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Loss of NLRP6 expression increases the severity of acute kidney injury

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ABSTRACT

Background. Nlrp6 is a nucleotide-binding oligomerization domain-like receptor (NLR) that forms atypical inflammasomes. Nlrp6 modulates the gut epithelium interaction with the microbiota. However, the expression and function of Nlrp6 in the kidney, a sterile environment, have not been characterized. We explored the role of Nlrp6 in acute kidney injury (AKI).

Methods. In a transcriptomics array of murine nephrotoxic AKI, Nlrp6 and Naip3 were the only significantly downregulated NLR genes. The functional implications of Nlrp6 downregulation were explored in mice and in cultured murine tubular cells.

Results. Nlrp6 was expressed by healthy murine and human kidney tubular epithelium, and expression was reduced during human kidney injury or murine nephrotoxic AKI induced by cisplatin or a folic acid overdose. Genetic Nlrp6 deficiency resulted in upregulation of kidney extracellular signal–regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) phosphorylation and more severe AKI and kidney inflammation. In cultured tubular cells, Nlrp6 downregulation induced by specific small interfering RNA resulted in upregulation of ERK1/2 and p38 phosphorylation and chemokine messenger RNA expression and downregulation of the nephroprotective gene *Klotho*. MAPK inhibition prevented the inflammatory response in Nlrp6-deficient cells.

Conclusion. Nlrp6 dampens sterile inflammation and has a nephroprotective role during nephrotoxic kidney injury through suppression of MAP kinase activation.

Keywords: acute kidney injury, apoptosis, fibrosis, inflammasome, NLRP6

INTRODUCTION

Acute kidney injury (AKI) is associated with interstitial inflammation and tubular injury, and nephrotoxicity is a key cause of AKI [1, 2]. However, there is no satisfactory treatment for AKI. Additionally, kidney disease is among the fastest growing causes of premature death worldwide [3]. Renal tubular epithelial cells are a central cell type in renal inflammation [4]. Sublethal or lethal tubular cell injury leads to secretion of chemokines or inflammatory cytokines as well as the release of damage-associated molecular patterns (DAMPs) that, activate the inflammasome [4–7]. Inflammasomes are multiprotein molecular platforms that, in response to infection or sterile stressors, promote the activation of caspase-1 and maturation of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 to engage innate immune defenses [8, 9]. Inflammasomes have been linked to several autoinflammatory and autoimmune diseases [10].

The nucleotide-binding oligomerization domain (NOD)-like family of receptors [NOD-like receptors (NLRs)] represents a major class of pattern recognition receptors in inflammasomes [11]. NLR proteins have three conserved domains: a central nucleotide-binding and oligomerization domain (NACHT), a C-terminal leucine-rich repeat domain (LRR) and an N-terminal effector domain [11]. The N-terminal effector domain may contain a pyrin domain (NLRP subgroup) or a caspase activation and recruitment domain (CARD, NLRC subgroup). The NLRP3 inflammasome has been most extensively studied in the context of kidney disease, where it promotes kidney injury under conditions of sterile inflammation [5, 12–17]. However, additional NLRs can form inflammasomes and contribute to kidney injury. More recently, the NLRC4 inflammasome was suggested to be involved in diabetic nephropathy [18].

NLRP6 is a poorly characterized NLR that forms atypical inflammasomes [19]. NLRP6 is expressed by haematopoietic cells, including dendritic cells, neutrophils, macrophages and T cells [20–23]. It is also highly expressed by gut epithelium (duodenum, ileum and colon), where it plays an important role in intestinal homeostasis by regulating interactions with the local microbiota [20, 21, 24]. NLRP6 is downregulated in colitis and protects against the development of colitis and colitis-

associated tumorigenesis in mice through the regulation of IL-18 production [21, 22, 24]. NLRP6 was also required for gut epithelial repair [22]. In the non-sterile gut context, NLRP6 is a negative regulator of inflammatory signalling [20]. Infected Nlrp6-deficient macrophages produced increased levels of nuclear factor (NF)-κB- and mitogen-activated protein kinase (MAPK)-dependent cytokines and chemokines [20]. Additionally, altered autophagy in NLRP6-deficient mice leads to altered goblet cell function and mucus secretion, increasing the susceptibility to persistent infection by certain pathogens [25].

However, the role of NLRP6 during kidney injury remains poorly characterized and is not expected to be related to the microbiota, since under physiological conditions the kidney is a sterile environment. We have now shown for the first time that Nlrp6 plays a protective role in nephrotoxic AKI. During nephrotoxic AKI, Nlrp6 is downregulated, and Nlrp6 deficiency increases the severity of AKI. Functional studies have identified MAPK activation, chemokine expression and cell death as novel Nlrp6-regulated processes in tubular cells that may underlie the nephroprotective effect of Nlrp6 in nephrotoxic AKI.

MATERIALS AND METHODS

Animal models

Studies were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Acute folate nephropathy causes a reversible increase in serum creatinine and urea, tubular cell death, compensatory tubular cell proliferation, activation of an inflammatory response and eventual progression to mild fibrosis, processes that are characteristic of human nephrotoxic AKI [26]. Indeed, folic acid nephropathy has been reported in humans [27]. In a first set of experiments, female 12- to 14-week-old Nlrp6 wild-type (WT) C57BL/6 mice received a single intraperitoneal injection of folic acid (Sigma-Aldrich, St. Louis, MO, USA) 250 mg/kg in 0.3 mol/L sodium bicarbonate or vehicle and were euthanized 24 or 72 h after injection ($n = 6$ per group). Kidney samples from the 24 h time point were used for transcriptomics analyses.

Cisplatin is among the most nephrotoxic drugs in clinical use [28, 29]. AKI was induced by the intraperitoneal injection of a single dose of 25 mg/kg cisplatin (Sigma-Aldrich) dissolved in 0.9% saline solution in female 12- to 14-week-old C57BL/6 WT mice ($n = 5$). The cisplatin dose was based on literature analysis and results of preliminary experiments, showing renal function impairment at day 3 after cisplatin injection (urea in vehicle 37 ± 2 mg/dL versus cisplatin 116 ± 12 mg/dL).

To assess the role of Nlrp6 in nephrotoxic AKI, female 12- to 14-week-old WT or Nlrp6$^{-/-}$ C57BL/6 littermate mice received a single intraperitoneal injection of folic acid (Sigma-Aldrich) 250 mg/kg in 0.3 mol/L sodium bicarbonate or vehicle and were euthanized 72 h after injection ($n = 6$ per group), as serum creatinine peaks at 72 h. Nlrp6$^{-/-}$ mice were from Prof. Gabriel Nuñez, University of Michigan [21].

Kidneys were perfused *in situ* with cold saline before removal. Half a kidney was snap frozen in liquid nitrogen for RNA and protein studies and the other half was fixed in paraformaldehyde and paraffin-embedded for histological studies.

Cells and reagents

MCT cells are a cultured line of proximal tubular epithelial cells harvested originally from the renal cortex of SJL mice and have been extensively characterized [30]. 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% carbon dioxide at 37°C [30]. PD98059 (ERK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor; Stressgen Bioreagent, Ann Arbor, MI, USA) were dissolved in dimethyl sulfoxide. Only cells still attached to the plate after removing the supernatant and washing were used for messenger RNA (mRNA) and protein studies.

Western blot

Tissue and cell samples were homogenized in lysis buffer then separated by 10 or 12% sodium dodecyl sulfate–polyacrylamide gel under reducing conditions and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), blocked with 5% skimmed milk in phosphate-buffered saline (PBS)/0.5% v/v Tween 20 for 1 h, washed with PBS/Tween and incubated with goat polyclonal anti-human NLRP6 [Santa Cruz Biotechnology, Dallas, TX, USA (F20): sc-50636] or anti-murine NLRP6 antibody [Santa Cruz Biotechnology (E20): sc-50635, both at 1:500], mouse monoclonal anti-p-ERK (1:500), rabbit polyclonal anti-ERK1/2 (1:500), mouse monoclonal anti-p-p38 (1:500), mouse monoclonal anti-NLRP3 (1:1000; AdipoGen, San Diego, CA, USA), rabbit polyclonal anti-cleaved caspase-3 (1:1000; Cell Signaling Technology, Danvers, MA, USA), or goat polyclonal anti-p38 (1:2500; Santa Cruz Biotechnology) [26]. Rabbit polyclonal anti-NLRP6 (1:1000; Abcam, Cambridge, UK) yielded similar results. Antibodies were diluted in 5% milk PBS/Tween. Blots were washed with PBS/Tween and subsequently incubated with appropriate horseradish peroxidase–conjugated secondary antibody (1:2000; Amersham, Aylesbury, UK). After washing, blots were developed with the chemiluminescence method (ECL, Amersham). Blots were then probed with mouse monoclonal anti-α-tubulin antibody (1:5000; Sigma-Aldrich) and levels of expression were corrected for minor differences in loading.

