Inflammopharmacol (2018) 26:403–411
https://doi.org/10.1007/s10787-017-0317-4

Inflammopharmacology

ORIGINAL ARTICLE

CCR2 contributes to the recruitment of monocytes and leads to kidney inflammation and fibrosis development

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Received: 9 October 2016 / Accepted: 23 January 2017 / Published online: 6 February 2017

Abstract Chemokines are a large family of proteins that, once associated to its receptor on leukocytes, stimulate their movement and migration from blood to tissues. Once in the tissue, immune cells trigger inflammation that, when uncontrolled, leads to fibrosis development. Among the immune cells, macrophages take a special role in fibrosis formation, since macrophage depletion reflects less collagen deposition. The majority of tissue macrophages is derived from monocytes, especially monocytes expressing the chemokine receptor CCR2. Here, we investigated the role of infiltrating $CCR2^{+}$ cells in the development of fibrosis, and specifically, the dynamic of infiltration of these cells into kidneys under chronic obstructive lesion. Using liposome-encapsulated clodronate, we observed that macrophage depletion culminated in less collagen deposition and reduced chemokines milieu that were released in the damaged kidney after obstructive nephropathy. We also obstructed the kidneys of $CCL3^{-/-}$, $CCR2^{-/-}$, $CCR4^{-/-}$, $CCR5^{-/-}$, and C57BL/6 mice and we found that among all animals, $CCR2^{-/-}$ mice demonstrated the more robust protection, reflected by less inflammatory and Th17-related cytokines and less collagen formation. Next we evaluated the dynamic of $CCR2^{+/rfp}$ cell infiltration and we observed that they adhere onto the vessels at early stages of disease, culminating in increased recruitment of $CCR2^{+/rfp}$ cells at later stages. On the other hand, $CCR2^{rfp/rfp}$ animals exhibited less fibrosis formation and reduced numbers of recruited cells at later stages. We have experimentally demonstrated that inflammatory $CCR2^{+}$ cells that reach the injured kidney at initial stages after tissue damage are responsible for the fibrotic pattern observed at later time points in the context of UUO.

Keywords $CCR2^{+}$ monocytes · Fibrosis · UUO

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Introduction

The term "chemokine" is a contraction of "chemotactic cytokine" and refers to a large family of structurally homologous proteins that stimulate the movement of leukocytes and regulate their migration from blood to tissues (Griffith et al. 2014). Chemokines are classified into four families, based on the number and location of the N-terminal cysteine residues. The first two families are the CC chemokines, where residues are adjacent, and the CXC family, whose residues are separated by one amino acid. A small number of chemokines has a single cysteine (C family) or two cysteines separated by three amino acids (CX3C) (Rot and von Andrian 2004). Chemokine receptors, in turn, are differentially expressed on all leukocytes. The receptor occupancy of its respective chemokine immediately initiates intracellular responses (Bromley et al. 2008; Sallusto and Baggiolini 2008), such as

increasing cytoplasmic calcium and activation of protein kinase C (Rot and von Andrian 2004). Indeed, changes in the cytoskeleton as well as the polymerization of the actin and myosin filaments were observed, resulting in increased cell motility. These signals also alter the conformation of cell surface integrins, increasing the affinity of them with their ligands (Kufareva et al. 2015).

Activated leukocytes play critical roles in injury and fibrosis development (Bohle et al. 1992; Sean Eardley and Cockwell 2005). Subsets of inflammatory cells are recruited into injured compartments after the release of pro-inflammatory mediators by activated resident cells. Recruited inflammatory cells propagate and amplify the immune response through the release of cytokines and growth factors, modulate extracellular matrix synthesis by the release of proteases, further promote the lesion by reactive oxygen species (ROS), and also initiate the healing process and tissue repair via the removal of immune complexes or cellular debris (Eddy 2000; Segerer et al. 2000; Sean Eardley and Cockwell 2005). The release of pro-inflammatory mediators, the activation of resident cells, hypoxia, and ROS production characterize the inflammation process and ultimately promotes fibrosis (Porubsky et al. 2004). Moreover, the number of macrophages and T cells generally correlates with the loss of tissue function in various forms of diseases due to uncontrolled immune response initiated by these cells (Vielhauer et al. 2004; Segerer and Nelson 2005).

