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F4/80hi Resident Macrophages Contribute to Cisplatin-Induced Renal Fibrosis

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Key Points:
*Long-term impacts 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 while depletion of infiltrating macrophages had no effect

*This study highlights a pathogenic role for kidney resident M2 macrophages in 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. 30% 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 impacted development of renal fibrosis and kidney injury. Results: We found that Clodrosome treatment decreased collagen deposition, myofibroblast accumulation, and inflammatory cytokine production, while Ccr2 genetic knockout had no effect on these markers following cisplatin treatment. Additionally, Ccr2-/- mice had decreased levels of F4/80lo infiltrating macrophages in the kidney following cisplatin treatments, but Clodrosome treatment depleted F4/80hi resident and CD206+ M2 macrophages. Conclusions: These data suggest that Clodrosome depletion of F4/80hi and M2 macrophages in the kidney attenuates development of renal fibrosis following repeated, low doses of cisplatin.

Disclosures: The authors have nothing to disclose.


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Data Sharing Statement:

Clinical Trials Registration:

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**F4/80 hi Resident Macrophages Contribute to Cisplatin-Induced Renal Fibrosis**

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- Long-term impacts of cisplatin are understudied; this study is the first to examine the role of macrophages in cisplatin-induced fibrosis
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- This study highlights a pathogenic role for kidney resident M2 macrophages in 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. 30% 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 impacted development of renal fibrosis and kidney injury.

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

Conclusions: These data suggest that Clodrosome depletion of F4/80hi and M2 macrophages in the kidney attenuates development of renal fibrosis following 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 FDA approval in 1978. Unfortunately, the success in treating these cancers with cisplatin is still hindered by dose-limiting nephrotoxicity. 30% of patients treated with cisplatin develop acute kidney injury (AKI).\textsuperscript{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 chronic kidney disease (CKD).\textsuperscript{4-8} The development of fibrosis following AKI has only recently begun to be modeled in rodents.\textsuperscript{9, 10} Cisplatin-induced renal fibrosis is now modeled using a repeated low dose cisplatin (RLDC) regimen in mice.\textsuperscript{11, 12} In this model, 4 weekly doses of 7-9 mg/kg cisplatin leads to development of renal fibrosis accompanied by renal immune cell infiltration.\textsuperscript{11} Comparing the traditional, high-dose cisplatin model and the RLDC model highlights the biological differences occurring in acute and chronic injury processes.\textsuperscript{13} These differences necessitate more studies on the mechanisms of AKI-to-CKD transition following 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 following AKI.\textsuperscript{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.\textsuperscript{14-16} Macrophages respond to episodes of AKI and play important roles in clearing cellular debris and orchestrating timely immune responses.\textsuperscript{17} Chronic macrophage activity, however, can lead to excessive myofibroblast activation and collagen deposition, promoting progression of renal fibrosis.\textsuperscript{18, 19}

Macrophages constitute a diverse group of cells with various origins and functions. Resident macrophages are seeded in the kidney during embryonic development.\textsuperscript{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.\textsuperscript{21-23} Infiltrating macrophages differentiate from monocytes that are recruited to the kidney following injury.\textsuperscript{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.\textsuperscript{24-26} These macrophages can be distinguished from resident macrophages by their low cell surface expression of F4/80 in mice.\textsuperscript{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 following injury and are considered “pro-inflammatory.” 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.\textsuperscript{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 following 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 following depletion of either infiltrating or resident macrophages. Our results suggest that resident macrophages contribute to fibrosis development and M2 polarization following RLDC treatment. These data suggest targeting resident macrophages could ameliorate the AKI-to-CKD transition following cisplatin treatment.

**Materials and Methods**

**Animal Experiments.** Eight-week-old male Ccr2 knockout (Ccr2\textsuperscript{-/-}) mice on a C57BL/6 background were purchased from The Jackson Laboratory (stock #004999) and bred with eight-
week-old female C57BL/6 wild type (WT) mice also purchased from The Jackson Laboratory (stock 000664). Heterozygous offspring were then aged to eight-weeks-old and cross-bred to produce littermate WT and Ccr2−/− mice. 8–11-week-old, male WT and Ccr2−/− littermates were used for this study. WT, eight-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 prior to beginning experiments. All mice were maintained on a 12:12 hour light-dark cycle and provided 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 (i.p.) injected with either 0.9% N saline vehicle or cisplatin at 9 mg/kg at 8:00 AM once a week for four weeks and euthanized three 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. Standard Macrophage Depletion Kit (Clodrosome + Encapsome) was purchased from Encapsula Nanosciences (CLD-8901). 200 μL of either Clodrosome (liposome encapsulated clodronate) or Encapsome (empty liposomes as vehicle control) was intravenously (i.v.) administered one day before the third and fourth dose of cisplatin. 100 μL of either Clodrosome or Encapsome was i.v. administered the day after the third and fourth dose of cisplatin, as well as four 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.

