

Inhibition on neutrophil extracellular traps by oligomeric procyanidins alleviate chemotherapy-induced chronic kidney injury via gut-kidney axis

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This **important** study provides evidence for the role of neutrophil extracellular traps in chronic kidney damage (CKD) induced by chemotherapy and suggests a therapeutic approach to mitigate the kidney pathology caused by the NETs. The study utilizes a sound murine in vivo model of CKD with low-dose administration cisplatin and a genetic model for impairment of NET formation by deletion of the enzyme Pad4. In its current form, the study was seen as **incomplete** as there is not yet formal demonstration of NET production by neutrophils in the model of CKD used. Additionally, the accuracy and clarity of data presentation could be improved.

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Abstract

Cisplatin is one of the most widely used chemotherapeutic agents for various solid tumors in the clinic, but its use is limited by adverse effects in normal tissues. In particular, cisplatin administration often damages the kidneys. However, little is known about how to alleviate cisplatin-induced chronic kidney disease (CKD) specifically. Here, we found that repeated low-dose cisplatin (RLDC) recruited neutrophils to the proximal tubule, thereby promoting the progression of CKD in the mouse model. Mechanically, cisplatin destroyed the intestinal epithelium, which induced dysregulation of gut flora and intestinal leakage. It triggered Neutrophil extracellular traps (NETs) formation, accumulating in the proximal tubule and promotes chronic inflammation and fibrosis, and promotes chronic hypoxia, leading to poor regeneration that promotes CKD progression. NETs provided a scaffold for tissue factors (TF)

adhesion and metalloproteinase 9 (MMP-9) activation, which triggers local ischemia and hypoxia. In addition, NETs promoted inflammasome construction through NOD-like receptor thermal protein domain associated protein 3 (NLRP3) shear and secretion of mature interleukin-18 (IL18), which subsequently released interferon- γ (IFN- γ), contributing to renal interstitial fibrosis. We proposed that oligomeric procyanidins (OPC) ameliorated RLDC-induced CKD through multi-targeting damage induced by NETs. OPC ameliorated microcirculatory disorders and inhibited inflammation by protecting the intestinal mucosa barrier and subsequent bacterial endotoxin translocation. Furthermore, we found that OPC directly blocked LPS & cisplatin-induced NETs formation in vitro. In summary, NETs play a pivotal role in CKD, which OPC alleviates by inhibiting TF/MMP-9 and IL-18-NLRP3 pathways. OPCs protect the kidney by inhibiting NETs production through anti-inflammatory and antioxidant activities and restoring the balance of the intestinal flora

Introduction

Cisplatin is the first platinum-based antineoplastic drug approved by the FDA (Famurewa et al., 2022 [\[1\]](#)). As the backbone of countless treatment regimens across a broad spectrum of malignancies, cisplatin improves survival and cure rates. Despite this positive effect, nephrotoxicity still limits life-saving therapy (Sahni et al., 2009 [\[2\]](#)). This impedes the dose and intensity of cisplatin in the target region, impacting patients' long-term quality of life (Crona et al., 2017 [\[3\]](#)). To minimize side effects, clinicians often use low doses and multiple cycles of cisplatin treatment, also called repeated low-dose cisplatin (RLDC). It is more likely to induce chronic kidney disease (CKD) than acute kidney injury (AKI), as the current RLDC model shows some pathological manifestations in accordance with the critical features of CKD, including chronic inflammation (Fu et al., 2023 [\[4\]](#)), tubulointerstitial fibrosis (Li et al., 2023 [\[5\]](#)), atubular glomeruli and glomerulosclerosis (Sharp et al., 2018 [\[6\]](#)). However, the exact effects of RLDC on CKD and the corresponding mechanism are not fully understood. In this study, we used an RLDC regimen of weekly repetitive low-dose cisplatin (7 mg/kg), which allowed the mice to survive 6 months posttreatment without exhibiting clinical signs of AKI.

Neutrophils, a significant arm of the innate immune system, are the first leukocytes to be recruited to sites of inflammation as the initial host defense against an extensive range of pathogens, killing harmful microorganisms in three ways: phagocytosis (Nordenfelt and Tapper, 2011 [\[7\]](#)), degranulation of cytotoxic enzymes (Stapels et al., 2015 [\[8\]](#)) and neutrophil extracellular traps (NETs), which are DNA meshes with associated cytotoxic enzymes and histones that are released into the extracellular space where they trap microorganisms (Burgener and Schroder, 2020 [\[9\]](#)). However, NETs are a double-edged sword; they also lead to damage, acting as a cascade to amplify and maintain inflammation and prevent the diffusion of damage-associated molecular patterns (DAMPs). The opposing roles of NETs have been described in autoimmune diseases such as lupus nephritis (Hakim et al., 2010 [\[10\]](#)), diabetic kidney disease (Gupta et al., 2022 [\[11\]](#)), and ANCA-associated vasculitis (Yoshida et al., 2013 [\[12\]](#)).

During NETs formation, neutrophil elastase (NE) and myeloperoxidase (MPO) facilitate nuclear membrane rupture and chromatin unwinding. Protein arginine deiminase 4 (PAD4), a histone citrullination-associated protease whose activity is greatly enhanced by binding to Ca (Lewis et al., 2015 [\[13\]](#)), is essential for NETs formation. Recent studies have shown that treating lupus mice with PAD4 inhibitors modulates the autoimmune response and significantly improves disease progression (Knight et al., 2015 [\[14\]](#)). PAD4-deficient mice do not form network structures that induce thrombosis and inflammation (Leppkes et al., 2022 [\[15\]](#); Raup-Konsavage et al., 2018 [\[16\]](#); Seri et al., 2015 [\[17\]](#)), and our present studies revealed that PAD4 knockout (PAD4^{-/-}) in mice relieved RLDC-induced CKD.

Inflammation is inevitable in NOD-like receptor thermal protein domain-associated protein 3 (NLRP3)-inflammasomes. An increasing amount of data has revealed a close relationship between NETs and the NLRP3 inflammasome (Warnatsch et al., 2015; Westerterp et al., 2018). Alexandra and colleagues reported that cisplatin significantly induced the release of NETs with NLRP3 inflammatory vesicles involved in acute kidney injury (Mousset et al., 2023). However, NETs-NLRP3 often appear in acute inflammation, and their role in recurrent chronic inflammation is unclear. Our present data revealed that NETs cooperated with NLRP3 to increase the levels of interleukin-18 (IL18) and interferon γ (IFN γ), which accumulated in the renal interstitium to induce renal interstitial fibrosis (Chen et al., 2020; Tapmeier et al., 2010).

Patients with chronic kidney disease (CKD) frequently exhibit aberrant coagulation patterns in the face of an elevated risk for thrombosis (Matsushita et al., 2022). Similarly, the risk and aggravating factors for thrombosis are positively correlated with kidney injury in a cisplatin-induced model (Watanabe et al., 2019). Tissue factor (TF) pathway inhibitors (TFPIs) are responsible for inhibiting TF-induced coagulation. The hypoxic microenvironment has been shown to activate hypoxia inducible factor 1 α (HIF1 α) to induce coagulation and increase TF levels, and HIF1 α can directly inhibit TFPI promoter activity (Cui et al., 2016). Neutrophils release elastase to cleave TFPI, counteracting its strong inhibitory effect on TF activity and subsequent thrombosis (Massberg et al., 2010). Furthermore, neutrophil-released metalloproteinase 9 (MMP9) (Carmona-Rivera et al., 2015) is thought to be a HIF 1 α -dependent angiogenic gene and proangiogenic protease (Song et al., 2009). Thus, we aim to investigate whether TF, TFPI, and MMP9 are involved in NETs-mediated thrombosis.

Procyanidins are known to have antibacterial, anti-inflammatory (Pallarès et al., 2013) and oxidative stress-reducing effects that can repair gut damage to reduce LPS production (Nallathambi et al., 2020). Oligomeric proanthocyanidins (OPCs) are small-molecular-weight proanthocyanidins that are generally dimers to tetramers. It has been reported that OPCs extracted from grapes improves CKD progression by reducing oxidative stress (Zhu and Du, 2020). We previously reported that OPCs strongly inhibited morphine-induced NLRP3 inflammatory vesicles in a model of gout pain (Cai et al., 2016; Liu et al., 2017). Thus, does OPCs also have an effect on NETs? Is this role vital in CKD? Our present findings showed that OPCs directly inhibited the formation of NETs, indirectly improved intestinal leakage, and ultimately alleviated RLDC-induced kidney injury.

Results

NETs accumulated in the kidneys after RLDC treatment

In accordance with the literature and multiple preliminary experiments, we treated the mice with 7 mg/kg cisplatin weekly for four weeks, and this regimen resulted in no significant change in mortality but did significantly decrease body weight in the mice after 1 month of RLDC treatment (Figure 1C). Compared with those from control mice, kidneys from RLDC-treated mice presented reductions in volume and weight (Figure 1A and B), suggesting decreased repair function following renal injury after RLDC. Furthermore, pathological analysis revealed that RLDC caused dramatic changes in renal structure, including tubular dilatation and necrosis, brush border loss, tubular formation, tubular atrophy, and inflammatory cell infiltration (Figure 1D and E). The levels of serum creatinine and urea nitrogen were significantly increased (Figure 1F and G). All the above results demonstrated that RLDC could indeed induce renal injury. Tumor chemotherapy often induces NETs formation (Adrover et al., 2023; Mousset et al., 2023; Xiao et al., 2021), leading to sensory numbness of the distal limbs accompanied by cold-touch abnormalities (Lin et al., 2022). Numerous studies have shown the involvement of NETs in thrombosis in patients during chemotherapy, and our previous studies demonstrated that elevated NETs in the blood of chemotherapeutic patients lead to ischemia and hypoxia in the peripheral

circulation. Consistent with the above studies, Doppler flow data also revealed a significant decrease in the plantar blood of the mice after RLDC (**Figure 1L and M**). Both plasma CitH3 and cfDNA, which are components of NETs, were elevated (**Figure 1H-J**), and the release of NETs were detected in the blood (**Figure 1K**). These findings demonstrated the increased formation of NETs in the blood after RLDC, resulting in a decrease in plantar blood flow. Furthermore, elevated CitH3 colabeled with MPO in renal tissues suggested that cisplatin-induced NETs entered the kidneys along with the bloodstream, which was associated with kidney injury.

Blocking NETs prevented RLDC-induced renal tubular injury and apoptosis

We proceeded to investigate whether the presence of NETs was involved in kidney injury. It is an essential process for PAD4 to enter neutrophils, where PAD4 citrullinates histone arginine residues to stimulate chromatin decondensation, generating NETs. Thus, PAD4 knockout (PAD4^{-/-}) mice, which are not able to form NETs, were used. As shown in **Figure 2A and B**, RLDC induced renal atrophy with body weight loss in both WT and PAD4^{-/-} mice. However, in contrast to the kidneys of WT mice, which presented marked brush border loss, cast formation, massive loss of tubular epithelial cells, tubular dilatation, and tubular intratubular debris, PAD4 knockout inhibited tubular injury, with well-preserved brush border membranes and no loss of tubular epithelial cells (**Figure 2C**). There was a significant difference in collagen accumulation in the renal interstitial and perivascular areas between the WT and PAD4^{-/-} mice, and PAD4 knockout significantly reduced the collagen area. Moreover, analysis of Masson's trichrome staining revealed that the absence of NETs attenuated interstitial fibrosis in the kidneys after RLDC (**Figure 2D and E**). Further semiquantitative analysis of HE, PAS, and Masson's trichrome staining revealed that tubular injury, collagen deposition, and tubulointerstitial fibrosis scores were significantly greater in RLDC-treated WT mice than in control mice, whereas the scores in RLDC-treated PAD4^{-/-} mice were similar to those in control mice (**Figure 2G-I**). Given that renal tubular degeneration and atrophy are the major causes of kidney injury, we tested the expression of a kidney injury marker (KIM-1) and a proximal tubule marker (Lotus tetragonolobus lectin, LTL). Under RLDC treatment, WT mice presented proximal renal tubular degeneration with loss of the brush border and LTL tubules, which was accompanied by increased KIM-1 in these tubules, which disappeared in PAD4^{-/-} mice (**Figure 2F**). As shown in the semiquantitative analysis, the percentage of the KIM-1-positive area decreased from 2.43% to 0.58%, and the LTL-positive area recovered from 2.39% to 6.68% after PAD4 deficiency (**Figure 2J and K**). Consistent with the improvement in renal function, the expression of KIM-1 and BAX was increased in WT mice but not in PAD4^{-/-} mice, suggesting that NETs are involved in CKD induced by RLDC (**Figure 2L-N**).