CCR2 and its main ligand, CCL2, an inflammatory chemokine also known as monocyte chemotactic protein 1 (MCP-1) (Segerer and Nelson 2005) have been implicated in the pathogenesis of several different disease processes (O'Connor et al. 2015). It has been shown that CCR2 is responsible for Ly6C^{high} monocyte recruitment (Kitagawa et al. 2004; Peng et al. 2015a, b) and regulation of bone marrow-derived fibroblasts (Xia et al. 2013) in kidneys underwent ureteral obstruction. Besides chemotactic function, CCL2 presents pro-survival properties once deficiency for CCL2 aggravates tubular damage in the ischemia/reperfusion model of disease (Stroo et al. 2015). CCR2 is, however, mainly related to the influx of inflammatory monocytes into the injured kidney (Giunti et al. 2010; Chousterman et al. 2016). In addition to inflammatory monocytes, CCR2 is also expressed in mesenchymal stem cell-derived exosomes. In this context, CCR2 regulates inflammation and renal injury repair by acting as a decoy to suppress CCL2 activity (Shen et al. 2016). Indeed, blocking CCR2 in renal ischemia/reperfusion injury (Furuichi et al. 2003a, b) and unilateral ureter obstruction (UUO) model (Lefebvre et al. 2016) led to decreased renal damage and reduced influx of macrophages and neutrophils. Also, it was observed that pharmacological blockage of CCR2 reduces the percentage of cells co-expressing F4/80 and CD206 accumulating in the kidneys, a reduction that is related to kidney protection in experimental renal artery stenosis (Kashyap et al. 2016). CCL2 mRNA and protein were also measured in the urine of patients with lupus nephritis, and its levels correlated with the clinical disease activity and histological score (Nakashima et al. 2004). Accordingly, CCR2 inhibition with CCX140-B has renoprotective effects on top of current standard medication in patients with type 2 diabetes and nephropathy (Weir 2015). In the present study, we aimed to investigate the role of infiltrating monocytes for the development of fibrosis, and specifically, the milieu of chemokines released in the context of UUO. We confirmed the involvement of CCR2 in renal pathology by analyzing kidney damage and fibrosis formation in animals deficient for CCR2. In addition, we investigated the dynamic of CCR2^{+} cell infiltration in kidneys under chronic obstructive lesion. We observed that inflammatory CCR2^{+} cells that reach the injured kidney at initial stages after tissue damage are responsible for the fibrotic pattern observed at later time points in the context of UUO.

Materials and methods

Animal studies

Male CCL3$^{-/-}$, CCR2$^{-/-}$, CCR4$^{-/-}$, CCR5$^{-/-}$, CCR2$^{+/rfp}$, and CCR2$^{rfp/rfp}$, all in C57BL/6 background, and control mice aging 6–8 weeks, from Jackson Laboratory, were bred and housed in a pathogen-free facility. CCR2$^{rfp/rfp}$ animals express red fluorescein protein (RFP) instead of CCR2 and CCR2$^{+/rfp}$ animals express both CCR2 and RFP. Mice were kept on a 12 h light/dark cycle in a temperature-controlled room at 21–23 °C, with free access to water and food. On day 0, mice were anesthetised with Ketamine–Xylazine (Agribrands do Brazil, Sao Paulo, Brazil) and UUO was performed by complete ligation of the left ureter. Animals were placed in single cages and warmed by indirect light until completely recovered from anesthesia. Seven days later, mice were sacrificed for biochemical, histological, protein, and genetic analyses. All procedures were approved by the internal ethics committee of the University of Sao Paulo (CEUA 45/2009).

Liposome preparation and macrophage depletion

The macrophage depletion kinetics was previously evaluated (Braga et al. 2016). Briefly, an ethereal solution (0.5 mL) containing phosphatidylcholine (50 mg), and cholesterol (8 mg) were injected (0.2 mL/min) into 5 mL of a 50 mM clodronate (Schering, São Paulo, Brazil) aqueous solution maintained at 42 °C. The liposome

suspension was centrifuged at 22,800*g* for 30 min (Hitachi Himac CR20B2 centrifuge; Hitachi Ltd.) at 25 °C, and the liposome-containing pellet was washed by centrifugation. The final pellet was resuspended in 2 mL of saline solution. The final phosphatidylcholine and clodronate concentrations in the liposomes were 10 and 0.5 mmol/L, respectively. Mice were injected i.p. with 100 μL of liposome preparation (6 μg of clodronate) 24 h before the UUO surgery and with 200 μL of liposome preparation (12 μg of clodronate) during surgery and 3 d later. Animals were killed after 7 days of surgery.

Renal function outcomes

Proteinuria was measured in samples collected from the pelvis of obstructed mice 7 days post-surgery and from mice before surgery. All samples were analyzed by colorimetric assays using commercially available kits for creatinine and protein measurement (Labtest, Minas Gerais, Brazil). Normalization was achieved by dividing the protein/creatinine ratio at day 7 by the ratio before surgery in each animal in each group.

Sirius red staining

Kidneys were harvested and placed in 10% buffered formaldehyde for fixation. Summarily, the slides were de-paraffinized, rehydrated, and immersed in saturated picric acid solution for 15 min and then in Picrosirius for 20 min. Counterstaining was performed using Harris hematoxylin. Picrosirius-stained sections were analyzed by an Olympus BX50 microscope and camera. Manual photographs were taken of the cortex, magnified at 20×, and observed under polarized light. Images of at least 20 different fields in each slide were taken, and structures such as the glomeruli, subcapsular cortex, large vessels, and medulla were excluded. For the morphometric analysis, image processing and analysis in Java Image J software was used. The result of the analysis was represented by percentage and refers to the proportion of the stained volume to the total cortical interstitial volume.