**Blood urea nitrogen (BUN) and neutrophil gelatinase associated lipocalin (NGAL) determination.** BUN was measured in the plasma of mice using a kit from AMS Diagnostics.
(80146, AMS Diagnostics) per the manufacturer’s instructions, and as previously published.\textsuperscript{12} ELISA for NGAL (DY1857, R&D Systems) was performed on mouse urine as previously published.\textsuperscript{12}

**Gene expression.** Total RNA was isolated from kidney cortex, and cDNA was made as previously published.\textsuperscript{12} The following pre-designed TAQman primers (Life Technologies) were used: \textit{Tnfa} (Mm00443258\_m1), \textit{Il-6} (Mm00446190\_m1), \textit{Cxcl1} (Mm04207460\_m1), \textit{Ccl2} (Mm00441242\_m1), \textit{B2m} (Mm00437762\_m1), and \textit{Arg-1} (Mm00475988\_m1). The following self-designed primers were used: \textit{Kim-1} forward AGATCCACACATGTACCAACATCAA and reverse CAGTGCCATTCCAGTCTGGTTT; \textit{Col1a1} forward CGATGGATTCCCGTTCGAGTA and reverse GTGGACATTGCCGAGGAGGAA; \textit{Tgfb} forward CAACATGTGAAGCGTGCAAAATAG and reverse ACAACTCCAGTGACGTCGAAAAGAC; \textit{Timp-1} forward GCAACTCGGAGCTGGTAAAAACGTCTTGTA. Real-time qRT-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 (SR/FG), and Masson Trichrome staining.** Alpha smooth muscle actin (\(\alpha\)SMA) immunohistochemistry for myofibroblasts and SR/FG stain for total collagen deposition was performed on paraffin embedded kidney sections as previously published.\textsuperscript{12} Masson 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 in running tap water for 10 minutes. Sections were then stained with Weigert’s iron hematoxylin solution (HT1079, Sigma-Aldrich) for 5 minutes, washed in running
tap water for 5 minutes, and stained in Biebrich Scarlet-Acid Fuschin for 5 minutes. Sections were rinsed briefly in distilled water 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. Cells were blocked with CD16/32 (101321, BioLegend) and extracellularly stained with: CD45-PerCP (103130, BioLegend), CD3e-PE-Cy7 (552774, BD Biosciences), CD19-PE-CF594 (562329, BD Biosciences), CD4-FITC (100406, BioLegend), CD8a-UV737 (612759, 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 LSRFortessa, collecting 1 million events per sample. Hierarchical gating was performed as depicted in Supplemental Figure 1. Data is 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+CD3−CD19−CD11b+F4/80hi. Infiltrating macrophages are identified as CD45+CD3−CD19−CD11b+F4/80lo.

**Statistical analysis.** Data are expressed as means ± standard error of the mean (SEM) for all experiments. Comparisons of normally distributed data sets were analyzed by either a one-way or two-way ANOVA as appropriate and group means were compared using Tukey post-tests. Correlations were determined using a linear regression model. The criteria for statistical differences were p<0.05.

**Results**
Renal macrophage infiltration correlates with kidney injury and fibrosis markers but not BUN elevation following 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.\textsuperscript{11} To further determine if macrophages were playing a role in promoting the AKI-to-CKD transition following 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 (Fig 1A). However, macrophage infiltration did correlate with elevation of mRNA kidney injury markers \textit{Kim-1} and \textit{Ccl2} (Fig 1B-C), as well as elevation of mRNA fibrotic markers \textit{Timp-1} and \textit{Col1a1} (Fig 1D-E). These data suggest that there is a relationship between macrophage accumulation and kidney injury and fibrosis following 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.

