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TNF-α from the Proximal Nephron Exacerbates Aristolochic Acid Nephropathy

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Key Points

- Proximal tubular TNF aggravates kidney injury and fibrogenesis in aristolochic acid nephropathy.
- Tubular TNF disrupts the cell cycle in injured tubular epithelial cells.
- TNF-mediated toxic renal injury is independent of systemic immune responses.

Abstract

Background Aristolochic acid nephropathy (AAN) presents with tubular epithelial cell (TEC) damage and tubulointerstitial inflammation. Although TNF-α regulates cell apoptosis and inflammatory responses, the effects of tubular TNF in the progression of AAN require elucidation.

Methods Floxed *TNF* mice on the 129/SvEv background were crossed with *PEPCK-Cre* mice to generate *PEPCK-Cre*$^{+}$ *TNF*$^{flox/flox}$ (TNF PTKO) mice or bred with *Ksp-Cre* mice to generate *KSP-Cre*$^{+}$ *TNF*$^{flox/flox}$ (TNF DNKO) mice. TNF PTKO, TNF DNKO, and wild-type controls (Cre negative littermates) were subjected to acute and chronic AAN.

Results Deletion of TNF in the proximal but not distal nephron attenuated kidney injury, renal inflammation, and tubulointerstitial fibrosis after acute or chronic aristolochic acid (AA) exposure. The TNF PTKO mice did not have altered numbers of infiltrating myeloid cells in AAN kidneys. Nevertheless, kidneys from AA-treated TNF PTKO mice had reduced levels of proteins involved in regulated cell death, higher proportions of TECs in the G0/G1 phase, and reduced TEC proportions in the G2/M phase. Pifithrin-α, which restores the cell cycle, abrogated differences between the wild-type and PTKO cohorts in G2/M phase arrest of TECs and kidney fibrosis after AA exposure.

Conclusions TNF from the proximal but not the distal nephron propagates kidney injury and fibrogenesis in AAN in part by inducing G2/M cell cycle arrest of TECs.

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Introduction

AKI is a complex clinical disorder with limited treatment strategies, deriving from diverse etiologies such as toxicity and ischemia.[1] Ongoing renal pathophysiological processes occur in AKI survivors that increase the long-term risk of CKD.[2] The pathogenic mechanisms of AKI to CKD transition remain unclear, but failed recovery of tubular epithelial cells (TECs) from injury may amplify the production of proinflammatory and profibrotic cytokines. Aristolochic acid (AA) is a toxic ingredient in traditional herbs that can trigger acute and chronic kidney disease grouped under the rubric of aristolochic acid nephropathy (AAN).[3] AA mainly induces the death of TECs acutely and aggravates tubulointerstitial inflammation and fibrosis over the longer term. However, the molecular mechanisms through which injured TECs mediate kidney inflammation and fibrosis during the pathogenesis of AAN require elucidation.

TNF-α is a pleiotropic proinflammatory cytokine that exerts multiple pathophysiological effects implicated in kidney disease.[4,5] AA exposure significantly increases renal TNF expression levels.[3,6] Inversely, treatment with TNF inhibitors or neutralizing

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antibodies attenuates renal function decline and histological damage after toxic AKI,[7] and global TNF deficiency can improve survival rates after AKI. Tsuruya *et al.*[8] found that TNF receptor 1 deficiency attenuates TEC apoptosis and necrosis after toxic AKI, establishing TECs as a primary target of TNF in this setting. However, TNF has various sources, such as TECs, mesangial cells, macrophages, dendritic cells, and T lymphocytes, which may influence TNF-associated renal damage and fibrosis. For example, in T lymphocytes, TNF exhibits protective immunoregulatory effects by promoting T-lymphocyte exhaustion.[9] Thus, the cell-specific actions of TNF during renal injury require additional scrutiny for therapeutic manipulation.

We previously reported that angiotensin receptor activation on renal epithelial cells exaggerates the severity of kidney injury by stimulating renal TNF production after cisplatin-induced AKI.[10] We therefore posited that TEC-derived TNF would contribute to tubulointerstitial inflammation and fibrogenesis during AAN. We tested this hypothesis by subjecting conditionally mutant mice that harbor selective TNF deficiency in the proximal tubule (PT) or distal nephron (DN) to acute and chronic AAN. We find that TNF deletion from the PT but not the DN attenuates AA-induced kidney injury and fibrosis without affecting the renal recruitment of inflammatory cells.

Methods

Animal Experiments

Floxed *TNFa* mice[11] on the C57BL/6 background strain were backcrossed for at least 6 generations to the 129/SvEv background and then crossed with 129/SvEv *PEPCK-Cre* mice[12] to generate *Pepck-Cre*$^{+}$ *TNFa*$^{flox/flox}$ (TNF PTKO) mice or crossed with 129/SvEv *Ksp-Cre* mice[13] to generate *Ksp-Cre*$^{+}$ *TNFa*$^{flox/flox}$ (TNF DNKO) mice. Cre negative littermates were used as wild-type (WT) controls for our experiments. Animals had free access to standard rodent chow and water. Eight-week-old to 12-week-old male mice were used for experiments. For genotyping, DNA from ear tissues was harvested and used for PCR amplification. Primers specific for the *Cre transgene* (sense: 5′-ACGAG TGATG AGGTT CGCAA G-3′; antisense: 5′-CAATC CCCAG AAATG CCAGA-3′) or the *TNF flox* gene (sense: 5′-GTTCC CACAC CTCTC TCT-3′; antisense: 5′-CTTCA TTCTC AAGGC ACA-3′) were included in the reaction system. The PCR products were analyzed by a Bio-Rad CFX Touch 96 Real-time Thermocycler to determine presence of the *C. transgene* through cycles or the *TNF flox* gene through high-resolution melting as illustrated in Supplemental Figure 1. According to National Institutes of Health guidelines, mice were bred and maintained in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facilities at the Durham Veterans Affairs Medical Center. The Durham Veterans Affairs Medical Center Institutional Animal Care and Use Committee approved all the animal studies following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Serum and urine TNF levels were determined by ELISA per manufacturer's instructions (88-7324-22, ThermoFisher Scientific).

Aristolochic Acid Model

TNF PTKO, DNKO, and WT control male mice were injected with aristolochic acid I (AA) (5 mg/kg, IP) (Sigma-Aldrich, A9451) for 3 consecutive days during acute AAN, and kidney tissues and blood samples were obtained on day 4 for analysis. In the chronic AAN model, TNF PTKO, TNF DNKO, and WT male mice were injected with AA every other day for 12 days. Kidney tissues and blood samples were obtained 5 weeks after the last injection in the chronic model. For the examination of cell cycle effects, TNF PTKO and WT male mice were injected intraperitoneally with 2.2 mg/kg/day of pifithrin-α on day 1 in acute AAN and every week for 3 injections in chronic AAN.[14] For scRNA-seq analysis, WT mice were injected with either vehicle or AA for 5 consecutive days, and kidneys were harvested the next day for analysis.

Single-Cell RNA Sequencing Analysis

The raw and processed single-cell RNA sequencing data interrogated in this study have been previously generated and deposited in a repository (https://gitlab.oit.duke.edu/jrp43/ren-crowley-pt-il1r-aki),[15] but the analysis presented herein is entirely new. For this study, the vehicle condition used both kidneys from one WT mouse, and the AA-challenged condition combined three kidneys from three AA-challenged WT mice to minimize potential biological and technical variability of the model. After library preparation, samples were sequenced on a Novaseq 6000 (Illumina) S2 flow cell by the Duke Center for Genomic and Computational Biology Sequencing Core. Cell Ranger software from 10× Genomics was used to transform raw base call files into FASTQ files (cellranger mkfastq) and align reads with reference mouse genome (cellranger count). After QC, filtering, normalization, and integration, the cells were originally assigned to 13 clusters showing individual nephron segments and infiltrating mononuclear cells. To examine expression of signaling pathways in the PT and DN as a whole, the clusters constituting the DN were combined ("DN"=ATL+TAL+DCT+CNT+DTL). Gene Set Enrichment Analysis (GSEA) was performed with clusterProfiler which supports statistical analysis and visualization of functional profiles for genes and gene clusters.[16]

Assessment of Renal Function

Collected blood was centrifuged (6000 rpm, 10 minutes) in Microtainer Tubes (Cat: VT365967, VWR international). The supernatant was collected, and BUN levels were measured in individual samples per kit instructions (Cat: EIABUN, ThermoFisher Scientific).

Histologic Analyses

Harvested kidneys were fixed with 10% neutral-buffered formalin overnight and embedded in paraffin. Kidney sections (5 μm) were used for PAS staining by the Duke Department of Pathology. The kidney injury score was quantitated by evaluating morphological changes including tubular necrosis, dilation, cast formation, epithelial swelling, and vacuolar degeneration (0, <5%; 1, 5%–25%; 2, 26%–50%; 3, 51%–75%; 4, >75%). The observer was blinded to experimental conditions.

Real-Time PCR Analyses of mRNA Expression

Total mRNA was extracted using an RNeasy Mini Kit per manufacturer's instructions (Cat: 74136, Qiagen). After determining mRNA concentrations, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Cat: 4168813, Thermo Fisher). Gene expression levels were determined by the ABI 7900HT system using TaqMan probes for *Lcn2*, *Col1a1*, *Tgfb1*, *FN1*, *havcr1*, *Tnfa*, *IL1b*, and *IFNg* and SYBR green primers for *IL17a*.