Human tissue

Normal and diseased kidney tissue was obtained from the IIS-Fundacion Jimenez Diaz Biobank, which is part of the Spanish Biobanks Platform (PT17/0015/0006). Samples are incorporated into the biobank after written informed consent has been obtained from patients. The biobank and its consent form were approved by the ethics committee of the IIS-FJD and comply with the Helsinki Declaration and Spanish law. Nephrectomy tissue was studied. As control kidney, tissue was obtained from one male, 76 years old, with serum creatinine 0.9 mg/dL, negative dipstick proteinuria and preserved kidney structure on optical microscopy. For kidney injury tissue, we studied kidney from a 74-year-old male with serum creatinine

1.6 mg/dL, 50 mg/dL dipstick proteinuria and signs of acute tubular injury and interstitial fibrosis.

Kidney transcriptomics

Two different transcriptomics studies were performed.

In three heathy adult WT C57BL/6 female mice, RNA-Seq was performed to assess the relative gene expression of members of the NLR family, including the NLRP and NLRC subfamilies. From 1 μg total kidney RNA, the polyA+ fraction was purified and randomly fragmented, converted to double-stranded complementary DNA (cDNA) and processed through subsequent enzymatic treatments of end repair, dA tailing and ligation to adapters as in Illumina's TruSeq Stranded mRNA Sample Preparation Part #15031047 Rev. D kit. The adapter-ligated library was completed by polymerase chain reaction (PCR) with Illumina PE primers (eight 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). Next-generation sequencing data analysis was performed with the nextpresso pipeline [31] as follows: sequencing quality was first checked with FastQC [32]. Reads were then aligned to the mouse genome (GRCm38/mm10) with TopHat-2.0.1050, using Bowtie 1.0.051 and Samtools 0.1.1952, allowing two mismatches and five multihits. Transcripts assembly, estimation of their abundance and differential expression were calculated with Cufflinks 2.2.150, using the mouse genome annotation data set (GRCm38/mm10).

Additionally, to assess changes in gene expression in nephrotoxic AKI, Affymetrix (Santa Clara, CA, USA) transcriptomics arrays were performed at Unidad Genomica Moncloa (Fundacion Parque Cientifico, Madrid, Spain) in three vehicles and three folic acid WT AKI mice at 24 h post-injection. Image files were initially obtained through Affymetrix GeneChip Command Console software. Subsequently Robust Multichip Analysis was performed using the Affymetrix Expression Console software. Starting from the normalized Robust Multichip Analysis, the Significance Analysis of Microarrays was performed using the limma package (Babelomics, http://www.babelomics.org) and a false discovery rate of 5% to identify genes that were significantly differentially regulated between the analysed groups [33]. The full results of these arrays have been previously published [34].

Quantitative reverse transcription-PCR

One μg RNA isolated by Trizol (Invitrogen UK) was reverse transcribed with the High Capacity cDNA Archive Kit and real-time PCR was performed on a ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA, USA) using the delta-delta Ct method. Expression levels are given as ratios to glyceraldehyde-3-phosphate dehydrogenase. Pre-developed primer and probe assays were from Applied Biosystems [26].

Transfection of small interfering RNA (siRNA)

Cells were grown in six-well plates (Costar, Cambridge, MA, USA) and transfected with a mixture of 40 nmol/mL siRNA (Ambion, Applied Biosystems, Foster City, CA), Opti-MEM I Reduced Serum Medium and Lipofectamine 2000 (Invitrogen) [35]. After 18 h, cells were washed and cultured for 6 h in complete medium and serum-depleted for 24 h before collection. This time point was selected from a time course of decreasing NLRP6 protein expression in response to siRNA. A negative control scrambled siRNA provided by the manufacturer did not reduce NLRP6 protein. Kinase inhibitors were added 24 h after siRNA addition and samples were collected 24 h later (48 h after siRNA addition).

Immunohistochemistry

Immunohistochemistry was performed as previously described on paraffin-embedded 5 μm tissue sections [35]. Primary antibodies were goat polyclonal anti-human NLRP6 [Santa Cruz Biotechnology (F20): sc-50636] or anti-murine NLRP6 antibody [Santa Cruz Biotechnology (E20): sc-50635], both at 1:75, rat polyclonal anti-F4/80 antigen (macrophages, 1:50; Serotec, Oxford, UK), rabbit polyclonal anti-phospho-p44/42 MAPK (ERK1/2), anti-phospho-p38 MAPK (1:100; Cell Signaling Technology), goat polyclonal anti-MCP-1 (1:50; Santa Cruz Biotechnology), rabbit polyclonal anti-RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted; 1:100; Millipore), anti-type I collagen (1:100; Abcam), or anti-fibronectin (1:250; Abcam), mouse monoclonal anti-caspase-1 (1:100; AdipoGen) and rat polyclonal anti-Ly-6G (neutrophils, 1:100; BioLegend, San Diego, CA, USA). Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody. Sections were counterstained with Carazzi's haematoxylin. Apoptosis was assayed by deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL, In Situ Cell Death Detection Kit; Roche, Basel, Switzerland) [26] costaining with rabbit polyclonal anti-type IV collagen (1:100; Abcam).

The image was quantified in cortical tissue with ImageProPlus software (Media Cybernetics, Bethesda, MD, USA) by a researcher blinded as to the nature of the samples. The results are shown as the percentage of positive-stained area versus total quantified area from 10 fields per kidney (×200 magnification). For macrophages, neutrophils and TUNEL-positive nuclei, these were counted in 10 fields (×200 magnification) per kidney and reported as the number of positive cells or nuclei per high-power field.

Cell death and apoptosis

Cells were cultured to subconfluence in six-well plates and transfected with Nlrp6 or scrambled siRNA as previously described [36]. Apoptosis was assessed by flow cytometry of DNA content. Adherent cells were pooled with spontaneously detached cells and stained in 100 μg/mL propidium iodide, 0.05% NP-40 and 10 μg/mL RNAse A in PBS at 4°C for >1 h. This assay permeabilizes the cells, allowing propidium iodide entry into all cells, dead or alive. Apoptotic cells are characterized by a lower DNA content (hypodiploid cells) because of nuclear fragmentation. Thus this assay is not based on the known ability of propidium iodide to enter dead cells. The percentage of apoptotic cells with decreased DNA content (A_o) was counted [36].

For assessment of cell death by annexin V/7-amino-actinomycin D (7-AAD) staining, 5×10^5 cells were washed with ice-cold PBS, resuspended in 100 µL binding buffer and stained with 2.5 µL PE-Annexin V and 5 µL 7-AAD. Cells were incubated for 15 min at 37°C in the dark. Then 400 µL of binding buffer was added just before flow cytometry. Cells were analysed using an FACS Canto cytometer and FACS Diva Software (BD Biosciences, Franklin Lakes, NJ, USA). Early and late cell death was evaluated on PE fluorescence (Annexin V) versus PerCP (7-AAD) plots. Cells stained only with Annexin V were evaluated as being in early cell death; cells stained with both Annexin V and 7-AAD were evaluated as being in late cell death [37]. Cell death was assessed 48 h post-transfection. Nlrp6 siRNA-specific cell death was estimated by subtracting death under control siRNA conditions from death under Nlrp6 siRNA conditions.

Enzyme-linked immunosorbent assay (ELISA)

Murine plasma IL-18 was determined by ELISA (R&D Systems, Minneapolis, MN, USA).

Statistics

Statistical analysis was performed using SPSS 11.0 statistical software (SPSS, Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD). Significance at the $P < 0.05$ level was assessed by Student's t test for two groups of data and analysis of variance for three or more groups with Bonferroni correction.

RESULTS

Identification of Nlrp6 as a downregulated gene in nephrotoxic AKI

Nephrotoxic AKI is characterized by increased serum creatinine, tubular cell death and interstitial inflammation and may evolve to mild fibrosis [1, 26]. NLR family members have been implicated in kidney injury through the modulation of cell death and inflammation [5, 12–18]. Specifically, excessive NLRP3 activity promoted kidney injury [5, 12–17]. A kidney transcriptomics array in preclinical nephrotoxic AKI induced by a folic acid overdose in mice confirmed an increased Nlrp3 mRNA expression (Supplementary data, Table S1). Strikingly, mRNA encoding for two NLRs (Nlrp6 and Naip3) was downregulated in injured kidneys (Supplementary data, Table S1). Of these, Nlrp6 expression was almost halved in AKI kidneys (Figure 1A). Additionally, healthy kidney RNA-Seq transcriptomics disclosed that Nlrp6 is the member of the NLR family with the highest constitutive expression in adult mouse kidney (Supplementary data, Table S2). Western blot confirmed the presence of an expected size 96-kDa protein reactive with anti-human NLRP6 antibodies in human kidney and a 96-kDa protein reactive with anti-murine Nlrp6 antibodies in normal murine kidney but not in Nlrp6 knockout (KO) mice (Supplementary data, Figure S1). Anti-human NLRP6 did not react with murine Nlrp6 and anti-murine Nlrp6 did not react with human NLRP6.