Blocking NETs prevented the activation of NLRP3 inflammatory vesicles

Our previous study demonstrated that NETs induced the maturation of macrophage NLRP3 inflammatory vesicles (Lin et al., 2022). To investigate whether the NLRP3 inflammasome was involved in NETs-mediated renal injury, the levels of NLRP3 and related inflammatory factors were detected in PAD4^{-/-} mice. First, with RLDC treatment, the mice exhibited severe renal dysfunction, as evidenced by elevated plasma creatinine and urea nitrogen, which were significantly lower in the PAD4^{-/-} mice (**Figure 3A and B**). Like the creatinine and urea nitrogen levels, NETosis was also decreased in PAD4^{-/-} mice (**Figure 3C-E**). Next, western blot analysis revealed that RLDC increased the expression of Cit H3, NLRP3, Casp-1, interleukin 1 β (IL1 β), interleukin 18 (IL18) and interferon γ (IFN γ) (**Figure 3F-L**). IL18, a Th1 cytokine, significantly induces natural killer (NK) cells and T cells to produce interferon gamma (IFN γ), which is involved in cytotoxicity and type I immunity (Landy et al., 2024). In addition to its well-known antifibrotic effects, IFN γ is involved in the repair of renal tubulointerstitial fibrosis. This is a double-edged sword, as it can also induce fibrosis (Kim et al., 2022). As demonstrated by immunofluorescence,

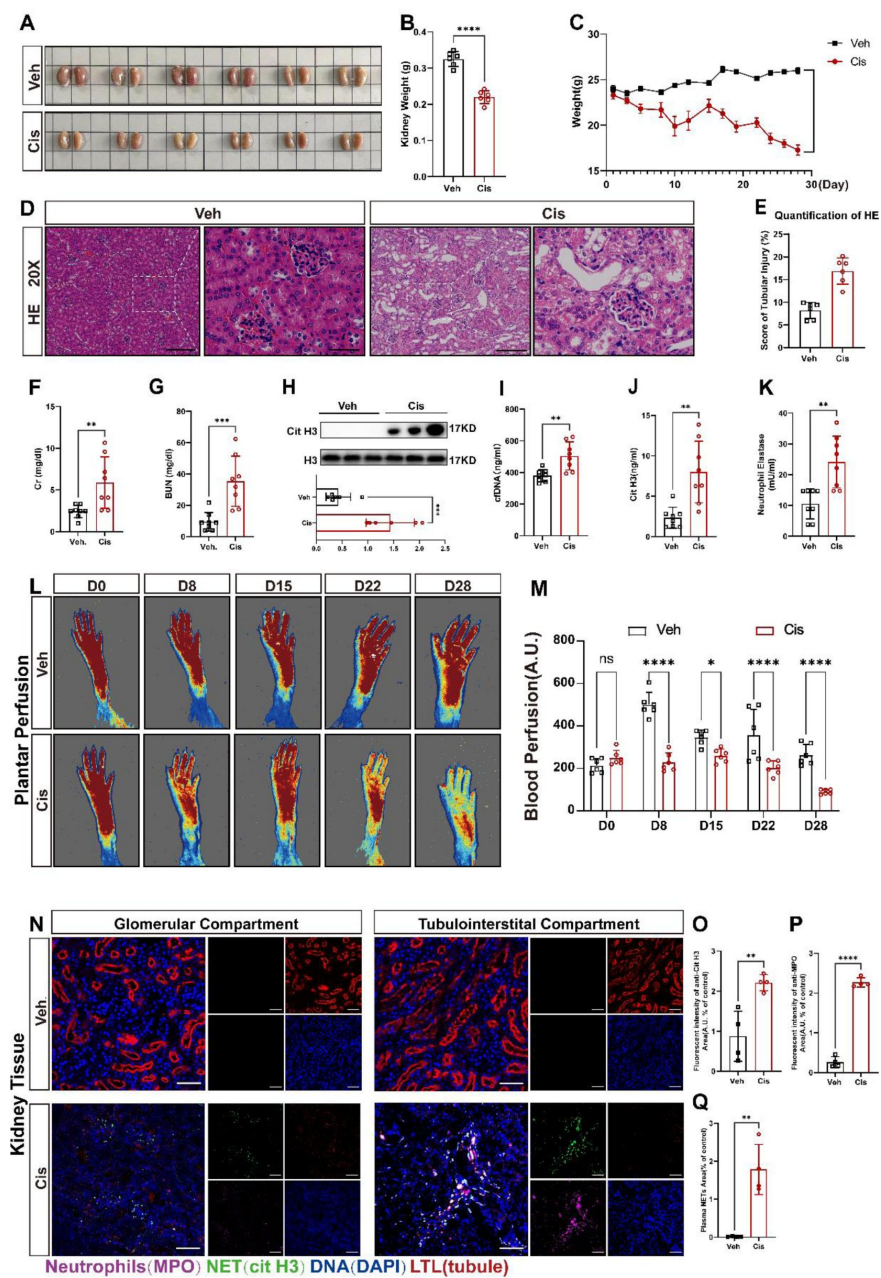


Figure 1.

RLDC triggered NETs formation.

The mice were injected weekly intraperitoneally with 7 mg/kg cisplatin for four weeks and analyzed by execution after 1 month (Cis group) or the untreated control (Veh group). Blood and kidney tissues were collected one week after the last cisplatin injection. **A**. Representative image of kidney size. **B**, **C**. Quantitative analysis of kidney weight and body weight ($n = 6$, **** $p < 0.0001$). **D**. Representative HE-stained images (bar = 100 μ m). **E**. Pathological tubular atrophy score ($n = 6$, **** $p < 0.0001$). **F**, **G**. The concentrations of serum creatinine and blood urea nitrogen ($n = 8$, ** $p = 0.0083$, and *** $p = 0.0008$). **H**. Detection of CitH3 protein levels in the kidney via western blotting ($n = 8$, ** $p = 0.0026$). **I**–**K**. The contents of H3Cit, NE, and cfDNA in plasma after intraperitoneal injection of cisplatin were evaluated via the H3Cit ELISA kit, NETosis assay, and dsDNA ELISA kit ($n = 8$, ** $p < 0.01$). **L**, **M**. Blood flow in the lower limbs of the mice was measured at the start of cisplatin treatment and at the end of each week via a MoorFLPI2 blood flow scatter hemodynamometer. ($n = 6$, **** $p < 0.0001$, D28). **N**–**O**. NETs (confocal immunofluorescence microscopy images; stained for MPO, Cit H3, LTL, and DNA) visible in kidney samples from chemotherapeutic nephritic mice ($n = 4$, ** $p < 0.01$, *** $p < 0.001$). Scale bar, 50 μ m. Significant differences were revealed via one-way ANOVA vs. vehicle (A, B, E, F–K, M, and O–Q).

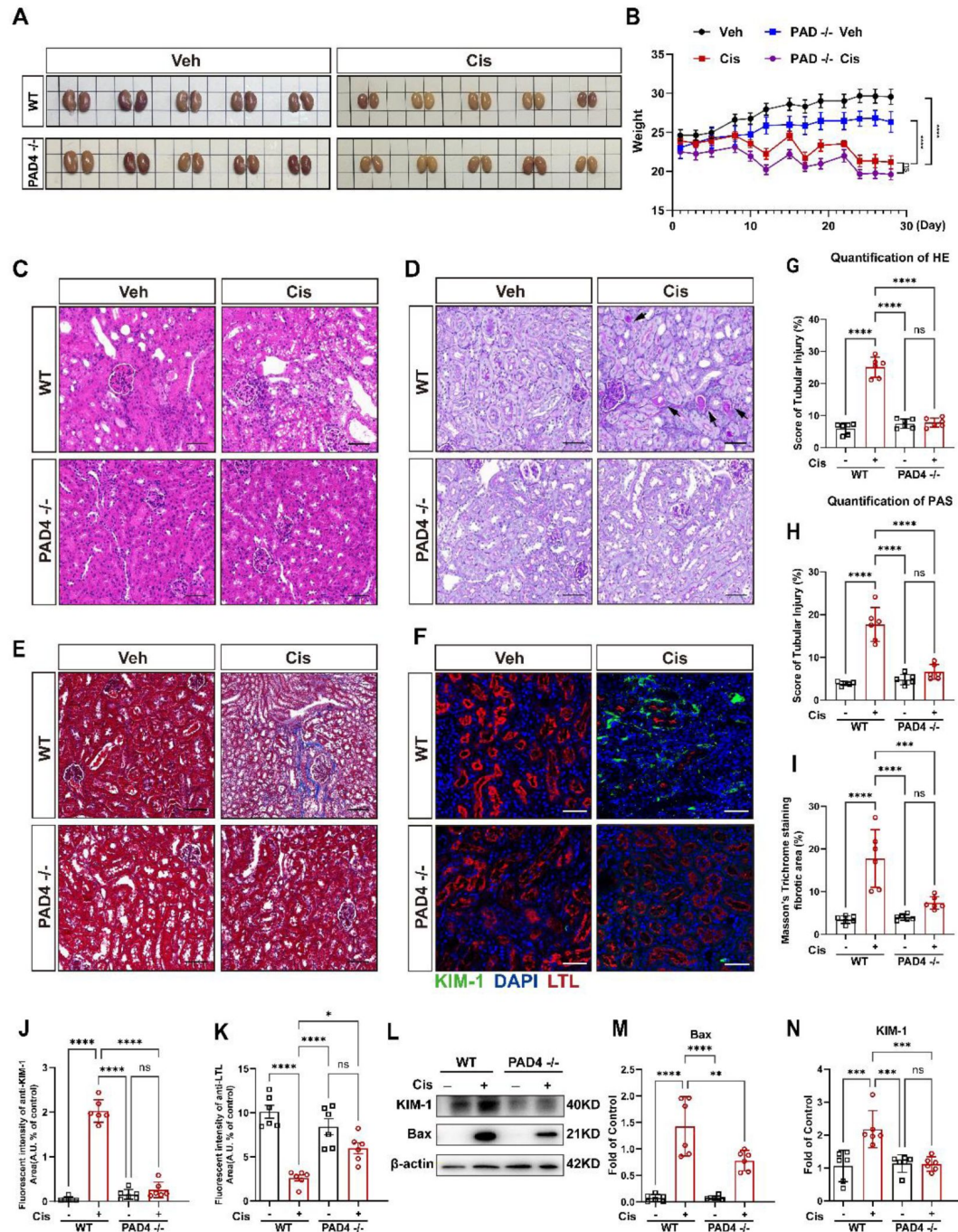


Figure 2.

NETs mediate kidney injury.

A. Gross observation of kidneys from RLDC-induced WT or PAD4^{-/-} mice. A representative image of kidney size is shown ($n = 5$). **B.** Trends in the body weights of the mice during the four weeks of modeling ($n = 10$). **C-E.** HE, PAS, and Masson's trichrome staining of kidney slices (scale bars 50 μm , $n = 6$). **F.** Representative confocal images of KIM-1+ and LTL+ tubules (scale bars 50 μm , $n = 6$). **G-I.** Quantification of renal tubular damage and renal fibrosis in the mice in each group ($n = 6$, **** $p < 0.0001$). **J, K.** Quantification of KIM-1-positive and LTL-positive areas of the kidney ($n = 6$, **** $p < 0.0001$). **L-N.** WB analysis and quantification of KIM-1 and BAX expression in the kidney in each group as indicated ($n = 6$, **** $p < 0.0001$, and **** $p < 0.0001$). Significant differences were revealed via one-way ANOVA vs. Vehicle/PAD4^{-/-} (B, J-K, M, and N).

IFN γ was present in almost every lumen of lumen-tethered dye LTL-labeled tubules following RLDC (**Figure 3M**). Compared with that of WT mice, the area favorable for IFN γ was 20.54% smaller in PAD4 $^{-/-}$ mice (**Figure 3O**). As expected, RLDC induced renal tubulointerstitial fibrosis in WT mice, accompanied by an increase in α -SMA. These changes were not obvious in PAD4 $^{-/-}$ mice (**Figure 3N and P**). The above results suggest that NETs participate in RLDC-mediated CKD, in which the NLRP3-IFN γ pathway is involved.

NETs-mediated functional TF-MMP9 activation was required for renal ischemia and hypoxia

In the kidney, the proximal tubules are the most susceptible to hypoxic injury, and the extent of tubular injury is a key determinant of the prognosis of renal disease. Hypoxia plays a key role in the pathogenesis of CKD and its promotion of coagulation and thrombotic events (Oe and Takahashi, 2022). Our present data revealed that RLDC led to microcirculation disorders (**Figure 1L**). Furthermore, recent studies have demonstrated that hypoxia-inducible factor 1 alpha (HIF-1 α) is involved in renal fibrosis in the RLDC mouse model (Zhao et al., 2021). To explore whether RLDC-induced NETs cause microthrombi in the kidneys and their roles in renal tubular injury, we examined the levels of HIF1 α and relevant microthrombus indicators. As shown in **Figure 4A-E**, RLDC significantly elevated the levels of HIF-1 α , TF, and MMP9 and reduced the levels of TFPI in the renal tissues of WT mice. In contrast, RLDC had no discernible effect on PAD4 $^{-/-}$ mice. Surprisingly, most NETs colocalized with TF in the renal tubular mesenchyme at the more pronounced corticomedullary junction, which is also the most sensitive to hypoxia, where there are more marked changes in a state of hypoxia. In contrast, due to the absence of NETs scaffolds, no obvious TF was deposited in the PAD4 $^{-/-}$ mice (**Figure 4F-H**). Long-term hypoxia usually results in interstitial hyperplasia. Ki67 is a marker of cell proliferation reflecting hyperplasia. A significant increase in the number of Ki67-positive cells was observed in the kidneys of WT mice following RLDC treatment, with a particularly notable increase in the region of the junction between the renal cortex and the medulla (**Figure 4I**). In contrast, the number of Ki67-positive cells in PAD4 $^{-/-}$ mice decreased by 55.29% (**Figure 4K**), suggesting that NETs cooperating with TF-stimulated interstitial hyperplasia. Finally, we employed laser Doppler speckle flowmetry to assess plantar blood flow in the mice. Compared with that in WT mice, the mean lower limb blood flow in PAD $^{-/-}$ mice was not influenced by RLDC (**Figure 4J and L**). This finding aligns with the hypothesis that NETs contribute to the exacerbation of thrombus formation.

