HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

C-C Motif Chemokine Receptor 9 Positive Macrophages Activate Hepatic Stellate Cells and Promote Liver Fibrosis in Mice

Po-sung Chu,[1] Nobuhiro Nakamoto,[1] Hirotoshi Ebinuma,[1] Shingo Usui,[1] Keita Saeki,[1] Atsuhiro Matsumoto,[1] Yohei Mikami,[1] Kazuo Sugiyama,[1] Kengo Tomita,[1,2] Takanori Kanai,[1] Hidetsugu Saito,[1,3] and Toshifumi Hibi[1]

Chemokine receptors mediate migration of immune cells into the liver, thereby promoting liver inflammation. C-C motif chemokine receptor (CCR) 9$^+$ macrophages are crucial in the pathogenesis of acute liver inflammation, but the role and underlying mechanisms of this macrophage subset in chronic liver injury and subsequent liver fibrosis are not fully understood. We confirmed that tumor necrosis factor alpha (TNF-α)-producing CCR9$^+$ macrophages accumulated during the initiation of carbon tetrachloride (CCl_4)-induced liver injury, and CCR9 deficiency attenuated the degree of liver damage. Accumulation of CCR9$^+$ macrophages persisted prominently during the process of liver fibrosis induced by repetitive CCl_4 or thioacetamide (TAA)/leptin administration. Increased CCR9 expression was also found on activated hepatic stellate cells (HSCs). Importantly, experimental liver fibrosis was significantly ameliorated in *CCR9*$^{-/-}$ mice compared with wild-type (WT) mice, assessed by α-smooth muscle actin (α-SMA) immunostain, Sirius red staining, and messenger RNA (mRNA) expression levels of *α-SMA, collagen 1α1, transforming growth factor (TGF)-β1*, and *tissue inhibitor of metalloproteinase (TIMP)-1*. Accumulated CD11b$^+$ macrophages in CCl_4-treated WT mice showed marked increases in *TNF, NO synthase-2*, and *TGF-β1* mRNA expression compared with *CCR9*$^{-/-}$ mice, implying proinflammatory and profibrogenic properties. Hepatic CD11b$^+$ macrophages from CCl_4-treated WT mice (i.e., CCR9$^+$ macrophages), but not CD8$^+$ T lymphocytes or non-CD11b$^+$ cells, significantly activated HSCs *in vitro* compared with those from *CCR9*$^{-/-}$ mice. TNF-α or TGF-β1 antagonism attenuated CCR9$^+$ macrophage-induced HSC activation. Furthermore, C-C motif chemokine ligand (CCL) 25 mediated migration and, to a lesser extent, activation of HSCs *in vitro*. *Conclusion*: Accumulated CD11b$^+$ macrophages are critical for activating HSCs through the CCR9/CCL25 axis and therefore promote liver fibrosis. CCR9 antagonism might be a novel therapeutic target for liver fibrosis. (HEPATOLOGY 2013;58:337-350)

Cirrhosis, the endstage of hepatic fibrosis subsequent to chronic liver inflammation, is one of the leading causes of morbidity and mortality worldwide, and is caused by various etiologies including viral, metabolic, autoimmune, and cholestatic diseases.[1,2] Progression of liver fibrosis occurs due to repeated hepatic wound healing and regeneration, with a prominent feature being recruitment of

Abbreviations: ALT, alanine aminotransferase; CCL, C-C motif chemokine ligand; CCl_4, carbon tetrachloride; CCR, C-C motif chemokine receptor; CD, cluster of differentiation; DC(s), dendritic cell(s); H&E, hematoxylin and eosin; HSC(s), hepatic stellate cell(s); IFN, interferon; LSEC(s), liver sinusoid endothelial cell(s); MACS, magnet-associated bead sorting; MFI, mean fluorescence intensity; Ly6C, lymphocyte antigen 6C; mAb(s), monoclonal antibodies; NK, natural killer cells; NKT, natural killer T cells; NOS, NO synthase; pDC(s), plasmacytoid dendritic cell(s); PD-L2, programmed death-ligand 2; PDGF, platelet-derived growth factor; qPCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SMA, smooth muscle actin; TAA, thioacetamide; TECK, thymus-expressed chemokine; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; Th(s), T-helper cell(s); TNF, tumor necrosis factor; TNFRsf1a, TNF receptor super family 1a; WT, wild type.

From the [1]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; [2]Division of Gastroenterology and Hepatology, Department of Internal Medicine, National Defense Medical College, Saitama, Japan; [3]Division of Pharmacotherapeutics, Keio University School of Pharmacy, Tokyo, Japan.

Received October 17, 2012; accepted February 19, 2013.

Supported in part by grants-in-aid for Scientific Research from the Japan Ministry of Education, Culture, Sports, Science and Technology and Keio Gijuku Academic Development Funds.

immunomodulatory cells including monocytes, lymphocytes, and hepatic stellate cells (HSCs) to the site of liver injury.[3,4] Hepatic resident macrophages (i.e., Kupffer cells) and recruited inflammatory monocytes release factors including tumor necrosis factor alpha (TNF-α), platelet-derived growth factor (PDGF), reactive oxygen species (ROS), and transforming growth factor beta (TGF-β) to activate HSCs.[3,5,6] These monocytes/macrophages can influence liver fibrogenesis.[7,8]

Several chemokine/chemokine receptor pathways have been reported to be crucial for the occurrence of liver fibrosis. C-C motif chemokine receptor (CCR)1, CCR2, CCR5, and CCR8 mediate monocyte and/or simultaneous HSC recruitment and promote liver fibrosis.[9-11] In contrast, C-X3-C motif chemokine receptor (CX3CR)1 mediates antifibrotic processes.[12-14]

Thymus-expressed chemokine (TECK, later designated CCL25) was identified as a novel chemokine in 1997, and is chemotactic for lymphocytes, dendritic cells (DCs), and activated macrophages.[15] Together with CCR9,[16] the CCR9/CCL25 chemokine axis has been a focus of studies investigating its functions on lymphocytes and DCs in terms of maturation,[15,17] gut-homing characteristics,[18,19] and the maintenance of immunological tolerance.[20] In humans, CCR9-expressing lymphocytes may be involved in the pathogenesis of Crohn's disease[21] and primary sclerosing cholangitis.[22] The immunological roles of CCR9-expressing monocytes/macrophages were not elucidated until recently, when we demonstrated an essential role of $CCR9^+$ macrophages to establish acute liver injury in multiple murine models.[23] In the present study, using murine experimental models of liver fibrosis, we demonstrated an essential role of the CCR9/CCL25 axis in chronic liver inflammation and liver fibrogenesis, and examined how $CCR9^+$ macrophages activate HSCs and promote liver fibrosis.

Materials and Methods

Mice. C57BL/6 wild-type (WT) mice were purchased from Japan CLEA (Tokyo, Japan), and *TNFRsf1a*$^{-/-}$ mice (C57BL/6 background) from Jackson Laboratory (Bar Harbor, ME). *CCR9*$^{-/-}$ mice (C57BL/6 background) were previously described.[24] Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Keio University School of Medicine. Experiments were performed with age- and sex-matched mice at 8-12 weeks of age. All experiments were approved by the regional Animal Study Committees and were performed according to institutional guidelines.

Induction of Acute and Chronic Liver Injury and Liver Fibrosis. To induce acute liver injury, mice received an intraperitoneal (IP) injection of 0.6 mL/kg body weight of carbon tetrachloride (CCl_4, Sigma Aldrich, St. Louis, MO) mixed with olive oil, and were sacrificed at 24 hours after IP. To induce liver fibrosis, 0.2 mL/kg CCl_4 was injected three times weekly for 6 weeks. As a second model, 200 mg/kg thioacetamide (TAA; Sigma Aldrich) plus 1 mg/kg leptin (R&D Systems, Minneapolis, MN) was injected three times weekly for 4 weeks as previously described.[25] Mice were sacrificed 24 hours after the last administration.

Assessment of Liver Injury and Liver Fibrosis. Livers were removed, fixed in 10% formalin, and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E), or with silver (Ag) for reticular fibers. Serum alanine aminotransferase (ALT) levels were measured using a lactate dehydrogenase (LDH)-UV kinetic method (SRL, Tokyo, Japan). Hepatic collagen contents were evaluated by Sirius red staining of paraffin-embedded sections. Sirius red-positive areas were quantified in five nonoverlapping random fields (magnification 100×) on each slide using the imaging software ImageJ (NIH, Bethesda, MD).

Isolation of Liver Nonparenchymal Mononuclear Cells. Liver nonparenchymal mononuclear cells were isolated from the liver as previously described.[26] Details are described in the Supporting Methods.

