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Chemokine Receptor CXCR6-Dependent Hepatic NK T Cell Accumulation Promotes Inflammation and Liver Fibrosis

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Chronic liver injury characteristically results in hepatic inflammation, which represents a prerequisite for organ fibrosis. Although NKT cells are abundantly present in liver and involved in hepatic inflammation, molecular mechanisms of their recruitment in liver fibrosis remained elusive. We hypothesized that chemokine receptor CXCR6 and its ligand CXCL16 control NKT cell migration and functionality in liver fibrosis. In patients with chronic liver diseases ($n = 58$), CXCR6 and CXCL16 expression was intrahepatically upregulated compared with controls. In murine liver, Cxcl16 was strongly expressed by endothelium and macrophages, whereas lymphocyte populations (NKT, NK, CD4 T, CD8 T cells) expressed CXCR6. Intravital two-photon microscopy imaging of $Cxcr6^{+/gfp}$ and $Cxcr6^{gfp/gfp}$ mice and chemotaxis studies in vitro revealed that CXCR6 specifically controls hepatic NKT cell accumulation during the early response upon experimental liver damage. Hepatic invariant NKT cells expressed distinct proinflammatory cytokines including IFN-γ and IL-4 upon injury. CXCR6-deficient mice were protected from liver fibrosis progression in two independent experimental models. Macrophage infiltration and protein levels of inflammatory cytokines IFN-γ, TNF-α, and IL-4 were also reduced in fibrotic livers of $Cxcr6^{-/-}$ mice, corroborating that hepatic NKT cells provide essential cytokine signals perpetuating hepatic inflammation and fibrogenesis. Adoptive transfer of NKT cells, but not CD4 T cells, isolated from wild type livers restored hepatic fibrosis in $Cxcr6^{-/-}$ mice upon experimental steatohepatitis. Our results demonstrate that hepatic NKT cells accumulate CXCR6-dependent early upon injury, thereby accentuating the inflammatory response in the liver and promoting hepatic fibrogenesis. Interfering with CXCR6/CXCL16 might therefore bear therapeutic potential in liver fibrosis. *The Journal of Immunology*, 2013, 190: 5226–5236.

C hronic liver diseases are typically based on insults such as metabolic disorders, viral hepatitis, alcohol abuse, autoimmunity, and cholestasis. Chronic liver injury leads to a characteristic remodeling of the organ, resulting in liver fibrosis, an ongoing wound healing response that provokes excessive deposition of extracellular matrix and replacement of functional parenchyma by scar tissue (1). Progression of hepatic fibrosis often results in liver cirrhosis and hepatocellular carcinoma as life-threatening complications of end-stage liver disease (1). Inflammation in the liver has been clearly identified as the driving force

in chronic liver injury, because the inflammatory response critically promotes the development and aggravation of hepatic injury (e.g., hepatocyte apoptosis or steatosis) as well as induction and progression of organ fibrosis (2–4). On a cellular level, the activation of liver-resident macrophages, traditionally called "Kupffer cells," and the vast infiltration of monocytes into the injured liver have been identified as major pathogenic factors (4). Because this pool of hepatic macrophages releases essential proinflammatory cytokines (e.g., TNF), they promote hepatocellular stress responses and lipid accumulation (5–8). In addition, CD8 T cells endorse liver fibrosis via activating hepatic stellate cells (HSC) (9), whereas NK cells are capable of promoting HSC apoptosis and are thus considered antifibrotic (10, 11). The functional role of NKT cells, which are abundantly present in the liver and constitute ~30% of the hepatic lymphocytes, in liver fibrogenesis is less clear (12, 13).

NKT cells are a heterogeneous population of unconventional T cells that express markers of NK cells and TCRs. NKT subsets with different functions exist in humans and mice, classical (type I) invariant (iNKT) and other CD1d-dependent (type II) NKT cells, as well as CD1d-independent (NKT-like) T cells. Proinflammatory iNKT cells in mice and humans can be reliably detected by CD1d-tetramers loaded with α-galactosylceramide (α-GalCer) (14). NKT cells were found to crawl within liver sinusoids during homeostasis; functionally, they might partake in immune surveillance in liver, because NKT cells were found to contribute to T cell–dependent, Concanavalin A (Con A)–induced hepatitis in mice (15, 16). Upon activation, NKT cells can produce a variety of different cytokines, including IFN-γ and IL-4. However, most experimental data indicate that NKT cells undergo activation-induced cell death within hours after stimulation, such as with CD3-specific

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Ab or α-GalCer (17), resulting in low hepatic NKT cell numbers in chronic injury, such as fibrosis (12). In experimental models, $Cd1d^{-/-}$ mice lacking NKT cells were less susceptible to inflammation, hepatocyte apoptosis, and fibrosis (18, 19). Concordantly, the preactivation of hepatic NKT cells by α-GalCer accelerated liver fibrosis in experimental murine models (12). However, particularly in early phases of carbon tetrachloride (CCl_4)–induced fibrosis, NKT cells can also be protective by restricting HSC activation, suggesting that the net effect of NKT cells in liver scarring depends on the balance between protective and detrimental consequences of NKT cell activation (12, 20).

Nevertheless, there is sufficient evidence that inhibition of NKT cell infiltration to the liver might be valid in restricting hepatic inflammation and fibrosis, but the mechanisms by which NKT cell recruitment is regulated during liver fibrogenesis are incompletely understood. Liver inflammation in general is tightly controlled by chemokines, which are peptide mediators that stimulate chemotaxis of target cells through specific G protein–coupled receptors (4). Prior studies in mice indicated that the chemokine receptor CXCR6 and its cognate ligand CXCL16 can control the patrolling of NKT cells on liver sinusoids during homeostasis (15). In humans, high levels of CXCR6+ T cells have been detected in blood of hepatitis C–infected patients compared with healthy controls, and >60% of intrahepatic human T cells expressed CXCR6, including CD4, CD8, and CD56+ (NK) T cells (21). The one known ligand of CXCR6, the chemokine CXCL16, exists in a transmembrane and soluble form. Expression of CXCL16 has been detected in hepatocytes and bile ducts of patients with liver disease (21), as well as in murine liver sinusoidal endothelial cells (15) and in macrophages or dendritic cells of other organs (22).

In this study, we comprehensively analyzed the role of CXCR6 and its ligand CXCL16 for the migration and functionality of NKT cells in liver inflammation and fibrosis. We demonstrate that this chemokine–chemokine receptor axis is upregulated in patients with chronic liver disease. By comparing wild type (WT) and $Cxcr6^{-/-}$ mice in long-term models of toxic liver injury and nonalcoholic steatohepatitis, we provide experimental evidence for a critical CXCR6-dependent accumulation of NKT cells upon liver injury, which exerts proinflammatory actions and promotes hepatofibrogenesis in vivo.

Materials and Methods
Human liver samples

Liver biopsies or explants of cirrhotic livers were scored and processed as described previously (7). Tissue from tumor-free margins of resected hepatic metastasis within normal liver parenchyma ($n = 5$) served as controls.

Mouse models and phenotyping analyses

C57bl/6 WT, congenic CD45.1, and $Cxcr6^{-/-}$ mice (backcrossed to c57bl/6 background) were housed in a specific pathogen-free environment. In $Cxcr6^{+/-}$ and $Cxcr6^{-/-}$ mice, one or both alleles of $Cxcr6$ were replaced with GFP cDNA (15, 23). All experiments were performed with animals at 6–8 wk of age under ethical conditions according to German legal requirements. Mice received 0.6 ml/kg body weight CCl_4 (Merck, Darmstadt, Germany) mixed with corn oil i.p. and were sacrificed at indicated time points. For induction of liver fibrosis, CCl_4 was injected twice per week for 6 wk, and mice were sacrificed 48 h after the last injection (8). The control population of animals received the same volume of tracer (corn oil) i.p. For induction of steatohepatitis and fibrosis, mice were fed with a methionine-choline–deficient (MCD) diet for 4 or 8 wk (cat. no. 390439; MP Biomedicals, Solon, OH) (8). Biochemical assays and gene and protein expression studies were performed as described previously (8, 24, 25).

