

## Antifibrotic Effects of CXCL9 and Its Receptor CXCR3 in Livers of Mice and Humans

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**BACKGROUND & AIMS:** Fibrosis is the hallmark of chronic liver diseases, yet many aspects of its mechanism remain to be defined. Chemokines are ubiquitous chemotactic molecules that mediate many acute and chronic inflammatory conditions, and CXC chemokine genes co-localize with a locus previously shown to include fibrogenic genes. We investigated the roles of the chemokine CXCL9 and its receptor CXCR3 in liver fibrosis. **METHODS:** The effects of CXCL variants on fibrogenesis were analyzed using samples from patients with hepatitis C virus infection and by induction of fibrosis in CXCR3<sup>-/-</sup> and wild-type mice. In mice, intrahepatic immune cell subsets were investigated and interferon gamma messenger RNA levels were measured at baseline and after injury. Human serum CXCL9 levels were measured and correlated with CXCL9 variant and fibrosis severity. The effects of stimulation with CXCL9 were investigated on human hepatic stellate cells (LX-2). **RESULTS:** Specific CXCL9 variants were associated with liver fibrosis in mice and humans; CXCL9 serum concentrations correlated with genotypes and levels of fibrosis in patients. In contrast to other chemokines, CXCL9 exerted antifibrotic effects in vitro, suppressing collagen production in LX-2 cells. CXCR3<sup>-/-</sup> mice had increased liver fibrosis; progression was associated with decreased numbers of intrahepatic interferon gamma-positive T cells and reduced interferon gamma messenger RNA, indicating that CXCL9-CXCR3 regulates Th1-associated immune pathways. **CONCLUSIONS:** This is the first description of a chemokine-based antifibrotic pathway in the liver; antifibrotic therapies might be developed to modulate CXC chemokine levels.

among the most prominent examples of the need to develop novel treatment options to halt progressive liver scarring that might otherwise result in liver cirrhosis and hepatocellular carcinoma. Notably, the reversibility of fibrosis in animal models has stimulated the enthusiasm for antifibrotic treatment strategies targeting critical regulatory pathways of liver fibrosis.<sup>2,3</sup>

The molecular mechanisms underlying liver fibrosis are complex and modulated by exogenous and endogenous factors.<sup>4</sup> Although some of these factors (eg, sex, alcohol consumption, and presence of diabetes) are well recognized, the identification of genes that regulate liver fibrosis in humans is challenging.<sup>5,6</sup> Most genetic studies lack reproducibility and do not provide functional data. Hence, we have proposed to identify modifier genes of polygenic diseases (such as liver fibrosis) in animal models first and validate these genes in human studies.<sup>7</sup> The identification of such conserved key regulatory genes and molecular pathways might help to identify new antifibrotic strategies for the regression of tissue injury.

Chemokines are ubiquitous chemotactic molecules that play major roles in acute and chronic inflammatory conditions.<sup>8</sup> They are divided into 4 families defined by the number of amino acids between the N-terminal cysteine residues (CC, CXC, CCX, and CX3C). The largest families are the CC and the CXC chemokines, members of which are expressed in acutely inflamed and fibrotic livers.<sup>9</sup> The CXC chemokine family is a pleiotropic family of molecules that are involved in the trafficking of various leukocyte subsets, angiogenesis, and vascular remodeling. This class of chemokines has been shown to play a pivotal role in pulmonary fibrosis.<sup>10</sup> Notably, the genes encoding CXC chemokines show an exceptional genomic organization, that is, the majority of them are clustered on

Liver fibrosis is a leading cause of morbidity and mortality worldwide. The main causes for advanced fibrosis are viral hepatitis, alcoholic liver disease, and metabolic conditions predisposing to nonalcoholic steatohepatitis.<sup>1</sup> Among these, chronic hepatitis C virus (HCV) infection and nonalcoholic steatohepatitis are

**Abbreviations used in this paper:** HCV, hepatitis C virus; HSC, hepatic stellate cell; hSNP, haplotype-tagging single nucleotide polymorphism; IFN, interferon; SNP, single nucleotide polymorphism.