OPCs inhibited LPS leakage caused by intestinal barrier damage

Cisplatin and its metabolites cause severe damage to the intestinal mucosa during chemotherapy, disrupting the intestinal mucosal barrier, leading to leaky gut, and causing long-term chemotherapeutic colitis (Hu et al., 2021). These findings prompted us to speculate that intestinal damage is related to cisplatin-induced CKD. To investigate whether OPCs can alleviate cisplatin-induced CKD through the intestine, we treated the mice with OPCs and used berberine (BBR) as an anti-inflammatory and antibacterial positive control (Chen et al., 2017; Pallarès et al., 2013; Pan et al., 2023; Zhang et al., 2019; Zhu et al., 2022). Colon shortening is a feature of colon inflammation in cisplatin-induced colitis. As shown in **Figure 5A and B**, the intestinal length significantly decreased after RLDC treatment but was restored by BBR and OPCs. HE staining revealed that RLDC resulted in the disappearance of villi due to atrophy and detachment, as well as inflammatory cell infiltration, in addition to the disappearance of glands, cup cells and crypts. Notably, inflammatory cell infiltration was absent in both the BBR and OPCs treatment groups, and OPCs effectively restored the intestinal villi (**Figure 5C and D**), suggesting that both BBR and OPCs significantly inhibited cisplatin-induced colonic inflammation. We also used laser Doppler imaging to detect the intestinal blood flow of mice with chemotherapeutic enteritis. RLDC significantly reduced intestinal blood flow, which was restored by both BBR and OPCs (**Figure 5E and F**). To further assess the status of intestinal damage, the intestinal barrier was

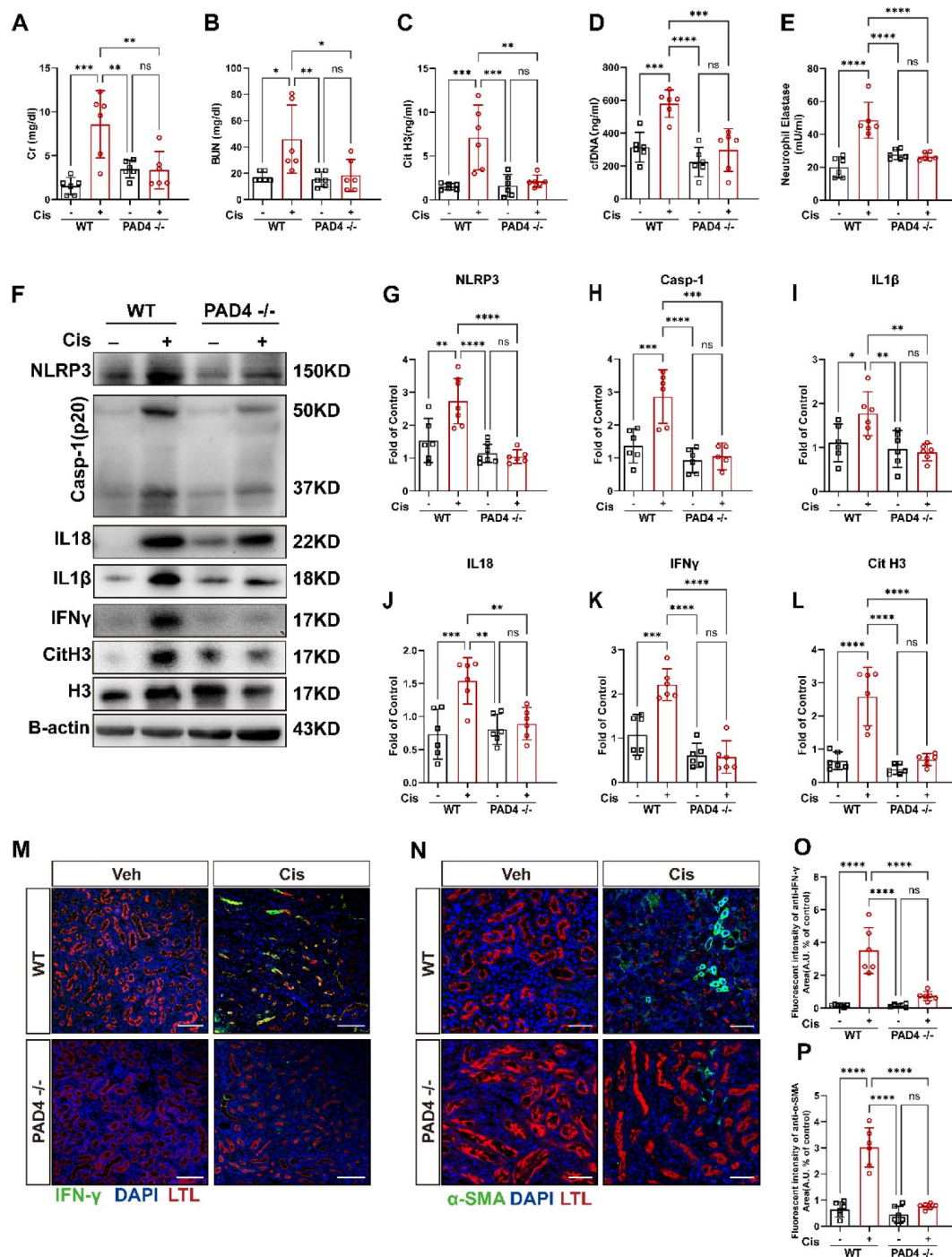


Figure 3.

NETs activate the NLRP3 inflammasome and subsequent renal fibrosis.

A, B. Induction of CKD in WT and Pad4^{-/-} mice treated with 7 mg/kg cisplatin for four weeks. Serum creatinine and blood urea nitrogen ($n = 6$, *** $p < 0.0001$, and ** $p = 0.0049$). **C-E.** The contents of Cit H3, NE, and cfDNA in plasma after intraperitoneal injection of cisplatin were evaluated via the Cit H3 ELISA kit, NETosis assay, and dsDNA ELISA kit ($n = 6$, **** $p < 0.0001$). **F-L.** Western blot analysis of NLRP3, Casp-1, IL18, IL-1β, IFNγ and Cit H3 in kidney tissues. β-actin was used as a loading control ($n = 6$, ** $p = 0.0029$, *** $p = 0.0004$, **** $p < 0.0001$). **M.** Representative images of costaining for IFNγ (green), LTL (red), and DAPI (blue). Scale bar, 100 μm. **N.** Representative images of costaining for α-SMA (green), LTL (red), and DAPI (blue). Scale bar, 50 μm. **O, P.** Quantification of IFNγ-positive and α-SMA-positive areas in the kidney ($n = 6$, **** $p < 0.0001$). Significant differences were revealed via one-way ANOVA vs. vehicle/PAD4^{-/-} (A-E, J-L, O and P).

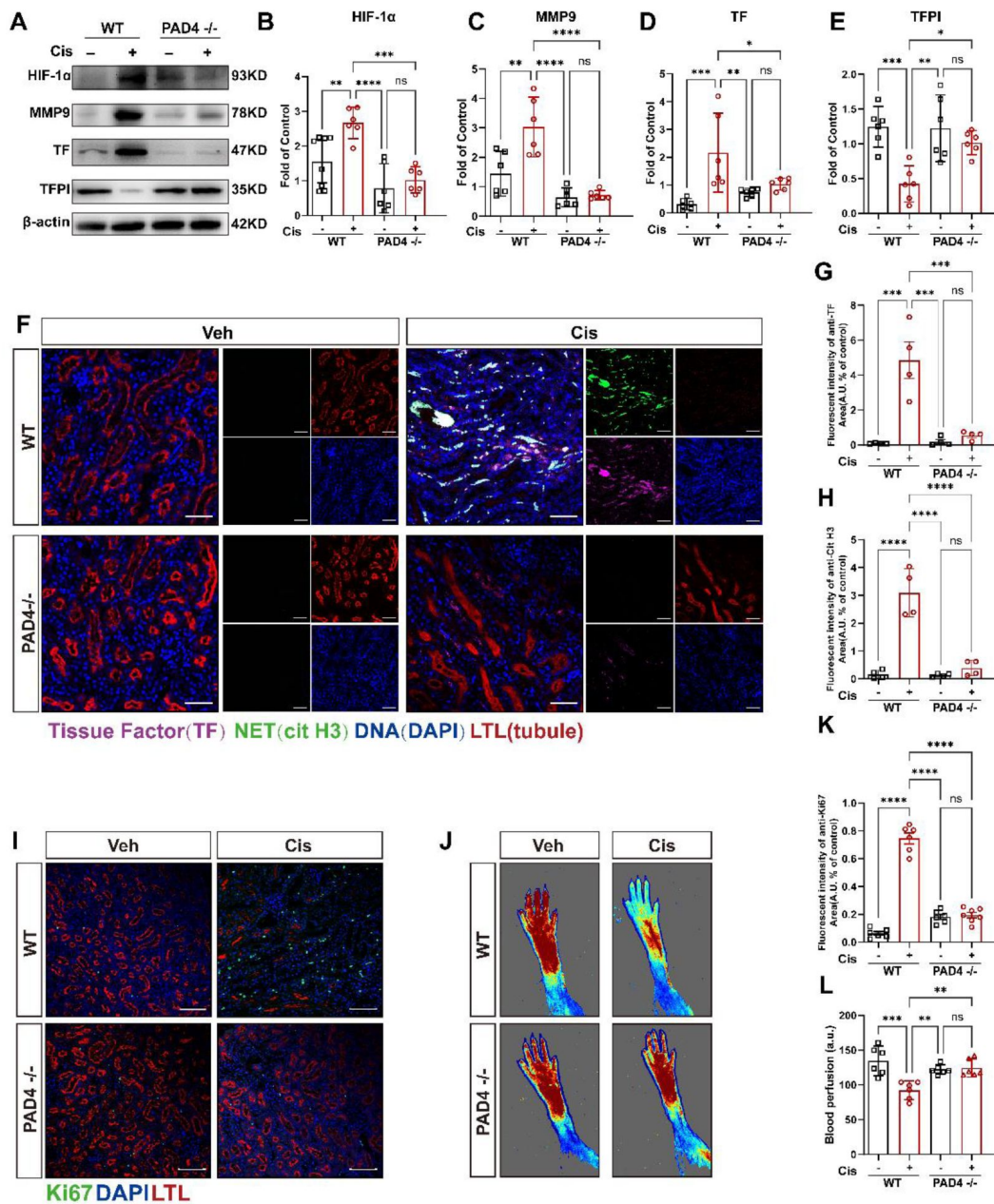


Figure 4.

NETs trigger thrombus formation, leading to local ischemia and hypoxia.

A-E. Western blot for HIF-1α, TF, MMP9 and TFPI in kidney tissues. β-actin was used as a loading control ($n = 6$, $**p = 0.0012$, and $***p = 0.0004$, $****p < 0.0001$). **F.** Confocal immunofluorescence microscopy images of kidney samples from chemotherapeutic nephritic mice ($n = 4$) stained for TF, Cit H3, LTL and DNA. Scale bar, 50 μm. **G, H.** Quantification of Cit H3-positive and TF-positive areas in the kidney ($n = 4$, $****p < 0.0001$). **I.** Representative images of Ki67 immunofluorescence staining and costaining with LTL. Scale bar, 100 μm. **K.** Quantification of the Ki67 area in the kidney ($n = 6$, $****p < 0.0001$). **J, L.** Measurement of lower limb blood flow in WT and PAD4^{-/-} mice via a MoorFLPI2 blood flow scattering hemodynamometer ($n = 6$, $***p = 0.0003$). Significant differences were revealed via one-way ANOVA vs. vehicle/PAD4^{-/-} (B-E, G, H, K and L).

quantitatively analyzed through Claudin-1, ZO-1, and Occludin, which are essential for maintaining tight junction stability and barrier function. The levels of Claudin-1, ZO-1, and Occludin were reduced by RLDC and restored by BBR and OPCs (**Figure 5G-L**, **Figure 5J-M**). Next, we detected plasma LPS levels in the mice. Elevated LPS after RLDC suggested that bacteria entered the bloodstream after intestinal leakage. BBR and OPCs attenuated the level of cisplatin-induced LPS (**Figure 5N**). In addition, IVIS spectrum imaging directly revealed that RLDC stimulated FITC-dextran leakage from the intestine and elevated serum FITC-dextran levels, which were prevented by both OPCs and BBR (**Figure 5K and L**). These results indicate that OPCs might play a role in preventing cisplatin-associated CKD by restoring the intestinal barrier.

OPCs maintained intestinal flora homeostasis

The destruction of the intestinal barrier by cisplatin may be accompanied by disruption of the gut microbiota. We investigated whether cisplatin altered the composition of the gut flora via 16S rDNA gene sequencing. First, there were significant changes in the Chao1 index, Shannon index, phylogenetic diversity (PD) and species of bacteria in the cisplatin group, demonstrating that the species diversity and homogeneity of the intestinal flora were significantly reduced by RLDC, whereas OPCs restored the richness of the intestinal flora (**Figure 6A-D**). Moreover, intergroup analysis by Metastats revealed a significant difference between the control and cisplatin groups, indicating that RLDC led to a clear separation between the biological communities (**Figure 6E**). β diversity analysis was subsequently performed, and unweighted UniFrac distance PCoA revealed that the composition of the intestinal flora was completely different between the control and cisplatin groups (**Figure 6F**). The composition of the OPCs-treated group was intermediate, suggesting the unique ability of OPCs to regulate the intestinal flora, which may be related to the mechanism of OPCs in CKD. To further confirm the specific composition of the intestinal flora in the RLDC state, we analyzed the intestinal flora at the phylum and genus levels in each group. The abundances of Firmicutes and Bacteroidetes were lower in the cisplatin group than in the control group. In contrast, OPCs contained an abundance of Bacteroidetes. The Firmicutes/Bacteroidetes (F/B) ratio is reportedly related to the microbiological state of the gut in chronic colitis (Kieffer et al., 2016). Unfortunately, OPCs did not obviously affect this ratio (**Figure 6G and H**). On the basis of absolute species abundance information, Muribaculaceae, Akkermansia, and Clostridia-UCG-014 declined after RLDC treatment and were significantly recovered by OPCs (**Figure 6L**). The same evolutionary map generated via LEfSe revealed differences among the three groups of taxa (from phylum to genus), with the model significantly different from the control. The dominant group present in the OPCs was c_Clostridia p_Firmicutes (**Figure 6J and K**). We also performed TAX4fun analysis to predict gut microbial function. CIS was enriched in several pathways, including the nervous system metabolism of cofactors and vitamins, whereas it inhibited several functions, including the cellular community, circulatory system, and signaling molecules. OPCs restored, as much as possible, the original function of the gut flora (**Figure 6I**). According to FAPROTAX functional analysis, xylanolysis and sulfate respiration were decreased, and hydrocarbon degradation and aliphatic_non_methane_hydrocarbon_degradation were elevated in the Mod group, whereas these changes were reverted to usual by OPCs (**Figure 6M**). To investigate the effects of OPCs on potential metabolic pathways in the intestinal microbiota, a t test was conducted on the annotated abundance data of secondary pathways within the KEGG metabolic pathway for a specific set of two samples. As shown in **Figure 6N**, the metabolism of the intestinal flora in the model group was markedly aberrant, e.g., reduced carbohydrate metabolism and increased amino acid metabolism. In conclusion, the significant changes mentioned above confirmed the modulatory effect of OPCs on intestinal flora homeostasis, which resisted the intestinal damage induced by RLDC.