Based on these observations, we focused on Nlrp6 expression and function during renal injury. For this we studied mouse models of nephrotoxic AKI. AKI induced by a single injection of folic acid is characterized by increased serum creatinine, cell death and interstitial inflammation, features shared with human AKI [33]. The transcriptomic findings of decreased Nlrp6 were validated by real-time PCR in a second preclinical model of nephrotoxic AKI, cisplatin-induced AKI (Figure 1B). The findings of Nlrp6 mRNA downregulation were also validated in nephrotoxic AKI induced by a folic acid overdose and decreased protein was confirmed by Western blot at 72 h following induction of AKI (Figure 1C and D). Immunohistochemistry localized Nlrp6 expression to the cytoplasm of epithelial cells in normal proximal tubules and was consistent with Nlrp6 protein downregulation during AKI (Figure 1E). Interstitial macrophages did not stain for Nlrp6 (Supplementary data, Figure S2A). Indeed, tubular cell NLRP6 protein was also downregulated during human AKI, supporting the potential clinical relevance of the findings (Supplementary data, Figure S2B).

Nlrp6 deficiency increases the severity of nephrotoxic AKI

Since kidney Nlrp6 expression was reduced during kidney injury, we focused on the consequences of reduced Nlrp6 expression. To address the role of Nlrp6 in nephrotoxic AKI, we used Nlrp6$^{-/-}$ mice [21]. At baseline, kidneys from Nlrp6$^{-/-}$ mice appeared histologically normal and did not differ from baseline WT kidneys in chemokine and extracellular matrix gene expression or the number of TUNEL-positive cells or interstitial macrophages.

Nlrp6$^{-/-}$ mice developed a more severe nephrotoxic AKI characterized by higher serum creatinine and urea levels than WT mice with AKI (Figure 2A and B). Furthermore, Nlrp6$^{-/-}$ mice with AKI had more severe renal inflammation than WT mice with AKI. Kidney expression of chemokine mRNA (Figure 2C) and protein, which localized mainly to tubular and interstitial cells (Supplementary data, Figure S3A and B), and infiltration by interstitial macrophages (Figure 2D) and neutrophils (Supplementary data, Figure S3C) were higher in Nlrp6$^{-/-}$ mice with AKI than in WT mice with AKI. In contrast, plasma IL-18 levels were lower in AKI Nlrp6$^{-/-}$ than in AKI WT mice (Supplementary data, Figure S3D), similar to findings in Nlrp6$^{-/-}$ mice during colon inflammation [21, 24]. Klotho mRNA downregulation during AKI was more severe in Nlrp6$^{-/-}$ with AKI than in WT mice with AKI (Figure 2C). Additionally, the number of TUNEL-positive tubular cells representing dying cells (Figure 2E) and the expression of extracellular matrix genes involved in kidney fibrosis were also higher in Nlrp6$^{-/-}$ mice with AKI than in WT mice with AKI (Supplementary data, Figure S4). These results suggest that Nlrp6 downregulation during AKI may activate an amplification loop of inflammation and cell death potentially leading to kidney fibrosis.

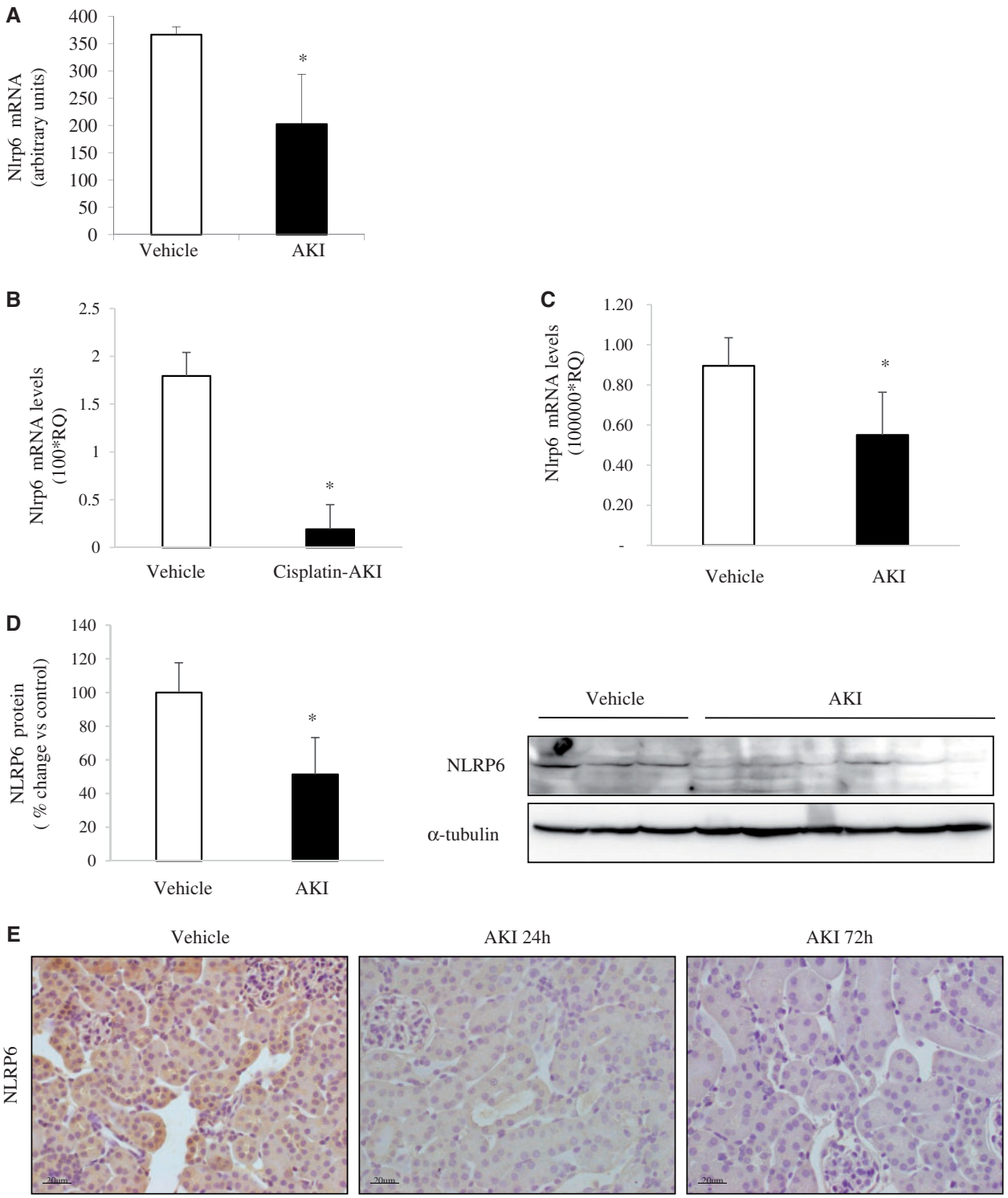

FIGURE 1: Decreased kidney Nlrp6 mRNA and protein expression in experimental AKI in WT mice. **(A)** Kidney transcriptomics results. Kidney tissue from AKI and vehicle-treated mice was studied 24 h following injection of folic acid or vehicle. **(B)** Nlrp6 mRNA expression in WT mice with cisplatin-induced AKI at 72 h following injection of cisplatin or vehicle. *$P < 0.0001$ versus vehicle. **(C)** Kidney Nlrp6 mRNA assessed by reverse transcription quantitative PCR 72 h following injection of folic acid or vehicle. *$P < 0.009$ versus vehicle. **(D)** Kidney Nlrp6 protein assessed by western blot at 72 h of injury. *$P < 0.01$ versus vehicle. **(E)** Immunohistochemistry localized NLRP6 expression to tubular cells. Original magnification 40×. Data expressed as mean ± SD of $n = 3$ mice [(A) kidney transcriptomics] or six mice per group (B)–(D). RQ, relative quantitation.