We further divided the data to assess how F4/80\textsuperscript{hi} (resident) and F4/80\textsuperscript{lo} (infiltrating) macrophages correlated with these markers. Only resident macrophage accumulation correlated with elevation of mRNA injury markers \textit{Kim-1} and \textit{Ccl2} (Fig 2A-D). However, infiltrating macrophage accumulation correlated with fibrotic mRNA markers \textit{Timp-1} and \textit{Col1a1} (Fig 2E-H). These data suggest that resident and infiltrating macrophages may be playing different roles in the AKI-to-CKD transition following RLDC.

**Ccr2\textsuperscript{-/-}** mice have reduced renal F4/80\textsuperscript{lo} infiltrating macrophages following RLDC.
We assessed immune cell infiltration in kidneys of littermate C57BL/6 wild type and Ccr2\(^{-/-}\) mice following RLDC treatment. Cisplatin treated Ccr2\(^{-/-}\) mice had reduced infiltration of CD45\(^+\) immune cells in the kidneys compared to cisplatin treated wild type mice, although results were not significant (Fig 3A). F4/80\(^{hi}\) resident macrophage levels remained unchanged in the kidneys of all groups (Fig 3B). F4/80\(^{lo}\) infiltrating macrophages were significantly increased in the kidneys of cisplatin treated wild type mice compared to vehicle treated animals. Kidneys from Ccr2\(^{-/-}\) mice did not have increased F4/80\(^{lo}\) infiltrating macrophages following RLDC treatment (Fig 3C). CD206\(^+\) M2 macrophages were similarly elevated in the kidneys of cisplatin treated wild type and Ccr2\(^{-/-}\) mice compared to vehicle treated mice (Fig 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 Ly6C\(^{hi}\) inflammatory monocyte recruitment to the kidney compared to cisplatin treated wild type mice (Fig 3E). No changes in other immune cell populations in the kidneys of Ccr2\(^{-/-}\) cisplatin treated mice compared to wild type mice were observed (Table 2 and Supplemental Figure 3). Ccr2\(^{-/-}\) mice also had basally reduced circulating macrophages and inflammatory monocytes in the blood compared to wild type mice (Supplemental Figure 4). These data suggest that Ccr2\(^{-/-}\) mice have inhibited monocyte recruitment to the kidney following RLDC treatment, leading to decreased levels of F4/80\(^{lo}\) infiltrating macrophages. It also suggests CD206\(^+\) M2 macrophage accumulation in the kidney can occur without significant contributions from the infiltrating macrophage population.

**Clodrosome depleted renal F4/80\(^{hi}\) resident macrophages and CD206\(^{+}\) M2 macrophages following RLDC.** After observing that Ccr2\(^{-/-}\) depleted F4/80\(^{lo}\) infiltrating macrophages with no effect on F4/80\(^{hi}\) renal resident populations, we sought to deplete F4/80\(^{hi}\) renal resident macrophages using liposome encapsulated clodronate (Clodrosome). Empty liposomes
(Encapsome) were also used as a vehicle control. Overall CD45+ immune cell infiltration in the kidneys was similarly elevated in all cisplatin treated groups compared to vehicle (Fig 4A). Interestingly, Clodrosome (liposome encapsulated clodronate) treatment led to a significant decrease in F4/80^{hi} resident macrophages in the kidneys compared to all other groups, including vehicle treated mice (Fig 4B). In contrast, F4/80^{lo} infiltrating macrophage populations were not affected by Clodrosome following RLDC treatment (Fig 4C). CD206+ M2 macrophage accumulation in the kidneys following cisplatin treatment was significantly reduced by Clodrosome (Fig 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 to other cisplatin treated groups (Fig 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 to other cisplatin treated mice (Table 3 and Supplemental Figure 6).

Clodrosome also increased circulating myeloid cells in the blood following RLDC treatment (Supplemental Figure 7). It is important to note that Clodrosome was given systemically and is designed to deplete all phagocytic cells. While it is possible that immune cell alterations are occurring in unobserved organs or at different timepoints in the kidney, these data suggest that Clodrosome treatment decreased resident macrophage populations in the kidney, leading to reduced CD206+ M2 macrophage accumulation following RLDC.

