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This information is current as
of February 25, 2015.

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J Immunol 2015; 194:1628-1638; Prepublished online 16
January 2015;

doi: 10.4049/jimmunol.1402149

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CX₃CR1 Reduces Kidney Fibrosis by Inhibiting Local Proliferation of Profibrotic Macrophages

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A dense network of macrophages and dendritic cells (DC) expressing the chemokine receptor CX₃CR1 populates most tissues. We recently reported that CX₃CR1 regulates the abundance of CD11c⁺ DC in the kidney and thereby promotes renal inflammation in glomerulonephritis. Given that chronic inflammation usually causes fibrosis, we hypothesized that CX₃CR1 deficiency should attenuate renal fibrosis. However, when we tested this hypothesis using the DC-independent murine fibrosis model of unilateral ureteral obstruction, kidney fibrosis was unexpectedly more severe, despite less intrarenal inflammation. Two-photon imaging and flow cytometry revealed in kidneys of CX₃CR1-deficient mice more motile Ly6C/Gr-1⁺ macrophages. Flow cytometry verified that renal macrophages were more abundant in the absence of CX₃CR1 and produced more of the key profibrotic mediator, TGF- β . Macrophages accumulated because of higher intrarenal proliferation, despite reduced monocyte recruitment and higher signs of apoptosis within the kidney. These findings support the theory that tissue macrophage numbers are regulated through local proliferation and identify CX₃CR1 as a regulator of such proliferation. Thus, CX₃CR1 inhibition should be avoided in DC-independent inflammatory diseases because it may promote fibrosis. *The Journal of Immunology*, 2015, 194: 1628–1638.

The renal mononuclear phagocyte system consists of an extensive network of mononuclear phagocytes (i.e., dendritic cells [DCs] and macrophages) (1, 2). Although there is no clear demarcation between these cell types, DCs are typically specialized at regulating other immune effector cells, especially T cells, whereas macrophages preferentially act as immune effectors in innate immune responses or contribute to tissue repair (3–6). In the healthy kidney, most resident immune cells display phenotypic and functional characteristics of DCs and can be distinguished from macrophages by expression of the CD11c molecule (7). Furthermore, CD11c⁺ kidney cells possess a transcriptome that resembles that of DCs but not of macrophages (8, 9). Although kidney DCs have been shown to perform sentinel and regulatory functions in inflammation and infection (10), macrophages contributed to host protection from infection and maintained tissue homeostasis (1, 11, 12). In contrast, inappropriate macrophage functions promoted fibrosis in various organs (13, 14). In

the kidney, macrophages but not DCs mediated fibrosis in unilateral ureter ligation (UUO) and after ischemia/reperfusion (15–18). Several mechanisms contribute to renal fibrosis, including the production of the main profibrotic molecule TGF- β (14, 19). In the kidney, TGF- β promotes fibrosis by activating fibroblasts and myofibroblasts, modulating the expression of tissue inhibitors of matrix metalloproteases and by directly promoting synthesis of collagens (20). TGF- β secretion has been linked to monocytes and macrophages (14), suggesting that macrophages might mediate kidney fibrosis through this mediator.

Tissue macrophages partially originate from embryonic progenitors of the yolk sac or the fetal liver (21, 22), and from infiltrating bone marrow–dependent progenitors such as monocytes, whereas DCs can develop from monocytes or pre-DCs (23–25). The exact contribution of the different precursors to the renal mononuclear cell system is unclear. The chemokine receptor CX₃CR1, also known as fractalkine receptor, is ubiquitously expressed on most tissue macrophages and DCs but does not play a major role for their ontogeny, homeostatic migration, or colonization of tissues with resident phagocytes (26, 27), except kidney DCs (3) and intestinal macrophages (28–30). There is evidence that the survival of monocytes and tissue macrophages depends on CX₃CR1 (31–33), and this required interaction with cell surface–bound CX₃CL1 (34). CX₃CL1⁺ cells have been shown to be present within glomeruli and in the tubular compartment (35), but their exact identity is unclear.

Under inflammatory conditions, CCR2-dependent monocytes expressing the markers Ly6C and Gr1 enter inflamed tissues (36–38) and rapidly give rise to DCs and macrophages (31, 39–41). Inflammatory Gr1⁺ macrophages possess profibrotic properties (42), and their depletion or genetic CCR2 deficiency attenuated tissue damage and fibrosis in UUO (16), lupus nephritis (43), and ischemia/reperfusion (44). Also, CX₃CR1 has been implicated in monocyte recruitment into inflamed tissues, like arteriosclerotic vessels (45), the listeria-infected spleen (46), the eye (47), in colitis models (28), or the skin (48). Furthermore, CX₃CR1 may exert profibrotic functions (49–55).

DCs and macrophages of the kidney abundantly express CX₃CR1 (56). CX₃CR1 deficiency reduced entry of their progenitors into the kidney and attenuated symptoms and fibrosis

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Received for publication August 22, 2014. Accepted for publication December 14, 2014.

This work was supported by the Deutsche Forschungsgemeinschaft (Grants SFB Transregio 57, Klinische Forschergruppe 228, and BO 3755/1-1) and the Gottfried-Wilhelm Leibniz Award (to C.K.) and the Australian National Health and Medical Research Council (Project Grants 565033 and 1045065). C.K. and D.R.E. are members of the Deutsche Forschungsgemeinschaft Excellence Cluster ImmunoSensation.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; Δ MI, change in morphology index; α SMA, α -smooth muscle actin; UUO, unilateral ureter ligation.

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in ischemia/reperfusion injury (44, 49), hypertensive kidney damage (54), and crescentic glomerulonephritis (3, 57). In contrast, the loss of renal phagocytes aggravated renal candidiasis (58) but not pyelonephritis (3). Also, studies on human biopsy material from fibrotic kidneys described upregulation of CX₃CR1, supporting a pathogenic role (59). However, in toxin- and in obstruction-induced liver fibrosis, CX₃CR1 was protective (50). The role of CX₃CR1 in the obstruction-induced standard kidney fibrosis model, UUO, has not been clarified yet. In this study, we have addressed this open question, assuming that the marked loss of renal DCs in CX₃CR1 deficiency would attenuate inflammation and fibrosis. However, we found that CX₃CR1 was protective in UUO because of a novel inhibitory effect on the other CX₃CR1-expressing mononuclear phagocyte type of the kidney, the macrophages.

Materials and Methods

Mice

Eight- to 14-wk-old *Cd45.1*, *Cd11c^{EGFP}* (60), *Cx₃cr1^{GFP/+}*, and *Cx₃cr1^{GFP/GFP}* mice (61), all on a C57BL/6 background, were bred and kept under specific-pathogen-free condition at the animal facilities of the University of Bonn and Monash Medical Centre. Institutional and Government Review Boards have approved all animal studies.

Induction of UUO

Adult male mice were anesthetized by inhalation of isoflurane. UUO was performed through a midline incision. The left ureter was tied with silk suture at two points and permanently ligated. Sham-operated mice underwent an identical midline incision but without ureteric ligation. At 3, 5, and 7 d postobstruction, mice were sacrificed, and kidneys were collected.