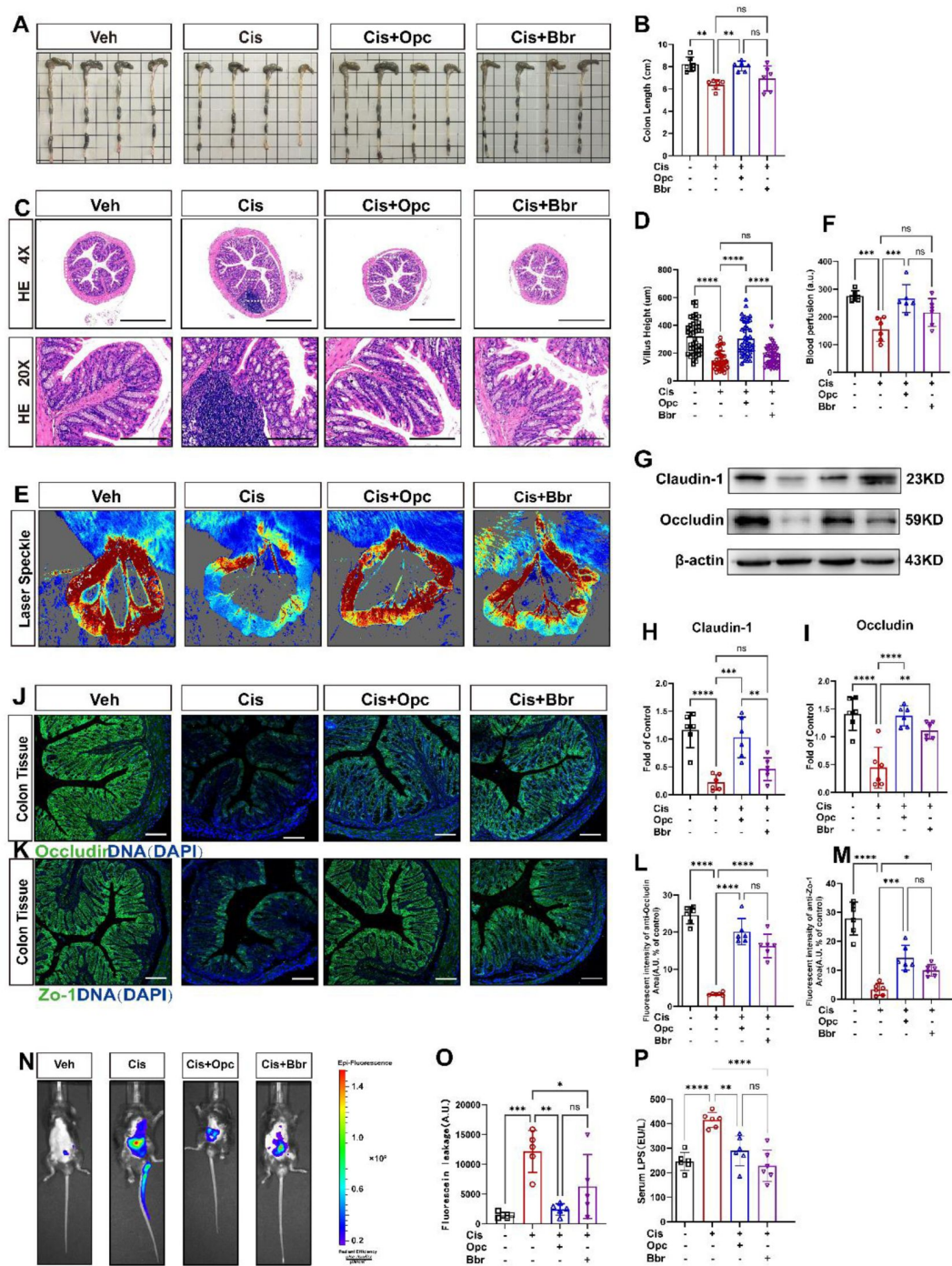


Figure 5.

OPCs retain the integrity of the cisplatin-treated intestinal barrier.

A, B. Macroscopic images and the length of the colon from each group were measured ($n = 6$, $***p = 0.0005$). **C.** HE staining of colon sections (scale bars $50 \mu\text{m}$, $n = 6$). **d.** Colonic villus length (40 villi per group, $****p < 0.0001$). **E, F.** Intestinal blood flow and perfusion indices were measured via a laser speckle blood flow analysis system ($n = 6$). **G-I.** Immunoblot analysis of tight junctions in the colons of cisplatin-treated mice ($n = 6$, $****p < 0.0001$). **J-M.** The expression levels of the tight junction proteins ZO-1 and Occludin were observed via immunofluorescence (scale bars $100 \mu\text{m}$, $n = 6$, $****p < 0.0001$). **N.** LPS levels in the serum of the mice ($n = 6$, $p < 0.0001$). **O.** FITC-dextran distribution in the mice with colitis was observed via small animal imaging. **P.** Content of FITC-dextran in serum ($n = 5$, $***p = 0.0003$). Significant differences were revealed via one-way ANOVA (B, D, F, H, I, K, M, N, and P).

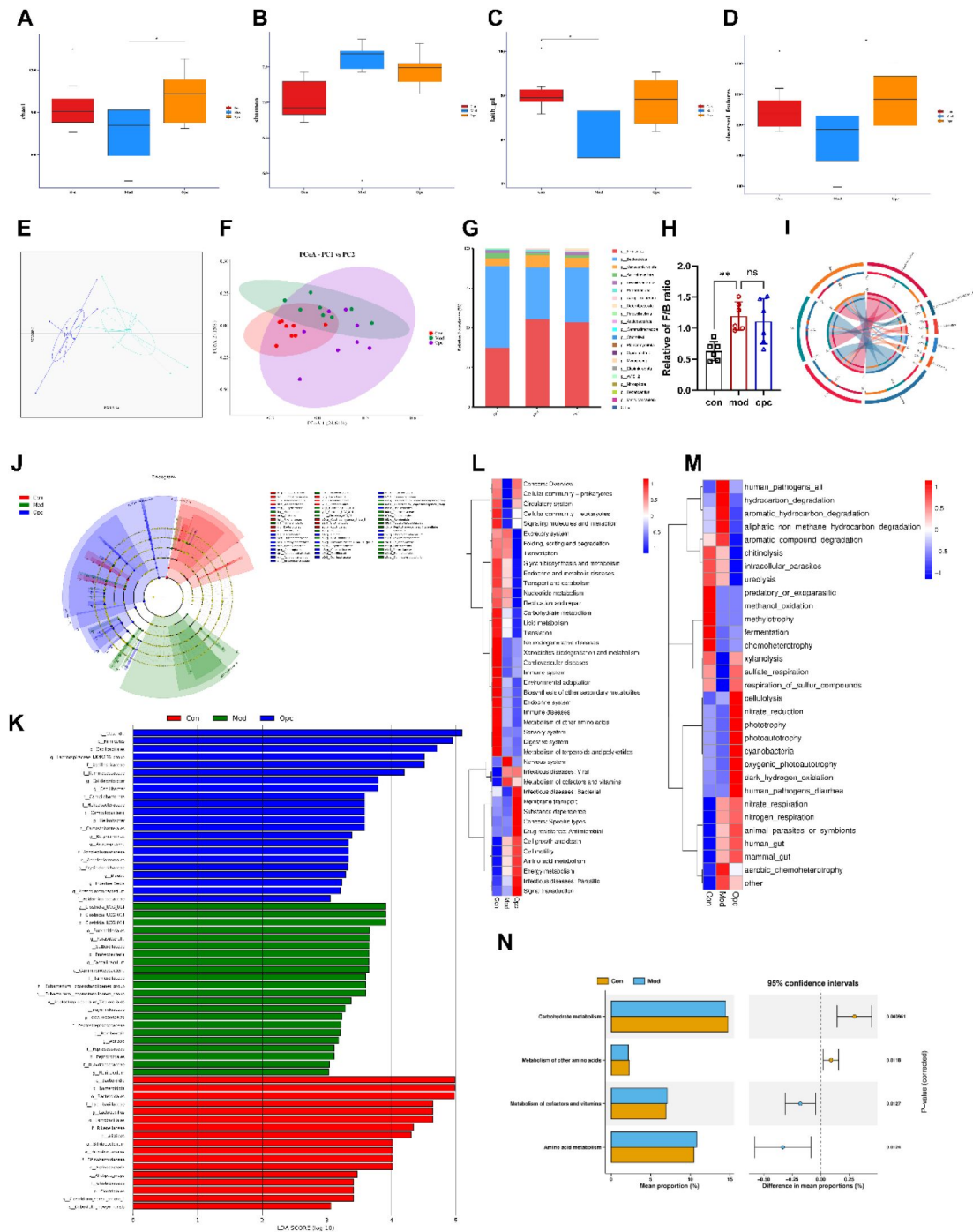


Figure 6.

Analysis of the mouse gut microbiota via 16S rDNA gene sequencing.

A-D. α -Diversity indicated by the Chao index, Shannon index, PD_whole_tree index and observed features (interquartile range, IQR, $n = 8$, $*p < 0.05$). **E.** Principal component analysis (PCA) revealed significant differences at the phylum level. **F.** Effect of OPCs on the β diversity of the gut microbiota assessed via principal coordinate analysis (PCoA). **G, H.** Relative abundance of Bacteroidetes and Firmicutes at the phylum level ($n = 6$, $***p = 0.0002$). **I.** Abundances of the top 5 species at different taxonomic levels and absolute species abundance information on the basis of ASVs within samples. **J.** LDA analysis. **K.** LEfSe analysis. **L, M.** Classification of functions based on Tax4Fun and FAPROTAX analysis. **N.** Analysis of differences in KEGG metabolic pathways. ($n = 8$, $*p < 0.05$).

OPCs prevented RLDC kidney injury by inhibiting the formation of NETs

To investigate whether OPCs inhibited cisplatin-induced renal injury through NETs, kidneys were collected from mice treated concomitantly with OPCs (100 mg/kg) and RLDC. As expected, OPCs significantly decreased the expression of NETs marker Cit-H3 (**Figure 7A and M**); the ischemia- and hypoxia-related factors HIF-1 α , MMP9, and TF (**Figure 7A-D**); and the inflammation- and fibrosis-related factors NLRP3, Casp-1, IL1 β , IL18, and IFN γ (**Figure 7A**, G, and I-K), all of which were increased by RLDC. In addition, the expression of TFPI, which is inhibited by RLDC, was elevated by OPCs. OPCs also inhibited renal injury signals, such as caspase-1, BAX and KIM-1, demonstrating its ability to alleviate ischemia-hypoxia and inflammation-fibrosis (**Figure 7A**–L). The potential of OPCs to mitigate renal impairment was subsequently explored. OPCs reduced the deposition of serum NETs (**Figure 7M-P**). Consistent with this trend, there was a notable decline in the serum creatinine and urea nitrogen levels. (**Figure 7Q and R**). Furthermore, renal tubular atrophy was significantly reduced by OPCs. The collagen area and degree of fibrosis in senescent kidneys were significantly decreased concurrently (**Figure 7S-X**).

RLDC elevated renal KIM-1 expression, which was restored by OPCs. However, LTL expression was not significantly restored by OPCs (**Figure 8M-N**). This may be attributed to the loss of the proximal tubular brush border as a consequence of long-term renal injury (Kishi et al., 2019; Livingston et al., 2023). OPCs also reduced α -SMA deposition (**Figure 8P and Q**), thereby alleviating the susceptibility to fibrosis produced by repeated kidney injury, which is a significant contributing factor in the development of CKD in older individuals (Ferenbach and Bonventre, 2015). Immunofluorescence staining revealed that OPCs inhibited the deposition of NETs (**Figure 8A**, **Figure 8C-E**) and TF-NETs microthrombosis (**Figure 8B**, **Figure 8F-H**) in the kidney induced by RLDC. These findings indicated that OPCs might act to prevent renal injury by inhibiting the production of NETs and subsequent ischemia-hypoxia and inflammatory pathways.

Proanthocyanidins, which are natural plant polyphenols, are widely recognized for their antioxidant effects (Liu et al., 2023). Reactive oxygen species (ROS) are also the primary substances involved in NETs release from neutrophils (Lee et al., 2017; Xi Zhan et al., 2023). Since the effect of OPCs on the kidney was better than that of berberine (**Figure 5**), we speculated that OPCs not only works through the intestine but also has antioxidant effects on neutrophils directly. The results of the in vitro study demonstrated that OPCs suppressed the cisplatin-stimulated increase in ROS in neutrophils (**Figure 8I and J**). The in vivo experiments demonstrated that OPCs restored the activities of SOD and GSH enzymes and alleviated the oxidative stress caused by long-range cisplatin (**Figure 8K and L**). Both in vitro and in vivo experiments demonstrated the direct antioxidant effect of OPCs on neutrophils. To determine whether LPS from the gut was also a factor contributing to the formation of NETs, neutrophils were isolated from the bone marrow and stimulated in vitro. Interestingly, neither LPS nor cisplatin alone caused NETs formation, while coadministration of both resulted in many NETs (**Figure 9A**). OPCs significantly reduced the number of NETs at the same stimulus intensity (**Figure 9B**), further demonstrating the direct inhibitory effect of OPCs on NETs formation.