Cytokines profile

Renal tissue was lysed in RIPA buffer with protease inhibitor. CCL2, CCL3, CCL4, CCL5, and CXCL2 were measured in a Bio-Plex mouse Plex cytokine assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The assay was read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager software version 4.0. Standard curves ranged from 32,000 to 1.95 pg/mL.

Intravital microscopy

Mice were anesthetized by i.p. injection of Ketamine–Xylazine, and during the experiments the animals were maintained at 37 °C on a microscopy heating chamber (PeCon GmbH, German). $CCR2^{+/rfp}$ or $CCR2^{rfp/rfp}$ animals were used to study the recruitment of monocytes in the microcirculation of the obstructed kidneys. The obstructed and normal kidneys were exteriorized through a lateral incision, and immobilized in a heated well incorporated into a custom-built stage. They were observed with a confocal microscope (LSM 780 Carl Zeiss) under a 20× and 40× objective (20/0.50 NA). The experiments were performed at days 1, 2, 6, and 14 after obstruction using a 488- and 543-nm laser lane of excitation. The images were collected using a single *z* step and a time-lapse course was acquired every 4 s over a 45 min period. The emitted fluorescence was detected by scanned detectors with 490–520, 575–605-nm and emission filters. Pre-defined settings for laser power and detector gain were used for all experiments (Sipos et al. 2007).

The images were analyzed according to a previously described protocol using ImageJ software (Menezes et al. 2008). Briefly, the numbers of $CCR2^{+/rfp}$ or $CCR2^{rfp/rfp}$ rolling, adherent, and recruited cells were determined off-line during video playback analysis. The rolling CCR2 (RFP) cells were considered as those cells moving at a velocity less than that of erythrocytes within a given vessel and they were considered as the number of rolling leukocytes passing at a given point in the venule per minute. The adherent leukocytes were considered to remain as stationary cells for at least 15 s. and total leukocyte adhesion was considered as the number of recruited cells within a 5 μm length of tissue in 15 min.

Statistics

The data were described in terms of the mean and S.E.M. Differences among groups were compared using ANOVA (with Tukey's post-test). Significant differences were considered as $p < 0.05$. All statistical analyses were performed using GraphPad Prism® 5. ANOVA one way with Tukey's post hoc test was performed to verify significant differences between the groups.

Results

Macrophage role in kidney fibrosis development

Several groups have shown that macrophage depletion leads to less collagen deposition and further protection in

UUO model (Sung et al. 2007; Kitamoto et al. 2009; Braga et al. 2012). We confirmed such data after depleting macrophages in C57BL/6 mice that underwent UUO using liposome-encapsulated clodronate. We observed that macrophage depletion led to reduced proteinuria and less collagen deposition, as seen in Fig. 1a–c. Next, we investigated specific chemokines and their receptors that macrophages use to its process of infiltration in injured kidneys. Macrophage-depleted animals showed lower levels of MCP1/CCL2, MIP1α/CCL3, MIP1β/CCl4, and MIP2α/CXCL2 protein, as compared to non-treated animals after UUO. Otherwise, RANTES/CCL5 did not change among the groups (Fig. 2a–e). Such data demonstrate that macrophages either amplify the inflammation pattern through the recruitment of additional immune cells, or provide cytokines and chemokines to activate residential cells, leading to fibrosis development in obstructive nephropathy.

CCR2 and CCR4 are important in fibrosis development

We next investigated the development of fibrosis and the production of inflammatory pattern-related cytokines into different chemokine and chemokine receptor-deficient animals. In addition to CCL3-deficient mice, we obstructed the ureter of $CCR2^{-/-}$ mice (the receptor of CCL2, CCL7, CCL12, CCL13, and CCL16), $CCR4^{-/-}$ (the CCL17 and CCL22 receptor), and $CCR5^{-/-}$ animals (the receptor of CCL3, CCl4, CCL5, CCL8, and CCL14). $CCR2^{-/-}$ and $CCR4^{-/-}$ animals were protected against the development of renal fibrosis, since these animals showed decreased proteinuria and less collagen deposition when compared to WT animals, as seen in Fig. 3a, b. On the other hand, mice deficient in CCL3 or CCR5 were not protected against fibrosis development.

Thereafter, we investigated the inflammatory pattern in obstructed kidneys of these animals. $CCR2^{-/-}$ and $CCR4^{-/-}$