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the short arm of chromosome 4q12-21 in humans and chromosome 5 in mice, respectively. This CXC chemokine gene cluster drew our attention, because it colocalizes with a chromosomal region that we identified in earlier studies by *in silico* mapping to include fibrogenic genes.<sup>11</sup>

We show here that the gene encoding the interferon (IFN)-inducible chemokine CXCL9 (MIG) underlies this association in mice and humans, uncovering a new immunologic pathway of liver fibrosis by functional studies *in vitro* and *in vivo*.

## Materials and Methods

### *Inbred Mice, Experimental Crosses, and Fibrosis Models*

F<sub>1</sub> hybrids of fibrosis-susceptible BALB/cJ and resistant A/J mice (Jackson Laboratory, Bar Harbor, ME) were crossed to obtain 358 F<sub>2</sub> progeny ([A/J × BALB/cJ]F<sub>1</sub> × [A/J × BALB/cJ]F<sub>1</sub>). Liver fibrosis in these mice was induced with CCl<sub>4</sub>.<sup>11</sup> The generation of CXCR3<sup>-/-</sup> mice on the C57BL/6J background has been described previously.<sup>12</sup> Male CXCR3<sup>-/-</sup> mice and wild-type littermates were challenged intraperitoneally with CCl<sub>4</sub> (0.6 mL/kg twice weekly) or with thioacetamide (150 mg/kg body wt thrice a week) for 6 weeks. Staging of liver fibrosis was performed as described.<sup>7</sup>

### *Quantitative Trait Locus Analysis*

A quantitative trait locus analysis of the *Cxc* chemokine cluster on murine chromosome 5 was performed in the F<sub>2</sub> progeny. Single nucleotide polymorphisms (SNPs) that tag the proximal, middle, and distal part of the *Cxc* chemokine cluster were identified at <http://snp.gnf.org/>. The identified SNPs (Figure 1A) were genotyped using 5'-nuclease (TaqMan) assays on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Supplementary Table 1 provides the primer and probe sequences for the allele-specific polymerase chain reactions. Regression analysis was calculated with Map Manager QTX (<http://www.mapmanager.org/>).

### *Expression Analysis of Murine Chemokines and Cytokines*

Total RNA was isolated from livers of mice and reversely transcribed using SuperScript (Invitrogen, Carlsbad, CA). Quantitative reverse-transcription polymerase chain reaction was performed for *Cxcl9*, *Cxcl10* and *Cxcl11*, *Cxcr3*, and *Ifn-γ* with Assays on Demand from <http://www.appliedbiosystems.com>.

### *Fluorescence-Activated Cell Sorter Analysis of Intrahepatic IFN-γ-Positive Immune Cells*

At baseline and after treatment of mice with CCl<sub>4</sub>, we performed flow cytometry analysis. Livers were flushed with phosphate-buffered saline and homogenized. Mononuclear cells were isolated by density gradi-

ent centrifugation. After 4-hour stimulation with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL; both Sigma, Hamburg, Germany), 10 μg/mL brefeldin was added. The cells were stained with CD45-APC-Cy7 and CD3-APC (BD Biosciences, Heidelberg, Germany). After permeabilization, the cells were incubated with IFN-γ-PE or rat immunoglobulin G/PE (isotype specific control) and analyzed by flow cytometry using the FACS Canto II flow cytometer (BD Biosciences).

