F4/80\textsuperscript{hi} Resident Macrophages Contribute to Cisplatin-Induced Renal Fibrosis

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Key Points
- Long-term effects of cisplatin are understudied. This study is the first to examine the role of macrophages in cisplatin-induced fibrosis.
- Depletion of kidney-resident macrophages ameliorated cisplatin-induced fibrosis, whereas depletion of infiltrating macrophages had no effect.
- This study highlights a pathogenic role for kidney-resident M2 macrophages in the development of fibrosis with repeated nephrotoxic injury.

Abstract
Background Cisplatin-induced kidney injury remains a major obstacle in utilizing cisplatin as a chemotherapeutic for solid-organ cancers. Thirty percent of patients treated with cisplatin develop acute kidney injury (AKI), and even patients who do not develop AKI are at risk for long-term declines in kidney function and development of chronic kidney disease (CKD). Modeling cisplatin-induced kidney injury in mice has revealed that repeated low doses of cisplatin lead to development of kidney fibrosis. This model can be used to examine AKI-to-CKD transition processes. Macrophages play a role in some of these processes, including immune response, wound healing, and tissue remodeling. Depleting macrophage populations in the kidney reduced fibrosis development in other models of renal fibrosis.

Methods We used either C57BL/6 mice with a Ccr2 genetic knockout or liposome encapsulated clodronate (Clodrosome) to deplete macrophage populations during repeated 9 mg/kg cisplatin treatments. We assessed how immune cell populations were altered in the blood and kidney of these mice and how these alterations affected development of renal fibrosis and kidney injury.

Results We found that Clodrosome treatment decreased collagen deposition, myofibroblast accumulation, and inflammatory cytokine production, whereas Ccr2 genetic knockout had no effect on these markers after cisplatin treatment. Additionally, Ccr2\textsuperscript{-/-} mice had decreased levels of F4/80\textsuperscript{lo} infiltrating macrophages in the kidney after cisplatin treatments, but Clodrosome treatment depleted F4/80\textsuperscript{hi} resident and CD206\textsuperscript{+} M2 macrophages.

Conclusions These data suggest that Clodrosome depletion of F4/80\textsuperscript{hi} and M2 macrophages in the kidney attenuates development of renal fibrosis after repeated low doses of cisplatin.

Introduction Cisplatin (cis-diamminedichloroplatinum[II]) has become a widely used chemotherapeutic for the treatment of many solid-organ cancers since its approval by the US Food and Drug Administration in 1978. Unfortunately, the success in treating these cancers with cisplatin is still hindered by dose-limiting nephrotoxicity. Thirty percent of patients treated with cisplatin develop AKI (1–3). AKI not only warrants suspension of cisplatin treatment, but also puts patients at risk for long-term decline in renal function, fibrosis development, and CKD (4–8). The development of fibrosis after AKI has only recently begun to be modeled in rodents (9,10). Cisplatin-induced renal fibrosis is now modeled using a repeated low-dose cisplatin (RLDC) regimen in mice (11,12). In this model, four weekly doses of 7–9 mg/kg cisplatin lead to the development of renal fibrosis accompanied by renal immune cell infiltration (11). Comparing the traditional high-dose cisplatin model and the RLDC model highlights the biologic differences occurring in acute and chronic injury processes (13). These differences
necessitate more studies on the mechanisms of AKI-to-CKD transition after cisplatin treatment.

Fibrosis development is a hallmark of the progression of AKI to CKD. Processes of maladaptive repair such as tubule G2/M cell-cycle arrest, cellular senescence, chronic inflammation, and chronic vascular impairment drive the development of fibrosis after AKI (14). With repeated insults, these maladaptive processes become more and more likely as normal repair processes are interrupted and immune cells are chronically recruited to sites of injury (14-16). Macrophages respond to episodes of AKI and play important roles in clearing cellular debris and orchestrating timely immune responses (17). Chronic macrophage activity, however, can lead to excessive myofibroblast activation and collagen deposition, promoting progression of renal fibrosis (18,19).

Macrophages constitute a diverse group of cells with various origins and functions. Resident macrophages are seeded in the kidney during embryonic development (20). These macrophages help maintain renal homeostasis by silently clearing cellular debris and aiding in tissue remodeling. They can be distinguished by high cell surface expression of F4/80 in mice (21–23). Infiltrating macrophages differentiate from monocytes that are recruited to the kidney after injury (19). The C-C motif chemokine receptor 2 (CCR2)/C-C motif chemokine ligand 2 (CCL2; also known as monocyte chemoattractant protein 1 [MCP-1]) axis plays a major role in inflammatory monocyte recruitment and accumulation of infiltrating macrophages (24–26). These macrophages can be distinguished from resident macrophages by their low cell surface expression of F4/80 in mice (23). Both resident and infiltrating macrophages can adopt different phenotypes to perform a variety of functions. For simplicity, we will refer to the extreme ends of the functional spectrum as M1 and M2 macrophages. M1 macrophages appear in the kidney rapidly after injury and are considered proinflammatory. M2 macrophages appear later in kidney injury and have a pro-repair and anti-inflammatory phenotype. Although M1 macrophages may still be present in chronic injury, development of renal fibrosis is associated with a M1-to-M2 phenotype transition (19,27).

