Selective depletion of a CD64-expressing phagocyte subset mediates protection against toxic kidney injury and failure

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Edited by Kenneth M. Murphy, Washington University in St. Louis School of Medicine, St. Louis, MO, and approved August 4, 2021 (received for review October 25, 2020)

Dendritic cells (DC), macrophages, and monocytes, collectively known as mononuclear phagocytes (MPs), critically control tissue homeostasis and immune defense. However, there is a paucity of models allowing to selectively manipulate subsets of these cells in specific tissues. The steady-state adult kidney contains four MP subsets with Clec9a-expression history that include the main conventional DC1 (cDC1) and cDC2 subsets as well as two subsets marked by CD64 but varying levels of F4/80. How each of these MP subsets contributes to the different phases of acute kidney injury and repair is unknown. We created a mouse model with a Cre-inducible lox-STOP-lox-diphtheria toxin receptor cassette under control of the endogenous CD64 locus that allows for diphtheria toxin–mediated depletion of CD64-expressing MPs without affecting cDC1, cDC2, or other leukocytes in the kidney. Combined with specific depletion of cDC1 and cDC2, we revisited the role of MPs in cisplatin-induced kidney injury. We found that the intrinsic potency reported for CD11c+ cells to limit cisplatin toxicity is specifically attributed to CD64+ MPs, while cDC1 and cDC2 were dispensable. Thus, we report a mouse model allowing for selective depletion of a specific subset of renal MPs. Our findings in cisplatin-induced injury underscore the value of dissecting the functions of individual MP subsets in kidney disease, which may enable therapeutic targeting of specific immune components in the absence of general immunosuppression.

Mononuclear phagocytes (MP) critically control barrier integrity, tissue homeostasis, and immune responses and include monocytes, macrophages, and dendritic cells (DCs) (1). Despite access to several “pan-macrophage” and “pan-DC” models for gene manipulation and cell depletion, there is a paucity in models to target macrophage and DC subsets selectively and in specific tissues (2, 3). Additionally, macrophages, monocytes, and DCs are notoriously difficult to distinguish based on surface markers, such as CD11c, which can be up- or down-regulated (1, 3, 4). As a result, the unique functions of MP subtypes in immunity, inflammation, wound repair, and specific tissues remain debated.

Most macrophages arise during embryogenesis, whereas conventional or classical DCs (cDCs), plasmacytoid DCs (pDCs), and monocytes arise from committed bone marrow progenitors (1, 2, 5). We have previously shown that precursors of cDCs in mice express the C type lectin receptor DNGR-1 (also known as Clec9a, encoded by the Clec9a gene) (6). Although DNGR-1 is also expressed in differentiated type 1 cDCs (cDC1) and, to a lower level, on pDCs, it is not expressed in other lymphoid and myeloid lineages, including precursors for monocytes, granulocytes, or lymphoid cells (6–8). By crossing Clec9a+cre mice to Rosalox-stop-lox-yellow fluorescent protein (YFP) or Rosalox-stop-lox-TOMATO mice (6, 9)

we have demonstrated that the adult kidney contains four subsets of MPs with prominent Clec9a-expression history, indicative of cDC origin (6, 9). These include the main cDC1 and cDC2 subtypes as well as CD64-expressing CD11b+ and F4/80+ cells (6, 9). These subsets are phenotypically, functionally, and transcriptionally distinct, express CD11c, and are uniformly marked by MHC (major histocompatibility complex) II (6, 9). Although F4/80+ MPs phenotypically and transcriptionally resemble embryonic-derived macrophages (9, 10) in adulthood they acquire prominent Clec9a-expression history (9). Because their affiliation as macrophages or DCs remains controversial (9–15), we henceforth refer to these cells as F4/80hi MPs.

Cell depletion using “pan-macrophage” and “pan-DC” models that would affect multiple kidney MP subsets has shown that MPs are critically involved in kidney immune defense but also contribute to kidney injury and subsequent tissue repair (4, 13, 16, 17). Thus, defining the functions of specific subsets of MPs in kidney disease could help to dissect their contributions to kidney pathology and repair, which could subsequently be targeted for

Significance

There continues to be a paucity in models to target mononuclear phagocyte (MP) subsets selectively and in specific tissues. We report a mouse model allowing for selective depletion of a specific subset of kidney MPs that is based on a Cre-inducible lox-STOP-lox-diphtheria toxin receptor gene controlled by the endogenous CD64 promoter. Combined with specific targeting of conventional DC1 (cDC1) and cDC2, we revealed that CD64+ MPs account for the reported ability of CD11c+ cells to limit cisplatin nephrotoxicity, while cDC1 and cDC2 are dispensable. Our study highlights that individual MP subsets have unique functions in kidney pathology. Combined with various CRE drivers, CD46-lox-STOP-lox-diphtheria toxin receptor receptor mice might be used to study CD64-expressing cells in other tissues.

Author contributions: B.U.S. performed research; D.P. designed and cloned the CD64-iDTR targeting vector; N.S. and B.U.S. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2022311118/-/DCSupplemental.

Published September 13, 2021.
therapeutic purposes. Toxic acute kidney injury (AKI) is a common side effect resulting from cisplatin nephrotoxicity during cancer therapy (15). In mice, cisplatin treatment similarly induces nephrotoxicity, but the role of individual MP subsets in disease remains unclear. Preemptive depletion of CD11c+ cells with diphtheria toxin (DT) in mice in which the CD11c promoter drives expression of a DT receptor (DTR; CD11cCreDTR mice) aggregates cisplatin-induced AKI (18). Because CD11c is not exclusively restricted to DCs but can also be found on some tissue macrophages as well as activated B and T cells, depletion in this model affects multiple cell types (1, 3). Notably, preemptive depletion of all phagocytic cells, including CD11c+ cells, using clodronate liposomes, does not alter the severity of cisplatin-induced AKI (19). These results seem conflicting when assuming that both models affect similar MPs. However, clodronate liposome treatment also depletes blood monocytes and both interventions may deplete multiple cell types with partially overlapping phenotype but distinct functions in toxic tissue injury. Thus, more specific models are needed to investigate the role of MP subsets in kidney disease.