**Inflammatory cytokine and chemokine expression in the renal cortex is reduced by Clodrosome treatment but not Ccr2^{−/−}.** We measured mRNA expression of *Tnfa*, *Il6*, *Cxcl1*, and *Ccl2* in the kidney cortex following RLDC treatment. Ccr2^{−/−} had no effect on cisplatin-induced elevation of *Tnfa* and *Ccl2* (Fig 5A-B). Unexpectedly, the kidneys of cisplatin treated
Ccr2\(^{-/-}\) mice had increased Il6 and Cxcl1 expression compared to cisplatin treated wild type mice (Fig 5C-D). Clodrosome treatment reduced expression of Tnfa, Ccl2, and Il6 in the renal cortex following cisplatin treatment as compared to other cisplatin treated groups, but the results were not statistically significant (Fig 5E-G). No effect was observed on induction of Cxcl1 expression in the renal cortex with Clodrosome treatment (Fig 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 while Ccr2\(^{-/-}\) had no effect.** Collagen deposition was observed via Sirius Red/Fast Green (SRFG) and Masson trichrome staining. Cisplatin treated Ccr2\(^{-/-}\) mice had similar levels of collagen deposition in the kidneys compared to cisplatin treated wild type mice (Fig 6A-B). In contrast, both stains revealed decreased collagen deposition in the kidneys of Clodrosome treated mice compared to other cisplatin treated groups (Fig 6C-D). Myofibroblast accumulation was assessed using IHC staining for alpha smooth muscle actin (αSMA). RLDC treatment causes 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 wild type mice (Fig 6E). In contrast, Clodrosome treatment reduced αSMA staining in the kidneys compared to other cisplatin treated groups. αSMA staining in kidney sections of Clodrosome treated mice was also less striated and more scattered through the kidneys (Fig 6F). PDGFRα expression was also measured via flow cytometry to assess fibroblast and myofibroblast levels in the kidney following RLDC treatment.\(^{28}\) Cisplatin treated Ccr2\(^{-/-}\) mice had the same level of PDGFRα expression in the kidneys as cisplatin treated wild type mice (Fig 6G). Clodrosome treatment caused a significant
reduction in PDGFRα positive cells in the kidneys compared to Encapsome (empty liposome) treated mice, but levels were the same as cisplatin only treated mice (Fig 6H).

mRNA expression of fibrotic makers *Timp-1*, *Col1a1*, and *Tgfβ* in the kidney cortex were also assessed following RLDC treatment. Ccr2<sup>-/-</sup> had no effect on *Timp-1* elevation following RLDC (Fig 7A), while Clodrosome treatment significantly reduced *Timp-1* mRNA expression compared to other cisplatin treated groups (Fig 7B). Similarly, Ccr2<sup>-/-</sup> had no effect on elevation of *Col1a1* and *Tgfβ* mRNA in the renal cortex following RLDC treatment (Fig 7C and 7E), while Clodrosome treatment reduced mRNA expression of *Col1a1* and *Tgfβ* compared to other cisplatin treated groups, although results were not significant (Fig 7D and 7F). These data indicate that Clodrosome treatment reduced collagen deposition, myofibroblast accumulation, and expression of mRNA fibrotic markers in the kidney following RLDC while genetic deletion of Ccr2 had no effect.

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

**Discussion**
In this study, we examined how depletion of macrophage populations using either liposome encapsulated clodronate (Clodrosome) or Ccr2 genetic knockout impacted development of renal fibrosis following repeated low dose cisplatin (RLDC) treatment. The differential effects these conditions had on immune cell populations allowed us to assess potential roles of F4/80$^{hi}$ resident and F4/80$^{lo}$ infiltrating macrophages in the development of fibrosis. We observed that Clodrosome depleted F4/80$^{hi}$ renal resident and M2 macrophages following RLDC treatment but had no effect on F4/80$^{lo}$ infiltrating macrophages in the kidney. This depletion was accompanied by reduced renal fibrosis following RLDC treatment. In contrast, Ccr2$^{-/-}$ mice had reduced F4/80$^{lo}$ infiltrating macrophages in the kidney following RLDC treatment with no change in F4/80$^{hi}$ renal resident or M2 macrophage accumulation. Ccr2 genetic knockout also had no effect on fibrosis development following 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{35} It is important to consider that Clodrosome may be depleting F4/80\textsuperscript{lo} infiltrating macrophage populations at earlier unobserved timepoints.

