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Dual effect of chemokine CCL7/MCP-3 in the development of renal tubulointerstitial fibrosis

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Abstract

Most end-stage renal disease kidneys display accumulation of extracellular matrix (ECM) in the renal tubular compartment (tubular interstitial fibrosis – TIF) which is strongly correlated with the future loss of renal function. Although inflammation is a key event in the development of TIF, it can also have a beneficial anti-fibrotic role depending in particular on the stage of the pathology. Chemokines play an important role in monocyte extravasation in the inflammatory process. CCL2 has already been shown to be involved in the development of TIF but CCL7, a close relative of CCL2 and able to bind to similar receptors, has not been studied in renal disease. We therefore studied chemokine CCL7 in a model of unilateral ureteral obstruction (UUO)-induced TIF. We observed that the role of CCL7 differs depending on the stage of the pathology. In early stages (0–8 days), CCL7 deficient (CCL7-KO) mice displayed attenuated TIF potentially involving two mechanisms: an early (0–3 days) decrease of inflammatory cell infiltration followed (3–8 days) by a decrease in tubular ECM production independent of inflammation. In contrast, during later stages of obstruction (10–14 days), CCL7-KO mice displayed increased TIF which was again associated with reduced inflammation. Interestingly, the switch between this anti- to profibrotic effect was accompanied by an increased influx of immunosuppressive regulatory T cells. In conclusion, these results highlight for the first time a dual role for CCL7 in the development of renal TIF, deleterious in early stages but beneficial during later stages.

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Keywords

Inflammation; Fibrosis; Kidney

1. Introduction

End-stage renal disease is the consequence of a variety of renal disorders characterized by, among others, accumulation of extracellular matrix (ECM) accumulation in the interstitial space (tubulointerstitial fibrosis, TIF) that leads to impaired kidney function. The development of TIF can be schematically described as a succession of three steps. The process is initiated by an inflammatory phase [1,2] where tissue injury induces secretion of chemoattractant proteins that recruit immune cells, mainly monocytes/macrophages and T lymphocytes, from blood to the damaged site [1,3]. These cells produce profibrotic cytokines including transforming growth factor beta (TGFβ) and connective tissue growth factor (CTGF) [2,4,5]. The second step, partly induced by these cytokines, leads to appearance of a new cell type in the kidney: myofibroblasts. The precise origin of the myofibroblasts is still not clear [6,7]. In the third step, myofibroblasts induce the accumulation of ECM by their ability to produce high quantities of matrix proteins like collagens and fibronectin [2].

Although inflammation is a key event in the development of TIF, it can also have a beneficial anti-fibrotic role in some settings of nephropathy, depending in particular on the stage of the pathology. Indeed, it has been shown that macrophages are deleterious in the early phase of fibrosis while renoprotective in the chronic phase [3,8–11]. Therefore, insights into the mechanism responsible for monocyte/macrophage recruitment and subsequent activation are important for a better understanding of the development of TIF.

Monocyte extravasation is induced by a class of cytokines called chemokines [12–14]. CCL chemokines are the most potent mediators of the monocyte influx [12,13,15]. CCL2, and the corresponding CCR2 receptor, are the most largely characterized. In particular, CCL2 blockade decreased fibrosis associated renal lesions in a number of experimental nephropathies including ureteral obstruction [16], diabetic nephropathy [17] and glomerulonephritis [18]. CCL7, a close relative of CCL2, is less studied. Nevertheless, CCL7 is potentially involved in a number of processes related to inflammation and/or fibrosis. Indeed CCL7 is, as CCL2, an agonist of CCR2. In addition, CCL7 is crucial for monocyte mobilization from bone marrow to blood and from blood to the inflammatory site [19]. CCL7 is also able to directly stimulate type I collagen expression by skin fibroblasts *in vitro* and to work in concert with TGFβ on collagen synthesis [20,21]. Finally, at the intersection of these proinflammatory and profibrotic effects, CCL7 appears to be an homing factor in the heart for mesenchymal stem cells [22]. Collectively, these observations lead us to investigate the role of CCL7 in the development of TIF. For this purpose we studied the role of CCL7 in the progression of TIF induced by unilateral ureteral obstruction (UUO). UUO is a well-characterized model of non-immune mediated disease mimicking the different stages of the development of TIF in an accelerated and highly reproducible manner [2–4].