Renal multiphoton microscopy

Intravital multiphoton microscopy of the intact kidney was performed on mice 3 d post-UUO, anesthetized by i.p. injection of 150 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, as described previously (18, 62). The jugular vein was cannulated, and the left kidney was exteriorized through a lateral incision, immersed in bicarbonate buffered saline at 37°C, and

covered with a coverslip. Mice were kept at 37°C. Time lapse recordings of GFP⁺ cells in the kidney of *Cx₃cr1^{GFP/+}* and *Cx₃cr1^{GFP/GFP}* mice were captured using a Leica SP5 multiphoton microscope system (Leica Microsystems, Mannheim, Germany) with a ×20 1.0 NA water immersion objective (18, 63). Fluorophores were excited by a SpectraPhysics MaiTai pulsed infrared laser tuned to 860 nm, with emissions captured by non-descanned detectors with 525/50 nm (GFP), 585/40 nm (tetramethylrhodamine isothiocyanate), and 650/50 nm (Alexa 633) filters. Celltracker Orange CMRA (C34551; Molecular Probes) was used as a counterstain to provide visual context. Images were collected with ~120 μm Z-depth in 6-μm steps, with one Z-stack every 30s for 20–30 min and analyzed using Imaris (version 7.0; Bitplane AG). To determine GFP⁺ cell motility, individual cells were tracked in vivo and a motile cell defined as one that had moved at least the diameter of the cell body. To determine cell activity, a morphology index was used as previously described (18), relating to the sphericity of cells described by the rate of change in morphology index (ΔMI). Cells with relatively low ΔMI values change morphology less than cells with higher ΔMI values.

Histology and immunohistochemistry

Methyl Carnoyl's fixed paraffin-embedded murine kidneys were sectioned at 1 μm and stained with periodic acid–Schiff's (PAS) for evaluation of hydronephrosis and overall injury. For the evaluation of fibrosis, Sirius Red stained sections as well as α-smooth muscle actin (αSMA) immunohistochemistry were evaluated using computer-based morphometric analyses by a pathologist in blinded fashion as described previously (64). In short, for both stainings, the percentage of positively stained area was calculated from 20 cortical fields per section, representing nearly the whole cortex. Indirect immunoperoxidase procedure was performed as described previously (64). HRP coupled to human αSMA (clone 1A4; DakoCytomation, Glostrup, Denmark), which cross-react with murine αSMA was used. Fluorescence microscopy was performed on 5-μm paraffin sections from 4% PFA-fixed kidneys. GFP-expressing cells in *Cx₃cr1^{GFP/+}* mice were identified by nuclei staining and quantified using the IX71 microscope and the CellR software (Olympus).

Isolation of murine kidney leukocytes

Kidneys were digested with collagenase and DNase-I (both from Sigma-Aldrich) as described previously (7). Briefly, kidneys were digested for 45 min, and tubular fragments from digested kidneys were removed by filtration through a 100-μm nylon mesh.

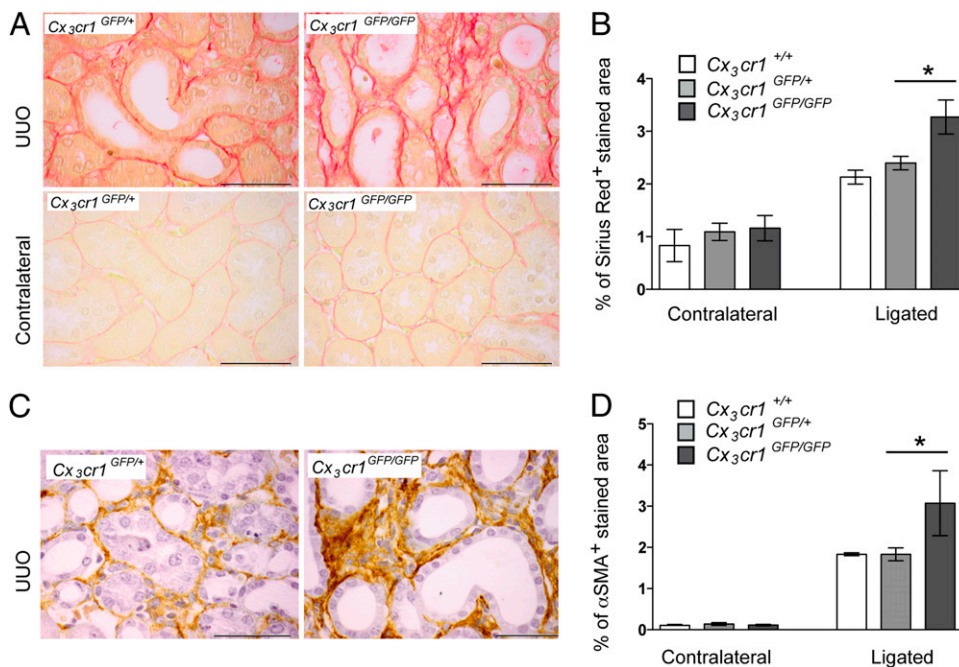


FIGURE 1. Lack of CX₃CR1 increases renal fibrosis in UUO. (A–D) Fibrosis was quantified by Sirius red staining (A and B) and αSMA immunostaining (C and D) of sections of ligated or contralateral kidneys from CX₃CR1-competent (*Cx₃cr1^{+/+}*, *Cx₃cr1^{GFP/+}* [left images or white and light gray bars]) and CX₃CR1-deficient (*Cx₃cr1^{GFP/GFP}* [right images or dark gray bars]) mice 7 d after ureter ligation. (A and C) Scale bars, 50 μm. Data are mean ± SEM from three (A and B) and two (C and D) experiments in groups of four to seven mice. **p* < 0.05.

Flow cytometry

After treatment with Fc-blocking Abs (2.4G2) cells were stained with fluorochrome-labeled mAbs against F4/80 (CI:A3-1), CD45 (30-F11), CD11c (N418), KI67 (SolA15; isotype control: eBR2a), Gr1 (RB6-8C5), and Annexin V (number 550474; BD Biosciences). For intracellular cytokine staining, single-cell suspensions were incubated in RPMI 1640 medium (10% FCS, penicillin/streptomycin, and glutamine) with 1 μ l/ml GolgiPlug and 1 μ l/ml GolgiStop (BD Biosciences) for 4 h at 37°C. After cell surface staining, cells were fixed in Cytofix (BD Biosciences) and permeabilized with PermWash (BD Biosciences). Intracellular staining was performed for 30 min at room temperature using fluorochrome-labeled mAbs against TGF β (TW7-16B4; isotype control: MOPC-21). For analysis of proliferation, BrdU was administered 3 and 4 d after ligation, and incorporation of BrdU was analyzed on day 5 after ligation by flow cytometry (number 557892; BD Biosciences). Cells were analyzed with a BD Canto II and a BD Fortessa (BD Biosciences), and data were analyzed using Flowjo (Tree Star). For concatenated data presentation, data from all mice within the same group was combined using Flowjo.

Transfer of bone marrow monocytes

Single-cell suspensions from the bone marrows were prepared by flushing the bones with sterile and cold PBS. Bone marrow monocytes were enriched by excluding CD19⁺CD3⁺CD11c⁺ cells. Bone marrow cells ($1-5 \times 10^6$) were transferred into mice.

Detection of cytokines

The protein levels of cytokines in the supernatant of mechanically homogenized kidneys were determined by Luminex assay. The growth factors M-CSF and GM-CSF were determined by using ELISA (R&D Systems).

All other levels were determined by a flow cytometry-based bead assay (eBioscience).

RNA isolation and RT-PCR

Whole-tissue RNA from kidneys was extracted using NucleoSpin RNA II kit (Macherey-Nagel), according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the High-Capacity Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed for 40 cycles using SYBR Green (Applied Biosystems). QuantiTect Primer assays (Qiagen) were used to detect mouse gene expression of *Stat1* (QT00162183), *Stat6* (QT01042503), *Arg1* (QT00134288), *Hmox* (QT00159915), *Irf5* (QT00252623), *Retnla* (QT00254359), and *Mrc1* (QT00103012). All samples were run in duplicates and normalized to *Hprt* primers (Invitrogen), forward primer (5'-GTCCCAGCGTCGTGATTAGC-GAT-3') and reverse primer (5'-GGGCCACAATGTGATGGCCTCC-3').

