Dual Role of CCR2 in the Constitution and the Resolution of Liver Fibrosis in Mice

Claudia Mitchell,^{*†} Dominique Couton,^{*†}
Jean-Pierre Couty,^{*†‡} Marie Anson,^{*†‡}
Anne-Marie Crain,^{*†‡} Vinciane Bizet,^{*†}
Laurent Rénia,^{*†¶} Stanislas Pol,^{*†§}
Vincent Mallet,^{*†§} and Hélène Gilgenkrantz^{*†§}

From the Institut Cochin,^{*} Université Paris Descartes, Centre National de Recherche Scientifique (UMR 8104), Paris, France; INSERM U567,[†] Paris, France; the Unité d'Hépatologie,[‡] Hôpital Cochin, Paris, France; Université Paris Diderot,[§] Paris, France; and the Singapore Immunology Network,[¶] Agency for Science, Technology, and Research, Biopolis, Singapore

Inflammation has been shown to induce the progression of fibrosis in response to liver injury. Among inflammatory cells, macrophages and lymphocytes play major roles in both the constitution and resolution of liver fibrosis. The chemokine receptor CCR2 is involved in the recruitment of monocytes to injury sites, and it is known to be induced during the progression of fibrosis in humans. However, its specific role during this process has not yet been unveiled. We first demonstrated that, compared with wild-type mice, CCR2 knockout animals presented a delay in liver injury after acute CCl₄ injection, accompanied by a reduction in infiltrating macrophage populations. We then induced fibrosis using repeated injections of CCl₄ and observed a significantly lower level of fibrotic scars at the peak of fibrosis in mutant animals compared with control mice. This diminished fibrosis was associated with a reduction in F4/80⁺CD11b⁺ and CD11c⁺ populations at the sites of injury. Subsequent analysis of the kinetics of the resolution of fibrosis showed that fibrosis rapidly regressed in wild-type, but not in CCR2^{-/-} mice. The persistence of hepatic injury in mutant animals was correlated with sustained tissue inhibitor of metalloproteinase-1 mRNA expression levels and a reduction in matrix metalloproteinase-2 and matrix metalloproteinase-13 expression levels. In conclusion, these findings underline the role of the CCR2 signaling pathway in both the constitution and resolution of liver fibrotic scars. (Am J Pathol 2009, 174:1766–1775; DOI: 10.2353/ajpath.2009.080632)

Liver fibrosis and its progression toward cirrhosis is the consequence of chronic liver aggressions provoked by various agents such as viruses, bile acids, or alcohol. It is characterized by an accumulation of extracellular matrix mainly secreted by activated myofibroblasts and hepatic stellate cells (HSCs).^{1,2} Although originally considered as an irreversible process, cirrhosis has now been shown to be potentially reversible, provided that the causative agent is removed.³ Current evidence suggests that the process of hepatic fibrosis is driven primarily by the development of an inflammatory reaction in response to parenchymal injury. In agreement with this hypothesis, deletion of specific components of the inflammatory response modifies or attenuates the fibrotic scar *in vivo*. It has been demonstrated that Kupffer cell inactivation,⁴ B lymphocyte deficiency,⁵ or macrophage depletion⁶ result in lower scarring and reduced activation of HSCs.

Molecular mechanisms responsible for reversibility of liver fibrosis are only now starting to be deciphered. Experimental depletion of macrophages at the onset of fibrosis resolution retards extracellular matrix degradation and the loss of activated hepatic stellate cells.⁶ Therefore, macrophages seem to be crucial in both processes: fibrogenesis and fibrosis resolution.

Chemokines are known to be critical mediators of inflammatory cell trafficking into sites of injury. Monocyte chemoattractant proteins (MCPs) attract cells through activation of their cognate receptor. MCP-1 is expressed by hepatocytes, endothelial cells, Kupffer cells, and HSCs in response to injury. CC chemokine receptor 2 (CCR2), which is expressed on monocytes/macrophages and on a subpopulation of T lymphocytes, has been found progressively up-regulated during the progression of liver fibrosis in humans.⁷ Moreover, comparison of serial sections of liver biopsies from patients with various degrees of necroinflammatory activity showed that infiltration with monocytes/macrophages is directly corre-

Supported by the Association pour la Recherche contre le Cancer and the Agence Nationale de la Recherche, France.

Accepted for publication January 13, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Dr. Hélène Gilgenkrantz, 24, rue du Faubourg Saint-Jacques, 75014 Paris, France. E-mail: helene.gilgenkrantz@inserm.fr.

lated with the expression of MCP-1,⁸ for which CCR2 is the main receptor.