Western Blots

Kidney tissues were homogenized in RIPA buffer, and the protein concentrations were quantitated using the DC protein assay kit (Bio-Rad). Equal amounts of protein samples were subjected to electrophoresis with Bis-Tris Gels and transferred to PVDF membranes. The blots were blocked with 5% milk in TBST and incubated with anti-collagen type I antibody (Cat: 1310-01, Southern Biotech), anti-Bax (2772S, Cell Signaling Technology), anti-Bcl2 (2870S, Cell Signaling Technology), anti-p53 (2524S, Cell Signaling Technology), anti-RIP3 (95702S, Cell Signaling Technology), and anti-GAPDH antibody (Cat: 2118, Cell Signaling Technology) overnight in 4°C. The blots were then washed and incubated with secondary antibodies for 1 hour at room temperature. Target bands were detected using ECL solution and quantified by Image J analysis.

Flow Cytometry

On day 4 of acute AAN, kidneys were harvested and minced into single-cell suspensions.[17] These single-cell suspensions were blocked for 30 minutes and incubated with fluorescent antibodies: anti-CD11b, anti-CD45, anti-Ly6C, anti-F4/80, anti-CD11c, anti-MHCII, anti-CD103, and anti-CD64 for 30 minutes at 4°C. Before analysis, cells were washed and fixed with Fix/Perm buffer (Cat: 554655, BD Biosciences), and 20 μl CountBright absolute counting beads (Cat: C36950, Invitrogen) were added to the cells. For the measurement of cell cycle distribution, cells were prepared for propidium iodide (Cat: 421301, BioLegend) staining according to standard protocols.[18] Samples were analyzed on an LSRII flow cytometer (BD). Flow cytometry data were analyzed using FlowJo software version 10.2 (Tree Star, Inc., Ashland, OR). Total cell numbers were obtained per CountBright manufacturer's instructions.

Cellular Respiration Analysis

According to the manufacturer's instructions, the oxygen consumption rate (OCR) was assessed using the Seahorse XF Cell Mito Stress Test Kit (Cat: 103015-100, Agilent Technologies). Before the assay, primary renal tubular cells (RTCs) (10^4 cells/well, 10–12 wells/condition) pooled from 3 WT mice and cultured for 7 days were transferred into the Seahorse plate, treated with vehicle, AA (10 μM), AA+pifithrin-α (30 μM), TNF-α (50 ng/ml), or TNF-α+pifithrin-α for 12 hours, respectively, and then washed twice with Seahorse XF Media to remove the chemical compounds. For assay substrates, glucose (10 mM), pyruvate (1 mM), and glutamine (2 mM) were added. During the assay, 2 μM oligomycin, 1.5 μM FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone], and 0.5 μM rotenone/0.5 μM antimycin were added at 14, 33, and 53 minutes, respectively, to assess OCR. Maximal respiration is defined as the peak OCR after FCCP administration. Spare respiratory capacity is the difference between maximal respiration and basal respiration achieved after rotenone and antimycin A.

Statistical Analysis

All data of each group are expressed as mean±SEM. Comparisons between two groups were assessed using an unpaired Student's *t*-test. For non-normally distributed values, a Mann–Whitney test was used. Comparison among groups was performed with one-way ANOVA. All statistical analyses were calculated using GraphPad Prism software.

Results

TNF and Cognate Receptor Expression in the Kidney during AA-Induced Tubular Injury

To examine expression of TNF and its receptors in the kidney during AA-induced injury, we interrogated a single-cell RNA sequencing dataset generated from the kidneys of WT mice that were injected with AA for 5 consecutive days and sacrificed on day 6 (Figure 1A). As shown in the dot plots, expression of the *Tnfa* gene encoding TNF was hardly detectable in the nephron segments compared with infiltrating myeloid cells. The *Tnfrsf1a* gene encoding TNF receptor 1 was expressed at high levels in infiltrating myeloid cells and the endothelium and at lower levels in several nephron segments, whereas the *Tnfrsf1b* gene encoding TNF receptor 2 was expressed in infiltrating myeloid cells but only faintly detectable in some cells within nephron segments (Figure 1B). Despite limited expression of TNF and its receptors in the nephron, GSEA revealed upregulation of pathways for TNF signaling and TNF receptor 1 signaling in the PT cluster but not in a DN cluster compiled from all nephron segments beyond the PT, including the loop of Henle, distal convoluted tubule, connecting tubule, and distal tubule/collecting duct. Further GSEA also detected upregulated signaling for several regulated cell death pathways targeted by TNF including apoptosis, regulated cell death, RIP1-mediated regulated necrosis, and *tp53* signaling in the PT as well as apoptosis, *tp53* regulates transcription of cell cycle genes, and *tp53* targets in the DN (Figure 1, C–E). We therefore posited that TNF in the PT or DN may propagate AA-induced tubular injury despite limited tubular expression because the PT is the site of initial AA uptake, and chronic AA-induced damage causes distal tubular dysfunction. Separately, RT-PCR detected considerably higher levels of *Tnfa* mRNA induction in the renal cortex compared with the medulla with either acute or chronic AA exposure (Supplemental Figure 2), corroborating greater *Tnfa* upregulation at the site of initial AA-induced injury.

TNF Deficiency in DN Does Not Affect AA-Induced Acute Tubular Damage

We previously found that TNF from the DN exacerbates cisplatin nephrotoxicity.[10] To measure the contribution of TNF in the DN to acute AAN, we intraperitoneally injected mice harboring DN-specific TNF deletion

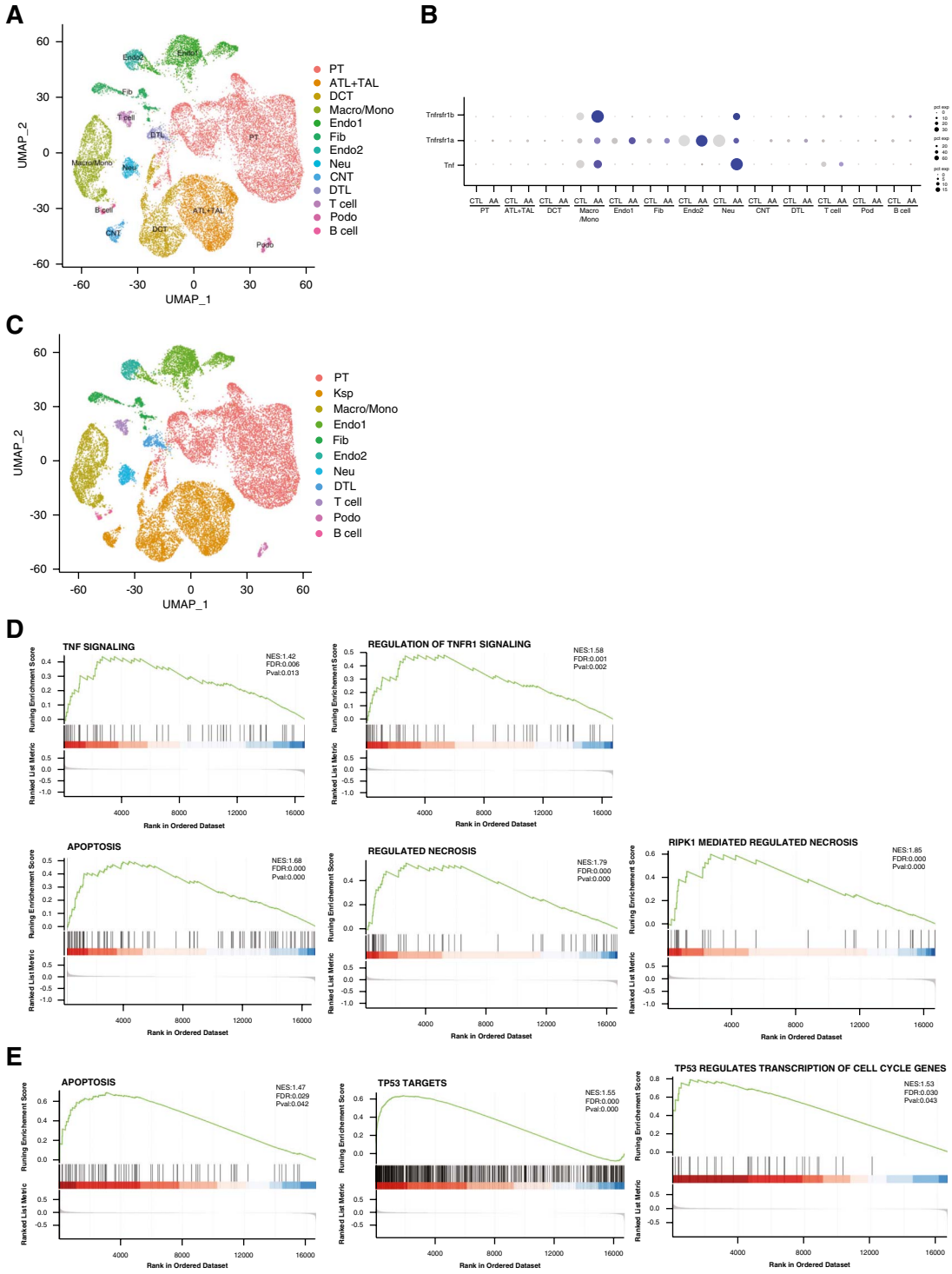

Figure 1. TNF and cognate receptor expression in the kidney during AA-induced tubular injury. (A) Integrated single-cell transcriptome map of kidneys from vehicle and AA-treated wild-type mice. (B) Dot plot of single-cell RNA sequencing analyses showing the *Tnfa*, *Tnfrsf1a*, and *Tnfrsf1b* genes encoding TNF, TNFR1, and TNFR2, respectively, in multiple cell lineages from kidneys of vehicle and AA-treated wild-type animals. CTL, control. AA, aristolochic acid. (C) Integrated single-cell transcriptome map from kidneys featuring a consolidated distal nephron (DN) cluster where DN=ATL+TAL+DCT+CNT+DTL. (D) Significantly enriched GO biological processes in gene set enrichment analysis (GSEA): TNF signaling, regulation of TNF receptor 1 signaling, apoptosis, regulated necrosis, and RIPK1-mediated regulated necrosis in the proximal tubular cell cluster, where a significant enrichment score and skewing of the peak to left reflects a higher frequency and strength of gene induction in the labeled signaling pathways comparing the PT cluster during AA-induced injury with the vehicle condition, (E) GSEA of apoptosis, *tp53* targets (*tp53* encodes p53), and *tp53* regulates transcription of cell cycle genes in the DN cluster.