Statistical analysis

Results are expressed as mean \pm SEM. Comparisons were drawn using parametric T-Test and nonparametric Mann-Whitney test (Prism 5; Graphpad Software, San Diego, CA). Significant *p* values were expressed as follows: **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Results

CX₃CR1 deficiency enhances renal fibrosis

The chemokine receptor CX₃CR1 has been shown to promote renal inflammation in several models (3, 49, 54, 57–59, 65). Given that chronic kidney inflammation often leads to fibrosis, we hy-

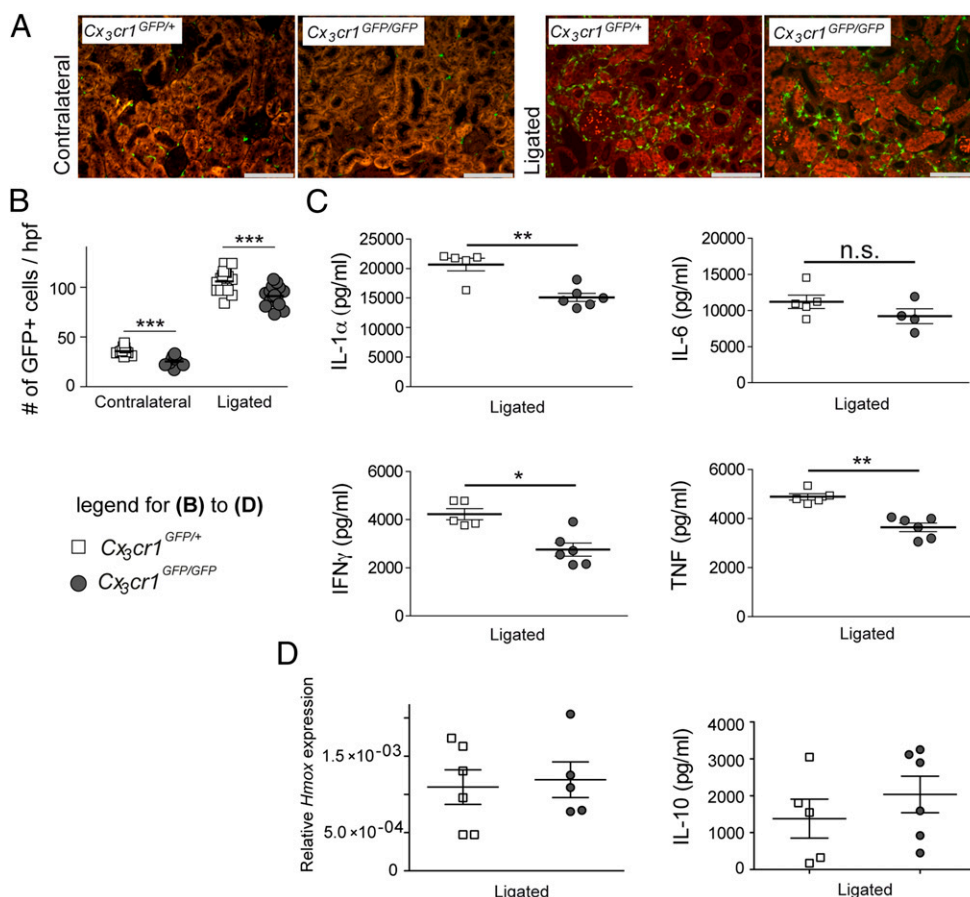


FIGURE 2. Lack of CX₃CR1 decreases inflammation and the abundance of renal mononuclear phagocytes in UUO. **(A)** Sections from contralateral *Cx3cr1*^{GFP/+} (left images) and ligated *Cx3cr1*^{GFP/GFP} (right images) mice 7 d after ureteric ligation. The white bars indicate 100 μ m. **(B)** The graph shows the numbers of GFP⁺ cells in *Cx3cr1*^{GFP/+} (open squares) and *Cx3cr1*^{GFP/GFP} (gray circles) mice determined by counting GFP⁺ cells per high power field (hpf) (one representative hpf is depicted in (A)). **(C)** Ligated kidneys from *Cx3cr1*^{GFP/+} (white squares) or *Cx3cr1*^{GFP/GFP} (gray circles) mice were homogenized, and levels of the cytokines IL-1 α (top left), IL-6 (top right), IFN- γ (bottom left), and TNF (bottom right) were determined 7 d after ureteric ligation. **(D)** Ligated kidneys from *Cx3cr1*^{GFP/+} (white squares) or *Cx3cr1*^{GFP/GFP} (gray circles) mice were analyzed 7 d after ureteric ligation for relative *Hmox* RNA expression and of IL-10 protein expression. Data in (B) to (D) are mean \pm SEM from two experiments in groups of four to seven mice. **p* < 0.05, ***p* < 0.05, ****p* < 0.001.

pothesized that CX₃CR1 deficiency should attenuate renal fibrosis. To test this hypothesis, we induced one of the standard kidney fibrosis models, UUO in mice expressing or lacking CX₃CR1. We used *Cx3cr1*^{GFP/GFP} mice that homozygously express a GFP reporter under the control of the CX₃CR1 promoter and are consequently CX₃CR1 deficient. As controls, we used *Cx3cr1*^{GFP/+} mice, which express the GFP reporter heterozygously and CX₃CR1-competent wild-type mice (*Cx3cr1*^{+/+} mice). Surprisingly, kidney fibrosis in CX₃CR1-deficient mice was notably aggravated compared with control mice 7 d after UUO, as evidenced by higher Sirius Red collagen staining in the ligated kidneys of *Cx3cr1*^{GFP/GFP} mice (Fig. 1A, 1B). Also, αSMA staining for profibrotic myofibroblasts was stronger in ligated kidneys of CX₃CR1-deficient mice (Fig. 1C, 1D). These findings revealed an unexpected antifibrotic role of CX₃CR1 in renal fibrosis.

CX₃CR1 alters the abundance, morphology, and migration of renal mononuclear phagocytes

To define the mechanisms underpinning the protective role of CX₃CR1, we focused on the cells that express CX₃CR1 in the kidney, namely mononuclear phagocytes, including DCs and macrophages. As reported before (3), CX₃CR1-deficient (*Cx3cr1*^{GFP/GFP}) mice normally possess far less GFP⁺ renal phagocytes than CX₃CR1-competent (*Cx3cr1*^{GFP/+}) mice (Fig. 2A, 2B). Also, on day 7, after ureter ligation, fewer phagocytes were seen, albeit only 10% less (Fig. 2A, 2B), suggesting the appearance of new phagocytes that partially compensated for their deficiency under homeostatic conditions. Of note, the message for the Th1 mediator Stat1 was below detection limit, and the message for the inflammatory cytokines IL-1α, IL-6, IFN-γ, and TNF and of the proresolving mediators, IL-10 and *Hmox*, did not differ between ligated kidneys of CX₃CR1-competent and -deficient mice (Fig. 2C). Thus, the aggravated fibrosis in CX₃CR1-deficient mice cannot be explained merely by stronger renal inflammation.

These findings led us to examine in more detail the renal GFP⁺ phagocytes in UUO using multiphoton microscopy, which allows analyzing their morphology and motility over time. In both healthy CX₃CR1-competent and CX₃CR1-deficient mice, GFP⁺ cells with dendritic protrusions were present within the interstitium (see Supplemental Videos 1, 2).

