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Proximal tubule-derived Colony Stimulating Factor-1 mediates polarization of renal macrophages and dendritic cells, and recovery in acute kidney injury

Yinqiu Wang[1], **Jian Chang**[1], **Bing Yao**[1], **Aolei Niu**[1], **Emily Kelly**[1], **Matthew C. Breeggemann**[1], **Sherry L. Abboud Werner**[4], **Raymond C. Harris**[1,2,5], and **Ming-Zhi Zhang**[1,3]

[1]Department of Medicine, University of Texas Health Science Center at San Antonio

[2]Molecular Physiology and Biophysics, University of Texas Health Science Center at San Antonio

[3]Cancer Biology, University of Texas Health Science Center at San Antonio

[4]Vanderbilt University School of Medicine, University of Texas Health Science Center at San Antonio

[5]Nashville Veterans Affairs Hospital

Abstract

Infiltrating cells play an important role in both the development of and recovery from acute kidney injury (AKI). Macrophages and renal dendritic cells are of particular interest because they can exhibit distinctly different functional phenotypes, broadly characterized as proinflammatory (M1) or tissue reparative (M2). Resident renal macrophages and dendritic cells participate in recovery from AKI in response to either ischemia/reperfusion or a model of selective proximal tubule injury induced by diphtheria toxin-induced apoptosis in transgenic mice expressing the human diphtheria toxin receptor on proximal tubule cells. Colony Stimulating Factor-1 (CSF-1) is an important factor mediating the recovery from AKI, and CSF-1 can stimulate macrophage and dendritic cell proliferation and polarization during the recovery phase of AKI. The kidney, and specifically the proximal tubule, is a major source of intrarenal CSF-1 production in response to AKI. We induced selective deletion of proximal tubule CSF-1 to determine its role in expansion and proliferation of renal macrophages and dendritic cells and in recovery from AKI. In both models of AKI, there was decreased M2 polarization, delayed functional and structural recovery and increased tubulointerstitial fibrosis. Thus, intrarenal CSF-1 is an important mediator of macrophage/dendritic cell polarization and recovery from AKI.

Correspondence: R.C. Harris M.D, Division of Nephrology, C3121 MCN, Vanderbilt University School of Medicine and Nashville Veterans Affairs Hospital, Nashville, TN 37232, Tel: (615) 322-2150, ; Email: ray.harris@vanderbilt.edu, or, Ming-Zhi Zhang, M.D. Division of Nephrology S-3223, Vanderbilt University School of Medicine, Nashville, TN 37232, ; Email: ming-zhi.zhang@vanderbilt.edu

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Keywords

AKI; macrophage; dendritic cell; diphtheria toxin; proximal tubule; CSF-1; c-fms

Introduction

Acute kidney injury (AKI) is defined as an abrupt decrease in renal function. The reported incidence of AKI varies from 5% in all hospitalized patients to 30–50% in intensive care units [1]. Renal tubule epithelia, and especially cells of the proximal tubule, are the primary targets for injury. Depending upon the nature and extent of the injurious stimuli, tubular cells lose functional integrity transiently or die by necrosis or apoptosis. It is currently thought that epithelial cell repair in response to AKI is accomplished primarily by the de-differentiation, migration and proliferation of surviving tubular cells, with ultimate restoration of tissue integrity [2, 3]. However, it remains uncertain for the major factor to stimulate the epithelial repair. Also unclear is the cell type(s) generating the stimulating factor in response to AKI.

There is increasing evidence that infiltrating cells play an important role in the initiation and propagation of the tubule dysfunction and structural injury [4, 5, 6, 7–12]. The role of macrophages is of particular interest because they can exhibit distinctly different functional phenotypes, broadly characterized as proinflammatory (M1 or “classically activated”) and tissue reparative (M2 or “alternatively activated”) phenotypes [13, 14].

Recent studies have indicated that Colony Stimulating Factor-1 (CSF-1) is an important factor mediating the recovery from acute kidney injury (AKI) [15, 16]. CSF-1 can stimulate macrophage and dendritic cell proliferation and polarization [16, 17, 18, 19–21], and recent studies have indicated an important role for these cells in recovery from AKI [16, 20, 22].

Previous studies [15, 23, 16] have also indicated that the kidney, and specifically the proximal tubule, is a major source of intrarenal CSF-1 production in response to AKI. However, CSF-1 has also been shown to stimulate renal epithelial cell proliferation directly after ischemia/reperfusion injury to the kidney[15]. Therefore, to determine the role of proximal tubule CSF-1 expression in macrophage/dendritic cell-mediated proliferation and differentiation following acute tubule injury, we utilized a previously described model of selective proximal tubule injury (proximal tubule DTR transgenic mice[16]) as well as ischemia-reperfusion-induced AKI. For both models of AKI, we induced selective deletion of proximal tubule CSF-1 in order to determine the role of proximal tubule-generated CSF-1 in expansion and proliferation of renal macrophages and dendritic cells and in recovery from acute injury.

Results

In order to determine the effect of proximal tubule CSF-1 on recovery from acute proximal tubule injury, we generated transgenic mice expressing the human diphtheria toxin receptor in proximal tubule (DTR) with selective proximal tubule CSF-1 deletion (γ-*GT Cre:CSF-1*$^{f/f}$ with littermate CSF-*1*$^{f/f}$ as wild type control) as described in MATERIALS AND

METHODS. In *CSF-1$^{f/f}$* mice, immunoreactive CSF-1 expression was detected primarily in proximal tubules (Figure 1A); immunostaining was inhibited by preincubation of the antibody with a blocking peptide or in the absence of primary antibody (Figure 1B&C). In γ-*GT Cre:CSF-1$^{f/f}$* mice, CSF-1 immunoreactivity was markedly decreased (Figure 1D).

As we have previously described, administration of diphtheria toxin (DT, 200 ng/g, i.p) resulted in functional renal dysfunction, as manifested by increased BUN, which peaked at 4 days after DT administration (Figure 2A). Six days after DT administration, *CSF-1$^{f/f}$* mice demonstrated phosphorylation of the CSF-1 receptor (c-fms) in tubules and interstitial cells, which was markedly decreased in γ-*GT Cre:CSF-1$^{f/f}$* mice (Figure 1E&F&G). Immunofluorescent staining showed that there was a marked increase in F4/80 and p-c-fms/CSF-1R double positive cells (monocytes/macrophages/dendritic cells) in *CSF-1$^{f/f}$* mice compared to γ-*GT Cre: CSF-1$^{f/f}$* mice (Figure 1F). The γ-*GT Cre:CSF-1$^{f/f}$* mice also had delayed functional recovery (Figure 2A) and increased expression of the proximal injury marker (Kim-1) (Figure 2B). In addition, γ-*GT Cre:CSF-1$^{f/f}$* mice had increased evidence of oxidative stress, as indicated by nitrotyrosine staining (Figure 2C).

Our previous study had indicated that the primary injury induced by DT administration in the proximal tubule DTR transgenic mice is epithelial cell apoptosis, with minimal necrosis, but secondary necrosis occurs if there is inadequate clearance of apoptotic cells and epithelial cell regeneration [16]. At six days following DT administration, there was marked increased expression of a marker of secondary necrosis, the DAMP, HMGB1, in the γ-*GT Cre:CSF-1$^{f/f}$* mice (Figure 2D). There was also increased neutrophil infiltration in the kidneys of γ-*GT Cre:CSF-1$^{f/f}$* mice (Figure 2E).