We hypothesized that signaling through CCR2 is involved in inflammatory cell recruitment during fibrogenesis and/or regression of fibrosis. To test this hypothesis, we compared the establishment and resolution of fibrosis after chronic liver aggression in CCR2-deficient mice versus wild-type mice. Our data show that the absence of CCR2 has opposite consequences during the constitution and the resolution of fibrosis.

Materials and Methods

Animal Models

CCR2^{-/-} mice were generated as previously described⁹ and were back-crossed into the BALB/c Ola background. Liver injury was induced by injection of 0.1 ml/20 g of CCl₄ dissolved in a 1:10 ratio with mineral oil. Control animals were injected with the same volume of mineral oil. For acute studies, animals were injected once with CCl₄ and euthanized after 12 (*n* = 6 animals per group), 18, 24, or 48 hours (*n* = 3). For chronic studies, animals were treated twice a week for 6 weeks and were sacrificed 2, 7, or 14 days after the last CCl₄ injection (*n* = 6 animals per group). Blood was collected just before sacrifice and the activity of serum alanine aminotransferases was measured on a Hitachi (Tokyo, Japan) 747 analyzer. Livers were harvested either into 10% buffered formalin for histological evaluation or snap-frozen into liquid nitrogen for mRNA or cellular lysate preparations. For flow cytometry studies, the liver was prepared as indicated below. All experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals.

Histological Analysis

Five- μ m-thick paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E), with Sirius Red, or Von Kossa stain. For immunohistochemical studies, the following antibodies were used: α -smooth muscle actin (α -SMA) (M 0851; DAKO, Glostrup, Denmark), CD68 (MCA1957; AbD Serotec, Oxford, UK), F4/80 (MF 48000; Caltag, Invitrogen, Carlsbad, CA). For α -SMA immunohistochemistry, 5- μ m paraffin sections were used, whereas 5- μ m cryostat sections were used for CD68 and F4/80 immunohistochemistries. For morphometric analysis, 15 images per animal, from at least two different lobes, were taken at $\times 100$ magnification. The areas of staining were quantitated using the software ImageJ 1.37v (National Institutes of Health, Bethesda, MD).

Nonparenchymal Liver Cells, Blood, and Bone Marrow Preparation

Livers from treated animals (with oil or CCl₄) of both genotypes (*n* = 3 per group) were harvested and resident nonparenchymal cells were harvested following the

protocol previously described.¹⁰ Blood was collected by intracardiac puncture at the moment of sacrifice. Bone marrow cells were collected by flushing the femur. Red blood cells present in the marrow and in the blood were lysed by incubating the suspension with lysis buffer (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 1 mmol/L ethylenediaminetetraacetic acid, 17 mmol/L Tris, pH 7.3) for 2 minutes. The suspension was then spun down for 10 minutes at 1500 rpm and resuspended in 10 ml of RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum.

Flow Cytometry

Flow cytometry was performed on a FACS-Calibur flow cytometer (BD Biosciences, Erembodegem, Belgium). Cells (2×10^5 nonparenchymal cells and blood cells or 1×10^6 bone marrow cells) were incubated for 30 minutes on ice with 1 μ g of anti-CD16/CD32 monoclonal antibody (anti-Fc γ RIII/II, clone 2.4G2) to reduce nonspecific binding of antibodies to Fc receptors. Cells were then incubated with a saturating amount of labeled antibodies (diluted in ice-cold phosphate-buffered saline containing 3% fetal calf serum, 0.1% w/vol sodium azide) or isotype-matched control immunoglobulin according to the manufacturer's recommendations for 45 minutes on ice, protected from light. We used the following monoclonal antibodies to study various cell surface markers: anti-CD3-PE, anti-DX-5-biot, anti-CD11c-PE, anti-CD-8-APC, anti-CD-4-PerCP, anti-CD11b-PE (all purchased from BD Biosciences), or anti-F4/80-APC (e-Biosciences, San Diego, CA). Biotinylated antibodies (DX-5-biot) were detected with fluorescein isothiocyanate-labeled streptavidin (BD Biosciences, Pharmingen). CELLQuest software (BD Biosciences) was used for analysis of flow cytometry data.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was purified from livers using the Trizol method (Invitrogen). cDNA synthesis was undertaken using the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). Quantitative RT-PCR was performed in duplicate with the QuantiTect SYBR Green PCR kit (Qiagen, Mainz, Germany) using the LightCycler apparatus. QuantiTect primer assays (Qiagen) were used to amplify the following mRNAs: CCR5, MCP-1, CCL-5 (RANTES), CXCL9, IP-10, tumor necrosis factor- α , interleukin-6, interleukin-1 β , transforming growth factor- β , MIP-1 α , α -SMA, collagen-1 α , matrix metalloproteinase (MMP-2, MMP-9, MMP-13), tissue inhibitors of metalloproteinase (TIMP-1, TIMP-2, HPRT, using the standard QuantiTect protocol. Relative concentrations were calculated and normalized to control gene *HPRT*. A wild-type mouse injected with excipient (oil) served as reference for the calculations of fold inductions in gene expression.