2. Materials and methods

2.1. Unilateral ureteral obstruction

Eight weeks of age CCL7 knockout (CCL7-KO [19]) and control wild type (WT) male C57Bl6 mice were used in all experiments. Unilateral ureteral ligation (UUO) was performed as previously described [23]. Control groups consisted of mice without experimentation. At the end of the protocol, mice were anesthetized, sacrificed by cervical dislocation and the kidneys were removed. For mRNA and protein analysis, kidney sections were snap frozen in liquid nitrogen and stored at −80 °C. For renal histology, kidney sections were fixed in Carnoy's solution and embedded in paraffin.

2.2. Cell culture

Immortalized human tubular proximal (HK-2) cells were cultured at 37 °C in 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) with 4.5 g/L glucose and 10% fetal calf serum (InVitrogen). HK-2 cells were seeded in 6-well plates and were treated for 4, 8 or 24 h with 100 ng/ml of human recombinant CCL7 (R&D systems) in the same medium but without fetal calf serum.

2.3. CCL7 protein

To measure renal CCL7 content, total proteins were extracted from snap frozen renal tissue in RIPA buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 0.1% SDS, 1% NP40, 1% sodium deoxycholate, 0.36 μg/ml PMSF, 0.01% SBTI, 0.01% leupeptin, and 0.01% aprotinin). CCL7 quantities were determined by ELISA (Gentaur), as described by the manufacturer.

2.4. Analysis of mRNA expression

Total RNA was isolated from snap frozen mouse tissue or HK-2 cells using the Qiagen RNeasy Plus Mini kit, eluted in 50 μl RNase-free water according to the manufacturer's protocol. 500 ng of total mRNA were retrotranscribed in cDNA using the Superscript II reverse transcriptase (Initrogen). After a pre-amplification step, cDNA quantification was analyzed by semi-quantitative PCR using the Fluidigm dynamic array (Fluidigm) in accordance with the manufacturer's protocol. The Fluidigm 96 × 96 qPCR thermocycling program on a BioMark (Fluidigm) was used for amplification. The relative mRNA copy number was calculated using Ct value and was normalized to GAPDH and 18S RNA in the experiments with UUO mice and HK-2 cells, respectively.

2.5. Immunohistochemistry analysis

After endogenous peroxidase blockage (S2001, DakoCytomation), 4-μm paraffin-embedded sections were incubated at room temperature with primary antibodies for the detection of F4/80 positive inflammatory cells (1/100, 45 min, RM2900, Caltag Laboratories), fibronectin (1/400, 30 min, F3648, Sigma), type I collagen (1/250, 30 min, CL50151AP, Cedarlane) and Foxp3 (1/100, 45 min, 14-5773-82, eBioscience) followed by anti-rabbit IgG Dako Envision HRP system (30 min, K4002, DakoCytomation) and a DAB substrate (10 min, TA-125-HDX, Thermoscientific). Histomorphometric analyses were performed as

previously described [23]. For F4/80, type I collagen and fibronectin stainings, results were expressed in percent of the total area. For Foxp3, results were expressed as mean of foxp3+ cells per field.

2.6. Statistical analysis

Data are expressed as mean ± SEM. For results in Tables 1 and 2, a Kruskal–Wallis test with *post hoc* Dunns test was performed for comparison to the control condition. For results presented in Figs 1–4, differences between WT and CCL7-KO mice were tested using a two-way ANOVA with a Bonferroni post-test. A $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. UUO induces CCL7 chemokine expression

We first analyzed CCL7 expression in the obstructed kidney and observed that UUO induced CCL7 mRNA expression at both 3 and 8 days after obstruction (Table 1). This was associated with a parallel increase of CCL7 protein 8 days after UUO (Table 1). It was already known that UUO increased the expression of CCL chemokines such as CCL2 and CCL5 [24]. Here we show that CCL7, another chemokine, is also induced in obstructive nephropathy.