Our previous studies indicated an important role for CSF-1 in renal macrophage and dendritic cell proliferation and differentiation after acute tubule injury [16]. As determined by flow cytometry, the number of renal F4/80-positive cells (monocytes/macrophages/dendritic cells) was similar between γ-*GT Cre:CSF-1$^{f/f}$* mice and *CSF-1$^{f/f}$* mice before induction of AKI by DT injection (10^4 cells/g tissue: 15.83 ± 1.74 vs. 16.31 ± 1.06 of *CSF-1$^{f/f}$* mice, n = 4, $p = 0.78$). However, 6 days after DT administration, there were significantly fewer F4/80-positive cells in γ-*GT Cre:CSF-1$^{f/f}$* mice compared to *CSF-1$^{f/f}$* mice (10^4 cells/g tissue: 19.35 ± 1.85 vs. 32.01 ± 2.45, n = 4, $p < 0.01$). In addition, there were significant differences in F4/80-positive cells expressing CD11b and CD11c (10^4 cells/g tissue: 16.61 ± 1.90 vs. 28.50 ± 2.20 of *CSF-1$^{f/f}$* mice, n = 4, $p < 0.01$) (Figure 3A), consistent with our previous results indicating that in this model, the predominant population of F4/80+ cells express both CD11b and CD11c, markers of intrinsic renal dendritic cells [16]. We utilized immunoreactive CD11b magnetic beads to isolate renal macrophages and dendritic cells 6 days after DT administration, as we have previously described [16] and determined that γ-*GT Cre:CSF-1$^{f/f}$* mice had significantly decreased mRNA expression of M2 markers, mannose receptor (CD206), IL-4Ra , TGF-s and Arginase 1 (Arg-1), but had comparable mRNA expression of M1 markers, including iNOS, TNF-α, CCL-2 and IL-23, compared to wild type mice (Figure 3B). Similar results were achieved using macrophages/dendritic cells isolated with FACS (Supplementary Figure 1). In addition, there was significantly decreased CD206 immunoreactivity in kidneys of γ-*GT Cre:CSF-1$^{f/f}$* mice (cells/hpf: cortex: 0.72

± 0.15 vs. 2.29 ± 0.13, n = 4, $p < 0.01$; outer medulla: 1.67 ± 0.24 vs. 4.32 ± 0. 47, n = 4, $p < 0.01$) (Figures 3C).

Consistent with the important role of renal M2 macrophages/dendritic cells in mediating recovery from acute tubule injury and preventing development of long term fibrotic sequelae, there was a significant increase in tubulointerstitial fibrosis, indicated by picrosirius red staining of fibrillary collagen in γ-*GT Cre:CSF-1*$^{f/f}$ mice at 4 weeks after administration of DT (Figure 4A). There were also marked increases in α-SMA, CTGF, fibronection, and nitrosylated tyrosine (a marker of oxidative stress) in γ-*GT Cre:CSF-1*$^{f/f}$ mice (Figure 4B).

In addition to CSF-1, GM-CSF produced in renal proximal tubule has also been reported to promote macrophage M2 polarization and to accelerate functional recovery from ischemia/reperfusion AKI [24]. Therefore, we investigated renal GM-CSF expression in both *CSF-1*$^{f/f}$ mice and *γ-GT Cre:CSF-1*$^{f/f}$ mice six days after DT injection. As indicated in Supplementary Figure 2, there were minimal differences in renal GM-CSF mRNA levels between *CSF-1*$^{f/f}$ mice and *γ-GT Cre:CSF-1*$^{f/f}$ mice six days after DT injection.

Although γ-GT is highly expressed in kidney, its low activity has been reported in lymphoid organs, such as spleen [25]. In order to exclude the possibility that reduced CSF-1 produced in spleen and bone marrow could contribute to delayed functional recovery from AKI as well as decreased macrophage/dendritic cell proliferation and polarization in γ-*GT Cre:CSF-1*$^{f/f}$ mice, we measured γ-GT and CSF-1 expression in kidney, spleen and bone marrow using qPCR. As indicated in Supplementary 3, although CSF-1 mRNA levels were relatively close in all three organs, γ-GT mRNA levels were extremely low in both spleen and bone marrow compared to kidney (γ-GT mRNA levels: kidney: 124.532 ± 27.035; spleen: 0.007 ± 0.002; bone marrow: 0.026 ± 0.008, n = 3 in each group).

In order to investigate the relative importance of CSF-1 produced in renal proximal tubular epithelial cells in the role of macrophages in recovery from AKI, *γ-GT Cre:CSF-1*$^{f/f}$ mice were treated with DT with or without macrophage depletion (clodronate treatment). As indicated in Supplementary Figure 4, macrophage depletion augmented the severity of kidney injury, suggesting that macrophage polarization and proliferation in response to AKI does not only depend on CSF-1 produced in proximal tubular epithelial cells.

Previous studies by us and others[16, 22] have also demonstrated an important role for M2 macrophages/dendritic cells in recovery from I/R injury. Our preliminary study showed that severe ischemia/reperfusion injury led to high mortality in *γ-GT Cre:CSF-1*$^{f/f}$ mice. Therefore, we chose to induce moderate injury in our study. Three days after I/R injury, γ-*GT Cre:CSF-1*$^{f/f}$ mice had more severe kidney injury as indicated by higher BUN compared to *CSF-1*$^{f/f}$ mice (mg/dl: 65.00 ± 2.31 vs. 47.75 ± 4.71 of *CSF-1*$^{f/f}$ mice, n = 7, $p < 0.05$) (Figure 5A). Five days after I/R injury, there was more renal neutrophil infiltration in γ-*GT Cre:CSF-1*$^{f/f}$ mice (10^4 cells/g tissue: 12.23 ± 2.25 vs. 5.14 ± 0.29 of *CSF-1*$^{f/f}$ mice, n = 4, $p < 0.05$). Five days after I/R injury, renal monocytes/macrophages/dendritic cells isolated from γ-*GT Cre:CSF-1*$^{f/f}$ mice had significantly decreased mRNA expression of M2 markers, mannose receptor (CD206), IL-4Ra , TGF-β and arginase 1 (Figure 5C), but unchanged M1

markers, similar to that seen in DT-mediated AKI. Four weeks after I/R injury, there was significantly increased tubulointerstitial fibrosis (Figure 6A) and increased α-SMA immunoreactivity (Figure 6B) in γ-*GT Cre:CSF-1*$^{f/f}$ mice.

Discussion

These studies definitively show the important role of proximal tubule-generated CSF-1 production in recovery from acute kidney injury and further indicate the proximal tubule to be an important source of CSF-1 that is necessary for renal macrophage/dendritic cell proliferation and differentiation to an M2 phenotype. With selective proximal tubule CSF-1 deletion, there was not only delayed functional recovery from injury but also development of persistent tubulointerstitial fibrosis, indicating incomplete recovery from injury.

