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Fibrocytes develop outside the kidney but contribute to renal fibrosis in a mouse model

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Collagen-producing bone marrow–derived cells (fibrocytes) have been detected in animal models and patients with fibrotic diseases. *In vitro* data suggest that they develop from monocytes with the help of accessory cells and profibrotic soluble factors. Using a mouse model of renal fibrosis, unilateral ureteral obstruction, we found the number of circulating fibrocytes was not reduced when monocytes were depleted with a monoclonal antibody against CCR2 or when CCR2 −/− mice with very low numbers of circulating or splenic monocytes were analyzed. The absence of CCR2, however, interfered with migration of fibrocytes into the kidney. The phenotype of splenic and renal fibrocytes was very similar and distinct from classical monocytes as fibrocytes expressed no CD115, medium levels of CCR2, and high levels of CD11b and Ly-6G. Using a depleting monoclonal antibody against Ly-6G or bone marrow chimeric mice expressing the diphtheria toxin receptor under the control of CD11b, we could efficiently deplete fibrocytes from the kidney. Depletion of fibrocytes or reduced migration of fibrocytes into the kidney resulted in lower renal expression of collagen-I. Thus, fibrocytes develop outside the kidney independent of infiltrating monocytes and rely on CCR2 for migration into target organs.

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Fibrocytes are defined as bone marrow–derived cells that produce collagen type I and to a lower degree other connective tissue proteins.[1,2] In addition, they express markers of hematopoietic cells like CD45, CD11b, and CD34.[3] It was shown by several groups that fibrocytes develop *in vitro* from a subpopulation of $CD14^+$ human monocytes or $Gr\text{-}1^+$ murine monocytes.[1,2,4,5] In culture, only a small fraction of these monocytes is able to differentiate under appropriate conditions to fibrocytes. The supporting factors are T helper type 2 cytokines, especially interleukin-13, transforming growth factor-β1, semaphorin7A, and angiotensin II, whereas the blocking factors are T helper type 1 cytokines, serum amyloid P, and crosslinked immunoglobulin G (IgG).[5–13] Fibrocytes accumulate in various tissues following injury, are preferentially found in areas of fibrosis, and are often accompanied by an inflammatory infiltrate consisting of monocytes and T cells.[14,15] Fibrocytes have been shown to depend on the chemokine receptors CCR1, CCR2, CCR5, and CCR7 as well as on the chemokine CXCL16 (chemokine (C-X-C motif) ligand 16) for migration into target organs.[16–24] CCR2 (C-C chemokine receptor type 2) also plays a major role in bone marrow release of monocytes, trafficking of these cells into the injured kidney, and establishment of the renal mononuclear phagocytic system.[25–28] Fibrocytes have been shown to contribute to tissue fibrosis in models of lung, renal, and cardiac fibrosis, and are detectable in patients with a variety of fibrotic diseases.[29–35] The differentiation of monocytes into fibrocytes *in vitro* suggests that *in vivo* fibrocytes also develop from classical monocytes that have entered injured tissues.[1,2,4,5] However, fibrocytes are also found in the peripheral blood and the spleen of healthy humans and mice, opening up the possibility that fibrocytes do not develop locally from monocytes but migrate as already differentiated or partially differentiated collagen-producing cells from the peripheral blood or the spleen into injured tissues. To analyze this question and to investigate the contribution of fibrocytes to renal collagen type I expression, we differentially depleted monocytes and fibrocytes with monoclonal antibodies and diphtheria toxin (DT) in bone marrow chimeric mice and analyzed the number of fibrocytes, the time course of their

appearance, and the outcome of fibrosis. We used the model of unilateral ureteral ligation that results in rapid infiltration of the obstructed kidney with monocytes, T cells, and neutrophils. Fibrocytes can readily be detected in the obstructed kidney and fibrosis develops within 5–7 days after ureteral ligation.[5,23]