Intravital time-lapse laser-scanning microscopy

Intravital microscopy was performed after surgical exposure of the liver using a LaVision multiphoton laser-scanning microscope (BioTec, Biele-feld, Germany) over 3–4 h per animal in video sequences of 30–60 min on different view fields to minimize laser damage to the tissue and to increase the area observed for statistical analysis. Mice were anesthetized followed by tracheotomy and controlled respiration with 2.5% isoflurane in 100% O_2. The liver was exposed, followed by extracorporal immobilization of the organ on a custom-made stage and embedding in 3% PBS agarose. Video sequences were generated and analyzed using Imaris 7.4 (Bitplane, Zurich, Switzerland). Automated cell tracking was performed with Imaris 7.4 to determine cellular motility and track displacements. Cells were identified by GFP signals and followed over a period of 1–2 h for the creation of movement tracks. Displacements were calculated from the tracked paths and shown as maximal displacement per cell. Cell motility index was calculated by comparison of moving (>5 μm displacement per hour) versus stationary (<5μm displacement per hour) cells.

Isolation of primary hepatic cell populations

Hepatocytes were isolated from murine livers by conventional collagenase perfusion methodology (6). Hepatic stellate cells were obtained by FACS sorting via negative staining for CD45, F4/80, CD11b, Ly6G, and positive autofluorescence in UV-channel (26). Inflammatory macrophages (Ly6G⁻ F4/80⁺CD11b⁺), Kupffer cells (Ly6G⁻F4/80⁺⁺CD11b⁻), and endothelial cells (CD45⁻ autofluorescence⁻ CD146⁺) were obtained from single-cell suspensions of livers by FACS-sorting.

Migration assay

Total leukocytes from spleen were subjected to CXCL16-dependent migration assays (100 ng/ml; eBioscience, San Diego, CA) through a semipermeable membrane (pore size, 5 μm) (24). The migrated population was analyzed using FACS. Numbers of migrated cells were assessed as the ratio of cells migrated toward CXCL16 compared with background (no chemokine).

FACS analysis of blood and intrahepatic leukocytes

Circulating and intrahepatic leukocytes were isolated and subjected to FACS analysis as described previously (24). Multicolor stainings were conducted using monoclonal Abs: F4/80 (Serotec, Raleigh, NC), CD115, CD4, CD11b (all eBioscience), CD45, CD146, Gr1/Ly6C, Ly6G, CD19, NK1.1, CD8 and CD3 (all BD, Heidelberg, Germany), CD1d tetramer loaded with αGalCer (ProImmune, Oxford, U.K.). Intrahepatic leukocytes were identified by positive staining for CD45, and dead cells were excluded by Hoechst-33258 dye (Sigma-Aldrich, St. Louis, MO). Macrophages were negative for Ly6G and positive for CD11b and F4/80. NKT cells were positive for CD4, NK1.1, and CD1d tetramer (Supplemental Fig. 1C).

Adoptive transfer of hepatic NK T and CD4 T cells

NKT and CD4 T cells were sorted from livers of CD45.1 congenic mice as CD45⁺CD4⁺NK1.1⁺ (NKT) and CD45⁺CD4⁺NK1.1⁻ (CD4) populations by FACSAria at purities >90%; 3–5 × 10⁵ cells were injected i.v. once per week into WT and $Cxcr6^{-/-}$ recipients that were fed an MCD diet for 4 wk.

Statistical analysis

All experimental data are expressed as mean ± SD. Differences between groups of mouse experiments were assessed by two-tailed unpaired Student t test and marked in the figures by *$p < 0.05$, **$p < 0.005$, or ***$p < 0.001$. Differences between groups of human data were assessed by Mann–Whitney U test.

Results
CXCR6 and its ligand CXCL16 are up-regulated in chronic liver diseases in humans and expressed by distinct cellular compartments in murine liver