Flow Cytometry. After blocking with anti-FcR (CD16/32, BD Pharmingen, Franklin Lakes, NJ) for 20 minutes, cells were incubated with specific fluorescence-labeled monoclonal antibodies (mAbs) at 4°C for 30 minutes.[27] Antimouse mAbs used are listed in Supporting Table 1. Irrelevant antirat isotype antibodies (BD Pharmingen) were used to assess background

Address reprint requests to: Nobuhiro Nakamoto or Toshifumi Hibi, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582 Japan. E-mail: nobuhiro@z2.keio.jp or thibi@z5.keio.jp; fax: +81-3-3353-6247.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.26351
Potential conflict of interest: Nothing to report.
Additional Supporting Information may be found in the online version of this article.

fluorescence. Cells for intracellular staining were pretreated with brefeldin A (10 μg/mL) and permeabilized. Stained cells were analyzed using FACS Canto II (Becton Dickinson, NJ), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Dual-Color Immunofluorescence and Immunohistochemistry. Liver specimens were fixed with 10% formalin, paraffinized, and sectioned to 6 μm thickness, then deparaffinized and rehydrated. Antigen retrieval was obtained by boiling in 10 nM citrate buffer solution. After blocking with 20% goat serum, sections were embedded with primary antibodies against CCR9 (Abcam, ab1662, Cambridge, UK) and alpha smooth-muscle actin (α-SMA) (DakoCytomation, clone 1A4, Carpinteria, CA) or CCR9 and F4/80 (eBioscience, clone BM8, San Diego, CA) overnight. Sections were then stained with secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 568 (Life Technologies, Carlsbad, CA), and nuclei counterstained with DAPI (Vector Laboratories, Burlingame, CA). Immunohistochemistry was performed with mAb to α-SMA (DakoCytomation) using an M.O.M. kit (Vector Laboratories).[9]

Real-Time Quantitative Polymerase Chain Reaction (qPCR) and Reverse Transcription. Total RNA was extracted from liver homogenates or cultured cells using Trizol reagent (Gibco-BRL, Grand Island, NY). Complementary DNA was synthesized from 100 ng of total RNA by reverse transcription. PCRs were performed using AmpliTaq Gold Fast PCR MasterMix (Applied Biosystems, Foster City, CA) and the predesigned primers listed in Supporting Table 2. To quantify the products, real-time PCR was performed using TaqMan Universal Master Mix and StepOne Plus systems (Applied Biosystems). The level of target gene expression was normalized to β-actin expression in each sample.

Isolation of Liver Sinusoid Endothelial Cells (LSEC). LSECs were isolated as previously described.[28] Details are described in the Supporting Methods.

HSC Coculture Experiments. Isolation of HSCs and information regarding coculture or treatment are described in the Supporting Methods. The cells were cocultured for 24 hours and HSCs were harvested from a plate using 0.25% EDTA trypsin, after macrophages or other cocultured cells were washed away. Purity over 97% of HSCs was confirmed by flow cytometry. HSC RNA was isolated for qPCR as described above.

Migration Assay. Cell-migration assays were performed using 8 μm-pore 96-well Transwell plates (Corning, Corning, NY). Serum-starved HSCs or isolated hepatic $CD11b^+$ cells from WT or *CCR9*$^{-/-}$ mice were placed in the upper chamber and exposed to CCL25 (R&D Systems, no. O35903.1) at the indicated concentrations in the lower chamber. After 48 hours of incubation at 37°C, cells that migrated to the lower chamber were counted.

Statistics. Data were analyzed using JMP9 (SAS Institute, Cary, NC) and expressed as mean ± standard error of the mean (SEM). The Mann-Whitney *U* test, the unpaired Student *t* test, and the Kruskal-Wallis test were used to assess the differences between groups, as appropriate. Differences were considered statistically significant when $P < 0.05$.

Results

Initiation of CCl_4-Induced Liver Injury and Inflammatory Macrophage Infiltration Is CCR9-Dependent. To elucidate how CCR9-expressing macrophages infiltrate and affect livers during the initiation of injury, a single IP administration of CCl_4 to WT mice was used to induce acute liver injury. The number, frequency, and phenotype of infiltrating mononuclear cells were assessed by flow cytometry. The number of $CD11b^+CCR9^+$ macrophages increased significantly 24 hours after CCl_4 administration compared with controls (Fig. 1A). Infiltrating $CD11b^+CCR9^+$ macrophages were positive for F4/80 and expressed $Ly6C^{hi}$, a marker of inflammatory macrophages recruited to inflamed sites (Fig. 1B).[29] Compared with CCR9-negative cells, $CD11b^+CCR9^+$ macrophages expressed higher levels of CD80 and CD86, and produced more TNF-α (Fig. 1B,C), suggesting an activated phenotype. Importantly, CCl_4-treated *CCR9*$^{-/-}$ mice showed less periportal necrosis and less leukocyte infiltration, as well as significantly lower serum ALT levels (Fig. 1D). The level of *TNF* mRNA in the whole liver of CCl_4-treated *CCR9*$^{-/-}$ mice was significantly lower than in WT mice (Fig. 1E). These results suggested a crucial role for CCR9 in the initiation of CCl_4-induced liver injury.

CCR9 Critically Mediates Macrophage Accumulation During Liver Fibrogenesis. Because accumulated $CCR9^+$ macrophages are crucial for the initiation of CCl_4-induced liver injury, they may also regulate intrahepatic processes in response to persistent liver injury, which leads to liver fibrosis. Therefore, we examined the role of $CCR9^+$ macrophages in murine liver fibrosis models. Repetitive administration of CCl_4 to WT mice three times per week for 6 weeks resulted in overt liver fibrosis (Fig. 2A) and a significant increase in $CD11b^+CCR9^+$ macrophage accumulation in

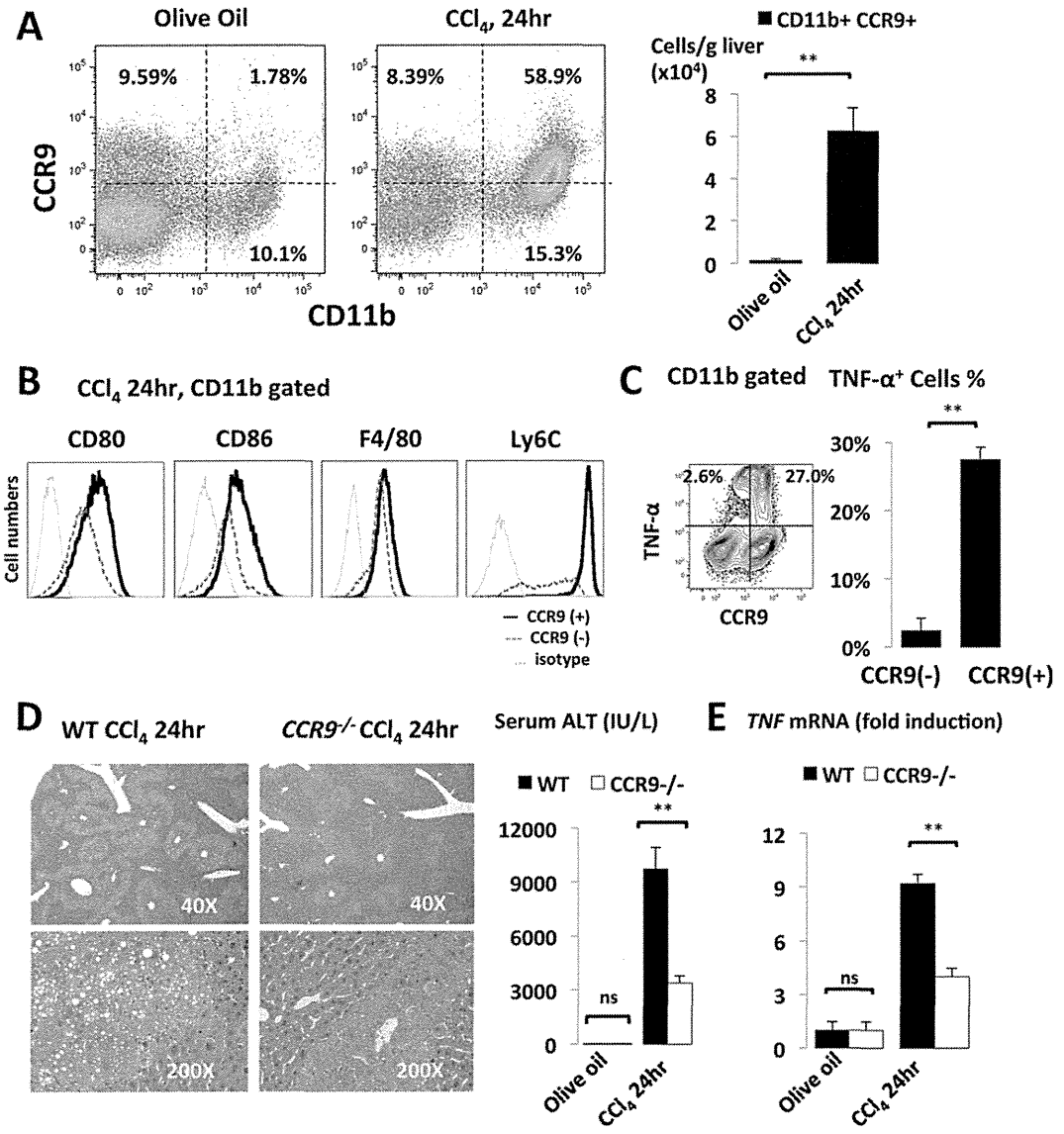

Fig. 1. CCR9$^+$ macrophages accumulate following acute liver injury. Mice were IP injected with CCl_4 (0.6 mL/kg) to induce acute liver injury and were sacrificed 24 hours later. (A) Left: Representative CD11b and CCR9 staining on total isolated mononuclear liver cells from olive oil- or CCl_4-treated WT mice. Right: Absolute cell numbers of CD11b$^+$CCR9$^+$ macrophages ($\times 10^4$ per gram of liver). Data show mean ± SEM (n = 4). (B) Surface CD80, CD86, F4/80, and Ly6C expression in CCR9 positive (black lines) and CCR9 negative (dashed lines) subsets of CD11b$^+$ macrophages. Isotype controls are shown as gray dotted lines. Data are representative of four independent experiments. (C) Left: Representative intracellular TNF-α and surface CCR9 expression in CD11b$^+$ macrophages. Right: Mean percentage of TNF-α$^+$ cells in CCR9$^-$CD11b$^+$ and CCR9$^+$CD11b$^+$ macrophages. Data show mean ± SEM (n = 3). (D) Left: Representative photomicrographs of H&E-stained sections of the liver (upper; 40×, lower; 200× magnification). Right: Serum ALT levels of olive oil- or CCl_4-treated WT and *CCR9*$^{-/-}$ mice. Data show mean ± SEM (n = 4). (E) Expression of *TNF* mRNA in whole liver cells. Data show mean ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$. ns, not significant.

fibrotic livers (Fig. 2B). Compared with CCR9-negative cells, the phenotypes of accumulated CD11b$^+$CCR9$^+$ macrophages resembled those that infiltrated livers with acute injury, which were F4/80$^+$ and mostly Ly6C^{hi}, with high expression levels of CD80, CD86 (Fig. 2C) and TNF-α (Fig. 2D). The levels of *TNF* mRNA of whole liver were significantly increased in fibrotic livers compared with controls (Fig. 2E). In the TAA/leptin liver fibrosis model, similar results were observed (Supporting Fig. 1A,B).

