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Hepatic Macrophage Migration and Differentiation Critical for Liver Fibrosis Is Mediated by the Chemokine Receptor C-C Motif Chemokine Receptor 8 in Mice

Felix Heymann[1], **Linda Hammerich**[1], **Dunja Storch**[1], **Matthias Bartneck**[1], **Sebastian Huss**[2], **Vanessa Rüsseler**[2], **Nikolaus Gassler**[3], **Sergio A. Lira**[4], **Tom Luedde**[1], **Christian Trautwein**[1], and **Frank Tacke**[1]

[1]Department of Medicine III, RWTH University-Hospital Aachen, Aachen, Germany

[2]Department of Pathology, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany

[3]Department of Pathology, RWTH University-Hospital Aachen, Aachen, Germany

[4]Immunology Institute, Mount Sinai Medical Center, New York, NY

Abstract

Chemokines critically control the infiltration of immune cells upon liver injury, thereby promoting hepatic inflammation and fibrosis. The chemokine receptor CCR8 can affect trafficking of monocytes/macrophages, monocyte-derived dendritic cells (DCs) and T-helper cell (Th) subsets, but its role in liver diseases is currently unknown. To investigate the functional role of CCR8 in liver diseases, *ccr8*$^{-/-}$ and wild-type (WT) mice were subjected to chronic experimental injury models of carbon tetrachloride (CCl_4) administration and surgical bile duct ligation (BDL). CCR8 was strongly up-regulated in the injured liver. *Ccr8*$^{-/-}$ mice displayed attenuated liver damage (e.g., ALT, histology, and TUNEL) compared to WT mice and were also protected from liver fibrosis in two independent injury models. Flow cytometry revealed reduced infiltrates of liver macrophages, neutrophils and natural killer cells, whereas hepatic CD4$^+$ T cells increased. The main CCR8-expressing cells in the liver were hepatic macrophages, and CCR8 was functionally necessary for CCL1-directed migration of inflammatory but not for nonclassical monocytes into the liver. Moreover, the phenotype of liver macrophages from injured *ccr8*$^{-/-}$ animals was altered with increased expression of DC markers and enhanced expression of T-cell-attracting chemokine macrophage inflammatory protein 1-alpha (MIP-1*α*/CCL3). Correspondingly, hepatic CD4$^+$ T cells showed increased Th1 polarization and reduced Th2 cells in CCR8-deficient animals. Liver fibrosis progression, but also subsequent T-cell alterations, could be restored by adoptively transferring CCR8-expressing monocytes/macrophages into *ccr8*$^{-/-}$ mice during experimental injury.

Address reprint requests to: Frank Tacke, M.D., Ph.D., Department of Medicine III, University Hospital Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany. frank.tacke@gmx.net; fax: -49-241-80-82455.

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Conclusions—CCR8 critically mediates hepatic macrophage recruitment upon injury, which subsequently shapes the inflammatory response in the injured liver, affecting macrophage/DC and Th differentiation. CCR8 deficiency protects the liver against injury, ameliorating initial inflammatory responses and hepatic fibrogenesis. Inhibition of CCR8 or its ligand, CCL1, might represent a successful therapeutic target to limit liver inflammation and fibrosis progression.

Liver cirrhosis following chronic liver inflammation is the most common cause for end-stage liver disease leading to liver failure and, ultimately, death. Recently, much has been learned about the cellular and molecular links between chronic injury, hepatic inflammation and progression of liver fibrosis.[1] As such, the chemokine-directed infiltration of immune cells has gained much attention.[2] Several studies have emphasized the crucial role of infiltrating monocytes/macrophages for the progression of liver inflammation and fibrosis in experimental mouse models[3,4] and in patients with liver cirrhosis.[5] It has become clear that the macrophage compartment of the liver, traditionally called Kupffer cells, is greatly augmented by an overwhelming number of infiltrating monocytes upon acute or chronic liver injury.[6] In conditions of liver damage, C-C motif chemokine receptor (CCR)2 and its ligand, monocyte chemotactic protein-1/C-C motif chemokine ligand (MCP-1/CCL)2, promote monocyte subset infiltration into the liver,[4,7,8], whereas the chemokine receptor, CX_3CR1, and its ligand, fractalkine (CX_3CL1), are important negative regulators of monocyte infiltration in hepatic inflammation.[9,10] Moreover, chemokines also attract adaptive immune cells, namely T cells, into the inflamed liver.[11] In this respect, the chemokine receptors, CXCR3 and CCR5, have been identified as important pathways,[12] which appear to be also promising therapeutic approaches, at least in experimental fibrosis models.[13]

In this study we investigated the possible involvement of CCR8, the chemokine receptor for CCL1 (TCA-3 in mice, I-309 in humans), in the pathogenesis of chronic liver injury. CCR8 has been originally identified on monocytes,[14] but later also linked to T helper cell (Th)2-type polarized T cells.[15,16] The *in vivo* functions of CCR8 are incompletely understood, at present. On one hand, CCR8 appears to be important for innate immune responses because it functionally impacts macrophage migration and activation in inflammatory disease models of sepsis,[17] peritonitis,[18] and diabetes,[19] in which CCR8-deficient mice are protected. On the other hand, *ccr8*$^{-/-}$ mice demonstrated impaired Th2 responses in animal models of granuloma and allergic airway inflammation,[16,20] suggesting that CCR8 is also essential for developing fully functional Th2-type adaptive immunity. The role of CCR8 during liver inflammation and fibrosis is currently unknown. Based on the essential role of (infiltrating) macrophages for the perpetuation of hepatic inflammation during fibrogenesis[6] and on the critical profibrogenic function of Th2-polarized Ths,[21] we hypothesized that CCR8 is an important, yet unrecognized, chemokine-receptor pathway involved in the pathogenesis of chronic liver disease.

Materials and Methods

Mice

C57Bl/6 and *ccr8*$^{-/-}$ mice[16] were housed in a specific pathogen-free environment. Experiments were performed with age- and sex-matched animals at 6–8 weeks of age under

ethical conditions approved by the appropriate authorities, according to German legal requirements.

Induction of Acute and Chronic Liver Injury or Liver Fibrosis

Mice received 0.6 mL/kg body weight of CCl_4 (Merck, Darmstadt, Germany), mixed with corn oil intraperitoneally (IP), and were sacrificed at indicated time points. For induction of liver fibrosis, CCl_4 was injected three times weekly for 8 weeks. Mice were sacrificed 36 hours after the last injection. Surgical bile duct ligation (BDL) was performed by tying the common biliary duct using a nonabsorbable filament (Ethicon, Inc., Somerville, NJ)[9]; mice were sacrificed after 3 weeks.

Assessment of Liver Injury and Fibrosis

Conventional hematoxylin and eosin (H&E) and Sirius red stainings, TUNEL staining (TdT-mediated dUTP nick-end labeling; Promega, Madison, WI), as well as measurements of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were performed according to standard protocols.[4] Morphometric analysis of Sirius red staining was performed in a blinded fashion, using ImageJ (National Institutes of Health, Bethesda, MD). Hepatic hydroxyproline content was quantified as previously described.[4] Immunohistochemistry (IHC) was performed on paraffin-embedded sections using a peroxidase-conjugated avidin-biotin method. Deparaffinized and rehydrated sections were incubated with anti–cluster of differentiation (CD) 45 (1:50; BD Biosciences, Franklin Lakes, NJ). Immuno-reactions were visualized by using 3-amino-9-ethylcarbazole (AEC) as a substrate (Dako A/S, Glostrup, Denmark).