After UUO, GFP⁺ cells tended to form clusters around damaged tubules (see Supplemental Videos 3, 4). In addition to GFP⁺ cells with dendritic morphology, the kidneys of CX₃CR1-deficient mice with UUO contained larger cells (Fig. 3A) devoid of dendrites (Fig. 3B), which were more motile (Fig. 3C). Furthermore, the phagocytes in CX₃CR1-deficient mice contained a subset that underwent little shape change over the time of analysis (Fig. 3D). Although these data did not reach statistical significance, they prompted us to hypothesize that GFP⁺ phagocytes with monocyte/macrophage properties might be involved in the increased fibrosis in CX₃CR1-deficient mice with UUO.

CX₃CR1 differentially regulates the numbers of renal DCs and Gr1⁺ macrophages in UUO

We hypothesized that higher numbers of renal macrophages might explain the profibrotic effect of CX₃CR1 deficiency in UUO (Fig. 1) because it has been previously reported that renal macrophages but not DCs mediate fibrosis in this model (16, 18, 66). However, *Cx3cr1*^{GFP/GFP} mice possessed similar or lower numbers of GFP⁺ renal phagocytes under homeostatic conditions, in experimental glomerulonephritis and in kidney infection compared with CX₃CR1-competent controls (3, 58), consistent with our present findings in UUO (Fig. 2A, 2B). We therefore discriminated between GFP⁺ kidney macrophages and DCs by costaining

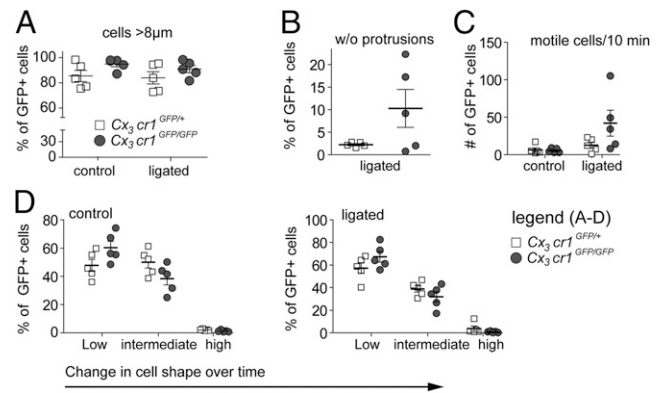


FIGURE 3. In vivo multiphoton microscopy of *Cx3cr1*^{GFP/+} and *Cx3cr1*^{GFP/GFP} mice. **(A)** Analysis shows the proportion of cells with a diameter larger than 8 μm (*Cx3cr1*^{GFP/+} [open squares]; *Cx3cr1*^{GFP/GFP} [gray circles]). **(B)** and **(C)** In the absence of CX₃CR1, there is a trend ($p = 0.09$) toward a higher proportion of GFP⁺ cells without protrusions (**B**) and higher number of motile cells (**C**) (*Cx3cr1*^{GFP/+} [open squares]; *Cx3cr1*^{GFP/GFP} [gray circles]). **(D)** Change in morphology index (ΔMI). Analysis of cellular shape change over time shows that CX₃CR1-deficient mice tend to have more cells that change shape < 30 min. The x-axis in **(D)** represents groups of cells that are changing their shape more over time (*Cx3cr1*^{GFP/+} [open squares]; *Cx3cr1*^{GFP/GFP} [gray circles]). Videos can be seen in the Supplemental Videos 1–4; data (A–D) represent the mean \pm SEM of five to six mice per group.

with F4/80 and CD11c. We found that CX₃CR1 deficiency reduced renal CD11c⁺ DC numbers in the ligated and contralateral kidney (Fig. 4A, upper gate in the dot plots) consistent with the morphological changes shown in Fig. 3. Next, we focused on CD11c[−]GFP⁺ cells (Fig. 4A, lower gate in the dot plots). We found that >90% of these cells expressed F4/80 (Fig. 4B) identifying these GFP/CX₃CR1-expressing cells as macrophages. Furthermore, these data also exclude considerable contaminations of other cells than monocytes/macrophages and DCs in the microscopy data (Figs. 2, 3). Because the microscopy findings above (Fig. 3) hinted at the appearance of monocytes/macrophages, we focused on the analysis of macrophages expressing the Gr1 marker, which designates mononuclear phagocytes recently recruited from the circulation (67, 68) and which is related to helper macrophages expressing Ly6C (4), a component of Gr1. Gr1⁺ macrophages were considerably more numerous already on day 3 after UUO (Fig. 4B, 4C). In the absence of CX₃CR1, these cells were more abundant than in CX₃CR1-competent mice, whereas the abundance of kidney-resident Gr1[−] macrophages was unchanged (Fig. 4C). On day 7, fewer recruited macrophages were seen, whereas numbers of Gr1[−] macrophages had further increased (Fig. 4C). Thus, the lack of CX₃CR1 reduced the number of CD11c⁺ DCs, but increased the numbers of CD11c[−] Gr1⁺ macrophages in UUO. This shift from DCs to macrophages explains why the total number of GFP⁺ phagocytes was only somewhat reduced in CX₃CR1-deficient mice (Fig. 2), and the relative increase of macrophages might explain the increased kidney fibrosis in the mice.

CX₃CR1 reduces TGF-β production by macrophages

Next, we examined whether CX₃CR1 also modulates the functionality of macrophages. To this end, we analyzed the production of TGF-β, the key profibrotic molecule in the kidney (65, 69). Most of the TGF-β-producing leukocytes indeed expressed F4/80 (Supplemental Fig. 1). Importantly, the frequencies (Supplemental Fig. 1) and numbers (Fig. 4D) of TGF-β-producing Gr1[−] and Gr1⁺ macrophages were increased in ligated kidneys of CX₃CR1-deficient mice. M1/M2 differentiation markers such as *Arg1*, *Mrc1*, *Irf5*, *Stat6*, and *Retnla1* were little altered in CX₃CR1