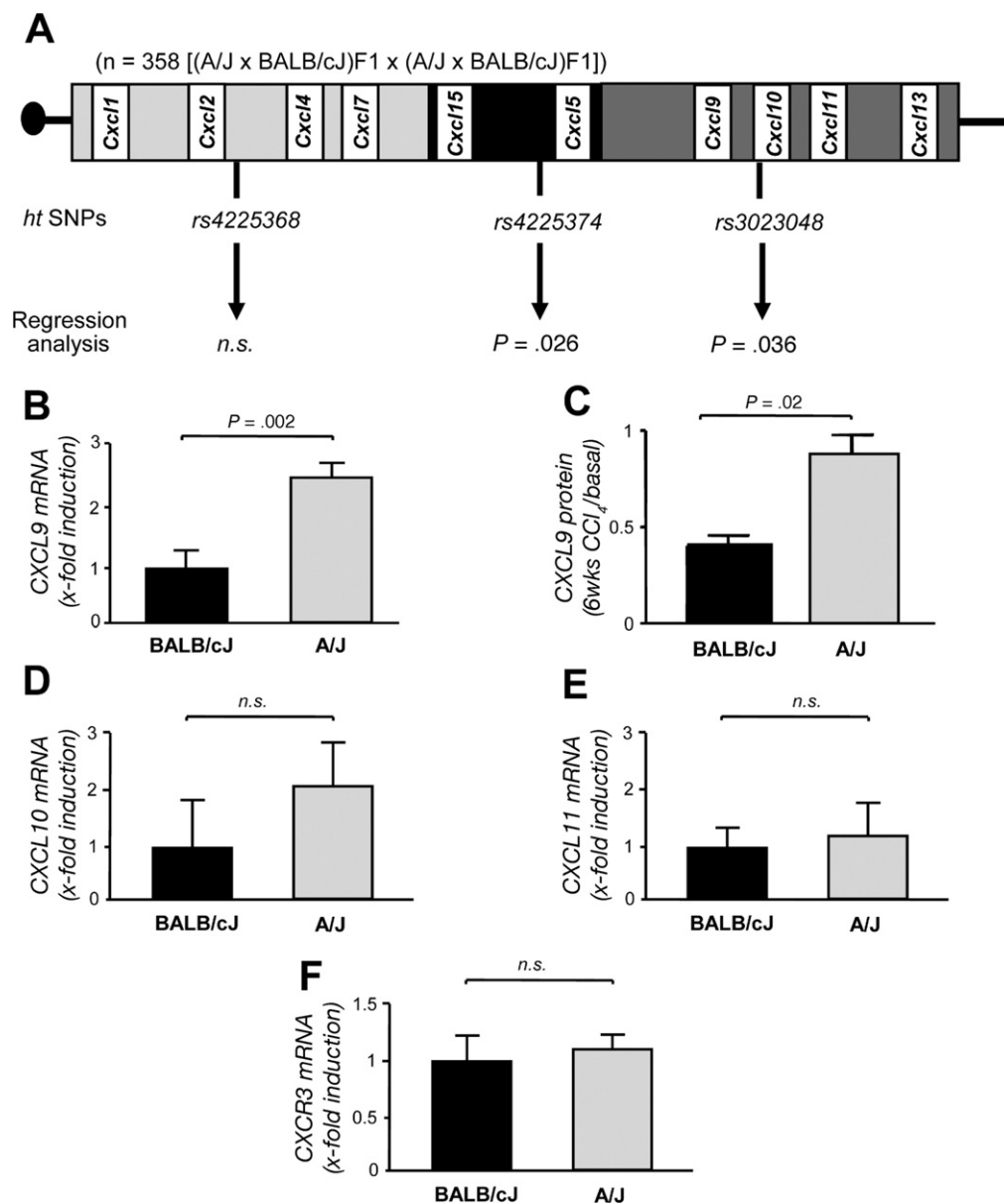
### *Human Study Populations*

Two independent cohorts of white HCV-infected patients were enrolled in the study (together 441 individuals). The first cohort was from the university hospitals of Aachen, Bonn, and Regensburg, and the second cohort was recruited at Charité in Berlin. HCV infection was confirmed by a positive HCV RNA test result (Roche Diagnostics, Mannheim, Germany). Other chronic liver diseases were excluded by appropriate serologic tests. We also genotyped 232 healthy white individuals for the evaluation of CXC chemokine cluster haplotypes and Hardy-Weinberg equilibrium. The demographic data of the cohorts are given in Supplementary Table 2. All subjects were asked for the ethnicity of their second-degree relatives to minimize systematic differences in ancestry that might lead to population stratification.<sup>13</sup> Informed consent was obtained from patients, and the protocol was approved by the local ethics committees. Liver biopsy specimens were obtained from all HCV-infected patients before antiviral treatment and were analyzed according to Desmet et al.<sup>14</sup>

### *Human Genetic Analysis*

All haplotype-tagging SNPs (htSNPs) of the chromosomal region that harbors the genes for IFN-inducible chemokines (4q21: 77,380–77,420 kilobases) were selected from the International HapMap Project (Supplementary Figure 1). Using the HapMap data, we identified 8 SNPs with minor allele frequencies ≥0.05, which cover the genetic region of interest (Figure 2A). In our control cohort, 5 haplotypes with a frequency >5% were identified (Supplementary Table 3). These SNPs were genotyped in 404 white patients with mild (F0–F1) or severe (F2–F4) liver fibrosis. Thirty-seven patients of the total cohort were excluded due to insufficient amounts of DNA for genotyping all 8 SNPs. The haplotypes were named in order of frequency (the most prevalent haplotype was *dCXC\_1*) (Figure 2B).