CCR9$^+$ Macrophages and CCR9$^+$ HSCs Colocalize During Liver Fibrosis. Flow cytometry of cells from nonfibrotic livers showed CCR9 expression was detected mainly in a subset of plasmacytoid dendritic cells (pDCs, defined by Siglec H$^+$), with some expression in CD11b$^+$ macrophages or CD3$^+$CD8$^+$ T lymphocytes. Little CCR9 expression was detected in CD3$^+$CD4$^+$ T lymphocytes. In contrast to the significant increase of CCR9$^+$ macrophages in persistent liver injury and liver fibrosis, pDCs and CD8$^+$ T

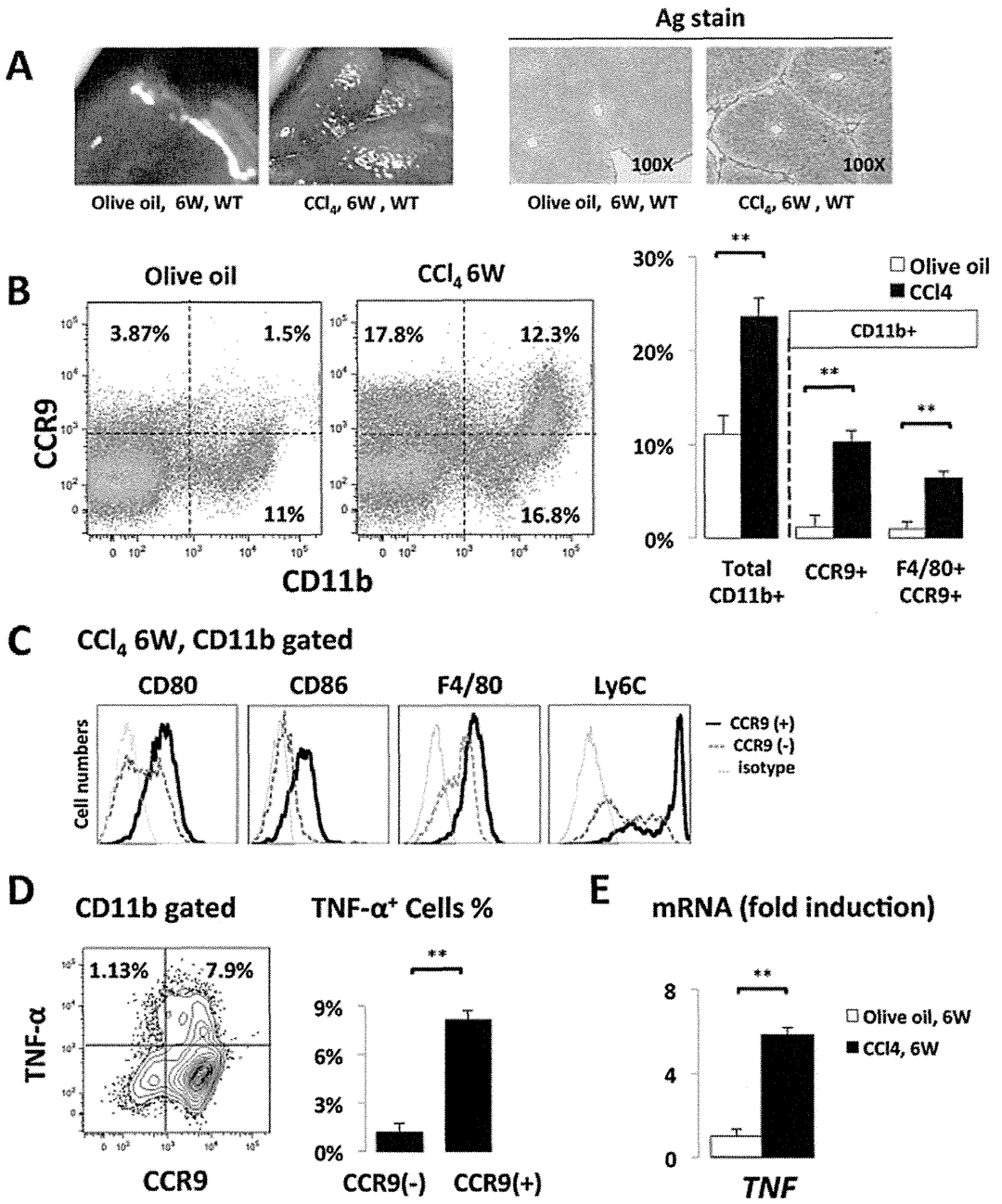

Fig. 2. CCR9 mediates macrophage accumulation during liver fibrogenesis. WT mice were injected IP with CCl_4 (0.2 mL/kg) three times per week for 6 weeks to induce liver fibrosis. (A) Left: Representative gross photograph of the surface of WT livers treated with olive oil alone or CCl_4. Right: Ag stain of WT livers (100× magnification) treated with olive oil or CCl_4. (B) Left: Representative CD11b and CCR9 staining on total isolated mononuclear liver cells from olive oil- or CCl_4-treated WT mice. Right: Mean percentages of isolated liver mononuclear cells of total $CD11b^+$ cells, $CD11b^+CCR9^+$ cells and $CD11b^+CCR9^+$ $F4/80^+$ cells. Data show mean ± SEM (n = 4). (C) Surface CD80, CD86, F4/80, and Ly6C expression in CCR9 positive (black lines) and CCR9 negative (dashed lines) subsets of $CD11b^+$ macrophages. Isotype controls are shown as gray dotted lines. Data are representative of four independent experiments. (D) Left: Representative intracellular TNF-α and surface CCR9 expression in $CD11b^+$ macrophages. Right: Mean percentage of TNF-α^+ cells in $CCR9^-CD11b^+$ and $CCR9^+CD11b^+$ macrophages. Data show mean ± SEM (n = 3). (E) Expression of *TNF* mRNA from whole liver cells isolated from olive oil- or CCl_4-treated WT mice. Data show mean ± SEM (n = 4). $^*P < 0.05$, $^{**}P < 0.01$.

lymphocytes were relatively unchanged in frequency compared with controls (Fig. 2B; Supporting Fig. 2). Comparison of the ratio of increased mRNA expression from cell fractions including hepatic immune cells, HSCs, LSECs, and hepatocytes between CCl_4- and olive oil-treated livers demonstrated a significant up-regulation in *CCR9* mRNA only in macrophages and HSCs, and a significant up-regulation of *CCL25* mRNA only in activated LSECs (Fig. 3A). Activation of LESCs was implicated by the simultaneous increase