Currently, tools for cell depletion do not accurately distinguish between the different types of kidney MPs. We hypothesized that specific depletion of the various subsets of kidney resident MPs before exposing mice to cisplatin would help to identify the MP subset that can mitigate nephrotoxic kidney injury and failure. We generated a mouse model with expression of a lox-STOP-lox-DTR construct under control of the endogenous Cd44 locus. Through an intercross with Clec9aCre mice, these mice allow for DT-mediated depletion of F4/80hi MPs in the kidney, while cDC1, cDC2, and other leukocytes are not affected. Importantly, DT treatment did not affect CD64-expressing macrophages/monocyte-derived cells, DCs, or other leukocytes in various other organs. In combination with models to deplete cDC1 and cDC2, we demonstrate that the protective effect previously attributed to CD11c+ cells in cisplatin AKI is predominantly mediated by CD64 expressing MPs but independent of cDC1 and cDC2.

Results

MPs with Clec9a-Expression History in Cisplatin Nephrotoxicity. We first investigated leukocyte dynamics in Clec9aCre RosaDTR mice with cisplatin nephrotoxicity to define the phenotype of MPs with Clec9a-expression history in toxic AKI. Cisplatin treatment of mice leads to acute tubular necrosis and kidney failure evident by a rapid increase in serum creatinine and urea levels after cisplatin exposure. We have shown that 3 d after cisplatin treatment, kidneys contain five prominent Clec9a-expression history: cDC1, cDC2, CD11bhi, and F4/80hi cells as well as a small population of MHCIIloF4/80hi cells that arises from down-regulation of MHCII on F4/80hi cells (9). Similarly, Clec9a-expression history was found in cDC1, cDC2, CD11bhi, and F4/80hi cells 48 h after cisplatin treatment but MHCIIloF4/80hi cells were barely detectable at this time (SI Appendix, Fig. S1 A–C and F). Notably, the frequency and number of cDC1 and cDC2 were reduced in kidneys from cisplatin-treated mice compared to controls 48 and 72 h after treatment, whereas frequency and number of F4/80hi and CD11bhi cells remained constant (Fig. 1 A–C and SI Appendix, Fig. S1 B–F). CD19 B cells and CD3+ T cells remained unchanged, while monocytes (MHCII+CD11b+Ly6Ghi cells) and neutrophils (MHCII+CD11b+Ly6Chi cells) were increased as sign of necroinflammation (20). Thus, in addition to our previous observation that F4/80hi cells alter their phenotype and transcriptional profile in response to cisplatin (9), we observed that cDC1 and cDC2 are lost from the inflamed kidney, leading us to hypothesize that MP subsets may play different roles in this disease model.

To assess the involvement of cells with Clec9a-expression history in cisplatin-induced AKI, we used Clec9a-cre mice crossed to Rosa26loxSTOP-loxDTR mice (Clec9aCre RosaDTR) (21, 22). A total 24 h after a single injection of DT, kidney cDC1 and cDC2 as well as F4/80hi cells were efficiently depleted in Clec9aCre RosaDTR mice, whereas CD11bhi DCs and other leukocytes were not affected (Fig. 1 D–F). We next subjected Clec9aCre RosaDTR and Clec9aCre RosaDTR control mice to a single injection of DT and 24 h later treated the mice with cisplatin (Fig. 1 G–K). Several DT-treated Clec9aCre RosaDTR mice showed a body weight loss exceeding 20% within 48 h after cisplatin treatment and thus reached experimental termination criteria. All mice were therefore analyzed 48 h after cisplatin treatment. cDCs and F4/80hi MPs were efficiently depleted at this time point (Fig. 1 F–K), and we observed a trend for higher serum urea and creatinine levels in Clec9aCre RosaDTR mice than in Clec9aCre RosaDTR control mice, indicating more severe kidney failure (Fig. 1H). Of note, CD11cCreDTR mice show higher mortality upon cisplatin exposure and have to be analyzed 48 h after disease induction (18). These data suggested that the protective effect previously attributed to CD11c+ leukocytes during cisplatin nephrotoxicity is mediated by MPs with Clec9a-expression history, leading us to investigate the cellular interplay of these MPs in more detail.

Loss of cDC1 Does Not Influence the Severity of Cisplatin-Induced AKI.

To specifically deplete cDC1 in renal disease, we obtained Xcr1DTR mice (Xcr1DTR). XCR-1 is a chemokine receptor specifically expressed by cDC1 across tissues and Xcr1DTR mice allow for efficient depletion of cDC1 in lymphoid tissues (23). Indeed, DT treatment of Xcr1DTR mice efficiently and selectively depleted cDC1 from kidneys 24 h later, whereas other MP or leukocyte populations remained unaltered (Fig. 2 A–D). We next assessed the influence of cDC1 depletion on the severity of cisplatin-induced AKI. Xcr1DTR and littermate control mice were treated with DT 24 h prior to injection with cisplatin. A total 72 h after cisplatin treatment, all mice showed clear signs of tubular necrosis and loss of kidney function, but serum urea and creatinine levels were similar between the two groups (Fig. 2F). A reduction of cDC1 in kidneys from DT- and cisplatin-treated Xcr1DTR compared to control mice was confirmed by flow cytometry (Fig. 2 G–I), indicating that a single injection of DT is sufficient to deplete cDC1 for the course of the 3-d experiment. As expected, the frequency and number of other DC subsets was unaltered (Fig. 2 H and I). Notably, Xcr1DTR mice showed reduced frequency and number of neutrophils (Fig. 2 H and I), suggesting that cDC1 depletion may affect neutrophils in the treated kidney although neutrophils are dispensable for the development for cisplatin AKI (20, 24). Taken together, these data indicate that selective loss of cDC1 does not influence disease severity in cisplatin-induced AKI.