Analysis of Blood and Intrahepatic Leukocytes

Flow cytometric analysis of intrahepatic leukocytes was performed as previously described.[9] In brief, livers were digested with collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ) at 37°C. Extracts were filtered and either stained directly for CD45 and lymphocyte antigen 6G (Ly6G) or subjected to density-gradient centrifugation (LSM-1077; PAA Laboratories GmbH, Pasching, Austria). Six-color staining was conducted using combinations of the following monoclonal antibodies: F4/80 (AbD Serotec, Düsseldorf, Germany), nitric oxide synthase 2 (NOS2; Santa Cruz Biotechnology, Santa Cruz, California), CD3, CD4, CD11b, CD69, CD86, CD45, interferon (IFN)-γ, interleukin (IL)-4, IL-12, IL-13, tumor necrosis factor alpha (TNF-α), forkhead box protein 3 (FoxP3; eBioscience, Inc., San Diego, CA), CD8α, CD40, I-A^b, IL-4, IL-10, Ly6G, natural killer (NK)1.1 (BD Biosciences), rat immunoglobulin G (IgG)2a, or hamster IgG isotype controls (BD Biosciences). CCR8 was stained using a polyclonal sheep IgG antibody (R&D Systems, Minneapolis, MN), followed by a polyclonal donkey antisheep Alexa Fluor 647 antibody (Invitrogen, Carlsbad, CA). Total cell numbers were determined by adding fixed numbers of Calibrite APC beads (BD Biosciences) before measurement as the internal reference. Analysis was performed using a FACSCanto-II (BD Biosciences), with cell sorting using FACSAria-II-SORP (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Intracellular Staining of Cytokine Expression

For intracellular staining, cells were cultured overnight in Roswell Park Memorial Institute (RPMI)-1640 containing 10% fetal calf serum. Lipopolysaccharide (LPS; 100 ng/mL) was added to the culture for macrophage restimulation. T cells were stimulated for 5 hours with 10 ng/mL of PMA (phorbole 12-myristate-13-acetate; AppliChem GmbH, Darmstadt, Germany) and 250 ng/mL of ionomycin (Sigma-Aldrich, St. Louis, MO). Cytokine secretion was blocked using GolgiPlug (BD Biosciences). After surface staining, cells were fixed using 2% formalin and permeabilized using 0.5% saponin (Sigma-Aldrich). Intracellular staining was performed using antibodies against TNF-α, IL-12, NOS2, FoxP3, IFN-γ, IL-4, IL-10, or IL-13 respectively.

Gene Expression

RNA was purified from sorted cells using the ArrayPure RNA isolation kit (Epicentre, Madison, WI), and complementary DNA was generated (Roche, Basel, Switzerland). Quantitative real-time polymerase chain reaction (qPCR) was performed using SYBR Green reagent (Invitrogen). Reactions were done in duplicates, and β-actin was used to normalize gene expression.[9] Primer sequences are available upon request.

Cell-Culture Experiments

Liver leukocyte transmigration—Liver-infiltrating leukocytes were isolated by collagenase type IV digestion with subsequent density-gradient centrifugation as described above. Then, 1×10^5 liver leukocytes were added into 24-well hanging Transwell cell-culture inserts with a pore size of 5 μm (Millipore, Billerica, MA) in a volume of 200 μL of RPMI-1640. Bottom wells were filled with 1 mL of RPMI-1640, adding 100 ng/mL of recombinant CCL1 (PAN Biotech GmbH, Aidenbach, Bavaria). Cells were incubated for 4 hours under standard cell-culture conditions (37°C, 5% CO_2). Subsequently migrated cells from bottom wells were analyzed by flow cytometry. Maturation/DC differentiation of macrophages by CCL1 was addressed by culturing 2×10^5 macrophages in a 12-well plate overnight in 1 mL of RPMI-1640, adding 100 ng/mL of recombinant CCL1 to the culture. Cytokine/chemokine release of hepatic macrophages was investigated by culturing primary macrophages, isolated by magnet-associated bead sorting (MACS) using anti-F4/80 and streptavidin microbeads (Miltenyi Biotec, Cologne, Germany) from repetitively CCl_4-treated livers, overnight without further stimulation. Next, 10^5 cells/well were plated, and cytokines and chemokines were measured from supernatant after 12 hours using a multiplex assay (FlowCytomix; eBioscience).[22]

Adoptive Monocyte Transfer

Monocytes were purified from mouse bone marrow by MACS of $CD115^+$ cells using a CD115-biotin (BD Biosciences) antibody, followed by a streptavidin-MACS conjugate (Miltenyi Biotec).[4] Purity of isolated monocytes was checked by flow cytometry analysis and was >80%. Then, 1×10^6 cells were injected intravenously (IV) weekly into WT and *ccr8*$^{-/-}$ recipients between CCl_4 challenges. Mice were sacrificed after 6 weeks.

Statistical Analysis

Data were analyzed using PRISM software (GraphPad Software, Inc., La Jolla, CA) and are expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed by the two-tailed, unpaired Student's *t* test.

Results

Ccr8$^{-/-}$ Mice Are Protected From Chronic Liver Injury and Fibrosis

To investigate the functional role of CCR8 in liver disease, *ccr8*$^{-/-}$ and C57/Bl6 WT animals were challenged with 0.6 mg/kg of CCl_4 IP repetitively over 8 weeks to induce chronic liver inflammation and fibrosis. *Ccr8*$^{-/-}$ mice, in comparison to WT animals, showed reduced perivascular clusters of immune cells in the liver and less leukocytes surrounding the portal fields by conventional histology. Also, liver necrosis was diminished at early and late stages of CCl_4-induced injury in *ccr8*$^{-/-}$ animals (Fig. 1A). In line with these results, reduced serum ALT (Fig. 1B) and AST (not shown) levels could be detected in *ccr8*$^{-/-}$ mice. Hepatocyte apoptosis was found to be significantly reduced by TUNEL staining in *ccr8*$^{-/-}$ mice (Fig. 1C,D), corroborating that hepatic injury is attenuated in CCR8-deficient animals. Importantly, this phenotype was unlikely to reflect a different response of hepatocytes to the toxic agent CCl_4 itself, because primary murine hepatocytes did not express significant levels of CCR8 and initial liver injury was not different after a single injection of CCl_4 (data not shown).

We next investigated the development of liver fibrosis in *ccr8*$^{-/-}$ versus WT mice, analyzing liver paraffin sections by Sirius red staining followed by a qualitative and quantitative assessment of liver fibrosis. After 8 weeks of repetitive CCl_4 injections, *ccr8*$^{-/-}$ mice displayed ameliorated liver fibrosis with significantly reduced amounts of collagen and also less prominent bridging between portal fields (Fig. 1E,F). Total liver collagen content was measured by quantifying hepatic content of hydroxyproline, an amino acid abundantly found in collagen fibers. *Ccr8*$^{-/-}$ mice showed significantly lower collagen deposition and hydroxyproline levels compared to WT animals (Fig. 1F). To exclude model-specific confounding effects, the development of liver fibrosis was assessed in a second model of chronic liver disease, using surgical BDL over 3 weeks as an established model for cholestatic fibrosis.[9] Consistently, *ccr8*$^{-/-}$ animals developed less prominent inflammatory infiltrates around affected bile ducts as well as significantly reduced liver fibrosis (Supporting Fig. 1).