The inhibition of CSF-1R activation in the proximal tubule in mice with selective proximal tubule CSF-1 deletion indicates an autocrine effect and is consistent with the previous studies of Menke et al. that CSF-1 may directly act on renal tubule epithelial cells to promote recovery after injury [15]. CSF-1 administration accelerates recovery from ischemia-induced AKI and reduces subsequent fibrosis [20]. Furthermore, the decreased number of renal macrophages/dendritic cells and decreased expression of M2 markers in the γ-*GT Cre:CSF-1*$^{f/f}$ mice during recovery is consistent with our previous study indicating an important role for these cells in recovery [16]. A similar role for CSF-1 to promote an M2 phenotype in renal macrophages was observed with CSF-1 administration with ischemia-reperfusion injury [20]. The current studies indicate that both the proliferation and the polarization of the renal macrophages and dendritic cells during the reparative phase are regulated in part by proximal tubule CSF-1 production.

In models of renal fibrosis, such as obstructive uropathy, macrophage proliferation is an important component of macrophage accumulation, and inhibition of CSF-1 signaling with an anti-c-fms antibody or selective c-fms tyrosine kinase inhibitors decreased macrophage proliferation[26, 27]. Our previous studies indicated that following tubule injury in the DTR model, the major increases in renal macrophages/dendritic cells result from increased renal macrophage/dendritic cell proliferation rather than circulating monocytes[16]. In I/R-induced AKI, a subset of M2 macrophages may arise from circulating monocytes [22], but there is also an important role for *in situ* renal proliferation and polarization [16]. Similarly, recent studies have also indicated that CSF-1 can mediate proliferation and polarization of macrophages and dendritic cells in uterine tissues [18]. Of note, proximal tubule generated GM-CSF has also been reported to promote macrophage alternative activation after ischemia/reperfusion injury[28]. Proximal tubule may also promote recovery from acute tubular injury through other mechanisms[29].

We are aware that the extent of kidney injury in response to either ischemia/reperfusion AKI or DT-induced AKI in DTR mice was less severe in γ-*GT Cre:CSF-1*$^{f/f}$ mice than in global CSF-1 knockout mice[16]. Our current studies suggest that the maximal macrophage/dendritic cell proliferation and M2 polarization during recovery from AKI need CSF-1 produced in proximal tubular epithelial cells as well as other sources. In addition, CSF-1 from proximal

epithelial cells and non-proximal tubule epithelial sources may also directly stimulate the proximal tubular epithelial cell proliferation in response to AKI.

In summary, our results demonstrate that deletion of CSF-1 expression in the proximal tubule delays renal recovery from AKI induced by either selective proximal tubule injury or in response to ischemia-reperfusion injury, and ultimately results in increased tubulointerstitial fibrosis. With this selective CSF-1 inhibition, there is decreased activation of the CSF-1 receptor in proximal tubule and decreased numbers of renal macrophages/dendritic cells during the recovery period, and these cells express decreased markers consistent with polarization to an M2 phenotype. Therefore, these studies demonstrate that activation of the CSF-1 receptor, c-fms by CSF-1 produced in the proximal tubule mediates increases in renal M2 macrophage/dendritic cells necessary for recovery from acute kidney injury and indicate that tubule expression of CSF-1 in response to acute kidney injury plays an essential role in regeneration and differentiation of renal epithelial cells after injury.

MATERIALS AND METHODS

Animals

CSF-1 floxed mice were generated as described in a previous report [30]. Generation of transgenic mice expressing diphtheria toxin receptor in proximal tubule (DTR) was reported previously [16]. Both DTR transgenic mice and *γ-GT Cre* mice, in which Cre is expressed in proximal tubule in the kidney [31], were crossed with CSF-1 floxed mice to generate *DTR:CSF-1*$^{f/+}$ and *γ-GT Cre:CSF-1*$^{f/+}$ mice, which were further crossed with original *CSF-1*$^{f/f}$ mice to generate *DTR:CSF-1*$^{f/f}$ and *γ-GT Cre:CSF-1*$^{f/f}$ mice, respectively. The resultant *DTR:CSF-1*$^{f/f}$ and *γ-GT Cre:CSF-1*$^{f/f}$ mice were crossed to generate *CSF-1*$^{f/f}$*:DTR* mice (*CSF-1*$^{f/f}$, wild type control) and *γ-GT Cre:CSF-1*$^{f/f}$*:DTR* mice (*γ-GT Cre:CSF-1*$^{f/f}$, CSF-1 knockout in proximal tubule). Only age-matched littermates were used for the experiments.

Acute kidney injury (AKI) models

DT-mediated AKI was initiated by a single i.p. injection of DT at a dose of 200 ng/kg (Sigma-Aldrich). Ischemia-reperfusion (I/R)-induced AKI was performed as previously described [16]. Briefly, the animal was uninephrectomized, immediately followed by unilateral ischemia-reperfusion with renal artery clamping for 31 min. Monocyte/macrophage depletion was induced by i.p. administration of clodronate (Encapsula NanoSciences) at a dose of 40 mg/kg at day 0, then 20 mg/kg every 3 days thereafter throughout the experiment. As controls, mice were given the liposome carrier alone. For experiments with macrophage depletion, DT was given at a dose of 200 ng/kg.

Measurement of serum BUN

AKI was monitored by measuring serum BUN using a Urea Assay Kit (BioAssay Systems).

Antibodies

The primary antibodies that were used for immunohistochemistry and immunoblotting included rabbit anti-p-c-fms/CSF-1R(Tyr723) and nitrotyrosine (marker of oxidative stress),

goat anti-CSF-1 and human connective tissue growth factor (CTGF) from Santa Cruz Biotechnology; rat anti-F4/80 (a marker of macrophage/dendritic cell) and Ly6G (Gr-1, a marker of neutrophils) from AbD Serotec; rabbit anti-HMGB1 from Cell Signaling Technology; mouse anti-Kim-1 (a marker of renal tubular injury, MAB1817) and mannose receptor (MR, CD206) from R&D Systems, mouse anti-α-smooth muscle actin (α-SMA, a marker of myofibroblasts) and rabbit anti-human fibronectin from Sigma-Aldrich.

Immunofluorescence/Immunohistochemistry staining and quantitative image analysis

The animals were anesthetized with Nembutal (70 mg/kg, i.p.) and given heparin (1,000 units/kg, i.p.) to minimize coagulation. In studies in which injury was induced by DT administration, one kidney was taken out for immunoblotting, flow cytometry, FACS, qRT-PCR, and histologic analysis, and the other was perfused with FPAS (3.7% formaldehyde, 10 mM sodium *m*-periodate, 40 mM phosphate buffer, and 1% acetic acid) through the aortic trunk cannulated by means of the left ventricle. The fixed kidney was dehydrated through a graded series of ethanols, embedded in paraffin, sectioned (4 μm), and mounted on glass slides. Immunostaining was carried out as in a previous report [32]. For p-c-fms/CSF-1R(Tyr723) immunostaining, antigen retrieval was achieved by boiling in citric acid buffer (pH 6.0, 100 mM) for 3 x5 min. For mouse anti-α-SMA, MR and Kim-1 staining, a M.O.M Kit for detecting mouse primary antibodies on mouse tissue peroxidase was used (Catalog# PK-2200, Vector Laboratories). CSF-1 is a secreted cytokine. We used two different controls to determine the specificity of CSF-1 immunostaining: neutralization of CSF-1 antibodies with CSF-1 peptide or omission of primary antibodies. For immunofluorescent staining, deparaffinized sections were treated with trypsin for 15 min, washed with PBS, blocked with 10% normal donkey serum for 1 hr, then stained with F4/80 primary antibody, followed by Texas Red labeled anti-rat-IgG. After washing with PBS, antigen retrieval was performed as above, and stained with p-c-fms/CSF-1R(Tyr723) primary antibody followed by FITC conjugated anti-rabbit IgG. Sections were viewed and imaged with a Nikon TE300 fluorescence microscope and spot-cam digital camera (Diagnostic Instruments). On the basis of the distinctive density and color of immunostaining in video images, the number, size, and position of stained cells were quantified using the BIOQUANT true-color windows system (R & M Biometrics) as previously described [33]. Six representative fields from each animal were quantified, and their average was used as data from one animal sample.