Western Blotting

Liver tissues mice were homogenized in protein lysis buffer (50 mmol/L Hepes, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0, 2.5 mmol/L ethyleneglycotetraacetic acid, pH 8.0, 0.1% Tween 20, 10% glycerol, 0.1% Nonidet P-40, 1 mmol/L NaF, 1 mmol/L dithiothreitol, 1 mmol/L sodium orthovanadate, 1 mmol/L β -glycerophosphate) containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Fifty μ g of protein were run on a 12% acrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed with the anti-MMP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence was revealed using a horseradish peroxidase-conjugated secondary anti-mouse antibody (DAKO).

Statistics

Statistical analyses were performed using the software Statview 4.5 (Abacus Concepts, Piscataway, NJ). Data sets were compared with analysis of variance *t*-test. Data are represented as mean $\pm$ SEM. Error bars indicate SEM. *P* values inferior to 0.05 were considered significant.

Results

Liver Expression of Inflammation Markers after Acute CCl₄ Injury

We first analyzed the modulation of various inflammation markers after acute CCl₄ challenge in wild-type and CCR2-deficient mice, compared with excipient-injected animals (Figure 1). MCP-1 mRNA expression increased by 40-fold in wild-type animals 12 hours after CCl₄ injection, whereas the increase in MCP-1 expression in CCR2^{-/-} mice was less important (10-fold) and seemed slightly delayed (Figure 1A). Corroborating these results, MIP-1 α , a chemokine induced by MCP-1,¹¹ also presents a reduced and delayed induction. Other inflammatory cytokines, such as tumor necrosis factor- α , interleukin-6, and interleukin-1 β , showed the same pattern of induction, that is, a diminished induction in CCR2^{-/-} animals compared with wild-type mice. Also, an up-regulation of interferon- γ -inducible genes, such as CXCL9 and IP-10, was detected at 12 hours after injection in wild-type animals, but not in CCR2^{-/-} mice. On the other hand, no significant up-regulation of CCR5 (another chemokine receptor commonly expressed in response to liver injury)^{12,13} or its ligand CCL5 was found either in wild-type mice or in CCR2^{-/-} mutant mice, suggesting that the couple CCR5/CCL5 does not mediate the response to the acute injury after CCl₄ injection. Finally, transforming growth factor- β , a crucial factor for hepatic stellate cell activation¹ that has been shown to be up-regulated by MCP-1 via the CCR2 receptor,¹⁴ is increased earlier in wild-type mice than in CCR2^{-/-} animals.

Serum transaminases (alanine aminotransferases) were measured 12, 18, 24, and 48 hours after CCl₄ acute

challenge and were found to be significantly lower in CCR2^{-/-} mutant mice than in wild-type littermates until 24 hours after injection (Figure 1B). These results show that CCR2^{-/-} mice present diminished inflammation and delayed hepatocyte injury after CCl₄ injection compared with wild-type mice.

Reduced Liver Fibrosis in CCR2^{-/-} Mice

To investigate the consequences of CCR2 receptor inactivation on fibrosis progression, we chronically injected CCl₄ in CCR2^{-/-} and control mice twice a week for 6 weeks. Liver sections were stained at the peak of fibrosis (2 days after the last CCl₄ injection) for markers of collagen and activated HSCs. Fibrotic area was quantified by Sirius Red staining and was found to be significantly decreased by 27% in CCR2^{-/-} mice compared with wild-type mice (Figure 2, A–C). We next analyzed the activation of fibrogenic cells through the expression of α -SMA and demonstrated that CCR2^{-/-} mice showed a significant decrease in this marker compared with wild-type mice (Figure 2, D–F). The reduced fibrosis seen in CCR2^{-/-} mice correlated with a milder parenchymal injury (Figure 3, A and B) and a reduction in the proportion of scar-associated macrophages present in the injured area, as defined by CD68 and F4/80 immunostaining (Figure 3, C–F).