Infiltration of Innate Immune Cells Into Injured Liver Is Dependent on CCR8

The protection of *ccr8*$^{-/-}$ mice in two independent models of liver injury and fibrosis led us to hypothesize that CCR8 was involved in controlling leukocyte infiltration into the liver upon hepatic damage. IHC staining for the panleukocyte marker CD45 indeed revealed significantly decreased amounts of CD45^{+} immune cells upon liver injury in *ccr8*$^{-/-}$ compared to WT mice (Fig. 2A). In other inflammatory conditions, such as asthma or peritonitis, CCR8 expression has been observed on monocytes/macrophages and on Th2-type CD4^{+} T cells.[15,18] We hence thoroughly analyzed the hepatic inflammatory infiltrate using flow cytometry (Fig. 2B), assessing the relative (Fig. 2C) as well as the absolute

amounts (Supporting Fig. 2) of different leukocyte subpopulations within the chronically injured liver tissue. *Ccr8*$^{-/-}$ mice showed significant reductions in innate immune cells, specifically liver macrophages, neutrophils, and NK cells, whereas CD4^{+} T cells increased both in relative amounts and in absolute cell numbers (Fig. 2B,C; Supporting Fig. 2). Notably, CD8^{+} T cells did not vary between *ccr8*$^{-/-}$ and WT mice after CCl_4-induced liver damage, indicating that trafficking of these cells was independent from direct or indirect CCR8 signaling. Collectively, CCR8 appeared important for infiltration of innate immune cells, whereas CCR8 deficiency induced a distinct increase in CD4^{+} T cells.

CCR8 Is Specifically Expressed by Hepatic Macrophages and Controls Macrophage Migration

Upon chronic liver injury induced by repetitive CCl_4 injections, *ccr8* messenger RNA (mRNA) expression was strongly (approximately 14-fold) up-regulated in injured compared to control liver tissue (Fig. 3A), corroborating the relevance of this pathway in chronic liver damage. Because the trafficking pattern of several leukocyte subsets was altered in *ccr8*$^{-/-}$ mice upon injury, we next aimed at identifying the immune cell populations expressing CCR8 in the liver. We performed real-time qPCR to assess the expression levels of *ccr8* mRNA from purified leukocyte populations. Leukocytes were isolated from liver or spleen of WT and *ccr8*$^{-/-}$ animals (as negative controls) using fluorescence-activated cell sorting (FACS). Macrophages, especially when isolated from the injured liver, expressed high levels of *ccr8* mRNA, whereas NK and T cells both only showed low-level expression (Fig. 3B). The *ccr8* expression pattern was also resembled by leukocyte subpopulations isolated from the spleen, albeit the overall expression of *ccr8* was lower compared to liver leukocytes (Fig. 3B). CCR8 expression was confirmed on protein level by FACS analysis. The only population that was detected to express CCR8 in the liver were macrophages, whereas none of the other immune cells, nor CD45^{-} cells, stained positive for CCR8 (Supporting Fig. 3A).

To test whether the CCR8 expression on hepatic macrophages was also functionally relevant, we tested the migratory response of isolated liver leukocytes using an *in vitro* transmigration assay with recombinant CCL1, the specific CCR8 ligand. Hepatic leukocytes were isolated from livers of WT and *ccr8*$^{-/-}$ mice 48 hours after CCl_4 challenge, subjected to an *in vitro* transmigration assay and migrated cells were phenotyped by FACS. After 4 hours of incubation against a CCL1 gradient, leukocytes derived from WT mice showed significantly higher migratory activity compared to cells isolated from *ccr8*$^{-/-}$ mice, and all migrating cells were CD45^{+}Ly6G^{-}CD11b^{+}F4/80^{+}, fully resembling the phenotype of liver macrophages (Fig. 3C). Migration of other leukocyte subpopulations, such as neutrophils, T cells, or NK cells, in response to CCL1 could not be detected (not shown). To exclude that CCL1 had additional direct effects on macrophage maturation, we also purified macrophages by MACS from liver tissue and cocultured them overnight with recombinant CCL1. CCL1 did not induce the differentiation of macrophages into DCs *in vitro*, as evidenced by analysis of major histocompatibility complex class II (MHC-II) expression (I-A^{b}) and costimulation molecule CD86 (Supporting Fig. 3B). Together, these experiments revealed that CCR8 was strongly up-regulated upon liver injury, specifically expressed by

macrophages in the liver, and indicated an important function of CCR8-CCL1 interactions for macrophage migration into the injured liver.

Altered Phenotype of Hepatic Macrophages/DCs and T-Helper Cells in ccr8$^{-/-}$ Mice

The *in vitro* data strongly suggested that the amelioration of hepatic inflammation and fibrosis observed in *ccr8*$^{-/-}$ mice upon liver injury was primarily driven by the reduced monocyte/macrophage migration. Murine monocytes consist of at least two major subsets, in which Gr-1/Ly6C^{high}-expressing cells are considered as classical "inflammatory" monocytes, whereas Gr-1/Ly6C^{low}-expressing, nonclassical monocytes have been linked to repair processes.[6] By thoroughly characterizing the phenotype of liver macrophages and DCs from *ccr8*$^{-/-}$ and WT mice upon CCl_4 injury *in vivo*, the reduction in hepatic macrophage accumulation could be fully attributed to reduced numbers of CD11b$^+$F4/80$^+$Ly6C$^+$ macrophages, whereas Ly6C^{low} monocytes/macrophages were not altered between *ccr8*$^{-/-}$ and WT mice (Fig. 4A). Interestingly, *ccr8*$^{-/-}$ mice not only displayed reduced numbers of inflammatory macrophages, characterized as CD45$^+$Ly6G$^-$CD11b$^+$F4/80$^+$CD11c$^-$ cells, in injured livers, but also a relative abundance of classical CD11b$^-$CD11c$^+$DCs (Fig. 4A,B). Moreover, liver macrophages isolated from *ccr8*$^{-/-}$ mice also showed increased signs of differentiation toward DCs. Hepatic *ccr8*$^{-/-}$ macrophages expressed significantly higher levels of the MHC-II molecule, I-A^b, and CD86, compared to WT cells (Fig. 4C), clearly indicating antigen-presentation capacity. Upon intracellular staining, *ccr8*$^{-/-}$ macrophages also displayed increased synthesis of IL-12, an important inducer of T-cell activation (Fig. 4C), corroborating that *ccr8*-deficient hepatic macrophages have an altered, more DC-like differentiated phenotype. We next investigated whether CCR8 also functionally effected the cytokine and chemokine secretion capacity of hepatic macrophages. CD11b$^+$F4/80$^+$ macrophages were isolated from chronically injured livers of *ccr8*$^{-/-}$ and WT mice, followed by overnight culture without further stimulation. Though the release of "classical" proinflammatory mediators, such as MCP-1, IL-6, or TNF, did not differ between ccr8-deficient and WT macrophages (Fig. 4D), hepatic macrophages from *ccr8*$^{-/-}$ mice secreted significantly higher amounts of the chemokine macrophage inflammatory protein (MIP)1α (CCL3), an important attractor for T cells (Fig. 4E). No differences in IFN-γ or IL-4 secretion, cytokines directly promoting T-helper 1/2 cell differentiation, were detected (data not shown).