(*Ksp Cre*$^{+}$ *TNF*$^{flox/flox}$=TNF DNKO) with AA for 3 consecutive days. We have previously reported that TNF DNKO mice have normal BUN, serum creatinine, and circulating serum TNF-α levels at baseline, despite significant reductions in kidney *Tnfa* mRNA expression.[10] Further *Tnfa* expression profiling in nonrenal tissues revealed preserved expression in the liver and heart of DNKO mice but blunted levels of *Tnfa* in the lung at baseline (Supplemental Figure 3). PAS-stained sections of TNF DNKO kidneys demonstrated similar renal architecture to WT (*Ksp Cre*$^{-}$ *TNF*$^{flox/flox}$=WT) controls after AA exposure with comparable injury scores (Figure 2, A and B). Moreover, on day 4, the TNF DNKO mice had similar mRNA levels for *lipocalin2* encoding NGAL (Figure 2C) and BUN levels compared with WT controls (Figure 2D). These results demonstrate that TNF from the DN does not influence the severity of kidney damage after acute AA exposure.

TNF Deficiency in Proximal Nephron Attenuates AA-Induced Acute Tubular Injury

We next generated mice with PT-specific *TNF* deletion (*Pepck Cre*$^{+}$ *TNF*$^{flox/flox}$=TNF PTKO) to evaluate the role of TNF from the proximal nephron in mediating AAN. Whole kidney from TNF PTKO mice showed significant reductions in *Tnfa* mRNA expression at baseline compared with controls (0.45±0.15 versus 1.00±0.12 au; $P < 0.05$) as analyzed by RT-PCR (Supplemental Figure 4A), despite preserved *Tnfa* mRNA levels in the liver, heart, and lung, normal TNF-α protein levels in the serum and urine, normal BUN levels, and normal renal mRNA levels for genes encoding NGAL, Kim-1, collagen 1, and fibronectin (Supplemental Figure 4, B–F). Blinded injury scores showed that TNF PTKO mice had less renal injury than WTs (*Pepck Cre*$^{-}$ *TNF*$^{flox/flox}$) on day 4 after the first AA injection (Figure 3, A and B). Compared with WTs, TNF PTKO kidneys expressed markedly lower mRNA levels for *lipocalin2*/NGAL (Figure 3C). These differences in injury were corroborated by lower BUN levels in the TNF PTKO cohort, suggesting a small but significant preservation in kidney function after acute AAN (Figure 3D). Interestingly, serum TNF concentrations after AAN were similar between groups, and urine TNF concentrations were slightly higher in the PTKO cohort compared with controls (Supplemental Figure 4, C–D). These results suggest that specific deletion of TNF in the proximal nephron attenuates renal functional decline and kidney damage after acute AA exposure, despite no detectable decrement in urine or circulating TNF levels.

TNF from the PT Mediates AA-Induced Renal Inflammation

TNF is known to drive inflammatory cytokine generation in several cell lineages. To examine the capacity of TNF in the PT to influence the inflammatory milieu, we measured renal gene expression for a panel of cytokines after acute AA exposure. Compared with WTs, kidneys from the TNF PTKO cohort showed lower mRNA expression levels for the genes encoding IL-1β (Figure 4A), a key driver of tubular damage in multiple models,[19,20] and the profibrotic cytokine TGF-β (Figure 4B). By contrast, TNF PTKO kidneys had similar mRNA levels for the genes encoding IFN-γ and IL-17A compared with WT kidneys (Figure 4, C and D). Thus, TNF deletion in the PT blunts the local expression of injury and fibrosis mediators after AA administration.

TNF from the PT Does Not Influence Myeloid Cell Infiltration into Kidney

To assess the effect of TNF in the PT on myeloid cell accumulation in the kidney during acute AAN, we isolated infiltrating CD45^{+} cells from the injured kidneys and delineated subpopulations through flow cytometry (Figure 5A). Macrophages contribute

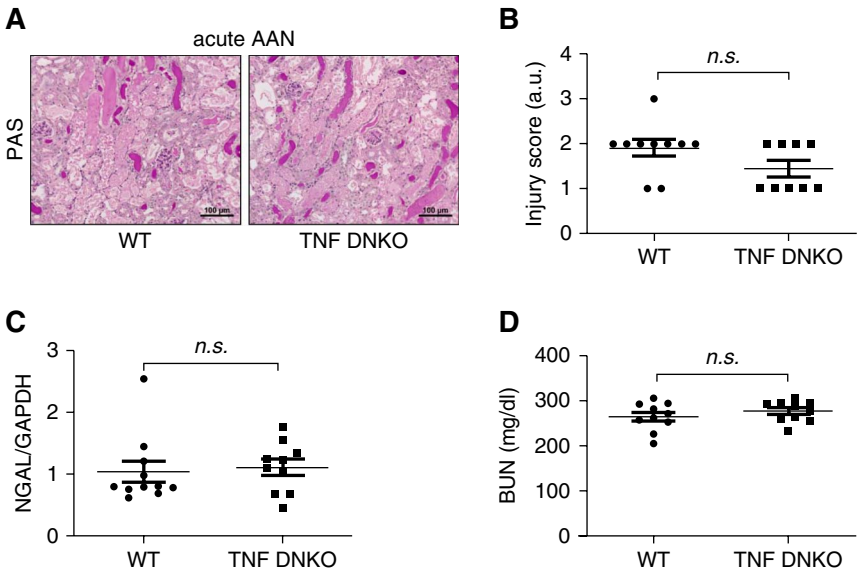

Figure 2. TNF deficiency in distal nephron does not affect AA-induced acute tubular damage. (A) Representative images of kidney sections from wild-type (WT) and TNF DNKO mice at day 4 after AA injection. Scale bar=100 μm (B) WT and TNF DNKO kidney pathology scores ($n \geq 8$). (C) Renal mRNA expression of *lipocalin2*/NGAL ($n \geq 8$). (D) BUN ($n \geq 8$). Values are mean±SEM (*$P < 0.05$).

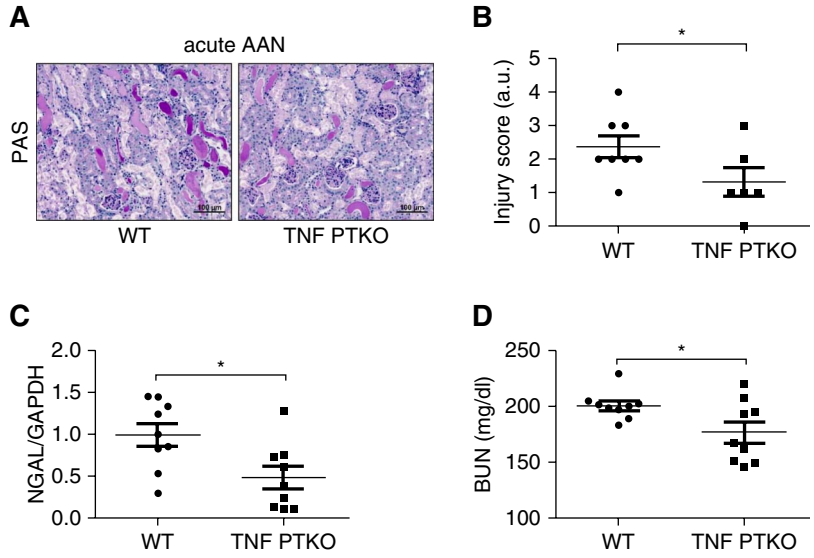

Figure 3. TNF deficiency in proximal nephron attenuates AA-induced acute tubular injury. (A) Representative images of kidney sections from WT and TNF PTKO mice at day 4 after AA-induced injury. Scale bar=100 μm (B) WT and TNF PTKO kidney pathology scores ($n \geq 6$). (C) Renal mRNA expression of *lipocalin2*/NGAL ($n \geq 9$). (D) BUN ($n \geq 9$). Values are mean±SEM (*$P < 0.05$).

to the injury and repair phase of toxic AKI. However, the number of MHCII$^+$CD11b$^+$CD64$^+$F4/80^{hi} macrophages from TNF PTKO kidneys after AA injection was similar to WTs (Figure 5B). Injured kidneys from TNF PTKO mice also had similar numbers of MHCII$^+$CD11c$^+$CD103$^+$ and MHCII$^+$CD11c$^+$CD11b^{hi} dendritic cells and CD64$^-$CD11b$^+$ monocytes compared with WTs (Figure 5, C–E). Thus, TNF from the PT aggravates kidney injury without affecting the renal accumulation of myeloid cells.