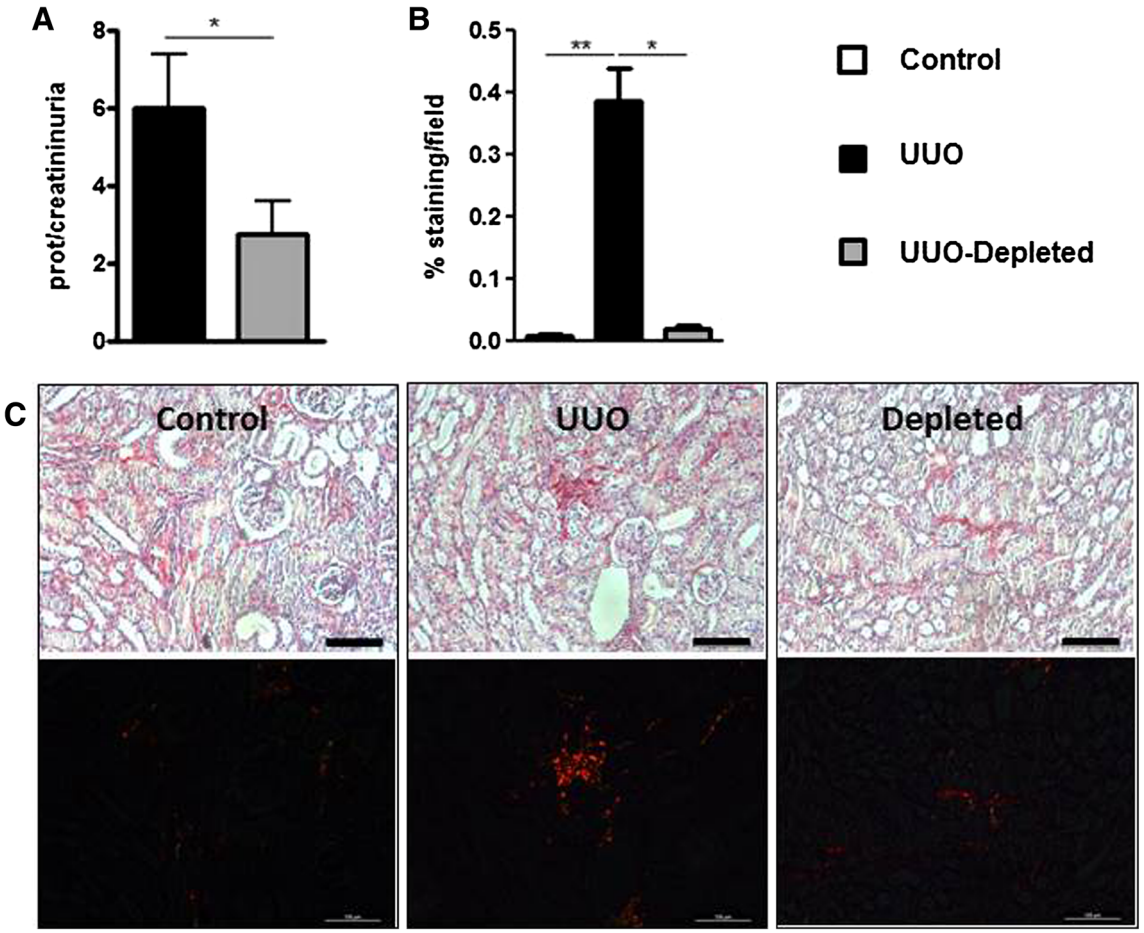

Fig. 1 Macrophage depletion leads to kidney protection. WT animals underwent UUO and treated with liposome-encapsulated clodronate at one day before, on the day of surgery, and three days after UUO. **a** Ratio protein/creatinine in urine of pelvis, normalized by the ratio before surgery, and **b** deposition of collagen by Sirius red staining in obstructed kidneys on the seventh day following UUO. **c** Representative micrographs of collagen deposition. The *bars* represent 100 μm. The statistical test used was ANOVA. $*p < 0.5$; $**p < 0.1$. $n = 5$ animals per group