Immunoblotting

Kidney samples were homogenized with buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS, 100 μM Na_3VO_4, 100 mM NaF, 0.5% sodium deoxycholate, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The homogenate was centrifuged at 15000×g for 20 min at 4°C. An aliquot of supernatant was taken for protein measurement with BCA protein assay kit (Thermo scientific, Rockford, IL). Immunoblotting was carried out as in a recent report [34].

Flow cytometry and fluorescence-activated cell sorting (FACS)

After perfusion of the kidneys with cold PBS, one kidney was removed, minced into fragments, and digested in RMPI 1640 containing 2 mg/ml collagenase type D and 100 μ/ml DNase I for 1 hour at 37°C, with intermittent agitation. Kidney fragments were passed through a 70 μm mesh (Falcon; BD Biosciences), yielding single cell suspensions. Cells were centrifuged (800×g, 10 minutes, 8°C), resuspended in FACS buffer, kept on ice and counted. 10^7 cells were incubated in 2.5 μg/ml Fc blocking solution, centrifuged again (800x g, 10 minutes, 8°C), and resuspended with FACS buffer. 10^6 cells were stained for 20 minutes at room temperature with antibodies including FITC rat anti-mouse CD45 (clone 30-F11), PE/Cy7 anti-mouse F4/80 (clone BM8), PE anti-mouse CD11b (clone M1/70), APC anti-mouse CD11c (clone N418), Pacific Blue anti-mouse CD3 (clone 17A2) (BD Biosciences), Alexa fluor647 anti-mouse neutrophils (clone 7/4) (AbD Serotec), then washed in sterile PBS and resuspended with 7-AAD to permit exclusion of nonviable cells. Finally, the cells were washed and resuspended in FACS buffer. After immunostaining, cells were analyzed immediately on a Facs Canto II cytomer with DIVA software (Becton Dickinson) and off-line list mode data analysis was performed using Winlist software from Verity Software House (Topsham, ME). Cell sorting was performed on a FACSAria cell sorter (BD) to purify the cells expressing $F4/80^+$ and $CD11b^+$ cells.

Isolation of renal monocytes/macrophages/dendritic cells

CD11b-expressing cells in the kidney single cell suspensions were enriched using mouse CD11b Microbeads and MACS columns (Milteni Biotec Auburn, CA) following the manufacturer's protocol.

RNA isolation and qRT-PCR

Total RNA from kidney or isolated macrophages was isolated using TRIzol reagents (Invitrogen), and cDNA was synthesized from equal amounts of total RNA from each individual sample using SuperScript III First-Strand Synthesis System kit (Invitrogen). With GAPDH as internal normalization control, qRT-PCR was performed using TaqMan real time PCR (7900HT, Applied Biosystems). The Master Mix and all gene probes were also purchased from Applied Biosystems. The probes used in the experiments included mouse GAPDH (Mm99999915), IL-4Rα (Mm01275139), mannose receptor (Mm01329362), TGF-β (Mm00441726), arginase 1 (Mm00475991), iNOS (Mm00440502), TNF-α (Mm99999068), CCL-3 (Mm00441258), IL-23 (Mm00518984), CSF1 (Mm00432686), CSF2 (GM-CSF, Mm00438328) and GGT1 (γ-GT, Mm00492322).

Micrography

Bright-field images from a Leitz Orthoplan microscope with DVC digital RGB video camera were digitized and saved as computer files. Contrast and color level adjustment (Adobe Photoshop) were performed for the entire image; i.e., no region- or object-specific editing or enhancements were performed.

Statistics

All values are presented as means, with error bars representing ± s.e. Fisher exact test, analysis of variance (ANOVA) and Bonferroni *t* tests were used for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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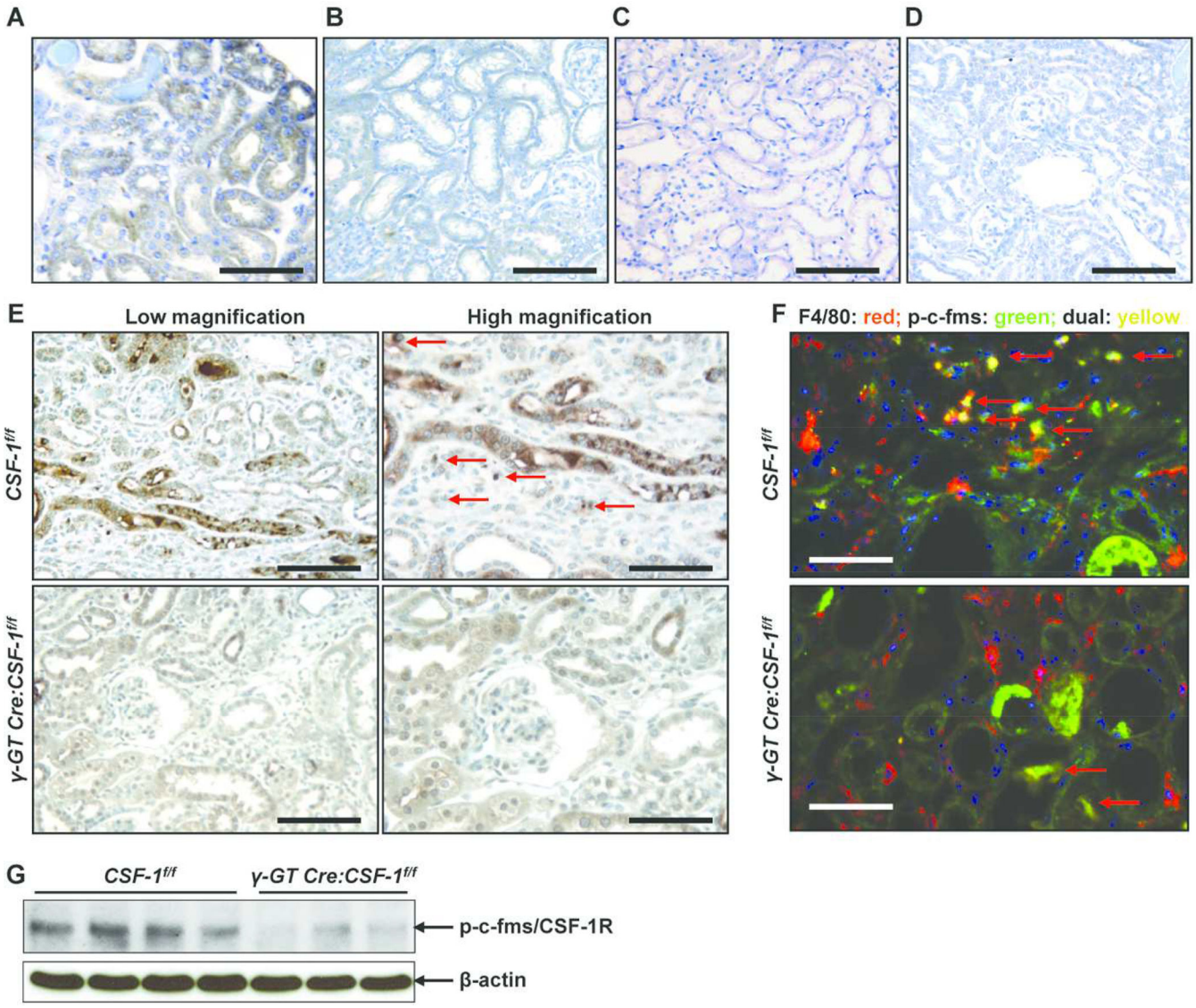