Haplotype distributions between the patient cohorts were compared by permutation testing, as implemented in PHASE 2.0.<sup>15</sup> We reproduced our haplotype results with the Haploview 2.0 program, which supports linkage disequilibrium and haplotype block analysis; haplotype population frequency estimation; single SNP, global, and haplotype-specific association tests; and permutation testing for association significance.<sup>16</sup> Subsequently, the frequencies of the most likely haplotypes as inferred by PHASE were compared between patients with mild and



**Figure 1.** Murine quantitative trait locus and gene expression analysis. (A) Schematic diagram of the *Cxcl* chemokine cluster on chromosome 5 (90,207,953 to 94,974,241 base pairs). The SNPs *rs4225368*, *rs4225374*, and *rs3023048* tag the proximal, middle, and distal part of the gene cluster. Regression analysis in the F<sub>2</sub> progeny showed that the SNPs tagging the middle and distal part of the cluster are significantly associated with the histologic stage of liver fibrosis induced by CCl<sub>4</sub> ( $P = .026$  and  $P = .036$ , respectively). (B–F) Quantitative expression of IFN-inducible chemokines and their receptor *Cxcr3* in BALB/cJ and A/J mice after challenge with CCl<sub>4</sub>. Whereas the mRNA expression levels of (D) *Cxcl10*, (E) *Cxcl11*, and (F) *Cxcr3* do not differ between the strains, the expression of *Cxcl9* was 2.3-fold increased in the resistant strain ( $P = .002$ , B). (C) The increased mRNA expression of CXCL9 was confirmed by CXCL9 protein analysis (C).

severe fibrosis. To further validate the robustness of the genetic data, the *dCXCL4* htSNP rs3733236 C>T was analyzed independently in both cohorts of patients (Supplementary Table 4). Tests for association were performed by Fisher exact test for comparison of allele frequencies and with Cochran–Armitage trend test (<http://ihg.gsf.de>) for comparison of genotypes. We chose the stage of fibrosis as the main outcome variable in the analysis. However, the exact duration of infection was available in a subgroup of study subjects (n = 243). In these subjects, we also analyzed the association of the *CXCL9* genotype with progression of fibrosis (fibrosis stage/duration in years).