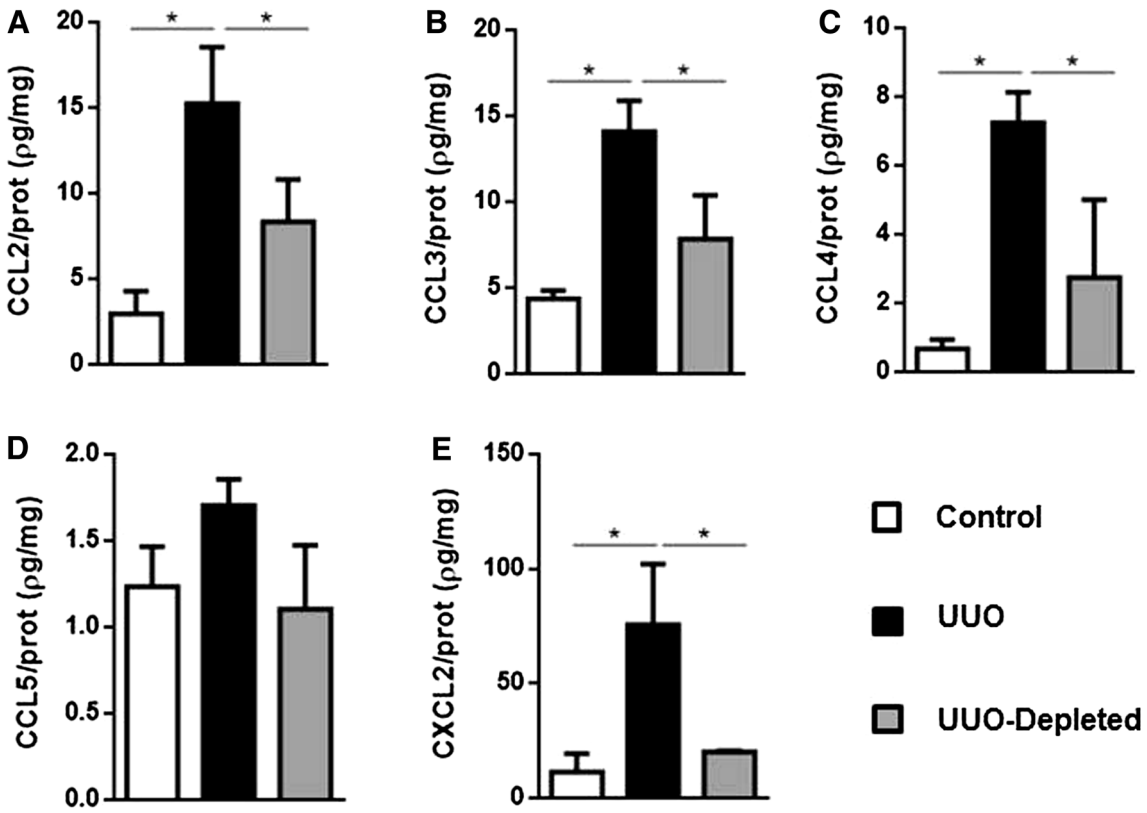

Fig. 2 Depletion of macrophages leads to reduction in renal chemokine levels. Protein levels of **a** CCL2, **b** CCL3, **c** CCL4, **d** CCL5, and **e** CXCL2 in the kidneys of non-obstructed animals, kidneys of UUO-submitted mice, and those undergoing UUO and treated with liposome-encapsulated clodronate. The graphs express the amount of chemokines in picograms, normalized by the total amount of protein. The statistical test used was ANOVA. $*p < 0.5$. $n = 5$ animals per group

mice showed lower TNFα and p40(IL12) protein levels, both inflammatory cytokines, when compared to WT animals (Fig. 4a, b). Indeed, only CCR2$^{-/-}$ animals showed decreased VEGF protein levels (Fig. 4c), the vascular and endothelial growth factor, in spite of no differences in IL-10 (Fig. 4d), an anti-inflammatory-related protein. Interestingly, both CCR2$^{-/-}$ and CCR4$^{-/-}$ animals showed reduced amounts of Th17-related cytokine profile: IL6, IL17, and GM-CSF (Fig. 4e–g). CCR5$^{-/-}$ and CCL3$^{-/-}$ mice, in turn, showed no difference in the levels of cytokine levels when compared to WT animals, except for IL17. These data demonstrated that among all investigated animals, CCR2$^{-/-}$ mice presented the more robust protection. It has been shown that CCR2 is responsible for pro-inflammatory monocyte recruitment to damaged sites (Kitagawa et al. 2004; Tsou et al. 2007; Peng et al. 2015a, b) and it is important for the development of chronic kidney disease (Furuichi et al. 2009; Giunti et al. 2010; Sezgin et al. 2011).

Dynamic of CCR2^{+} cell infiltration

We next tracked CCR2$^{+/rfp}$ cells into obstructed kidneys throughout different days. To assess and to study the recruited CCR2$^{+/rfp}$ cells, kidneys after UUO were visualized by confocal time-lapse microscopy. The dynamic of CCR2$^{+/rfp}$ cell infiltration indicates that such cells adhere onto vessels since the first day after UUO, corresponding to the peak of adherent cells (Fig. 5a). Indeed, the number of CCR2$^{+/rfp}$ rolling cells also reached the peak at the initial stages of UUO, as seen in Fig. 5b. The increased number of adherent cells on days one and two culminates in increased CCR2$^{+/rfp}$ cells recruited at day six and day fourteen into damaged kidneys, as quantified in Fig. 5c. A representative image of kidney RFP cells recruited at day 14 is shown in Fig. 5d. On the other hand, CCR2 deficiency, observed in CCR2$^{rfp/rfp}$ animals, led to decreased ability of these cells to adhere and to roll through the blood vessels, reflecting reduced numbers of recruited cells at later stages. Altogether, our data indicated that the absence of CCR2, previously demonstrated as being predictive for less fibrosis formation, led to reduced number of CCR2^{+} cells recruited to obstructed kidneys at chronic phase.

Discussion

Fibrosis is defined as a process of excessive amount of connective tissue production in the course of a reactive process (Wick et al. 2013). It has been demonstrated that