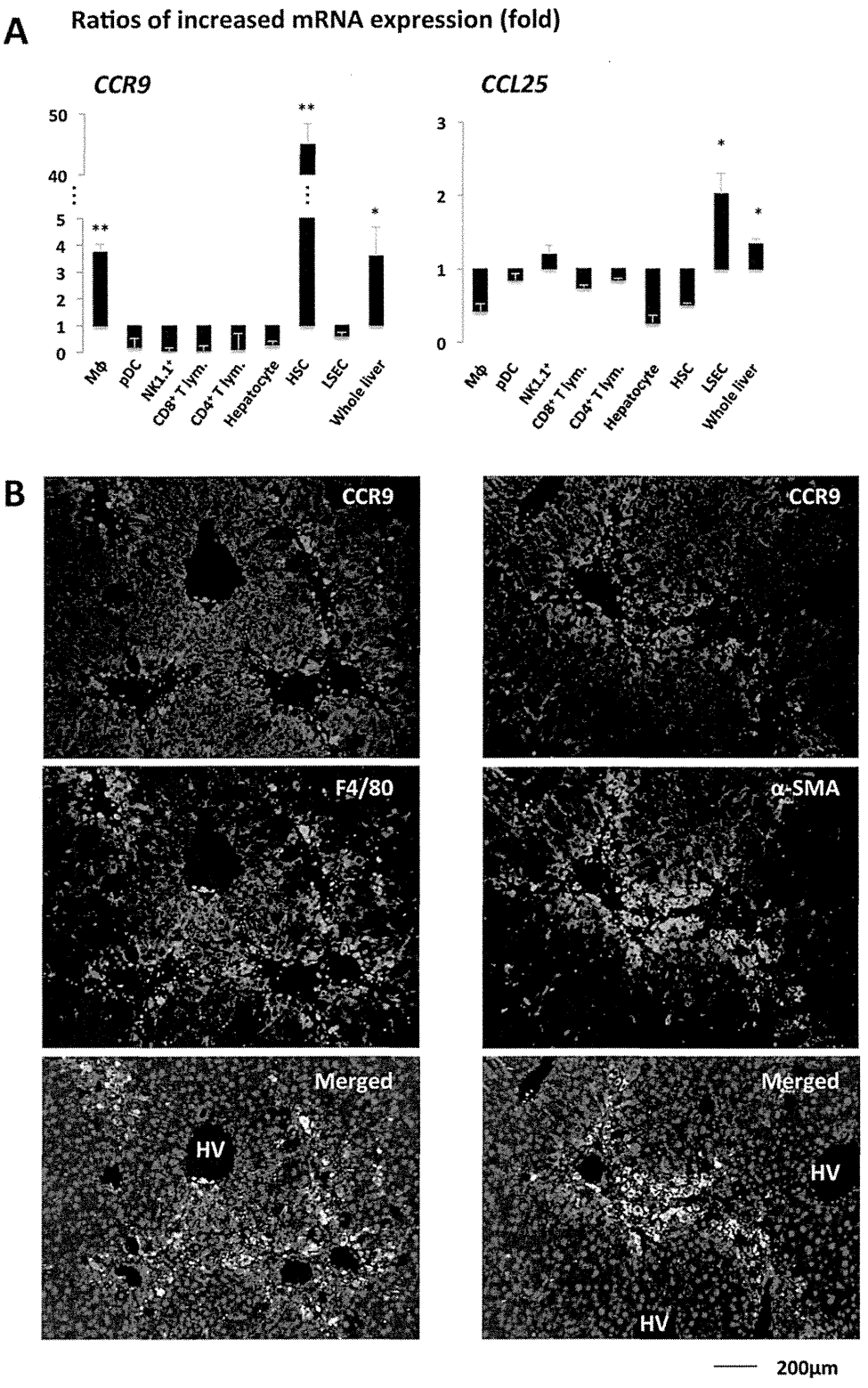

Fig. 3. CCR9 expression on macrophages and hepatic stellate cells. WT mice were injected IP with CCl_4 (0.2 mL/kg) three times per week for 6 weeks to induce liver fibrosis. (A) Ratios of increased expression of mRNAs of *CCR9* (left) and *CCL25* (right) of cell fractions including macrophages (Mψ, $CD11b^+$), pDCs (Siglec H^+), NK/NKT cells ($NK1.1^+$), CD8 lymphocytes, CD4 lymphocytes, hepatocytes, HSCs, LSECs, and whole liver cells isolated from olive oil- or CCl_4-treated WT mice. Expression of mRNAs from the olive oil-treated group was used as controls in each fraction. Data show mean ± SEM ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$. (B) Liver specimens were fixed with 10% formalin, paraffinized, and sectioned to 6 μm thickness, then deparaffinized and rehydrated, and then stained with primary and secondary antibodies for CCR9 (red) and F4/80 (green, marker for macrophages), or CCR9 and α-SMA (green, marker for hepatic stellate cells, HSCs). Merged coimmunofluorescence is indicated as yellow. Nuclei were counterstained with DAPI (blue). Representative photomicrographs are shown (100× magnification). HV indicates hepatic vein.

of *ICAM-1* and *VCAM-1* mRNAs (Supporting Fig. 3). These results suggest that CCL25-expressing LSECs in livers, as suggested in previous study,[22] might mediate the accumulation of $CCR9^+$ macrophages, as well as HSCs during liver fibrosis, in persistent liver injury. We further analyzed fibrotic livers, caused by repetitive administration of CCl_4, with dual-color immunofluorescence staining for CCR9 and F4/80, or CCR9 and α-SMA. This confirmed that $CCR9^+$ HSCs colocalized with accumulated $CCR9^+$ macrophages around periportal areas during liver fibrosis (Fig. 3B).

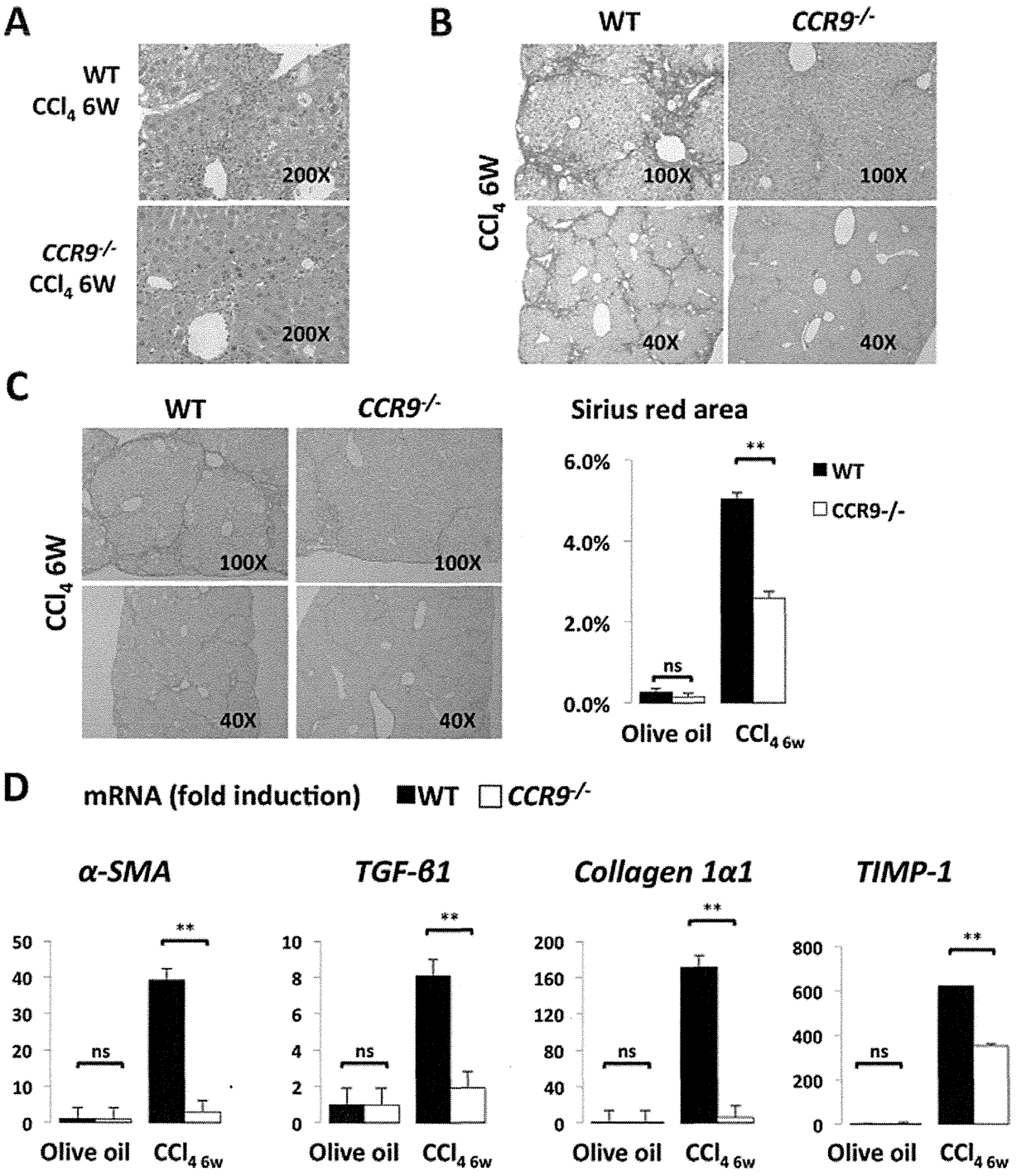

Fig. 4. CCR9 deficiency inhibits liver fibrosis after CCl_4 treatment. WT and $CCR9^{-/-}$ mice were injected IP with CCl_4 (0.2 mL/kg) three times per week for 6 weeks to induce liver fibrosis. (A) Representative photomicrographs of H&E-stained sections of the liver (200× magnification) from CCl_4-treated WT (upper) and $CCR9^{-/-}$ (lower) mice. (B) Representative photomicrographs of α-SMA immunohistochemistry stained sections of liver (upper: 100×; lower: 40× magnification) from CCl_4-treated WT (left) and $CCR9^{-/-}$ (right) mice. (C) Left: Representative photomicrographs of Sirius red-stained sections of liver (upper: 100×; lower: 40× magnification) from CCl_4-treated WT (left) and $CCR9^{-/-}$ (right) mice. Right: Mean percentage Sirius red positive areas measured from five randomly nonoverlapping fields (100× magnification). Data show mean ± SEM (n = 4). (D) Expression of mRNAs of *α-SMA*, *TGF-β1*, *collagen 1α1*, and *TIMP-1* from whole liver cells isolated from olive oil- or CCl_4-treated WT or $CCR9^{-/-}$ mice. Data show mean ± SEM (n = 4). $^{**}P < 0.01$. ns, not significant.

CCR9 Deficiency Attenuates Liver Fibrosis. To further investigate the functional roles of $CCR9^{+}$ macrophages in liver fibrogenesis, $CCR9^{-/-}$ and WT mice were repetitively administered CCl_4. Less periportal fibrosis and leukocytic infiltration in the liver of $CCR9^{-/-}$ mice was observed by H&E staining (Fig. 4A).