The increased numbers of hepatic DCs in *ccr8*$^{-/-}$ mice, DC-like differentiation of *ccr8*$^{-/-}$ macrophages, and their enhanced secretion of the T-cell-attracting chemokine, MIP1α, suggested that hepatic T-cell activation and differentiation might be affected, as well. Thus, we next investigated the increased number of hepatic CD4$^+$ T cells observed in CCl_4-challenged *ccr8*$^{-/-}$ mice with respect to their functionality. CD4$^+$ T cells isolated from *ccr8*$^{-/-}$ animals showed increased expression of CD69 compared to WT animals which serves as an early activation marker expressed on stimulated T cells, whereas CD8$^+$ T cells did not show signs of differential activation (data not shown). To assess whether these T cells would also exhibit alternate functional properties, we tested for different Th subtypes.[21] CD4$^+$ T cells isolated from livers of *ccr8*$^{-/-}$ mice expressed significantly higher levels of IFN-γ (Fig. 5A), indicating a polarization into Th1 T cells. For the other marker cytokines, the results were less pronounced. The Th2 cytokine, IL-13, was found at low

levels on $CD4^+$ T cells, but significantly reduced in *ccr8*$^{-/-}$ mice, whereas the reduction in IL-10 did not reach statistical significance (Fig. 5A). Accordingly, higher levels of the Th1-specific transcription factor, t-bet, but not GATA-3, could be detected in intrahepatic $CD4^+$ T cells from *ccr8*$^{-/-}$ mice compared to WT animals after CCl_4 challenge (not shown). Regulatory T cells (Tregs), as identified by CD4, CD25, and Foxp3 expression, were reduced in *ccr8*$^{-/-}$ mice (Fig. 5B). Collectively, in agreement with the preferential DC phenotype of hepatic CCR8-deficient macrophages, the intrahepatic T-cell response was skewed toward a Th1-biased direction in *ccr8*$^{-/-}$ mice, with reduced Th2- and Treg-cells.

Adoptively Transferred WT Monocytes Restore Liver Fibrosis in ccr8$^{-/-}$ Mice In Vivo

Our experiments indicated that ameliorated liver damage and fibrosis upon experimental chronic injury in *ccr8*$^{-/-}$ mice was the result of a CCR8-dependent recruitment of inflammatory monocytes into the injured liver and their subsequent differentiation into classical M1-type macrophages. The alterations of the hepatic inflammatory microenvironment, including reduced neutrophils and NK cells, more DC-type differentiated cells, as well as pronounced Th1- and reduced Th2-response, could, in principle, be well explained by the lack of CCR8-dependent macrophage recruitment in *ccr8*$^{-/-}$ mice. To prove this hypothesis, we employed an adoptive transfer model that we had developed previously.[4,23] $CD45.1^+$ WT $CD115^+$ $Gr1^+$ bone marrow monocytes were isolated by MACS and adoptively transferred into CCl_4-challenged $CD45.2^+$ WT or *ccr8*$^{-/-}$ mice. First, 1×10^6 monocytes were injected IV weekly during a 6-week chronic CCl_4 treatment course. Staining for CD45.1 allowed the identification of adoptively transferred cells in livers of WT or *ccr8*$^{-/-}$ recipient mice (Fig. 6A), providing evidence that the $Gr1^+$ bone marrow monocytes were indeed recruited into injured livers of WT or *ccr8*$^{-/-}$ mice. The vast majority (approximately 75%) of transferred monocytes differentiated into typical $CD11b^+F4/80^+$ inflammatory macrophages in the injured liver (Fig. 6A), whereas the remaining cells were mainly $CD11b^-F4/80^+$, likely resembling more mature macrophages.[4] Clearly, the adoptive transfer of CCR8-expressing monocytes into *ccr8*$^{-/-}$ mice during ongoing experimental liver injury increased liver fibrosis of *ccr8*$^{-/-}$ mice, so that collagen deposition was restored to the same level as observed in WT animals upon CCl_4 without adoptive transfer (Fig. 6B,C). Interestingly, *ccr8*$^{-/-}$ mice also showed reduced intrahepatic $CD4^+$ T cells upon adoptive monocyte transfer, compared to nontransferred mice (not shown), suggesting that the increased intrahepatic T cells in *ccr8*$^{-/-}$ mice without transfer were an epiphenomenon directly linked to the impaired macrophage recruitment. WT mice that had received monocytes also showed increased signs of fibrosis, as observed previously,[4] but differences did not reach statistical significance. Taken together, these experiments demonstrated that the chemokine receptor, CCR8, mediates hepatic macrophage migration and differentiation in experimental liver injury, which, critically, promotes hepatic inflammation and fibrosis progression.

Discussion

Recruitment of immune cells, such as macrophages and T cells, to the site of injury is an important event in the initiation of inflammation, but also for wound healing and hepatic fibrosis.[2] Recent work from both clinical and experimental studies provided evidence that

chemokines, small chemotactic cytokines, are critically involved in acute and chronic liver diseases. The fine-tuned network of distinct chemokine/chemokine receptor interactions in response to liver injury, which subsequently results in the controlled influx of immune cells into the liver, their differential activation, and communication with different cell populations in the liver (i.e., hepatocytes, Kupffer cells, endothelium, or hepatic stellate cells; HSCs), has only started to be unraveled.[11] Given the considerable redundancy within the chemokine system (several receptors may respond to one chemokine and vice versa) and the promiscuous chemokine-receptor expression pattern by many immune cells, identifying unique, functionally relevant chemokine pathways during hepatic inflammation is important for understanding this network and for developing novel targeted therapeutic strategies against liver fibrosis.

Our study now provides compelling evidence for an important functional role of the chemokine receptor, CCR8, in regulating the inflammatory response toward liver injury and the hepatic wound-healing reaction. Knockout of CCR8 leads to an attenuated development of liver disease with reduced numbers of innate immune cells, specifically macrophages, and increased numbers of $CD4^+$ T cells. Consequently, CCR8-deficient mice were protected from liver fibrosis development in two independent experimental fibrosis models.

In liver tissue, macrophages were the main cells expressing CCR8, whereas we failed to detect expression on hepatic T cells or other immune cells, indicating a predominant role of CCR8 for macrophage recruitment into the liver. The specific expression of CCR8 on infiltrating monocytes and activated macrophages has been also observed in models of bacterial[17] and sterile peritonitis.[18] In both peritonitis studies, CCR8-deficient animals were protected, because they showed reduced lethality in the septic and diminished adhesion development in the aseptic model.[17,18] Interestingly, *ccr8*$^{-/-}$ mice not only showed diminished intrahepatic macrophage infiltration upon chronic CCl_4 administration in our study, but also striking differences in macrophage/DC differentiation and T-cell functionality. Convincingly, the adoptive transfer of CCR8-expressing (i.e., bone marrow) monocytes into *ccr8*$^{-/-}$ mice during liver injury restored liver fibrosis progression, providing direct evidence for a functional role of CCR8-dependent hepatic macrophage recruitment in chronic liver damage. Moreover, also, the intrahepatic $CD4^+$ T-cell numbers were restored by monocyte transfer, suggesting that the observed alterations in macrophage/DC differentiation and T-cell functionality likely reflect an altered hepatic inflammatory microenvironment caused by the reduced inflammatory macrophage recruitment.