Clec9aCre Irf4fl/fl Mice Do Not Show Altered Disease Severity in Cisplatin-Induced AKI. Loss of Irf4 inhibits cDC2 development in some organs and strongly impacts the function of the remaining cDC2 (25–31). Kidney cDC2 and CD11bhi MPs express higher levels of interferon regulatory factor 4 (IRF4) compared to cDC1 and F4/80hi MPs, in which IRF4 expression is low (9). To specifically deplete cDC1 in renal disease, we obtained Clec9a-cre mice crossed to Irf4floxed mice (32). As expected (27), cDC2 in kidney and spleen of Clec9aCre Irf4floxed mice were reduced compared to littermate controls, whereas other MPs or leukocytes were unaffected (Fig. 3 A–C and SI Appendix, Fig. S2). Irf4floxed mice carry an enhanced green fluorescent protein (eGFP) reporter expressed upon Cre-mediated recombination of the lox sites, allowing to trace cells with successful DNA recombination by eGFP fluorescence (32). As expected, recombination efficiency was high in the four MP subsets that show Clec9a-expression history but not in other leukocytes (Fig. 3 D and E), confirming specificity and efficiency of Clec9aCre-mediated DNA recombination. CD11bhi DCs and the few remaining cDC2 in kidneys from Clec9aCre Irf4floxed mice showed reduced levels of IRF4 protein compared to littermate controls (Fig. 3F), suggesting...
**Fig. 1.** MPs with Clec9a-expression history in cisplatin nephrotoxicity. (A–E) Clec9a<sup>Cre</sup>Rosa<sup>YFP</sup> mice were injected with NaCl or cisplatin. A total 72 h later, renal leukocytes were analyzed by flow cytometry. Total leukocytes (A) as well as number (B) and frequency (C) of the indicated populations per kidney were calculated and plotted. Data are combined from two independent experiments. (D–F) Clec9a<sup>Cre</sup>Rosa<sup>YFP</sup> mice were treated with PBS or DT, and littermate controls (Clec9a<sup>Cre</sup>Rosa<sup>YFP</sup>, Clec9a<sup>Cre</sup>Roza<sup>YFP</sup>) were injected with DT. A total 24 h later, total leukocytes (D) per kidney were quantified, and the number (E) and frequency (F) of the indicated populations per kidney were calculated. Data are combined from two independent experiments. (G) Schematic representation of experimental design. (H) Serum creatinine and blood urea nitrogen (BUN) levels. (I–K) Kidneys were analyzed by flow cytometry. Total leukocytes (I) as well as number (J) and frequency (K) of the indicated populations per kidney were calculated and plotted. Data are representative of two independent experiments with n = 3 and similar results. Each dot represents one mouse. Horizontal bars represent mean, error bars represent SD. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Fig. 2. Loss of cDC1 does not influence susceptibility to cisplatin-induced AKI. (A–D) Xcr1<sup>DTIR</sup> mice were injected with PBS or DT, and 24 h later, kidney leukocytes were analyzed by flow cytometry. (A) Live CD45.2<sup>+</sup> MHCII<sup>+</sup> cells were gated and subdivided into CD11c<sup>−</sup>CD64<sup>−</sup> and CD64<sup>+</sup> cells as well as CD11c<sup>+</sup>CD64<sup>−</sup> cells representing B cells. CD24<sup>+</sup> cDC1 and CD11b<sup>+</sup> cDC2 were identified in the CD11c<sup>−</sup>CD64<sup>−</sup> fraction. CD64<sup>+</sup> cells were further divided into F4/80<sup>hi</sup> and CD11b<sup>hi</sup> DCs. MHCII<sup>−</sup> cells were subdivided into CD11b<sup>+</sup> cells (entailing mostly neutrophils and monocytes) and CD11b<sup>−</sup> cells (containing mostly T cells). Total leukocytes per kidney (B) as well as number (C) and frequency (D) of indicated populations per kidney are shown. Data are combined from two independent experiments. (E–I) Xcr1<sup>DTIR</sup> or littermate control mice were injected with DT followed by cisplatin treatment (15 mg/kg body weight [b. w.]) 24 h later. A total 72 h after cisplatin injection, mice were analyzed. (E) Schematic representation of experimental design. (F) Serum creatinine and BUN levels were quantified. Total leukocytes per kidney (G) as well as number (H) and frequency (I) of indicated populations per kidney are shown (Fig. 1A). Data are combined from two independent experiments. Each dot represents one mouse. Horizontal bars represent mean, error bars represent SD, **<i>P</i> < 0.01, and ****<i>P</i> < 0.0001.