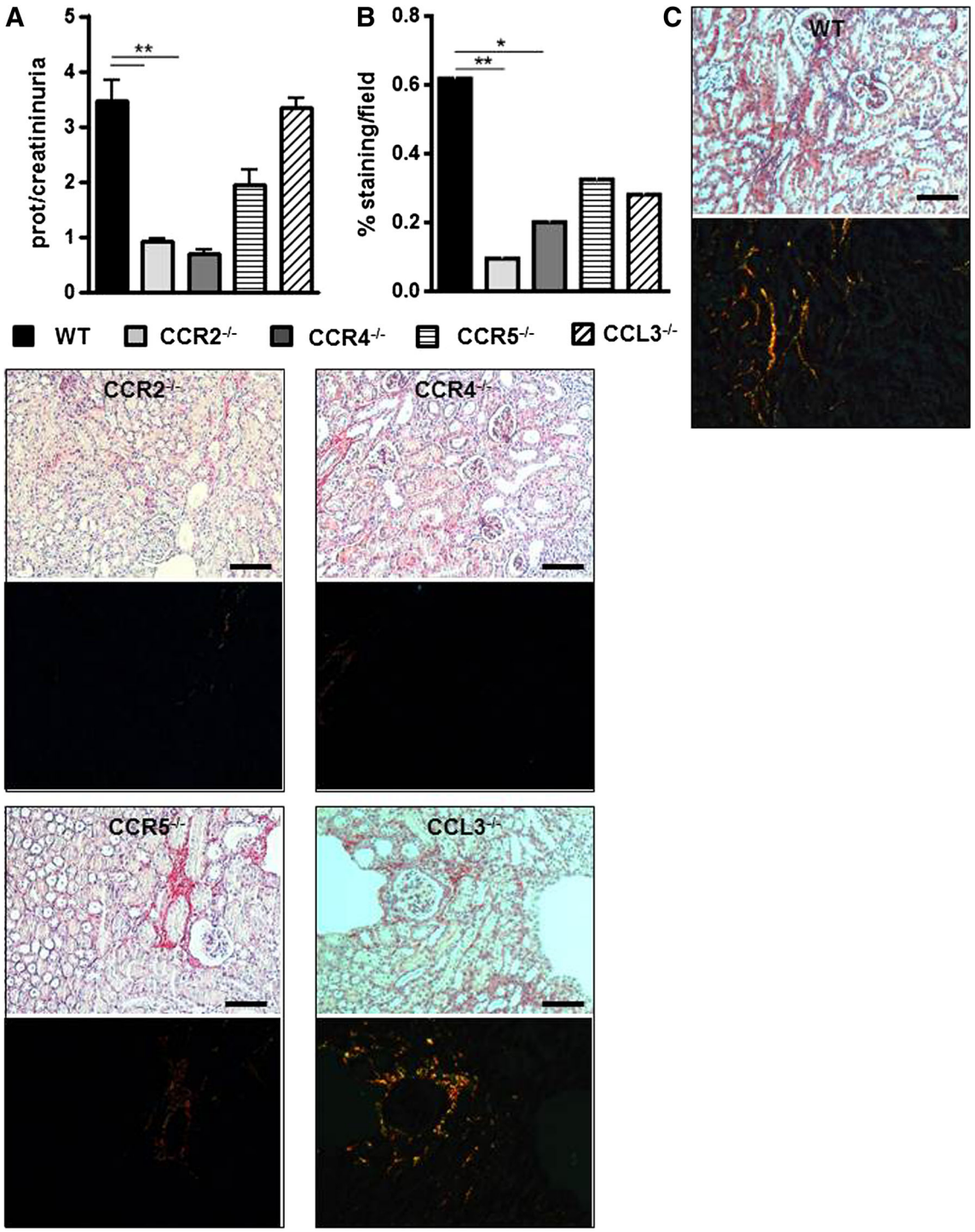

Fig. 3 CCR2$^{-/-}$ and CCR4$^{-/-}$ mice present reduced fibrosis within UUO. CCR2$^{-/-}$ CCR4$^{-/-}$, CCR5$^{-/-}$, CCL3$^{-/-}$, and WT animals that underwent UUO were euthanized after seven days. **a** Protein/creatinine ratio in the urine of kidney pelvis and **b** deposition of collagen by Sirius red staining at obstructed kidneys. **c** Representative images of collagen deposition seen in bright field and polarized light are shown. The *bars* represent 100 μm. The statistical test used was ANOVA. *$p < 0.5$; **$p < 0.1$. $n = 5$ animals per group

macrophages are crucial cells for the development of renal fibrosis (Braga et al. 2012). Macrophages are heterogeneous cells that can develop from different sources (Geissmann et al. 2010; Wynn et al. 2013; Dey et al. 2014; Cassado Ados et al. 2015), however, the majority of them are derived from the infiltration of monocytes expressing chemokine receptors (Geissmann et al. 2003; Serbina and Pamer 2006; Wynn et al. 2013). In the present study, it has been demonstrated that liposome-encapsulated clodronate leads to reduced release of chemokines into obstructed