3.2. CCL7 deficiency reduces renal inflammation in early stages of UUO

The main role of chemokines is to attract the inflammatory cells from the vessels to the damaged site. Among the chemokines, CCL7 has been described as a potent chemoattractant for monocytes [19,25]. As monocytes/macrophages infiltration plays a pivotal role in the progression of TIF, the induction of CCL7 during UUO suggested that CCL7 could be an important factor in the development of TIF *via* macrophage accumulation. To test this hypothesis, CCL7-KO mice were subjected to UUO and the expression of macrophage marker F4/80 was analyzed by immunohistochemistry. This showed a decrease of F4/80 positive cells in CCL7-KO compared to WT mice 3 days after UUO (Fig. 1). However, this difference disappeared 8 days after UUO. These results suggest that during early stages (3 days) of UUO, CCL7 contributes to the recruitment of monocytes/macrophages in renal tissue, leading to early tubulointerstitial inflammation. But at later stages (*e.g.* 8 days), monocyte/macrophage infiltration in UUO is most likely not controlled by CCL7.

3.3. CCL7 deficiency attenuates tubulointerstitial fibrosis in early stages of UUO

Next we examined whether decreased inflammation in CCL7-KO mice was accompanied with reduced TIF. TGFβ and CTGF are the main profibrotic cytokines [2,4,5]. They are involved in fibroblast activation and epithelial-to-mesenchymal transition, which are potential mechanisms leading to myofibroblast accumulation [6,26]. By secreting extracellular matrix proteins, including collagens and fibronectin, myofibroblasts are the major ECM producing cells [6,26]. Therefore the effect of CCL7 knockout on UUO-induced TIF development was studied by analyses of the expression of TGFβ and CTGF, αSMA and vimentin as myofibroblast markers, and type I collagen and fibronectin as ECM markers. In CCL7-KO mice, reduced mRNA expression levels of TGFβ, αSMA and vimentin were

observed after 3 days of UUO compared to WT mice. This was accompanied by decreased fibronectin accumulation, without changing CTGF mRNA levels and type I collagen expression (Fig. 2). Thus, following the sequence of events in UUO [2,7], CCL7-induced inflammation can lead to increased expression of TGFβ which in turn stimulates myofibroblast accumulation and fibronectin synthesis. Our results are therefore consistent with the large number of studies highlighting that macrophages are deleterious for the kidney during the early phase of UUO-induced TIF [16,27,28].

Interestingly, 8 days after UUO, CTGF mRNA expression and type 1 collagen deposition were decreased in CCL7-KO mice compared to WT mice while TGFβ, αSMA and vimentin mRNAs were not modified (Fig. 2). Thus in the period from 3 to 8 days post- UUO, CCL7 potentially contributes to ECM remodeling by stimulating type 1 collagen deposition, independently of the number of macrophages (Fig. 1) and myofibroblasts (Fig. 2). At least two explanations are available for this discrepancy. First, decreased CTGF expression and type I collagen synthesis can be a delayed response to the early (3 days) reduced inflammation. On the other hand, it can be due to a direct effect of CCL7 in renal tubular cells. Indeed, (i) although macrophages and myofibroblasts are the main profibrotic cytokines and matrix proteins producing cells, injured tubular cells also contribute to this process [29,30]; (ii) CCL7 is able to induce ECM protein production by fibroblasts [20,21]; (iii) CCL7 is an agonist for CCR2 and CCL2 activated- CCR2 increases ECM protein production, including collagen, in mesangial cells and pulmonary fibroblasts [31,32]. To test this second hypothesis, we studied the effect of human recombinant CCL7 on CTGF and type I collagen mRNA in HK-2 cells. We observed that CCL7 y stimulated both CTGF (4 h) and type I collagen mRNA (24 h) expression (Table 2), thereby suggesting for the first time that CCL7 is a direct profibrotic cytokine for tubular cells in the kidney.

Taken together, these *in vitro* and *in vivo* data suggested that CCL7 promotes early renal inflammation and tubular cell activation at a later stage, leading thereby to fibrogenesis.

3.4. Role of CCL7 in regulatory T cell influx

CCL7 is not only a CCR1, CCR2 and CCR3 agonist but also a CCR5 antagonist [33,34]. Since these receptors are expressed by a wide range of immune cells [14,35], including T lymphocytes shown to participate in the development of TIF [36], we explored the possibility that CCL7 modifies the type of immune cell recruited in the kidney during UUO. To assess this hypothesis, we screened the renal expression of other immune cell markers (CDs, cytokines, chemokines, chemokine receptors). We observed that mRNA levels of CD3e, a T lymphocyte lineage marker [37], were increased 8 days after UUO and this effect was amplified in CCL7-KO mice (Fig. 3A). Since CD3e does not discriminate between the different T subfamilies, we also analyzed mRNA expression of CD4, CD25, foxp3 and anti-inflammatory cytokine IL-10, hallmarks of regulatory T cells (Tregs) [38]. Interestingly, mRNA expression of those four markers was increased by UUO, but was more pronounced in CCL7-KO mice than in WT mice (Fig. 3A), suggesting that the absence of CCL7 increases the presence of regulatory T cells in renal tissue. Finally, to confirm these results, foxp3 immunostaining was performed. Although mRNA levels were different between WT and CCL7-KO mice, foxp3 protein levels were not modified 8 days after obstruction.