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functional impairment of these cells (25–29). It is noteworthy that despite efficient excision of the floxed allele in Clec9acreIrf4fl/fl mice (Fig. 3D and E), staining with the anti-IRF4 antibody was higher than with the isotype-matched control antibody. It is possible that this reflects residual IRF4 protein resulting from transcription though the internal stop of the GFP reporter (32), but it could also reflect IRF4 protein expression from an earlier state of cell development or nonspecific staining of the anti-IRF4 antibody compared to isotype-matched control antibody. A total 3 d after treatment of Clec9acreIrf4fl/fl mice and Clec9awtIrf4fl/fl littermate controls with cisplatin, serum creatinine and urea levels were similar between the two groups (Fig. 3G and H). Thus, cDC2 play a negligible role in the induction of cisplatin-induced AKI. We therefore speculated that the protective effect previously attributed to CD11c+ cells in cisplatin-induced renal injury must be mediated by CD64 expressing MPs.
Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> Mice Allow to Deplete F4/80<sup>hi</sup> MPs from Kidneys. To specifically deplete CD64<sup>+</sup> MPs from kidneys, we constructed a mouse model in which the endogenous Cd64 locus drives expression of a lox-STOP-lox-DTR construct (SI Appendix, Fig. S34). In these mice, DTR expression is expected in cells with active transcription of the Cd64 locus but only when the lox sites were previously excised. Crossing these mice to Clec9a<sup>cre</sup> mice would therefore lead to specific expression of DTR on CD64-expressing cells with Clec9a<sup>cre</sup> expression history, allowing for specific depletion of kidney CD11b<sup>hi</sup> and F4/80<sup>hi</sup> MPs (Fig. 4A). Successful generation of Cd64<sup>iDTR</sup> mice was confirmed using Southern Blot (SI Appendix, Fig. S3B). To circumvent inflammatory defects associated with loss of CD64, we inserted the DTR transgene using a self-cleaving 2A sequence leaving CD64 intact, as confirmed by flow cytometry (SI Appendix, Fig. S3C). In Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice, we detected unidomal DTR expression on renal F4/80<sup>hi</sup> MPs to a lower extent on CD11b<sup>hi</sup> MPs but not on cDC1 and cDC2 or monocytes (SI Appendix, Fig. SSD), as expected. Accordingly, a single injection of DT in Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice lead to efficient depletion of F4/80<sup>hi</sup> MPs (stxford reduction, Fig. 4 D and E) from kidneys 24 h later without affecting cDC1, cDC2, or other leukocytes, including Ly6C<sup>+</sup> monocytes, neutrophils, B cells, and T cells (Fig. 4 B–D and SI Appendix, Fig. S3E). Notably, treatment of Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice with DT induced a mild loss of CD11b<sup>hi</sup> MPs, despite the fact that these cells express CD64 and have Clec9a-expression history (9). This indicates a reduced sensitivity of CD11b<sup>hi</sup> MPs to DT treatment, consistent with low DTR expression, which is likely a consequence of low CD64 expression (SI Appendix, Fig. S3 C and D). Notably, DT treatment of Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice caused a significant increase in MHCII<sup>+</sup>Ly6C<sup>+</sup> cells (Fig. 4 B–E), that also expressed CD11b and CD64 and could thus constitute tissue-infiltrating monocytes (33, 34). Loss of F4/80<sup>hi</sup> MPs was also confirmed by microscopy, which revealed a fourfold reduction of F4/80<sup>hi</sup> cells in kidney cortex and a 2.2-fold reduction in medulla of DT-treated Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice (Fig. 4 F and G).

We additionally performed depletion analysis in Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice across various lymphoid and nonlymphoid tissues. DT injection in Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice did not alter frequency or number of cDC1, cDC2, and CD64<sup>+</sup> cells, which include various types of macrophages and monocytes in spleen, skin, lung, brain, and liver when compared to DT-treated controls (SI Appendix, Figs. S4 and S5). Other leukocytes were also unaltered (SI Appendix, Figs. S4 and S5). We did not observe signs of systemic neutrophilia or monocytosis (35) apart from a slight increase of neutrophils in liver of Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice (SI Appendix, Figs. S4 and S5). Serum levels of TNF and CCL2 (MCP-1), which promote monocyte recruitment, were increased in DT-treated Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> compared to control mice (Fig. 4F). Thus, Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice allow for selective depletion of CD64<sup>+</sup> cells with Clec9a-expression history in kidney but do not affect cDC1, cDC2, or other CD64<sup>+</sup> cells across lymphoid and nonlymphoid organs.

Despite depleting a major population of leukocytes from the kidney during homeostasis, DT treatment of Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice did not cause significant alterations of serum urea and creatinine levels (Fig. 4I), indicating normal excretory kidney function. DT-treated Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice showed similar kidney architecture to DT-treated littermates as judged by Periodic Acid Schiff (PAS) staining (Fig. 4H and SI Appendix, Fig. S3F). Thus, DT treatment of Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice selectively depletes renal F4/80<sup>hi</sup> MPs and to a lower extent CD11b<sup>hi</sup> DCs without affecting kidney function and architecture. Taken together, Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice can be used to specifically assess the role of F4/80<sup>hi</sup> MPs in kidney injury.