It is well established that (blood and bone marrow) monocytes are circulating precursors of macrophages and (myeloid) DCs, especially in inflammatory conditions.[24] In this respect, CCR8 appeared functionally important to guide the migration of the inflammatory Gr1/$Ly6C^{hi}$ monocyte subset (but not of nonclassical Gr1/$Ly6C^{low}$ monocytes) from blood into the injured liver, where these monocytes usually develop into classical proinflammatory macrophages.[4,9] This is well in agreement with a recent study that demonstrated preferentially proinflammatory functions of organ-infiltrating monocytes/macrophages, whereas resident macrophages may be more important for anti-inflammatory actions.[25] Interestingly, we observed that macrophages in chronically injured *ccr8*$^{-/-}$ livers displayed a preferential DC-type differentiation.[26] Our finding is consistent with the hypothesis that

CCR8 is primarily needed for the migration of classical macrophages into the liver, but that the migration of nonclassical monocytes and other nonmonocytic precursors that give rise to DCs is not affected by CCR8. Consequently, we observed that the Th response is clearly shifted toward IFN-γ-producing Th1-type $CD4^+$ T cells in *ccr8*$^{-/-}$ livers. These results are in full agreement with current paradigms of Th responses in fibrosis, because Th1-type cytokines are thought to be antifibrogenic, whereas Th2-type cytokines are considered to be profibrogenic.[27] However, it is important to note that the role of IFN-γ in liver fibrosis is controversially discussed. Though several animal studies clearly showed attenuated fibrosis development upon IFN-γ treatment with impaired activation of HSCs, inhibition of transforming growth factor beta signaling in HSCs, and reduced extracellular matrix deposition,[28–30] IFN-γ can also have proinflammatory effects in distinct conditions, thereby possibly also aggravating disease progression and organ dysfunction.[31]

Although we did not observe strong Th2 cell responses in either WT or *ccr8*$^{-/-}$ livers, it is important to note that we cannot rule out additional effects of CCR8 on Th2 cells. Experimental and clinical models of allergic asthma and granulomatous lung diseases had suggested that CCR8 might be an important factor for Th2 cell migration into the lung as well as their activation in disease.[15,16,20] In our model, even in c57bl/6 control animals, Th2 cells could only be detected at very low frequency in fibrotic livers. The Th2 response (e.g., IL-13) was even further reduced in livers of *ccr8*$^{-/-}$ mice, supporting the interpretation of an augmented Th1 phenotype and diminished Th2 response in *ccr8*$^{-/-}$ animals. Even though we could not detect expression on CCR8 on T cells on a cellular level, we cannot rule out an impaired migration of Th2 cells, because with the low frequency of Th2 cells, the amount of $CD4^+$ T cells carrying CCR8 was just too small to be detected reliably.

Neutralization of chemokines has been recently proposed as a novel therapeutic strategy for hepatic fibrosis.[11–13] Because of the specific expression of CCR8 on hepatic macrophages and its significant functional involvement in the progression of injury and fibrosis, CCR8 may represent an attractive target for the treatment of chronic liver diseases. Interestingly, several small molecular agonistic and antagonistic drugs have been developed for either CCR8 or CCL1.[32,33] Thus, based on our results, CCR8 antagonism by small-molecule inhibitors may represent a feasible, promising novel antifibrogenic approach in inflammatory liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AEC	3-amino-9-ethylcarbazole
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BDL	bile duct ligation
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	cluster of differentiation
DC(s)	dendritic cell(s)
FACS	fluorescence-activated cell sorting
FoxP3	forkhead box protein 3
H&E	hematoxylin and eosin
HSC(s)	hepatic stellate cell(s)
IFN	interferon
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IP	intraperitoneally
IV	intravenously
LPS	lipopolysaccharide
Ly6G	lymphocyte antigen 6G
MACS	magnet-associated bead sorting
MCP-1	monocyte chemotactic protein-1
MHC-II	major histocompatibility complex class II
MIP	macrophage inflammatory protein
mRNA	messenger RNA
NK	natural killer
NOS2	nitric oxide synthase 2
PMA	phorbole 12-myristate-13-acetate
qPCR	quantitative real-time polymerase chain reaction
RPMI-1640	Roswell Park Memorial Institute medium
SEM	standard error of the mean

Th(s)	T-helper cell(s)
TNF-α	tumor necrosis factor alpha
Treg(s)	regulatory T cell(s)
TUNEL	TdT-mediated dUTP nick-end labeling
WT	wild type

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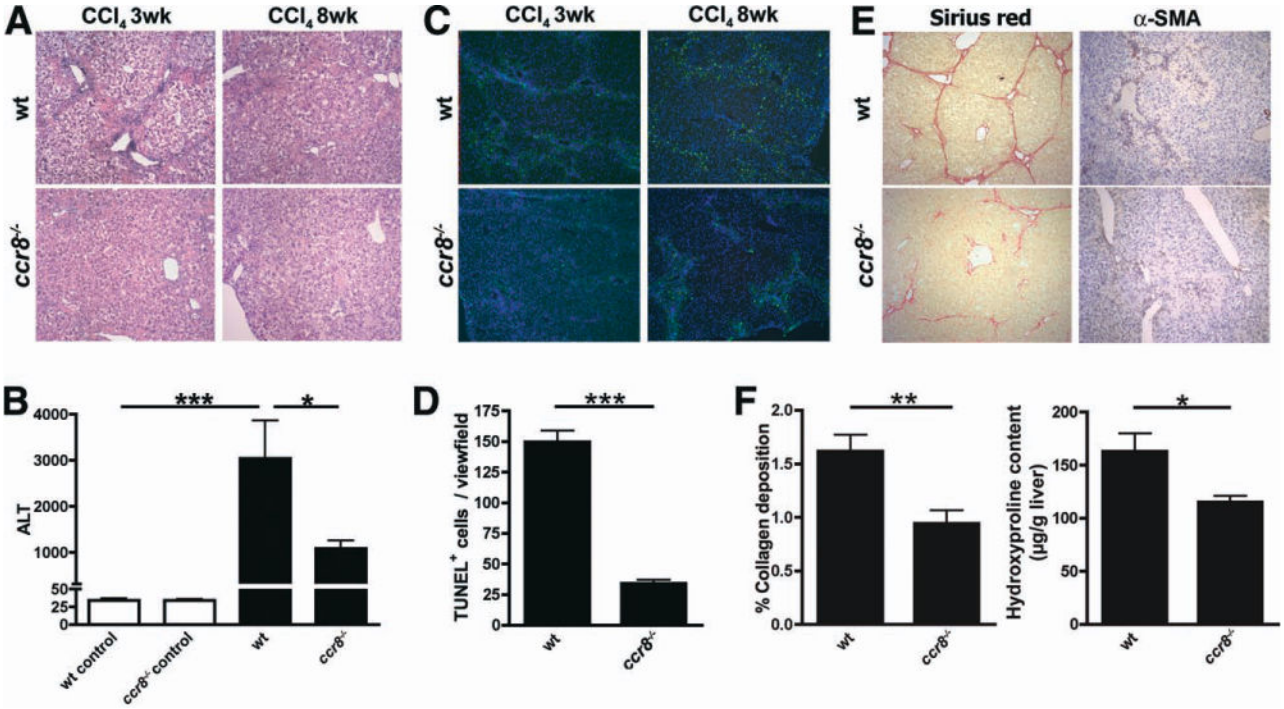

Fig. 1.