TNF in the Proximal Nephron Promotes Cell Cycle Arrest

Cell cycle arrest features prominently in the AKI to CKD transition.[21] We therefore posited that TNF modulates proximal tubular injury by regulating the cell cycle. To test this possibility, injured kidneys underwent collagenase digestion and fixation, followed by propidium iodide staining and flow cytometric analysis (Figure 6A).[18] Consistent with protection from cell cycle arrest, TNF PTKO kidneys showed higher proportions of cells in the G0/G1 phase and reduced percentages of cells in the G2/M phase

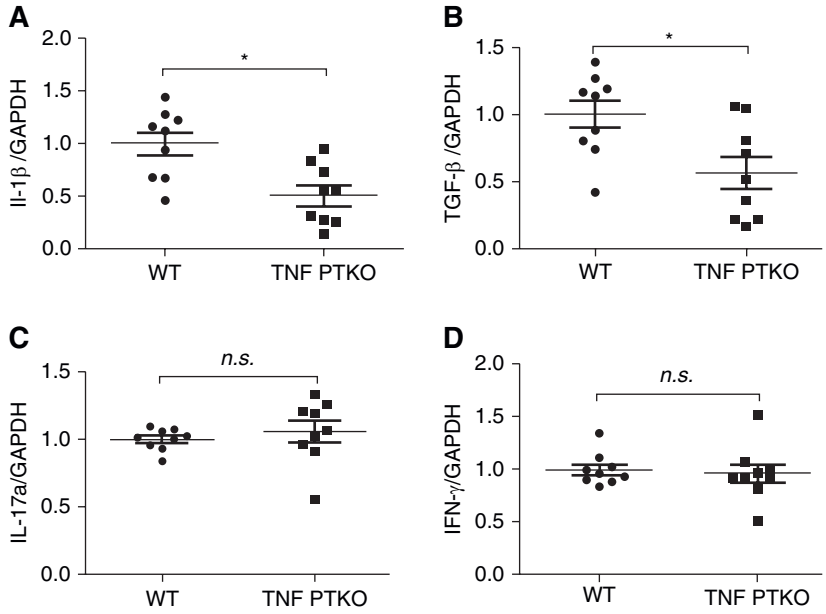

Figure 4. TNF from the proximal tubule mediates AA-induced kidney inflammation. Quantitative PCR determination of renal mRNA levels for genes encoding (A) IL-1β, (B) TGF-β, (C) IL-17A, and (D) IFN-γ at day 4 after AA exposure ($n \geq 9$). Values are mean±SEM (*$P < 0.05$).

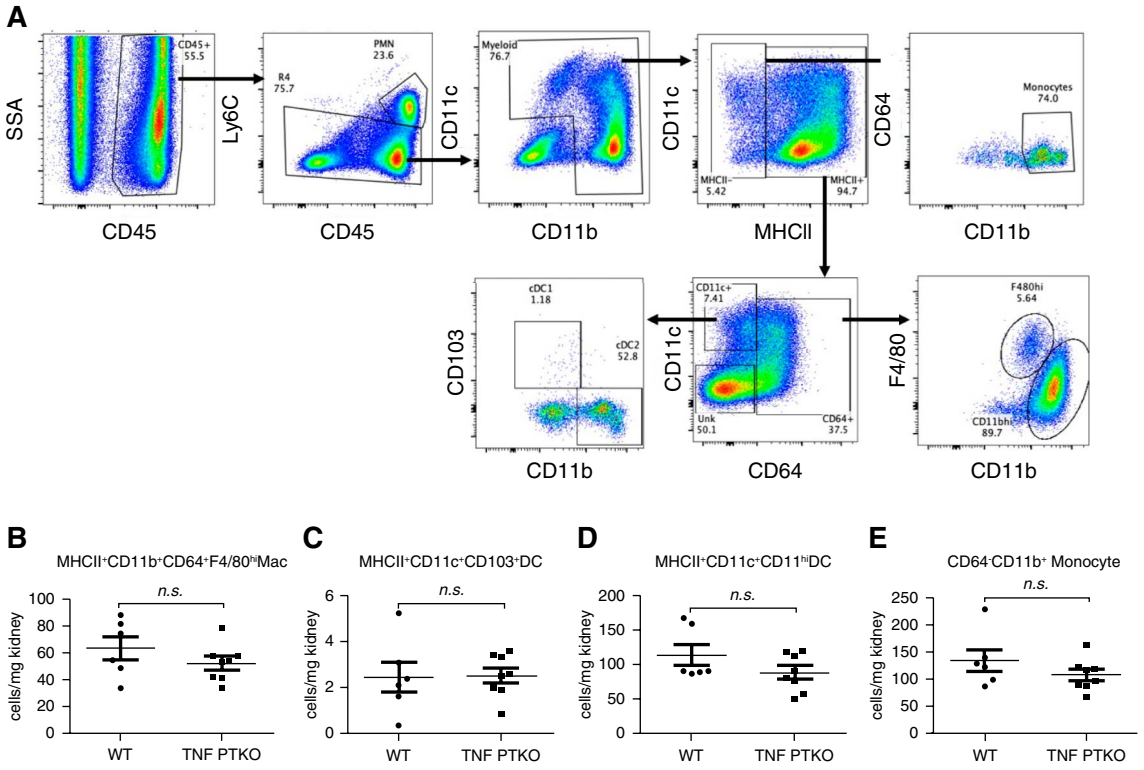

Figure 5. Proximal tubular TNF does not influence renal accumulation of myeloid cells. (A) Representative flow cytometry gating strategy for single-cell suspensions from aristolochic acid nephropathy (AAN) kidneys. (B–E) Number of (B) MHCII^{hi}CD11b^{+}F4/80^{hi} macrophages, (C) MHCII^{hi}CD11c^{+}CD103^{+} dendritic cells, (D) MHCII^{hi}CD11c^{+}CD1lb^{hi} dendritic cells, and (E) CD64^{-}CD11b^{+} monocytes in kidneys from WT and TNF PTKO mice at day 4 after AA injection ($n \geq 6$). Values are mean±SEM (*$P < 0.05$).

(Figure 6, B and C). To explore whether TNF in the PT contributes to these cell cycle differences, we administered pifithrin-α, which preserves cell cycle progression, to the cohorts on day 1 of AAN. Pifithrin-α treatment raised and equalized the proportions of cells in G0/G1 in the WT and PTKO kidneys and correspondingly reduced and equalized the proportions of cells at the G2/M phase in the groups (Figure 6, B and C). We found that renal injury was reduced and equalized in the TNF PTKO and WT groups with pifithrin-α treatment as reflected by blinded scoring of PAS staining kidney sections, mRNA levels for *lipocalin2*/NGAL (1.00±0.25 versus 0.88±0.24 au; P = NS), and BUN levels (146±7 versus 141±7 mg/dl; P = NS) (Figure 6, D–G). These data suggest that TNF elaborated by the PT instigates injury after AA exposure in part by contributing to cell cycle arrest.

Pifithrin-α Enhances Cellular Respiration in AA-Injured Kidney Tubular Epithelium

Pifithrin-α also affects the function of mitochondria that maintain the health of proximal tubular cells, a key target of acute AA-induced injury. Using Seahorse analysis, we examined the effect of pifithrin-α (30 M) on OCRs in WT murine primary RTCs treated with AA (10 μM) or vehicle for 12 hours (Supplemental Figure 5A). Pifithrin-α at least partially restored spare respiratory capacity and maximal respiration in AA-treated cells (Supplemental Figure 5B-C). Pifithrin-α also enhanced the spare respiratory capacity in RTCs exposed to TNF (50 ng/ml) (6.72±0.89 versus 4.37±0.41 pmol/min) (Supplemental Figure 5, D–F).

Deletion of TNF from the Proximal Nephron Attenuates Renal Fibrosis in Chronic AAN

To determine whether TNF in the PT contributes to kidney scar formation after chronic AAN, we administered AA 4 mg/kg IP every other day for 6 injections and examined renal injury parameters 4 weeks later.[22] PAS-stained sections of TNF PTKO kidneys demonstrated less architectural disruption than WTs (Figure 7A) and blunted mRNA levels for *lipocalin2*/NGAL (Figure 7B). BUN levels were also lower in TNF PTKO mice than in the WTs (70±7 versus 97 ± 9 mg/dl; $P < 0.05$) after chronic AAN (Figure 7C), having been reduced approximately 39% back toward baseline BUN levels. In the chronic AAN model, we found that mRNA levels for genes encoding TGF-β, fibronectin, and collagen I (Col-I) were lower in injured kidneys from TNF PTKO mice than in WTs (Figure 7, D–F) as were protein levels of Col-I (Figure 7, G and H). By contrast, no differences were seen between the groups in serum or urine TNF levels (Supplemental Figure 4, C–D). Treating the mice during chronic AA exposure concomitantly with pifithrin-α reduced and equalized BUN levels (36 ± 3 versus 30 ± 2 mg/dl; P = NS) (Figure 7C), renal mRNA expression for kidney injury and inflammatory mediators, and markers of fibrosis in the groups (Figure 7, B–H). Proportionately, treatment with pifithrin-α reduced BUNs