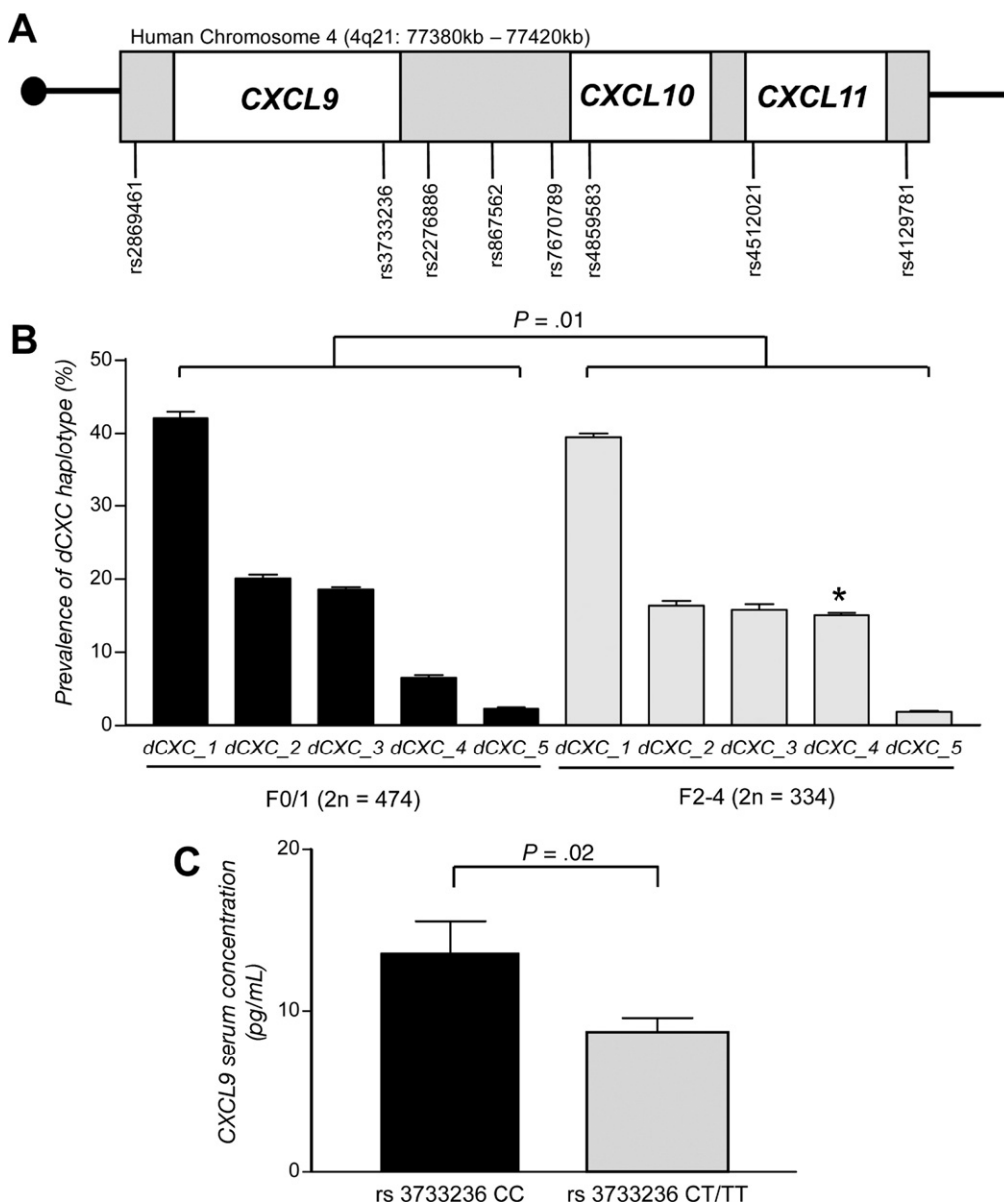
#### Genotyping of Human SNPs and Direct Sequencing of CXCL9

Genotyping was performed with 5′-nuclease (TaqMan) assays on the ABI PRISM 7000 System. Assays for

genotyping were obtained from Applied Biosystems. Polymerase chain reaction amplification of the 4 exons and adjacent splice sites plus 300 base pairs upstream of the start codon of the human *CXCL9* gene was performed using primers and annealing temperature conditions as listed in Supplementary Table 5.

#### Isolation and Stimulation of Stellate Cells

Culture of the human hepatic stellate cell (HSC) line LX-2 has previously been described.<sup>17</sup> For chemokine stimulation, the serum content was reduced, and cells were stimulated with recombinant human IFN- $\gamma$ , CXCL9, or CXCL4 (R&D Systems, Wiesbaden, Germany) for 48 hours. Total messenger RNA (mRNA) was isolated and collagen protein levels were determined by Western blot analysis after isolation of total protein from cultures. CXCR3 isoform mRNA was amplified with primers



**Figure 2.** Human genetic analyses. (A) Schematic diagram of the distal part of the human CXC chemokine cluster on chromosome 4q21 (77,380–77,420 kilobases). Eight SNPs cover the genes for the chemokines CXCL9, CXCL10, and CXCL11. (B) These SNPs were genotyped in 404 patients with mild (stage F0/F1, 2n = 474) or advanced (stage F2–F4, 2n = 334) liver fibrosis. The overall haplotype distribution is different between patients with mild fibrosis and subjects with severe fibrosis ( $P = .01$ ). Specifically, the fourth most common haplotype, dCXC\_4, is significantly more prevalent in individuals with advanced fibrosis (15.0%) compared with patients with mild fibrosis (6.4%;  $P = .001$ ). (C) dCXC\_4 is tagged by rs3733236 in the 3'-untranslated region of CXCL9. The minor (risk) allele of this SNP is functionally associated with reduced serum concentrations in patients with HCV infection and mild fibrosis (n = 60,  $P = .02$ ).

specific for the A and B splice variants.<sup>18</sup> Quantification of mRNA was performed with SYBR green and  $\beta$ -actin as internal control. Crucial experiments performed in LX-2 cells were reproduced in primary myofibroblasts.