However analysis of a later time point (10 days) showed that foxp3 protein expression was higher in CCL7-KO mice (Fig. 3B). Therefore, potentially, CCL7 contributes to a reduced Treg influx in later stages of UUO.

3.5. Late stage effects of CCL7 deficiency on inflammation and tubulointerstitial fibrosis

Tregs are the most potent anti-inflammatory cells that contribute to the ending of inflammation in the kidney [39,40]. Because CCL7 deficiency modified the renal inflammatory environment in the late stages of UUO, we re-evaluated macrophage infiltration at 10 and 14 days post-UUO in CCL7-KO mice. We observed that F4/80 staining decreased in CCL7-KO compared to WT mice 14 days after UUO (Fig. 4A). Inflammation has been considered for a long time as deleterious in the development of TIF. However, recent studies showed that this is true for early time-points but probably incorrect for later stages in UUO-induced TIF since the number of macrophages recruited in the kidney and degree of fibrosis are inversely correlated 14 days post UUO [8–11]. Moreover, it was shown that Tregs can be involved in the development of fibrosis but their pro- or antifibrotic role still needs further investigation [39,41,42]. As for macrophage infiltration, Tregs could have a dual role, either beneficial or detrimental, depending on the stage of the pathology [43]. Therefore, we also re-evaluated type I collagen deposition 10 and 14 days after UUO. Interestingly, CCL7-KO mice displayed increased type I collagen immunostaining compared to WT mice (Fig. 4B). Thus, in contrast with its effects in early stage disease, CCL7 in late stages could be involved in reduction of TIF by limiting Treg recruitment, thereby maintaining late and protective inflammation.

Results of the present study argue for a dual role of inflammation in TIF. In addition, they suggest that the CCL7 ambivalent effects – i.e. profibrotic during early phase and antifibrotic during later phases of UUO-induced TIF – are probably a consequence of this inflammation duality. Such opposite effects have been shown for other molecules such as matrix metalloproteinase [44], hypoxia- inducible factor activator [45] or plasminogen activator inhibitor- 1 [46]. These and our studies highlight the concept of a therapeutic time window and the need to study effects of pharmacological treatments or knockouts on the different stages of a disease. Our data are also consistent with a detrimental role of Tregs, since we showed increased Treg recruitment (10 days) which is followed by reduced macrophage infiltration associated with increased TIF in UUO-subjected KO-CCL7 mice (14 days). Further studies should be thus performed to assess the precise role and kinetic of Tregs in the development of UUO-induced TIF.

In conclusion, the present study demonstrated for the first time a role for CCL7, an underestimated chemokine, in the development of inflammation-associated renal tubulointerstitial fibrosis. Therefore, targeting renal CCL7 or related pathways may be an interesting strategy to attenuate or revert fibrosis in chronic kidney diseases.