Nlrp6$^{-/-}$ mice have been reported to display increased ERK1/2 phosphorylation after peripheral nerve injury and in infected leucocytes and liver [20, 38]. Thus we explored whether differences in ERK1/2 and p38 phosphorylation were present in the kidney. Nlrp6$^{-/-}$ mice showed an increased baseline phosphorylation of ERK1/2 and p38 MAPK compared with WT mice (Supplementary data, Figure S5). Following induction of AKI, ERK1/2 phosphorylation in total

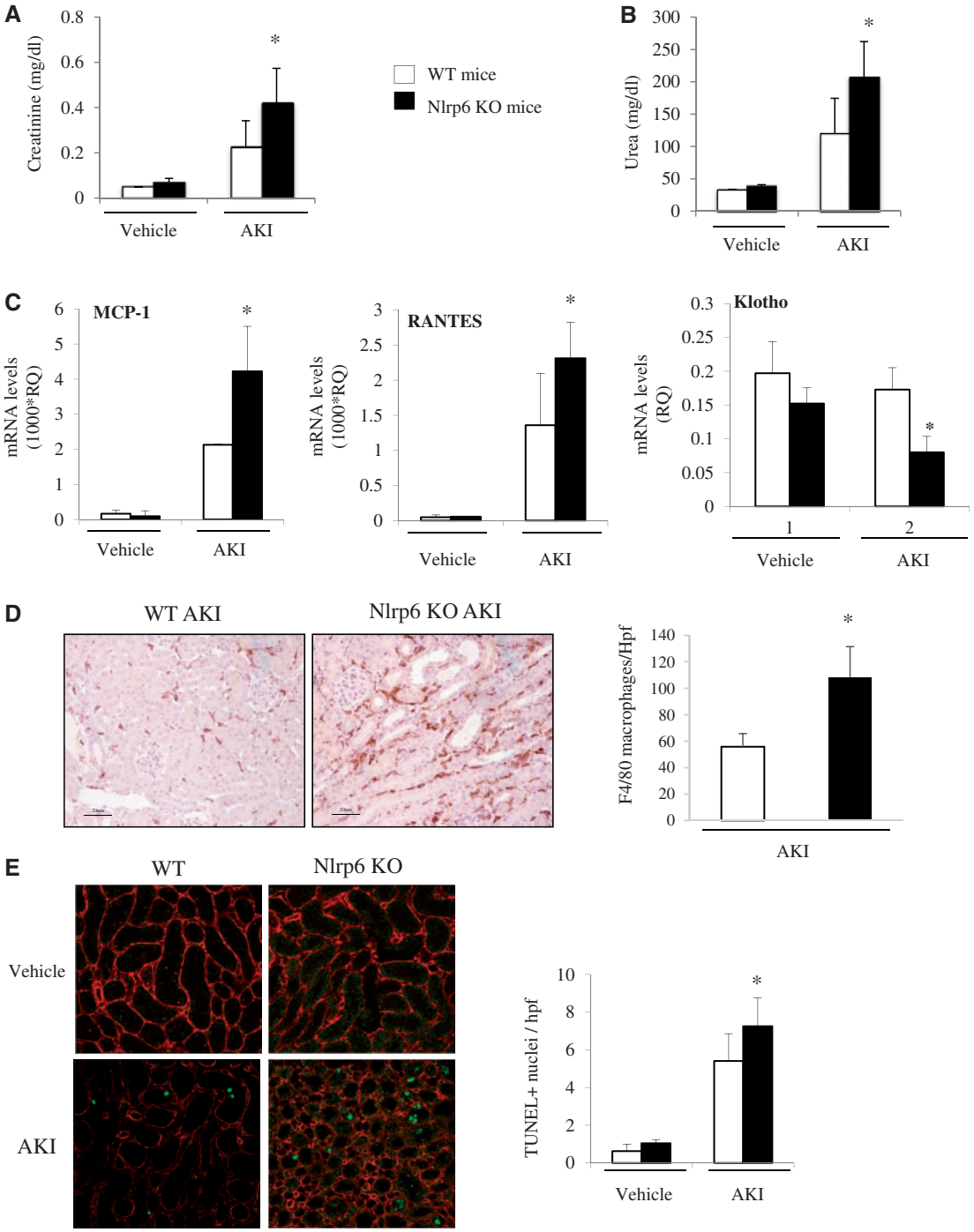

FIGURE 2: Nlrp6 deficiency results in more severe folic acid–induced AKI. (**A**) Serum creatinine. *P < 0.005 versus WT mice with AKI. (**B**) Serum urea. *P < 0.005 versus WT mice with AKI. (**C**) Higher whole kidney MCP-1, RANTES and lower Klotho mRNA expression in Nlrp6-deficient mice with AKI compared with WT mice with AKI. Reverse transcription quantitative PCR. *P < 0.05 versus WT AKI mice. (**D**) F4/80 macrophage immunohistochemistry. Macrophage infiltration is higher in Nlrp6-deficient mice with AKI than in WT mice with AKI. *P < 0.0001 versus WT AKI mice. Original magnification ×20. (**E**) Type IV collagen (red) and TUNEL staining (green) for fragmented DNA characteristic of cell death. The number of TUNEL-positive cells was higher in Nlrp6-deficient mice with AKI. *P < 0.03 versus WT AKI mice. Original magnification ×20. Data expressed as mean ± SD of $n = 6$ animals per group at 72 h.

kidney extracts was higher in Nlrp6$^{-/-}$ than in WT mice (Figure 3A). A similar observation was made for phosphorylated p38. During AKI, whole kidney extract p38 phosphorylation was higher in Nlrp6$^{-/-}$ mice than in WT mice (Figure 3C). Immunohistochemistry also localized the increased ERK1/2 (Figure 3B) and p38 (Figure 3D) phosphorylation to tubular cells, the key kidney cells in which Nlrp6 is expressed in WT mice. Since ERK1/2 and p38 MAPK activation contribute to kidney inflammation and injury [39–45], these results suggest that dysregulation of MAPK activation may contribute to the increased sensitivity of Nlrp6$^{-/-}$ mice to kidney injury.

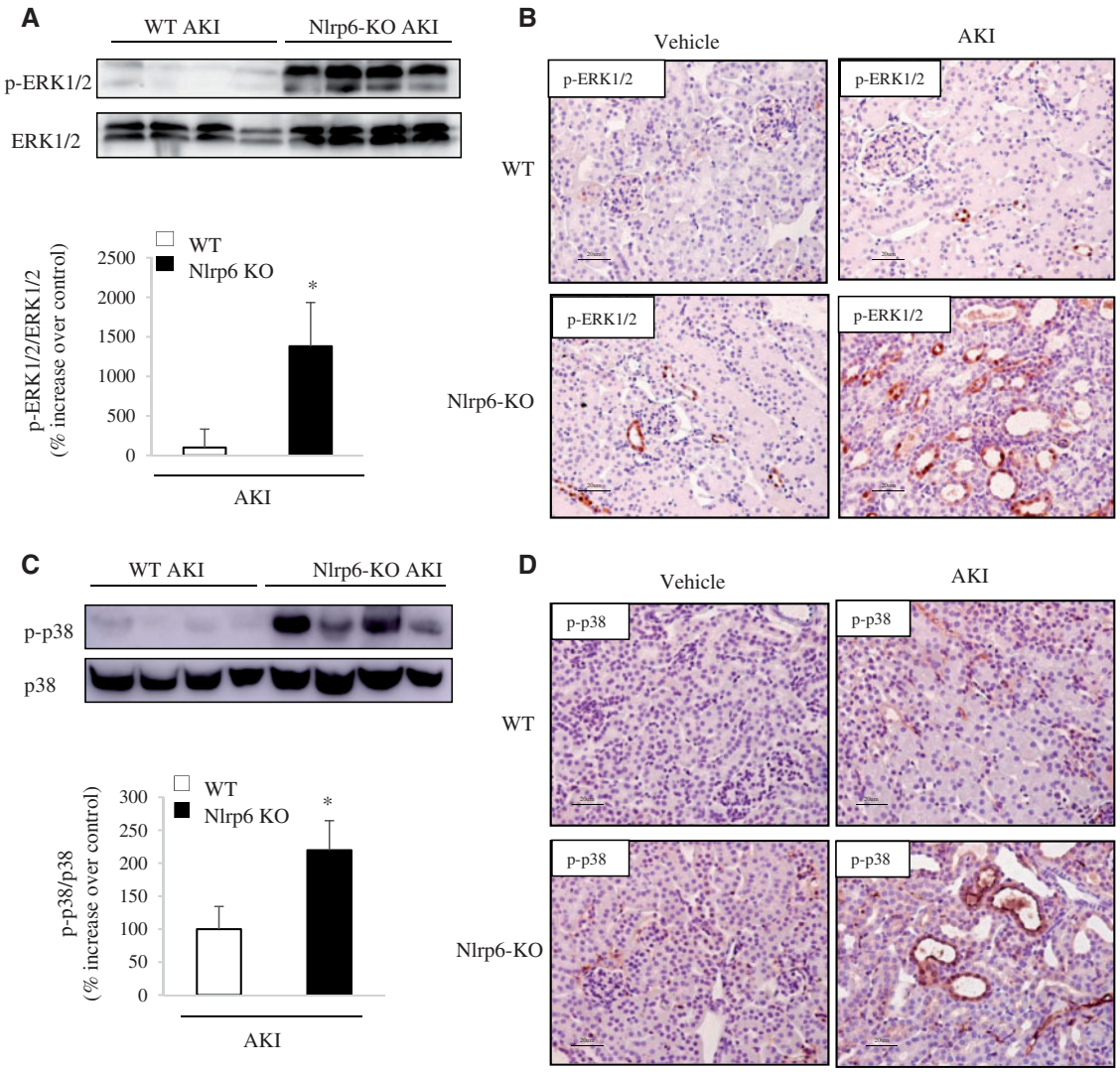

FIGURE 3: Nlrp6 deficiency results in higher ERK and p38 phosphorylation in folic acid–induced AKI. (**A**) ERK phosphorylation and (**C**) p38 phosphorylation were assessed by western blot in whole kidney extracts 72 h following administration of folic acid or vehicle. Representative western blot and quantification. *$P < 0.01$ versus WT mice. Data expressed as mean ± SD of $n = 6$ animals per group at 72 h. Immunohistochemistry showing (**B**) ERK phosphorylation and (**D**) p38 phosphorylation in tubular cells in Nlrp6$^{-/-}$ or WT mice with AKI. Original magnification ×20.