Although macrophages have been implicated in development of fibrosis in other models of kidney injury, their role in the AKI-to-CKD progression after RLDC treatment remains unknown. In this study, we examined the response of resident and infiltrating macrophages in the kidney after RLDC treatment. We also evaluated development of fibrosis and injury in the RLDC model after depletion of either infiltrating or resident macrophages. Our results suggest that resident macrophages contribute to fibrosis development and M2 polarization after RLDC treatment. These data suggest targeting resident macrophages could ameliorate the AKI-to-CKD transition after cisplatin treatment.

Materials and Methods

Animal Experiments

Eight-week-old male Ccr2 knockout (Ccr2<sup>−/−</sup>) mice on a C57BL/6 background were purchased from The Jackson Laboratory (stock 000664). Heterozygous offspring were then aged to 8 weeks and cross-bred to produce littermate WT and Ccr2<sup>−/−</sup> mice. Eight- to 11-week-old male WT and Ccr2<sup>−/−</sup> littersmates were used for this study. WT 8-week-old male C57BL/6 mice were purchased from The Jackson Laboratory (stock 000664) for liposome encapsulated clodronate experiments. These mice were acclimated for 1 week before beginning experiments. All mice were maintained on a 12-hour/12-hour light/dark cycle and provided with food and water ad libitum. Animals were maintained under standard laboratory conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville (Protocol ID 19568) and followed the guidelines of the American Veterinary Medical Association. Mice were intraperitoneally injected with either 0.9% N saline vehicle or cisplatin at 9 mg/kg at 8:00 AM once a week for 4 weeks and euthanized 3 days after the last dose. Pharmaceutical-grade cisplatin purchased from the University of Louisville hospital pharmacy (1 mg/ml in 0.9% N saline from Intas Pharmaceuticals) was used for all experiments. A Standard Macrophage Depletion Kit (Clodrosome+Encapsome) was purchased from Encapsula Nanosciences (CLD-8901). Two hundred microliters of either Clodrosome (liposome encapsulated clodronate) or Encapsome (empty liposomes as vehicle control) was intravenously administered 1 day before the third and fourth dose of cisplatin. One hundred microliters of either Clodrosome or Encapsome was intravenously administered the day after the third and fourth dose of cisplatin, and 4 days after the third dose of cisplatin (Table 1). Mice were monitored for weight loss or evidence of high levels of discomfort/stress. Upon euthanasia, plasma was prepared and stored at −80°C. One kidney was divided into sections to be flash-frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin. The other kidney was taken for immune cell analysis by flow cytometry.

BUN and Neutrophil Gelatinase Associated Lipocalin Determination

BUN was measured in the plasma of mice using a kit from AMS Diagnostics (80146) according to the manufacturer’s instructions and as previously published (12). ELISA for neutrophil gelatinase associated lipocalin (NGAL; DY1857; R&D Systems) was performed on mouse urine as previously published (12).

Gene Expression

Total RNA was isolated from kidney cortex, and cDNA was made as previously published (12). The following pre-designed TAQman primers (Life Technologies) were used: Tnf-α (Mm00443258_m1), Il-6 (Mm00446190_m1), Cxcl1 (Mm04207460_m1), Cd2 (Mm04124242_m1), B2m (Mm00475988_m1), and Arg-1 (Mm00475988_m1). The following self-designed primers were used: Kim-1 forward AGATCCACACATGTCACCAATCACA and reverse CAGTGCCATTCAGTCTGGTTT; Colla1 forward CGATGGATTCCCGTG; Tgf-β forward CAAATGGGAACTCTACCCAGAAG; Tgf-β reverse GAGTCCACCATGACTCCAGAAATATAG and reverse ACAACTCCAGTGACTCAAAGAC; and Timp-1 forward GCAACTGCGAATGGTACAA and reverse...
TTAGT CATCTTGATCTTATAACGCTTGTA. Real-time quantitative RT-PCR was performed using iTaq Universal Probes Supermix (172–5134; Bio-Rad) or iTaq Universal SYBR Green Supermix (172–5124; Bio-Rad). Beta-2-microglobulin was used as the reference gene for expression analysis. Data are expressed as the fold change in relative expression of the tested gene from vehicle-treated mice.

Immunohistochemistry, Sirius Red/Fast Green, and Masson’s Trichrome Staining

Alpha smooth-muscle actin (αSMA) immunohistochemistry for myofibroblasts and Sirius Red/Fast Green stain for total collagen deposition was performed on paraffin-embedded kidney sections as previously published (12). Masson’s trichrome staining was performed using the Trichrome Stain (Masson) Kit (HT15; Sigma–Aldrich). Paraffin-embedded tissue was deparaffinized, rehydrated, and placed in Bouin’s solution (HT10132; Sigma–Aldrich) at 56°C for 15 minutes under chemical hood. Sections were washed under running tap water for 10 minutes. Sections were then stained with Weigert’s iron hematoxylin solution (HT1079; Sigma–Aldrich) for 5 minutes, washed under running tap water for 5 minutes, and stained in Biebrich Scarlet-Acid Fuschin for 5 minutes. Sections were rinsed briefly in distilled water and then stained with phosphotungstic/phosphomolybdic acid solution for 15 minutes before being directly placed into Aniline Blue solution for 10 minutes. Lastly, sections were rinsed briefly in distilled water before dehydrating and clearing sections for mounting.