Discussion

Patients who receive multiple cycles of cisplatin chemotherapy may suffer from CKD (Latcha et al., 2016). However, the underlying mechanisms and corresponding drugs for alleviating CKD are largely unknown. The objective of our experiments was to elucidate the role of NETs in RLDC-induced CKD and search for helpful measures. In this study, we employed PAD4 $^{-/-}$ mice to demonstrate the involvement of NETs in RLDC-induced CKD. Cisplatin continuously reduces

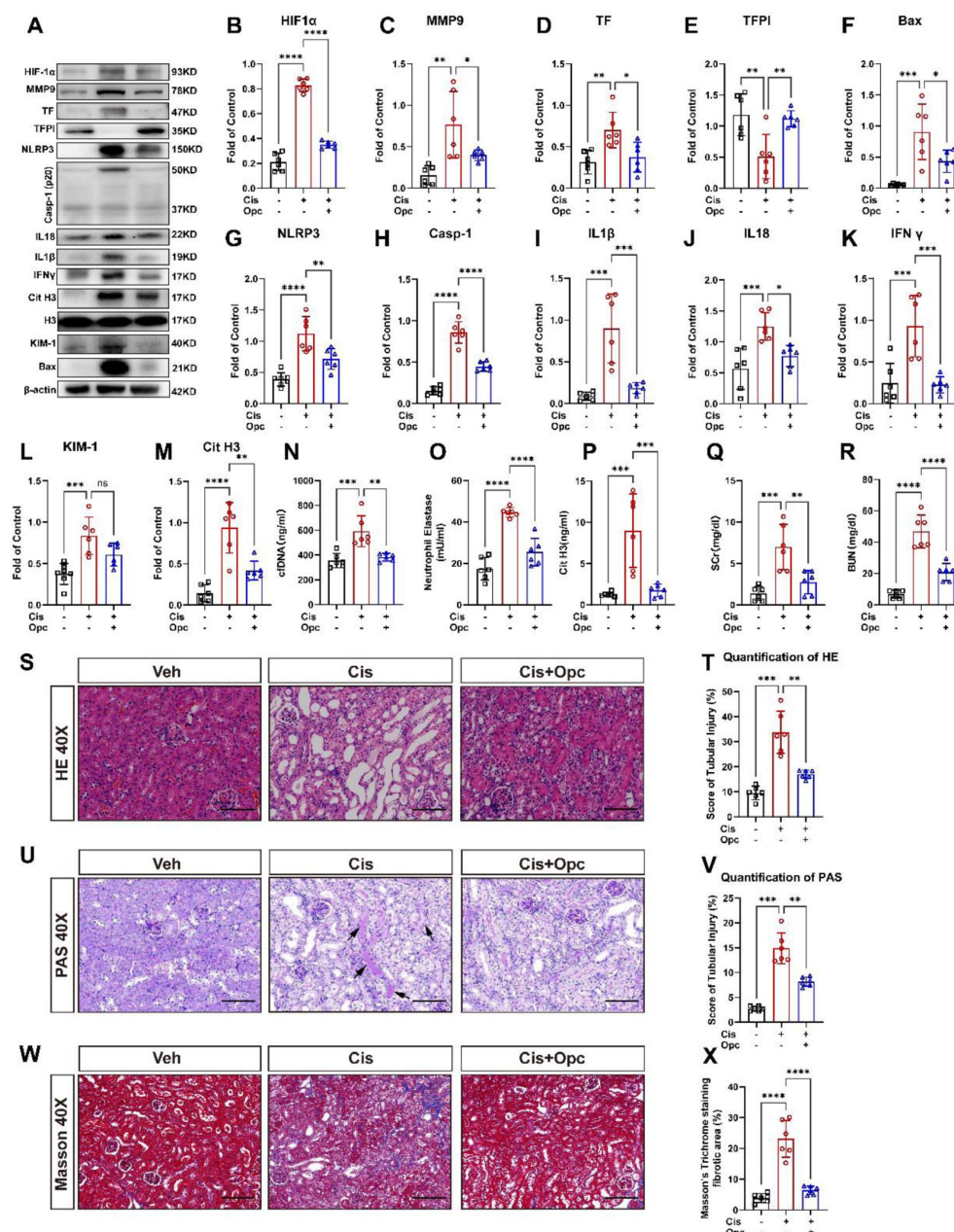


Figure 7.

OPCs reduces kidney damage by inhibiting NETs.

The administration of OPCs (100 mg/kg, i.g./3 d) was conducted in conjunction with the initial intraperitoneal cisplatin injection. RLDC treatment was administered with or without OPCs treatment and was administered one week after the last cisplatin treatment. **A–M.** Immunoblot analysis of HIF-1 α , TF, MMP9, TFPI, BAX, NLRP3, Casp-1, IL18, IL1 β , IFN γ , Cit H3 and KIM-1 in kidney tissues. For quantification, the protein was analyzed through densitometry and then normalized to β -actin ($n = 6$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). **N–P.** The content of Cit H3, NE, and cfDNA in plasma was evaluated via the Cit H3 ELISA kit, NETosis Assay, and dsDNA ELISA kit ($n = 6$, *** $p < 0.001$, **** $p < 0.0001$). **Q, R.** Serum creatinine (SCr) and blood urea nitrogen (BUN) levels ($n = 6$, *** $p = 0.0002$, **** $p < 0.0001$). **S.** Representative HE-stained kidney slices (scale bars = 50 μ m). **T.** Quantification of renal tubular damage in the mice in each group ($n = 6$, **** $p < 0.0001$). **U.** Representative images of PAS-stained kidney slices (scale bars = 50 μ m). **V.** Quantification of renal tubular damage in the mice in each group ($n = 6$, **** $p < 0.0001$). **W.** Masson's trichrome staining of kidney cortex sections (scale bars = 50 μ m). **X.** Quantification of the collagen-positive area according to Masson staining ($n = 6$, **** $p < 0.0001$). Significant differences were revealed via one-way ANOVA vs. Vehicle/Cis+OPCs (B–R, T, V, and X).

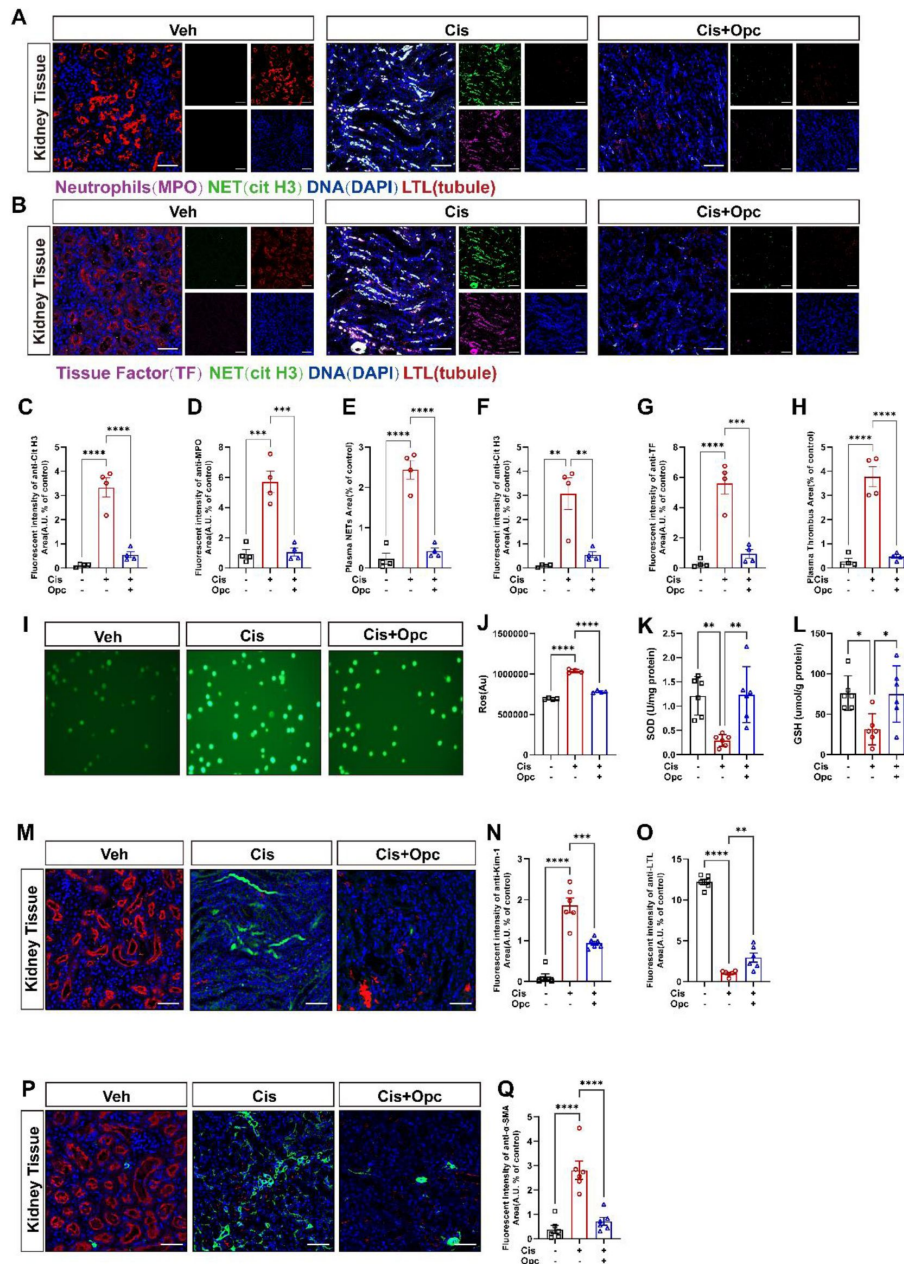


Figure 8.

OPCs inhibit NETs production, with subsequent thromboembolism and inflammatory fibrosis.

A. NETs (confocal immunofluorescence microscopy images; stained for MPO, Cit H3, LTL and DNA) visible in kidney samples from chemotherapeutic nephritic mice (scale bar = 50 μ m). **B.** Thrombus (confocal immunofluorescence microscopy images; stained for TF, Cit H3, LTL and DNA) visible in kidney samples from chemotherapeutic nephritic mice (scale bar = 50 μ m). **C-E.** Quantification of renal Cit H3 positivity, MPO positivity and the colabeled area of the kidney ($n = 4$, **** $p < 0.0001$). **F-H.** Quantification of renal Cit H3 positivity, TF positivity and colabeled area of the kidney ($n = 4$, **** $p < 0.0001$). **I.** ROS analysis by DCFH-DA staining in neutrophils pretreated with OPCs for 1 h followed by cisplatin for 12 h. **J.** Quantification of DCFH-DA staining of neutrophils by luminescence zymography ($n = 4$, **** $p < 0.0001$). **K.** Serum SOD levels ($n = 6$, ** $p = 0.0015$). **L.** Serum GSH levels ($n = 6$, * $p = 0.0142$). **m.** Representative images of KIM-1- and LTL-stained sections of mouse kidneys (scale bar = 50 μ m). **n, o.** KIM-1-positive and LTL-positive areas of the kidney ($n = 6$, **** $p < 0.0001$, ** $p = 0.0077$). **P.** Representative images of costaining for α -SMA (green), LTL (red), and DAPI (blue). Scale bar = 50 μ m. **Q.** Quantification of the α -SMA-positive area of the kidney ($n = 6$, **** $p < 0.0001$). Significant differences were revealed via one-way ANOVA vs. Vehicle/Cis+OPCs (C-H, J-L, N, O, and Q).

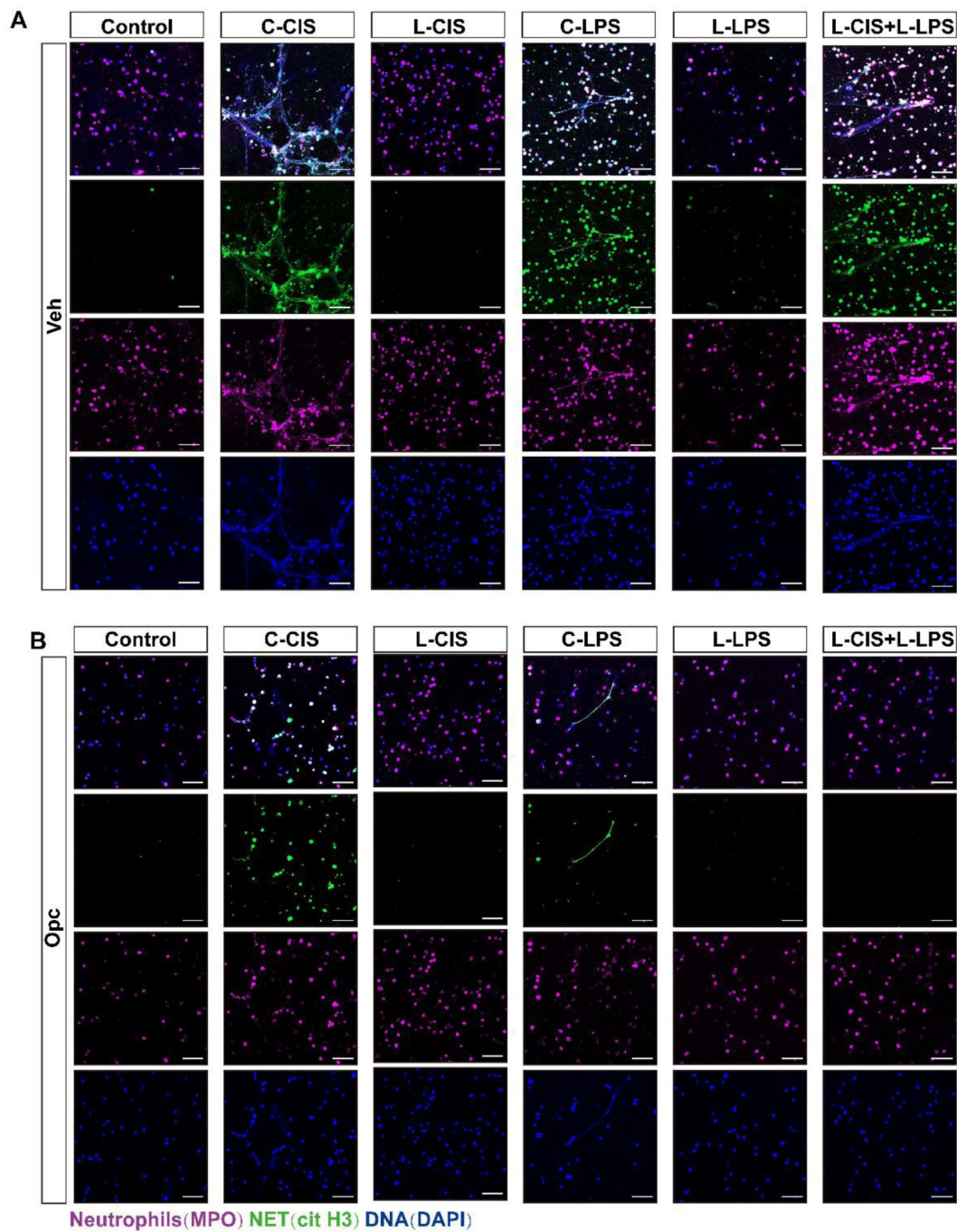


Figure 9.

OPCs inhibits the cisplatin- and LPS-induced release of NETs from neutrophils.