Figure 1. Renal CSF-1-CSF-1R pathway was blunted *in* γ–*GT Cre:CSF-1*$^{f/f}$ mice

(a-d) CSF-1 expression in renal proximal tubule was decreased in γ–*GT Cre:CSF-1*$^{f/f}$ mice (CSF-1 knockout in renal proximal tubule). Immunohistochemical staining indicated strong CSF-1 immunostaining in tubule epithelia in kidney of *CSF-1*$^{f/f}$ mice (wild type control) at 6 days after DT injection (a), which was eliminated by pre-incubation of primary antibody with CSF-1 peptide (b) or omission of primary antibody (c). (d) The effectiveness of CSF-1 deletion in proximal tubule was confirmed by very weak renal CSF-1 immunostaining in γ–*GT Cre:CSF-1*$^{f/f}$ mice 6 days after DT injection. (e) Kidney p-c-fms/CSF-1R immunostaining in both epithelial cells (arrows) and interstitial cells (arrow heads) was much lower in γ–*GT Cre:CSF-1*$^{f/f}$ mouse than in *CSF-1*$^{f/f}$ mice at 6 days after DT injection. (f) Double immunofluorescent staining indicated that activation of c-fms/CSF-1R in macrophages (F4/80^{+} and p-c-fms/CSF-1R^{+} cells) was attenuated in γ–*GT Cre:CSF-1*$^{f/f}$ mouse (arrows). (g) Immunoblotting indicated reduced protein levels of phosphorylated c-fms/CSF-1R in kidneys from γ–*GT Cre:CSF-1*$^{f/f}$ mice at 6 days after DT injection. Each

lane represented a sample from individual animal. (f) Kidney p-c-fms/CSF-1R immunostaining in both epithelial cells (arrows) and interstitial cells (arrow heads) was much lower in γ–*GT Cre:CSF-1*$^{f/f}$ mouse than in *CSF-1*$^{f/f}$ mice at 6 days after DT injection. Scale bar: 62 μm in A and E high magnification, 96 μm in B-D and E low magnification, and 32 μm in F.

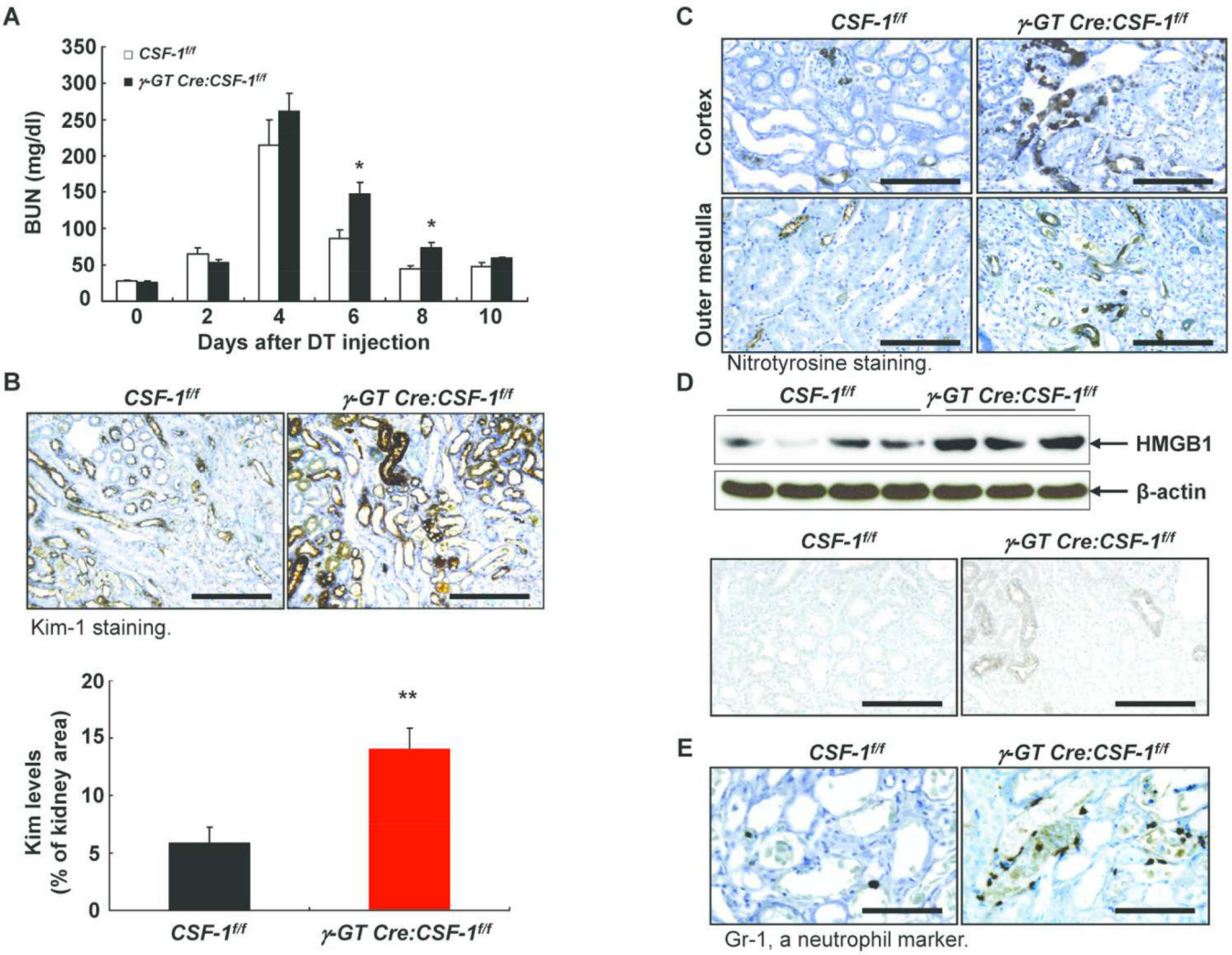
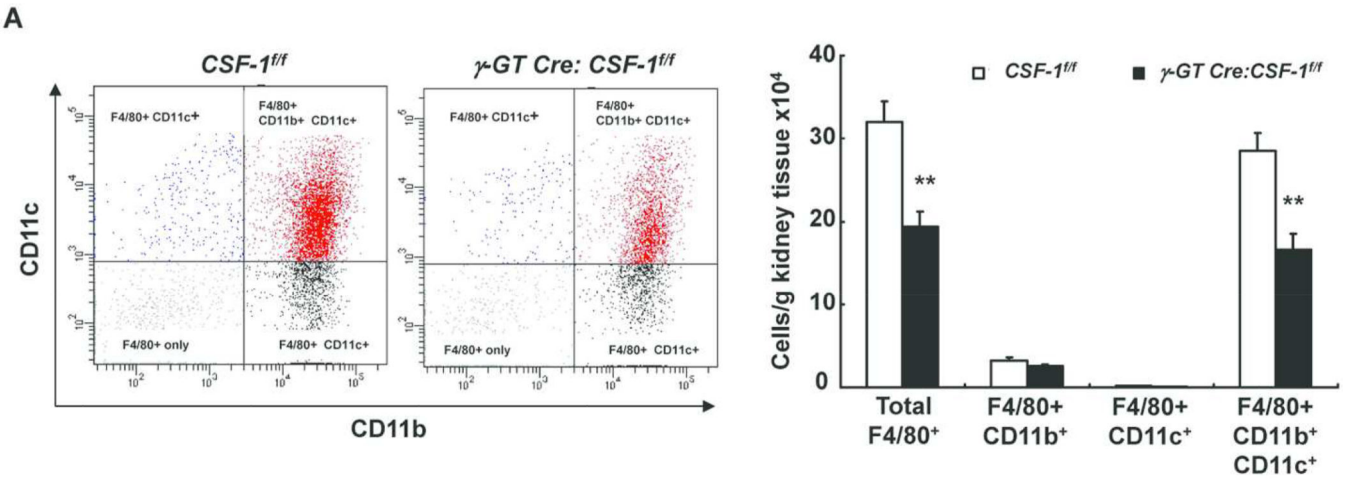