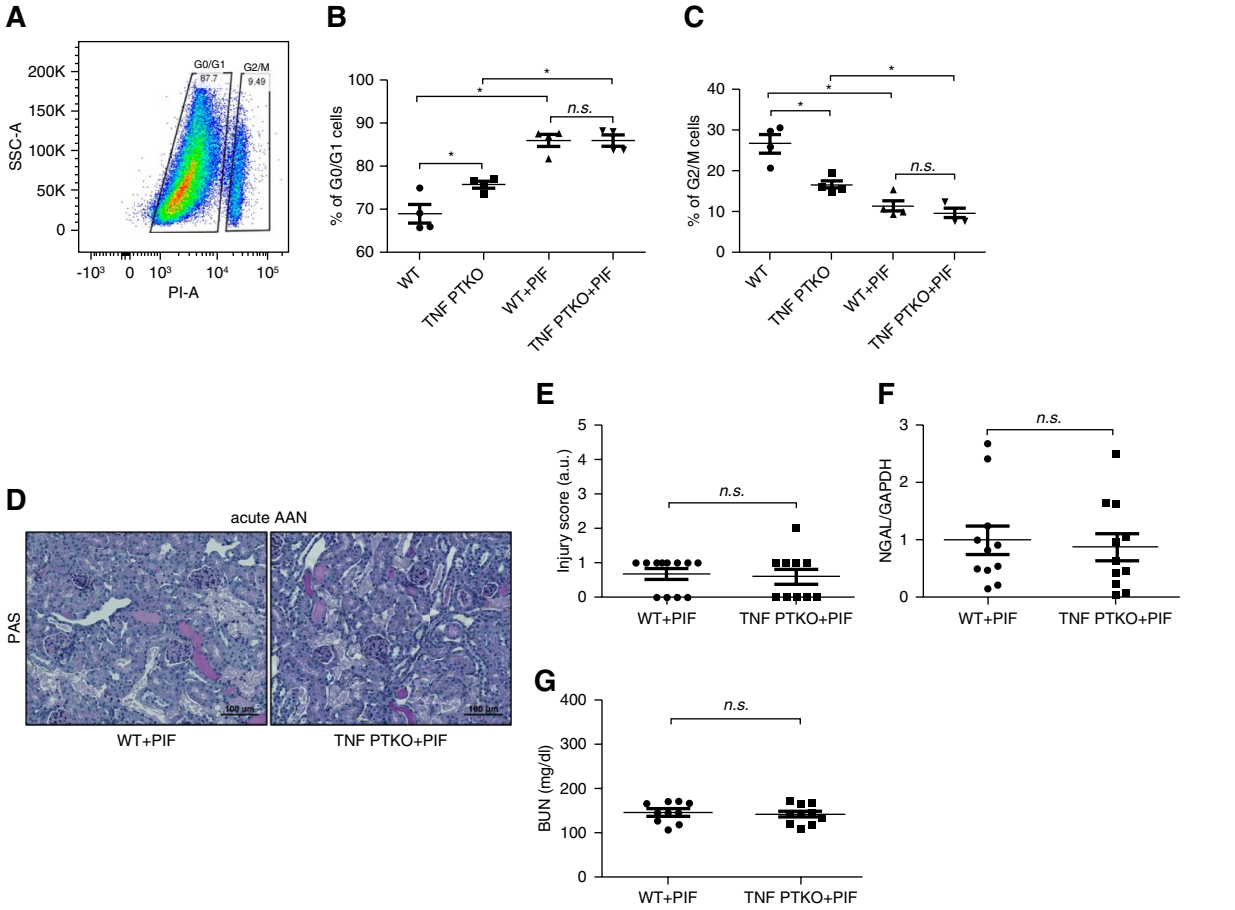

Figure 6. TNF in the proximal nephron promotes cell cycle arrest. (A) Representative flow plots of cell cycle arrested tubular epithelial cells from kidneys of AA-treated WT and TNF PTKO mice. (B–C) Percentage of tubular epithelial cells in (B) G0/G1 phase and (C) G2/M phase in AAN kidneys from WT and TNF PTKO mice with/without pifithrin-α treatment (n=4). (D) Representative images of kidney sections from pifithrin-α–treated WT and TNF PTKO mice at day 4 after AA injection. Scale bar=100 μm (E) Kidney pathology scores of pifithrin-α–treated WT and TNF PTKO mice at day 4 after AA injection ($n\geq10$). (F) Renal mRNA expression of *lipocalin2*/NGAL ($n\geq10$). (G) BUN ($n\geq10$). Values are mean±SEM (*$P < 0.05$).

by 96% toward the baseline level in WTs but by 79% toward baseline in the TNF PTKO cohort. By contrast, subjecting our TNF DNKO mice to the chronic AAN model, we found that deletion of TNF from the DN did not affect renal architecture, BUN levels, or mRNA levels for the genes encoding NGAL and PAI-1 (Supplemental Figure 6A-D). Thus, TNF in the proximal nephron, but not DN, propagates kidney injury and fibrosis after chronic AA exposure.

TNF in the PT Drives Expression of Regulated Cell Death Proteins in Chronic AAN

Because AA exposure induced signaling pathways for regulated cell death in the PT in our scRNA-seq analysis, and TNF influences activation of these pathways,[23,24] we measured renal expression of several TNF-regulated proteins that trigger apoptosis and/or necroptosis in the WT and PTKO cohorts after acute or chronic AAN. At the protein level, we found that, compared with WT controls, PTKO kidneys expressed similar levels of Bax, Bcl2, and p53, all linked to apoptosis, but lower levels of RIPK3, an early trigger for necroptosis, in our acute AAN model (Figure 8A). By contrast, renal levels of all these proteins were blunted in the PTKO cohort in our chronic AAN model (Figure 8B).

Discussion

Kidney damage deriving from AA exposure occurs endemically in Balkan countries and as a spectrum of herbal nephropathies in Asia. Whereas early reports suggested that AA could curtail inflammation,[25] more recently inhibition of the cytokine TNF attenuated the severity of chronic AAN in an experimental model, highlighting the proinflammatory effects of AA in kidney disease.[6] However, TNF is produced by a broad range of cell lineages including TECs and macrophages, and whether the capacity of TNF to drive AA-induced renal fibrosis relates to TNF's effects on acute tubular injury is not clear. This study therefore discriminates the acute and chronic effects of TEC-derived TNF on renal inflammation, injury, fibrosis, and activation of regulated cell death pathways after AA exposure. Our studies were restricted to males because female mice are reportedly more resistant to AA-induced renal injury.[26] We find that TNF from the proximal but not the DN promotes

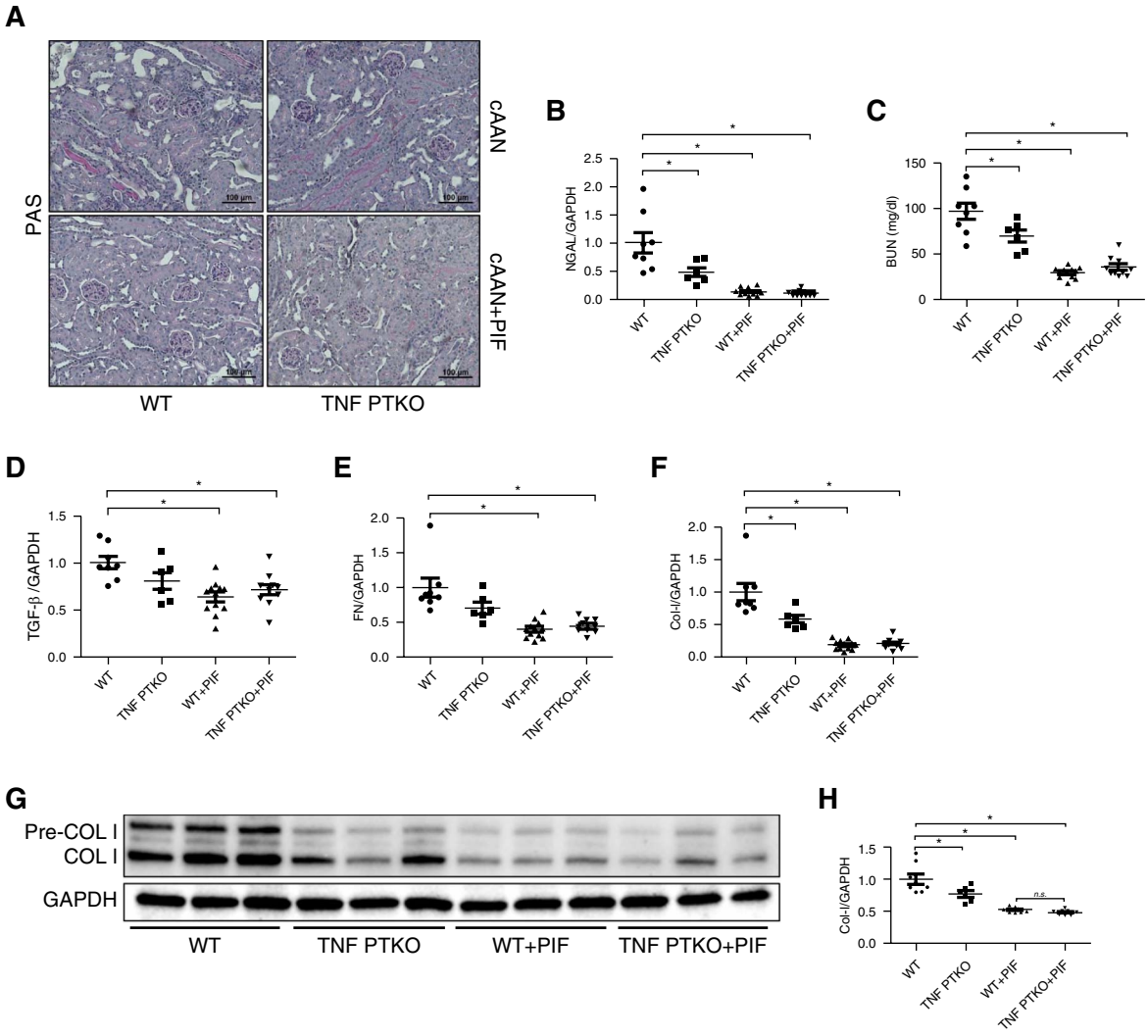

Figure 7. Deletion of TNF from the proximal nephron attenuates renal fibrosis in chronic AAN. (A) Representative images of kidney sections from WT and TNF PTKO mice with/without pifithrin-α treatment at day 35 after first AA injection. Scale bar=100 μm (B) Renal mRNA expression of *lipocalin2*/NGAL ($n \geq 6$). (C) BUN ($n \geq 6$). (D–F) Renal mRNA expression of genes encoding (D) TGF-β, (E) fibronectin (FN), (F) Col-I ($n \geq 6$). (G) Western blot for Col-I in each group as indicated. (H) Semiquantification of Col-I from (G) ($n \geq 6$). Values are mean±SEM (*$P < 0.05$).

acute AA-induced injury in part by inducing G2/M cell cycle arrest, which ultimately aggravates kidney fibrosis with chronic exposure. Accordingly, treatment with pifithrin-α, which restores the cell cycle, attenuates kidney fibrosis in chronic AAN and abrogates differences in AA-induced injury between WT mice and those with PT-specific TNF deletion (PTKOs). Interestingly, TNF generated in the PT seems to promote tubulointerstitial injury and inflammation independently of immune cell recruitment.