Although increased numbers of CXCR6-expressing circulating and hepatic lymphocytes have been described in cases of hepatitis C–infected patients (21), the relevance of this chemokine-chemokine receptor axis in the pathogenesis of liver diseases has remained elusive. Therefore, we first tested whether CXCR6 or CXCL16 are activated in human liver diseases. By analyzing liver tissue from 58 liver disease patients in comparison with controls, hepatic *CXCR6* and *CXCL16* mRNA expression was found upregulated in chronic liver diseases, dependent of the
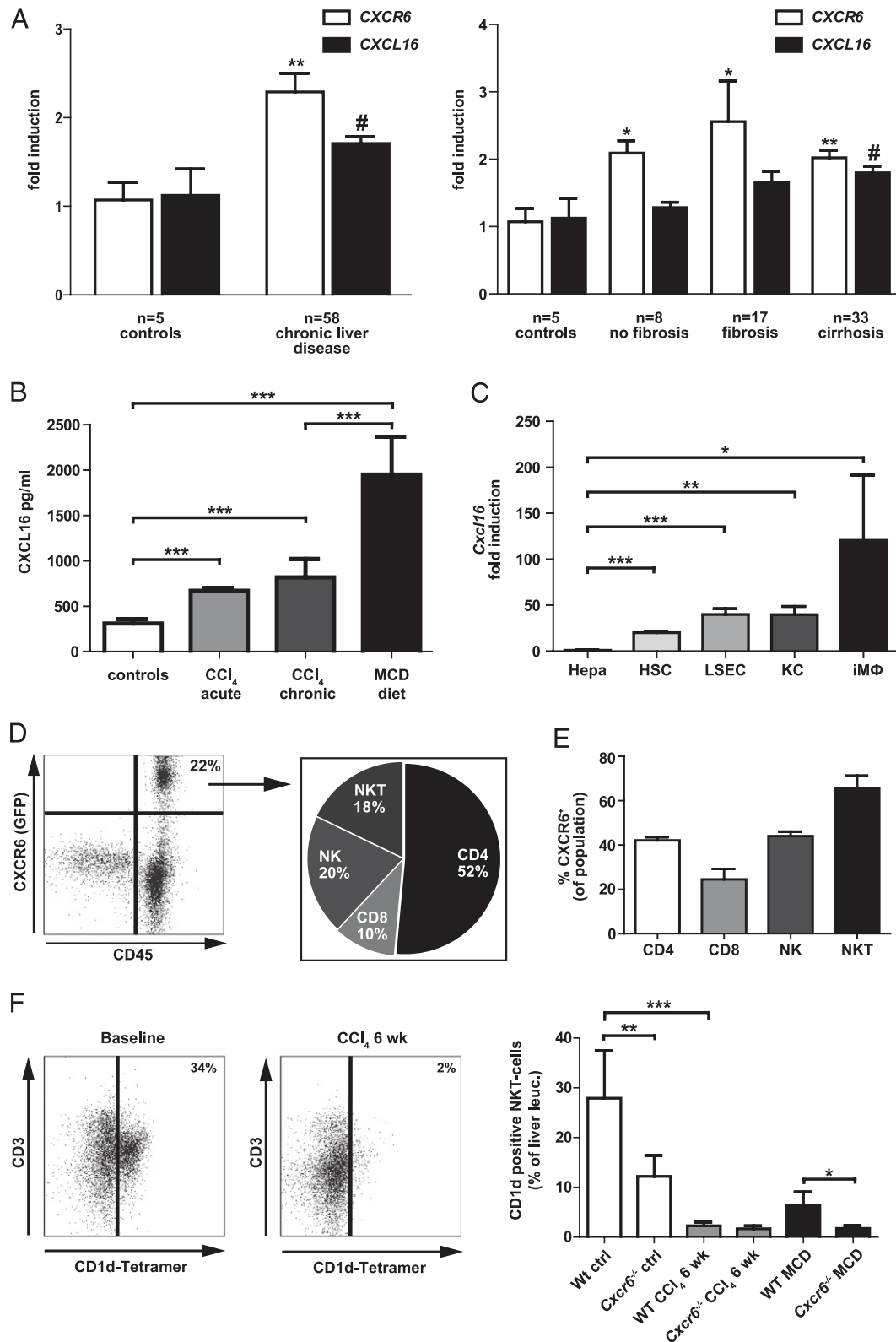
FIGURE 1. CXCR6 and its ligand CXCL16 are upregulated in chronic liver diseases in humans and are expressed by distinct cellular compartments in murine liver. (**A**) Gene expression of *CXCR6* and *CXCL16* (normalized to β-actin) by quantitative PCR from the livers of 58 patients with chronic liver disease compared with five healthy controls. Bars express fold-induction, and error bars represent SEM. (**B**) CXCL16 serum levels by ELISA from control mice and experimental acute (48 h after a single CCl_4 injection) or chronic (6 wk repeated CCl_4 or an 8-wk MCD diet, respectively) liver injury. (**C**) *CXCL16* expression from different isolated primary murine liver cell populations of healthy animals. Hepa, hepatocytes; LSEC, liver sinusoidal endothelial cells; KC, Kupffer cell; iMΦ, inflammatory macrophages. (**D** and **E**) CXCR6 protein expression by FACS from livers of untreated *Cxcr6*[+/gfp] mice. Distributions of lymphocyte populations among CXCR6-expressing cells (D) and percentage of CXCR6-expressing cells within individual hepatic immune cell compartments (E) are displayed. (**F**) When hepatic NKT cells were identified by CD3 and α-GalCer–loaded CD1d-tetramer staining, it was apparent that NKT cell numbers were drastically reduced upon chronic liver injury (representative FACS plots are shown). *Cxcr6*^{−/−} mice showed significantly reduced hepatic NKT cell counts at baseline, (*Figure legend continues*)
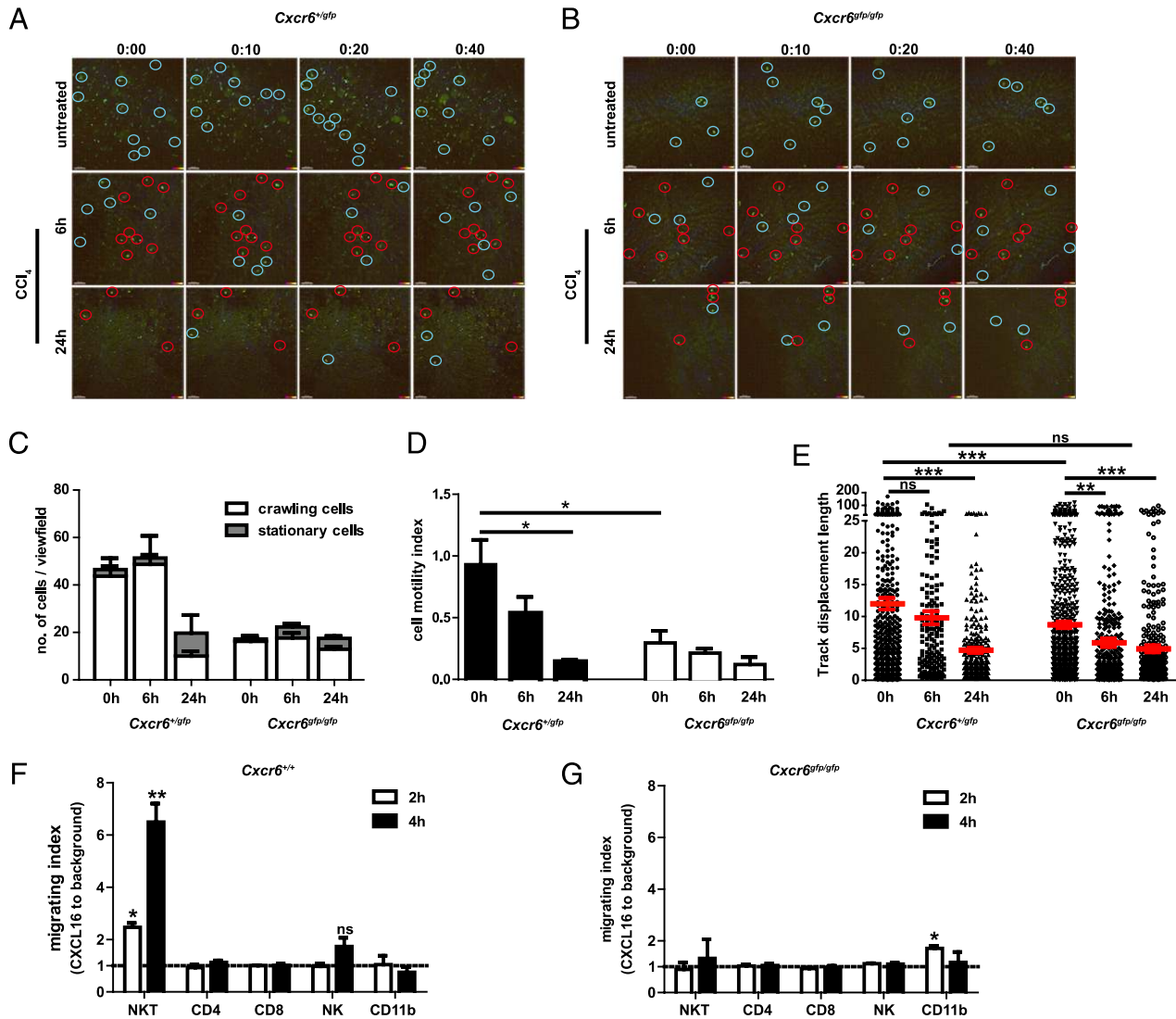
FIGURE 2. CXCR6 regulates hepatic lymphocyte migration in homeostasis and experimental liver injury. (**A**, **B**) Intravital time-lapse, multiphoton microscopy of $Cxcr6^{+/gfp}$ and $Cxcr6^{gfp/gfp}$ ($Cxcr6^{-/-}$) in homeostasis (0 h) and after acute CCl$_4$-induced injury (6 and 24 h). Sample images of video sequences (at 0, 10, 20, and 40 min) taken from $Cxcr6^{+/-}$ (A) or $Cxcr6^{-/-}$ (B) animals. Blue circles highlight crawling cells (displacement > 10μm within 15 min), and red circles highlight stationary cells. In heterozygous $Cxcr6^{+/gfp}$ mice, only a few exemplary cells are marked by circles for clarity. (**C**) Statistics for (A) and (B) ($n = 3$ per genotype and time point). Cell numbers were calculated by averaging cell counts of 15-min video sequences derived from movies up to 1 h long. Time points refer to baseline (0 h) or hours after a single CCl$_4$ injection, as indicated. (**D**) Cell motility index (ratio of cells with movement > 5μm per 1 h video analysis to cells with < 5 μm/h movement) revealed reduced cell motility in $Cxcr6^{-/-}$ animals at baseline and effects of injury on the motility index. (**E**) Track displacement length (distance over 1 h) of individual GFP$^+$ cells in the liver as a measure of average cell movements at baseline and upon injury induction by CCl$_4$. (**F**, **G**) Chemotaxis assay ($n = 3$) using spleen leukocytes of $Cxcr6^{+/+}$ (WT, F) and $Cxcr6^{gfp/gfp}$ ($Cxcr6^{-/-}$, G) mice toward 100 ng/ml recombinant murine CXCL16. The dotted line indicates nonspecific migration (ratio = 1).

disease stage as characterized by presence of fibrosis or cirrhosis (Fig.1A). The underlying etiology of liver disease, such as viral hepatitis, alcoholism, or cholestatic disorders, did not significantly effect *CXCR6* or *CXCL16* expression (Supplemental Fig. 1A, 1B), suggesting that the upregulation of the CXCR6-CXCL16 axis is a general, etiology-independent mechanism in chronic liver diseases in humans.