Flow Cytometry

Whole kidneys were homogenized into single-cell suspensions and prepared for staining as previously described (11). Cells were blocked with CD16/32 (101321; BioLegend) and extracellularly stained with CD45-PerCP (103130; BioLegend), CD3e-PE-Cy7 (552774; BD Biosciences), CD11b-BV650 (563402; BD Biosciences), F4/80-BV421 (565411; BioLegend), CD8a-BUV737 (612759; BD Biosciences), CD4-FITC (100406; BioLegend), CD19-PE-CF594 (562329; BD Biosciences), Ly-6G-Alexa Fluor 700 (561236; BD Biosciences), Ly-6C-APC-Cy7 (560596; BD Biosciences), F4/80-BV421 (565411; BD Biosciences), CD11b-BV650 (563402; BD Biosciences), and PDGFRα-BUV737 (741789; BD Biosciences). After staining, cells were fixed and permeabilized with FoxP3/Transcription Factor Staining Buffer Set (00-5523-00; Invitrogen). Intracellular staining was done with CD206-PE (141705; BioLegend). Flow cytometry was done using a BD LSRSFortessa, collecting 1 million events per sample. Hierarchical gating was performed as depicted in Supplemental Figure 1. Data are represented as the percentage of positively stained cells for the indicated population from the total number of single cells observed in that sample. Resident macrophages are identified as CD45<sup>-</sup>CD3<sup>-</sup>CD19<sup>-</sup>CD11b<sup>-</sup>F4/80<sup>hi</sup>. Infiltrating macrophages are identified as CD45<sup>-</sup>CD3<sup>-</sup>CD19<sup>-</sup>CD11b<sup>-</sup>F4/80<sup>lo</sup>.

Statistical Analyses

Data are expressed as the mean±SEM for all experiments. Comparisons of normally distributed data sets were analyzed by either a one- or two-way ANOVA as appropriate, and group means were compared using Tukey post tests. Correlations were determined using a linear regression model. The criterion for statistical difference was P<0.05.

Results

Renal Macrophage Infiltration Correlates with Kidney Injury and Fibrosis Markers but not BUN Elevation after RLDC

Previously, our lab demonstrated that RLDC leads to significant infiltration of immune cells in the kidney, and Ccl2 mRNA expression in the renal cortex correlates with other biomarkers of kidney injury (11). To determine further if macrophages were playing a role in promoting the AKI-CKD transition after RLDC treatment, we first performed an analysis on WT C57BL/6 mice. We compared the degree of macrophage infiltration with a variety of factors, including changes in kidney function, degree of kidney injury, and mRNA markers of renal fibrosis. These relationships were assessed in available data from 20 male cisplatin-treated WT C57BL/6 mice. Significance was determined as P<0.05 using a linear regression model. Total macrophage infiltration did not significantly correlate with BUN elevation, which was used as a marker of functional loss (Figure 1A). However, macrophage infiltration did correlate with elevation of mRNA kidney injury markers Kim-1 and Ccl2 (Figure 1, B and C) and elevation of mRNA fibrotic markers Timp-1 and Col1al (Figure 1, D and E). These data suggest that there is a relationship between macrophage accumulation and kidney injury and fibrosis after RLDC treatment; however, we cannot assume causality. Increased macrophage numbers could be causing increased levels of kidney injury, or there could be more macrophages responding to greater levels of kidney damage.

Table 1. Clodrosome/encapsome dosing schedule with repeated low-dose cisplatin treatments

| Sunday, µL Clodrosome or Encapsome | Monday, mg/kg Cisplatin or Saline | Tuesday, µL Clodrosome or Encapsome | Wednesday | Thursday | Friday, µL Clodrosome or Encapsome | Saturday |
|------------------------------------|-----------------------------------|-------------------------------------|-----------|---------|-----------------------------------|----------|
| 9                                  | 9                                 | 9                                   | 100       | 100     | 100                               | Euthanize |
| 200                                | 9                                 | 100                                 |           |         |                                   |          |
| 200                                | 9                                 | 100                                 |           |         |                                   |          |
We further divided the data to assess how F4/80hi (resident) and F4/80lo (infiltrating) macrophages correlated with these markers. Only resident macrophage accumulation correlated with elevation of mRNA injury markers Kim-1 and Ccl2 (Figure 2, A–D). However, infiltrating macrophage accumulation correlated with fibrotic mRNA markers Timp-1 and Col1a1 (Figure 2, E–H). These data suggest that resident and infiltrating macrophages may be playing different roles in the AKI-to-CKD transition after RLDC.