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

In other models of renal fibrosis, liposome encapsulated clodronate depletion of macrophages has been protective.\textsuperscript{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 F4/80\textsuperscript{hi} 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 following ureteral obstruction and ischemia reperfusion.\textsuperscript{25,36} Additionally, M2 macrophage depletion by repeated administration of liposome encapsulated clodronate attenuated development of fibrosis following ureteral obstruction,\textsuperscript{40} and adoptive transfer of M2 macrophages to liposome encapsulated clodronate treated mice reversed protection from ischemia reperfusion induced fibrosis.\textsuperscript{33} In this study, we observed that Clodrosome treatment reduced F4/80\textsuperscript{hi} resident and M2 macrophages while Ccr2 genetic knockout reduced F4/80\textsuperscript{lo} infiltrating macrophages with no
effect on M2 macrophage accumulation. These data suggest that F4/80$^{hi}$ kidney resident macrophages may be more susceptible to M2 polarization than F4/80$^{lo}$ infiltrating macrophages. Although more studies are needed to examine this relationship, a predisposition towards 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 NK 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. CCR2 expression has not been found to be necessary for embryonic development or maintenance of renal resident macrophages. Therefore, the Ccr2 genetic knockout model allows us to assess how blocking renal infiltration of immune cells, predominantly monocytes and macrophages, effects development of injury and fibrosis.

Ccr2 genetic knockout has been shown to be protective in some models of renal fibrosis. In contrast, Yang et. al. demonstrated that Ccr2 genetic knockout, while protective from ischemia reperfusion-induced AKI, worsened fibrosis development. Our study demonstrated that Ccr2$^{-/-}$ in mice had no effect on development of fibrosis following 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, but this signaling axis may not be as important in the AKI-to-CKD transition following RLDC treatment. The CCR2/CCL2 independence of RLDC-induced fibrosis highlights unique mechanisms of fibrosis development as compared to 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.\textsuperscript{45, 46} Furthermore, KIM-1 has been implicated in mediating macrophage activity in models of both
AKI and renal fibrosis.\textsuperscript{46, 47} Macrophages are also known to be important regulators of
myofibroblasts.\textsuperscript{48, 49} It is possible 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.\textsuperscript{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.\textsuperscript{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

The authors have nothing to disclose.

Funding

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Kidney Diseases Grants (R01DK124112 and 3 R01 DK124112-01S1 to L. J. Siskind and
F31DK126400 to S. M. Sears).
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The authors would like to thank Dr. James George and all those who participated in the UAB-UCSD O’Brien Center Workshop on Flow Cytometry for the Nephrology Researcher (P30 DK079337) for their help in establishing a protocol for analysis of immune cells in the kidney.

Author Contributions

S.M.S., L.J.B., and L.J.S. conceived and designed research; S.M.S. and A.A.V. performed animal experiments; S.M.S., A.A.V., A.M.K., and M.A.D. performed animal dissections; S.M.S., Z.K., and R.M. designed and optimized flow cytometry analysis protocol; S.M.S., M.A.D., P.P.S., and G.B.O. analyzed data; S.M.S., L.J.B., and L.J.S. interpreted results of experiments; S.M.S. prepared figures and drafted manuscript; S.M.S., M.A.D., L.J.B., and L.J.S. edited and revised manuscript; all authors approved final version of manuscript.

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Supplemental Figure 5. Immune Cell Depletion in the Kidney following Clodronate Treatment as Percentage of CD45+ Cells

Supplemental Figure 6. Immune Cell Depletion in the Kidney Following Clodronate Treatment
Supplemental Figure 7. Immune Cell Depletion in the Blood Following Clodronate Treatment

References


Table 1. Clodrosome/Encapsome dosing schedule with RLDC treatments.

<table>
<thead>
<tr>
<th>Sunday</th>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
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<tbody>
<tr>
<td></td>
<td>9mg/kg CIS</td>
<td></td>
<td></td>
<td></td>
<td>100 uL Clod</td>
<td>Euthanize</td>
</tr>
<tr>
<td></td>
<td>or Saline</td>
<td></td>
<td></td>
<td></td>
<td>or Encap</td>
<td></td>
</tr>
<tr>
<td>200 uL Clod</td>
<td>9mg/kg CIS</td>
<td>100 uL</td>
<td>100 uL</td>
<td></td>
<td>or Encap</td>
<td></td>
</tr>
<tr>
<td>or Encap</td>
<td>or Saline</td>
<td>Clod</td>
<td>Clod</td>
<td></td>
<td>or Encap</td>
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</tr>
<tr>
<td>200 uL Clod</td>
<td>9mg/kg CIS</td>
<td>100 uL</td>
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<td>Euthanize</td>
<td>or Encap</td>
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<tr>
<td>or Encap</td>
<td>or Saline</td>
<td>Clod</td>
<td></td>
<td></td>
<td>or Encap</td>
<td></td>
</tr>
</tbody>
</table>

CIS= Cisplatin, Clod= Clodrosome, Encap= Encapsome
Table 2. Immune cell alterations in the kidney of cisplatin treated Ccr2−/− compared to wild type mice.