CCR8 deficiency attenuates chronic liver injury and fibrosis. (A) H&E staining of WT and *ccr8*$^{-/-}$ mice challenged with 0.6 mL/kg of CCl_4 IP repetitively three times per week. Magnification 10×. (B) Serum ALT levels (U/L) from WT and *ccr8*$^{-/-}$ animals after repetitive CCl_4 challenge over 8 weeks (36 hours after last CCl_4 injection). Experiments were performed in groups of 3 animals, and results were confirmed in two independent experiments. (C) TUNEL assay of liver cryosections from WT and *ccr8*$^{-/-}$ animals after 3 and 8 weeks of CCl_4 treatment. Apoptotic cells were stained green with FITC-dUTP, and nuclei were stained blue with DAPI. Magnification 10×. (D) Quantitative analysis of C. Five to seven independent view fields with 10× magnification were counted per animal in a blinded fashion. This experiment was performed in groups of 3 mice. (E) Sirius Red staining and IHC staining of alpha smooth muscle actin were performed on liver paraffin sections derived from WT and *ccr8*$^{-/-}$ mice after 8 weeks of CCl_4 treatment. Magnification ×10. (F) Collagen deposition was quantified from Sirius Red stains using polarization microscopy (10 view fields per animal were counted from at least 6 animals per group and quantified by in silico morphometric analyis), as well as by measurement of hepatic hydroxyproline content. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are presented as mean ± SEM. All results are representative of two independent experiments with 3 animals per group. FITC-dUTP, fluorescein isothiocyanate/deoxyuridine triphosphate; DAPI, 4′,6-diamidino-2-phenylindole; *α*-SMA, alpha smooth muscle actin.

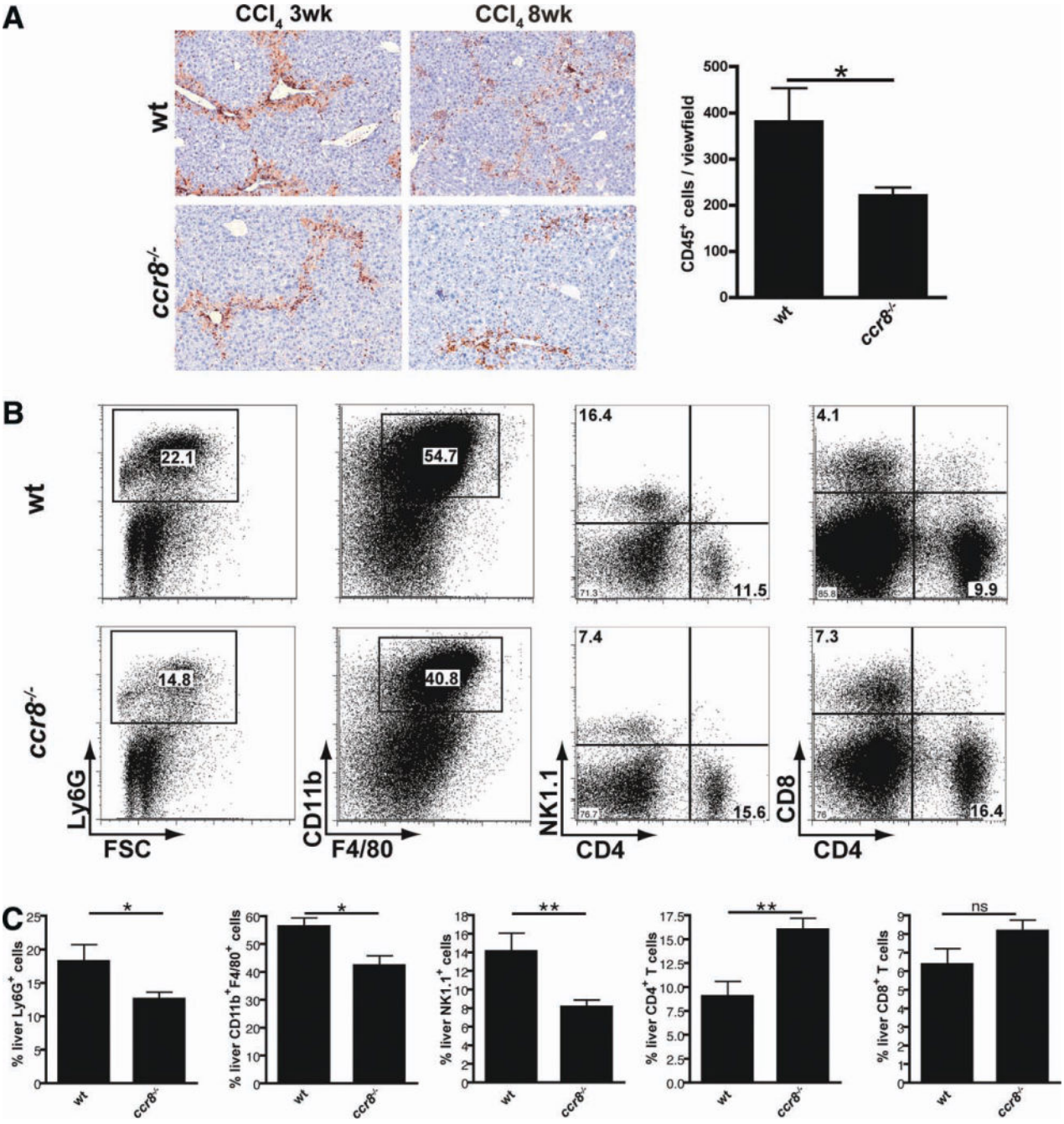

Fig. 2.

CCR8 deficiency ameliorates infiltration of innate immune cells. (A) Pan-leukocyte marker CD45 was stained by IHC on 4-μm-thick paraffin-embedded sections. Magnification 10×. $CD45^+$ cells were quantified manually by a pathologist who was blinded to experimental data. (B) Leukocytes were isolated from liver tissue of WT and *ccr8*$^{-/-}$ animals after repetitive CCl_4 challenge over 8 weeks. Cells were stained for CD45 to discriminate between liver parenchymal cells and leukocytes. Dead cells were excluded by Hoechst 33258. Cells were stained for Ly6G to identify neutrophils, CD11b, and F4/80 for liver macrophages, NK1.1 for NK cells, and $CD4^+$ and $CD8^+$ for helper and cytotoxic T

lymphocytes. (C) Statistical analysis of (B). Percentage values for total CD45 cells. This experiment was performed in groups of 3 animals, and results were confirmed in two independent experiments. Absolute cell counts of these hepatic leukocyte subsets are presented in Supporting Fig. 2. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are presented as mean ± standard deviation.

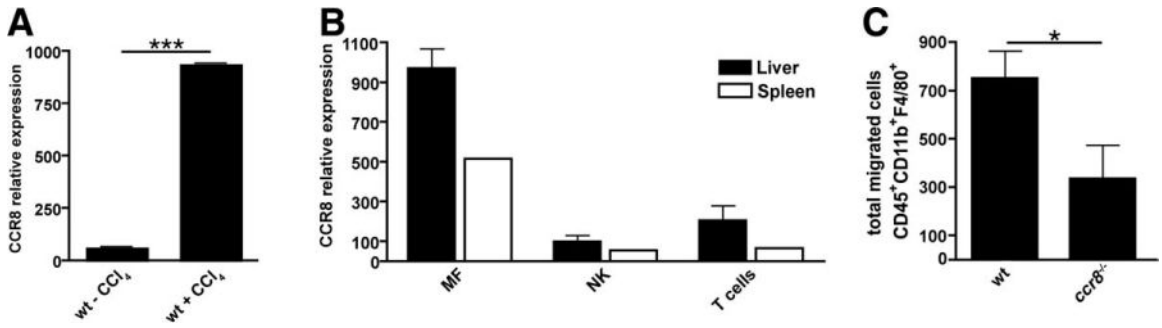

Fig. 3.