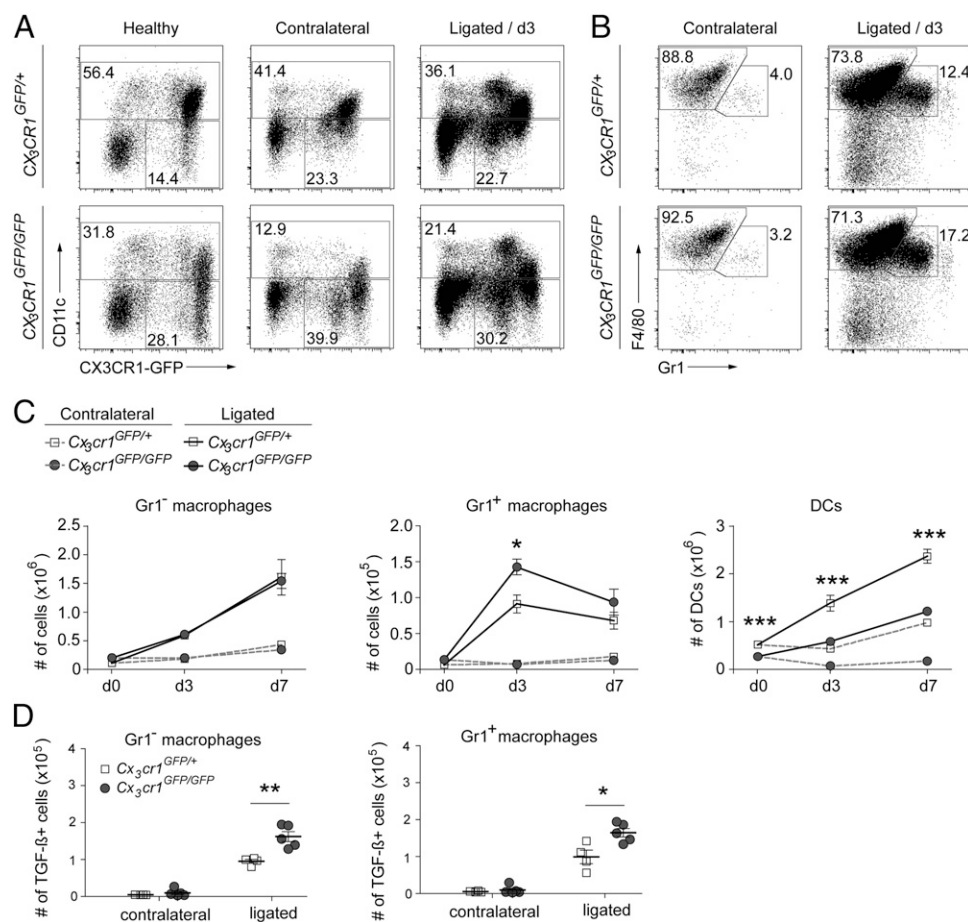


FIGURE 4. CX₃CR1 differentially regulates the numbers of renal DCs and Gr1⁺ macrophages and TGF-β production in UUO. **(A)** Flow cytometric analysis of single-cell suspensions from nonligated (healthy; left), contralateral (middle), and ligated kidneys (right) of CX₃CR1^{GFP/+} (upper row) and CX₃CR1^{GFP/GFP} (lower row) mice. Shown is CD11c staining for DCs versus CX₃CR1 expression levels (GFP) on CD45⁺ immune cells. Note that homozygous CX₃CR1^{GFP/GFP} mice show two times stronger GFP signals. **(B)** The CD11c⁺GFP⁺ macrophages [bottom right region in (A)] were gated and their F4/80 versus Gr1 expression was displayed to identify Gr1⁻ and Gr1⁺ macrophages (top right and left region). **(C)** Enumeration of CD11c⁺CD45⁺GFP⁺F4/80⁺ macrophages (Gr1⁻ macrophages in the left panel, Gr1⁺ macrophages in the middle panel) and CD11c⁺CD45⁺ DCs (right panel) in healthy mice (d0) and on days 3 and 7 after UUO. **(D)** Three days after ureteric ligation, intracellular content of the profibrotic factor TGF-β was determined on F4/80⁺GFP⁺CD11c⁺ macrophages in ligated and contralateral kidneys as indicated and the numbers of these TGF-β⁺F4/80⁺GFP⁺CD11c⁺ macrophages expressing Gr1 or not were displayed. Dot plots in (A) represent concatenated dot plots from four to seven mice. Data represent the mean ± SEM from three (A–C) and four (D) individual experiments in groups of four to seven mice. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

deficiency (Fig. 5), indicating that the functional changes of macrophages could not be explained by effects on M1/M2 differentiation (70, 71). These data demonstrate that CX₃CR1 not only negatively regulates the numbers of profibrotic macrophages but also their fibrogenic potential.

CX₃CR1 regulates neither recruitment nor differentiation of kidney macrophages

The inhibitory effects of CX₃CR1 on renal macrophages could theoretically be due to reduced recruitment of macrophage precursors such as monocytes, reduced intrarenal differentiation into macrophages, reduced proliferation, increased survival or be due to a combination of these factors. Therefore, we examined all of these potential mechanisms.

To establish a system to analyze macrophage precursor recruitment to the kidney, we first adoptively transferred bone marrow–derived monocytes expressing a YFP reporter for CD11c, which allows convenient discrimination between DCs and macrophages, into CD45.1-congenic mice with or without UUO. Only a few monocytes reached the kidneys of healthy mice and the contralateral kidney of mice with UUO (Fig. 6A), suggesting

that renal phagocytes possess a low turnover rate. In contrast, ligated kidneys contained clearly detectable monocytes 2 d after transfer, and some of them were YFP⁺ (Fig. 6A), indicating differentiation into DCs.

To investigate whether CX₃CR1 is required for the entry of these precursors into the kidney, we transferred monocytes from CX₃CR1^{GFP/GFP} mice or CX₃CR1^{GFP/+} mice into CX₃CR1-competent wild-type recipients 1 d after ureter ligation (Fig. 6B, 6C). After an additional 2 d, less CX₃CR1-deficient phagocytes had entered the ligated kidneys than did CX₃CR1-competent phagocytes (Fig. 6C). Notably, no difference was seen in the blood (Supplemental Fig. 2), excluding an effect of CX₃CR1 on the abundance of circulating macrophage precursors. Thus, CX₃CR1 promoted, rather than inhibited, phagocyte precursor recruitment. Furthermore, the levels of the chemokine MIP-1α that can attract macrophage precursors were decreased in ligated and also in contralateral kidneys of CX₃CR1-deficient mice, and there were trends toward reduced levels of RANTES, MCP-1, and MCP-3 (Fig. 6D). This is consistent with reduced macrophage precursor recruitment and may be explained by the reduction in chemokine-producing DCs in CX₃CR1-deficient mice (3).

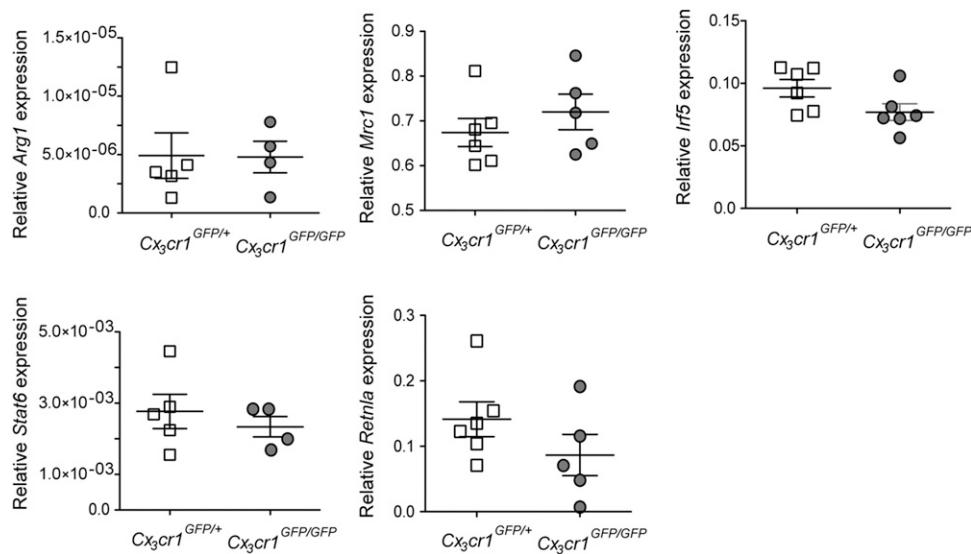


FIGURE 5. Polarization of macrophages does not depend on CX₃CR1. Gene expression profiles in contralateral and ligated kidneys 3 d after ureteric ligation. Data are normalized to the housekeeping gene Hprt and represent the mean ± SEM from two individual experiments in groups of four to six mice.

To study whether CX₃CR1 regulated the differentiation of monocytes into macrophages versus DCs, we determined the ratio between donor-derived DCs and macrophages after entering the ligated kidney. This parameter was not significantly changed in CX₃CR1 deficiency (Fig. 7A). Furthermore, the intrarenal levels of cytokines that affect DC/macrophage differentiation, GM-CSF or M-CSF, were CX₃CR1 independent (Fig. 7B). Taken together, these findings argue against changes in macrophage recruitment or differentiation as an explanation for their increased intrarenal numbers in CX₃CR1-deficient mice.