**Loss of F4/80<sup>hi</sup> MPs Increases Susceptibility to Cisplatin-Induced Renal Injury.** To address the role of F4/80<sup>hi</sup> MPs in cisplatin-induced AKI, we injected Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice with DT (Fig. 5A). As controls, we used cohorts of Clec9a<sup>cre</sup>Cd64<sup>wt</sup> and Clec9a<sup>cre</sup>Cd64<sup>−/−</sup> mice that were injected with phosphate buffered saline (PBS) or DT, respectively. Mice were treated with cisplatin or NaCl as control 24 h later (Fig. 5A). Mice were analyzed 48 h after cisplatin treatment because weight loss of many DT-treated Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice had reached 20% at this point, necessitating termination of the experiment. Analysis of kidney leukocytes showed a reduction of total leukocytes per kidney in Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice, which predominately reflected the loss of increased apoptosis of renal tubular cells (Fig. 5C–E). We also observed a twofold reduction of CD11b<sup>hi</sup> DCs and cDC2 in kidneys from DT-treated Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice compared to controls (Fig. 5 D and E). Importantly, compared to NaCl treatment, cisplatin did not induce CD64 expression on immune cell populations that normally do not express it, although higher levels of CD64 were observed on monocytes, F4/80<sup>hi</sup>, and CD11b<sup>hi</sup> MPs (SI Appendix, Fig. S6A). These data indicate that the intersection of Clec9a<sup>cre</sup> and Cd64<sup>iDTR</sup> remains restricted to F4/80<sup>hi</sup> and CD11b<sup>hi</sup> MPs (SI Appendix, Fig. S6A). Thus, a single DT treatment is sufficient to deplete F4/80<sup>hi</sup> MPs for the course of this 3-d experiment and that cells resembling F4/80<sup>hi</sup> MPs are not generated by compensatory mechanisms of emergency hematopoiesis, such as monocyte differentiation. Despite alterations in frequencies, the total numbers of monocytes, neutrophils, and B and T cells per kidney were not affected (Fig. 5D). No differences were found in splenic DCs, macrophages, monocytes, neutrophils, and B and T cells between these two groups, indicating that depletion in Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice does not cause systemic neutrophilia, as observed in other DC depletion models (SI Appendix, Fig. S6B). MHCII<sup>+</sup>Ly6C<sup>+</sup> cells resembling monocytes were found in kidneys from both Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> and control mice after cisplatin treatment (Fig. 5D). Serum creatinine and urine levels were significantly increased in cisplatin- compared to NaCl-treated mice, confirming induction of kidney damage and loss of tubular function (Fig. 5B). Induction of kidney damage was further confirmed by measuring expression of kidney injury molecule 1 (also known as T cell Ig and mucin-containing 1/TIM-1), which was significantly increased in cisplatin compared to NaCl treatment (Fig. 5F). Serum urea was higher in Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice compared to control-treated littermates 48 h after cisplatin treatment, suggesting increased susceptibility to disease (Fig. 5B), whereas creatinine levels showed no difference between the two groups at this time (Fig. 5B). In line with increased serum urea in DT-treated Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice, these mice also showed a more pronounced apoptosis of renal tubular cells, as assessed by cleaved Caspase 3 staining (Fig. 5G) and more severe tubular damage (Fig. 5H). In conclusion, loss of F4/80<sup>hi</sup> MPs leads to more pronounced cisplatin AKI.

**Discussion**

Kidney MP subtypes exhibit a prominent phenotypic overlap, and these cells have predominately been studied as a functionally homogeneous entity. In the present study, we had hypothesized that specific depletion of MP subsets before exposing mice to cisplatin would help to identify the subtype that can mitigate nephrotoxic kidney injury and failure. Indeed, the generation of a Cd64<sup>Cre-loxloxsato-DTR</sup> mouse model allowed us to selectively deplete a subtype of CD64-expressing MPs but not cDC1, cDC2, or other leukocytes in the kidney. Importantly, CD64-expressing leukocytes in spleen, liver, lung, skin, and brain were unaffected by DT treatment of Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice. In combination with models to specifically deplete cDC1 and cDC2, we could demonstrate that kidney CD64<sup>+</sup> MPs but not cDC1 or cDC2 attenuate kidney injury upon cisplatin exposure. Clec9a<sup>cre</sup>Cd64<sup>iDTR</sup> mice provide a powerful model to study the specific functions of F4/80<sup>hi</sup> MPs in kidney pathology. Importantly, combined with macrophage- or monocyte-specific CRE drivers, Cd64<sup>iDTR</sup> mice may also serve as a valuable tool to study the functions of other CD64-expressing leukocytes in immunity.

cDC1, though best known for their ability to promote cytotoxic T cell and Th1 responses (36–38), also influence inflammatory
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Fig. 4. Clec9acreCd64iDTR mice allow for specific depletion of renal F4/80+ MPs. (A) Schematic representation of cell depletion in Clec9acreCd64iDTR mice. (B–H) Clec9acreCreCD64oiDTR and Clec9acreCreCd64wt mice were injected with DT and analyzed 24 h later. (B) Kidney leukocytes were analyzed by flow cytometry. Live CD45.2+MHCI+ cells were gated and divided into Ly6C+ and Ly6C− cells, as indicated. Ly6C+ cells were further divided into CD11c+CD64+ and CD64− cells, and within the CD11c+CD64+ fraction, CD24+ cDC1 and CD11b+ cDC2 were gated. CD64+ cells were divided into F4/80+ and D11b+ DCs. (C) Total kidney leukocytes as well as number (D) and frequency (E) of the indicated populations per kidney are shown. (F and G) Kidney cryosections from DT-treated Clec9acreCreCd64iDTR and Clec9acreCreCd64wt mice were stained for MHCl (blue), CD64 (red), and F4/80 (green). MHCl+CD64+F4/80+ cells were identified, and the number of these cells per field in renal cortex and medulla were quantified and plotted. (Scale bar: 100 μm.) The average number of cells per six fields of view is shown (G). (H) Normal kidney architecture and function in DT-treated Clec9acreCreCd64iDTR mice. Representative PAS staining of renal medulla 24 h after DT treatment is shown. (Scale bar: 50 μm.) (I) Serum creatinine and BUN levels 24 h after DT treatment. (J) Serum levels of indicated cytokines 24 h after DT treatment. Each dot represents one mouse. Horizontal bars represent mean, error bars represent SD. *P < 0.05, **P < 0.01, and ****P < 0.0001. Data from are combined from at least two independent experiments.
Fig. 5. Loss of F4/80hi MPs increases susceptibility to cisplatin-induced AKI. (A–G) Cisplatin-induced AKI in Clec9a™Cd64™ mice. (A) Experimental design. Clec9a™Cd64™ mice were injected with DT to induce cell depletion. As controls for cell depletion and DT treatment, Clec9a™Cd64™ and Clec9a™Cd64™ mice were injected with PBS or DT, respectively. Control mice were treated with either NaCl or cisplatin to induce AKI 24 h later, as indicated. Cisplatin/NaCl injected mice were analyzed 48 h later. (B) BUN and serum creatinine levels are shown. (C–E) Kidneys from mice were analyzed 48 h after cisplatin treatment by flow cytometry. Total leukocytes per kidney (C) as well as number (D) and frequency (E) of the indicated populations per kidney was calculated. (F) mRNA from kidney was isolated and analyzed for expression of Kim-1. Expression was normalized to Hprt. (G) Kidneys were analyzed for cleaved Caspase-3 by immunohistochemistry. The average number of cleaved Caspase-3-positive tubular epithelial cells per 10 high power fields of view was calculated. (H) Representative histology of kidney cortex by hematoxylin and eosin staining and histopathological score of tubular damage. (Scale bar: 30 μm.) Each dot represents one mouse. Horizontal bars represent mean, error bars represent SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and only statistically significant differences are indicated. Data are combined from three independent experiments.
processes by controlling neutrophils (39–41). Selective depletion of CD1c in Clec9aCreCd64iDTR mice prior to cisplatin treatment did not alter serum creatinine and urea levels, demonstrating that these cells do not influence severity of cisplatin-induced AKI. This is possibly to be expected because few CD1c remain in kidneys 3 d after cisplatin treatment. Notably, neutrophils were reduced in kidneys from DT-treated Clec9aCreCd64iDTR mice after cisplatin exposure, suggesting that CD1c may regulate neutrophil survival or recruitment into the kidney (39–41). Although neutrophils are dispensable in cisplatin AKI, they contribute to the pathology of kidney ischemia reperfusion injury (20, 24, 42). Thus, CD1c may contribute to necroinflammation in situations when neutrophils contribute to pathology.