Generated by macrophages, T lymphocytes, TECs, and podocytes, TNF could affect kidney damage by regulating inflammation and/or cell survival. We previously reported that macrophage-derived TNF aggravates kidney injury and inflammation by stimulating TEC necroptosis during nephrotoxic serum nephritis.[27] During experimental hypertension, increased TNF production from renal parenchymal cells promotes BP elevation by modulating tubular function,[28,29] and in hypertensive humans, elevated TNF receptor signals mark increased risk of CKD progression.[30] Levels of TNF increase in renal tissue during toxic AKI,[31,32] and TNF generated in the kidney clearly promotes toxic injury.[33] Accordingly, serum TNF is a promising biomarker in predicting AKI severity.[34] We previously reported that TNF from the DN exacerbates cisplatin nephrotoxicity.[10] By contrast, in this study, we found that TNF from the DN does not influence kidney injury and inflammation after AA injection. The reason for this discrepancy is not clear but may relate to a less vigorous renal inflammatory cell infiltrate during acute AA versus cisplatin exposure. Next, we examined the effects of TNF from the proximal nephron on the pathophysiology of AAN and found that PTEC-specific deletion of TNF attenuates AA-induced tubular damage after 4

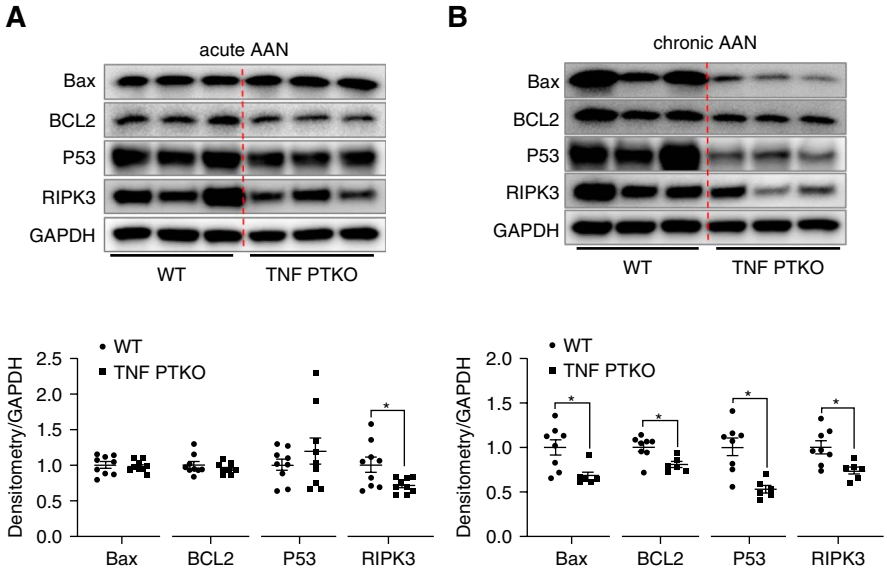

Figure 8. TNF in the proximal tubule drives renal expression of regulated cell death proteins in chronic AAN. (A) Representative Western blot for BAX, BCL2, P53, and RIPK3 in WT and TNF PTKO kidneys at day 4 after acute AA exposure with summary data of semiquantification ($n \geq 9$). (B) Representative Western blot for BAX, BCL2, P53, and RIPK3 in WT and TNF PTKO kidneys at day 35 after chronic AA exposure with summary data for semiquantification ($n \geq 6$). Values are mean±SEM ($^*P < 0.05$).

days. Moreover, kidneys from TNF PTKO mice had lower levels of mRNA encoding IL-1β and TGF-β compared with WTs. Thus, TNF can drive renal IL-1β generation in experimental AKI, converse to our previous report that IL-1 receptor stimulation induces renal TNF.[20] Moreover, the early divergence between our experimental groups in profibrotic cytokine generation may help to explain the protection from tubulointerstitial fibrosis detected in the TNF PTKOs in our chronic AAN model.

Given that TNF from the proximal nephron stimulated generation of these cytokines after AA exposure, we evaluated the infiltration of myeloid immune cells at 4 days after AA injection. Here, we found that PTEC-specific TNF deficiency does not influence the number of dendritic cells, macrophages, or monocytes accumulating in the kidney during acute AAN. Admittedly, we did not measure capacity of intrarenal myeloid cells for cytokine production or phagocytosis in the current studies. Nevertheless, the lack of differences in immune cell accumulation between the WT and PTKO kidneys in the AAN model may have permitted detection of the clear detrimental effects of proximal tubular TNF here because we have found that TNF produced by T lymphocytes, for example, can actually protect the kidney by regulating IL-17A generation and renal infiltration of neutrophils.[35] Others have even reported that TNF blockade can exacerbate lupus nephritis and also glomerulonephritis associated with rheumatoid arthritis.[36,37] Thus, TNF from the proximal nephron may aggravate acute AAN independently of immune cell functions, allowing the development of future therapies targeting proximal tubular TNF with limited off-target systemic side effects.

Whereas AKI survivors exhibit an increased risk of developing CKD, the mechanisms underlying this susceptibility warrant definition. Maladaptive repair after AKI is characterized by persistent inflammation, activation of myofibroblasts, and increased extracellular matrix deposition.[38] Because fate tracing studies failed to provide incontrovertible evidence of epithelial to fibroblast transition, attention turned to other alterations in TEC behavior during the AKI to CKD transition. Yang *et al.*[21] demonstrated that TEC arrest in the G2/M phase drives fibrogenesis in multiple rodent models of kidney injury, including severe bilateral ischemic AKI, unilateral ischemic AKI, and AAN. However, the initiating mechanisms of cell cycle arrest after toxic AKI remained unclear. After ischemia, TNF produced by macrophages drives inflammation and fibrosis, promoting the AKI to CKD transition.[39] However, during AAN, we found that TNF from the proximal nephron does not affect macrophage infiltration. We therefore focused on the role of proximal nephron-derived TNF to alter the cell cycle of TECs.

In our hands, TNF from the proximal nephron augments the proportions of TECs stuck in the G2/M phase with consequent exacerbations in kidney fibrosis after chronic AAN. p53 is a tumor suppressor that regulates cell death and metabolism in response to injurious stimuli, including hypoxia and DNA damaging agents,[40] and plays a fundamental role in instigating proximal tubular cell injury.[41] In turn, post-translational activation of p53 promotes the apoptosis of TECs after AA injection.[14] Activation of p53 protein inhibits CDC2/cyclin B complexes essential for the G2/M transition, yielding cell cycle arrest of TECs at the G2 phase.[42–44] Accordingly, pharmacological inhibition or genetic deletion of p53 attenuates tubular injury and apoptosis.[45–48] We found that restoring the cell cycle with pifithrin-α treatment equalizes levels of kidney injury and renal function between the TNF PTKO and WTs during acute AAN, with similar effects on renal fibrosis in our chronic AAN model.

Thus, TNF from the proximal nephron may exacerbate AA-induced G2/M cell cycle arrest.

Mitochondria are highly complex organelles that fulfill cellular energy demands and therefore govern the proliferation and death of cells. Proximal TECs reabsorb solutes in an ATP-dependent manner leading to substantial requirements for mitochondrial content and function.[49] Mitochondrial dysfunction allows chronic inflammation, toxic injury, and metabolic derangements, promoting the AKI to CKD transition. As such, mitochondrial dysfunction contributes to proximal tubular cell death in experimental AAN.[50,51] Human proximal tubular epithelial (HK-2) cells treated with AA have decreased levels of ATP production, mitochondrial membrane depolarization, and cytochrome C release.[52,53] Jiang *et al.*[54] reported that respiratory control ratios and ATP content are reduced in tubular cells by AA treatment in a dose-dependent manner, highlighting dysfunction of mitochondrial energy metabolism in the nephron. In our current *in vitro* studies with primary kidney tubular cells, the basal levels of cellular respiration are quite low and actually rise rather than fall with AA exposure. We speculate that these discrepancies may arise from our use of cultured primary TECs rather than a cell line. However, consistent with the earlier studies, we find that spare respiratory capacity and maximal respiration in renal TECs are reduced by AA treatment.