<table>
<thead>
<tr>
<th>Immune Cell</th>
<th>Vehicle</th>
<th>Cisplatin</th>
<th>Vehicle</th>
<th>Cisplatin</th>
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<tr>
<td></td>
<td>WT</td>
<td>Ccr2−/−</td>
<td>WT</td>
<td>Ccr2−/−</td>
</tr>
<tr>
<td>CD45+ Immune Cells</td>
<td>1.677±0.190</td>
<td>1.825±0.162</td>
<td>6.573±0.633</td>
<td>4.989±0.367</td>
</tr>
<tr>
<td>CD19+ B Cells</td>
<td>0.223±0.031</td>
<td>0.242±0.030</td>
<td>0.541±0.056</td>
<td>0.454±0.039</td>
</tr>
<tr>
<td>CD3+ T Cells</td>
<td>0.178±0.024</td>
<td>0.200±0.019</td>
<td>0.569±0.048</td>
<td>0.519±0.046</td>
</tr>
<tr>
<td>CD4+ T Cells</td>
<td>0.075±0.012</td>
<td>0.087±0.008</td>
<td>0.253±0.021</td>
<td>0.235±0.022</td>
</tr>
<tr>
<td>CD8+ T Cells</td>
<td>0.040±0.004</td>
<td>0.048±0.005</td>
<td>0.149±0.016</td>
<td>0.136±0.012</td>
</tr>
<tr>
<td>CD3+CD4−CD8−</td>
<td>0.065±0.008</td>
<td>0.063±0.010</td>
<td>0.157±0.012</td>
<td>0.142±0.015</td>
</tr>
<tr>
<td>CD11b+ Myeloid Cells</td>
<td>0.608±0.087</td>
<td>0.687±0.108</td>
<td>1.209±0.096</td>
<td>0.963±0.064</td>
</tr>
<tr>
<td>Ly6G+ Neutrophils</td>
<td>0.037±0.011</td>
<td>0.087±0.051</td>
<td>0.183±0.021</td>
<td>0.231±0.034</td>
</tr>
<tr>
<td>Ly6C hi Inflammatory Monocytes</td>
<td>0.035±0.006</td>
<td>0.017±0.006</td>
<td>0.079±0.012</td>
<td>0.030±0.005*</td>
</tr>
<tr>
<td>Ly6C lo Monocytes</td>
<td>0.045±0.010</td>
<td>0.088±0.049</td>
<td>0.271±0.027</td>
<td>0.258±0.033</td>
</tr>
<tr>
<td>F4/80 hi Resident Macs</td>
<td>0.375±0.067</td>
<td>0.392±0.046</td>
<td>0.527±0.050</td>
<td>0.447±0.028</td>
</tr>
<tr>
<td>F4/80 lo Infiltrating Macs</td>
<td>0.122±0.015</td>
<td>0.098±0.012</td>
<td>0.345±0.031</td>
<td>0.151±0.013*</td>
</tr>
<tr>
<td>CD206+ M2 Macrophages</td>
<td>0.223±0.050</td>
<td>0.232±0.036</td>
<td>0.846±0.087</td>
<td>0.679±0.055</td>
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<tr>
<td>PDGFRα Expression</td>
<td>14.167±0.885</td>
<td>14.533±1.834</td>
<td>35.733±1.848</td>
<td>33.014±1.700</td>
</tr>
</tbody>
</table>

Data presented as percentage of indicated immune cell population of single cells collected per sample. Values are mean ± SEM. * indicates a significant reduction in Ccr2−/− cisplatin treated kidneys compared to wild type cisplatin treated kidneys. WT= wild type C57BL/6, Ccr2−/−= Ccr2 genetic knockout.
Table 3. Immune cell alterations in the kidney of CIS+Clod treated mice compared to CIS alone and CIS+Encap.