A, B. Immunostaining of mouse neutrophils cultured as indicated. Anti-MPO (purple), anti-Cit H3 (green), and DAPI (blue) staining was used to assess NETs formation. Scale bar = 100 μ m ($n = 3$).

endotoxemia *in vivo* by disrupting the intestinal barrier, leading to the ongoing induction of NETosis by LPS. Cisplatin consistently maintains low endotoxemia *in vivo* by impairing the intestinal barrier, leading to continuous induction of NETosis by LPS. Furthermore, we elucidated the roles of the NETs-TF-MMP9 and NETs-NLRP3-IFN γ pathways in CKD. In the context of cisplatin stimulation, OPCs have the capacity to improve renal dysfunction and tubular injury. This effect was consistent with the observed consequences of NETs inhibition and was accompanied by decreased microthromboembolism and chronic inflammation. Elevated levels of NETs in the kidney result in NLRP3-associated inflammation on the one hand and ischemia and hypoxia on the other, which ultimately lead to apoptosis and interstitial fibrosis in renal parenchymal cells. Thus, we believe that OPCs may act as a protective agent against cisplatin nephropathy by inhibiting NETs-mediated inflammation and ameliorating ischemia and hypoxia (**Figure 10** [↗](#)).

LPS represents a significant component of the outer membrane phospholipids of the majority of gram-negative bacteria and is continuously produced in the gut. It elicits a range of inflammatory immune responses in the host, including the production of proinflammatory cytokines. The integrity of the intestinal barrier represents a fundamental line of defense, preventing the penetration of LPS into the internal environment. Tight junction proteins play essential roles in maintaining the structural integrity of the intestinal barrier. Their functions are carried out through transmembrane proteins such as claudin, Occludin, and tricellulin, as well as intracellular scaffolding proteins, including ZO-1, ZO-2, and ZO-3 ([Ghosh et al., 2021](#) [↗](#); [Mouries et al., 2019](#) [↗](#)). Our small animal image directly revealed that OPCs blocked cisplatin-induced intestinal leakage. These findings, combined with the finding that OPCs restored the serum LPS level and intestinal tight junction proteins, suggest that targeting the intestinal barrier via OPC inhibits LPS leakage. Consistent with previous reports, in some systemic inflammation-related diseases, OPCs can also restore intestinal tight junction proteins, thereby inhibiting increased permeability ([González-Quilen et al., 2019](#) [↗](#); [Xu et al., 2019](#) [↗](#)).

In rodents and humans, alterations in the composition and metabolism of the gut microbiota (ecological dysbiosis) have also been associated with colonic inflammation, characterized by decreases in the overall diversity of the microbiota and the abundance of Bacteroidetes and increases in the relative abundance of Firmicutes. It has been demonstrated that diets rich in fiber increase the abundance of Bacteroidetes, which in turn decreases the F/B ratio. This is accompanied by a reduction in the concentrations of indophenol sulfate and p-cresol sulfate-based uremic toxins, which can improve renal function ([Kieffer et al., 2016](#) [↗](#)). Furthermore, gut microbiota dysbiosis with bacterial translocation increases trimethyl orthoacetate (TMAO) production, which is accompanied by a reduced filtration capacity in the kidney. This leads to the accumulation of these uremic, gut-derived metabolites in the serum, which significantly accelerates the progression of CKD and its concomitant comorbidities. Our flora study demonstrated that the most critical flora, including Muribaculaceae, Akkermansiaceae, Lachnospiraceae_NK4A136, and Glostridia_UCG-014, exhibited a decline in response to RLDC, after which the OPCs subsequently recovered. These findings demonstrated that OPCs could increase the abundance of Bacteroidetes. Furthermore, the increase in Muribaculaceae and Clostridia-UCG-014 has been shown to limit LPS levels in the bloodstream and ameliorate metabolic endotoxemia ([Li et al., 2016](#) [↗](#)), which is attributed to decreased intestinal mucus secretion, which suppresses intestinal mucus barrier function ([Wang et al., 2023](#) [↗](#)). The Lachnospiraceae NK4A136 group also demonstrated a significant inverse correlation with intestinal permeability and plasma LPS levels and a positive correlation with colon length and Cldn1 expression. Thus, it is reasonable to believe that OPCs upregulation of the above flora is required for intestinal integrity to inhibit LPS leakage.

On the basis of the functional analyses, the effect of OPCs on amino acid metabolism piqued our interest. As the majority of uremic toxins originate from intestinal amino acids that can be metabolized by the colonic microbiota, we surmised that OPCs decreased uremic toxins through the colonic microbiota, which mediate amino acid metabolism. Amino acid metabolism by the gut

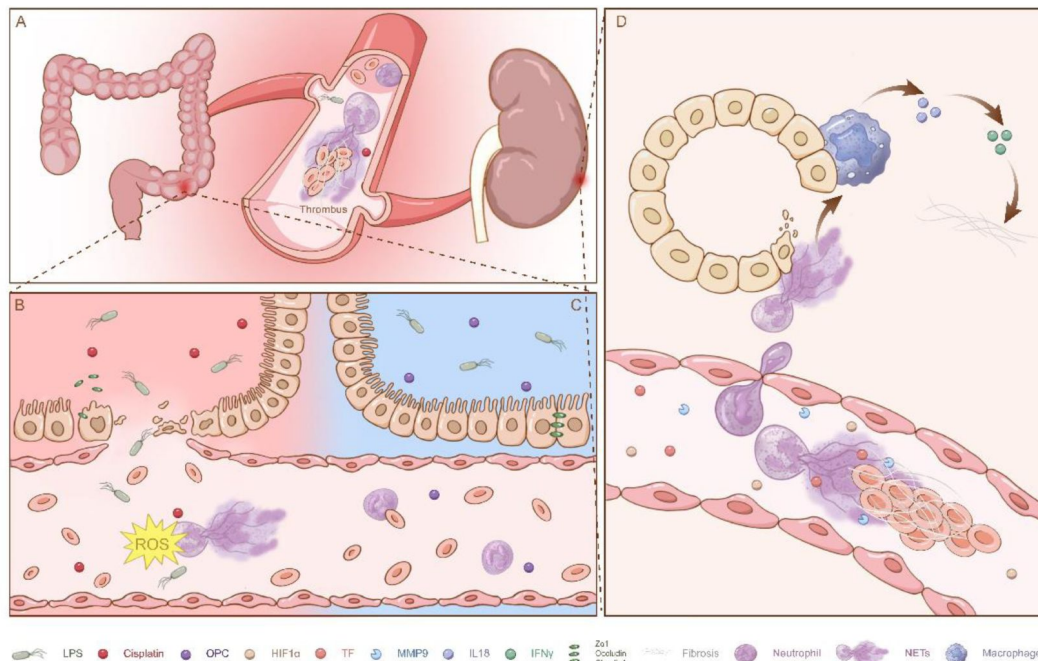


Figure 10.

Schematic illustration showing that RLDC-induced NETs promote the development of CKD by disrupting the gut barrier and the therapeutic role of OPCs.

A. The combination of cisplatin and intestine-derived LPS induces NETs formation, leading to CKD via the gut–kidney axis. **B.** Cisplatin-induced gut barrier dysfunction facilitates NETosis caused by both LPS and cisplatin, which disturb microcirculation. **C.** The inhibition of NETosis by OPCs is attributed to its anti-inflammatory and antioxidant activities and ability to maintain a balanced intestinal flora. **D.** NETs induce local ischemia and fibrosis, which are involved in the pathogenesis of kidney damage.

flora and CKD can interact and be promoted mutually. The intestinal amino acid metabolic profile is also disrupted as CKD progresses (Y. Liu et al., 2018 [↗](#)). Along with CKD, the gut bacterial metabolites of trimethylamine oxide change into trimethylamine, which predicts poorer long-term survival. In animal models, chronic dietary exposure to trimethylamine oxide directly leads to progressive renal fibrosis and dysfunction (Tang et al., 2015 [↗](#)). Similarly, uremic toxins from CKD patients increase intestinal permeability, resulting in marked deficiencies in crucial protein components of epithelial tight junctions (Claudin-1, Occludin, and ZO-1). This may subsequently lead to bacterial and endotoxin translocation across the intestinal wall (Vaziri et al., 2013 [↗](#), 1985 [↗](#)). These findings further demonstrate that the gut and kidney act interdependently, amplifying damage to the organism through the gut–kidney axis. The kidney is one of the organs susceptible to dysregulation of intestinal microbial homeostasis. Therefore, intestinal-derived factors contribute to the pathological process of AKI and CKD (Ramezani and Raj, 2014 [↗](#)), revealing the mechanism of OPCs in CKD. In our studies, OPCs protected the kidneys by restoring the physical and ecological structure of the gut, thereby disrupting the positive feedback pathway of the gut–kidney axis.

Notably, in our study, we used BBR as a positive control drug to kill intestinal bacteria and found that BBR reduced intestinal inflammation and alleviated CKD, but this effect was not as effective as that of OPCs. These findings suggested that BBR inhibited the growth of all bacteria (data not shown), whereas OPCs adjusted the gut microbiota. Another reason could be the direct effect of OPCs being absorbed into the circulation. It has been reported that PAs can reduce cisplatin-induced oxidative stress and inflammatory infiltration, alleviating acute nephrotoxicity (Sayed, 2009 [↗](#)). The results of our study indicated that OPCs depleted antioxidant enzymes and prevented renal collagen deposition and fibrosis formation over time (**Figure 5** [↗](#)). Additionally, cellular experiments demonstrated that both cisplatin and LPS were capable of stimulating the NETs formation. L-LPS plus L-CIS imitates the *in vivo* microenvironment under the dual effects of hypoendotoxemia. Surprisingly, both L-LPS plus L-CIS could activate the production of NETs, whereas neither L-LPS nor L-CIS alone could unequivocally induce NETs formation (**Figure 9** [↗](#)). This finding prompted us to associate L-LPS-accumulated reactive oxygen species with increased NETs formation. The activation of reactive oxygen species plays a key role in NETosis, which can be reduced by inhibiting ROS activation (Amara et al., 2021 [↗](#)). Consistent with this viewpoint, our *in vitro* data revealed that OPCs decreased the levels of neutrophil ROS and NETs, demonstrating that OPCs directly inhibited NETosis through an antioxidative effect. Collectively, our findings may reveal an important mechanism underlying cisplatin-induced CKD that can be alleviated by OPCs.

With respect to the roles of NETs in cisplatin-treated mice with AKI, it has been reported that targeting NETs can improve renal function (Mousset et al., 2023 [↗](#)), which is consistent with the recently discovered effects of NETs in ischemia (Raup-Konsavage et al., 2018 [↗](#)) and sepsis (Ni et al., 2021 [↗](#)). However, the presence of NETs in a mouse model of CKD caused by RLDC and the related mechanisms in kidney injury remain obscure. Cisplatin is primarily cleared by the kidneys through glomerular filtration and tubular excretion, resulting in higher concentrations in the kidneys than in other organs; thus, renal accumulation is associated with a high incidence of acute and chronic nephrotoxicity. In general, the high affinity of high-dose cisplatin for DNA results in the direct induction of necrosis and endoplasmic reticulum stress-related apoptotic pathways, leading to cell death (Wei et al., 2007 [↗](#)). In contrast to the nephrotoxic effects of high-dose cisplatin, low-dose cisplatin, which leads to accumulation of the drug in regeneratively repaired renal tubular epithelial cells, does not result in severe toxicity. However, low-dose cisplatin maintains a proinflammatory state, which ultimately leads to chronic, irreparable nephropathy at the next cycle of cisplatin treatment (Yamashita et al., 2021 [↗](#)). In this study, we found that NETs were indispensable in this chronic vicious cycle. We believe that we have provided the first evidence that RLDC induces CKD, which is positively correlated with the elevation of NETs. Furthermore, the results verify that NETs are crucial targets for RLDC-induced CKD in PAD4^{-/-} mice (**Figure 2** [↗](#)).

Neutrophils are sentinels in response to the acute phase of inflammation, and they rapidly subside over 24 hours. In the case of RLDC-induced chronic inflammation, many NETs are deposited in the kidneys through the circulation. This can be harmful, as it intensifies systemic or local inflammation, leading to tissue damage and thrombosis, which in turn encourages increased neutrophil recruitment and aggregation. In our study, the inhibition of NETs by OPCs resulted in the suppression of chronic inflammation and fibrosis, which was characterized by the downregulation of NLRP3 inflammatory vesicles, IL-1 β and IL-18, and subsequently IFN γ . OPCs also inhibited TF and MMP9 enrichment on NETs scaffolds at the source, thereby alleviating microcirculatory embolism and regenerative disorders (**Figure 7**, **Figure 8**). Similar to our findings, recent evidence has indicated that the consumption of OPCs is inversely correlated with inflammatory activity and oxidative stress (Wang et al., 2013; Zhou et al., 2018). Experimental animal studies have demonstrated that grape seed proanthocyanidin extract can enhance osteoblast activity in experimental periodontitis diabetic rats by reducing MMP-8 and HIF-1 α levels, thereby reducing periodontal inflammation and alveolar bone loss (Toker et al., 2018). Additionally, procyanidins relieve neuropathic pain by inhibiting ROS-mediated activation of MMP2/MMP9 (Pan et al., 2018). We postulate that neutrophil-originated NETs modulate inflammation through NLRP3-associated factors, highlighting a critical target in RLDC-induced CKD.

NETs interact with NLRP3 in cardiovascular diseases such as atherosclerosis, thereby promoting disease progression (Westertorp et al., 2018; Yalcinkaya et al., 2023). NETs also drive the activation of the NLRP3 inflammasome, which in turn leads to sterile inflammation. This phenomenon is also an essential contributor to kidney injury (Gupta et al., 2022). Notably, NLRP3 inhibition has no effect on cisplatin-induced AKI (Kim et al., 2013) but appears to play a role in cisplatin-induced CKD (Li et al., 2019). Previous research in our lab revealed that NETs act as upstream activators of the NLRP3 inflammasome and are released from macrophages via the TLR7 and TLR9 receptors. (Lin et al., 2022). Consistent with this view, we found that PAD4 knockout inhibited NLRP3 expression, accompanied by decreases in CASP1, IL18, and IFN γ expression, in the RLDC model, demonstrating that the NETs-NLRP3-CASP1-IL18-IFN γ pathway plays a significant role in the progression of cisplatin-induced renal fibrosis during the development of CKD (**Figure 3**). CASP1 and IL18 are involved in the process of membrane damage in hypoxic proximal tubules (Edelstein et al., 2007). Additionally, IL18 can promote mesenchymal transition, which in turn induces fibrosis in obstructive renal injury (Bani-Hani et al., 2009). Previous studies conducted in our laboratory have demonstrated that OPCs might inhibit NLRP3 inflammasome activation by scavenging ROS. This process significantly reduces CASP1 and IL-1 β levels, thereby relieving gout pain (Liu et al., 2017). We found that OPCs inhibited the cisplatin-induced expression of NETs, ROS, NLRP3, CASP1, IL18 and IFN γ . Our study revealed that the NETs-NLRP3-CASP1-IL18-IFN γ pathway was involved in RLDC-induced CKD, which was blocked by OPCs through the scavenging of ROS.