Immunostaining disclosed that caspase-1 was not detectable in tubular cells. Rather, it was present in interstitial cells, likely macrophages, which were more common in Nlrp6$^{-/-}$ mice (Supplementary data, Figure S6), confirming prior reports of inefficient Nlrp3 inflammasome activation in tubular cells [46].

Nlrp6 protects tubular cells from apoptosis and dampens inflammatory responses

Following the findings of Nlrp6 downregulation in tubular cells and of a deleterious effect of Nlrp6 deficiency on inflammation and tubular cell death *in vivo*, the function of Nlrp6 was explored in cultured murine tubular epithelial cells. In these cells, siRNA targeting resulted in downregulation of Nlrp6 protein (Figure 4A) but did not modify Nlrp3 levels (Supplementary data, Figure S7).

Deprivation of the survival factors from serum is a classical inducer of cell death [36]. Nlrp6 silencing increased cell death in serum-deprived tubular cells (Figure 4B) as well as activation of the effector caspase, caspase-3 (Figure 4C). Furthermore, Nlrp6 silencing amplified responses previously shown to be NF-κB-mediated in these cells [4, 47], such as the increased expression of the canonical NF-κB targets and pro-inflammatory chemokines MCP-1 and RANTES, and the downregulation of the anti-inflammatory gene Klotho (Figure 4D). These cell culture findings are in line with *in vivo* observations in Nlrp6$^{-/-}$ mice and suggest that uncontrolled NF-κB-mediated pro-inflammatory responses and sensitization to tubular cell death may contribute to the more severe kidney injury in Nlrp6$^{-/-}$ mice.

The pro-inflammatory and lethal responses in Nlrp6-deficient tubular cells are driven by MAPK activation

We next explored the state of ERK1/2 and p38 phosphorylation in cells targeted by specific Nlrp6 siRNA. Nlrp6 siRNA resulted in increased phosphorylation of both ERK1/2 and p38 (Figure 5A). Thus Nlrp6 downregulation in cultured tubular

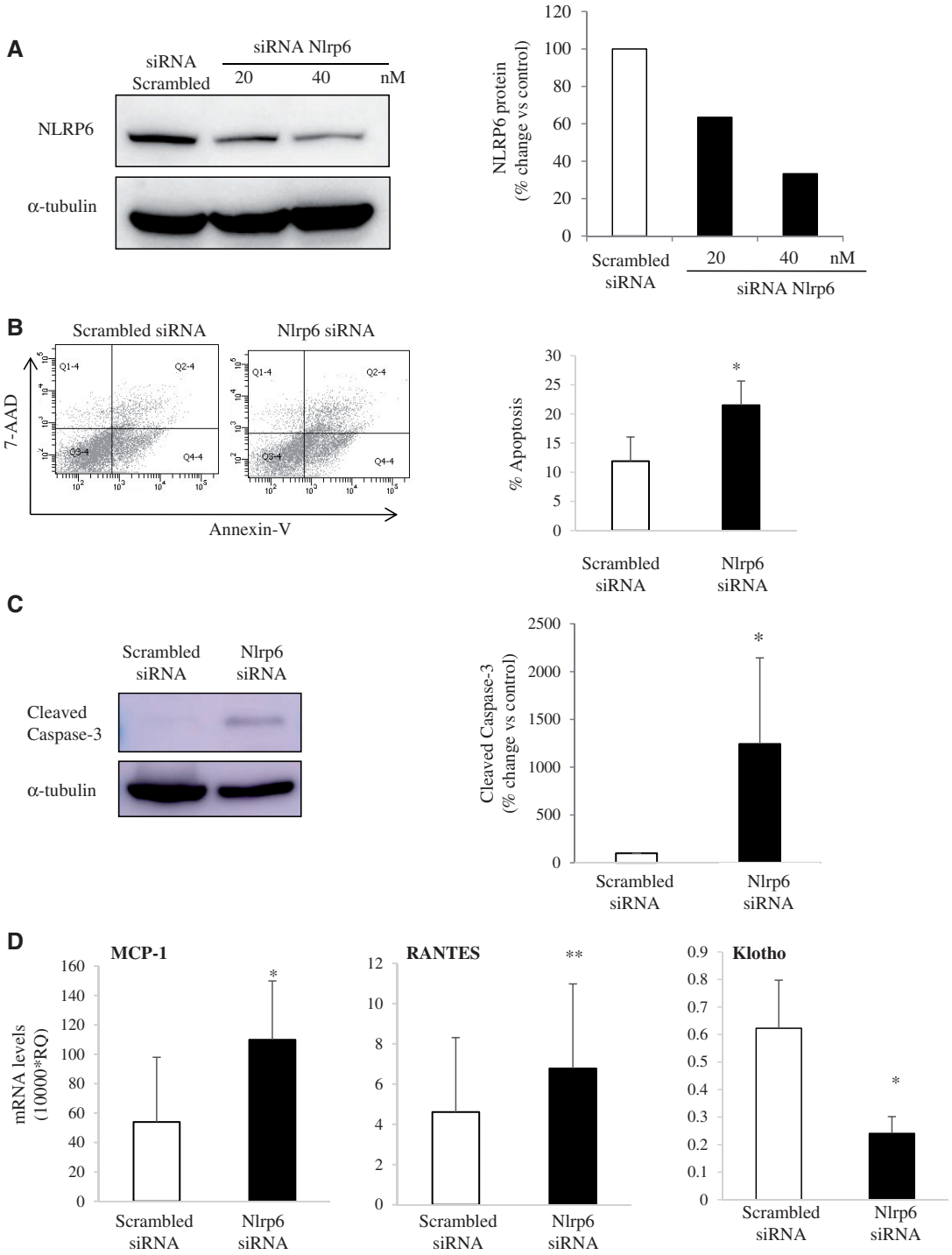

FIGURE 4: Functional characterization of Nlrp6 actions on cultured proximal tubular cells. (**A**) Nlrp6 siRNA silencing in cultured tubular cells suppressed Nlrp6 protein expression. Representative western blot and quantification. (**B**) Nlrp6 silencing increases spontaneous cell death of serum-deprived tubular cells. Representative flow cytometry diagrams and quantification of cell death. *$P < 0.03$ versus scrambled siRNA. Mean ± SD of three independent experiments. (**C**) Nlrp6 silencing promotes caspase-3 activation. *$P = 0.037$ versus scrambled siRNA mRNA upregulation. (**D**) Nlrp6 silencing promotes MCP-1 and RANTES mRNA upregulation and Klotho mRNA downregulation. *$P < 0.004$ and **$P < 0.05$ versus scrambled siRNA. Reverse transcription quantitative PCR. Mean ± SD of three independent experiments involving duplicate wells. Experiments collected 48 h after silencing.

cells reproduced the *in vivo* observation of excess MAPK activation observed in tubular cells during AKI. Given that excess ERK1/2 or p38 phosphorylation has been reported to contribute to kidney inflammation [39–45], we assessed whether ERK1/2 or p38 phosphorylation mediated the increase in chemokine mRNA expression observed in Nlrp6-deficient cells. Treatment with a specific ERK1/2 (PD98059) (Figure 5B) or p38 inhibitor (SB203580) (Figure 5C) resulted in milder