Loss of IRF4 inhibits cDC2 development in some organs and strongly impacts cDC2 function (25–31); we crossed Clec9aCre mice to Irf4flo/flo mice. Because CDc2 and CD11bhi cells in kidneys express high levels of IRF4 (9), we expected to generate mice lacking functional cDC2 and CD11bhi cells. As observed when using Clec9aCre as a driver to delete IRF4 (27), Clec9aCreIrf4flo/flo mice exhibited a modest reduction of cDC2 in steady-state kidneys. In the remaining cDC2 and in CD11bhi DCs, Irf4 protein expression was decreased, as expected, suggesting functional impairment (25–31). Clec9aCreIrf4flo/flo mice did not show altered susceptibility to cisplatin-induced AKI, indicating that cDC2 and possibly CD11bhi cells do not mitigate nephrotoxic kidney injury. Constitutive lack of IRF4 leads to increased tubular necrosis, neutrophil influx, and inflammatory cytokine expression 24 h after renal artery clamping (43). Although the cellular mediator of IRF4 action in this model is unclear, a CD11c+ population is involved (43). Because CDc2 and the CD11c+ cell type in kidney expressing highest levels of IRF4 (9), it is likely that IRF4 acts in these cells. This can be addressed in Clec9aCreIrf4flo/flo mice independent of secondary effects of loss of IRF4 in other immune populations, such as B or T cells.

To be able to address the specific functions of renal CD64-expressing DCs in immunity and kidney pathology, we generated a mouse model, in which the endogenous CD64 locus drives expression of a lox-STOP-lox-DTR construct. To circumvent inflammatory defects associated with loss of CD64 (44, 45), we inserted the DTR transgene using a self-claving 2A sequence behind the full-length Cd64 coding sequence, leaving CD64 intact. By crossing Clec9aCre to Cd64flo/flo mice, we expected to gain DTR expression on cells in which CD64 is actively transcribed and which show evidence of Clec9a-expression history, rendering these cells susceptible to DT treatment. Indeed, DT treatment of Clec9aCreCd64iDTR mice specifically and efficiently depleted CD64-expressing DCs from the kidney, while cDC1, cDC2, and other leukocytes were unaffected. CD64-expressing cells in other organs, which constitute mainly macrophages or monocyte-derived cells (15) were also unaffected. Importantly, F4/80hi cells showed a stronger reduction following DT treatment than CD11bhi DCs, despite the fact that CD11bhi cells also exhibit Clec9aCre expression history and express CD64. We attribute this reduced susceptibility of CD11bhi DCs to DT treatment to lower DTR expression. Interestingly, the efficiency of F4/80hi MP depletion was increased following cisplatin treatment compared to steady state, which could be related to a loss in kidney architecture during nephrotoxicity. After cisplatin treatment, kidney CDc2 numbers were twofold lower in DT-treated Clec9aCreCd64iDTR mice than in control mice. Because cisplatin causes a 10-fold reduction of kidney cDC1 and cDC2 over NaCl treatment within 48 h, we believe these differences to be a consequence of inflammatory changes associated with disease rather than an indicator of nonspecific cell depletion. Whether cisplatin induces cDC1/cDC2 death or whether these cells actively migrate out of the organ is unclear. Taken together, Clec9aCreCd64iDTR mice specifically deplete renal F4/80hi MPs. Because CD64 is also induced on some DCs during infection with listeria and respiratory viruses (46, 47), Clec9aCreCd64iDTR mice will provide a useful tool to study the specific functions of inflammatory DCs.