<table>
<thead>
<tr>
<th>Immune Cell</th>
<th>VEH</th>
<th>CIS</th>
<th>CIS+Clod</th>
<th>CIS+Encap</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45+ Immune Cells</td>
<td>1.744±0.1817</td>
<td>4.627±0.3563</td>
<td>4.509±0.3763</td>
<td>5.335±0.4519</td>
</tr>
<tr>
<td>CD19+ B Cells</td>
<td>0.192±0.0434</td>
<td>0.543±0.0497</td>
<td>0.567±0.0505</td>
<td>0.573±0.0445</td>
</tr>
<tr>
<td>CD3+ T Cells</td>
<td>0.422±0.0752</td>
<td>0.901±0.0832</td>
<td>0.666±0.0656</td>
<td>0.903±0.1156</td>
</tr>
<tr>
<td>CD4+ T Cells</td>
<td>0.128±0.0206</td>
<td>0.268±0.0241</td>
<td>0.265±0.0261</td>
<td>0.257±0.0252</td>
</tr>
<tr>
<td>CD8+ T Cells</td>
<td>0.074±0.0098</td>
<td>0.218±0.0144</td>
<td>0.210±0.0174</td>
<td>0.247±0.0306</td>
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<tr>
<td>CD3+CD4-CD8-</td>
<td>0.216±0.0548</td>
<td>0.412±0.0472</td>
<td>0.190±0.0480*</td>
<td>0.396±0.0665</td>
</tr>
<tr>
<td>CD11b+ Myeloid Cells</td>
<td>0.742±0.0792</td>
<td>1.59±0.1117</td>
<td>1.183±0.2094</td>
<td>1.821±0.1324</td>
</tr>
<tr>
<td>Ly6G+ Neutrophils</td>
<td>0.106±0.0250</td>
<td>0.272±0.0380</td>
<td>0.403±0.0461*</td>
<td>0.264±0.0178</td>
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<tr>
<td>Ly6C hi Inflammatory Monocytes</td>
<td>0.066±0.0098</td>
<td>0.155±0.0257</td>
<td>0.408±0.0608*</td>
<td>0.142±0.0317</td>
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<tr>
<td>Ly6C lo Monocytes</td>
<td>0.124±0.024</td>
<td>0.396±0.0392</td>
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<tr>
<td>F4/80 hi Resident Macs</td>
<td>0.388±0.0511</td>
<td>0.579±0.0820</td>
<td>0.088±0.0589*</td>
<td>0.515±0.0959</td>
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<tr>
<td>F4/80 lo Infiltrating Macs</td>
<td>0.184±0.0291</td>
<td>0.643±0.0595</td>
<td>0.634±0.1256</td>
<td>0.934±0.1085</td>
</tr>
<tr>
<td>CD206+ M2 Macrophages</td>
<td>0.182±0.0240</td>
<td>0.642±0.0934</td>
<td>0.123±0.0508*</td>
<td>0.576±0.1188</td>
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<tr>
<td>PDGFRα Expression</td>
<td>2.412±0.1959</td>
<td>8.31±0.9712</td>
<td>7.621±0.7666*</td>
<td>10.92±0.8561</td>
</tr>
</tbody>
</table>

Data presented as percentage of indicated immune cell population of single cells collected per sample. Values are mean ± SEM. * indicates a significant change in CIS+Clod treated kidneys compared to either CIS or CIS+Encap treated kidneys. VEH= vehicle, CIS= Cisplatin, CIS+Clod= Cisplatin + Clodrosome, CIS+Encap= Cisplatin + Encapsome.
**Figure Legends**

**Figure 1.** Macrophage infiltration correlates with kidney injury and fibrosis markers but not BUN following RLDC treatment. Renal infiltration of total F4/80 macrophages in cisplatin treated wild type C57BL/6 male mice (n=20) was plotted against (A) BUN, (B-C) kidney injury mRNA makers, and (D-E) fibrotic mRNA markers following RLDC treatment. Significant correlations were determined as p<0.05 using a linear regression model.

**Figure 2.** Resident macrophages correlate with kidney injury and fibrosis markers following RLDC treatment. The percentage of F4/80 hi resident and F4/80 lo infiltrating macrophages in the kidney of cisplatin treated C57BL/6 male mice (n=20) following 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.

**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 indentify (A) CD45+ total immune cells, (B) F4/80 hi resident macrophages, (C) F4/80 lo infiltrating macrophages, (D) CD206+ M2 macrophages, and (E) Ly6C hi inflammatory monocytes present in the kidney following 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.