**Ccr2$$^/-$$ Mice Have Reduced Renal F4/80lo Infiltrating Macrophages after RLDC**

We assessed immune cell infiltration in kidneys of littermate C57BL/6 WT and Ccr2$$^/-$$ mice after RLDC treatment. Cisplatin-treated Ccr2$$^/-$$ mice had reduced infiltration of CD45$$^+$$ immune cells in the kidneys compared with cisplatin-treated WT mice, although results were not significant (Figure 3A). F4/80lo$$^+$$ resident macrophage levels remained unchanged in the kidneys of all groups (Figure 3B). F4/80lo$$^+$$ infiltrating macrophages were significantly increased in the kidneys of cisplatin-treated WT mice compared with vehicle-treated animals. Kidneys from Ccr2$$^/-$$ mice did not have increased F4/80lo$$^+$$ infiltrating macrophages after RLDC treatment (Figure 3C). CD206$$^+$$ M2 macrophages were similarly elevated in the kidneys of cisplatin-treated WT and Ccr2$$^/-$$ mice compared with vehicle-treated mice (Figure 3D). This pattern of depletion was also observed when macrophage populations were analyzed as a percentage of CD45$$^+$$ cells in the kidney, indicating the depletion observed was not simply due to a decreased number of proximal tubule cells (Supplemental Figure 2). Cisplatin-treated Ccr2$$^/-$$ mice also had impaired Ly6Ch$$^+$$ inflammatory monocyte recruitment to the kidney compared with cisplatin-treated WT mice (Figure 3E). No changes in other immune cell populations in the kidneys of Ccr2$$^/-$$ cisplatin-treated mice compared with WT mice were observed (Table 2 and Supplemental Figure 3). Ccr2$$^/-$$ mice also had basally reduced circulating macrophages and inflammatory...
monocytes in the blood compared with WT mice (Supplemental Figure 4). These data suggest that Ccr2−/− mice have inhibited monocyte recruitment to the kidney after RLDC treatment, leading to decreased levels of F4/80lo infiltrating macrophages. It also suggests CD206+ M2 macrophage accumulation in the kidney can occur without significant contributions from the infiltrating macrophage population.

Figure 2. | Resident macrophages correlate with kidney injury and fibrosis markers after RLDC treatment. The percentage of F4/80lo resident and F4/80hi infiltrating macrophages in the kidney of cisplatin-treated C57BL/6 male mice (n=20) after RLDC treatment was plotted against (A–D) expression of kidney injury mRNA markers and (E–H) expression of fibrotic mRNA markers. Significance was determined at $P<0.05$ using a linear regression model.
Clodrosome Depleted Renal F4/80hi Resident Macrophages after RLDC

After observing that Ccr2^{−/−} depleted F4/80lo in infiltrating macrophages with no effect on F4/80hi renal resident populations, we sought to deplete F4/80hi renal resident macrophages using liposome encapsulated clodronate (Clodrosome). Empty liposomes (Encapsome) were also used as a vehicle control. Overall, CD45^{+} total immune cell infiltration in the kidneys was similarly elevated in all cisplatin-treated groups compared with vehicle (Figure 4A). Interestingly, Clodrosome treatment led to a significant decrease in F4/80hi resident macrophages in the kidneys compared with all other groups, including vehicle-treated mice (Figure 4B). In contrast, F4/80lo infiltrating macrophage populations were not affected by Clodrosome after RLDC treatment (Figure 4C). CD206^{+} M2 macrophage accumulation in the kidneys after cisplatin treatment was significantly reduced by Clodrosome (Figure 4D). Again, this pattern of depletion was observed when macrophage populations were analyzed as a percentage of CD45^{+} cells in the kidney, indicating the depletion observed was not due to a decreased number of proximal tubule cells (Supplemental Figure 5). Depletion of M2 macrophages was also indicated by a decrease in Arg-1 mRNA expression in the kidney cortex of Clodrosome-treated mice compared with other cisplatin-treated groups (Figure 4E). We also observed a significant decrease in CD3^{+}CD4^{+}CD8^{+} immune cells and an increase in inflammatory monocytes and neutrophils in the kidneys of Clodrosome-treated mice compared with other cisplatin-treated mice (Table 3 and Supplemental Figure 6). Clodrosome also increased circulating myeloid cells in the blood after RLDC treatment.

Figure 3. | Ccr2^{−/−} blocked inflammatory monocyte and macrophage infiltration with no effect on resident or M2 macrophages. Whole kidneys were homogenized into a single-cell suspension for flow cytometric analysis of immune cells. Hierarchical gating was performed to identify (A) CD45^{+} total immune cells, (B) F4/80hi resident macrophages, (C) F4/80lo infiltrating macrophages, (D) CD206^{+} M2 macrophages, and (E) Ly6C^{hi} inflammatory monocytes present in the kidney after RLDC treatment. Data are presented as percentage of positively labeled cells from the total number of single cells counted for each sample. Statistical analysis was determined by two-way ANOVA followed by Tukey post test. ▲Significance was determined at P<0.05. VEH, vehicle; CIS, cisplatin. *Significantly different from WT VEH; ▲significantly different from Ccr2^{−/−} VEH; #significantly different from Ccr2^{−/−} CIS.
Inflammatory Cytokine and Chemokine Expression in the Macrophage Accumulation after RLDC.