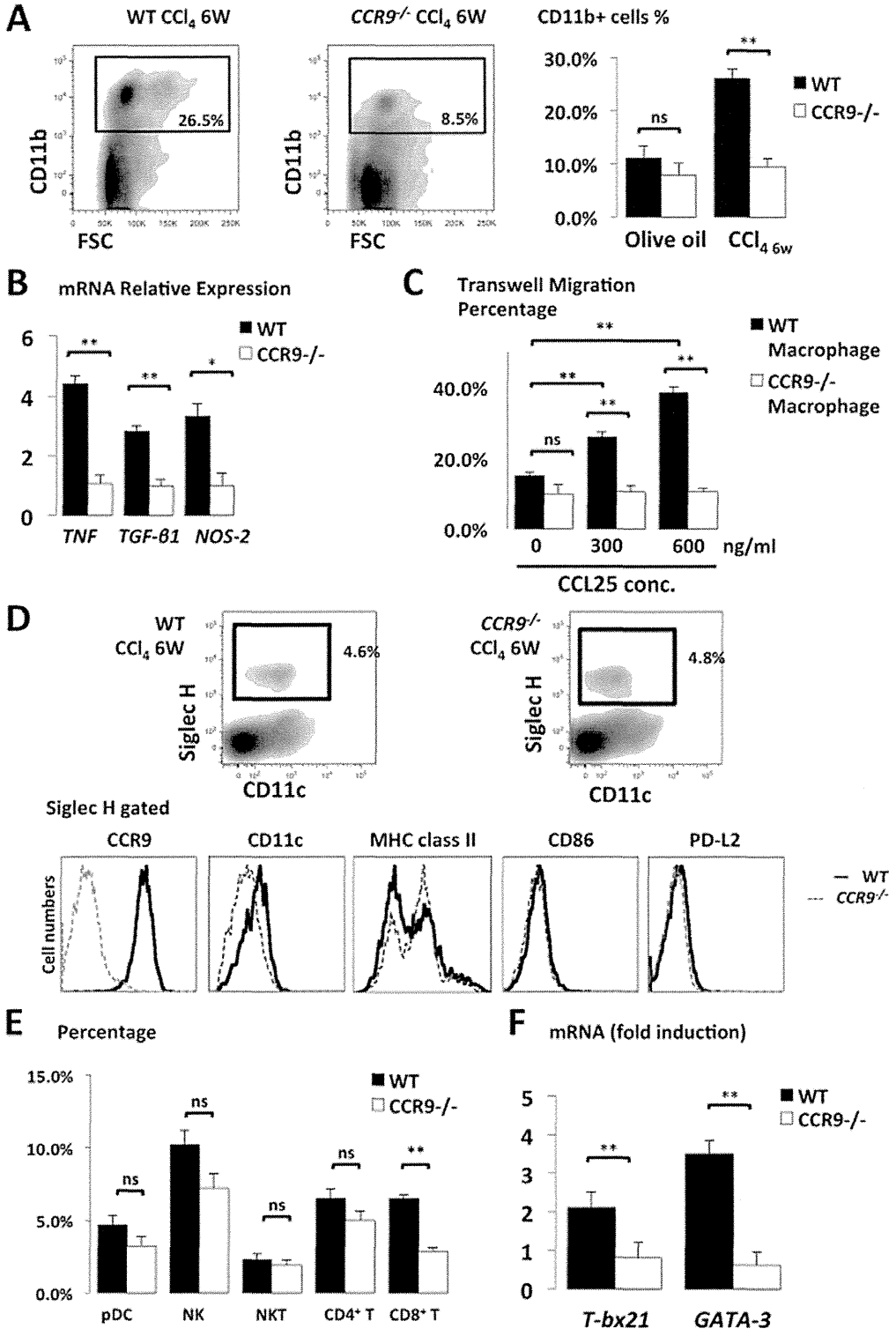

Fig. 5. CCR9 deficiency preferentially ameliorates macrophage infiltration. WT (black bars) and *CCR9*$^{-/-}$ (white bars) mice were injected IP with CCl_4 (0.2 mL/kg) three times per week for 6 weeks to induce liver fibrosis. Cells were stained for CD11b to identify macrophages, Siglec H for pDCs, NK1.1 for NK cells and NKT cells, CD3 for T lymphocytes, CD4 and CD8 for helper and cytotoxic T lymphocytes and were analyzed by flow cytometry. (A) Left: Representative CD11b and forward scatterplots of total isolated mononuclear liver cells from CCl_4-treated WT or *CCR9*$^{-/-}$ mice. Right: Mean percentages of isolated liver mononuclear cells from total CD11b$^+$ cells. Data show mean ± SEM (n = 4). (B) Expression of mRNAs of *TNF*, *TGF-β1* and *NOS-2* on isolated CD11b$^+$ cells from CCl_4-treated WT or *CCR9*$^{-/-}$ mice. Data show mean ± SEM (n = 4). (C) Chemotaxis of CD11b$^+$ macrophages from WT or *CCR9*$^{-/-}$ mice by CCL25 at different concentrations (0, 300, and 600 ng/mL) in 8-μm pore Transwell membranes. Data show mean ± SEM (n = 3). (D) Upper: Representative CD11c and Siglec H$^+$ staining on total isolated mononuclear liver cells from CCl_4-treated WT or *CCR9*$^{-/-}$ mice. Lower: Surface CCR9, CD11c, MHC class II, CD86 and PD-L2 expression in Siglec H$^+$ gated pDCs from CCl_4-treated WT (black lines) and *CCR9*$^{-/-}$ (dashed lines) livers. Data are representative of four independent experiments. (E) Mean percentages of isolated liver mononuclear cells of pDCs, NK cells, NKT cells, CD4$^+$, and CD8$^+$ T lymphocytes. Data show mean ± SEM (n = 4). (F) Expression of mRNAs of *T-bx21* and *GATA-3* from whole liver cells isolated from CCl_4-treated WT (black bars) or *CCR9*$^{-/-}$ (white bars) mice. Data show mean ± SEM (n = 4). *$P < 0.05$, **$P < 0.01$. ns, not significant.

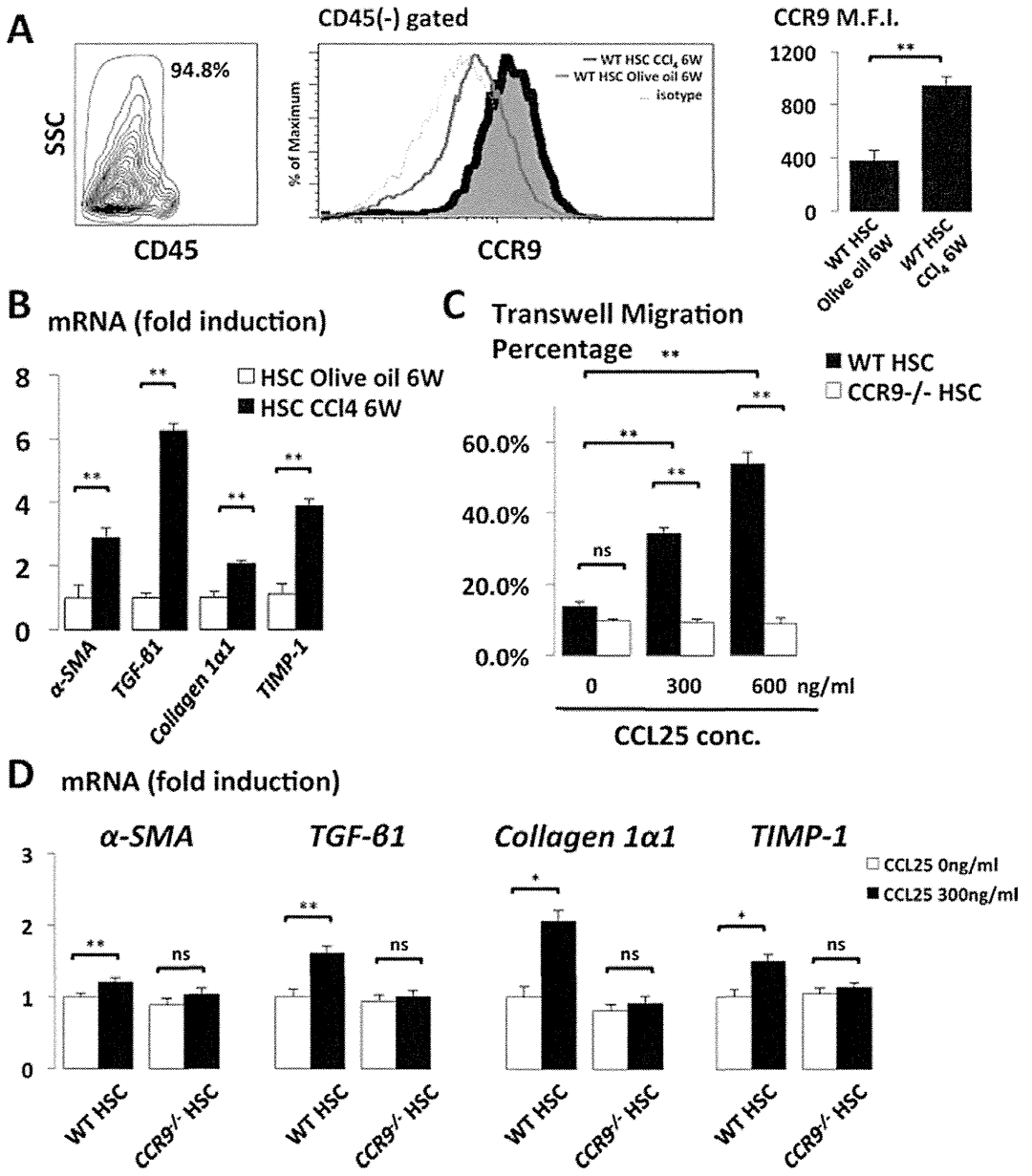

Fig. 6. CCL25-dependent migration of HSCs. (A) Left: After exclusion of $CD45^+$ cells, CCR9 expression was analyzed in isolated HSCs from olive oil-treated livers and CCl_4-treated livers by flow cytometry. Representative histogram (middle) and CCR9 MFI (right) for HSCs from the two groups are shown. Data show mean ± SEM (n = 4). (B) WT HSCs from olive oil- (white bars) and CCl_4-treated livers (black bars) were isolated. Messenger RNA levels of *α-SMA, TGF-β1, collagen 1α1,* and *TIMP-1* were measured by qPCR. Data show mean ± SEM (n = 4). (C) Chemotaxis of starved HSCs from WT (black bars) or *CCR9*$^{-/-}$ (white bars) mice by CCL25 at different concentrations (0, 300, and 600 ng/mL) in 8-μm pore transwell membranes. Data show mean ± SEM (n = 3). (D) Quiescent HSCs were isolated from WT or *CCR9*$^{-/-}$ livers, and cultured with CCL25 (300 ng/mL, black bars) or without CCL25 (white bars) for 48 hours. Messenger RNA levels of *α-SMA, TGF-β1, collagen 1α1,* and *TIMP-1* were measured by qPCR. Data show mean ± SEM (n = 4). *$P < 0.05$, **$P < 0.01$. ns, not significant.