Impairment of tubular mitochondrial respiration by insults including cisplatin or inhibition of hepatocyte nuclear factor-1β associates with increased local TNF generation.[55,56] We find that cell cycle rescue with pifithrin-α treatment may partially restore cellular respiration in these epithelial cells after AA or TNF exposure. Nevertheless, given the subtle *in vitro* effect in our studies of pifithrin-α on spare respiratory capacity in TNF-treated tubular cells, it seems more likely that the benefits of pifithrin-α treatment in our *in vivo* studies accrue from ameliorating TNF-dependent cell cycle arrest in the PT.

There are several important limitations to this study. First, in our proximal tubular deletion model, we were not able to detect any evidence of reduced TNF generation in the serum or urine at baseline or injured conditions and also did not detect an increase in circulating or excretion of TNF in the WT cohort with injury. The lack of TNF induction in the serum during injury is particularly surprising because we previously saw a 60-fold induction in circulating TNF during cisplatin nephrotoxicity, although in that model TNF is admittedly a fundamental pathogenic mediator.[7,10] Thus, the only evidence pointing to efficacy of deletion is the reduction in renal gene expression for TNF at baseline in the PTKO animals and the reductions in kidney damage in the acute and chronic models. Although we have looked for tubular TNF expression with our scRNA-seq approach, we acknowledge that this strategy may not be sufficiently sensitive to detect low levels of TNF expression. In this regard, our scRNA-seq data suggest that most TNF in the kidney during nephrotoxic injury comes from infiltrating inflammatory cells. On the other hand, we acknowledge off-target *Tnfa* mRNA deletion in the lungs of our DNKO animals as is plausible with the cadherin-16 promoter,[57] but submit that this did not affect the kidney injury response. We further acknowledge that the protection from chronic kidney injury in the PTKO animals likely occurs largely as a result of their mitigated acute AA-induced injury and that pifithrin-α treatment demonstrates both TNF-dependent and TNF-independent renoprotective effects in our models. Moreover, we recognize that any effects of pifithrin-α on cellular respiration that we have detected *in vitro* are quite small such that the extent to which these effects contribute to the diminished renal injury with proximal tubular deletion of TNF is not clear.

In summary, TNF from the proximal nephron drives AA-induced kidney injury and tubulointerstitial fibrosis. Proximal tubular TNF promotes kidney damage and fibrogenesis by driving the G2/M cell cycle arrest of TECs, which can be rescued by pifithrin-α treatment. In contrast to the effects of TNF on immune cells in other types of kidney disease, TNF from the proximal nephron does not modulate kidney accumulation of innate immune cells during AAN. These studies illustrate the distinct effects of TNF from the proximal nephron in AA-induced kidney disease. Given the inherent challenges in designing TNF-dependent therapies targeted to the nephron, particularly with the low level of intrinsic tubular TNF expression, future studies may identify tubule-specific targets downstream of TNF for the disruption of the AKI to CKD transition.

Disclosures

S.A. Nedospasov reports the following—Advisory or Leadership Role: Lomonosov Moscow State University; Russian Academy of Sciences; Sirius University of Science and Technology. J.R. Privratsky reports the following—Employer: PLLC, Private Diagnostic Clinic and Other Interests or Relationships: JRP is recipient of National Institutes of Health grants K08 GM132689 and R01 DK131065. N.P. Rudemiller reports the following—Employer: BioAgilytix Labs, Immunologix Labs and Ownership Interest: BioAgilytix Labs. All remaining authors have nothing to disclose.

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Data Sharing Statement

All data are included in the manuscript and/or supporting information.

Supplemental Material

This article contains the following supplemental material online at http://links.lww.com/KN9/A411, http://links.lww.com/KN9/A412.

Supplemental Figure 1. Illustration of genotyping to detect the presence of *flox* and *Cre* for *TNF flox* and *Pepck-Cre* determination.

Supplemental Figure 2. Robust *Tnfa* mRNA induction in renal cortex with acute or chronic AA exposure.

Supplemental Figure 3. *Tnfa* mRNA expression in nonrenal tissues from TNF DNKO mice at baseline.

Supplemental Figure 4. TNF levels and other parameters in naïve and injured TNF PTKO mice.

Supplemental Figure 5. Pifithrin-α enhances cellular respiration in AA-injured kidney tubular cells.

Supplemental Figure 6. TNF deficiency in distal nephron does not affect AA-induced chronic tubular damage.

References

1. Ronco C, Bellomo R, Kellum JA. Acute kidney injury. *Lancet.* 2019;394(10212):1949–1964. doi:10.1016/S0140-6736(19)32563-2
2. See EJ, Jayasinghe K, Glassford N, et al. Long-term risk of adverse outcomes after acute kidney injury: a systematic review and meta-analysis of cohort studies using consensus definitions of exposure. *Kidney Int.* 2019;95(1):160–172. doi:10.1016/j.kint.2018.08.036
3. Ren J, Rudemiller NP, Wen Y, Lu X, Privratsky JR, Crowley SD. The transcription factor Twist1 in the distal nephron but not in macrophages propagates aristolochic acid nephropathy. *Kidney Int.* 2020;97(1):119–129. doi:10.1016/j.kint.2019.07.016
4. Xu Y, Ma H, Shao J, et al. A role for tubular necroptosis in cisplatin-induced AKI. *J Am Soc Nephrol.* 2015;26(11):2647–2658. doi:10.1681/ASN.2014080741
5. Dong X, Swaminathan S, Bachman LA, Croatt AJ, Nath KA, Griffin MD. Resident dendritic cells are the predominant TNF-secreting cell in early renal ischemia-reperfusion injury. *Kidney Int.* 2007;71(7):619–628. doi:10.1038/sj.ki.5002132
6. Taguchi S, Azushima K, Yamaji T, et al. Effects of tumor necrosis factor-α inhibition on kidney fibrosis and inflammation in a mouse model of aristolochic acid nephropathy. *Sci Rep.* 2021;11(1):23587. doi:10.1038/s41598-021-02864-1
7. Ramesh G, Reeves WB. TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. *J Clin Invest.* 2002;110(6):835–842. doi:10.1172/JCI15606
8. Tsuruya K, Ninomiya T, Tokumoto M, et al. Direct involvement of the receptor-mediated apoptotic pathways in cisplatin-induced renal tubular cell death. *Kidney Int.* 2003;63(1):72–82. doi:10.1046/j.1523-1755.2003.00709.x
9. Beyer M, Abdullah Z, Chemnitz JM, et al. Tumor-necrosis factor impairs CD4(+) T cell-mediated immunological control in chronic viral infection. *Nat Immunol.* 2016;17(5):593–603. doi:10.1038/ni.3399
10. Zhang J, Rudemiller NP, Patel MB, et al. Competing actions of type 1 angiotensin II receptors expressed on T lymphocytes and kidney epithelium during cisplatin-induced AKI. *J Am Soc Nephrol.* 2016;27(8):2257–2264. doi:10.1681/ASN.2015060683
11. Grivennikov SI, Tumanov AV, Liepinsh DJ, et al. Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: protective and deleterious effects. *Immunity.* 2005;22(1):93–104. doi:10.1016/j.immuni.2004.11.016
12. Rankin EB, Tomaszewski JE, Haase VH. Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. *Cancer Res.* 2006;66(5):2576–2583. doi:10.1158/0008-5472.CAN-05-3241
13. Shao X, Somlo S, Igarashi P. Epithelial-specific Cre/lox recombination in the developing kidney and genitourinary tract. *J Am Soc Nephrol.* 2002;13(7):1837–1846. doi:10.1097/01.ASN.0000016444.90348.50
14. Zhou L, Fu P, Huang XR, Liu F, Lai KN, Lan HY. Activation of p53 promotes renal injury in acute aristolochic acid nephropathy. *J Am Soc Nephrol.* 2010;21(1):31–41. doi:10.1681/ASN.2008111133
15. Ren JLK, Wu B, Lu X, et al. Divergent actions of renal tubular and endothelial type 1 interleukin-1 receptor signaling in toxin-induced acute kidney injury. *J Am Soc Nephrol.* 2023;34(10):1629–1646. doi:10.1681/ASN.0000000000000191
16. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS.* 2012;16(5):284–287. doi:10.1089/omi.2011.0118
17. Rudemiller NP, Crowley SD. Characterization and functional phenotyping of renal immune cells via flow cytometry. *Methods Mol Biol.* 2017;1614:87–98. doi:10.1007/978-1-4939-7030-8_8
18. Manolopoulou M, Matlock BK, Nlandu-Khodo S, et al. Novel kidney dissociation protocol and image-based flow cytometry facilitate improved analysis of injured proximal tubules. *Am J Physiol Renal Physiol.* 2019;316(5):F847–F855. doi:10.1152/ajprenal.00354.2018
19. Haq M, Norman J, Saba SR, Ramirez G, Rabb H. Role of IL-1 in renal ischemic reperfusion injury. *J Am Soc Nephrol.* 1998;9(4):614–619. doi:10.1681/ASN.V94614
20. Privratsky JR, Zhang J, Lu X, et al. Interleukin 1 receptor (IL-1R1) activation exacerbates toxin-induced acute kidney injury. *Am J Physiol Renal Physiol.* 2018;315(3):F682–F691. doi:10.1152/ajprenal.00104.2018
21. Yang L, Besschetnova TY, Brooks CR, Shah JV, Bonventre JV. Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury. *Nat Med.* 2010;16(5):535–543. doi:10.1038/nm.2144
22. Nlandu-Khodo S, Neelisetty S, Phillips M, et al. Blocking TGF-beta and beta-catenin epithelial crosstalk exacerbates CKD. *J Am Soc Nephrol.* 2017;28(12):3490–3503. doi:10.1681/ASN.2016121351
23. Genestier L, Bonnefoy-Berard N, Rouault JP, Flacher M, Revillard JP. Tumor necrosis factor-alpha up-regulates Bcl-2 expression and decreases calcium-dependent apoptosis in human B cell lines. *Int Immunol.* 1995;7(4):533–540. doi:10.1093/intimm/7.4.533
24. Sundararajan R, Cuconati A, Nelson D, White E. Tumor necrosis factor-alpha induces Bax-Bak interaction and apoptosis, which is inhibited by adenovirus E1B 19K. *J Biol Chem.* 2001;276(48):45120–45127. doi:10.1074/jbc.M106386200
25. Moreno JJ. Effect of aristolochic acid on arachidonic acid cascade and in vivo models of inflammation. *Immunopharmacology.* 1993;26(1):1–9. doi:10.1016/0162-3109(93)90061-t
26. Chang MM, Lin CN, Fang CC, et al. Glycine N-methyltransferase inhibits aristolochic acid nephropathy by increasing CYP3A44 and decreasing NQO1 expression in female mouse hepatocytes. *Sci Rep.* 2018;8(1):6960. doi:10.1038/s41598-018-22298-6
27. Wen Y, Lu X, Ren J, et al. KLF4 in macrophages attenuates TNFα-mediated kidney injury and fibrosis. *J Am Soc Nephrol.* 2019;30(10):1925–1938. doi:10.1681/ASN.2019020111
28. Furusho T, Sohara E, Mandai S, et al. Renal TNFα activates the WNK phosphorylation cascade and contributes to salt-sensitive hypertension in chronic kidney disease. *Kidney Int.* 2020;97(4):713–727. doi:10.1016/j.kint.2019.11.021
29. Zhang J, Patel MB, Griffiths R, et al. Tumor necrosis factor-α produced in the kidney contributes to angiotensin II-dependent hypertension. *Hypertension.* 2014;64(6):1275–1281. doi:10.1161/HYPERTENSIONAHA.114.03863
30. Chen TK, Coca SG, Estrella MM, et al. Longitudinal TNFR1 and TNFR2 and kidney outcomes: results from AASK and VA NEPHRON-D. *J Am Soc Nephrol.* 2022;33(5):996–1010. doi:10.1681/ASN.2021060735
31. Kim JY, Leem J, Jeon EJ. Protective effects of melatonin against aristolochic acid-induced nephropathy in mice. *Biomolecules.* 2019;10(1):11. doi:10.3390/biom10010011

