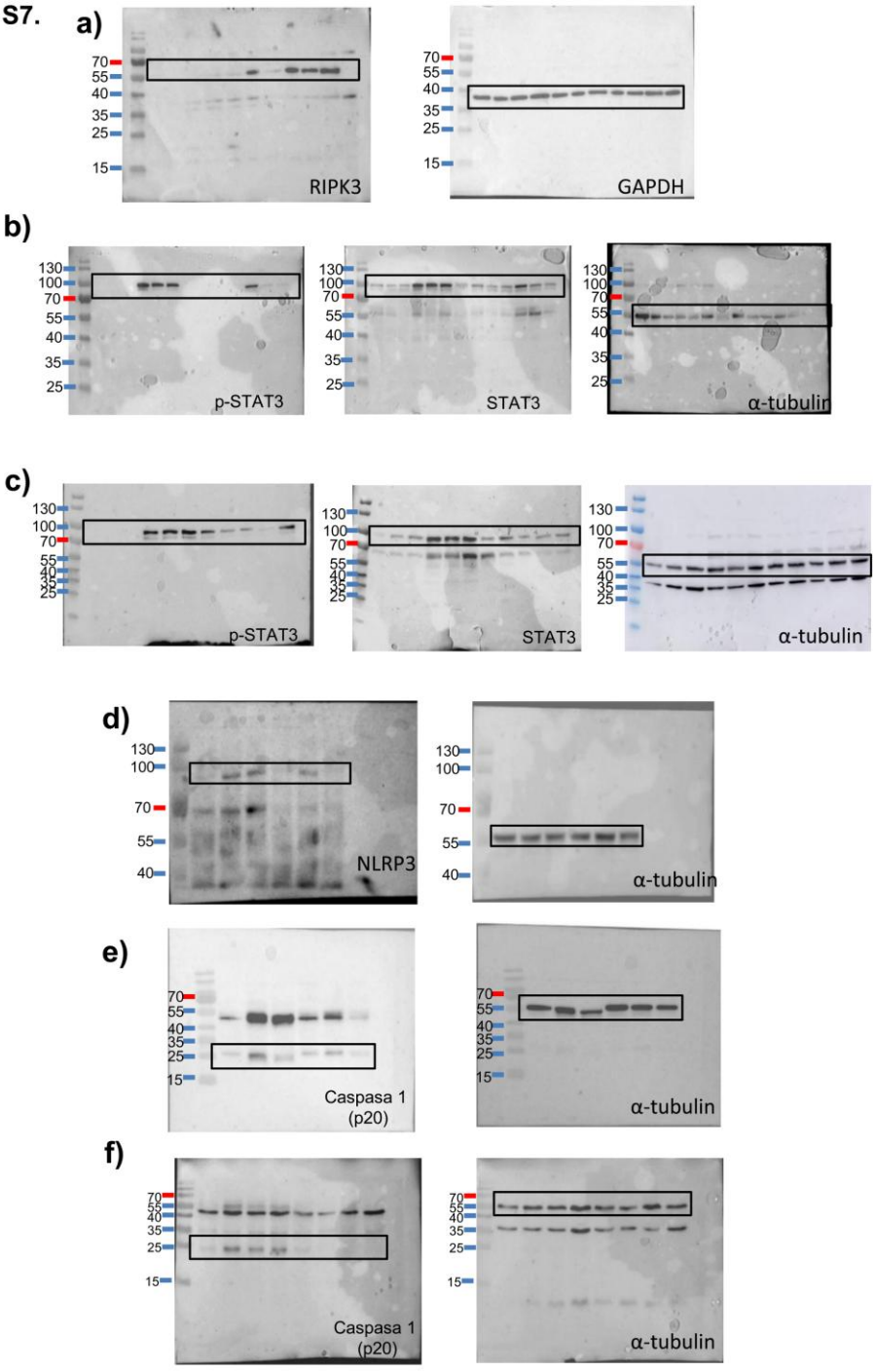

Fig. S7. Uncropped gel scans merged with molecular weight images for all presented western blots. **A)** Corresponds to Fig. 3.B**. B)** Corresponds to Fig. 6.B. **C)** Corresponds to Fig. 6.G. **D)** Corresponds to SF. 3.A. **E)** Corresponds to SF. 3.B. **F)** Corresponds to SF. 3.C

Supplementary Table 1. Correlation between kidney cytokine expression and severity of kidney failure in mouse chimera. Pearson correlation. R values shown.

	Creatinine	BUN
Il-6	0.630***	0.740***
Ccl5	0.451*	0.630***
Cxcl1	0.473*	0.443*
Cxcl9	0.358	0.439*

***p<0.001; **p<0.01