Analogous to human disease, CXCL16 serum levels were significantly elevated in murine models of experimental acute and chronic liver injury, induced by CCl$_4$ or MCD diet (Fig.1B). To identify *Cxcl16* expressing cells in liver, we isolated various pri-

mary parenchymal and nonparenchymal cell populations from murine liver. *Cxcl16* expression was clearly detected in liver sinusoidal endothelium, in line with prior reports (15), whereas hepatocytes and hepatic stellate cells expressed only low levels of *Cxcl16* (Fig. 1C). Interestingly, we found that Kupffer cells and inflammatory hepatic macrophages also expressed high levels of *Cxcl16* (Fig.1C), as observed before for tissue macrophages from other organs (22).

To study CXCR6 expression by different cells in the liver, we used $Cxcr6^{+/gfp}$ mice in which the *Cxcr6* coding region was replaced by GFP cDNA (23). GFP expression could be detected

but also upon chronic liver injury (CCl$_4$ injury over 6 wk, MCD diet over 4 wk). All murine data are expressed as mean $\pm$ SD, comprising $n = 3$–6 animals in each group. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ (Mann-Whitney U test for human data, and unpaired Student t test for mouse data).

only on liver leukocytes (staining positive for the pan-leukocyte marker CD45), but not on other resident hepatic parenchymal or nonparenchymal cells (Fig. 1D). Although CXCR6/GFP was also expressed by some hepatic NK and CD8 T cells, most of the $Cxcr6^{+/gfp}$ cells in liver were CD4 T or NKT cells (Fig. 1D). However, in contrast to hepatic CD4, CD8, or NK cells, in which only a fraction expressed this receptor, the vast majority of NKT cells expressed CXCR6/GFP, indicating that CXCR6 could be of distinct functional relevance for this lymphocyte subset (Fig. 1E). Of note, CXCR6 expression on other leukocytes like macrophages and neutrophils was <1% (data not shown). Intrahepatic NKT cells were identified as cells stained positive for CD1d tetramers loaded with α-GalCer as well as CD45$^+$CD4$^+$NK1.1$^+$ cells both in homeostasis and in conditions of chronic liver injury (Supplemental Fig. 1C) (14), confirming that this combination of stainings identifies specific hepatic iNKT cells (type I NKT cells) and not other populations of activated T cells. Notably, especially chronic injury induced by CCl$_4$ dramatically reduced hepatic CD4$^+$NK1.1$^+$ NKT cell counts, as confirmed by α-GalCer–loaded CD1d-tetramer staining (Fig. 1F).

Furthermore, to explore the functional relevance of CXCR6 for liver inflammation and fibrosis, $Cxcr6^{gfp/gfp}$ ($Cxcr6^{-/-}$) mice were analyzed. FACS analysis of intrahepatic leukocytes revealed significantly reduced numbers of NKT cells in $Cxcr6$-deficient

compared with WT mice already at baseline conditions and in chronic liver injury models, both by CD4$^+$NK1.1$^+$ and by α-GalCer– loaded CD1d-tetramer–based analysis (Fig. 1F).

CXCR6 controls hepatic NKT cell accumulation upon experimental liver injury

To investigate the functional involvement of CXCR6 for the migratory behavior of lymphocytes in injured liver, we established a novel experimental setup of intravital time-lapse multiphoton laser-scanning microscopy. This setup allowed visualization of CXCR6/GFP$^+$ immune cells in the liver and differentiation between crawling and stationary leukocytes in vivo (27). In steady-state conditions, it was apparent that $Cxcr6^{+/gfp}$ animals (Fig. 2A) had significantly higher constitutive levels of CXCR6/GFP$^+$ cells than $Cxcr6^{gfp/gfp}$ ($Cxcr6^{-/-}$) mice (Fig. 2B, 2C). Importantly, in heterozygous $Cxcr6^{+/gfp}$, almost all CXCR6/GFP$^+$ lymphocytes were crawling cells, because they were freely motile and patrolled the sinusoids in healthy liver (Supplemental Videos 1, 2), consistent with prior work using confocal intravital microscopy (15). In contrast, $Cxcr6^{gfp/gfp}$ mice not only had significantly lower numbers of intrahepatic GFP$^+$ cells; a lower fraction of these cells showed a motility >5 μm per 1 h video analysis (Fig. 2D) and the average track displacement was reduced compared with untreated $Cxcr6^{+/gfp}$ mice (Fig. 2E).
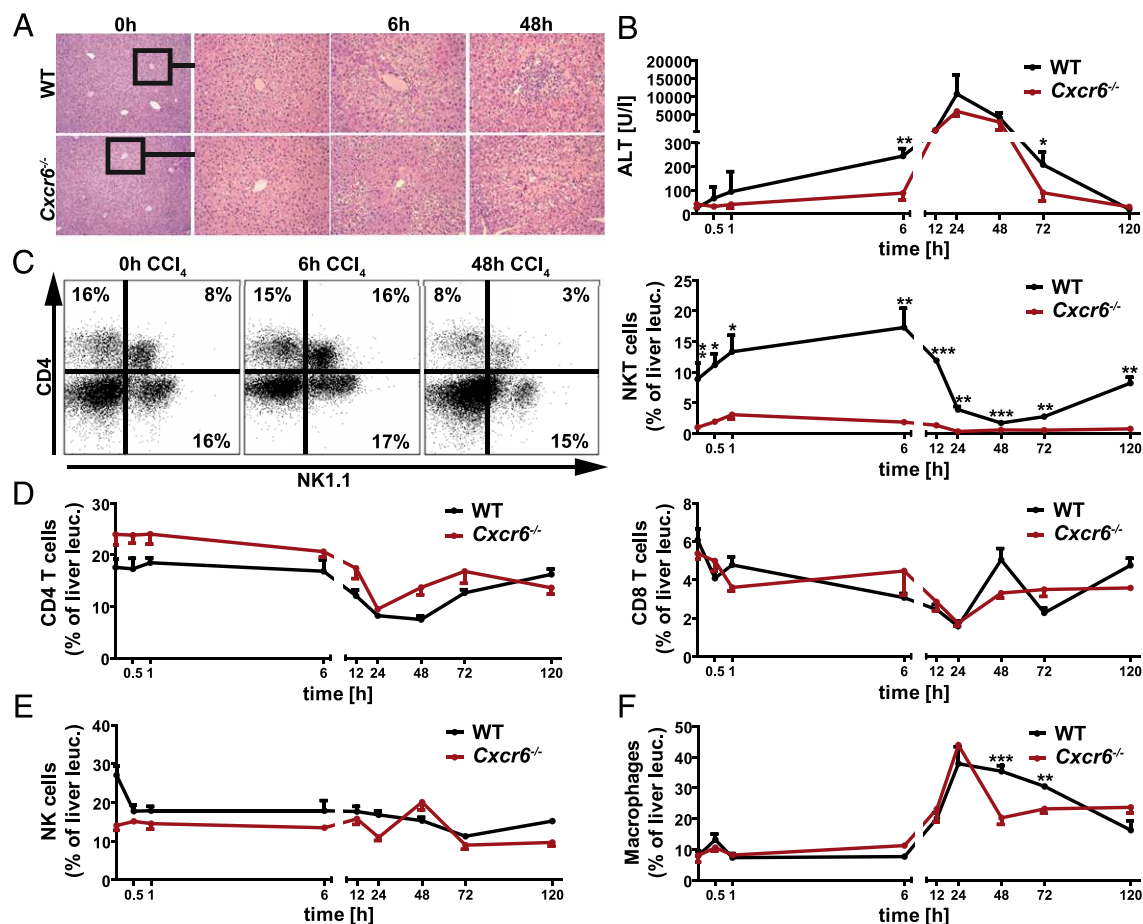

FIGURE 3. CXCR6 controls hepatic NKT cell accumulation in homeostasis and experimental liver injury. C57bl/6 (WT) and $Cxcr6^{-/-}$ mice analyzed after a single dose of CCl$_4$ i.p. (**A**). H&E staining showed necrosis and progressive mononuclear periportal infiltrates after acute liver injury. (**B**) Hepatic injury as indicated by serum ALT activity. *Left panels*, Original magnification ×50; *center and right panels*, original magnification ×200. (**C–F**) Flow cytometric analysis of intrahepatic leukocytes, pregated on CD45$^+$ and living (Hoechst dye negative). Representative FACS plots of CD4$^+$NK1.1$^+$ hepatic NKT cells in WT mice (**C**). Statistical summary of hepatic NKT cells (**C**), intrahepatic CD4 T and CD8 T cells (**D**), NK cells (**E**), and hepatic CD11b$^+$F4/80$^+$ macrophages (**F**) in WT versus $Cxcr6^{-/-}$ mice upon CCl$_4$-induced liver injury. All data are expressed as mean ± SD from three independent experiments, summarizing $n = 3$–6 animals per group. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (unpaired Student t test).