Unexpectedly, the kidneys of Etreatment compared with other cisplatin-treated groups, although the results were not statistically significant (Figure 5, E–G). No effect was observed on induction of Ccl2 expression in the renal cortex with Clodrosome treatment (Figure 5H). These data suggest that Clodrosome treatment-induced depletion of resident macrophages mildly blunted the inflammatory cytokine and chemokine response induced by RLDC treatment in the kidney cortex. In contrast, Ccr2−/− mice had reduced levels of infiltrating macrophages but displayed no changes in inflammatory cytokine and chemokine expression in the kidney cortex.

Clodrosome Treatment Ameliorated Cisplatin-Induced Renal Fibrosis, whereas Ccr2−/− Had No Effect

Collagen deposition was observed via Sirius Red/Fast Green and Masson's trichrome staining. Cisplatin-treated Ccr2−/− mice had similar levels of collagen deposition in the kidneys compared with cisplatin-treated WT mice (Figure 6, A and B). In contrast, both stains revealed decreased collagen deposition in the kidneys of Clodrosome-treated mice compared with other cisplatin-treated groups (Figure 6, C and D). Myofibroblast accumulation was assessed using immunohistochemical staining for αSMA. RLDC treatment caused an increase in αSMA staining in the kidneys, typically observed in striated patterns through the corticomedullary region (11,12). Cisplatin-treated Ccr2−/− mice had a similar amount and pattern of αSMA staining in the kidneys as observed in the cisplatin-treated WT mice (Figure 6E). In contrast, Clodrosome treatment reduced αSMA staining in the kidneys compared with other cisplatin-treated groups. αSMA staining in kidney sections of Clodrosome-treated mice was also less striated and more scattered through the kidneys (Figure 6F). PDGFRα expression was also measured via flow cytometry to assess fibroblast and myofibroblast levels in the kidney after RLDC treatment (28). Cisplatin-treated Ccr2−/− mice had the same level of PDGFRα expression in the kidneys as cisplatin-treated WT mice (Figure 6G). Clodrosome treatment caused a significant reduction in PDGFRα-positive cells in the kidneys compared with Encapsome-treated mice, but levels were the same as mice treated with cisplatin only (Figure 6H).

mRNA expression of fibrotic makers Temp-1, Col1a1, and Tgf-β in the kidney cortex were also assessed after RLDC treatment. Ccr2−/− had no effect on Temp-1 elevation after RLDC (Figure 7A), whereas Clodrosome treatment significantly reduced Temp-1 mRNA expression compared with other cisplatin-treated groups (Figure 7B). Similarly, Ccr2−/− had no effect on elevation of Col1a1 and Tgf-β mRNA in the renal cortex after RLDC treatment (Figure 7, C and E), whereas Clodrosome treatment reduced mRNA expression of Col1a1 and Tgf-β compared with other cisplatin-treated groups, although the results were not significant (Figure 7, D and F). These data indicate that Clodrosome treatment reduced collagen deposition, myofibroblast accumulation, and expression of mRNA fibrotic markers in the kidney after RLDC, whereas genetic deletion of Ccr2 had no effect.

Table 2. Immune cell alterations in the kidney of cisplatin-treated Ccr2 genetic knockout compared with wild-type mice

| Immune Cell | Vehicle | Ccr2 Genetic Knockout | Cisplatin | Ccr2 Genetic Knockout |
|-------------|---------|-----------------------|-----------|-----------------------|
| CD45+ immune cells | 1.677±0.19 | 1.825±0.162 | 6.573±0.633 | 4.989±0.367 |
| CD19− B cells | 0.223±0.031 | 0.242±0.03 | 0.541±0.056 | 0.454±0.039 |
| CD3+ T cells | 0.178±0.024 | 0.2±0.019 | 0.569±0.048 | 0.519±0.046 |
| CD4+ T cells | 0.075±0.012 | 0.087±0.008 | 0.253±0.021 | 0.235±0.022 |
| CD8+ T cells | 0.04±0.004 | 0.048±0.005 | 0.149±0.016 | 0.136±0.012 |
| CD3−CD4−CD8− | 0.065±0.008 | 0.063±0.01 | 0.157±0.012 | 0.142±0.015 |
| CD11b+ myeloid cells | 0.608±0.087 | 0.687±0.108 | 1.209±0.096 | 0.963±0.064 |
| Ly6G+ neutrophils | 0.037±0.011 | 0.087±0.051 | 0.183±0.021 | 0.231±0.034 |
| Ly6C+ inflammatory monocytes | 0.035±0.006 | 0.017±0.006 | 0.079±0.012 | 0.03±0.005 |
| Ly6C− monocytes | 0.045±0.01 | 0.088±0.049 | 0.271±0.027 | 0.258±0.033 |
| F4/80+ resident mcs | 0.375±0.067 | 0.392±0.046 | 0.527±0.05 | 0.447±0.028 |
| F4/80+ infiltrating mcs | 0.122±0.015 | 0.098±0.012 | 0.345±0.031 | 0.151±0.013 |
| CD206+ M2 macrophages | 0.223±0.05 | 0.232±0.036 | 0.846±0.087 | 0.679±0.055 |
| PDGFRα expression | 14.167±0.885 | 14.533±1.834 | 35.733±1.848 | 33.014±1.7 |