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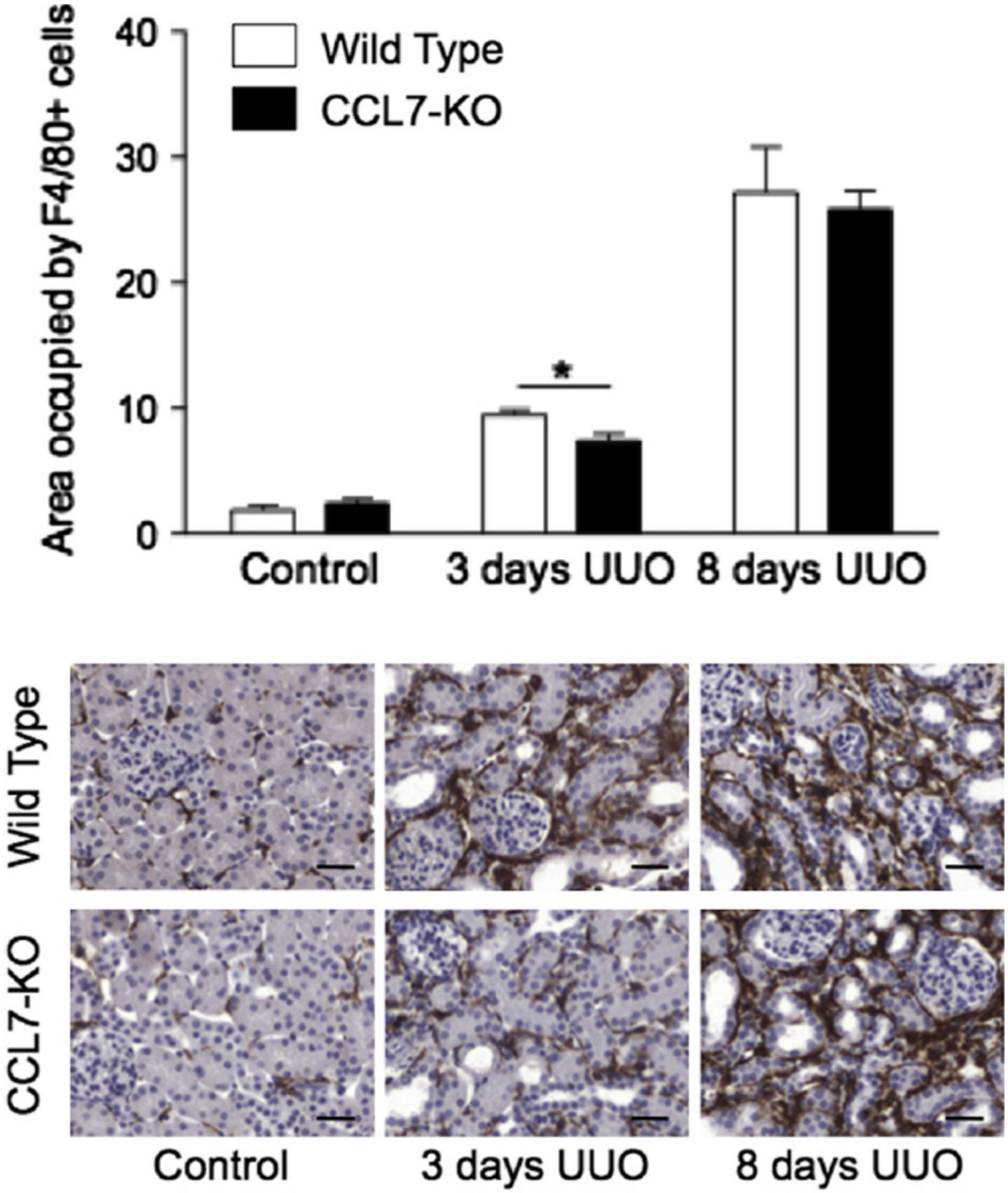

Fig. 1.

Inflammation is reduced in CCL7-KO mice after 3 days but not after 8 days of UUO. CCL7 knockout mice (CCL7-KO, black bars) and their wild-type littermates (WT, open bars) were subjected or not (Control) to 3 or 8 days of UUO. To characterize inflammation, the level of protein F4/80, a marker of monocytic lineage, was evaluated by immunohistological staining. Pictures display representative areas of F4/80 staining. Values are means ± SEM from 6 mice per group. $^{**}P < 0.01$.