Characterization of Immune Cell Populations after Acute and Chronic Injury

To quantify the proportion of various populations of inflammatory cells recruited to injured livers, we performed fluorescence-activated cell sorting analyses of nonparenchymal cells present in the liver after acute and chronic CCl₄ treatment. Because CCR2 has been shown to be responsible for the recruitment of inflammatory cells from bone marrow to injury sites^{15–17} we also investigated the blood and bone-marrow compartments of these animals. As shown in Figure 4A, we observed an increase in macrophages (F4/80⁺CD11b⁺) in wild-type animals at 12 hours after an acute CCl₄ injection as well as at the peak of fibrosis (day 2 after chronic CCl₄ treatment), whereas no difference was seen between the two types of animals at basal levels (oil injection). This reduced proportion of F4/80⁺CD11b⁺ in CCR2^{-/-} mice was also found in blood (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). In contrast, bone marrow of CCR2^{-/-} mice showed a higher proportion of F4/80⁺CD11b⁺ both at basal levels and after injury (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). We also analyzed the CD11c⁺ population in the liver, which corresponds to macrophages and dendritic cells, and showed that their recruitment was also impaired in CCR2^{-/-} mice (Figure 4B). Finally, no significant differences were found in the liver populations of CD4⁺ and CD8⁺ T lymphocytes (see Supplemental Figure S1, C and D, at <http://ajp.amjpathol.org>) or in NK (DX5⁺CD3⁻) and NKT (DX5⁺CD3⁺) cell populations (data not shown) between wild-type and CCR2^{-/-} mice. Altogether, these results demonstrate

Figure 1. Analysis of inflammation markers and liver injury in oil- or CCl₄-injected wild-type (white bars) or CCR2^{-/-} mice (black bars), at 12, 18, 24, or 48 hours after injection. **A:** Real-time RT-PCR analysis of the hepatic expression of various chemokines, chemokine receptors, and cytokines during acute liver injury after CCl₄ injection. **B:** Liver injury was assessed by measuring serum transaminases (alanine aminotransferases). Excipient (oil)-injected animals were sacrificed 12 hours after injury. **P* < 0.05, ***P* < 0.01.

that the reduced proportion of scar-associated macrophages observed in mutant mice is not attributable to a lower proportion of this population in quiescent CCR2^{-/-} livers, but rather to a weaker recruitment of F4/80⁺CD11b⁺ and CD11c⁺ cells to the liver after injury.

CCR2 Is Involved in the Resolution of Fibrosis

It has been previously shown that macrophages are also implicated in the resolution of liver fibrosis.^{6,18} We therefore analyzed the consequences of CCR2 deficiency dur-

Figure 2. Fibrotic markers at peak fibrosis (day 2) after 6 weeks of bi-weekly injections of CCl₄ in wild-type (WT) or CCR2^{-/-} mice. **A** and **B**: Sirius Red staining of liver sections showing less collagen deposit in CCR2^{-/-} mutant mice. **C**: Quantification of Sirius Red staining in oil- or CCl₄-injected wild-type or CCR2^{-/-} animals, showing a significant reduction in fibrosis in CCR2^{-/-} mutant mice compared with wild-type mice. **D** and **E**: α -SMA staining of liver sections with less staining in mutant mice. **F**: Quantification of α -SMA-positive area in oil- or CCl₄-injected animals. **P* < 0.05, ***P* < 0.01. Original magnifications, $\times 100$.

ing the process of fibrosis resolution. With this aim, we treated the animals for 6 weeks with CCl₄, as previously, but sacrificed them at 7 or 14 days after the last injection of CCl₄. Collagen deposition decreased by 54% after 7 days of spontaneous recovery in wild-type mice. In contrast,

CCR2^{-/-} mice showed only a 5% reduction during the same period (Figure 5, A–D, and G). This discrepancy was still significant at 14 days after the end of the treatment, with a 75% reduction in collagen deposition observed in wild-type mice compared with only 31% in mutant mice (Figure 5, E–G). CCl₄ toxicity induces necrosis mainly around central veins. We observed that these necrotic areas also corresponded to areas of calcium deposit, which could be detected by Von Kossa staining (Figure 6, A and B). As soon as 7 days of spontaneous resolution, necrotic areas represent only 10% of the necrosis observed at the peak of fibrosis in wild-type animals, whereas in CCR2^{-/-} mice these areas increased from days 2 to 7 and presented no significant reduction even at 14 days after the last CCl₄ injection (Figure 6, C–I). In contrast to what we observed 2 days after the end of the injections, during the resolution period we did not find a clear difference in the proportion of F4/80⁺CD11b⁺ macrophages (Figure 4A) nor in the distribution of these cells (F4/80 IHC, data not shown) between mutant and normal mice. However, CD11c⁺ cells were still found significantly increased in wild-type mice compared with CCR2^{-/-} at day 7 (Figure 4B). We therefore hypothesize that inflammatory cells recruited via CCR2 signaling during the constitution phase subsequently help eliminate necrotic cells from injured areas once the toxic compound is removed. Altogether, our results indicate that CCR2 is involved both in the constitution and in the resolution of the fibrotic scar.

Figure 3. Histological analysis of wild-type (WT) livers or CCR2^{-/-} livers at peak fibrosis (day 2) after 6 weeks of CCl₄ injury. **A** and **B**: H&E-stained sections show a greater inflammatory reaction and injury in wild-type mice. **C** and **D**: CD68 immunostainings showing positive cells underlying the bridging fibrosis that is less extensive in CCR2^{-/-} mutant mice.