Data presented as percentage of indicated immune cell population of single cells collected per sample. Values are mean±SEM. *A significant reduction in Ccr2−/− cisplatin-treated kidneys compared with WT cisplatin-treated kidneys.
Clodrosome Treatment Reduced \textit{Kim-1} mRNA Expression in the Renal Cortex after RLDC with No Effect on BUN or NGAL

Surprisingly, neither Clodrosome treatment nor Ccr2\(^{-/-}\) protected against changes in BUN after RLDC (Figure 8, A and B). Injury, as assessed by the urinary AKI biomarker NGAL, was also unchanged by Clodrosome and Ccr2\(^{-/-}\) (Figure 8, C and D). Ccr2\(^{-/-}\) had no effect on \textit{Kim-1} mRNA elevation in the kidney cortex after RLDC (Figure 8E); however, \textit{Kim-1} mRNA expression was significantly reduced in Clodrosome-treated mice compared with other cisplatin-treated groups (Figure 8F). This suggests less proximal tubule injury in mice receiving Clodrosome along with RLDC. These data suggest that macrophage depletion does not affect development of AKI after cisplatin treatment by traditional markers but may prevent development of subclinical pathologic changes such as fibrosis.

\textbf{Discussion}

In this study, we examined how depletion of macrophage populations using either liposome encapsulated clodronate (Clodrosome) or Ccr2 genetic knockout affected...
Table 3. Immune cell alterations in the kidney of cisplatin+clodrosome-treated mice compared with cisplatin alone and cisplatin+encapsome.

| Immune Cell | Vehicle | Cisplatin | Cisplatin+Clodrosome | Cisplatin+Encapsome |
|-------------|---------|-----------|----------------------|--------------------|
| CD45+ immune cells | 1.744±0.1817 | 4.627±0.3563 | 4.509±0.3763 | 5.335±0.4519 |
| CD19+ B cells | 0.192±0.0434 | 0.543±0.0497 | 0.5675±0.0505 | 0.573±0.0445 |
| CD3+ T cells | 0.422±0.0752 | 0.901±0.0832 | 0.666±0.0656 | 0.903±0.1156 |
| CD4+ T cells | 0.128±0.0206 | 0.268±0.0241 | 0.265±0.0261 | 0.257±0.0252 |
| CD8+ T cells | 0.074±0.0098 | 0.218±0.0144 | 0.21±0.0174 | 0.247±0.0306 |
| CD3+CD4+CD8- | 0.216±0.0548 | 0.412±0.0472 | 0.19±0.048 | 0.396±0.0665 |
| CD11b+ myeloid cells | 0.742±0.0792 | 1.59±0.1117 | 1.183±0.2094 | 1.821±0.1324 |
| Ly6G+ neutrophils | 0.106±0.025 | 0.272±0.038 | 0.403±0.0461* | 0.264±0.0178 |
| Ly6C+ inflammatory monocytes | 0.066±0.0098 | 0.155±0.0257 | 0.408±0.0608* | 0.142±0.0317 |
| Ly6C+ monocytes | 0.124±0.024 | 0.396±0.0392 | 0.499±0.0661 | 0.428±0.0397 |
| F4/80hi resident macs | 0.388±0.0511 | 0.579±0.082 | 0.08888±0.0589* | 0.515±0.0959 |
| F4/80hi infiltrating macs | 0.184±0.0291 | 0.643±0.0595 | 0.634±0.1256 | 0.934±0.1085 |
| CD206+ M2 macrophages | 0.182±0.024 | 0.642±0.0934 | 0.123±0.0508a | 0.576±0.1188 |
| PDGFRα expression | 2.412±0.1959 | 8.31±0.9712 | 7.621±0.7666* | 10.92±0.8561 |

Data presented as percentage of indicated immune cell population of single cells collected per sample. Values are mean±SEM. Cis, cisplatin; Cis+Clod, cisplatin+clodrosome; Cis+Encap, cisplatin+encapsome.

*Significant change in Cis+Clod-treated kidneys compared with either Cis- or Cis+Encap-treated kidneys.

The development of renal fibrosis after RLDC treatment. The differential effects these conditions had on immune cell populations allowed us to assess potential roles of F4/80hi resident and F4/80lo infiltrating macrophages in the development of fibrosis. We observed that Clodrosome depleted F4/80hi resident and M2 macrophages after RLDC treatment but had no effect on F4/80lo infiltrating macrophages in the kidney. This depletion was accompanied by reduced renal fibrosis after RLDC treatment. In contrast, Ccr2−/− mice had reduced F4/80lo infiltrating macrophages in the kidney after RLDC treatment with no change in F4/80hi resident or M2 macrophage accumulation. Ccr2 genetic knockout also had no effect on fibrosis development after RLDC treatment.