To explore the relevance of the CXCR6/CXCL16 pathway in conditions of liver injury, $Cxcr6^{+/gfp}$ and $Cxcr6^{gfp/gfp}$ ($Cxcr6^{-/-}$) mice were challenged with a single injection of CCl_4. This toxic injury results in a severe liver injury with maximal damage between 12 and 48 h, followed by a strong infiltration of inflammatory monocytes–macrophages peaking at 24–48 h after injection (6). Within the first 6 h after injury induction, an increase of crawling CXCR6/GFP$^+$ cells could be detected in $Cxcr6^{+/gfp}$ mice (Fig. 2A, 2C). Some of these crawling cells showed an arrest and diapedesis into the hepatic parenchyma, as visualized by major deformation of the cells passing through the sinusoidal capillary vessels (Supplemental Videos 3, 5). At 24 h after CCl_4 administration, a high proportion of CXCR6/GFP$^+$ cells had become stationary in the injured liver (Fig. 2A), whereas the numbers of motile cells was strongly decreased (Fig. 2C, 2D), and the average cell movement (track displacement length) was significantly reduced. Although no clear accumulation of CXCR6/GFP$^+$ lymphocytes could be observed in $Cxcr6^{gfp/gfp}$ mice upon liver injury (Fig. 2C), cell motility of the low numbers of GFP$^+$ cells decreased upon liver damage as well, indicating that the small number of remaining iNKT cells responded to injury induction (Fig. 2D, 2E; Supplemental Videos 1–6).

For identifying the CXCR6-dependent lymphocyte subsets, the migration of spleen cells toward CXCL16 was analyzed by chemotaxis assays in vitro. Only NKT cells migrated toward a CXCL16 gradient, whereas other CXCR6-expressing leukocyte populations (e.g., NK, CD4, or CD8 T cells; see Fig. 1D) were not affected by stimulation with CXCL16 (Fig. 2F). CXCL16-induced NKT cell migration was not observed with CXCR6-deficient leukocytes (Fig. 2G).

Next, we investigated the exact phenotype of CXCR6-dependent immune cell migration on CCl_4-induced injury in WT versus $Cxcr6^{-/-}$ mice. As evidenced by liver histology (Fig. 3A) and serum alanine aminotransferase (ALT) activity (Fig. 3B), liver injury appeared overall attenuated in $Cxcr6^{-/-}$ mice, with signifi-

cantly lower liver damage at 6 and 72 h after CCl_4 injection. Based on the lack of CXCR6 expression by hepatocytes (Fig. 1D, and data not shown), this effect was not related to different CCl_4 metabolism in $Cxcr6^{-/-}$ mice, but to differences in the inflammatory response. Thus, intrahepatic leukocytes were phenotypically characterized by FACS, and iNKT cells were identified as CD45$^+$ CD4$^+$NK1.1$^+$ cells (Fig. 3C, Supplemental Fig. 1C). In WT mice, NKT cells represented a distinctive population of hepatic leukocytes, which increased significantly within 6 h after injury, both as relative proportion of hepatic leukocytes (Fig. 3C) and in absolute numbers (Supplemental Fig. 1D). As anticipated from prior studies, the NKT cell pool decreased subsequently (Fig. 3C) because of an overwhelming influx of monocytes–macrophages and apoptosis of activated NKT cells (12, 28). In contrast, hepatic NKT cells were dramatically reduced already at baseline in $Cxcr6^{-/-}$ mice, and the distinct accumulation of hepatic NKT cells could not be established in injured liver in the absence of CXCR6 (Fig. 3C). The other CXCR6-expressing lymphocyte subsets, namely NK, CD4, and CD8 T cells, did not significantly differ between WT and $Cxcr6^{-/-}$ mice (Fig. 3D, 3E). Interestingly, WT and $Cxcr6^{-/-}$ mice showed a clear difference with respect to hepatic macrophage accumulation in injured livers, because the lack of NKT cells in $Cxcr6^{-/-}$ mice was accompanied by a reduced fraction of macrophages among hepatic leukocytes at 48 and 72 h after injury (Fig. 3F). These results indicate that an early hepatic iNKT cell activation (within the first 6 h after injury) is important for promoting the subsequent accumulation of infiltrating monocytes (after 48–72 h), as supported by lower ALT levels at late time points in $Cxcr6^{-/-}$ mice lacking this response.

$Cxcr6^{-/-}$ mice are protected from chronic liver injury and fibrosis

Our experiments indicated that CXCR6 controls the early accumulation of hepatic iNKT cells upon injury, thus possibly shaping the inflammatory response within the liver. To address the func-
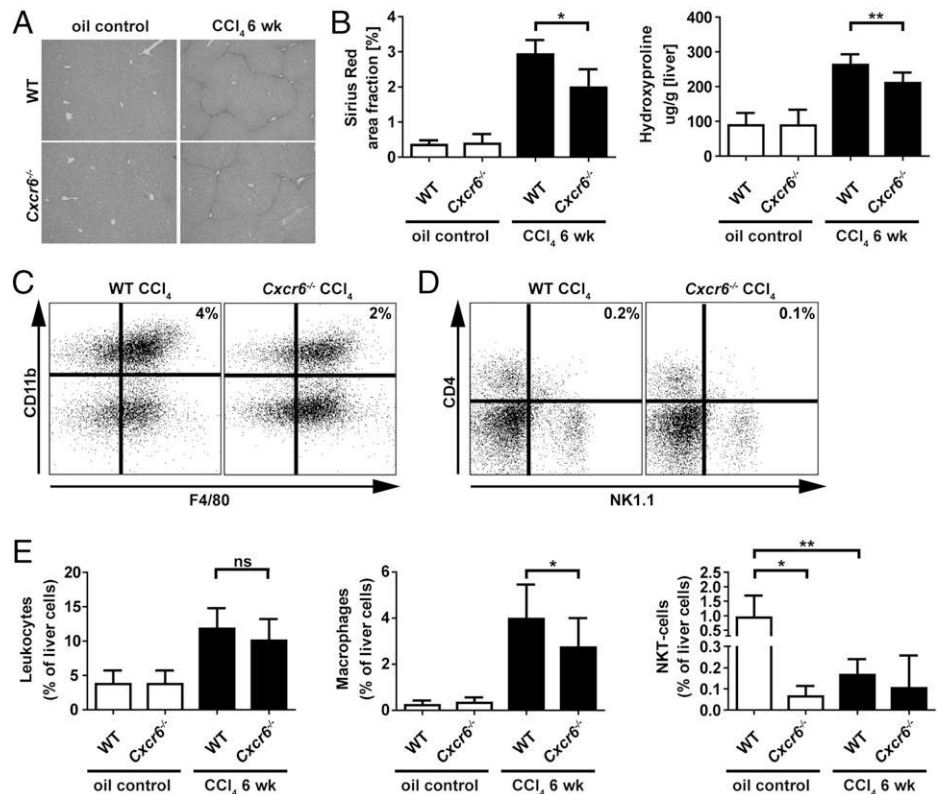
FIGURE 4. $Cxcr6^{-/-}$ mice are protected from CCl_4-induced liver fibrosis. C57bl/6 WT and $Cxcr6^{-/-}$ mice received CCl_4 i.p. twice weekly for 6 wk. (**A**) Representative Sirius Red stainings showing reduced deposition of extracellular matrix in $Cxcr6^{-/-}$ mice. Original magnification ×50. (**B**) Liver fibrosis was quantified as histologic collagen deposition and hepatic hydroxyproline content. (**C–E**) FACS analysis of intrahepatic immune cells. All data are expressed as mean ± SD from three independent experiments, summarizing $n = 9$–12 animals per group. *$p < 0.05$, **$p < 0.005$ (unpaired Student t test).