**Figure 4.** Clodrosome depleted renal resident and M2 macrophages with no effect on infiltrating macrophages. Whole kidneys were homogenized into a single cell suspension for flow cytometric analysis of immune cells. Hierarchical gating was performed to indentify (A) CD45+ total immune cells, (B) F4/80 hi resident macrophages, (C) F4/80 lo infiltrating macrophages, and (D) CD206+ M2 macrophages present in the kidney following RLDC treatment. Data are presented as percentage of positively labeled cells from the total number of single cells counted for each sample. (D) mRNA expression of Arg-1 was measured relative to B2m in the kidney cortex. Statistical analysis was determined by one-way ANOVA followed by Tukey post-test. Significance was determined at p<0.05. VEH= vehicle, CIS= cisplatin, CIS+Clod= cisplatin plus Clodrosome, CIS+Encap= cisplatin plus Encapsome. *= significantly different from VEH, ^= significantly different from CIS, #= significantly different from CIS+Encap.

**Figure 5.** Clodrosome reduced inflammatory cytokine and chemokine induction following RLDC while Ccr2-/- had no effect. RNA was isolated from kidney cortex tissue. mRNA expression of (A,E) Tnfa, (B,F) Ccl2, (C,G) Il6, and (D,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. VEH= vehicle, CIS= cisplatin, CIS+Clod= cisplatin plus Clodrosome, CIS+Encap= cisplatin plus Encapsome. (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 while Ccr2-/- had no effect. (A,C) Sirius red/fast green, (B,D) Masson trichrome, and (E-F) aSMA IHC staining of paraffin embedded kidney sections. (G-H) percent PDGFRα expressing cells of single cells...
counted in each sample run on 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. VEH= vehicle, CIS= cisplatin, CIS+Clod= cisplatin plus Clodrosome, CIS+Encap= cisplatin plus Encapsome. (G) * = significantly different from WT VEH, ^= significantly different from Ccr2-/- VEH. (H) *= significantly different from VEH, #= significantly different from CIS+Encap.

Figure 7. Clodrosome blunted RLDC induced fibrotic mRNA markers while Ccr2-/- had no effect. RNA was extracted from kidney cortex tissue. mRNA expression of (A-B) Timp-1, (C-D) Col 1a1, and (E-F) Tgfb 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. VEH= vehicle, CIS= cisplatin, CIS+Clod= cisplatin plus Clodrosome, CIS+Encap= cisplatin plus Encapsome. (A,C,E) *= significantly different from WT VEH, ^= significantly different from Ccr2-/- VEH. (B,D,F) *= significantly different from VEH, ^= significantly different from CIS+Encap.

Figure 8. Clodrosome reduced Kim-1 expression but neither Clodrosome or Ccr2-/- altered BUN or NGAL. (A-B) BUN was measured from plasma. (C-D) NGAL was measured from urine. (E-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. VEH= vehicle, CIS= cisplatin, CIS+Clod= cisplatin plus Clodrosome, CIS+Encap= cisplatin plus Encapsome. (A,C,E) *= significantly different from WT VEH, ^= significantly different from Ccr2-/- VEH. (B,D,F) *= significantly different from VEH, ^= significantly different from CIS+Encap.
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Figure 2. Resident macrophages correlate with kidney injury and fibrosis markers following RLDC treatment.
Figure 3

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Figure 4. Clodrosome depleted renal resident and M2 macrophages with no effect on infiltrating macrophages.
Figure 5. Clodrosome reduced inflammatory cytokine and chemokine induction following RLDC while Ccr2-/- had no effect.
Figure 6. Clodrosome ameliorated RLDC induced renal fibrosis while Ccr2-/ had no effect.
Figure 7. Clodosome blunted RLDC induced fibrotic mRNA markers while Ccr2/- had no effect.
Figure 8. Clodrosome reduced Kim-1 expression but neither Clodrosome or Ccr2-/ altered BUN or NGAL.
Supplemental Data

• Supplemental Figure 1. Hierarchical gating strategy
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Supplemental Figure 7. Immune Cell Depletion in the Blood Following Clodronate Treatment. Data are presented as percentage indicated immune cell population of single cells collected for each sample. Values in table are means ± SEM. VEH= vehicle, CIS= cisplatin, CIS+Clod= cisplatin plus Clodrosome, CIS+Encap= cisplatin plus Encapsome. *- significantly different from VEH, ^- significantly different from CIS, #- significantly different from CIS+Encap.