32. Jadot I, Colombaro V, Martin B, et al. Restored nitric oxide bioavailability reduces the severity of acute-to-chronic transition in a mouse model of aristolochic acid nephropathy. *PLoS One.* 2017;12(8):e0183604. doi:10.1371/journal.pone.0183604
33. Zhang B, Ramesh G, Norbury CC, Reeves WB. Cisplatin-induced nephrotoxicity is mediated by tumor necrosis factor-alpha produced by renal parenchymal cells. *Kidney Int.* 2007;72(1):37–44. doi:10.1038/sj.ki.5002242
34. Fatani SH, Alrefai AA, Al-Amodi HS, Kamel HF, Al-Khatieb K, Bader H. Assessment of tumor necrosis factor alpha polymorphism TNF-α_{-238} (rs 361525) as a risk factor for development of acute kidney injury in critically ill patients. *Mol Biol Rep.* 2018;45(5):839–847. doi:10.1007/s11033-018-4230-8
35. Wen Y, Rudemiller NP, Zhang J, et al. TNF-α in T lymphocytes attenuates renal injury and fibrosis during nephrotoxic nephritis. *Am J Physiol Renal Physiol.* 2020;318(1):F107–F116. doi:10.1152/ajprenal.00347.2019
36. Stokes MB, Foster K, Markowitz GS, et al. Development of glomerulonephritis during anti-TNF-alpha therapy for rheumatoid arthritis. *Nephrol Dial Transplant.* 2005;20(7):1400–1406. doi:10.1093/ndt/gfh832
37. Mor A, Bingham CO III, Barisoni L, Lydon E, Belmont HM. Proliferative lupus nephritis and leukocytoclastic vasculitis during treatment with etanercept. *J Rheumatol.* 2005;32(4):740–743. PMID: 15801034.
38. Grgic I, Campanholle G, Bijol V, et al. Targeted proximal tubule injury triggers interstitial fibrosis and glomerulosclerosis. *Kidney Int.* 2012;82(2):172–183. doi:10.1038/ki.2012.20
39. Lech M, Gröbmayr R, Ryu M, et al. Macrophage phenotype controls long-term AKI outcomes—kidney regeneration versus atrophy. *J Am Soc Nephrol.* 2014;25(2):292–304. doi:10.1681/ASN.2013020152
40. Sborchia M, Keun HC, Phillips DH, Arlt VM. The impact of p53 on aristolochic acid I-induced gene expression in vivo. *Int J Mol Sci.* 2019;20(24):6155. doi:10.3390/ijms20246155
41. Zhang D, Liu Y, Wei Q, et al. Tubular p53 regulates multiple genes to mediate AKI. *J Am Soc Nephrol.* 2014;25(10):2278–2289. doi:10.1681/ASN.2013080902
42. Cao JY, Wang B, Tang TT, et al. Exosomal miR-125b-5p deriving from mesenchymal stem cells promotes tubular repair by suppression of p53 in ischemic acute kidney injury. *Theranostics.* 2021;11(11):5248–5266. doi:10.7150/thno.54550
43. Gifford CC, Lian F, Tang J, et al. PAI-1 induction during kidney injury promotes fibrotic epithelial dysfunction via deregulation of klotho, p53, and TGF-β1-receptor signaling. *FASEB J.* 2021;35(7):e21725. doi:10.1096/fj.202002652RR
44. Qi R, Wang J, Jiang Y, et al. Snai1-induced partial epithelial-mesenchymal transition orchestrates p53-p21-mediated G2/M arrest in the progression of renal fibrosis via NF-κB-mediated inflammation. *Cell Death Dis.* 2021;12(1):44. doi:10.1038/s41419-020-03322-y
45. Fu S, Hu X, Ma Z, et al. p53 in proximal tubules mediates chronic kidney problems after cisplatin treatment. *Cells* 2022;11(4):712. doi:10.3390/cells11040712
46. Sutton TA, Hato T, Mai E, et al. p53 is renoprotective after ischemic kidney injury by reducing inflammation. *J Am Soc Nephrol.* 2013;24(1):113–124. doi:10.1681/ASN.2012050469
47. Xiang X, Zhu J, Zhang G, Ma Z, Livingston MJ, Dong Z. Proximal tubule p53 in cold storage/transplantation-associated kidney injury and renal graft dysfunction. *Front Med (Lausanne).* 2021;8:746346. doi:10.3389/fmed.2021.746346
48. Ying Y, Kim J, Westphal SN, Long KE, Padanilam BJ. Targeted deletion of p53 in the proximal tubule prevents ischemic renal injury. *J Am Soc Nephrol.* 2014;25(12):2707–2716. doi:10.1681/ASN.2013121270
49. Galvan DL, Green NH, Danesh FR. The hallmarks of mitochondrial dysfunction in chronic kidney disease. *Kidney Int.* 2017;92(5):1051–1057. doi:10.1016/j.kint.2017.05.034
50. Pozdzik AA, Salmon IJ, Debelle FD, et al. Aristolochic acid induces proximal tubule apoptosis and epithelial to mesenchymal transformation. *Kidney Int.* 2008;73(5):595–607. doi:10.1038/sj.ki.5002714
51. Liu X, Wu J, Wang J, et al. Mitochondrial dysfunction is involved in aristolochic acid I-induced apoptosis in renal proximal tubular epithelial cells. *Hum Exp Toxicol.* 2020;39(5):673–682. doi:10.1177/0960327119897099
52. Chou CK, Huang YS, Lin PY, et al. The ginsenoside Rg1 rescues mitochondrial disorders in aristolochic acid-induced nephropathic mice. *Life (Basel)* 2021;11(10):1018. doi:10.3390/life11101018
53. Qi X, Cai Y, Gong L, et al. Role of mitochondrial permeability transition in human renal tubular epithelial cell death induced by aristolochic acid. *Toxicol Appl Pharmacol.* 2007;222(1):105–110. doi:10.1016/j.taap.2007.03.029
54. Jiang Z, Bao Q, Sun L, et al. Possible role of mtDNA depletion and respiratory chain defects in aristolochic acid I-induced acute nephrotoxicity. *Toxicol Appl Pharmacol.* 2013;266(2):198–203. doi:10.1016/j.taap.2012.07.008
55. Bajwa A, Rosin DL, Chroscicki P, et al. Sphingosine 1-phosphate receptor-1 enhances mitochondrial function and reduces cisplatin-induced tubule injury. *J Am Soc Nephrol.* 2015;26(4):908–925. doi:10.1681/ASN.2013121351
56. Casemayou A, Fournel A, Bagattin A, et al. Hepatocyte nuclear factor-1β controls mitochondrial respiration in renal tubular cells. *J Am Soc Nephrol.* 2017;28(11):3205–3217. doi:10.1681/ASN.2016050508
57. Wertz K, Herrmann BG. Kidney-specific cadherin (cdh16) is expressed in embryonic kidney, lung, and sex ducts. *Mech Dev.* 1999;84(1–2):185–188. doi:10.1016/s0925-4773(99)00074-x

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