NETs provide a scaffold for the exposure of functional tissue factors in thrombus formation, providing vital support for cardiovascular events (Stakos et al., 2015; von Brühl et al., 2012) and accounting for the frequency of thromboembolic events in cancer patients (Thälén et al., 2019). Concurrently, neutrophils secrete the protease MMP9, which contributes to local ischemia-hypoxia (Carmona-Rivera et al., 2015). In a manner analogous to its function in renal development, MMP-9 inhibits apoptosis in the AKI model, which is renoprotective (Bengatta et al., 2009). In contrast to fibrin-degrading activity during AKI, MMP9 plays a negative role during the period from AKI to CKD progression and promotes EMT with fibrosis in a CKD model (Wang et al., 2019). Thus, MMP9 plays an important role in the late progression of fibrotic injury. Our previous data demonstrated that neutrophil infiltration was accompanied by elevated TF and MMP9 in a mouse model of chemotherapeutic enteritis and endotoxin (Li et al., 2021; Yu et al., 2020). In our study, we found that the levels of NETs were elevated, accompanied by elevated TF

and MMP9 and diminished TFPI levels during RLDC-induced CKD (**Figure 4**). Furthermore, OPCs reversed the effect of RLDC on CKD, revealing that OPCs inhibited the formation of microthrombi by inhibiting NETs formation.

In the kidney, proximal tubular cells are the most susceptible to hypoxic stimuli, and the extent of tubular injury is a crucial determinant of the prognosis of renal disease. Hypoxia-promoted coagulation and thrombotic events play important roles in the pathogenesis of CKD (Oe and Takahashi, 2022). Indeed, NETs can form large aggregates that may even be large enough to block small blood vessels without coagulation activation (Jiménez-Alcázar et al., 2017), further exacerbating the susceptibility of the kidney to hypoxia. The high oxygen consumption of the renal tubules, coupled with the relatively low blood circulation in the proximal tubules of the kidney, exacerbates renal injury further. During the period of renal repair, an increase in aerobic glycolytic oxygen consumption leads to relative hypoxia during the period of renal repair, causing a vicious cycle involved in renal injury (B.-C. Liu et al., 2018). Therefore, our study revealed that the increase in the renal hypoxia-inducible factor HIF1 α was proportional to the increase in the proliferative marker protein Ki67 after RLDC induction, which suggested that hypoxia and repair formed a malignant cyclic process in CKD, whereas the absence of NETs-associated pathological emboli after PAD4 knockout impeded the malignant positive feedback process at the source. Furthermore, in the process of CKD, interstitial fibrosis impairs oxygen diffusion efficiency because of the long distance between capillaries and tubular cells. The accumulation of the extracellular matrix increases the diffusion distance between functional vessels and renal units, exacerbating hypoxia. The presence of interstitial fibrosis impedes the diffusion of oxygen between the peritubular capillaries and the renal parenchyma. This, in turn, results in a state of hypoxia within the renal tissue, accelerating the process of fibrogenesis. This creates a vicious cycle that promotes the progression from AKI to CKD. In conclusion, the advantage of NETs is that they facilitate bidirectional communication between the immune response and coagulation, thereby enhancing the efficacy of the two major host protection systems, hemostasis, and innate immunity. Conversely, chronic inflammation-induced fibrosis and chronic hypoxia lead to poor regeneration, forming a vicious cycle that facilitates the progression from AKI to CKD.

In conclusion, prolonged chemotherapy leads to impaired intestinal barrier function, allowing LPS to enter the circulation and induce NETs, which are eventually deposited in the kidney, leading to inflammatory fibrosis via the NLRP3-CASP1-IL18-IFN γ pathway and necrosis due to ischemia and hypoxia via TF-MMP9, which promotes the progression of CKD in the kidney. In contrast, OPCs exert anti-inflammatory, antibacterial, and antioxidant effects, thereby preventing the progression of CKD.

Materials and methods

Key resources table

Antibody	Anti-BAX[N1N2]	GeneTex	GTX109683, RRID: AB_1949720	WB(1:2000)
Antibody	Anti-NLRP3	AdipoGen	AG-20B-0014-C100, RRID: AB_2490202	WB(1:1000)
Antibody	Anti-Caspase-1 (p20)	AdipoGen	AG-20B-0042-C100, RRID: AB_2490249	WB(1:1000)
Antibody	Anti-IL1 β	Abclonal	A16288, RRID: AB_2769945	WB(1:1000)
Antibody	Anti-IL18	Proteintech	10663-1-AP, RRID: AB_2123636	WB(1:2000)
Antibody	Anti-Interferon- γ Rabbit pAb	Abclonal	A12450, RRID: AB_2759294	WB(1:2000)
Antibody	Anti-Occludin	Proteintech	27260-1-AP, RRID: AB_2880820	WB(1:5000)
Antibody	Anti-Claudin-1	Proteintech	13050-1-AP, RRID: AB_2223021	WB(1:1000)
Antibody	HRP-conjugated AffiniPure goat anti-rabbit IgG	Sigma Aldrich	A6154, RRID: AB_258284	WB(1:5000)
Antibody	HRP-conjugated AffiniPure goat anti-mouse IgG	Sigma Aldrich	A4416, RRID: AB_258167	WB(1:5000)
Antibody	DAPI	Biosharp	BL739A	
Antibody	Anti-Myeloperoxidase Mouse mAb	Abmart	T62224	IF(1:1000)
Antibody	Anti-Tissue Factor	Santa	H-9:sc-374441	IF(1:200)
Antibody	Anti-Kidney Injury Molecule 1	Thermo Fisher Scientific	MA5-28211, RRID: AB_2745182	IF(1:200)
Antibody	Anti-Interferon- γ Rabbit pAb	Abclonal	A12450, RRID: AB_2759294	IF(1:200)
Antibody	Anti-Ki67	Proteintech	27309-1-AP, RRID: AB_2730911	IF(1:200)

AB_2756525				
Antibody	Anti- α smooth muscle Actin	Abcam	ab5694, RRID: AB_2223021	IF(1:1000)
Antibody	Anti-Zonula Occludens-1	Proteintech	21773-1-AP, RRID: AB_10733242	IF(1:100)
Antibody	Anti-Occludin	Proteintech	27260-1-AP, RRID:AB_2880820	IF(1:500)
Antibody	Anti-Lotus Tetragonolobus Lectin	Vector Labs	FL-1321, RRID: AB_2336560	IF(1:400)
Antibody	Alexa Fluor 647–conjugated donkey anti-mouse	Thermo Fisher Scientific	A-31571, RRID: AB_162542	IF(1:500)
Antibody	Alexa Fluor 488-conjugated donkey anti-rabbit	Thermo Fisher Scientific	A-21206, RRID: AB_2535792	IF(1:500)
Antibody	Alexa Fluor 488-conjugated donkey anti-mouse	Proteintech	SA00013-1, RRID: AB_2810983	IF(1:200)
Antibody	Rhodamine Red-X Streptavidin	Jackson ImmunoResearch	016--290—084, RRID: AB_2337247	IF(1:200)
Commercial assay or kit	Citrullinated Histone H3 ELISA Kit	Cayman	501620	
Commercial assay or kit	NETosis Assay Kit	Cayman	601010	
Commercial assay or kit	dsDNA ELISA Kit	JSBOSEN	BS-E8939M1	
Commercial assay or kit	SCr	NJJC Bioengineering Institute	C011-2-1	
Commercial assay or kit	BUN	NJJC Bioengineering Institute	C013-2-1	

Commercial assay or kit	LPS	JSBOSSSEN	BS-E9334M1
Commercial assay or kit	DCFH-DA	Beyotime	S0033S
Commercial assay or kit	SOD	Biosharp	BL903A
Commercial assay or kit	GSH	NJJC Bioengineering Institute	A006-2-1
Chemical compound, drug	Cisplatin	Qilu Pharmaceutical Co. Ltd.	CAS#H37021362
Chemical compound, drug	Proanthocyanidins	Tianrun Pharmaceutical Co. Ltd.	CAS#20315-25-7
Chemical compound, drug	Berberine	Tianrun Pharmaceutical Co. Ltd.	CAS#633-65-8
Chemical compound, drug	LPS	Sigma–Aldrich	L2630

Mouse model of RLDC and OPCs treatment

Adult male C57BL/6J mice (20–25 g, wild type) were obtained from the Animal Core Facility of Nanjing Medical University, Nanjing, China. Pad4^{-/-} mice on a C57BL/6J background were purchased from The Jackson Laboratory. The animals were housed five to six per cage under pathogen-free conditions with soft bedding at a controlled temperature (22°C±2°C) and photoperiod (12:12-hour light–dark cycle). Prior to the commencement of the experiment, the animals were permitted to acclimatize to the experimental conditions for a minimum of two days. The animals were matched for age and weight in each set of experiments. Eight-week-old male C57BL/6J background mice and PAD4^{-/-} mice were intraperitoneally injected with 7 mg/kg cisplatin (CIS, H37021362, Qilu Pharmaceutical Co. Ltd.) or 0.2 ml of saline via the intraperitoneal route on a weekly basis for a period of four weeks (Sears and Siskind, 2021 [↗](#)).

To assess the therapeutic effects of natural medicines, mice were administered OPCs. OPCs (100 mg/kg; Tianrun Pharmaceutical Co. Ltd.) was dissolved in 0.5% carboxymethyl cellulose sodium (A501427-0250; Sangon Biotech) and was given by gavage three times a week for four weeks, commencing with the initial cisplatin injection. Berberine (100 mg/kg; Tianrun Pharmaceutical Co. Ltd.) was administered via gastric lavage three times a week for four weeks, starting with the initial cisplatin injection as a positive control for treating the gut microbiota. The dosages of OPCs and BBR used in the experiments were previously described and were based on the results of preliminary experiments (Allameh et al., 2020 [↗](#); Gao et al., 2023 [↗](#); Gholampour et al., 2022 [↗](#)).

The purity of the OPCs was greater than 95%. The OPCs contained 1.1% monomeric procyanidins, 34.2% dimeric procyanidins, 24.9% trimeric procyanidins, 6.7% tetrameric procyanidins (66.9% oligomeric procyanidins in total), and 33.1% polymeric procyanidins.

Histological staining

Kidney tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μm . Renal tissues were subjected to hematoxylin and eosin (HE) staining. The extent of tubular damage was quantified at 40 $\times$ magnification, with a total of 200 cortical tubules examined and calculated in accordance with previously described methods (Li et al., 2018 [DOI](#); Tan et al., 2023 [DOI](#)). For periodic acid-Schiff (PAS) staining, the lumen of the tube containing glycogen components was stained red–purple. Masson's trichrome staining was employed to assess the presence and distribution of collagen fibrils within renal tissues. For quantification, 6 positive collagen-stained fields (40 $\times$ magnification) were randomly selected from each section and analyzed via ImageJ (Fu et al., 2019 [DOI](#)).

Colon Tissue Processing

The distal colon was removed and washed with a physiological saline solution. Segments of approximately 0.5 cm in length were then cut. A proportion of the colon samples were fixed in 4% paraformaldehyde solution, prepared in 0.1 M phosphate-buffered saline (PBS) with a pH of 7.4, and then embedded in paraffin wax for morphological analysis. A proportion of the colon samples were fixed in 4% paraformaldehyde solution, prepared in 0.1 M PBS at a pH of 7.4, and then embedded in paraffin wax for morphological analysis. A minimum of 40 villus lengths were quantified on selected HE-stained sections via ImageJ software (Xinlu Zhan et al., 2023).

Cells and treatment

After the mice were euthanized, the tibias and femurs were isolated via aseptic techniques. The ends of the bones were then cut after the muscle tissue was removed to expose the marrow cavity. A 5 ml syringe was filled with RPMI-1640 (KGM31800N-500, Keygen Biotech), 10% FBS (04-001-1ACS, Biological Industries), and 2 mmol/L EDTA (E9884, Sigma–Aldrich) to flush the bone marrow cells into a 50 ml centrifuge tube. After centrifugation at 1200 $\times$ g for 5 min at 4°C, the cells were resuspended in 3 ml of sodium chloride, and then the supernatant was added to the upper layer of 9 ml of Histopaque-1077 (density, 1.077 $\times$ g/mL, 10771, Sigma–Aldrich) and centrifuged at 2000 $\times$ g for 20 min at 4°C without braking. The supernatant was discarded, and the cells were resuspended in 5 ml of sodium chloride physiological solution. Neutrophils were collected at the junction of tissue water and sodium chloride physiological solution after the cells were placed in the upper layer of 10 ml of Histopaque-1119 (density, 1.119 g/mL, 11191, Sigma–Aldrich) and centrifuged for 20 min at 4°C and 2000 $\times$ g without braking. The collected neutrophils were washed twice with RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin (KGY0023, Keygen Biotech) and centrifuged at 1400 rpm for 7 min at 4°C.

To assess the promotion of NETs formation by LPS versus cisplatin and the inhibition of NETs formation by OPCs, neutrophils were incubated with C-LPS (1 mg/ml, L2630, Sigma–Aldrich), C-CIS (3 $\mu\text{g}/\text{ml}$, HY-17394, MedChemExpress) or L-LPS (10 ng/ml, L2630, Sigma–Aldrich), or L-CIS (0.15 $\mu\text{g}/\text{ml}$, HY-17394, MedChemExpress) for 4 hours, and OPCs (1 $\mu\text{g}/\text{ml}$, Zelang Pharmaceutical Co. Ltd.) or RPMI1640 was added to the medium 1 hour before LPS or cisplatin.