tional relevance of this mechanism on the outcome of chronic liver injury, WT and *Cxcr6*$^{-/-}$ mice received CCl$_4$ i.p. twice weekly for 6 wk to induce chronic inflammation and hepatic fibrosis. Strikingly, *Cxcr6*$^{-/-}$ mice were protected from liver fibrosis, as evidenced by reduced deposition of extracellular matrix by Sirius Red staining in histologic analysis (Fig. 4A) and by decreased hepatic hydroxyproline content as a quantitative measure of intrahepatic collagen (Fig. 4B). In line with the observations during acute injury (Fig.3), fibrotic livers of WT mice showed increased macrophage numbers compared with *Cxcr6*$^{-/-}$ mice, whereas

NKT cells were low in the livers of all chronically injured animals (Fig. 4C–E), confirming previous results that hepatic NKT cell numbers are strongly decreased in established CCl$_4$-induced liver fibrosis mostly because of NKT cell apoptosis (12). Other hepatic lymphocyte populations (NK, CD4 T, and CD8 T cells) did not differ in cell frequency or cell numbers between WT and *Cxcr6*$^{-/-}$ mice in CCl$_4$-induced liver fibrosis (Supplemental Fig. 1E).

To exclude model-specific confounding effects related to the toxic fibrosis model, mice were subjected to an MCD diet, leading to severe steatohepatitis and fibrosis over 4 and 8 wk (Fig. 5A) (8).
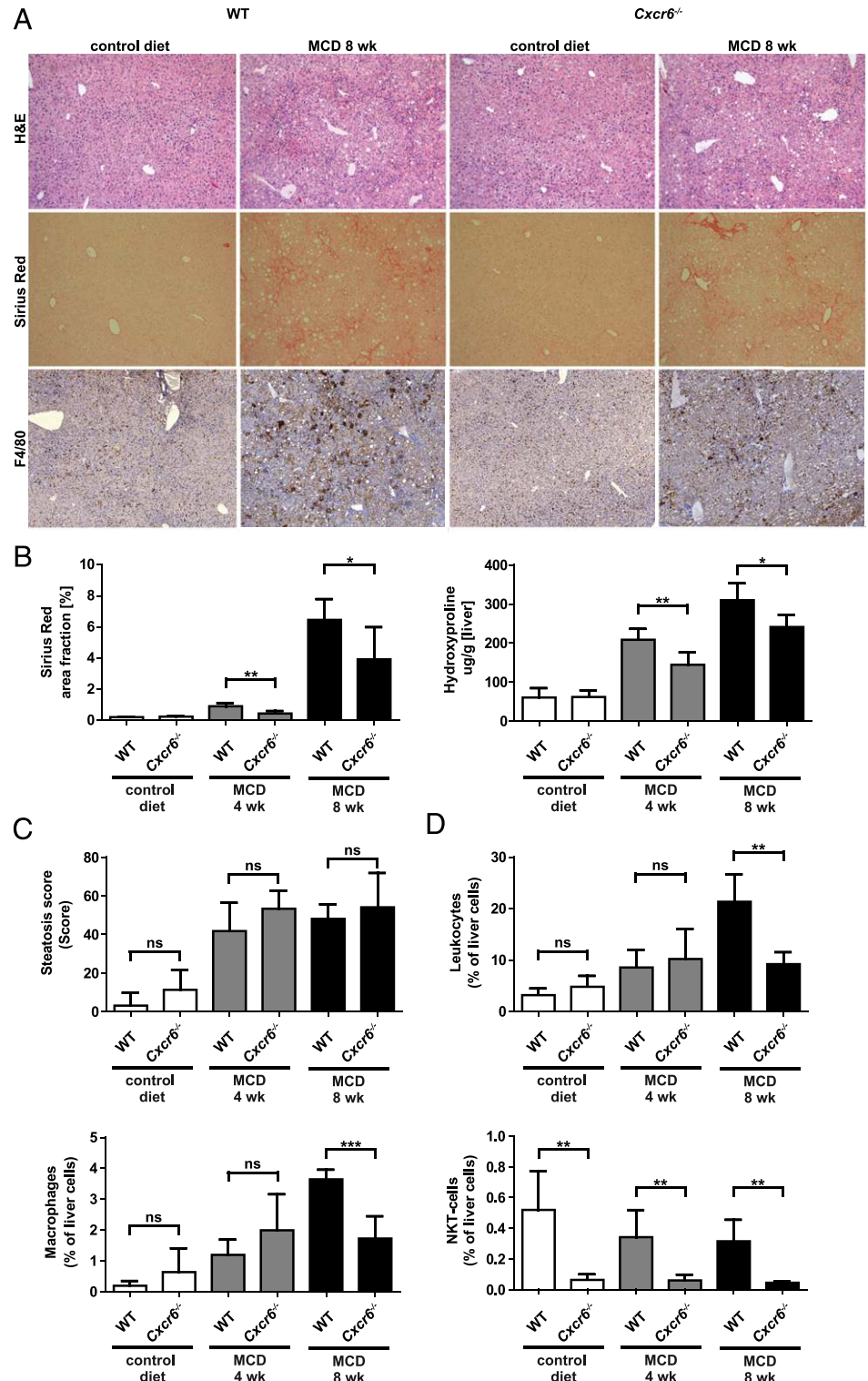

FIGURE 5. *Cxcr6*$^{-/-}$ mice are protected from liver fibrosis induced by MCD diet. C57bl/6 WT and *Cxcr6*$^{-/-}$ mice received an MCD diet for 4–8 wk to induce steatohepatitis and fibrosis. (**A**) Liver histology showed attenuated mononuclear periportal infiltrates in steatotic livers from *Cxcr6*$^{-/-}$ mice (H&E stainings, *upper row*), reduced collagen deposition (Sirius Red stainings, *middle row*) and attenuated macrophage infiltration (F4/80 immunohistochemistry, *lower row*). Original magnification ×50. (**B**) Collagen distribution quantified by Sirius Red stainings and intrahepatic hydroxyproline measurement. (**C**) Histologic scoring of fatty liver degeneration by a blinded pathologist. (**D**) FACS analysis of intrahepatic immune cells. All data are expressed as mean ± SD from three independent experiments, summarizing *n* = 5–6 animals per group. *p < 0.05, **p < 0.005, ***p < 0.001 (unpaired Student *t* test).

Again, $Cxcr6^{-/-}$ mice were protected from hepatic fibrosis, as demonstrated by lower collagen deposition in histologic analysis (Fig. 5A) and reduced Sirius Red–positive extracellular matrix and hepatic hydroxyproline quantification (Fig. 5B). Of note, fatty liver degeneration (steatosis) did not differ, indicating that the response of the hepatocytes to the metabolic injury was independent of CXCR6 (Fig. 5C). Regarding intrahepatic immune cells, hepatic NKT cells were clearly reduced in $Cxcr6^{-/-}$ compared with WT animals at all time points; this related to significantly reduced overall inflammatory leukocytes, macrophages, and lymphocytes in the liver at advanced stages (8 wk) of steatohepatitis and fibrosis (Fig. 5D, Supplemental Fig. 1F). These experiments reveal an essential role of CXCR6 for progression of liver fibrosis in two independent models, and our data indicate that hepatic NKT cells might be critical for amplifying inflammatory responses via ensuing macrophage accumulation in the liver upon injury.