Figure 2. Renal proximal tubule CSF-1 was involved in recovery from DT-mediated AKI

(a) Levels of serum BUN remained higher during recovery from DT-mediated AKI in γ–*GT Cre:CSF-1$^{f/f}$* mice. *: $p < 0.05$ vs. *CSF-1$^{f/f}$* mice, n = 6. (b) CSF-1 deletion in proximal tubule led to increased renal Kim-1 expression in mice at 6 days after DT injection. **: $p < 0.01$ vs. *CSF-1$^{f/f}$* mice, n = 4. (c) CSF-1 deletion in proximal tubule led to increased renal oxidative stress in both cortex and medulla as indicated by increased nitrotyrosine staining in mice at 6 days after DT treatment. (d) Both immunoblotting and immunohistochemical staining indicated that CSF-1 deletion in proximal tubule led to secondary necrosis as indicated by increased expression levels of HMGB1 (chromatin-associated protein high-mobility group box 1), a protein released by necrotic cells. (e) CSF-1 deletion in proximal tubule led to increased renal neutrophil infiltration in mice at 6 days after DT injection. Scale bar: 200 μm in B, 132 μm in C and D, and 80 μm in E.



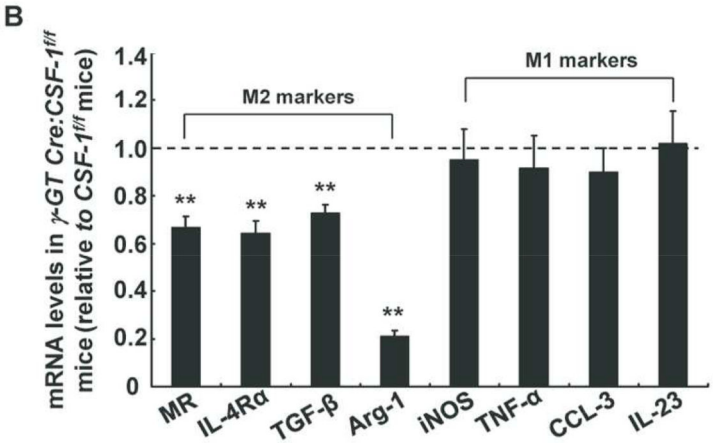


Figure 3a

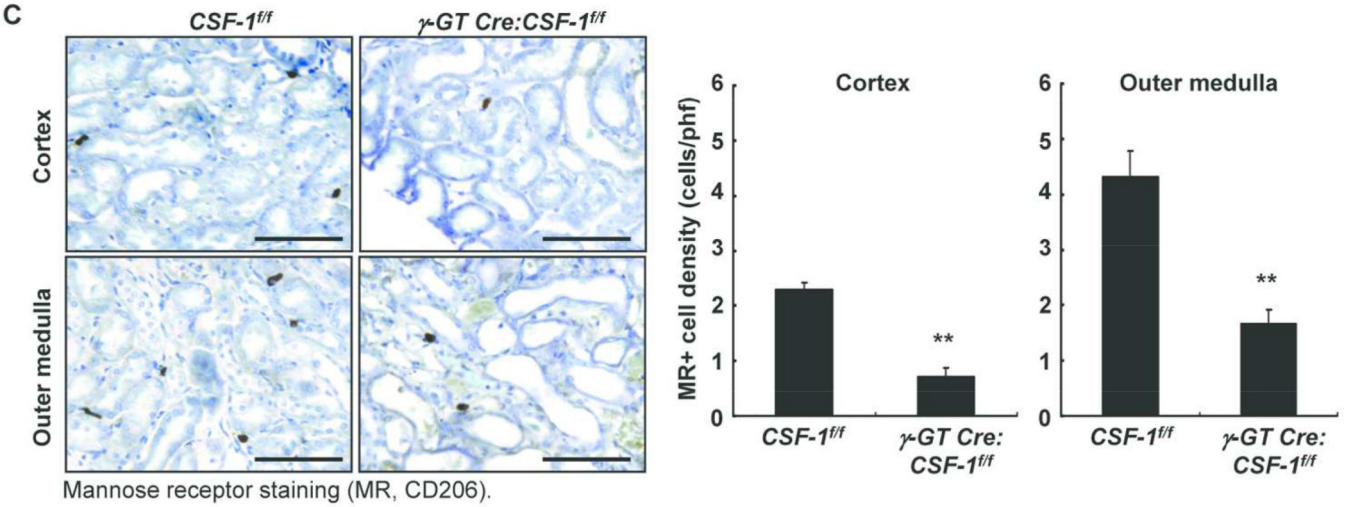


Figure 3b

Figure 3. Renal proximal tubule CSF-1 was essential for macrophage/dendritic cell proliferation and polarization in response to DT-mediated AKI

(a) Flow cytometric analysis gating with F4/80 indicated that there were less renal F4/80-positive cells (monocytes/macrophages/dendritic cells) in γ–*GT Cre:CSF-1*$^{f/f}$ mice than in *CSF-1*$^{f/f}$ mice at 6 days after DT injection, with the majority being dendritic cells, as indicated by co-expression of F4/80, CD11b, and CD11c. **: $p < 0.01$ vs. *CSF-1*$^{f/f}$ mice, n = 4. (b) CSF-1 deletion in proximal tubule led to decreased mRNA levels of markers of M2 phenotypic macrophages/dendritic cells, including mannose receptor (MR, CD206), IL-4Rα, TGF-β, and arginase 1 in isolated kidney macrophages/dendritic cells from mice with DT injection for 6 days. **: $p < 0.01$ vs. *CSF-1*$^{f/f}$ mice, n = 4. (c) Immunostaining indicated decreased renal MR-positive macrophages/dendritic cells in kidney cortex and outer medulla from γ–*GT Cre:CSF-1*$^{f/f}$ mice at 6 days after DT injection. **: $p < 0.01$ vs. *CSF-1*$^{f/f}$ mice, n = 4. Scale bar: 80 μm.