Other studies using liposome encapsulated clodronate have observed varied effects on macrophage populations in the kidney. These studies often report global depletion of macrophages, but do not differentiate between populations of resident and infiltrating macrophages (29-36). Interestingly, Yang et al. utilized liposome encapsulated clodronate to deplete Ly6C+ tissue resident macrophages that were not depleted in the kidneys of Ccr2−/− mice (36). These results indicated Ccr2 genetic knockout and liposome encapsulated clodronate alter different populations of macrophages. Additionally, Puranik et al. reported that a single high dose of liposome encapsulated clodronate-depleted blood monocytes, but populations were recovering by 48 hours and were fully replenished by 72 hours. This single high dose of liposome encapsulated clodronate was also unsuccessful in depleting kidney-resident macrophages. To achieve depletion of kidney-resident macrophages, repeated low doses of clodronate were used (35).

These studies suggest that the dosing schedule and time of observation play a role in determining immune populations altered by liposome encapsulated clodronate. In our study, we observed macrophage populations 48 hours after the final Clodrosome treatment. Bone marrow-derived immune cells may have had time to repopulate, causing an observed surge in inflammatory monocytes and neutrophils. Similar results have been reported (35). It is important to consider that Clodrosome may be depleting F4/80lo infiltrating macrophage populations at earlier unobserved time points.

We also acknowledge that Clodrosome is given systemically and designed to deplete all phagocytic cells. In the kidney, Clodrosome treatment also depleted CD3+CD4+CD8- cells and increased both Ly6C+ monocytes and Ly6G neutrophils (Table 3 and Supplemental Figures 6, B, D, and I). These cell populations have also been studied in the context of renal injury. For example, alterations in Ly6C+ monocytes have been suggested to be both protective in models of sepsis-induced AKI (37), and pathogenic in ischemia reperfusion-induced AKI (38). Therefore, the protective effects observed with Clodrosome treatment in this study cannot be solely attributed to F4/80hi resident macrophage depletion.

In other models of renal fibrosis, liposome encapsulated clodronate depletion of macrophages has been protective (27-29,36,39). Our study supports these findings, indicating liposome encapsulated clodronate treatment targets immune cell populations that are pathogenic in processes of renal fibrosis. We believe the depletion of F4/80hi resident and M2 macrophages by Clodrosome in our study is key to the protective effects observed. Kidney-resident macrophages have been specifically implicated in fibrotic development after ureteral obstruction and ischemia reperfusion (25,36). Additionally, M2 macrophage depletion by repeated administration of liposome encapsulated clodronate attenuated development of fibrosis after ureteral obstruction (40), and adoptive transfer of M2 macrophages to liposome encapsulated clodronate-treated mice reversed protection from ischemia reperfusion-induced fibrosis (33). In this study, we observed that Clodrosome treatment reduced F4/80hi resident and M2 macrophages, whereas Ccr2 genetic knockout reduced F4/80lo infiltrating macrophages with no effect on M2 macrophage accumulation.
Figure 5. Clodrosome reduced inflammatory cytokine and chemokine induction after RLDC, whereas Ccr2^{−/−} had no effect. RNA was isolated from kidney cortex tissue. mRNA expression of (A) and (E) Tnf-α, (B) and (F) Ccl2, (C) and (G) Il-6, and (D) and (H) Cxcl1 was assessed relative to B2m expression. Statistical analysis was determined by either one- or two-way ANOVA as appropriate followed by Tukey post test. Significance was determined at P<0.05. (A–D) *Significantly different from WT VEH; ^significantly different from Ccr2^{−/−} VEH; #significantly different from Ccr2^{−/−} CIS. (E–H) *Significantly different from VEH.
Figure 6. Clodrosome ameliorated RLDC-induced renal fibrosis, whereas Ccr2<sup>−/−</sup> had no effect. (A) and (C) Sirius Red/Fast Green, (B) and (D) Masson’s trichrome, and (E) and (F) αSMA immunohistochemical staining of paraffin-embedded kidney sections. (G) and (H) percent PDGFRα expressing cells of single cells counted in each sample run on a flow cytometer. Statistical analysis was determined by one- or two-way ANOVA as appropriate followed by Tukey post test. Significance was determined at P<0.05. (G) *Significantly different from WT VEH; ^significantly different from Ccr2<sup>−/−</sup> VEH. (H) *Significantly different from VEH; #significantly different from CIS+Encap. αSMA, alpha smooth-muscle actin.
These data suggest that F4/80hi kidney-resident macrophages may be more susceptible to M2 polarization than F4/80lo infiltrating macrophages. Although more studies are needed to examine this relationship, a predisposition toward M2 polarization could explain why kidney-resident macrophages are more likely to promote development of fibrosis.

This study diverges from the literature in observations regarding Ccr2 genetic knockout effects on renal fibrosis development. CCR2 is expressed predominantly by monocytes, dendritic cells, and natural killer cells. Lower levels of CCR2 expression can also be found on neutrophils, B cells, and T cells. The expression of CCR2 allows for mobilization of these immune cell populations from the bone marrow (41). CCR2 expression has not been found to be necessary for embryonic development or maintenance of renal resident macrophages (23). Therefore, the Ccr2 genetic knockout model allows us to assess how blocking renal infiltration of immune cells, predominantly monocytes and macrophages, affects the development of injury and fibrosis.