CX₃CR1 regulates intrarenal macrophage proliferation but not their survival

We next investigated whether CX₃CR1 regulates apoptosis by staining with Annexin V. We found that more Gr1[−] and Gr1⁺ macrophages were apoptotic within the ligated kidneys (Fig. 8A, 8B, black line versus gray line). However, lack of CX₃CR1 did not reduce but instead increased the frequency of Annexin V⁺Gr1[−] and Gr1⁺ macrophages (Fig. 8A, 8B), excluding apoptosis as the mechanism for the increased number of macrophages in CX₃CR1-deficient animals.

It has been previously reported that tissue macrophage numbers can be regulated by their local proliferation (72, 73). To investigate whether CX₃CR1 decreases intrarenal macrophage numbers by inhibiting their proliferation, we determined expression of the proliferation marker Ki67 on day 3 and uptake of BrdU from day 3 until day 5 after ureteric ligation. We found that Ki67 expression was significantly increased in macrophages of ligated kidneys from CX₃CR1-deficient mice compared with CX₃CR1-competent mice (Fig. 9A), and this was seen in both Gr1[−] and Gr1⁺ macrophages (Fig. 9B). Furthermore, more macrophages of CX₃CR1-deficient mice incorporated BrdU (Fig. 9C, 9D), and the uptake per macrophage was higher (Fig. 9E), verifying local CX₃CR1-dependent proliferation of macrophages. Notably, such increased proliferation was not detectable in DCs (Fig. 9D). Thus, CX₃CR1 inhibits local macrophage proliferation in UUO, and this can explain the higher numbers of profibrotic renal macrophages in the absence of CX₃CR1.

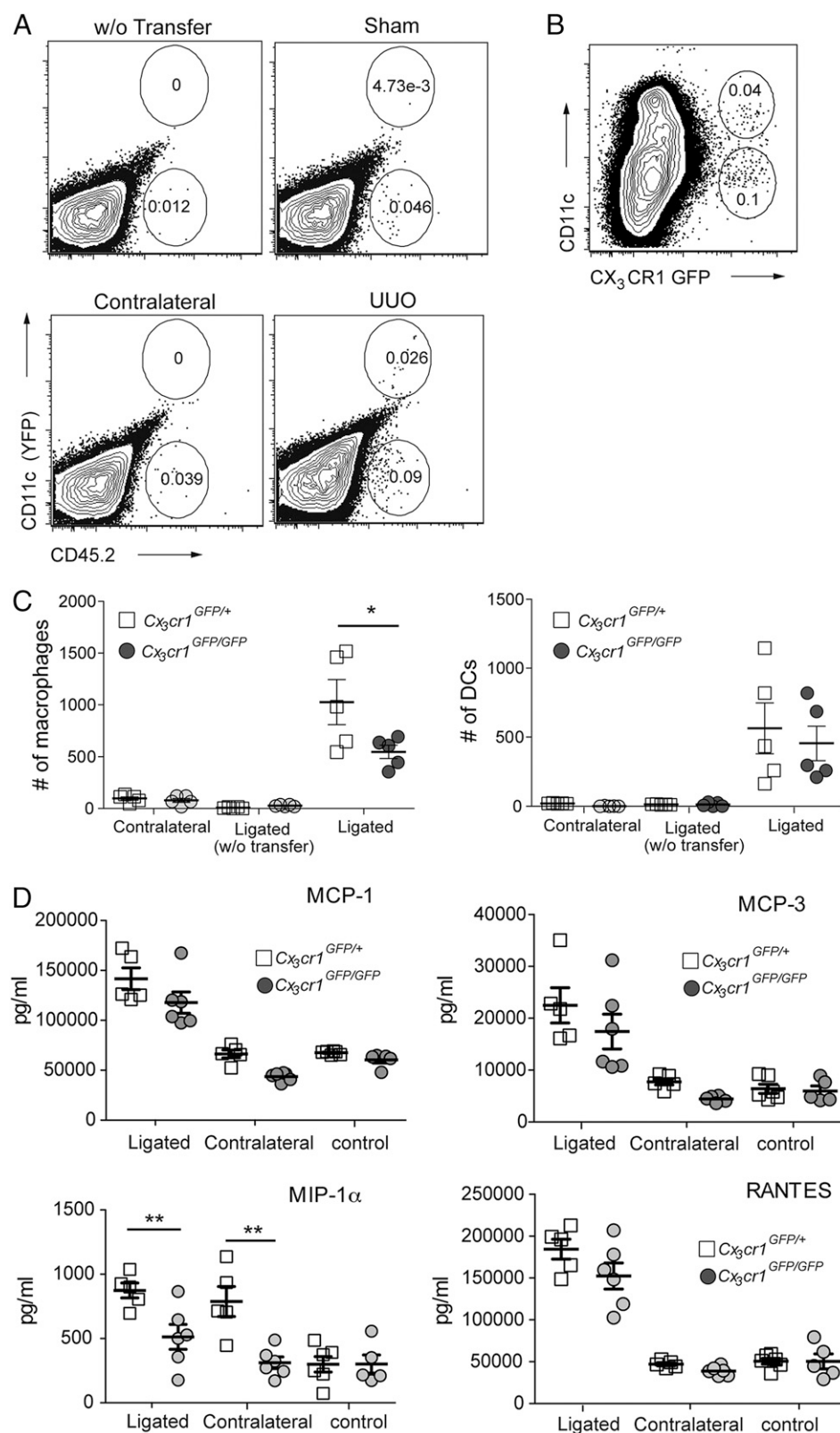
Discussion

The chemokine receptor CX₃CR1 is widely expressed by DCs and macrophages (74), but its functional role is still not fully clear. In this paper, we report a previously undescribed antifibrotic effect of

CX₃CR1: it reduced the inflammation-induced proliferation of TGF-β-producing renal macrophages, which are critically important in experimental kidney fibrosis (15–18). This finding was unexpected, given the proinflammatory and profibrotic properties of CX₃CR1 not only in diseases of the kidney (3, 49, 54, 57–59, 65), but also of the lung (75), liver (50), intestine (28), skin (55), arteriosclerotic vessels (76), and the CNS (33). CX₃CR1 has been shown to contribute to the inflammatory recruitment of monocytes from the circulation into all these organs (44). We found that CX₃CR1 had a similar effect in UUO so that fewer monocytes lacking this receptor entered the inflamed kidneys. The lower levels of monocyte-attracting chemokines and of inflammatory mediators that we detected in kidneys of CX₃CR1-deficient mice may be responsible for the reduced kidney recruitment of these cells. It is likely that these cells originated from monocytes, which have been shown to give rise to tissue macrophages under inflammatory conditions (5). The reduction of monocyte-attracting chemokines likely resulted from the paucity of DCs in kidneys of CX₃CR1-deficient animals because DCs are the principal producers of chemokines and inflammatory mediators in the kidney (77–79). However, these findings were hard to reconcile with the accumulation of macrophages in CX₃CR1-deficient mice, implying that other mechanisms must exist that overcompensated for the reduced attraction of macrophage precursors.