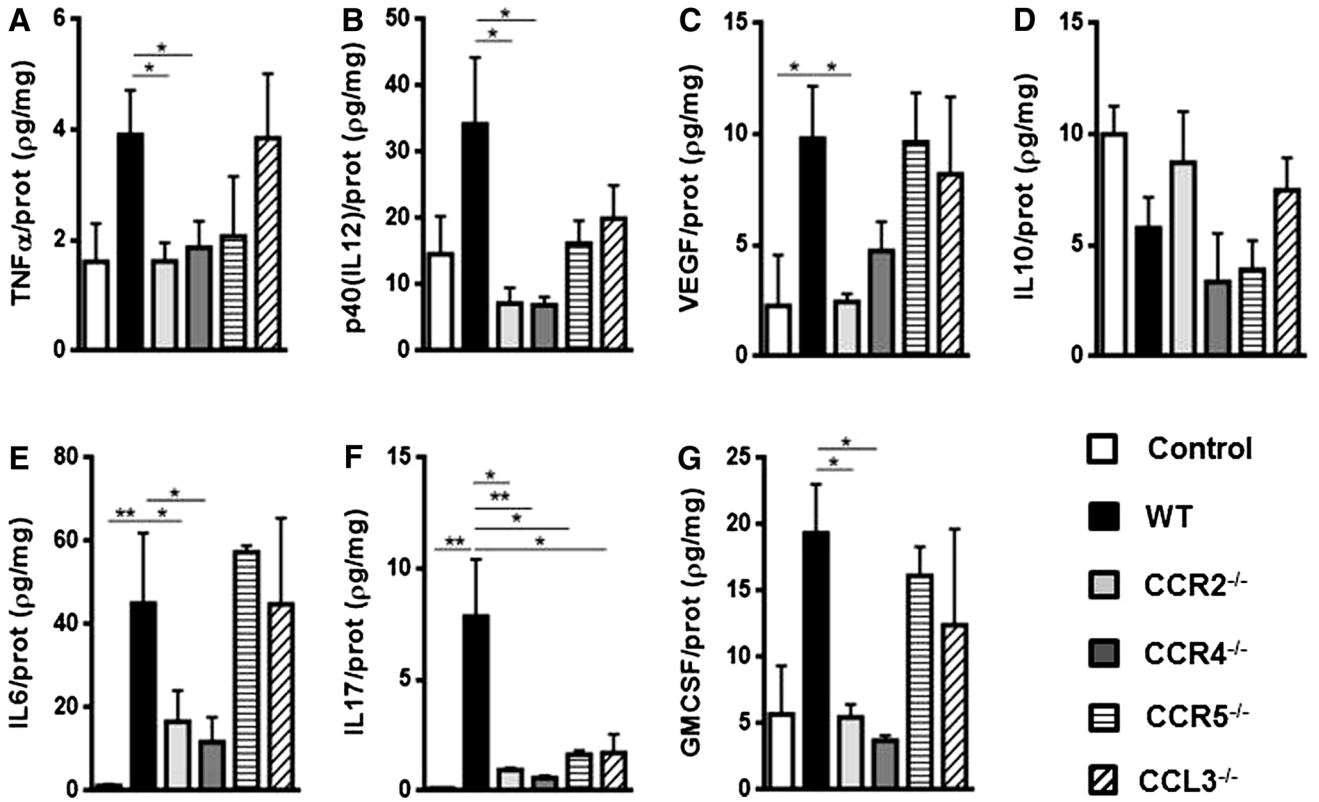

Fig. 4 CCR2$^{-/-}$ and CCR4$^{-/-}$ mice produce reduced levels of inflammatory cytokines. CCR2$^{-/-}$ CCR4$^{-/-}$, CCR5$^{-/-}$, CCL3$^{-/-}$, and WT animals were subjected to UUO and euthanized on day 7 for kidney removal, and non-obstructed animals were used as control. The charts show **a** TNFα, **b** p40IL12, **c** VEGF, **d** IL10, **e** IL6, **f** IL17, and **g** GM-CSF protein levels of obstructed kidneys. The results are expressed as picograms of cytokine normalized per milligram of renal tissue. The statistical test used was ANOVA. *$p < 0.5$; **$p < 0.1$. $n = 5$ animals per group

kidneys, a finding corroborated by the fact that macrophages amplify local inflammation through the production of chemokines (Cao et al. 2015).

Different chemokines and chemokine receptors have also been related to kidney lesions in obstructive nephropathy (Mia et al. 2015; Peng et al. 2015a, 2015b; Yuan et al. 2015; Yang et al. 2016). In this current report we demonstrated that, different from CCR5 and CCL3, the chemokine receptor CCR2 is directly related to the fibrotic pattern, a finding consistent with the data from a previous study indicating that CCR2 inhibition is beneficial in the context of fibrosis through a diminishing infiltration and accumulation of macrophages in obstructed kidneys (Kitagawa et al. 2004). We also observed a marginal role of CCR4 in renal fibrosis development, despite the fact that CCR4 is described as being related to pulmonary fibrosis, more than renal fibrosis (Trujillo et al. 2008; Kawamura et al. 2015; Adegunsoye et al. 2016).

CCR2 deficiency led to decreased Th17-related cytokines production, an immune response profile associated with nephropathy and progressive fibrosis (Mehrotra et al. 2015; Mehrotra et al. 2016), and also led to decreased VEGF production, a described molecule directly related to renal fibrosis, being therapeutically inhibition of VEGF under discussion in the clinical practice as a treatment of kidney fibrosis (Katagiri et al. 2016; Vanhove et al. 2016). After UUO injury, bone marrow CCR2^{+} monocyte egress of blood circulation and, within kidney, it is suggested that these cells switch their phenotype (Peng et al. 2015a, b), contributing for the formation of fibrosis. It could be possible that the cytokines milieu in the obstructed kidney leads to phenotype switching into the monocyte-derived macrophages or even to bone marrow-derived fibroblasts uptake (Xia et al. 2013). We demonstrated that CCR2^{+} cells adhere and become rolling onto vessels since the first day after UUO, and progressively, CCR2^{+} cells are recruited to obstructed kidneys, increasing the number of recruited cells over time.