RESULTS

Expression of surface markers on splenic and renal fibrocytes

We have shown previously that fibrocytes develop under appropriate culture conditions from splenic monocytes expressing the surface markers CD11b, CD115, and Gr-1.[5] *In vitro* differentiated fibrocytes still express high levels of CD11b and CD45 but only low levels of CD115. In order to characterize the expression of surface markers on fibrocytes *in vivo*, we performed unilateral ureteral ligation that leads to a rapid and pronounced infiltration of the obstructed kidney with fibrocytes. The gating strategy to identify fibrocytes is shown in Figure 1a. Single cells (Gate A, exclusion of cell doublets) and peripheral blood mononuclear cells (Gate B, exclusion of neutrophils) were identified by light scatter properties. After gating of CD45$^+$ cells (Gate C) expression of CD11b and intracellular collagen-I was displayed. Staining with an isotype control antibody (rabbit IgG) was performed to exclude unspecific staining. Before ureteral ligation (day 0), we detected only very low numbers of fibrocytes in the obstructed kidney, whereas there was a strong increase after ureteral ligation (Figure 1b). In the spleen, fibrocytes were clearly detectable on day 0 and increased on days 3–7 (Figure 1b). Fibrocytes in the spleen and the obstructed kidney were very similar regarding expression of surface markers and, interestingly, displayed a rather unusual combination of surface molecules (Figure 1c). Apart from CD11b and CD45, collagen-I$^+$ cells expressed medium to low levels of CCR2, medium levels of Gr-1 and Ly-6C, high levels of Ly-6G, and were negative for CD115. These markers distinguish fibrocytes clearly from monocytes (Table 1) that typically express CD115 and consist of two main subpopulations, one expressing high levels of CCR2, Gr-1, and Ly-6C, and the other expressing low to negligible levels of CCR2, low levels of Gr-1, and no Ly-6C.[36–38] We did not detect intracellular expression of collagen-I in CD115$^+$ monocytes (Figure 1c). These data show that fibrocytes have either changed their repertoire of surface markers after development from monocytes or constitute a distinctive subpopulation of cells in the spleen. In order to investigate the contribution of various cell populations for development of fibrocytes, we depleted these cell populations with monoclonal antibodies or DT.

Depletion of CCR2$^+$ Gr-1$^+$ monocytes with the anti-CCR2 antibody MC-21

CCR2$^+$ cells were depleted by daily application of the CCR2 antibody MC-21 (50 μg intraperitoneal (i.p.)) from day -1 to day 6 (Figure 2).[39,40] Unilateral ureteral obstruction (UUO) was performed on day 0 and mice analyzed on day 7. After the first injection of MC-21, Gr-1$^+$ monocytes were almost completely depleted from the peripheral blood as measured by flow cytometry on day 0 (Figure 2a). Further daily injection of MC-21 maintained the depletion of Gr-1$^+$ monocytes until day 3 and also resulted in a considerable reduction of Gr-1$^-$ monocytes that are known to develop from Gr-1$^+$ monocytes (Figure 2a).[41,42] However, on day 7, Gr-1$^+$ monocytes reappeared in the peripheral blood because the activity of MC-21 was neutralized by a humoral immune response against the antibody (Supplementary Figure S1 online). On day 7, there was also no significant depletion of monocytes in the spleen and obstructed kidney (Figure 2b). Fibrocytes, identified by expression of CD45, CD11b, and collagen-I, were not significantly diminished by treatment with MC-21 in the obstructed kidney or the spleen on day 7 (Figure 2c and d). In addition, there was no change in renal collagen-I expression as measured by reverse transcription–PCR for collagen type I (Figure 2e).

To exclude that neutralization of MC-21 is responsible for the failure of MC-21 to deplete the fibrocytes in the kidney on day 7, we treated mice with MC-21 for only a shorter period of time from day -1 to day 2 and analyzed peripheral blood, spleen, and kidneys on day 3 (Figure 3). There was a marked depletion of Gr-1$^+$ monocytes in the peripheral blood, spleen, and kidney, but no depletion of fibrocytes (Figure 3a and b). The CCR2 antibody MC-21 very efficiently depleted Gr-1$^+$ monocytes and interfered with the development of Gr-1$^-$ monocytes, but was unable to deplete fibrocytes from either the spleen or kidney, most likely because of their low expression of CCR2 (the mean fluorescence intensity for CCR2 was 17 ± 2.3 s.d. on fibrocytes and 115 ± 2.6 s.d. on Gr-1$^+$ monocytes.)

Similarly, treatment of mice with MC-21 from days 4 to 6 after UUO with analysis at day 7 efficiently depleted the monocytes, but did not reduce fibrocyte numbers or renal collagen-I expression (Supplementary Figure S2 online). The persistence of fibrocytes in the spleen and the accumulation of fibrocytes in the obstructed kidney despite considerable depletion of monocytes indicate that fibrocytes do not develop from monocytes within the kidney. Fibrocytes could either develop from other cell populations within the kidney or more likely migrate from the periphery (for example, spleen) via the peripheral blood into the kidney.

Development and migration of fibrocytes in CCR2$^{-/-}$ mice

To analyze whether monocytes play a role in the development of fibrocytes in the spleen and to investigate whether CCR2 plays a role in the migration of fibrocytes into the kidney, we analyzed CCR2$^{-/-}$ mice in comparison with wild-type mice (Figure 4). Because of the requirement of CCR2 for emigration of monocytes out of the bone marrow, CCR2$^{-/-}$ mice have low numbers of monocytes in the peripheral blood and spleen but increased numbers of monocytes in the bone marrow.[25,26] The numbers of monocytes were analyzed before (day 0) and on day 7 after UUO. As expected, CCR2$^{-/-}$ mice contained much lower numbers of monocytes in the peripheral blood, spleen, and kidney (Figure 4a–c).