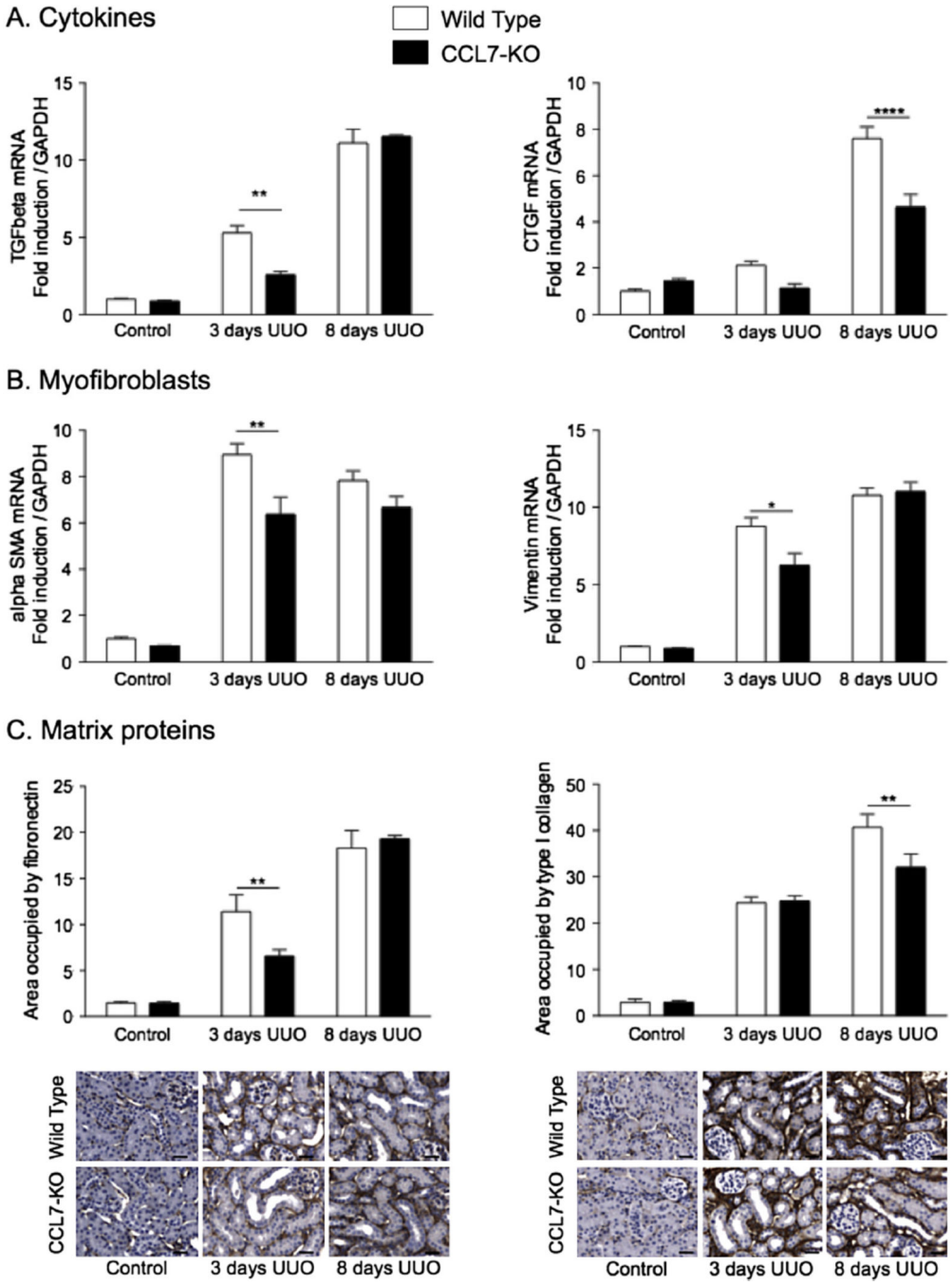

Fig. 2.

CCL7 depletion protects against tubulointerstitial fibrosis development in early stages (3 and 8 days) of UUO. CCL7 knockout mice (CCL7-KO, black bars) and their wild-type littermates (WT, open bars) were subjected or not (Control) to 3 or 8 days of UUO. (A) To evaluate the synthesis of profibrotic cytokines, renal mRNA encoding for TGFβ or CTGF was quantified by real-time PCR and results are expressed as the fold induction compared to control. (B) For the characterization of myofibroblast appearance, renal mRNA encoding for αSMA or Vimentin were quantified as in A. (C) For analysis of extracellular matrix

accumulation, Fibronectin and type 1 Collagen were detected by immunohistological staining. Pictures display representative areas of fibronectin and type 1 Collagen staining. Values are means ± SEM from 6 mice per group. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