TIMP and MMP Expression during the Resolution of Fibrosis

To better understand the molecular events behind the lack of prompt fibrosis resolution in CCR2^{-/-} animals, we

Figure 4. Fluorescence-activated cell sorting analysis showing increased percentages of macrophages and dendritic cells in the livers of wild-type (WT) compared with CCR2^{-/-} mice after acute and chronic CCl₄ injury. Nonparenchymal liver cells were collected after excipient (oil) injection, at 12 hours after acute CCl₄ injection, at 2 days (day 2) after the end of chronic treatment (peak fibrosis), or at 7 days (day 7) after the end of chronic treatment (recovery phase). **A:** Representative data of three animals per time point showing double-positive CD11b and F4/80 cell populations in wild-type and CCR2^{-/-} mice, with the corresponding mean percentages ± SE. **B:** Representative data of three animals per time point showing CD11c⁺ populations in wild-type and CCR2^{-/-} mice, with the corresponding mean percentages ± SE. Excipient (oil)-injected animals were analyzed 12 hours after acute injury. **P* < 0.05, ***P* < 0.01.

next analyzed the expression of various MMPs and TIMPs during the recovery phase. We first looked at MMP-2 expression by real-time RT-PCR and found it slightly more expressed in wild-type animals by 7 days after the end of the treatment, whereas at 14 days, the trend was reversed and MMP-2 was more expressed in CCR2^{-/-} animals (Figure 7A). These results were confirmed by Western blot analysis, which showed a lower expression of MMP-2 in CCR2-deficient mice at 2 and 7 days after the last CCl₄ injection, and an increase in expression by day 14 after the end of the treatment (Figure 7B). MMP-13 presented the same pattern of expression, both at mRNA (Figure 7C) and protein (data not shown) levels. TIMP-1, on the other hand, was highly expressed in CCR2^{-/-} animals 7 days after the end of the treatment and diminished to control levels by day 14 (Figure 7D). Our results show that at 7 days after treatment, while wild-type animals secrete MMPs to degrade the extracellular matrix and resolve fibrosis, CCR2^{-/-} mice present lower MMP expression accompanied by increased expression of TIMP-1. It is only later, at 14 days after treatment, that CCR2^{-/-} mice show consistent MMPs expression, with TIMP-1 levels returning back to control levels.

Discussion

The C-C chemokine receptor CCR2 has been identified as the primary receptor that mediates monocyte chemoattractant protein-1 (MCP-1 or CCL2) responses. Mice deficient for CCR2 develop normally and have no hematopoietic abnormalities but fail to recruit monocytes-macrophages to inflamed sites.^{15,19} MCP-1 and CCR-2 mRNAs are up-regulated during the progression of liver fibrosis in humans,^{7,8,20} and it is well documented that MCP-1 plays an essential role in the physiopathology of

chronic liver diseases.^{8,21} While in the lung^{22,23} and in the kidney²⁴ the absence of CCR2 protects from fibrosis, the role of this receptor during liver fibrosis has not yet been studied. To address this issue, we compared the fibrotic process induced by CCl₄ between CCR2 knockout and wild-type mice.

First, we investigated the kinetics of cytokine and chemokine induction after acute CCl₄ injury. We showed that the expression of MCP-1 in CCR2^{-/-} animals was significantly decreased in comparison with wild-type littermates after CCl₄ injection. This reduced expression of MCP-1 in the mutants was accompanied by a weaker induction of inflammatory cytokines and delayed liver injury.

MCP-1 is known to be involved in the recruitment of monocytes/macrophages to sites of inflammation through CCR2 receptor.²⁵ Indeed, we show here that both after acute injury or 6 weeks of bi-weekly injections of CCl₄, CCR2^{-/-} animals present milder macrophage infiltration than wild-type mice. Furthermore, we demonstrate that CCR2^{-/-} mice develop less fibrosis than wild-type animals. Accordingly, a mutated dominant-negative form of MCP-1 has been shown to prevent liver fibrogenesis in rats,²⁶ suggesting that MCP-1 could stimulate liver fibrogenesis by two mechanisms that are not mutually exclusive: by inducing monocyte/macrophage infiltration and by directly activating HSCs to modulate their function. MCP-1 has indeed been shown to activate HSCs in culture, but its effects seem to pass through other receptors than CCR2, which is not expressed in HSCs.²⁷ This could therefore explain the fact that the protection against liver fibrosis seen in our CCR2^{-/-} mice is not as drastic as in animals expressing a dominant-negative form of MCP-1.