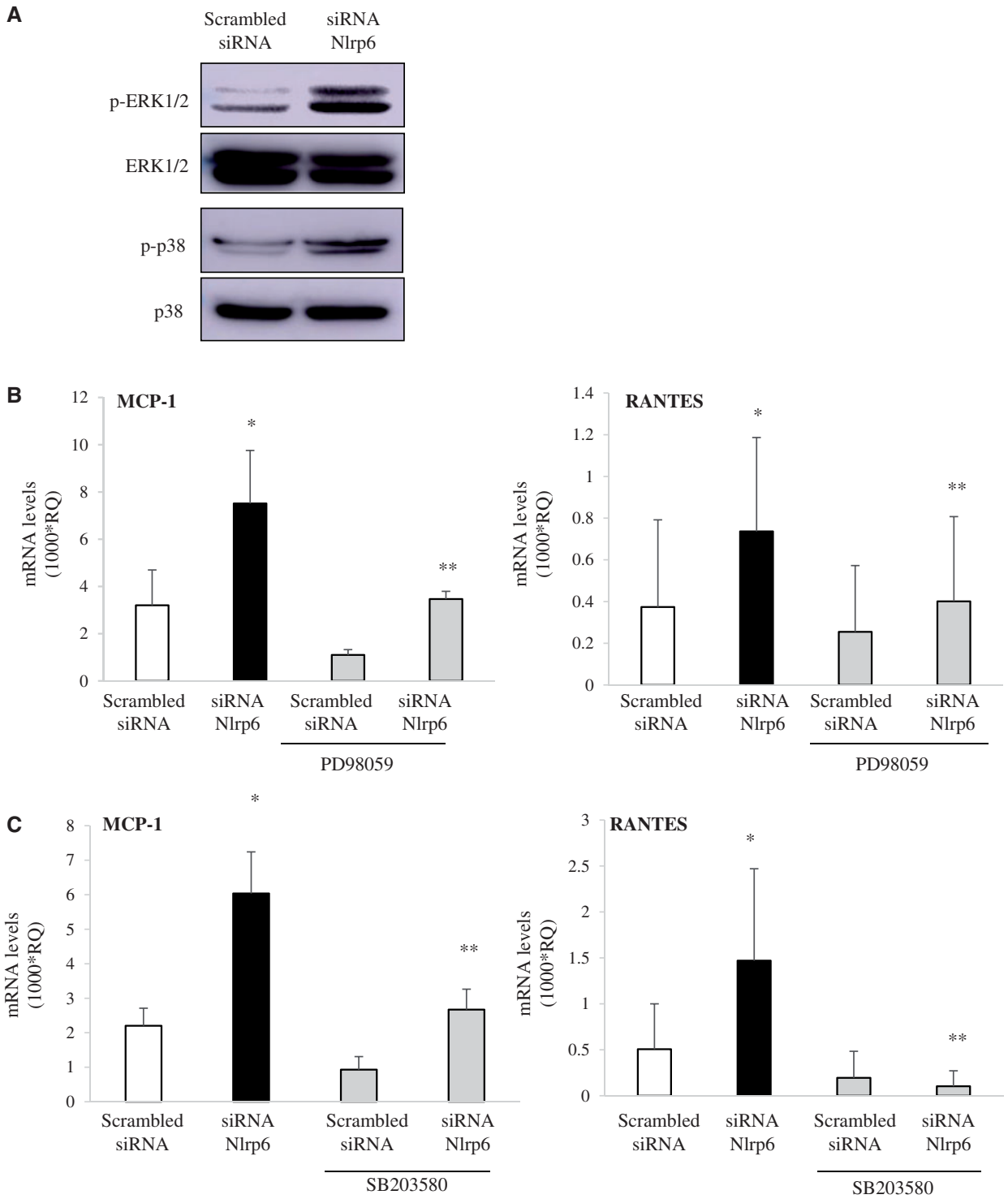

FIGURE 5: Nlrp6 downregulation in cultured proximal tubular cells promotes MAPK activation. (**A**) Nlrp6 siRNA silencing in cultured tubular cells promotes ERK1/2 and p38 phosphorylation as assessed by western blot. (**B** and **C**) In Nlrp6 silenced cells, pretreatment with inhibitors of ERK1/2 (20 μM PD98059) or p38 MAPK (5 μM SB203580) prevented the induction of MCP-1 and RANTES mRNA expression. *$P < 0.005$ versus corresponding scrambled siRNA, **$P < 0.01$ versus Nlrp6 siRNA alone. Reverse transcription quantitative PCR. Mean ± SD of four independent experiments involving duplicate wells. Experiments collected 48 h after silencing.

upregulation of MCP-1 and RANTES mRNA in response to Nlrp6 downregulation.

Finally, MAPK ERK1/2 inhibition also decreased tubular cell death in cells targeted by specific Nlrp6 siRNA (Supplementary data, Figure S8).

DISCUSSION

The main finding of this study is that tubular cell Nlrp6 expression is lost during nephrotoxic AKI and experiments using Nlrp6-deficient mice and cells suggest that Nlrp6 expression by kidney tubular cells is required to dampen sterile

inflammation and preserve the expression of nephroprotective molecules such as Klotho. Thus Nlrp6 deficiency results in more severe nephrotoxic AKI. This suggests a homeostatic function of Nlrp6 in kidney injury similar to its homeostatic role in gut epithelium. However, contrary to the gut situation, the kidney protective role of Nlrp6 occurs in a sterile environment.

The function of Nlrp6 has been studied in detail in the gut epithelium, where it is a key modulator of the interaction between the gut microbiota and the gut mucosa. Gut stress or injury leads to Nlrp6 downregulation and this, in turn, was thought to modify the microbiota (although this concept has been recently disputed [48–50]), sensitizing to chronic gut inflammation and carcinogenesis [19]. Interestingly, an abnormal microbiota itself may downregulate Nlrp6 expression through specific microbiota metabolites [51]. As a result, mice with dysbiosis secondary to Nlrp6 deficiency were thought to spread the abnormal microbiota and gut inflammation to mice bred in close contact [24]. Among the functions of Nlrp6 in this context, we find stimulation of goblet cell mucus secretion and of the secretion of antibacterial peptides [52, 53]. Additionally, the abnormal microbiota was thought to promote chemokine secretion by gut epithelial cells [19]. However, the concept of Nlrp6-deficiency-induced dysbiosis has been challenged [48–50]. Indeed, Mamantopoulos *et al.* [49] very strongly conclude that inflammasomes make the case for littermate-controlled experimental design. They found littermate-controlled $Nlrp6^{-/-}$ and $Nlrp6^{+/+}$ mice had no differences in gut microbiota, and thus in experimental colitis, as compared with previous studies showing more severe colitis due to altered microbiota in $Nlrp6^{-/-}$ mice than in non-littermate $Nlrp6^{+/+}$ mice. In this regard, the use of littermates supports that it might not be related to microbiota [48–50]. Furthermore, these mechanisms of action of Nlrp6 as an epithelial barrier protectant do not apply to the kidney, a sterile environment devoid of mucus-secreting cells where there is no microbiota to modulate epithelial cell chemokine expression. Initial Nrlp6 studies focused on the gut, as Nlrp6 was reported to be exclusively expressed in the intestinal tissues based on immunoblotting analysis of FLAG-Nlrp6 expression in tissues of FLAG-Nlrp6 knock-in mice, although liver expression was also noted [54]. However, anti-Nlrp6 antibodies were not tested and it was unclear whether exposure of the blot was enough to demonstrate Nrlp6 expression outside the intestine and liver. According to our data, Nlrp6 was the NLR family gene with the highest constitutive expression in healthy adult kidney and Western blot and immunohistochemistry studies using diverse antibodies against both human and murine NLRP6 confirmed tubular epithelial cell expression of the molecules in steady-state kidneys. Surprisingly, the role of Nlrp6 in the modulation of kidney inflammation in a sterile environment mimics its anti-inflammatory, homeostasis-preserving role in the gut and can be reproduced in tubular cells cultured in a sterile environment. In addition, Nlrp6 deficiency compromised the survival of cultured tubular cells. These observations are in line with the observed higher rate of cell death and more severe inflammation in Nlrp6-deficient mice during AKI. The consequences of the negative impact of Nlrp6 deficiency during AKI may be long-standing. In this regard, a signal was observed in experimental AKI suggestive of more severe activation of fibrogenic mechanisms, as there was increased expression of genes encoding fibrotic extracellular matrix.

Nlrp6 KO mice have lower serum and colon IL-18 during colon inflammation [21, 24]. This was pinpointed to result from decreased epithelial cell production of IL-18 directly related to Nlrp6 deficiency rather than to any change in hemopoietic cell–derived IL-18 production, and to contribute to gut inflammation, since IL-18 KO mice also displayed more severe colon inflammation [24]. Since we did not observe changes in kidney Nlrp3 expression or in macrophage expression of Nlrp6 in kidneys from WT mice with AKI and tubular cells, IL-18 production is well characterized [55, 56], we hypothesize that as in other organs, this is a direct consequence of Nlrp6 deficiency.