DT-mediated depletion of CD11c+ cells can induce functional neutrophilia that influences immune responses in the kidney (35). A total 24 h after DT treatment, Clec9aCreCd64iDTR mice did not show infiltration of neutrophils into the kidney or other organs, barring a small increase of monocytes and neutrophils in liver. Following DT treatment, Clec9aCreCd64iDTR mice exhibited normal excretory kidney function and architecture, validating their use to study the role of F4/80hi cells in kidney injury. Following depletion of F4/80hi cells, we observed a significant increase in MHCIiLy6c+ cells that also expressed CD64 and CD11b and therefore resembled monocytes, which acquire MHCIi expression upon differentiation in tissues (15, 48). It is possible that these MHCIiLy6c+ cells constitute monocytes attempting to fill the niche of F4/80hi cells in the kidney, similar to monocytes replenishing the niche of Kupffer cells following depletion (33, 49). However, F4/80hi MPs remain strongly reduced during cisplatin AKI even 3 d after DT treatment, suggesting that if replenishment of the cells by monocytes takes place, it is inefficient. Rather, MHCIiLy6c+ cells may constitute a distinct subtype of inflammatory monocytes, as reported to appear in spleen after depletion of CD11c+ cells (34).

Exposing DT-treated Clec9aCreCd64iDTR mice to cisplatin leads to more severe kidney damage and loss of excretory function, allowing to specifically attribute the protective effect previously reported for CD11c+ cells in cisplatin-induced AKI to the function of kidney F4/80hi MPs. The fact that serum urea and not creatinine levels were increased in DT-treated Clec9aCreCd64iDTR mice could be related to the time point of analysis. Overall, kidney damage was mild 48 h after cisplatin exposure, and serum urea is a more sensitive biomarker of renal excretory function in mice than creatinine. In seeming paradox to the increased susceptibility of DT-treated Clec9aCreCd64iDTR mice to cisplatin nephrotoxicity, these mice showed lower expression of the tubular damage marker Kim-1/Tim-1 compared to control mice. However, Kim-1/Tim-1 serves as a receptor for phosphatidylserine exposed on apoptotic cells (50–52), and in cisplatin-induced AKI, Kim-1/Tim-1 dampens inflammation by inhibiting inflammatory cytokine production from epithelial cells (53). These data suggest that F4/80hi cells may ameliorate cisplatin-induced renal injury in part through interacting with renal tubular cells and by reducing their inflammatory properties.

Cisplatin is a commonly used chemotherapeutic agent with a major limiting factor: cytotoxic side effects on normal tissues, including tubular necrosis, leading to loss of kidney function as well as neurotoxicity and damage of the gastrointestinal epithelium (54–56). Cisplatin additionally induces loss of adipocyte tissue (57). In our experiments, cisplatin-induced weight loss in all mice, but Clec9aCreCd64iDTR mice reached a critical weight loss defined by our animal license earlier, necessitating termination of the experiments after 48 h. This phenotype is reminiscent of the higher mortality reported for DT-treated CD11cDTR mice (18). Because weight loss in cisplatin AKI relates to side effects on other tissues, adverse effects of cell depletion in CD11cDTR and Clec9aCreCd64iDTR mice on tissue integrity and cisplatin-induced pathology in other organs will have to be investigated in future studies. In terms of kidney pathology, our studies demonstrate that distinct MP subsets have specific roles in renal disease. An increased understanding of the specific functions of DC and macrophage subsets in kidney pathology will pave the way to design therapeutic approaches to specifically target individual immune components during kidney injury and disease.

Materials and Methods

Mice. Clec9aCreCD64iDTR mice were backcrossed 10 generations onto C57BL/6J. Clec9aCreCD64iDTR mice were derived from crosses of Clec9aCreIrf4flo/flo males and Irf4flo/flo females. Genotyping was performed by PCR using primers Clec9aCre (5′-gaacattaacagccatccagaag-3′) and Clec9aCre (5′-cacttcagacgtgtgagcagactgt-3′) for the forward and reverse direction, respectively.

https://doi.org/10.1073/pnas.2022311118
pathogen-free conditions. Mice at the age of 8 to 14 wk were used for experiments. All animal procedures were performed in accordance with national and institutional guidelines for animal welfare and approved by the Regierung of Oberbayern.

**Cloning of CD64 Targeting Vector and Generation of CD64-lox-STOP-lox-DTR Mice and Immunofluorescence Microscopy.** Refer to SI Appendix.

**Southern Blot.** Southern blot was performed using the Digoxigenin system for labeling and detection of nucleic acid (Roche Applied Science) according to the manufacturer's instructions. Phil and KpnI-HF restriction enzymes were used. The probe1 located downstream of the 3' homology arm was generated using primes 5′-ACTTGGCCGCGGGAATCTCAT and 5′-TCTGCGTTACGTAGGACAGCGA. Neo probe located in the Neo cassette was generated using primes 5′-AATATCCGGGGTACGAAGC and 5′-CATTGAAAGATGCTGTACG.

**Cell Isolation.** Organs were isolated after perfusion with ice-cold PBS. Kidneys, lungs, and brains were cut into small pieces and digested in 2 ml of RPMI medium (Thermo Fisher Scientific) with 200 μM collagenase IV (Worthington) and 0.2 mg/ml DNase I (Roche) for 1 h at 37 °C with constant shaking (120 rpm). After digestion, cells were passed through a 70-μm strainer and washed with cold fluorescence-activated cell sorting (FACS) buffer (PBS [Sigma-Aldrich] containing 1% fetal bovine serum [FBS] [Sigma-Aldrich], 2.5 mM ethylenediaminetetraacetic acid [Invitrogen], and 0.02% sodium azide [Sigma-Aldrich]). Leukocytes were enriched with 70–37.3% Percoll gradient by centrifugation (935 × g for 30 min at room temperature), collected at the 37–37% interface, washed once, and resuspended in FACS buffer for analysis. Isotonic Percoll was made by adding 9 parts of Percoll to 1 part of 10× concentrated PBS.