One such mechanism might be a negative effect of CX₃CR1 on the survival of tissue macrophages. However, previous studies in disease models of liver, CNS, gut, and arteriosclerotic vessels (74) demonstrated that CX₃CR1 signaling increased rather than reduced the life span of macrophages. In contrast to these findings, one recent study reported increased macrophage numbers and liver fibrosis in CX₃CR1-deficient mice after surgical bile duct ligation, a model that resembles UUO by being induced by mechanic tissue damage (50). The discrepancy between macrophage accumulation and decreased macrophage life span in that study was suggested to have resulted from the release of proinflammatory mediators from CX₃CR1-deficient dying macrophages that attracted further profibrotic macrophages. Although we did observe more dying macrophages after UUO in CX₃CR1-deficient mice, we found neither increased monocyte-attracting chemokines and inflammatory cytokines, nor did we find a stronger influx of inflammatory monocytes, arguing against inflammation resulting

FIGURE 6. Lack of CX₃CR1 decreases phagocyte recruitment into the kidney. **(A)** Sorted CD19⁺CD3⁺CD11c⁺ bone marrow cells from CD45.2 CD11c^{YFP} mice were adoptively transferred into CD45.1 wild-type mice. The frequency of donor YFP⁺CD45.2⁺F4/80⁺CD11c⁺ DCs (upper region) and of YFP⁺CD45.2⁺F4/80⁺CD11c⁺ macrophages (lower region) in the kidney was determined 3 d after ureter ligation (bone marrow cells were transferred 1 d after ligation). **(B)** Sorted CD19⁺CD3⁺CD11c⁺ bone marrow cells from *Cx₃cr1*^{GFP/+} mice were transferred into wild-type mice 1 d after ligation. Three days after ureter ligation GFP⁺F4/80⁺CD11c⁺ DCs (upper region) and GFP⁺F4/80⁺CD11c⁺ macrophages (lower region) were detected by flow cytometry. **(C)** Sorted CD19⁺CD3⁺CD11c⁺ bone marrow cells from *Cx₃cr1*^{GFP/+} or *Cx₃cr1*^{GFP/GFP} mice were transferred into wild-type mice. Three days after ureter ligation, the number of F4/80⁺GFP⁺CD11c⁺ macrophages (left panel) and of GFP⁺F4/80⁺CD11c⁺ DCs (right panel) were determined by flow cytometry. The group designated without transfer was used to control for specific detection of transferred cells. **(D)** Kidneys from *Cx₃cr1*^{GFP/+} (white squares) or *Cx₃cr1*^{GFP/GFP} (gray circles) mice were homogenized and levels of the chemokines MCP-1 (top left), MCP-3 (top right), MIP-1α (bottom left), and RANTES (bottom right) were determined 3 d after ureter ligation. Control animals did not undergo unilateral ureteral ligation. Data represent three (A–C) and two (D) experiments in groups of five to six mice. Results in (D) are mean ± SEM. **p* < 0.05, ***p* < 0.01.



from premature macrophage death in the kidney as the underlying reason for macrophage accumulation.

Another hypothetical mechanism is that CX₃CR1 might cause immigrating monocytes to differentiate preferentially into macrophages rather than DCs. DC numbers are severely reduced in CX₃CR1-deficient mice (3), which would be consistent with altered differentiation. DCs do not play a major role in UUO,

whereas macrophages are critical (18, 49, 54). This is not unexpected given that DCs primarily regulate adaptive immune responses, explaining why they affected fibrosis in models that involve adaptive immune cells, such as ischemia/reperfusion injury, crescentic glomerulonephritis and hypertensive nephropathy (3, 49, 54, 57). By contrast, a model of mechanic tissue injury like UUO is more likely mediated by cells of the innate immune

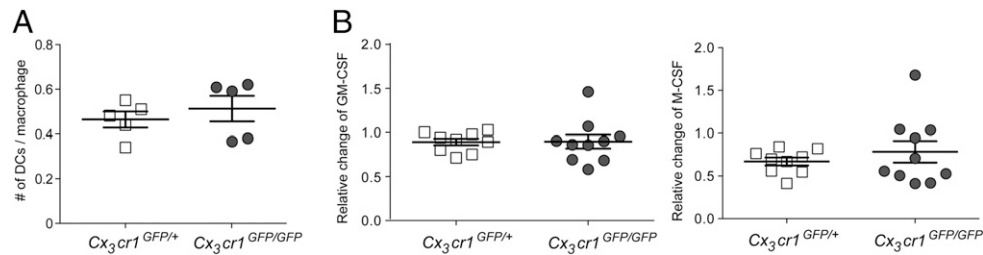


FIGURE 7. Differentiation of infiltrating monocytes into DCs is CX₃CR1-independent. **(A)** Sorted CD19⁺CD3⁺CD11c⁺ bone marrow cells from *Cx3cr1*^{GFP/+} or *Cx3cr1*^{GFP/GFP} mice were transferred into wild-type mice. Three days after ureter ligation, the number of F4/80⁺GFP⁺CD11c⁺ macrophages and of GFP⁺F4/80⁺CD11c⁺ DCs were determined by flow cytometry, and the ratio between these DCs and macrophages was calculated (number of DCs per macrophage). **(B)** Levels of GM-CSF (left) and M-CSF (right) in homogenates of ligated kidneys from *Cx3cr1*^{GFP/+} (white squares) and *Cx3cr1*^{GFP/GFP} (gray circles) mice 3 d after UUO in comparison with contralateral kidneys. Results are mean \pm SEM and represent three (A) and two (B) independent experiments in groups of 5–10 mice.

system, like macrophages. However, adoptive transfer experiments revealed that monocytes expressing or lacking CX₃CR1 gave rise to similar ratios between macrophages and DCs, arguing against an effect of CX₃CR1-signaling on the monocytes' decision to differentiate into either DCs or macrophages. This conclusion was corroborated by unchanged levels of the growth factors that usually govern this decision, M-CSF and GM-CSF.

In vivo multiphoton microscopic analysis revealed morphologic alterations of CX₃CR1-deficient renal phagocytes. Although there were slight changes in the morphology of CX₃CR1⁺ kidney DCs, such as more yet shorter dendrites (our unpublished observations), it is unlikely that these alterations impacted fibrogenesis given that DCs do not affect renal fibrosis (18, 49, 54). However, in addition to resident DCs, two-photon microscopy revealed larger and rounder CX₃CR1⁺ cells that were more motile and maintained their shape over time, which is consistent with the morphology

and dynamics of monocytes/macrophages. These cells were more abundant in CX₃CR1-deficient mice, prompting us to hypothesize about a functional link between macrophages and CX₃CR1. Indeed, we noted an inhibitory effect of CX₃CR1 on intrarenal macrophage proliferation. This can explain why these cells accumulated in CX₃CR1-deficient mice, although their precursor recruitment was less efficient and although more of them were apoptotic. As we excluded other potential causes, increased proliferation was most likely the cause for the accumulation of macrophages in the CX₃CR1-deficient kidney. It is possible that CX₃CR1 also prevents macrophage proliferation in DC-dependent models of renal disease (e.g., crescentic glomerulonephritis) but that this anti-inflammatory effect does not become manifest because its proinflammatory effect on the more numerous DCs prevails.

Traditionally, tissue macrophages were thought to arise from circulating monocytes recruited in response to locally produced chemokines (11). Recent findings have revealed that some tissue macrophages are derived from embryonic precursors (22). A sudden demand because of inflammatory conditions is met by the proliferation of such tissue-resident macrophages, in particular under Th2 or M2-associated conditions (72, 73, 80). We found that all macrophages, including those that had just recently been recruited to the kidney, showed stronger proliferation in the absence of CX₃CR1. These findings are consistent with the concept that local proliferation of tissue macrophages regulates their numbers and extend this concept by showing that also recently recruited macrophages are locally regulated in the inflamed kidney. It remains to be seen whether this mechanism also contributes to fibrosis in other organs.