CCR8 is specifically expressed on macrophages and promotes macrophage migration. (A) Quantitative RT-PCR analysis of ccr8 expression from whole liver of WT animals after repetitive CCl_4 challenge over 8 weeks or controls. Expression levels were normalized against β-actin and calculated against unspecific background amplification in *ccr8*$^{-/-}$ animals. (B) Quantitative RT-PCR analysis of leukocyte subpopulations after FACS sorting. Cells were sorted for CD45$^+$ and further divided into Ly6G$^-$CD3$^-$NK1.1$^-$CD11b$^+$F4/80$^+$ cells (macrophages, MF), CD3 NK1.1$^+$ cells (NK cells, NK), and NK1.1$^-$CD3$^+$ cells (T cells). Expression levels were normalized against β-actin and calculated against unspecific background amplification in *ccr8*$^{-/-}$ animals. (C) Transwell migration of liver leukocytes after CCl_4 challenge. Leukocytes were isolated 36 hours after the last CCl_4 injection and cultured *in vitro* in 5-μm Transwell chambers for 4 hours against a CCL1 gradient (100 ng/mL). Migrated cells were harvested from the bottom wells and characterized by FACS. Cells were stained for neutrophils, macrophages, NK cells, and CD4$^+$ and CD8$^+$ T-cells, and dead cells were excluded by Hoechst 33258. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are presented as mean ± SEM. All results are representative of two independent experiments with 3 animals per group.

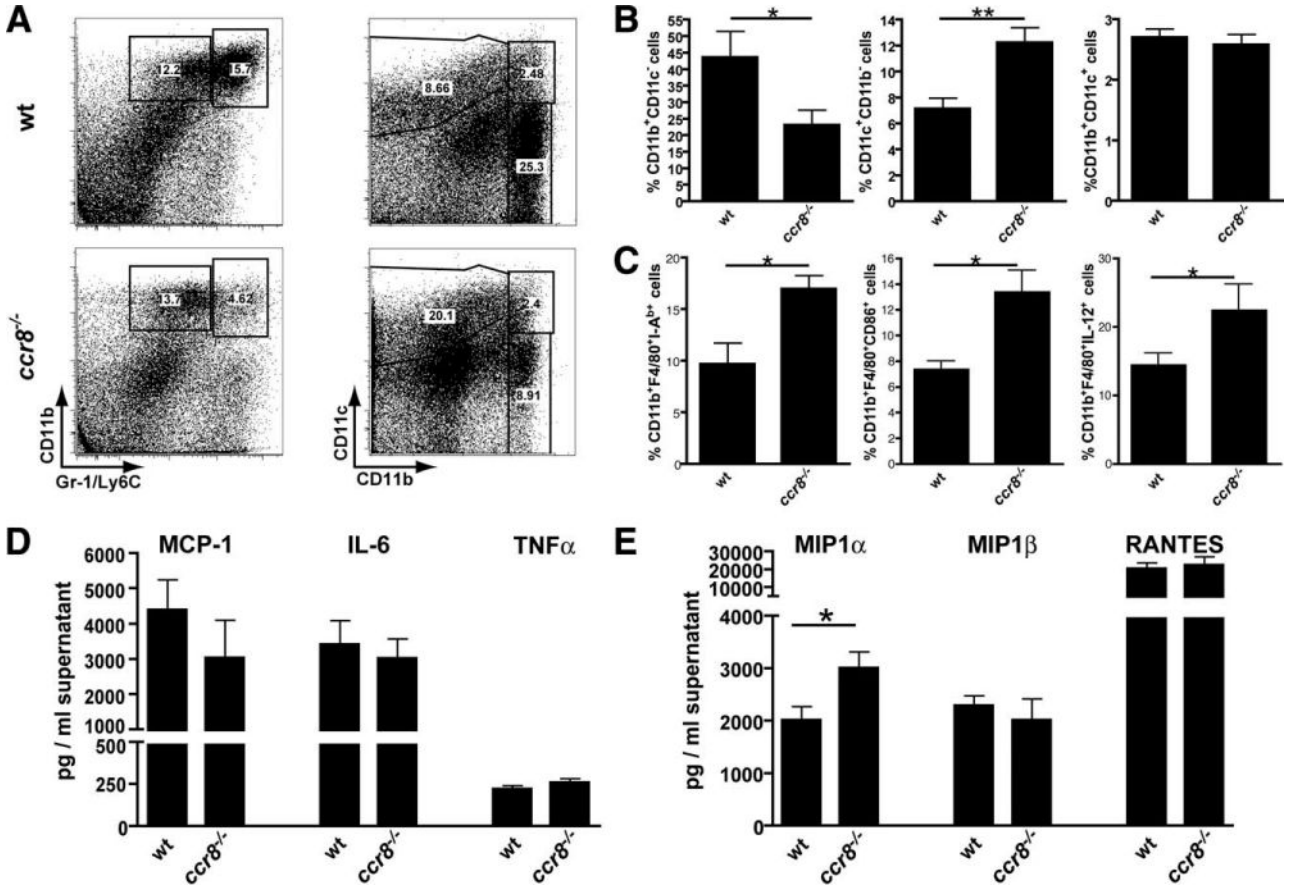

Fig. 4.

Phenotype of *ccr8*$^{-/-}$ liver macrophages and DCs. (A) Phenotypic analysis of liver macrophages and DCs after CCl_4 challenge in WT and *ccr8*$^{-/-}$ mice. Cells were isolated as described above and stained for CD45, Ly6G to gate out neutrophils, and CD11b, Gr-1/Ly6C, CD11c, and F4/80 for liver macrophage subtyping. (B) Statistical analysis of the macrophage/DC subtypes gated in (A). (C) Liver macrophages ($CD11b^+F4/80^+$) were isolated after 8 weeks of repetitive CCl_4 challenge by MACS purification against CD11b. Then, 2×10^5 cells were seeded into 24-well plates and restimulated overnight, adding 100 ng/mL of LPS to the culture. For intracellular cytokine staining, cells were cultured in the the presence of GolgiPlug (BD Biosciences, Franklin Lakes, NJ) additionally 4 hours before analysis. Macrophages were stained extracellularly for $I\text{-}A^b$ and CD86 (left, middle) and intracellularly for IL-12 (right). (D and E) Liver macrophages ($CD11b^+F4/80^+$) were isolated after repetitive CCl_4 challenge by MACS purification against F4/80, plated at 10^5 cells/well, and cultured overnight without further stimulation. The release of cytokines and chemokines typical for classical macrophages (D) as well as of the T-cell-attracting chemokines (E), MIP1a (CCL3), MIP1β (CCL4), and RANTES (CCL5), was measured from the supernatant by multiplex assay. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are presented as mean ± SEM. All results are representative of two independent experiments with 3 animals per group. RANTES, regulated upon activation normal T cell expressed and secreted.