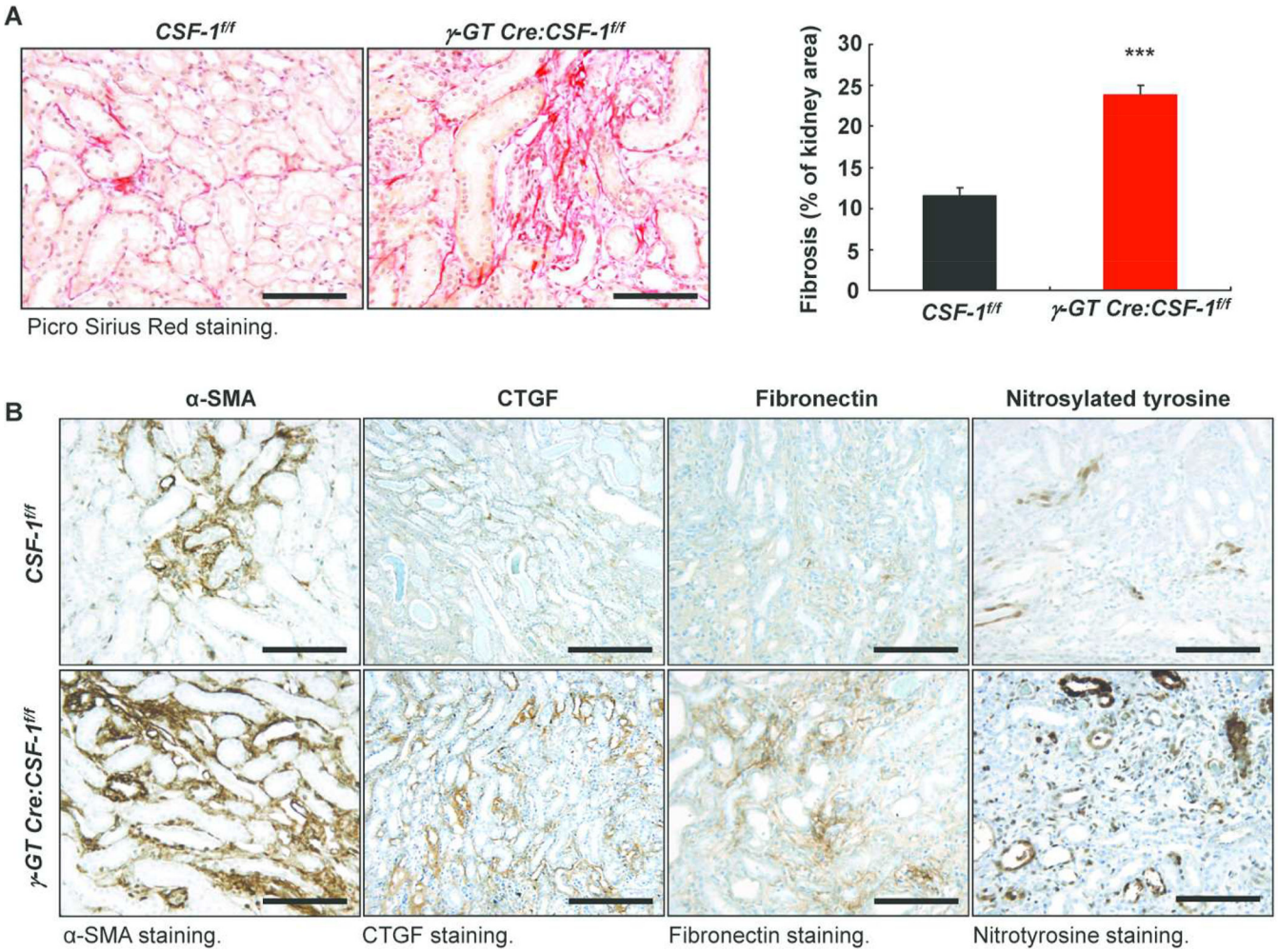


Figure 4. Renal proximal tubule CSF-1 protected against the development of chronic kidney injury after DT-mediated AKI

Both *CSF-1$^{f/f}$* and γ–*GT Cre:CSF-1$^{f/f}$* mice were injected with DT (200 ng/kg, i.p.) and sacrificed 4 weeks later. (a) CSF-1 deletion in proximal tubule led to increased renal fibrosis. ***: $p < 0.001$ vs. *CSF-1$^{f/f}$* mice, n = 4. (b) CSF-1 deletion in proximal tubule increased renal expression of α-SMA, CTGF, and fibronectin as well as increased oxidative stress as indicated by increased nitrotyrosine staining. Scale bar: 100 μm in (a) and (b: α-SMA, fibronectin and nitrotyrosine) and 160 μm in (b: CTGF).