Markedly attenuated liver fibrosis in *CCR9*$^{-/-}$ mice was demonstrated by α-SMA immunohistochemical staining (Fig. 4B), and quantitative analyses of Sirius red staining (Fig. 4C). CCR9 deficiency also resulted in significantly reduced mRNA expression of fibrosis markers including *α-SMA, TGF-β1, collagen 1α1,* and *tissue inhibitor of metalloproteinase 1* (*TIMP-1*) (Fig. 4D). In the TAA/leptin liver fibrosis model, similar results were observed (Supporting Fig. 1C).

CCR9 Deficiency Prominently Ameliorates Macrophage Infiltration. Because of the protective role of CCR9 deficiency in murine models of liver fibrosis, we hypothesized that CCR9 was critical for controlling immune cell infiltration into the liver upon chronic liver injury and fibrosis. First, we noticed that the

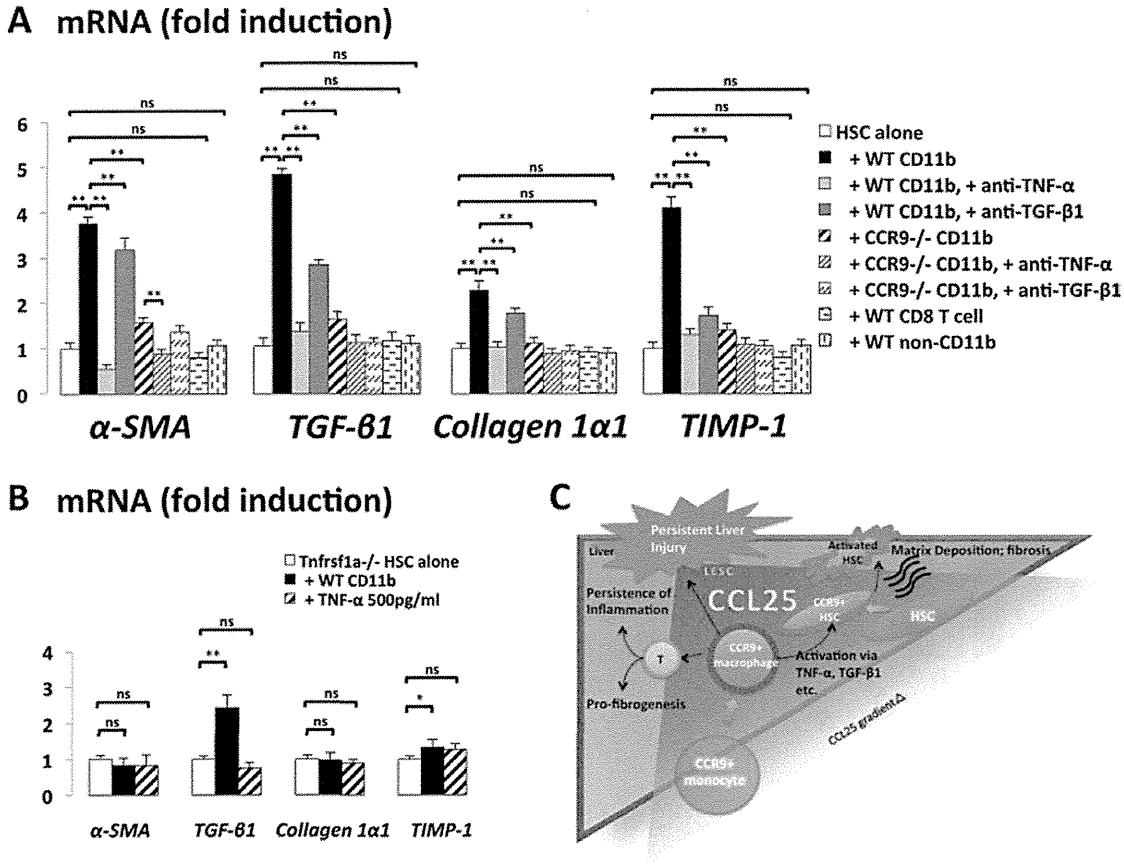

Fig. 7. HSC coculture experiments and summary scheme. (A) Twenty-four hours after IP administration of CCl_4, $CD11b^+$ macrophages or $CD8^+$ T cells from WT and *CCR9*$^{-/-}$ livers were isolated from perfused livers and cocultured with quiescent WT HSCs. Anti-TNF-α or anti-TGF-β1 antibody at a final concentration of 1 μg/mL was added to neutralize TNF-α or TGF-β1. After 24 hours of coculture, immune cells were washed away and HSCs were harvested at a purity greater than 97%. mRNA levels of *α-SMA, TGF-β1, collagen 1α1*, and *TIMP-1* in HSCs were evaluated. Results are representative of two independent experiments with three animals per group. $^{**}P < 0.01$. ns, not significant. (B) Twenty-four hours after IP administration of CCl_4, $CD11b^+$ macrophages from WT livers were isolated from perfused livers, then cocultured with quiescent HSCs isolated from *TNFRsf1a*$^{-/-}$ livers. TNF-α at a final concentration of 500 pg/mL was added as a control. After 24 hours of coculture, m RNA levels of *α-SMA, TGF-β1, collagen 1α1*, and *TIMP-1* in *TNFRsf1a*$^{-/-}$ HSCs were evaluated. Results are representative of two independent experiments with three animals per group. $^{**}P < 0.01$. ns, not significant. (C) Scheme summarizing the results. Under persistent liver injury, intrahepatic CCL25 levels are elevated. $CCR9^+$ macrophages and $CCR9^+$ HSCs undergo chemotaxis along the CCL25 concentration gradient to the liver. $CCR9^+$ macrophages activate HSCs via mediators including TNF-α and TGF-β1, and promote liver fibrosis.

frequency of infiltrating $CD11b^+$ macrophages significantly decreased in *CCR9*$^{-/-}$ livers compared with WT livers following repetitive CCl_4 injection (Fig. 5A). Isolated $CD11b^+$ macrophages from WT mice showed significantly higher *TNF, NO synthase (NOS)-2*, and *TGF-β1* mRNA expression compared with *CCR9*$^{-/-}$ mice (Fig. 5B). This indicated that accumulating $CCR9^+$ macrophages in chronic liver injury had both proinflammatory and profibrogenic phenotypes. Macrophages from WT fibrotic livers also showed significant concentration-dependent chemotaxis to CCL25 compared with *CCR9*$^{-/-}$ macrophages (Fig. 5C). In contrast, there was no significant difference in the frequency and phenotype of intrahepatic pDCs between WT and *CCR9*$^{-/-}$ mice under chronic CCl_4 administration (Fig. 5D,E). Notably, the frequency of $CD8^+$ cytotoxic T lymphocytes, but not $CD4^+$ helper T lymphocytes, was statistically lower in chronically injured *CCR9*$^{-/-}$ livers than in WT livers (Fig. 5E). This phenomenon might be due to the observation that some $CD8^+$ T lymphocytes, but not $CD4^+$ T lymphocytes, expressed CCR9 in nonfibrotic livers, as described above. The level of *IFN-γ* mRNA was not significantly different between WT and *CCR9*$^{-/-}$ mice with persistent liver injury (Supporting Fig. 4). The levels of *T-bx21* and *GATA-3* mRNA, representative of Th1 and Th2 transcription factors, respectively, were significantly increased in fibrotic livers from WT mice compared with *CCR9*$^{-/-}$ mice (Fig. 5F), suggesting that CCR9 might play roles in the maintenance of both Th1-related inflammation and Th2-related fibrogenesis.

CCL25 Mediates Migration of HSCs. As the up-regulation of *CCR9* mRNA in HSCs and the existence of $CCR9^+$ HSCs were confirmed in fibrotic livers (Fig. 3), this suggested that HSCs might be affected by the CCL25/CCR9 axis. By flow cytometry, significantly increased CCR9 expression was noticed in HSCs from fibrotic livers (Fig. 6A). Importantly, CCR9-expressing HSCs isolated from CCl_4-treated WT mice had activated and profibrogenic phenotypes, as shown by the increase of *α-SMA, TGF-β1, collagen 1α1*, and *TIMP-1* mRNA expression (Fig. 6B). A transwell migration assay demonstrated that WT HSCs had significant potential to migrate along CCL25 gradients compared with HSCs from $CCR9^{-/-}$ mice (Fig. 6C). After 48 hours of culture with 300 ng/mL CCL25, fibrosis marker mRNAs increased in HSCs from WT mice compared with HSCs from $CCR9^{-/-}$ mice (Fig. 6D), but to a lesser extent compared with activated HSCs *in vivo* (Fig. 6B).