**Spleen.** Spleens were minced and digested in 1 ml of RPMI for 30 min as above. Erythrocytes were lysed with ammonium-chloride-potassium lysing buffer for 2 min on ice, washed, and resuspended in FACS buffer for analysis.

**Liver.** Liver was digested in 2 ml PBS containing Mg2+ and Ca2+ (Sigma-Aldrich, Cat. DB662) with 1 mg/ml collagenase IV, 60 μU/ml DNase I, 2.4 mg/ml dispase II (Roche), and 3% FBS (Sigma-Aldrich) for 30 min at 37 °C with constant shaking (120 rpm). Cells were passed through a 100-μm strainer and centrifuged for 3 min at 50 × g and 4 °C to pellet hepatocytes. A supernatant containing liver leukocytes was washed with FACS buffer (320 × g, 7 min, 4 °C) and resuspended in FACS buffer for analysis.

**Trypsinization.** For skin isolation, mouse ears were split into dorsal and ventral parts and placed on PBS containing 2.5 mg/ml dispase II for 2 h at 37 °C or overnight at 4 °C. Epidermis and dermis were then separated and digested in 2 ml RPMI with 200 μU/ml collagenase IV and 0.2 mg/ml DNase I for 1 h at 37 °C with constant shaking (200 rpm). After digestion, cells were passed through a 70-μm strainer, washed with cold FACS buffer, and enriched using a Percoll gradient as described above.

**Flow Cytometry.** Flow cytometry was performed on an LSR Fortessa (BD Biosciences) with subsequent data analysis using FlowJo software (Tree Star, Inc.). Cells were quantified by using CountBright Absolute Counting Beads (Thermo Fisher Scientific).

**Real-Time qPCR.** Total messenger RNA (mRNA) from kidneys was isolated by using RNeasy Midi Kit (Qiagen) according to the manufacturer's protocol. Complementary DNA was synthesized by using SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol. Real-time polymerase chain reaction (PCR) analysis was performed using SYBR Green Fast master mix (Applied Biosystems) according to the manufacturer's manual on a StepOnePlus Real-Time PCR Systems (Applied Biosystems) using the relative standard curve method and primers for Kim-1 (Kim-1 forward: 5′-CTGGAATGGCACTGTGACATCC; Kim-1 reverse: 5′-GGGGCTGTACTGCTTAACCAG) (7). Hprt forward: 5′-Hprt forward: 5′-TCACTCGAAGGGGGAGCAATA; Hprt reverse: 5′-GGGGCCTGATCTGCCAAGC (7).

**Cytokine Detection.** Cytokines in mouse serum were quantified by flow cytometry using LEGENDplex Mouse Inflammation Panel (Biolegend).

**Statistical Analysis.** Statistical significance was calculated using two-tailed t test or Mann-Whitney U test (for nonparametric data) in Prism 7 software (GraphPad). Multiple comparison was performed by using one-way ANOVA with Bonferroni correction. A P value <0.05 was considered significant.

**Data Availability.** All study data are included in the article and/or SI Appendix.

**ACKNOWLEDGMENTS.** We thank members of B.U.S.’s laboratory and Peter Murray for helpful discussions and critical reading of the manuscript. We acknowledge the Core Facility Flow Cytometry and the Core Facility Bio-imaging at the Biomedical Center, Ludwig Maximilian University of Munich for providing equipment and expertise. This work was funded by the Deutshe Forschungsgemeinschaft (DFG, German Research Foundation) Emmy Noether grant: Schr 1444/1-1 (to B.U.S.) and Project-ID 360372040-SFB 1335 (project 8, to B.U.S.; project 201 to K.S.). Work in the Schraml laboratory is also funded by a European Research Council Starting Grant Awarded to B.U.S. (ERC-2016-STG-715182). H.J.A. was supported by grants from the DFG (AN372/4-4 and 30-1). We thank Natalio Garbi (IEI, Bonn) for providing XCR1-BV421 (clone ZET), anti-IRF4-AF647 (clone 1B1), anti-IL-5G3, anti-CD24-BUV395 (clone M1/69).

**siRNA.**siRNA transfected cells were scored on sections stained with Hematoxylin-Eosin and PAS according to standard protocols. Cleaved Caspase 3 immunohistochemistry was performed on a BondRx system (Leica, Wetzlar, Germany). Briefly, the slides were deparaffinized, and heat-mediated retrieval (ER1) was applied for 20 min. A primary antibody against cleaved Caspase 3 was used (1:150, Cell Signals Technology, Cat. CS-0155), and binding was visualized using a PolyRefine Detection Kit (Leica, Wetzlar, Germany) without postprimary antibody. All slides were scanned with an AT2 scanning system (Leica, Wetzlar, Germany).

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**ACKNOWLEDGMENTS.** We thank members of B.U.S.’s laboratory and Peter Murray for helpful discussions and critical reading of the manuscript. We acknowledge the Core Facility Flow Cytometry and the Core Facility Bio-imaging at the Biomedical Center, Ludwig Maximilian University of Munich for providing equipment and expertise. This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) Emmy Noether grant: Schr 1444/1-1 (to B.U.S.) and Project-ID 360372040-SFB 1335 (project 8, to B.U.S.; project 201 to K.S.). Work in the Schraml laboratory is also funded by a European Research Council Starting Grant Awarded to B.U.S. (ERC-2016-STG-715182). H.J.A. was supported by grants from the DFG (AN372/4-4 and 30-1). We thank Natalio Garbi (IEI, Bonn) for providing the plasmid containing the DTR sequence.
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