Hepatic NKT cells produce proinflammatory cytokines upon experimental liver injury

NKT cells are a heterogeneous population of T lymphocytes, which express NK and TCRs and are also capable of releasing different types of proinflammatory cytokines, including IL-4, IFN-γ, and TNF-α (14). We hypothesized that expression of these cytokines might be one of the main mechanisms by which NKT cells promote fibrogenesis. When we isolated hepatic NKT cells from control and injured livers by FACS sorting, WT NKT cells showed a significantly altered expression of $Ifn\gamma$ and $Il-4$, but not of $Tnf\alpha$ mRNA in CCl$_4$- and MCD-diet treated animals (Fig. 6A). $Cxcr6^{-/-}$ mice not only showed dramatically reduced hepatic NKT cell numbers (Fig. 1F); these NKT cells also lacked in $Il-4$ mRNA induction in both injury models (Fig. 6A).

Although we failed to detect significant protein concentrations of IL-4 in liver tissue, possibly because of low IL-4 levels in mice bred on a c57bl/6 background, we found significantly reduced IFN-γ protein levels in $Cxcr6^{-/-}$ mice at 8 wk of MCD diet (Fig. 6B). In line with attenuated macrophages in the MCD diet model, $Cxcr6^{-/-}$ mice also had significantly lower levels of intrahepatic TNF-α and MCP-1, suggesting that CXCR6-dependent NKT cell accumulation in liver injury represents an early event important for establishment and perpetuation of macrophage-dominated inflammatory responses in the injured liver.

Adoptive transfer of hepatic WT NKT cells restores liver fibrosis in $Cxcr6^{-/-}$ mice in vivo

We next aimed at providing functional evidence that hepatic NKT cells promote fibrogenesis in a CXCR6-dependent manner. Therefore, hepatic NKT cells were isolated from livers of congenic CD45.1 WT mice by FACS sorting (Fig. 7A). CD4 T cells, which also express CXCR6 to a considerable amount (Fig. 1E), were simultaneously sorted from livers as a control cell population (Fig. 7A). Isolated hepatic NKT or CD4 T cells were then adoptively transferred by i.v. injection once per week into either WT (CD45.2) or $Cxcr6^{-/-}$ mice that were subjected to 4 wk of an MCD diet. The adoptive transfer of hepatic NKT cells, but not of hepatic CD4 T cells, significantly increased collagen deposition in $Cxcr6^{-/-}$ mice, as indicated by Sirius Red staining and supported by trends in hydroxyproline content (Fig. 7B, 7C). Interestingly, NKT cell transfer did not increase fibrogenesis in WT mice (Fig. 7B, 7C), in contrast to experiments in which NKT cell activation was enhanced by α-GalCer (12), likely because of the nonphysiologic overstimulation of the NKT cells by α-GalCer or the low overall number of NKT cells (3–5 × 10^5 cells) that could be isolated for transfer from murine livers by sorting. Collectively, these data provide experimental evidence that the CXCR6-dependent accumulation of hepatic NKT cells in injured liver is an important pathogenic factor in modulating liver inflammation and promoting hepatofibrogenesis.

Discussion

Our study not only unravels an unrecognized function for chemokine receptor CXCR6 and its ligand CXCL16 in liver fibrosis; it also illuminates novel aspects of the central pathogenic involvement of CD1d-reactive iNKT cells in liver injury. Based on the finding that CXCR6 and CXCL16 were upregulated in livers of patients with chronic hepatic disorders, the experimental induction of liver fibrosis by two independent models in WT and $Cxcr6$-deficient mice revealed that CXCR6 critically controls the early accumulation of murine NKT cells in injured livers, which provide inflammatory cytokines, partake in early macrophage recruitment and late macrophage accumulation, promoting hepatofibrogenesis.

For this study, we used a novel two-photon laser-scanning microscopy imaging approach that allows in vivo real-time microscopic imaging with enhanced depth penetration, good optical sectioning, and excellent resolution in three dimensions. Its principle

FIGURE 6. Hepatic NKT cells produce proinflammatory cytokines upon experimental liver injury. (**A**) NKT cells were sorted from livers of c57bl/6 WT and $Cxcr6^{-/-}$ mice ($n = 3$) from untreated animals, 24 h after CCl$_4$ injection or after 4 wk of an MCD diet for gene expression analysis (quantitative PCR). (**B**) Cytokines were measured from total liver protein extracts by ELISA after 8 wk of an MCD diet ($n = 3$–5). All data are expressed as mean ± SD ($n = 3$–5 per group).*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (unpaired Student t test).
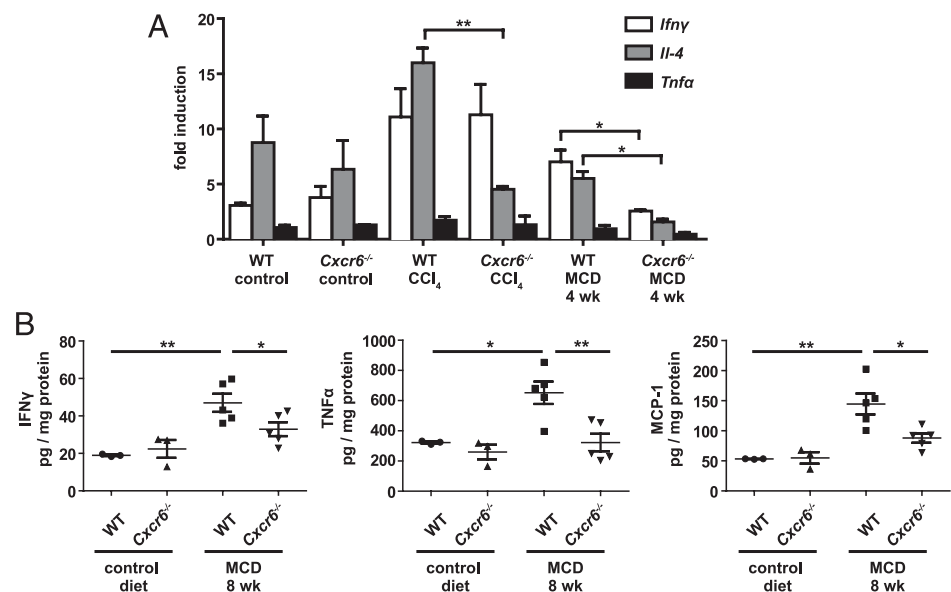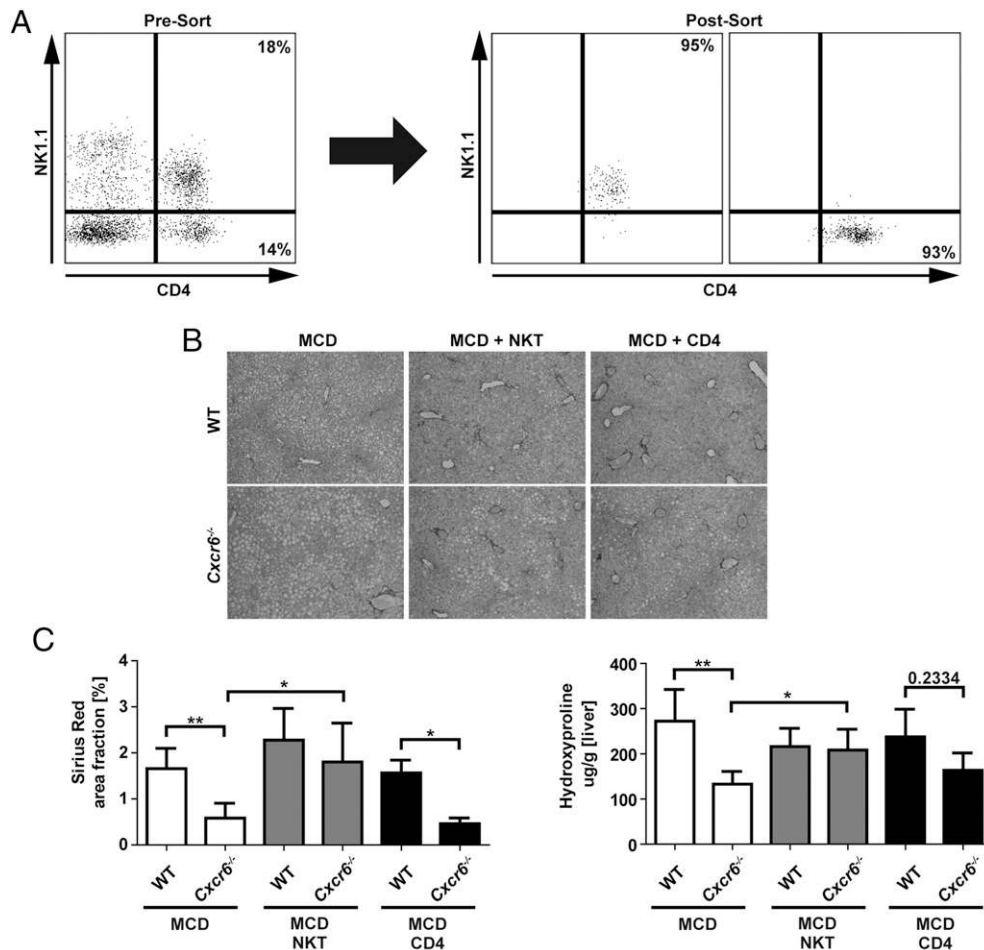
FIGURE 7. Adoptive transfer of hepatic WT NKT cells restores liver fibrosis in $Cxcr6^{-/-}$ mice in vivo. (**A**) NKT and CD4 T cells were sorted from congenic CD45.1 WT livers, representative FACS plots before and after sorting are displayed. (**B**, **C**) WT and $Cxcr6^{-/-}$ mice received 4 wk of an MCD diet and simultaneously once per week, $3–5 \times 10^5$ WT NKT or CD4 T cells i.v. Representative Sirius red stainings (B) and quantification of liver fibrosis (C). All data are expressed as mean $\pm$ SD ($n = 5$ animals in controls and $n = 6$ animals in NKT cell transfers per genotype). *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (unpaired Student t test). Original magnification $\times 50$.