Altogether, the data in the present study indicate that macrophage depletion reflects reduced fibrosis, as monocytes/macrophages consider central cells for fibrotic process (Han et al. 2017). Indeed, macrophages amplify the tissue damage through the activation of parenchymal cells and the production of chemokines, which are the attractive molecules for immune cells. CCR2 and CCR4 deficiency, specifically, led to reduced inflammatory and Th17-related

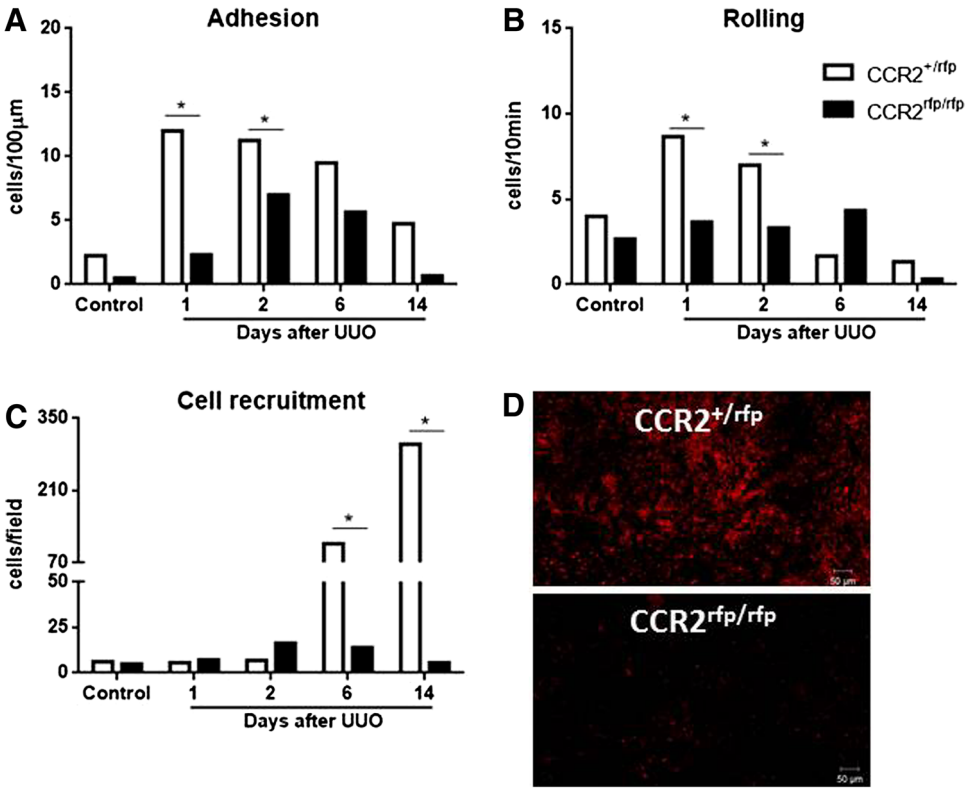

Fig. 5 $CCR2^{rfp/+}$ cells infiltrate obstructed kidneys at early stages and accumulate at later stage of UUO. $CCR2^{+/rfp}$ or $CCR2^{rfp/rfp}$ mice were submitted to UUO during different time points: 1, 2, 6, or 14 days **a** rolling and **b** adherent red fluorescent cells were counted under confocal microscopy. **c** Recruited cells were quantified in all fields around the venules. The control group is also indicated in the figure (first column). **d** Representative microscopies of recruited $CCR2^{+/rfp}$ or $CCR2^{rfp/rfp}$ cells at 14 days after UUO. *Open square* indicates a statistical difference between $CCR2^{+/rfp}$ and $CCR2^{rfp/rfp}$ animals at the same time point. The statistical test used was *t* test. $n = 5$ animals per group

cytokines—both these immune response patterns are associated with kidney fibrosis development (Kim et al. 2015; Mehrotra et al. 2015; Mehrotra et al. 2016; Silva et al. 2016). Among the chemokine receptors analyzed, CCR2 presents a special role in the context of fibrosis once $CCR2^{+}$ cells infiltrate the damaged kidney at initial stages, culminating in higher number of monocytes at later phase. Moreover, CCR2 deficiency reflected reduced loss of renal function and less collagen deposition. Together, these data provide new insight into the role of $CCR2^{+}$ cells and macrophages for renal fibrosis development following UUO, and provide the foundation for therapeutic strategies.

Acknowledgements We gratefully acknowledge funding provided by CNPq, Capes, and Fapesp (2014/06992-8 and 2012/02270-2).

Compliance with ethical standards

Conflict of interest None of the authors have any competing interests.

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