There is evidence that Nlrp3 may have inflammasome-independent functions in cells that do not readily form inflammasomes, such as epithelial cells. Thus primary mouse tubular epithelial cells lacking Nlrp3 displayed reduced apoptosis downstream of the tumour necrosis factor receptor and CD95. This was pinpointed to a non-canonical NLRP3 and ASC platform for caspase-8 activation, independent of the inflammasome that regulates apoptosis within epithelial cells [57]. However, this mechanism would not explain tubular cell death when Nlrp6 was targeted, since no changes in Nlrp3 were observed and the survival response was opposite that found for Nlrp3. While Nlrp6 has been hypothesized to elicit inflammasome-dependent responses, this has not been convincingly demonstrated [58].

It had been previously observed that myeloid NRLP6-deficient mice exhibit increased inflammatory responses, consistent with a negative regulatory role in NF-κB and MAPK signalling during infection [20]. Nlrp6 deficiency enhanced MAPK and canonical NF-κB pathway activation after Toll-like receptor ligation in infected cultured leucocytes, resulting in increased levels of NF-κB- and MAPK-dependent cytokines and chemokines [20]. We have now observed that activation of MAPK in response to genetic Nlrp6 downregulation is also observed in epithelial (renal tubular) cells and in a sterile inflammation environment. Additionally, in this sterile environment, MAPK activation contributed to the increased inflammatory responses observed in Nlrp6-deficient renal cells. These results are in line with the known deleterious effect of ERK1/2 and p38 in kidney inflammation, fibrosis and injury [39–45] as well as to the known pro-apoptotic role of p38 in kidney tubular cells [59, 60]. A protein potentially linking Nrlp6 to MAPK activation was recently described [54]. Nlrp6 binds Dhx15 [54], an activator of NF-κB and MAPK pathways [61] that inhibits cell death [62] and is expressed by kidney tubular cells [63]. However, the function of Dhx15 in the kidney is currently unknown. This or similar molecules may be the link between Nlrp6 and its cell protective effects.

A limitation of the present study is that it was not designed to directly evaluate *in vivo* the relative contribution of Nlrp6

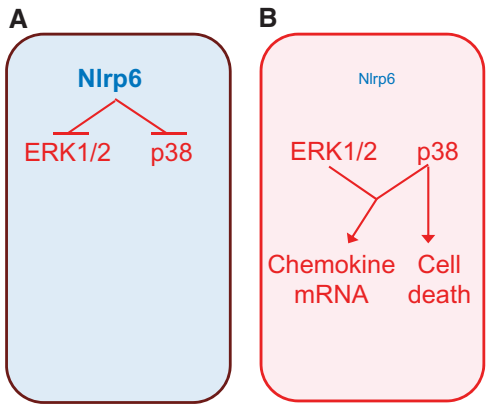

FIGURE 6: A mechanistic hypothesis on nephroprotection by Nlrp6. Hypothesis based on cell culture and *in vivo* interference with Nlrp6 expression during kidney cell injury. Nlrp6 knockdown led to ERK1/2 and p38 activation in tubular cells in culture and *in vivo* and inhibitors of these MAPKs prevented the pro-inflammatory consequences of Nlrp6 deficiency and decreased cultured tubular cell death. (**A**) We hypothesize that Nlrp6 keeps ERK1/2 and p38 inactive in healthy cells. (**B**) Nlrp6 downregulation or knockdown favours ERK1/2 and p38 activation and this leads to a pro-inflammatory response characterized by increased expression of chemokine mRNA as well as to tubular cell death.

deficiency in kidney cells versus non-renal cells. Thus, since we did not use tubular cell-specific Nlrp6 KO or overexpressing mice, there is no absolute certainty that the observed increased severity of AKI is directly related to tubular Nlrp6 expression. However, cell culture results suggest that Nlrp6 deficiency in cultured kidney cells reproduces the *in vivo* observations of increased activation of MAPK, more intense inflammatory responses, lower Klotho expression and higher rates of cell death. Indeed, activation of MAPK in Nlpr6-deficient mice was mainly observed in tubular epithelium *in vivo* during AKI. In this regard, the impact of NRLP6 deficiency in myeloid cells had already been previously studied [20].

Little is known about the factors downregulating Nlrp6 expression during gut injury [19]. Our studies did not identify the factors driving the downregulation of Nlrp6 expression. TWEAK is a key driver of AKI that reproduces many of the abnormalities observed during AKI at the gene expression level, including increased inflammatory gene expression and decreased Klotho expression [64–66]. However, in preliminary experiments, TWEAK did not downregulate Nlrp6 expression in murine tubular cells (not shown).

In conclusion, Nlrp6 is constitutively expressed by human and murine kidney tubular cells in culture, and *in vivo*, in a sterile environment, it contributes to quench inflammatory responses by downregulating ERK1/2 or p38 activation (Figure 6). During nephrotoxic kidney injury, Nlrp6 expression is lost and Nlrp6 deficiency aggravates the severity of nephrotoxic AKI. Further studies should address the therapeutic potential of preserving or increasing Nlrp6 expression during kidney disease. Our findings of biological actions of Nlrp6 on tissue injury in a sterile environment are in line with recent publications that question its role in modulating the gut microbiota [48–50].

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

1. Bellomo R, Kellum JA, Ronco C. Acute kidney injury. *Lancet* 2012; 380: 756–766
2. Webster AC, Nagler EV, Morton RL, Masson P. Chronic kidney disease. *Lancet* 2017; 389: 1238–1252
3. GBD 2015 Mortality and Causes of Death Collaborators. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 2016; 388: 1459–1544
4. Sanz AB, Justo P, Sanchez-Niño MD *et al.* The cytokine TWEAK modulates renal tubulointerstitial inflammation. *J Am Soc Nephrol* 2008; 19: 695–703
5. Anders H-J, Schaefer L. Beyond tissue injury-damage-associated molecular patterns, toll-like receptors, and inflammasomes also drive regeneration and fibrosis. *J Am Soc Nephrol* 2014; 25: 1387–1400
6. Martin-Sanchez D, Ruiz-Andres O, Poveda J *et al.* Ferroptosis, but not necroptosis, is important in nephrotoxic folic acid-induced AKI. *J Am Soc Nephrol* 2017; 28: 218–229
7. Mulay SR, Linkermann A, Anders H-J. Necroinflammation in kidney disease. *J Am Soc Nephrol* 2016; 27: 27–39
8. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol Cell* 2002; 10: 417–426
9. Agostini L, Martinon F, Burns K *et al.* NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 2004; 20: 319–325
10. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. *Nature* 2012; 481: 278–286
11. Chen G, Shaw MH, Kim Y-G, Nuñez G. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu Rev Pathol* 2009; 4: 365–398
12. Sanchez-Niño MD, Sanz AB, Ortiz A. Uromodulin, inflammasomes, and pyroptosis. *J Am Soc Nephrol* 2012; 23: 1761–1763
13. Shahzad K, Bock F, Dong W *et al.* Nlrp3-inflammasome activation in non-myeloid-derived cells aggravates diabetic nephropathy. *Kidney Int* 2015; 87: 74–84
14. Andersen K, Eltrich N, Lichtnekert J *et al.* The NLRP3/ASC inflammasome promotes T-cell-dependent immune complex glomerulonephritis by canonical and noncanonical mechanisms. *Kidney Int* 2014; 86: 965–978
15. Bakker PJ, Butter LM, Claessen N *et al.* A tissue-specific role for Nlrp3 in tubular epithelial repair after renal ischemia/reperfusion. *Am J Pathol* 2014; 184: 2013–2022
16. Bakker PJ, Butter LM, Kors L *et al.* Nlrp3 is a key modulator of diet-induced nephropathy and renal cholesterol accumulation. *Kidney Int* 2014; 85: 1112–1122
17. Lorenz G, Darisipudi MN, Anders H-J. Canonical and non-canonical effects of the NLRP3 inflammasome in kidney inflammation and fibrosis. *Nephrol Dial Transplant* 2014; 29: 41–48