$CCR9^+$ Macrophages Are Crucial for Activating HSCs and Inducing Hepatic Collagen Deposition. Accordingly, accumulation of $CD11b^+CCR9^+$ macrophages might be influential upon chronic liver injury and subsequent hepatic fibrosis. To examine the interactions of HSCs that produce the majority of collagen leading to liver fibrosis,[3] hepatic $CD11b^+$ macrophages were isolated from CCl_4-injected WT or $CCR9^{-/-}$ mice and cocultured with quiescent HSCs isolated from WT mice. The mRNA levels of fibrosis markers, including *α-SMA, TGF-β1, collagen 1α1*, and *TIMP-1*, were significantly higher in HSCs cocultured with $CD11b^+$ macrophages from WT mice compared with those with $CD11b^+$ macrophages from $CCR9^{-/-}$ mice (Fig. 7A). TNF-α is a key factor of HSC activation.[3,25] Addition of anti-TNF-α antibodies significantly decreased the levels of fibrosis marker mRNAs in HSCs from each group. Furthermore, the neutralization of TGF-β1 caused decreased levels of fibrosis marker mRNAs in HSCs as well. This suggested both TNF-α and TGF-β released from $CCR9^+$ macrophages are important for HSCs activation. We also confirmed that fibrosis marker mRNAs in HSCs were not affected by $CD8^+$ T lymphocytes or other non-CD11b immune cells (Fig. 7A). To confirm the significance of TNF-α-mediated HSC activation by $CD11b^+CCR9^+$ macrophages, quiescent HSCs isolated from mice deficient for TNF receptor super family 1a ($TNFRsf1a^{-/-}$) were cocultured with WT $CD11b^+$ macrophages from fibrotic livers, or cultured with TNF-α at 500 pg/mL, as TNF-α is known to activate HSCs through TNF receptor 1.[30] The addition of TNF-α did not activate $TNFRsf1a^{-/-}$ HSCs, and the degree of HSC activation evaluated by *α-SMA* and *collagen 1α1* mRNA expression when cocultured with WT $CD11b^+$ macrophages was significantly diminished, while that evaluated by *TGF-β1* and *TIMP-1* mRNA expression was only slightly elevated (Fig. 7B).

Discussion

Immune cells, including macrophages, play a critical role in the initiation of liver injury and subsequent liver fibrosis. Our previous work demonstrated that TNF-α-producing $CCR9^+$ macrophages initiate acute liver inflammation through interactions with Th1 cells.[23] The present study suggests a novel role for the CCR9/CCL25 axis in the process leading to persistent liver injury and subsequent liver fibrosis, as summarized in Fig. 7C.

Deficiency in CCR9 protected the liver from overt fibrosis in two different murine models, as well as causing decreased infiltration of macrophages into the liver. The crucial role of recruited macrophages has been emphasized previously in several experimental models.[3] Various chemokines are involved at different stages of inflammation and are highly tissue-specific.[14,29,31] In murine models of liver fibrosis, the essential roles of CCR2-dependent monocytes have been reported, and are similar to the monocytes recruited to livers with acute injury,[9] while CCR5-dependent fibrogenesis is prominent in the later process of fibrosis.[10] A possible role for the CCR9/CCL25 axis in the pathogenesis of experimental atherosclerosis, a chronic inflammatory state, was recently reported.[32] $CCR9^+$ macrophages in the synovial fluid may also play a role in the pathogenesis of rheumatoid arthritis, a chronic inflammatory disease.[33] These findings suggest a possible immunological role for $CCR9^+$ macrophages in chronic inflammation in various tissues. The present study is the first to demonstrate that $CCR9^+$ macrophages affect chronic inflammation and subsequent fibrosis in the liver.

It is important to clarify the relevance of the CCR9/CCL25 axis during the development of liver fibrosis in our model. First, we carefully evaluated the source of CCR9-positive cells by isolating each cell fraction in fibrotic livers and found that CCR9 expression was up-regulated only in macrophages and HSCs, together with the up-regulation of CCL25 in LSECs. Regarding the cellular location of CCR9, dual-color immunofluorescence analysis demonstrated the colocalization of CCR9 on macrophages and HSCs around

periportal areas where profound matrix deposition occurs in various liver fibrosis models.

Several observations support our hypothesis that $CCR9^+$ macrophages are key factors in processing wound healing and subsequent liver fibrosis. First, numbers of $CCR9^+CD11b^+$ macrophages with an activated phenotype and high TNF-α production dramatically increased in experimental fibrotic livers. Second, CCR9 deficiency resulted in reduced infiltration of $CD11b^+$ macrophages to the liver and subsequent attenuation of fibrosis. Third, and most important, *in vitro* coculture analysis revealed that $CD11b^+$ macrophages from CCl_4-treated WT mice (i.e., the existence of $CCR9^+$ macrophages), but not $CD11b^+$ macrophages from CCl_4-treated *CCR9*$^{-/-}$ mice ($CCR9^-$ macrophages) have the potential to activate HSCs by up-regulating *α-SMA, TGF-β1, collagen 1α1*, and *TIMP-1* mRNA.

Molecular interactions between macrophages and HSCs are important for promoting fibrosis. We demonstrated that TNF-α produced by $CCR9^+$ macrophages was a critical activator of HSCs in the process of liver fibrosis, by showing that (1) TNF-α neutralization significantly diminished HSC activation induced by $CCR9^+$ macrophages (Fig. 7A), and (2) HSCs deficient in TNF receptor 1 were only slightly activated by $CCR9^+$ macrophages (Fig. 7B). Furthermore, accumulating $CCR9^+$ macrophages also showed increased levels of *TGF-β1* and *NOS-2* mRNA (Fig. 5B). TGF-β1 antagonism significantly decreased HSCs activation induced by $CCR9^+$ macrophages (Fig. 7A). These results suggest that TGF-β1 or ROS produced by $CCR9^+$ macrophages may act in concert with TNF-α to activate HSCs and cause subsequent liver fibrosis.

Alternatively, it is possible that CCR9/CCL25 directly targets HSCs to promote activation and subsequent liver fibrosis. We demonstrated that in fibrotic livers, CCR9 expression increased in HSCs, and CCL25 had the potential to attract HSCs by *in vitro* transwell assay (Fig. 6A-C). Furthermore, CCL25 could up-regulate *α-SMA, TGF-β1, collagen 1α1*, and *TIMP-1* mRNA in HSCs *in vitro*, although to a lesser extent than *in vivo* (Fig. 6B) and in coculture experiments with the existence of $CCR9^+$ macrophages (Fig. 7A), indicating that CCL25 might play a more profound role in attracting HSCs to injured livers rather than directly activating HSCs. Although these results support our hypothesis that the CCR9/CCL25 axis contributes to liver fibrosis by (1) directly targeting HSCs in the injured liver, and (2) recruiting $CCR9^+$ macrophages and indirectly activating HSCs, the profound decrease of fibrosis observed due to CCR9 deficiency *in vivo* (Fig.4) and the superiority of HSC activation with $CCR9^+$ macrophages compared with CCL25 *in vitro* (Fig. 6D, 7A) may suggest a more prevailing potential of $CCR9^+$ macrophages to activate HSCs leading to fibrosis, compared with the direct effect of CCL25.

We also investigated the possibility that other immune cells might be involved in the process of liver fibrosis, since CCR9 expression was also detected in Siglec H^+ pDCs and $CD3^+CD8^+$ T lymphocytes. It is worth noting that decreased numbers of $CD8^+$ T lymphocytes were observed in the livers of CCl_4-treated *CCR9*$^{-/-}$ mice compared with WT mice. A previous study showed that $CD4^+$ T lymphocytes down-regulate CCR9 expression upon leaving the thymus, while $CD8^+$ T lymphocytes retain CCR9 expression.[34] We confirmed this by showing that only $CD8^+$ T lymphocytes expressed CCR9 in nonfibrotic murine livers (Supporting Fig. 2). Thus, the decrease in $CD8^+$ T lymphocytes in *CCR9*$^{-/-}$ mice may be the result of redistribution due to loss of CCR9. According to previous studies, the role of $CD8^+$ T lymphocytes in liver fibrogenesis is still controversial.[35-37] Here, we demonstrated that the activation of HSCs was not induced by isolated hepatic $CD8^+$ lymphocytes *in vitro* (Fig. 7A). Furthermore, there was no significant difference in the level of intrahepatic *IFN-γ* mRNA, a representative effector cytokine of $CD8^+$ T lymphocytes, between CCl_4-treated WT and *CCR9*$^{-/-}$ mice (Supporting Fig. 4). Taken together, $CCR9^+$ macrophages appeared to have a greater role in chronic liver injury and liver fibrosis than $CCR9^+CD8^+$ T lymphocytes.

With regard to the involvement of pDCs, we demonstrated that the number and the phenotype of hepatic pDCs did not differ between WT and *CCR9*$^{-/-}$ mice under experimental fibrosis in this study (Fig. 5D,E). A previous study showed that $CD11c^+$ TNF-α-producing DCs were pathogenic and activated HSCs in murine liver fibrosis models.[25] TGF-β can negatively regulate pDCs in the spleen or intestinal mucosa.[38,39] However, it is still not completely understood how pDCs interact with HSCs. Limitations of experimental models of liver fibrosis may be a reason for these discrepancies.