Ccr2 genetic knockout has been shown to be protective in some models of renal fibrosis (24, 26, 42–44). In contrast, Yang et al. demonstrated that Ccr2 genetic knockout,

Figure 7. Clodrosome blunted RLDC-induced fibrotic mRNA markers, whereas Ccr2–/– had no effect. RNA was extracted from kidney cortex tissue. mRNA expression of (A) and (B) Timp-1, (C) and (D) Col 1a1, and (E) and (F) Tgf-β was assessed relative to B2m expression. Statistical analysis was determined by either one- or two-way ANOVA as appropriate followed by Tukey post test. Significance was determined at P<0.05. (A), (C), and (E) *Significantly different from WT VEH; ^significantly different from Ccr2–/– VEH. (B), (D), and (F) *Significantly different from VEH; ^significantly different from CIS; #significantly different from CIS+Encap.
although protective from ischemia reperfusion–induced AKI, worsened fibrosis development (36). Our study demonstrated that Ccr2−/− in mice had no effect on development of fibrosis after RLDC treatment. We hypothesize that the CCR2/CCL2 signaling axis plays a role in cisplatin-induced AKI, as indicated by its correlation with other markers of kidney injury (11), but this signaling axis may not be as important in the AKI-to-CKD transition after RLDC treatment. The CCR2/CCL2 independence of RLDC-induced fibrosis highlights unique mechanisms of fibrosis development compared with ischemia reperfusion and ureteral obstruction models of fibrosis.

Future studies should examine the response of kidney-resident macrophages to RLDC in more depth. We believe determining the position of resident macrophages in relationship to both injured proximal tubule cells and activated myofibroblasts could provide vital clues about the function of these macrophages in fibrosis development. Other studies have shown that macrophages accumulate around injured proximal tubule cells in cases of both AKI and CKD (45,46). Furthermore, KIM-1 has been implicated in mediating macrophage activity in models of both AKI and renal fibrosis (46,47). Macrophages are also known to be important regulators of myofibroblasts (48,49). It is possible

Figure 8. | Clodrosome reduced Kim-1 expression, but neither Clodrosome nor Ccr2−/− altered BUN or NGAL. (A) and (B) BUN was measured from plasma. (C) and (D) NGAL was measured from urine. (E) and (F) Kim-1 mRNA expression in the kidney cortex was assessed relative to B2m expression. Statistical analysis was determined by either one- or two-way ANOVA as appropriate followed by Tukey post test. Significance was determined at P<0.05. (A), (C), and (E) *Significantly different from WT VEH; ^significantly different from Ccr2−/− VEH. (B), (D), (F) *Significantly different from VEH; ^significantly different from CIS; #significantly different from CIS+Encap. NGAL, neutrophil gelatinase associated lipocalin.
that resident macrophages, injured proximal tubules, and activated myofibroblasts form spatial units that may play a major role in the progression of RLDC-induced fibrosis. Disrupting this spatial relationship may prevent development of fibrosis and progression of CKD.

The diverse activity of macrophages in the kidney leads to differential roles in injury development. In the high-dose model of cisplatin-induced AKI, macrophage depletion with liposome encapsulated clodronate had no effect on development of AKI (50). Importantly, liposome encapsulated clodronate depletion of macrophages after development of ischemia reperfusion–induced AKI led to incomplete recovery, indicating essential roles for macrophages in the healing process (27). In our study, macrophage depletion had no effect on BUN or NGAL elevation, which are used as markers of AKI development. This indicates that macrophages are not a viable target to prevent cisplatin-induced AKI; however, the AKI-to-CKD transition processes may be prevented with macrophage depletion.

Disclosures
All authors have nothing to disclose.

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Author Contributions
L.J. Beverly, M.A. Doll, S.M. Sears, and L.J. Siskind edited and revised the manuscript; L.J. Beverly, S.M. Sears, and L.J. Siskind conceived and designed research and interpreted the results of the experiments; M.A. Doll, A. Krueger, S.M. Sears, and A.A. Vega performed the animal dissections; M.A. Doll, G.B. Ortopilla S.M. Sears, and P.P. Shah analyzed the data; Z. Kurlawala, R. Miller, and S.M. Sears designed and optimized the flow cytometry analysis protocol; S.M. Sears prepared the figures and drafted the manuscript; S.M. Sears and A.A. Vega performed animal experiments; and all authors approved the final version of manuscript.

Supplemental Material
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Supplemental Figure 1. Hierarchical gating strategy.

Supplemental Figure 2. Immune cell alterations in Ccr2−/− kidney as percentage of CD45+ cells.

Supplemental Figure 3. Immune cell alterations in Ccr2−/− kidney.

Supplemental Figure 4. Immune cell alterations in Ccr2−/− blood.

Supplemental Figure 5. Immune cell depletion in the kidney after clodronate treatment as percentage of CD45+ cells.

Supplemental Figure 6. Immune cell depletion in the kidney after clodronate treatment.

Supplemental Figure 7. Immune cell depletion in the blood after clodronate treatment.

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