18. Yuan F, Kolb R, Pandey G *et al.* Involvement of the NLRC4-inflammasome in diabetic nephropathy. *PLoS One* 2016; 11: e0164135
19. Levy M, Shapiro H, Thaiss CA, Elinav E. NLRP6: a multifaceted innate immune sensor. *Trends Immunol* 2017; 38: 248–260
20. Anand PK, Malireddi RKS, Lukens JR *et al.* NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. *Nature* 2012; 488: 389–393
21. Chen GY, Liu M, Wang F *et al.* A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol* 2011; 186: 7187–7194
22. Normand S, Delanoye-Crespin A, Bressenot A *et al.* Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci USA* 2011; 108: 9601–9606
23. Grenier JM, Wang L, Manji GA *et al.* Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-κB and caspase-1. *FEBS Lett* 2002; 530: 73–78
24. Elinav E, Strowig T, Kau AL *et al.* NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 2011; 145: 745–757
25. Djudjaj S, Lue H, Rong S *et al.* Macrophage migration inhibitory factor mediates proliferative GN via CD74. *J Am Soc Nephrol* 2016; 27: 1650–1664
26. Ortiz A, Husi H, Gonzalez-Lafuente L *et al.* Mitogen-activated protein kinase 14 promotes AKI. *J Am Soc Nephrol* 2017; 28: 823–836
27. Metz-Kurschel U, Kurschel E, Wagner K *et al.* Folate nephropathy occurring during cytotoxic chemotherapy with high-dose folinic acid and 5-fluorouracil. *Ren Fail* 1990; 12: 93–97
28. Sánchez-González PD, López-Hernández FJ, López-Novoa JM, Morales AI. An integrative view of the pathophysiological events leading to cisplatin nephrotoxicity. *Crit Rev Toxicol* 2011; 41: 803–821
29. Sancho-Martínez SM, Prieto-García L, Prieto M *et al.* Subcellular targets of cisplatin cytotoxicity: an integrated view. *Pharmacol Ther* 2012; 136: 35–55
30. Haverty TP, Kelly CJ, Hines WH *et al.* Characterization of a renal tubular epithelial cell line which secretes the autologous target antigen of autoimmune experimental interstitial nephritis. *J Cell Biol* 1988; 107: 1359–1368
31. next*presso*: Next generation sequencing expression analysis pipeline. http://bioinfo.cnio.es/nextpresso/ (30 March 2018, date last accessed)
32. Babraham Bioinformatics. FastQC. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (30 March 2018, date last accessed)
33. Izquierdo MC, Sanz AB, Mezzano S *et al.* TWEAK (tumor necrosis factor–like weak inducer of apoptosis) activates CXCL16 expression during renal tubulointerstitial inflammation. *Kidney Int* 2012; 81: 1098–1107
34. González-Guerrero C, Morgado-Pascual JL, Cannata-Ortiz P *et al.* CCL20 blockade increases the severity of nephrotoxic folic acid-induced acute kidney injury. *J Pathol* 2018; 246: 191–204
35. Sanchez-Niño MD, Poveda J, Sanz AB *et al.* 3,4-DGE is cytotoxic and decreases HSP27/HSPB1 in podocytes. *Arch Toxicol* 2014; 88: 597–608
36. Sanchez-Niño MD, Sanz AB, Lorz C *et al.* BASP1 promotes apoptosis in diabetic nephropathy. *J Am Soc Nephrol* 2010; 21: 610–621
37. Sanchez-Niño MD, Sanz AB, Sanchez-Lopez E *et al.* HSP27/HSPB1 as an adaptive podocyte antiapoptotic protein activated by high glucose and angiotensin II. *Lab Invest* 2012; 92138: 32–45
38. Ydens E, Demon D, Lornet G *et al.* Nlrp6 promotes recovery after peripheral nerve injury independently of inflammasomes. *J Neuroinflammation* 2015; 12: 143
39. Leonard M, Ryan MP, Watson AJ *et al.* Role of MAP kinase pathways in mediating IL-6 production in human primary mesangial and proximal tubular cells. *Kidney Int* 1999; 56: 1366–1377
40. Jo S-K, Cho WY, Sung SA *et al.* MEK inhibitor, U0126, attenuates cisplatin-induced renal injury by decreasing inflammation and apoptosis. *Kidney Int* 2005; 67: 458–466
41. Han Y, Masaki T, Hurst LA *et al.* Extracellular signal-regulated kinase-dependent interstitial macrophage proliferation in the obstructed mouse kidney. *Nephrology (Carlton)* 2008; 13: 411–418
42. Alderliesten M, de Graauw M, Oldenampsen J *et al.* Extracellular signal-regulated kinase activation during renal ischemia/reperfusion mediates focal adhesion dissolution and renal injury. *Am J Pathol* 2007; 171: 452–462
43. Korcheva V, Wong J, Corless C *et al.* Administration of ricin induces a severe inflammatory response via nonredundant stimulation of ERK, JNK, and P38 MAPK and provides a mouse model of hemolytic uremic syndrome. *Am J Pathol* 2005; 166: 323–339
44. Ma FY, Tesch GH, Flavell RA *et al.* MKK3-p38 signaling promotes apoptosis and the early inflammatory response in the obstructed mouse kidney. *Am J Physiol Renal Physiol* 2007; 293: F1556-F1563
45. Stambe C, Atkins RC, Tesch GH *et al.* The role of p38alpha mitogen-activated protein kinase activation in renal fibrosis. *J Am Soc Nephrol* 2004; 15: 370–379
46. Darisipudi MN, Thomasova D, Mulay SR *et al.* Uromodulin triggers IL-1β-dependent innate immunity via the NLRP3 inflammasome. *J Am Soc Nephrol* 2012; 23: 1783–1789
47. Moreno JA, Izquierdo MC, Sanchez-Niño MD *et al.* The inflammatory cytokines TWEAK and TNFα reduce renal klotho expression through NFκB. *J Am Soc Nephrol* 2011; 22: 1315–1325
48. Lemire P, Robertson SJ, Maughan H *et al.* The NLR protein NLRP6 does not impact gut microbiota composition. *Cell Rep* 2017; 21: 3653–3661
49. Mamantopoulos M, Ronchi F, Van Hauwermeiren F *et al.* Nlrp6- and ASC-dependent inflammasomes do not shape the commensal gut microbiota composition. *Immunity* 2017; 47: 339–348.e4
50. Elinav E, Henao-Mejia J, Strowig T, Flavell R. NLRP6 and dysbiosis: avoiding the luring attraction of over-simplification. *Immunity* 2018; 48: 603–604
51. Levy M, Thaiss CA, Zeevi D *et al.* Microbiota-modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. *Cell* 2015; 163: 1428–1443
52. Birchenough GMH, Nyström EEL, Johansson MEV, Hansson GC. A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion. *Science* 2016; 352: 1535–1542
53. Wlodarska M, Thaiss CA, Nowarski R *et al.* NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell* 2014; 156: 1045–1059
54. Wang P, Zhu S, Yang L *et al.* Nlrp6 regulates intestinal antiviral innate immunity. *Science* 2015; 350: 826–830
55. Novick D, Kim S, Kaplanski G, Dinarello CA. Interleukin-18, more than a Th1 cytokine. *Semin Immunol* 2013; 25: 439–448
56. Melnikov VY, Ecder T, Fantuzzi G *et al.* Impaired IL-18 processing protects caspase-1-deficient mice from ischemic acute renal failure. *J Clin Invest* 2001; 107: 1145–1152
57. Chung H, Vilaysane A, Lau A *et al.* NLRP3 regulates a non-canonical platform for caspase-8 activation during epithelial cell apoptosis. *Cell Death Differ* 2016; 23: 1331–1346
58. Radulovic K, Normand S, Rehman A *et al.* A dietary flavone confers communicable protection against colitis through NLRP6 signaling independently of inflammasome activation. *Mucosal Immunol* 2018; 11: 811–819
59. Liang H, Huang Q, Liao M-J *et al.* EZH2 plays a crucial role in ischemia/reperfusion-induced acute kidney injury by regulating p38 signaling. *Inflamm Res* 2019; 68: 325–336
60. An JN, Yang SH, Kim YC *et al.* Periostin induces kidney fibrosis after acute kidney injury via the p38 MAPK pathway. *Am J Physiol Renal Physiol* 2019; 316: F426–F437
61. Mosallanejad K, Sekine Y, Ishikura-Kinoshita S *et al.* The DEAH-box RNA helicase DHX15 activates NF-κB and MAPK signaling downstream of MAVS during antiviral responses. *Sci Signal* 2014; 7: ra40
62. Pan L, Li Y, Zhang H-Y *et al.* DHX15 is associated with poor prognosis in acute myeloid leukemia (AML) and regulates cell apoptosis via the NF-kB signaling pathway. *Oncotarget* 2017; 8: 89643–89654
63. Human Protein Atlas. DHX-15. https://www.proteinatlas.org/ENSG00000109606-DHX15/tissue/kidney#img (30 March 30 2019, date last accessed)
64. Ruiz-Andres O, Suarez-Alvarez B, Sánchez-Ramos C *et al.* The inflammatory cytokine TWEAK decreases PGC-1α expression and mitochondrial function in acute kidney injury. *Kidney Int* 2016; 89: 399–410
65. Sanz AB, Izquierdo MC, Sanchez-Niño MD *et al.* TWEAK and the progression of renal disease: clinical translation. *Nephrol Dial Transplant* 2014; 29 (Suppl 1): i54–i62
66. Poveda J, Sanz AB, Carrasco S *et al.* Bcl3: a regulator of NF-κB inducible by TWEAK in acute kidney injury with anti-inflammatory and antiapoptotic properties in tubular cells. *Exp Mol Med* 2017; 49: e352

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