When examining monocyte/macrophage populations present in the bone marrow of acutely or chronically

Figure 5. A–F: Sirius Red staining during the resolving phase after 2 (A and B), 7 (C and D), and 14 (E and F) days of spontaneous recovery after the last CCl₄ injection in wild-type (WT) and CCR2^{-/-} mutant mice. G: Persistence of the total area of fibrosis detected by Sirius Red staining after 7 and 14 days of recovery in CCR2^{-/-} mice compared with control mice. **P* < 0.05, ***P* < 0.01. Original magnifications, ×100.

treated animals, a larger population of F4/80⁺CD11b⁺ cells was found in CCR2^{-/-} mice compared with normal littermates, in accordance with previous data showing that monocytes from CCR2^{-/-} mice fail to exit the bone marrow.^{15–17} Corroborating this observation, there were fewer F4/80⁺CD11b⁺ cells released into the circulation of CCR2^{-/-} mice after injury. We also investigated several additional inflammatory cell populations present in the liver after injury and found no other difference between mutant and wild-type mice, except for the CD11c marker. This antigen is the most commonly used marker of dendritic cells, although it can also detect macrophages.²⁸ Again, the main difference was seen at the peak of fibrosis (day 2). Taken together, our data indicate that the reduction in liver fibrosis seen in CCR2^{-/-} mice is mainly

a result of a diminished monocyte/macrophage infiltration after injury.

We also demonstrated that CCR2^{-/-} mice presented reduced fibrosis regression. We hypothesize that the prolonged necrosis and diminished regression seen in CCR2^{-/-} mice is attributable to the lack of infiltrating macrophages seen at the peak of fibrosis. We cannot exclude that other cell types also participate to fibrosis resolution. In fact, we observed that the higher proportion of CD11c⁺ cells in wild-type mice seen at day 2 persisted at day 7, whereas the percentages of F4/80⁺CD11b⁺ cells had become equivalent between the two genotypes at this latter time point. These results suggest that a population of CD11c⁺ dendritic cells remained increased during the resolution phase and could have helped accelerate fibrosis regression in wild-type mice. Indeed, data from the literature showed that CCR2 is also implicated in the recruitment of dendritic cells.²⁹

To better define the role of CCR2 specifically during the regression phase, it would be necessary to block its action at the peak of fibrosis using neutralizing antibodies or specific CCR2 antagonists. This way, one would be able to assess whether the cells recruited during the establishment of fibrosis are the ones necessary for regression or if other cells also need to be recruited during fibrosis resolution.

Although not completely understood, fibrosis resolution has been temporally correlated with a diminution of TIMP-1 and TIMP-2 (mainly expressed by HSCs³⁰) and with an increased activity of MMP-2.^{31,32} HSCs seem to be the major source of MMP-2,^{33,34} although macrophages have also been shown to express this protein.² Furthermore, MMP-13, mainly secreted by scar-associated macrophages, has also been shown to facilitate the resolution of hepatic fibrosis.¹⁸ In our present study, we found an overexpression of TIMP-1 and a mirrored lower expression of MMP-2 and MMP-13 in CCR2^{-/-} mice compared with wild-type mice that could account for the delay observed in fibrosis regression. Although we saw no modulation in the expression of TIMP-2, we found that the expression of TIMP-1 during fibrosis resolution correlated with the expression of collagen 1 and α -SMA (data not shown), as described by Iredale and colleagues.³⁰ This result suggests that, contrary to wild-type mice, in CCR2^{-/-} animals HSCs are still activated at 7 days after the end of the treatment and therefore still express TIMP-1, collagen 1, and α -SMA.

It has been recently shown that autologous bone marrow cells contribute to the spontaneous regression of liver fibrosis by expressing matrix metalloproteinases.³⁵ Although it is beyond the scope of this article to determine whether the CCR2-positive cells that participate in the resolution of fibrosis come from the bone marrow, the fact that monocyte emigration from bone marrow requires signals mediated by chemokine receptor CCR2 argues for this hypothesis.^{15–17} The mirrored distribution pattern of F4/80⁺CD11b⁺ that we observed in blood and in bone marrow (ie, CCR2^{-/-} mice have higher proportions in bone marrow but lower in blood) supports this assumption.

Figure 6. Analysis of calcium deposit in necrotic areas. The same necrotic area was stained by H&E (A) and Von Kossa (B) staining. C: Quantification of Von Kossa-positive areas expressed in percent of total area in liver sections analyzed. Necrotic areas decreased significantly in wild-type (WT) animals between 2 and 7 days, but not in CCR2^{-/-} mice. **P* < 0.05. D–I: Von Kossa staining in liver sections of wild-type mice and CCR2^{-/-} mice 2 (D and E), 7 (F and G), and 14 (H and I) days after the last CCL₄ injection.

Overall, we demonstrated that the absence of CCR2 results in a diminished migration of inflammatory macrophages and consequently a delayed and diminished activation of HSCs, which translates into a reduced fibrosis. However, once the chronic injury stops, the spontaneous process of regression is also delayed, attesting of the dual role played by CCR2 during fibrogenesis.