Immunofluorescence

After administering anesthetic agents to the mice, the kidneys and intestinal tissues were excised via a transcardiac infusion of saline, fixed in 4% paraformaldehyde, and the embedded blocks were sectioned into 4 μm thick pieces. These samples were then blocked for one hour with 1% normal donkey serum (017–000–121, Jackson ImmunoResearch, RRID: AB_2337258) and 0.1% Triton-X in phosphate buffer solution (PBS). For immunofluorescence analysis, tissue sections

were subjected to incubation with primary antibodies against Cit H3 (ab281584, Abcam), MPO (T62224, Abmart), TF (H-9:sc-374441, Santa), KIM-1 (MA5-28211, Thermo Fisher Scientific, RRID: AB_2745182), Ki67 (27309-1-AP, Proteintech, RRID: AB_2756525), IFN- γ (A12450, ABclonal, RRID: AB_2759294), α -SMA (ab5694, Abcam, RRID: AB_2223021), ZO-1 (21773-1-AP, Proteintech, RRID: AB_10733242) and Occludin (27260-1-AP, Proteintech, RRID: AB_2880820) overnight at 4°C. The proximal tubules were labeled with LTL (FL-1321, Vector Labs, RRID: AB_2336560), and the nuclei were counterstained with DAPI. The secondary antibodies used were as follows: Alexa Fluor 647-conjugated donkey anti-mouse (A-31571, Thermo Fisher Scientific, RRID: AB_162542), Alexa Fluor 488-conjugated donkey anti-rabbit (A-21206, Thermo Fisher Scientific, RRID: AB_2535792), Alexa Fluor 488-conjugated donkey anti-mouse (SA00013-1, Proteintech, RRID: AB_2810983), and Rhodamine Red-X Streptavidin (016--290--084, Jackson ImmunoResearch, RRID: AB_2337247). Following three washes with PBS, the samples were examined under a fluorescence microscope (Leica DM2500) to ascertain the morphological details of the immunofluorescence staining. The examination was conducted in a blinded manner.

In vitro NETs assay

For immunofluorescence staining, freshly isolated polymorphonuclear neutrophils (PMNs) were seeded on poly-D-lysine-coated coverslips, and their adherence was permitted. After NETs production was induced, the cells were fixed for 15 minutes with 4% paraformaldehyde (PFA) and blocked with 1% BSA and 0.3% Triton X-100 in PBS for 30 minutes. Then, anti-Cit H3 and anti-MPO (ab90810, Abcam) primary antibodies were used overnight at 4°C. After three washes, Alexa Fluor 488-conjugated donkey anti-rabbit (A-21207, Invitrogen), Alexa Fluor 647-conjugated donkey anti-mouse (A-31571, Invitrogen), and DAPI (BL739A, Biosharp) were added for 2 hours at room temperature. NETs formation was visualized via fluorescence microscopy (Leica DM2500).

Quantification of NETs

Mouse plasma was collected from whole blood by centrifugation at 3,000 rpm for 5 minutes. The quantification of NETs in plasma was conducted in accordance with the Citrullinated Histone H3 ELISA Kit (501620, Cayman), NETosis Assay Kit (601010, Cayman), and dsDNA ELISA Kit (BS-E8939M1, JSBOSEN). The absorbance was quantified with a microplate reader (Multiskan FC, Thermo Fisher).

Renal function

In summary, blood samples were taken from the infraorbital region for anticoagulation and then centrifuged at room temperature to collect the serum. For Scr (C011-2-1, NJJC Bioengineering Institute), the reaction was conducted at 37°C for 5 min, and the absorbance at 546 nm was recorded at the end of the response. For BUN (C013-2-1, NJJC Bioengineering Institute), the sample was added to the preheated reaction mixture at 37°C, and the absorbance at 640 nm was monitored after 10 min of reaction. BUN and Scr levels (mg/dl) were calculated according to the assay kit.

LPS assay

All materials used for both sample preparation and testing were pyrogen-free. The lipopolysaccharide (LPS) concentration in the serum was quantified via a chromogenic endotoxin assay (BS-E9334M1, JSBOSEN) based on a Limulus amoebocyte extract. The samples were subjected to centrifugation at 3000 rpm for a period of 10 minutes to separate the supernatant. The endotoxin concentration was expressed in endotoxin units per milliliter (EU/mL). All the data were obtained from standard curves.

Measurement of ROS

Neutrophil collection was performed subsequent to the induction of NETs production, and DCFH-DA (S0033S, Beyotime) was used to quantify reactive oxygen species (ROS) as a fluorescence probe. Upon cell formation, DCFH is oxidized by intracellular ROS and converted to DCF. Consequently, the observed fluorescence signal was found to be proportional to the production of ROS. A laser confocal microscope was used to observe the staining results, and the fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm with a fluorescence enzyme marker (Cytation).

Measurement of SOD and GSH

Mouse blood was collected in an anticoagulation tube, mixed upside down, and centrifuged at 600 × g for 10 min at 4°C, and the supernatant was added to the working solution and incubated at 37°C for 30 min. The absorbance at 450 nm was then measured to detect the inhibition rate of the SOD enzyme in the blood (BL903A, Biosharp). For GSH (A006-2-1, NJJC Bioengineering Institute), 0.05 ml of 10-fold diluted heparin anticoagulated blood and 0.2 ml of working reagent were centrifuged at 3500 rpm for 10 min. The supernatant was removed, the subsequent working solution was added, the mixture was oscillated, the mixture was mixed for 5 min, and the absorbance at 405 nm was detected.

Western blotting

Following the euthanasia of the animals, their kidneys and colon tissues were rapidly excised and homogenized in RIPA lysis buffer after protein concentration was measured with a Pierce™ BCA protein assay kit (23225, Thermo Fisher Scientific). Equal amounts of protein (40 µg for each tissue lysate) were introduced, separated via SDS–PAGE under reducing conditions, and subsequently transferred to PVDF membranes (0000206738, Millipore Corp.) for the standard procedure of immunoblot analysis. The membranes were blocked with 5% bovine serum albumin for 1 h at room temperature and probed with primary antibodies, including anti-histone H3 (A2348, ABclonal, RRID: AB_2631273), anti-citrulline histone H3 (ab5103, Abcam, RRID: AB_304752), anti-KIM-1 (MA5-28211, Thermo Fisher Scientific, RRID: AB_2745182), anti-BAX (GTX109683, GeneTex, RRID: AB_1949720), anti-NLRP3 (AG-20B-0014-C100, AdipoGen, RRID: AB_2490202), anti-caspase-1 (AG-20B-0042-C100, AdipoGen, RRID: AB_2490249), anti-IL1β (A16288, ABclonal, RRID: AB_2769945), anti-IL18 (10663-1-AP, Proteintech, RRID: AB_2123636), anti-IFNγ (A12450, ABclonal, RRID: AB_2759294), anti-Occludin(27260-1-AP, Proteintech, RRID: AB_2880820), and anti-Claudin-1 (13050-1-AP, Proteintech, RRID: AB_2079881).

The following secondary antibodies were used: HRP-conjugated AffiniPure goat anti-rabbit IgG (A6154, Sigma–Aldrich, RRID: AB_258284) and HRP-conjugated AffiniPure goat anti-mouse IgG (A4416, Sigma–Aldrich, RRID: AB_258167). Data were acquired with a molecular imager ChemiDoc system (ChemiDoc XRS+, Bio-Rad) and analyzed with ImageJ.

Lower limb blood flow measurement

Lower limb blood flow was quantified via laser Doppler flowmetry (LDF). In particular, a computer-controlled optical scanner was employed to direct a low-power laser beam onto the exposed lower limb in a controlled fashion. Concurrently, the scanner head was positioned parallel to the exposed lower limb at a distance of approximately 20 cm. A colored image on the video monitor subsequently indicates the relative perfusion level in question. The values of blood flow are recorded and assessed by a Moor FLPIR view V40 program (Gene & I Scientific. Ltd), which has been designed for this purpose.

Small animal imaging

Before autopsy, all the animals were fasted for 12 h and orally administered fluorescein isothiocyanate (FITC) dextran (60842-46-8, Sigma-Aldrich). After a four-hour incubation period, the distribution of fluorescein isothiocyanate (FITC) dextran in the mice was observed via a small animal imaging system (IVIS Spectrum, PerkinElmer). The peripheral blood of the mice was collected and centrifuged at 3000 r/min, 4°C, and 10 min, after which 100 µl of serum was obtained from the supernatant and added to a 96-well plate. The fluorescence intensity of the serum was quantified via a fluorescence microplate reader with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

16S rDNA Sequencing Analysis

Stool samples were stored at 80°C until analysis. Total stool DNA was extracted and isolated from the fecal pellets. A set of primers was designed to amplify a specific region, the 16S V3-V4 region, and a 420 bp fragment was successfully amplified. The fragment was then subjected to splicing via the Illumina NovaSeq 6000 platform, resulting in 2×250 bp paired-end data. These data were then subjected to splicing, which enabled the generation of a more extended sequence, thus facilitating 16S analysis. DADA2 was employed for the removal of low-quality sequences and chimeras, as well as for the generation of characteristic sequences for QIIME2 (<https://forum.qiime2.org/t/qiime2-chinese-manual/838>) for clustering operational taxonomic units (OTUs), diversity analysis, difference analysis, correlation analysis, and function prediction analysis. The default option for 16S rRNA genes is to use the Silva 138 rRNA database (Edgar, 2013). The proportion of sequences at different taxonomic levels for each sample was calculated on the basis of the abundance and annotation information of the ASVs. This was done to assess the species annotation resolution of the sample and the species complexity of the sample.

Statistical analysis

The data from individual experiments are expressed as the mean ± standard error of the mean (SEM). To ascertain whether there were any significant differences between the groups, Student's *t* test or ANOVA was employed, with a *p* value of less than 0.05 deemed to indicate statistical significance. The statistical analyses were conducted via Prism 9 software.

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Author contributions

Yaqi Luan, Weiwei He and Kunmao Jiang designed and supervised the research. L., Shenghui Qiu, Lan Jin, Ying Huang and Xinrui Mao performed the experiments. Lai Jin and Wentao Liu wrote the original draft. Lai Jin, Jingyuan Cao and Rong Wang verified the data and edited the final draft. All the authors read and approved the final version of the manuscript, ensuring that this was the case.

Ethics

All procedures were performed in strict accordance with the regulations of the ethics committee of the International Association for the Study of Pain and the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006). All animal experiments were approved by the Nanjing Medical University Animal Care and Use Committee (No. IACUC: 2305042) and were designed to minimize suffering and the number of animals used. All animal experiments complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (<https://arriveguidelines.org> )

Data sharing statement

All data associated with this study are presented in the paper or the Supplementary Materials. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

Declaration of interests

The authors declare that they have no competing interests.

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Reviewer #1 (Public review):

Summary:

Chemotherapy-induced chronic kidney injury is a significant and growing concern, as it can lead to long-term renal damage and compromised kidney function. The authors have highlighted an important aspect of this issue by evaluating the potential protective effects of OPCs against cisplatin-induced kidney injury. They propose that OPCs may mitigate renal damage by reducing NET formation, which could improve kidney function.

Strengths:

The study addressed a significant issue in the field of chemotherapy-induced kidney injury. The use of multiple markers and experimental methods provided a comprehensive exploration of the impact of OPCs on kidney damage. This approach allowed for a nuanced understanding of how OPCs might mitigate renal injury by reducing NET formation and improving kidney function.

Weaknesses:

The hypothesis is intriguing and relevant. However, the study encounters challenges, such as incomplete evidence and discrepancies between the text and data. Addressing these issues is crucial to improving the overall study's conclusions. The paper can potentially advance the understanding of therapeutic strategies for chemotherapy-induced kidney injury. Nonetheless, a clearer presentation of the data is necessary for it to have a substantial impact.

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Reviewer #2 (Public review):**Summary:**

The authors aimed to understand the mechanisms underlying chronic kidney disease (CKD) induced by cisplatin treatment. Acute or chronic kidney diseases are major adverse effects of cisplatin chemotherapy for cancer, which limits the treatment's efficacy. Understanding the disease's genesis is fundamental to identifying targets for preventing or treating these conditions.

Strengths:

The authors employed an in vivo model of cisplatin-induced chronic kidney disease (CKD) in mice, which displayed similar adverse effects of the therapy as seen in humans. The model called repeated low-dose cisplatin (RLCD), caused similar tissue and functional damage in the kidneys, led to harmful effects on the intestines by altering the microbiota and epithelial cell barrier, and impaired systemic vascular blood flow.

The authors demonstrated that the detrimental effects on the intestinal barrier led to the release of bacterial compounds into the circulation, which, in association with reactive oxygen species formed by the inflammatory and oxidative action of cisplatin, activated blood, and kidney neutrophils to release neutrophil extracellular traps (NETs). In turn, they suggested circulating NETs migrated into kidney tissue, causing damage. Moreover, they showed NETs are capable of trapping coagulation factors responsible for impaired systemic blood flow.

These conclusions were primarily based on reduced CKD symptoms and vascular damage in genetically modified animals that do not form NETs, as well as the observation that a bacterial compound (lipopolysaccharide) associated with cisplatin induces NET formation in isolated neutrophils. Moreover, treating animals with an anti-inflammatory and antioxidant natural compound simultaneously with cisplatin administration abolished the harmful effects on the kidneys and intestines.

The authors conclude that the intestinal damage and inflammatory properties of cisplatin lead to NET release, which, in turn, is responsible for the kidney and vascular damage evoked by cisplatin treatment.

Hence, the manuscript employs a well-designed experimental model and covers several important manifestations of cisplatin toxicity. It also uses genetically deficient mice to demonstrate the involvement of NETs in the development of chronic kidney disease (CKD)

Weaknesses:

Overall, the work was well executed. However, a few aspects require additional experiments to confirm the conclusions. The involvement of NETs in the genesis of CKD is unquestionable; nonetheless, the roles of locally induced versus circulating NETs, as well as the translation of in vitro NET release to in vivo CKD genesis, need further evaluation. Additionally, the primary mechanism of the natural anti-inflammatory compound used appears to be antioxidative, which does not promote the formation of reactive oxygen species necessary for NET formation. It is not clear in the title.

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