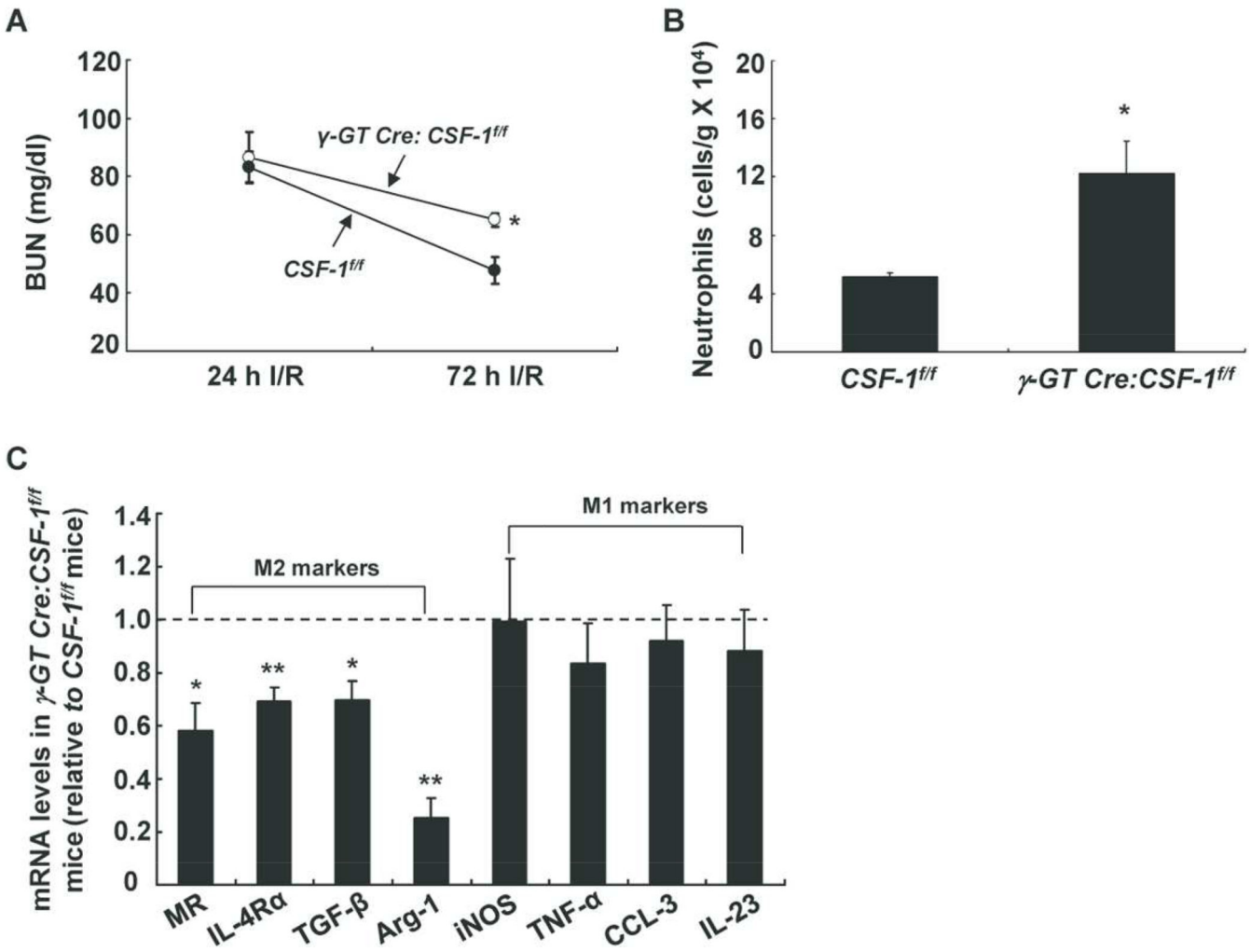


Figure 5. Renal proximal tubule CSF-1 was essential for recovery and macrophage/dendritic cell polarization in response to I/R-induced AKI

Both *CSF-1*$^{f/f}$ and γ-*GT Cre:CSF-1*$^{f/f}$ mice were uninephrectomized, immediately followed by unilateral I/R with renal artery clamping for 31 min. (a and b) CSF-1 deletion in proximal tubule led to slower recovery from I/R-induced AKI, as indicated by higher BUN 3 days after injury (**: $p < 0.05$ vs. *CSF-1*$^{f/f}$ mice, n = 7) and more renal neutrophil infiltration 5 days after I/R injury (*: $p < 0.05$ vs. *CSF-1*$^{f/f}$ mice, n = 4). (c) CSF-1 deletion in proximal tubule led to decreased mRNA levels of markers of M2 phenotypic macrophages/dendritic cells, including mannose receptor (MR, CD206), IL-4Rα, TGF-β, and arginase 1 in isolated kidney macrophages/dendritic cells from mice 5 days after I/R injury. *: $p < 0.05$, **: $p < 0.01$, n = 6.

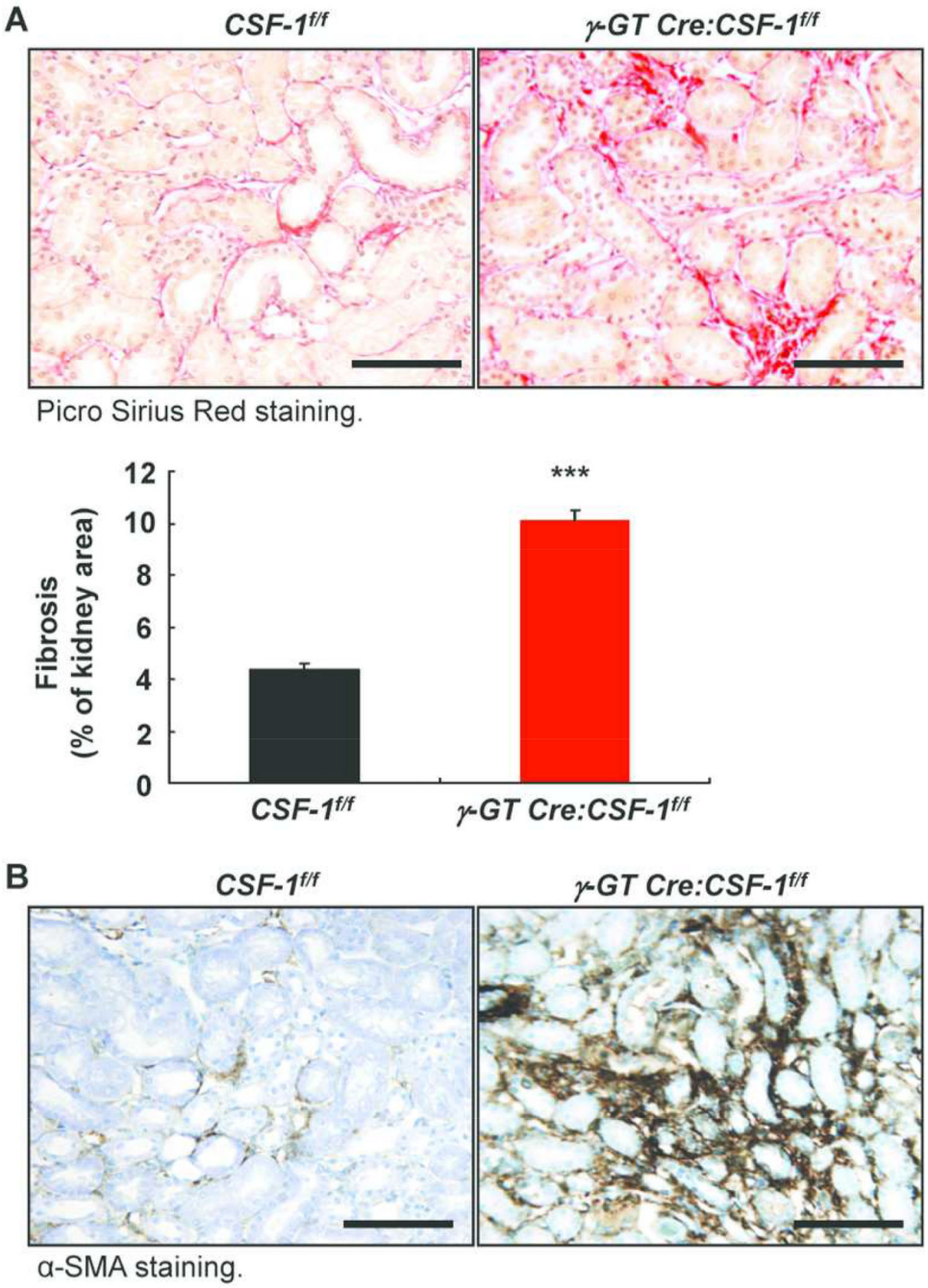


Figure 6. Renal proximal tubule CSF-1 protected against the development of chronic kidney injury after I/R-induced AKI

Both *CSF-1$^{f/f}$* and γ–*GT Cre:CSF-1$^{f/f}$* mice were uninephrectomized, immediately followed by unilateral I/R with renal artery clamping for 31 min, and sacrificed 4 weeks later. (a) CSF-1 deletion in proximal tubule led to increased renal fibrosis. ***: $p < 0.001$ vs. *CSF-1$^{f/f}$* mice, n = 4. (b) CSF-1 deletion in renal proximal tubule increased renal expression of α-SMA, a marker of myofibroblasts. Scale bar: 100 μM in (a) and (b).