Presently, there is still a serious need for therapeutic approaches that could impede fibrosis progression independently of disease etiology. Based on our results, one could thus propose the use of a pharmacological antagonist of CCR2 during the constitution phase of fibrosis. Such an approach has been proven efficient in improving insulin sensitivity by lowering macrophage content in a

Figure 7. Analysis of liver expression of MMP-2, MMP-13, and TIMP-1 during the recovery phase after CCL₄ treatment. A diminished expression of MMP-2 was seen in CCR2^{-/-} mice (black bars) by real-time RT-PCR (A) and Western blot (B), during the initial phase of recovery. C: MMP-13 expression was also diminished at 7 days but increased at 14 days after the last CCL₄ injection, whereas TIMP-1 (D) showed the inverted pattern of expression. **P* < 0.05.

mouse model of nonalcoholic steatohepatitis, one frequent cause of fibrosis.³⁶ However, one has to keep in mind that fibrosis is a dynamic process of matrix deposition and degradation. Hence, our results show that once the causes of fibrosis have been eradicated, such antagonists could hamper the natural process of fibrosis resolution.

Acknowledgments

We thank Dr. William A. Kuziel for the gift of CCR2^{-/-} mice, Dr. Pierre Bedossa for help with histological interpretation, and Dr. Patrick Jaffray for measurements of alanine aminotransferases.