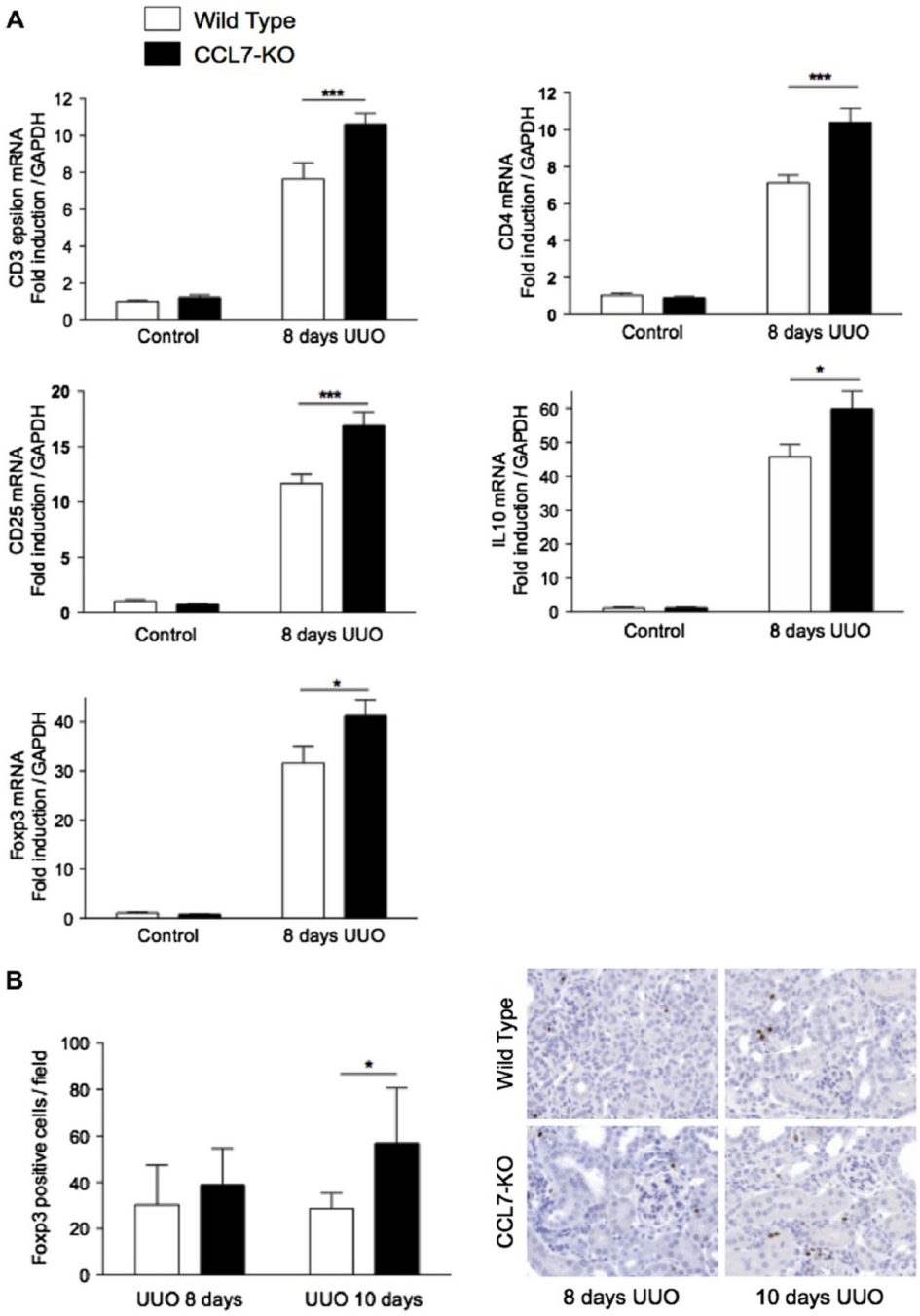

Fig. 3.

CCL7 blockade increases regulatory T cell influx. CCL7 knockout mice (CCL7-KO, black bars) and their wild-type littermates (WT, open bars) were subjected or not (Control) to 8 or 10 days of UUO. (A) The combined mRNA levels of CD3ε, CD4, CD25, foxp3 and IL-10, as hallmark of regulatory T cells, were quantified by real-time PCR. Results are expressed as the fold induction compared to Control. (B) Foxp3 proteins were detected by immunohistological staining. Pictures display representative areas of Foxp3 staining. Values are means ± SEM from 6 mice per group. **P* < 0.05, ****P* < 0.001.