CX₃CR1 not only affected the abundance, but also the functional state of renal macrophages. In its absence, more macrophages produced TGF- β , the key profibrotic mediator in the kidney (69). A previous study showed that CCR2-dependent macrophages produced TGF- β in UUO and that the lack of these cells significantly reduced renal fibrosis (16). Also, we found that both Gr1⁺ and Gr1⁺ macrophages produced TGF- β and that production was increased in the absence of CX₃CR1. TGF β production suggested acquisition of a profibrotic functional state change. This state could not be explained by M2 differentiation, nor was it associated with higher renal inflammation. Recently, the classical M1/M2 paradigm was refined by the discovery of an activation-independent functional state that does not fall into the M1 or M2 definition (71). Our findings are consistent with this notion by demonstrating that TGF- β -producing macrophages cannot be categorized as classical M1 and M2 macrophages. Future studies are required to clarify whether these profibrotic macrophages belong to a distinct functional state.

In summary, we report a novel inhibitory effect of CX₃CR1 on local macrophage proliferation and on fibrogenesis in the kidney.

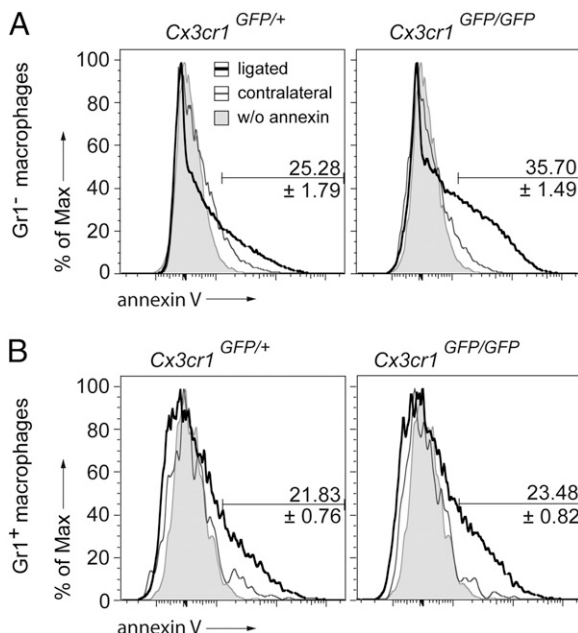


FIGURE 8. Lack of CX₃CR1 increases apoptosis of renal macrophages in UUO. **(A)** and **(B)** Gr1[−] (A) and Gr1⁺ (B) macrophages (CD45⁺CD11c[−]GFP⁺F4/80⁺; gating strategy, see Fig. 4A, 4B) from ligated (black thick line) and contralateral (gray line) kidneys from *Cx3cr1*^{GFP/+} (left panels) and *Cx3cr1*^{GFP/GFP} (right panels) mice were analyzed by flow cytometry for annexin V binding on day 3 after ureter ligation. The gray area shows fluorescence without addition of annexin V. Data are mean \pm SEM and the graphs represent concatenated histograms from seven to eight mice (concatenated = combined histograms from seven to eight mice).

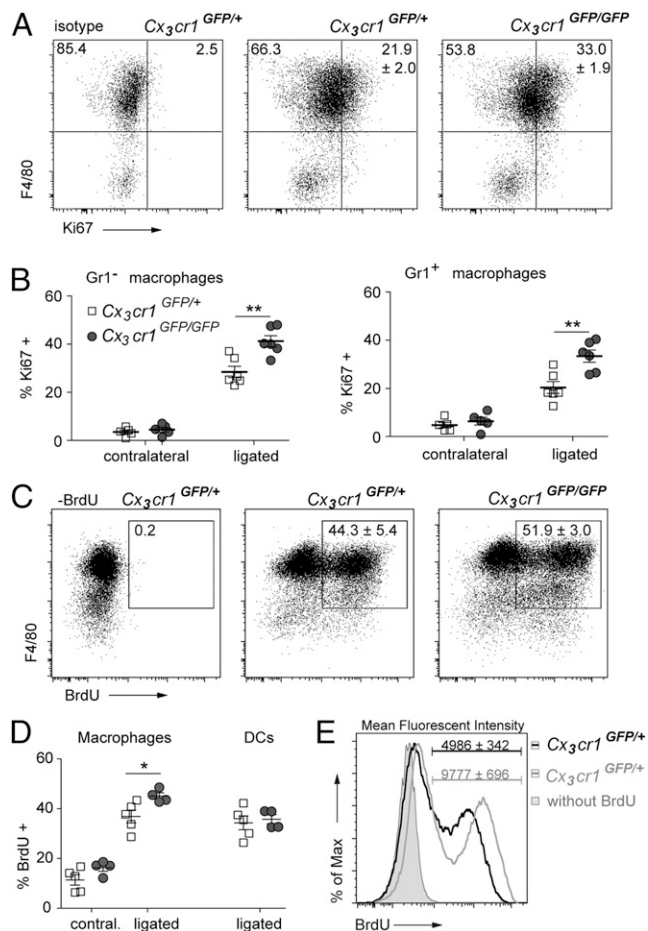


FIGURE 9. Lack of CX₃CR1 increases proliferation of Gr1⁺ and Gr1⁺ macrophages in UUO. **(A)** Three days after ureter ligation, intracellular expression of the proliferation marker Ki67 was determined on F4/80⁺GFP⁺CD11c⁺ macrophages in $Cx3cr1^{GFP/+}$ (middle dot plot) and $Cx3cr1^{GFP/GFP}$ (right dot plot) mice. Isotype Ab staining shows the negative control to ensure Ki67 specificity (left dot plot). **(B)** Percentages of Ki67⁺F4/80⁺GFP⁺CD11c⁺ macrophages expressing Gr1 (right) or not (left) in the ligated or contralateral kidneys of $Cx3cr1^{GFP/+}$ (white squares) and $Cx3cr1^{GFP/GFP}$ (gray circles) mice on day 3 after ureter ligation. **(C)** BrdU was injected into $Cx3cr1^{GFP/+}$ and $Cx3cr1^{GFP/GFP}$ mice 3 and 4 d after UUO. One day later (day 5), F4/80⁺GFP⁺CD11c⁺ macrophages in the ligated kidneys were analyzed for their BrdU incorporation. **(D)** Percentages of BrdU⁺F4/80⁺GFP⁺CD11c⁺ macrophages and BrdU⁺CD11c⁺ DCs of $Cx3cr1^{GFP/+}$ (white squares) and $Cx3cr1^{GFP/GFP}$ (gray circles) mice on day 5 after ureter ligation (BrdU was administered on day 3 and 4 after ligation). **(E)** BrdU was injected into $Cx3cr1^{GFP/+}$ and $Cx3cr1^{GFP/GFP}$ mice 3 and 4 d after UUO. One day later (day 5), F4/80⁺GFP⁺CD11c⁺ macrophages in the ligated kidneys were analyzed for their BrdU incorporation. Concatenated dot plots in (A) are combined data from six mice. Data (B–D) represent the mean \pm SEM in groups of four to six mice from three experiments. * $p < 0.05$, ** $p < 0.01$.

In crescentic GN, the previously described proinflammatory influence of CX₃CR1 on DCs prevailed (3) because macrophages are downstream of DCs in the pathomechanism of this disease and because DCs outnumber macrophages in the kidney. However, in DC-independent renal diseases like UUO, the inhibitory effect of CX₃CR1 on macrophages becomes manifest, as we demonstrated in the present study, so that CX₃CR1 inhibition in such diseases aggravates fibrosis. This may apply also to organs in which CX₃CR1 does not regulate DCs, like the liver or the lung (3). In this case, therapeutic CX₃CR1 inhibition in liver and lung disease may turn out profibrotic.

Acknowledgments

We thank F. Tacke for critically reading the manuscript and Dr. Camden Lo for assistance with in vivo imaging analytic parameters. We acknowledge support by the Flow Cytometry Core Facility and the House for Experimental Therapy of the medical faculty of Bonn University.

Disclosures

The authors have no financial conflicts of interest.

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