References

- Battaller R, Brenner DA: Liver fibrosis. *J Clin Invest* 2005, 115:209–218
- Iredale JP: Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 2007, 117:539–548
- Mallet V, Gilgenkrantz H, Serpaggi J, Verkarre V, Vallet-Pichard A, Fontaine H, Pol S: Brief communication: the relationship of regression of cirrhosis to outcome in chronic hepatitis C. *Ann Intern Med* 2008, 149:399–403
- Rivera CA, Bradford BU, Hunt KJ, Adachi Y, Schrum LW, Koop DR, Burchardt ER, Rippe RA, Thurman RG: Attenuation of CCl(4)-induced hepatic fibrosis by GdCl(3) treatment or dietary glycine. *Am J Physiol* 2001, 281:G200–G207
- Novobrantseva TI, Majeau GR, Amatucci A, Kogan S, Brenner I, Casola S, Shlomchik MJ, Kotliansky V, Hochman PS, Ibragimov A: Attenuated liver fibrosis in the absence of B cells. *J Clin Invest* 2005, 115:3072–3082
- Duffield JS, Forbes SJ, Constantinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang R, Iredale JP: Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 2005, 115:56–65
- Bièche I, Asselah T, Laurendeau I, Vidaud D, Degot C, Paradis V, Bedossa P, Valla DC, Marcellin P, Vidaud M: Molecular profiling of early stage liver fibrosis in patients with chronic hepatitis C virus infection. *Virology* 2005, 332:130–144
- Marra F, DeFranco R, Grappone C, Milani S, Pastacaldi S, Pinzani M, Romanelli RG, Laffi G, Gentilini P: Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. *Am J Pathol* 1998, 152:423–430
- Kuziel WA, Morgan SJ, Dawson TC, Griffin S, Smithies O, Ley K, Maeda N: Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc Natl Acad Sci USA* 1997, 94:12053–12058
- Couty JP, Crain AM, Gerbaud S, Labasque M, Marchiol C, Fradelizi D, Boudaly S, Guettier C, Vignuzzi M, van der Werf S, Escrion N, Viguier M: Delivery of mengovirus-derived RNA replicons into tumoural liver enhances the anti-tumour efficacy of a peripheral peptide-based vaccine. *Cancer Immunol Immunother* 2008, 57: 1161–1171
- Hokeness KL, Kuziel WA, Biron CA, Salazar-Mather TP: Monocyte chemoattractant protein-1 and CCR2 interactions are required for IFN-alpha/beta-induced inflammatory responses and antiviral defense in liver. *J Immunol* 2005, 174:1549–1556
- Moreno C, Gustot T, Nicaise C, Quertinmont E, Nagy N, Parmentier M, Le Moine O, Deviere J, Louis H: CCR5 deficiency exacerbates T-cell-mediated hepatitis in mice. *Hepatology* 2005, 42:854–862
- Murai M, Yoneyama H, Harada A, Yi Z, Vestergaard C, Guo B, Suzuki K, Asakura H, Matsushima K: Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. *J Clin Invest* 1999, 104:49–57
- Sakai N, Wada T, Furuichi K, Shimizu K, Kokubo S, Hara A, Yamahana J, Okumura T, Matsushima K, Yokoyama H, Kaneko S: MCP-1/CCR2-dependent loop for fibrogenesis in human peripheral CD14-positive monocytes. *J Leukoc Biol* 2006, 79:555–563
- Serbina NV, Pamer EG: Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 2006, 7:311–317
- Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, Charo IF: Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest* 2007, 117:902–909
- Ito A, Suganami T, Yamauchi A, Degawa-Yamauchi M, Tanaka M, Kouyama R, Kobayashi Y, Nitta N, Yasuda K, Hirata Y, Kuziel WA, Takeya M, Kanegasaki S, Kamei Y, Ogawa Y: Role of C-C chemokine receptor 2 in bone marrow cells in the recruitment of macrophages into obese adipose tissue. *J Biol Chem* 2008, 283:35715–35723
- Fallowfield JA, Mizuno M, Kendall TJ, Constantinou CM, Benyon RC, Duffield JS, Iredale JP: Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol* 2007, 178:5288–5295
- Kurihara T, Warr G, Loy J, Bravo R: Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med* 1997, 186:1757–1762
- Asselah T, Bieche I, Laurendeau I, Paradis V, Vidaud D, Degot C, Martinot M, Bedossa P, Valla D, Vidaud M, Marcellin P: Liver gene expression signature of mild fibrosis in patients with chronic hepatitis C. *Gastroenterology* 2005, 129:2064–2075
- Narumi S, Tominaga Y, Tamaru M, Shimai S, Okumura H, Nishioji K, Itoh Y, Okanoue T: Expression of IFN-inducible protein-10 in chronic hepatitis. *J Immunol* 1997, 158:5536–5544
- Moore BB, Paine III R, Christensen PJ, Moore TA, Sitterding S, Ngan R, Wilke CA, Kuziel WA, Toews GB: Protection from pulmonary fibrosis in the absence of CCR2 signaling. *J Immunol* 2001, 167:4368–4377
- Moore BB, Kolodtsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB: CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. *Am J Pathol* 2005, 166:675–684
- Kitagawa K, Wada T, Furuichi K, Hashimoto H, Ishiwata Y, Asano M, Takeya M, Kuziel WA, Matsushima K, Mukaida N, Yokoyama H: Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol* 2004, 165:237–246
- Boring L, Gosling J, Chensue SW, Kunkel SL, Farese Jr RV, Broxmeyer HE, Charo IF: Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 1997, 100:2552–2561
- Imamura M, Ogawa T, Sasaguri Y, Chayama K, Ueno H: Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats. *Gastroenterology* 2005, 128:138–146
- Marra F, Romanelli RG, Giannini C, Failli P, Pastacaldi S, Arrighi MC, Pinzani M, Laffi G, Montalto P, Gentilini P: Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. *Hepatology* 1999, 29:140–148
- Hume DA: Macrophages as APC and the dendritic cell myth. *J Immunol* 2008, 181:5829–5835
- Osterholzer JJ, Curtis JL, Polak T, Ames T, Chen GH, McDonald R, Huffnagle GB, Toews GB: CCR2 mediates conventional dendritic cell recruitment and the formation of bronchovascular mononuclear cell infiltrates in the lungs of mice infected with *Cryptococcus neoformans*. *J Immunol* 2008, 181:610–620
- Iredale JP, Benyon RC, Arthur MJ, Ferris WF, Alcolado R, Winwood PJ, Clark N, Murphy G: Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. *Hepatology* 1996, 24:176–184
- Preaux AM, D'Ortho MP, Bralet MP, Laperche Y, Mavrier P: Apoptosis of human hepatic myofibroblasts promotes activation of matrix metalloproteinase-2. *Hepatology* 2002, 36:615–622
- Watanabe T, Niioka M, Ishikawa A, Hozawa S, Arai M, Maruyama K, Okada A, Okazaki I: Dynamic change of cells expressing MMP-2 mRNA and MT1-MMP mRNA in the recovery from liver fibrosis in the rat. *J Hepatol* 2001, 35:465–473
- Théret N, Musso O, L'Héroguez A, Clement B: Activation of matrix metalloproteinase-2 from hepatic stellate cells requires interactions with hepatocytes. *Am J Pathol* 1997, 150:51–58
- Yang X, Lu P, Ishida Y, Kuziel WA, Fujii C, Mukaida N: Attenuated liver

- tumor formation in the absence of CCR2 with a concomitant reduction in the accumulation of hepatic stellate cells, macrophages and neovascularization. *Int J Cancer* 2006, 118:335–345
35. Higashiyama R, Inagaki Y, Hong YY, Kushida M, Nakao S, Niioka M, Watanabe T, Okano H, Matsuzaki Y, Shiota G, Okazaki I: Bone marrow-derived cells express matrix metalloproteinases and contribute to regression of liver fibrosis in mice. *Hepatology* 2007, 45:213–222
36. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante Jr AW: CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 2006, 116:115–124