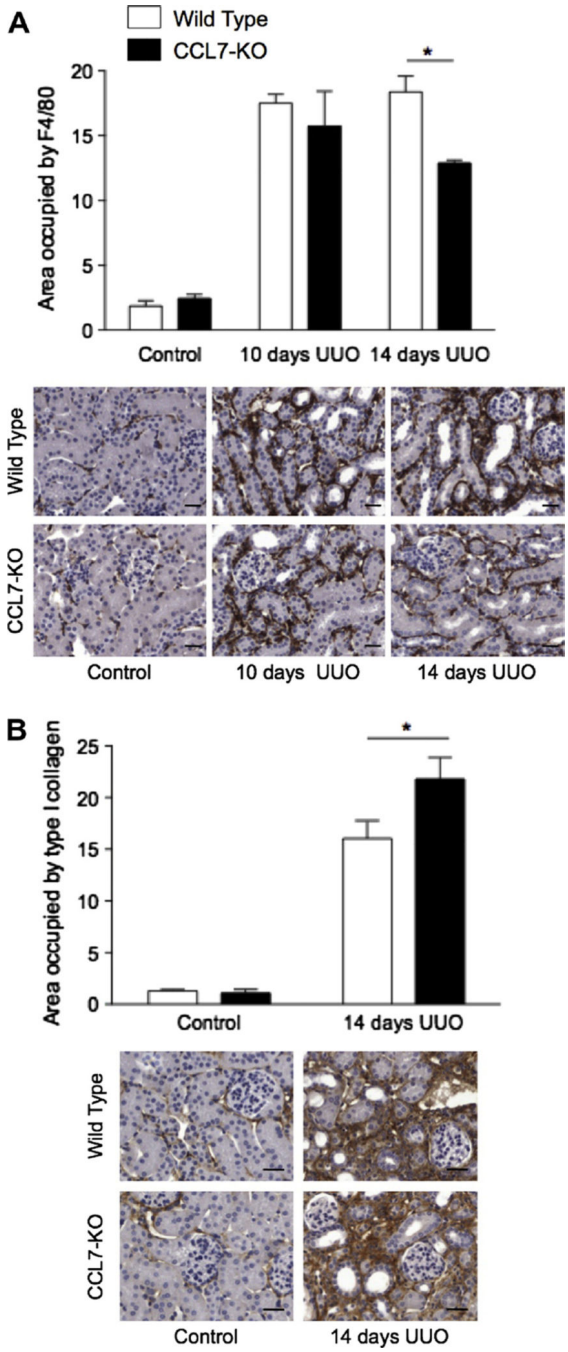

Fig. 4.

CCL7 depletion reduces inflammation and increases type I collagen accumulation in late stages (14 days) of UUO. CCL7 knockout mice (CCL7-KO, black bars) and their wild-type littermates (WT, open bars) were subjected or not (Control) to 10 or 14 days of UUO. (A) F4/80 protein and (B) type 1 Collagen were detected by immunohistological staining, as described in Figs. 2 and 3, respectively. Values are means ± SEM from 6 mice per group. *$P < 0.05$.

Table 1

UUO induces CCL7 chemokine expression.

	UUO duration (days)		
	0	**3**	**8**
CCL7 mRNA	1.14 ± 0.28	24.29 ± 3.68*	81.92 ± 11.53***
CCL7 protein	1.00 ± 0.31	1.19 ± 0.20	2.84 ± 0.37**

Wild-type mice were subjected or not (Control) to 3 or 8 days of UUO. Renal mRNA encoding for CCL7 and CCL7 protein were quantified by real-time PCR and ELISA, respectively. Results are expressed as the fold induction compared to Control. Values are means ± SEM from 6 mice per group.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$ versus Control.

Table 2

CCL7 induces expression of profibrotic cytokines and ECM proteins in tubular cells.

	Treatment duration (h)			
	0	**4**	**8**	**24**
CTGF mRNA	1.03 ± 0.18	1.68 ± 0.04*	1.41 ± 0.10	1.13 ± 0.10
Type I collagen mRNA	1.03 ± 0.17	2.86 ± 0.08	2.84 ± 0.20	4.88 ± 1.14**

Confluent monolayers of HK-2 cells were treated or not (Control) with 100 ng/ml of human recombinant CCL7 for 4, 8 or 24 h. mRNA encoding for CTGF and type I collagens were quantified by real-time PCR and results are expressed as the fold induction compared to Control. Values are means ± SEM from 3 experiments.

* $P < 0.05$.

** $P < 0.01$ versus Control.