application in liver has been demonstrated using frozen sections of human livers infected with hepatitis C virus (29). Few studies have applied this method for liver in living mice, mostly because of artifacts created by breath movements and the autofluorescence of hepatic tissue (27, 30). By using this novel, intravital multiphoton microscopy imaging approach that allows cell-specific kinetic studies upon injury induction alongside FACS phenotyping, it became apparent that CXCR6-expressing NKT cells contribute to establishing the inflammatory infiltrate and that numbers and migratory patterns of hepatic NKT cells are significantly affected by CXCR6 deficiency, at baseline and upon liver injury induction. The chemotaxis assays further corroborated that CXCR6 is directly involved in NKT cell migration.

Prior studies have suggested hepatic endothelium as the major source of CXCL16 in the liver in homeostasis (15), and cholangiocyte-derived CXCL16, secreted following Hedgehog-pathway-dependent crosstalk with HSC, as a strong stimulus for NKT cell migration in injury (31, 32). In our study, we could clearly demonstrate that also monocyte-derived inflammatory macrophages and resident Kupffer cells (defined as reported in Ref. 33) expressed high levels of CXCL16. We further demonstrated that *CXCL16* mRNA expression was enhanced in chronically injured, especially cirrhotic, human livers compared with control tissue, which is consistent with prior studies mainly conducted in hepatitis C–induced cirrhosis (21, 34). In its soluble form,

CXCL16 can be released by endothelial cells to mediate chemokine-dependent activation of leukocytes (35). In its transmembrane form, CXCL16 functions as an adhesion molecule, anchoring activated NKT cells (36). Our study revealed that these interactions are not only relevant in homeostatic conditions, in which $Cxcr6^{-/-}$ mice have considerably less hepatic NKT cells with a less motile migratory pattern. These interactions importantly provoke an NKT cell recruitment to the site of damage early in the course of hepatic injury. Of note, inflammatory cytokines like TNF or IFN-γ, which we found activated in our models, are capable of further inducing CXCL16 expression, suggesting an active amplification loop (35). The specificity of the CXCL16-dependent migration by iNKT cells is further corroborated by our in vitro migration experiments. These observations, and the fact that $Cxcr6^{-/-}$ mice also partially lack the massive macrophage accumulation observed later after damage, indicated that NKT cells initiate and accentuate the inflammatory response in the liver.

NKT cells are more abundant in the liver than in other organs, constituting up to 30% of the intrahepatic lymphocytes in mice (37). Their role in the pathogenesis of chronic liver injury and fibrosis is controversial. Experimental studies using Con A or α-GalCer demonstrated that NKT cell activation contributes to acute hepatitis (15, 16, 38), likely by releasing various proinflammatory cytokines and via Fas-mediated cell lysis (4). In HBV-transgenic mice, NKT cell activation promoted fibrosis progres-

sion by secreting HSC-activating cytokines IL-4 and IL-13 (39), similar to observations from patients with chronic hepatitis B (40) and consistent with the cytokine expression pattern we identified in hepatic NKT cells isolated from murine injury models in WT, but not $Cxcr6^{-/-}$ mice. In addition, $Cd1d^{-/-}$ mice lacking NKT cells were protected in thioacetamide-induced liver fibrosis (18) and in nonalcoholic steatohepatitis (19). Activation of NKT cells via α-GalCer promoted CCl_4-induced liver fibrosis (12). However, particularly in early phases of CCl_4-induced fibrosis (i.e., 2 wk of repetitive CCl_4 injections), NKT cells could also be protective by restricting HSC activation, thus assigning also antifibrotic functions to hepatic NKT cells in a distinct condition (12). In our study, $Cxcr6^{-/-}$ mice were protected from fibrosis progression in two independent models, alongside reduced hepatic macrophage infiltration and lower intrahepatic inflammatory cytokines, thereby indicating that the in vivo accumulation and activation of hepatic NKT cells upon injury accentuates the inflammatory response in the liver and promotes fibrogenesis. Importantly, the adoptive transfer of NKT cells isolated from WT livers restored the fibrosis phenotype of $Cxcr6^{-/-}$ mice.

Hepatic NKT cell numbers decreased during chronic liver injury, and this effect was most prominent in CCl_4-induced toxic fibrosis. The depletion of NKT cells during liver fibrosis progression has been observed previously (12) and could be related to either activation-induced NKT cell death or loss of cell markers such as NK1.1 (38, 41, 42). In the CCl_4 fibrosis model, NKT cell apoptosis in particular was found to account for reduced NKT cell numbers in chronic injury (12), and it had been speculated that the depletion of NKT cells abrogated their beneficial effects on stellate cells. Importantly, hepatic iNKT cell apoptosis has not been confirmed in human liver disease yet, possibly because of differences between the slow, decades-long, and continuous disease progression in humans compared with the harsh, rapid, and pulse-type injury induced by repeated CCl_4 injections in mice (4). In our study, we also observed depletion of NKT cells in both analyses using NK1.1/CD4 and the CD1d tetramer staining, thus supporting the hypothesis of NKT cell apoptosis upon liver injury in murine fibrosis models. However, hepatic NKT cell numbers were even lower by the NK1.1/CD4-based analysis compared with CD1d tetramer staining, indicating that not only hepatic NKT cell numbers and migration but also NKT cell maturation and activation might be regulated by CXCR6.

Our study further supports investigating CXCR6 or CXCL16 as therapeutic targets for fibrosis in subsequent studies. Preliminary experiments inhibiting CXCL16 by neutralizing Abs in acute liver failure models in mice indicated the general feasibility of such an approach (43). Nevertheless, interference with hepatic NKT cell functionality has to be investigated with great caution, because NKT cells influence the local inflammatory environment in the liver and are involved in systemic immune surveillance mechanisms (27).

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Disclosures

The authors have no financial conflicts of interest.

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