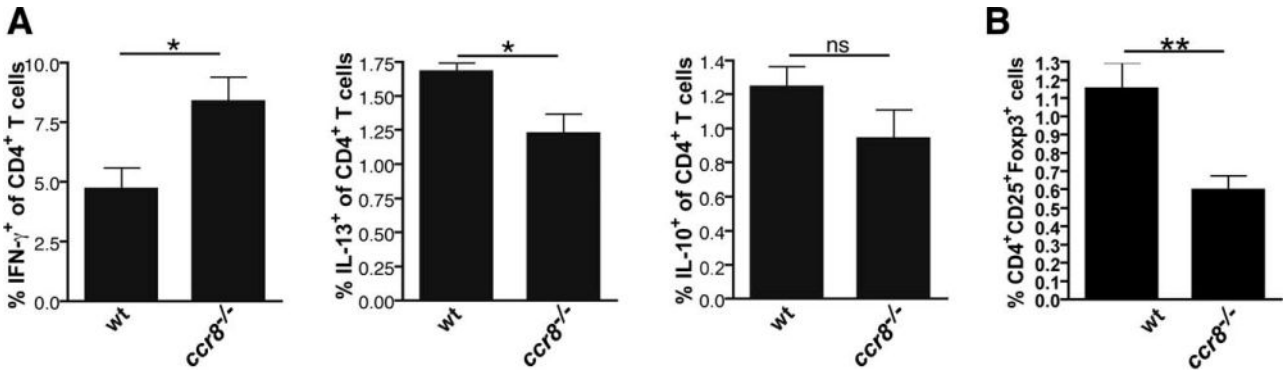

Fig. 5.

Preferential differentiation of Th1-type effector cells in livers of *ccr8*$^{-/-}$ mice. Liver-infiltrating helper T cells were analyzed regarding their activation and functional subtype after chronic CCl_4 challenge. (A) Intracellular cytokine staining of T cells isolated from inflamed liver tissue. $CD4^+$ T cells were phenotyped as described after restimulation with PMA/ionomycin for 4 hours in the presence of GolgiPlug (BD Biosciences, Franklin Lakes, NJ) and stained intracellularly after fixation and permeabilization to detect IFN-γ, IL-13, and IL-10. (B) Analysis of Tregs in WT and *ccr8*$^{-/-}$. T cells were stained extracellularly for CD4 and CD25 and after fixation and permeabilization intracellularly for Foxp3 to identify Tregs. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are presented as mean ± SEM. All results are representative of two independent experiments with 3 animals per group.

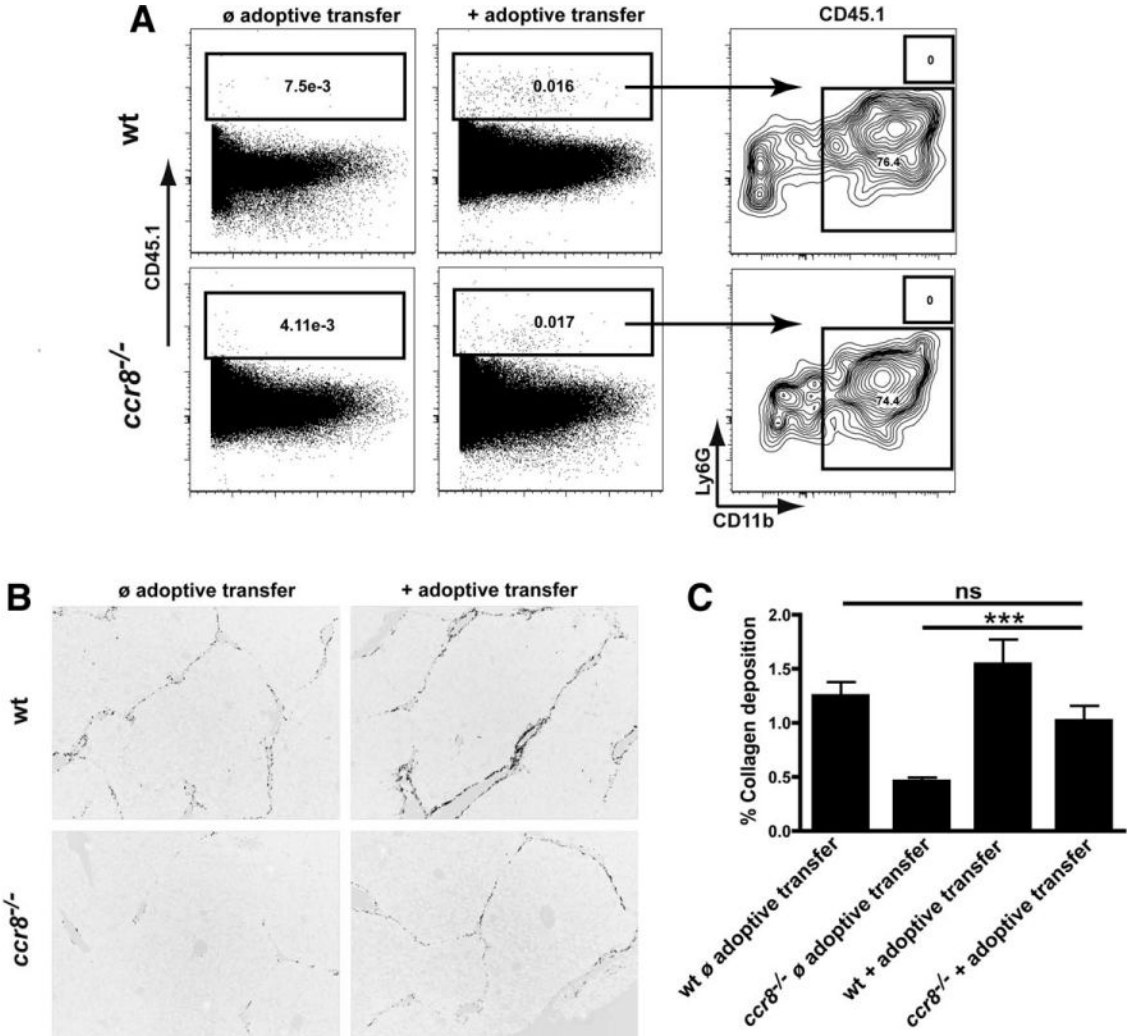

Fig. 6.

WT monocytes restore liver fibrosis in *ccr8*$^{-/-}$ mice after adoptive transfer during CCl4-mediated liver damage. WT and *ccr8*$^{-/-}$ mice were treated with CCl4 over 6 weeks, as described above, and were either injected weekly with 1×10^6 monocytes isolated from CD45.1 donor mice using CD115 MACS purification or left further untreated. (A) Flow cytometric analysis of CD45.1$^+$ cells isolated from the liver. Mice were sacrificed after 6 weeks of treatment with CCl_4 with or without additional monocyte transfer (72 hours before analysis). CD45.1$^+$ cells were further subcharacterized using Ly6G to identify neutrophils and CD11b do detect monocytes/macropages. (B) Sirius Red stainings from liver paraffin sections of WT and *ccr8*$^{-/-}$ mice challenged with CCl_4 and adoptively transferred WT monocytes. Collagen was visualized using polarization microscopy; pictures displayed are inverted and depicted as grayscale images. Magnification ×10. (C) Statistical analysis of (B). Four to six view fields per animal were counted and subsequently quantified by *in silico* morphometric analyis. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are presented as mean ± SEM. All results are representative of two independent experiments with 3 animals per group.