HSCs are abundant cellular reservoirs of retinoids.[5] Retinoic acid up-regulates CCR9 and α4β7 expression on T lymphocytes and mediates their gut-homing ability.[40] Although HSCs have been suggested to participate in this process,[41] quiescent HSCs failed to show superiority in stimulating CCR9 expression on naïve T lymphocytes *in vitro* compared with intestinal DCs.[42] In the current study, we also confirmed that addition of

retinoic acid up-regulated CCR9 expression in intrahepatic $CD11b^{+}$ macrophages from WT mice *in vitro* (data not shown). Taken together, this suggests that interactions between macrophages and HSCs by way of retinoic acid may indicate a highly privileged environment for CCR9 in the promotion of liver fibrosis.

Collectively, we demonstrated the prominent and specific expression of CCR9 in liver macrophages and their involvement in the process of fibrosis by interacting with HSCs in chronically injured livers. Neutralization of CCR9 has been proposed as a novel therapeutic strategy for Crohn's disease and ulcerative colitis.[43] Thus, based on our murine model results and subject to future verification in human samples, CCR9 antagonism may represent a promising novel therapeutic target for liver fibrosis.

Acknowledgment: We thank Dr. Paul E. Love (National Institute of Child Health and Human Development) for providing *CCR9*$^{-/-}$ mice, and Dr. Toshiaki Teratani and Dr. Takahiro Suzuki (Keio University School of Medicine) for technical assistance.

References

1. Gines P, Cardenas A, Arroyo V, Rodes J. Management of cirrhosis and ascites. N Engl J Med 2004;350:1646-1654.
2. Friedman SL. Liver fibrosis — from bench to bedside. J Hepatol 2003; 38(Suppl 1):S38-S53.
3. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115: 209-218.
4. Ramm GA. Chemokine (C-C motif) receptors in fibrogenesis and hepatic regeneration following acute and chronic liver disease. HEPATOLOGY 2009;50:1664-1668.
5. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 2008;88:125-172.
6. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. HEPATOLOGY 2009;50:261-274.
7. 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.
8. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 2005;115: 56-65.
9. Seki E, de Minicis S, Inokuchi S, Taura K, Miyai K, van Rooijen N, et al. CCR2 promotes hepatic fibrosis in mice. HEPATOLOGY 2009;50: 185-197.
10. Seki E, De Minicis S, Gwak GY, Kluwe J, Inokuchi S, Bursill CA, et al. CCR1 and CCR5 promote hepatic fibrosis in mice. J Clin Invest 2009;119:1858-1870.
11. Heymann F, Hammerich L, Storch D, Bartneck M, Huss S, Russeler V, et al. Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor C-C motif chemokine receptor 8 in mice. HEPATOLOGY 2012;55:898-909.
12. Aoyama T, Inokuchi S, Brenner DA, Seki E. CX3CL1-CX3CR1 interaction prevents carbon tetrachloride-induced liver inflammation and fibrosis in mice. HEPATOLOGY 2010;52:1390-1400.
13. Karlmark KR, Zimmermann HW, Roderburg C, Gassler N, Wasmuth HE, Luedde T, et al. The fractalkine receptor CX(3)CR1 protects against liver fibrosis by controlling differentiation and survival of infiltrating hepatic monocytes. HEPATOLOGY 2010;52:1769-1782.
14. Sahin H, Trautwein C, Wasmuth HE. Functional role of chemokines in liver disease models. Nat Rev Gastroenterol Hepatol 2010;7: 682-690.
15. Vicari AP, Figueroa DJ, Hedrick JA, Foster JS, Singh KP, Menon S, et al. TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. Immunity 1997;7:291-301.
16. Zaballos A, Gutierrez J, Varona R, Ardavin C, Marquez G. Cutting edge: identification of the orphan chemokine receptor GPR-9-6 as CCR9, the receptor for the chemokine TECK. J Immunol 1999;162:5671-5675.
17. Drakes ML, Stiff PJ, Blanchard TG. Inverse relationship between dendritic cell CCR9 expression and maturation state. Immunology 2009; 127:466-476.
18. Zabel BA, Agace WW, Campbell JJ, Heath HM, Parent D, Roberts AI, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. J Exp Med 1999;190:1241-1256.
19. Wendland M, Czeloth N, Mach N, Malissen B, Kremmer E, Pabst O, et al. CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine. Proc Natl Acad Sci U S A 2007;104:6347-6352.
20. Hadeiba H, Sato T, Habtezion A, Oderup C, Pan J, Butcher EC. CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. Nat Immunol 2008;9:1253-1260.
21. Saruta M, Yu QT, Avanesyan A, Fleshner PR, Targan SR, Papadakis KA. Phenotype and effector function of CC chemokine receptor 9-expressing lymphocytes in small intestinal Crohn's disease. J Immunol 2007;178:3293-3300.
22. Eksteen B, Grant AJ, Miles A, Curbishley SM, Lalor PF, Hubscher SG, et al. Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. J Exp Med 2004;200:1511-1517.
23. Nakamoto N, Ebinuma H, Kanai T, Chu PS, Ono Y, Mikami Y, et al. CCR9+ macrophages are required for acute liver inflammation in mouse models of hepatitis. Gastroenterology 2012;142:366-376.
24. Uehara S, Grinberg A, Farber JM, Love PE. A role for CCR9 in T lymphocyte development and migration. J Immunol 2002;168: 2811-2819.
25. Connolly MK, Bedrosian AS, Mallen-St Clair J, Mitchell AP, Ibrahim J, Stroud A, et al. In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF-alpha. J Clin Invest 2009;119:3213-3225.
26. Ojiro K, Ebinuma H, Nakamoto N, Wakabayashi K, Mikami Y, Ono Y, et al. MyD88-dependent pathway accelerates the liver damage of Concanavalin A-induced hepatitis. Biochem Biophys Res Commun 2010;399:744-749.
27. Mikami Y, Kanai T, Sujino T, Ono Y, Hayashi A, Okazawa A, et al. Competition between colitogenic Th1 and Th17 cells contributes to the amelioration of colitis. Eur J Immunol 2010;40:2409-2422.
28. Imaeda AB, Watanabe A, Sohail MA, Mahmood S, Mohamadnejad M, Sutterwala FS, et al. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. J Clin Invest 2009;119:305-314.
29. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. Immunobiology 2006;211:609-618.
30. Tarrats N, Moles A, Morales A, Garcia-Ruiz C, Fernandez-Checa JC, Mari M. Critical role of tumor necrosis factor receptor 1, but not 2, in hepatic stellate cell proliferation, extracellular matrix remodeling, and liver fibrogenesis. HEPATOLOGY 2011;54:319-327.
31. Dominguez PM, Ardavin C. Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. Immunol Rev 2010;234:90-104.
32. Abd Alla J, Langer A, Elzahwy SS, Arman-Kalcek G, Streichert T, Quitterer U. Angiotensin-converting enzyme inhibition down-regulates

the pro-atherogenic chemokine receptor 9 (CCR9)-chemokine ligand 25 (CCL25) axis. J Biol Chem 2010;285:23496-23505.
33. Schmutz C, Cartwright A, Williams H, Haworth O, Williams JH, Filer A, et al. Monocytes/macrophages express chemokine receptor CCR9 in rheumatoid arthritis and CCL25 stimulates their differentiation. Arthritis Res Ther 2010;12:R161.
34. Carramolino L, Zaballos A, Kremer L, Villares R, Martin P, Ardavin C, et al. Expression of CCR9 beta-chemokine receptor is modulated in thymocyte differentiation and is selectively maintained in CD8(+) T cells from secondary lymphoid organs. Blood 2001;97: 850-857.
35. Safadi R, Ohta M, Alvarez CE, Fiel MI, Bansal M, Mehal WZ, et al. Immune stimulation of hepatic fibrogenesis by CD8 cells and attenuation by transgenic interleukin-10 from hepatocytes. Gastroenterology 2004;127:870-882.
36. Weng H, Mertens PR, Gressner AM, Dooley S. IFN-gamma abrogates profibrogenic TGF-beta signaling in liver by targeting expression of inhibitory and receptor Smads. J Hepatol 2007;46:295-303.
37. Bansal R, Prakash J, Post E, Beljaars L, Schuppan D, Poelstra K. Novel engineered targeted interferon-gamma blocks hepatic fibrogenesis in mice. HEPATOLOGY 2011;54:586-596.
38. Contractor N, Louten J, Kim L, Biron CA, Kelsall BL. Cutting edge: Peyer's patch plasmacytoid dendritic cells (pDCs) produce low levels of type I interferons: possible role for IL-10, TGFbeta, and prostaglandin E2 in conditioning a unique mucosal pDC phenotype. J Immunol 2007;179:2690-2694.
39. Li L, Liu S, Zhang T, Pan W, Yang X, Cao X. Splenic stromal microenvironment negatively regulates virus-activated plasmacytoid dendritic cells through TGF-beta. J Immunol 2008;180:2951-2956.
40. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity 2004;21:527-538.
41. Winau F, Hegasy G, Weiskirchen R, Weber S, Cassan C, Sieling PA, et al. Ito cells are liver-resident antigen-presenting cells for activating T cell responses. Immunity 2007;26:117-129.
42. Eksteen B, Mora JR, Haughton EL, Henderson NC, Lee-Turner L, Villablanca EJ, et al. Gut homing receptors on CD8 T cells are retinoic acid dependent and not maintained by liver dendritic or stellate cells. Gastroenterology 2009;137:320-329.
43. Thomas S, Baumgart DC. Targeting leukocyte migration and adhesion in Crohn's disease and ulcerative colitis. Inflammopharmacology 2012; 20:1-18.