#### **CXCL9 Expression Analysis in Human Fibrotic Liver**

We isolated total RNA from formalin-fixed, paraffin-embedded liver biopsy specimens from 53 patients with HCV and 42 patients with nonalcoholic fatty liver disease (20 with fatty liver; 22 with steatohepatitis and fibrosis) as described.<sup>19</sup> Quantitative reverse-transcription polymerase chain reaction for CXCL9, CXCR3-A, and CXCR3-B was performed as described previously.

#### **CXCL9 and CXCL10 Serum Concentrations**

Serum levels of CXCL9 and CXCL10 were determined in 180 HCV-infected patients. Serum was taken at

the time of liver biopsy and analyzed by cytometric bead array (BD Pharmingen, Heidelberg, Germany). Serum levels of both chemokines were assessed with regard to stage of fibrosis by analysis of variance. For genotype-phenotype correlation, CXCL9 serum levels were measured in 60 patients with stage 0 and 1 fibrosis in which the CXCL9 rs3733236 genotype was determined as described.

#### **Immunohistochemistry of CXCL9 in Human Liver Tissue**

Immunohistochemical staining was performed with a mouse monoclonal anti-human CXCL9 antibody (R&D Systems).

#### **Statistical Analysis**

Statistical analysis was performed with GraphPad Prism and InStat (GraphPad Software, La Jolla, CA).<sup>19</sup>



Data are given as means and SEM. Categorical variables are compared with Fisher exact test and continuous variables with Student *t* test with Welsh correction in case of unequal variances. Multiple logistic regression analysis was performed with stage of fibrosis as the dependent variable and genotype, age, and sex as covariates. Continuous variables in more than 2 groups were compared by analysis of variance.

## Results

### *The Distal Cxc Chemokine Cluster Determines the Severity of Liver Fibrosis in Experimental Crosses of Inbred Mice*

We first performed a quantitative trait locus analysis in the F<sub>2</sub> progeny from an experimental cross between the fibrosis-susceptible mouse strain BALB/cJ and the resistant A/J strain (Figure 1A). In this cross, regression analysis showed that the SNPs tagging the middle and distal parts of the cluster (*rs4225374* -  $0.3 \pm 0.2$  cM - *rs3023048*) are significantly associated with the histologic stage of liver fibrosis ( $P = .026$  and  $P = .036$ , respectively; Figure 1A). Notably, the distal part of the cluster harbors the genes of the Ifn-inducible chemokines *Cxcl9*, *Cxcl10*, and *Cxcl11*, which are of particular interest because IFN- $\gamma$  is suggested to exert antifibrotic effects.<sup>20,21</sup>

### *The Chemokine Cxcl9 Is Differentially Expressed in Fibrosis-Susceptible and Fibrosis-Resistant Mice*

We therefore analyzed the hepatic mRNA expression of these genes and their receptor *Cxcr3* in the parental strains after CCl<sub>4</sub> treatment. As depicted in Figure 1B and C, we observed a significant difference in *Cxcl9* mRNA and protein expression between BALB/cJ and A/J mice ( $P = .002$  and  $P = .02$ , respectively), whereas mRNA levels of *Cxcl10*, *Cxcl11*, and *Cxcr3* did not significantly differ between the strains (Figure 1D–F).

### *The Distal Part of the Orthologous Human CXC Chemokine Cluster Is Associated With the Severity of Liver Fibrosis in Patients With Chronic Hepatitis C*

We next performed a haplotype cluster analysis of 8 SNPs in 404 HCV-infected patients with mild (F0/F1) and severe liver fibrosis (F2–F4). As in our control population (Supplementary Table 1), 5 common haplotypes (frequency >0.05) of the distal CXC (*dCXC*) cluster were identified in HCV-infected patients. The overall haplotype distribution did not differ between our total patients' cohort and the control cohort (data not shown). However, the distribution of *dCXC* haplotypes between patients with mild fibrosis and individuals with severe fibrosis differed significantly ( $P = .01$ ) by permutation testing with 2 independent programs.<sup>15,16</sup> Specifically, the fourth most frequent haplotype (*dCXC\_4*) is significantly

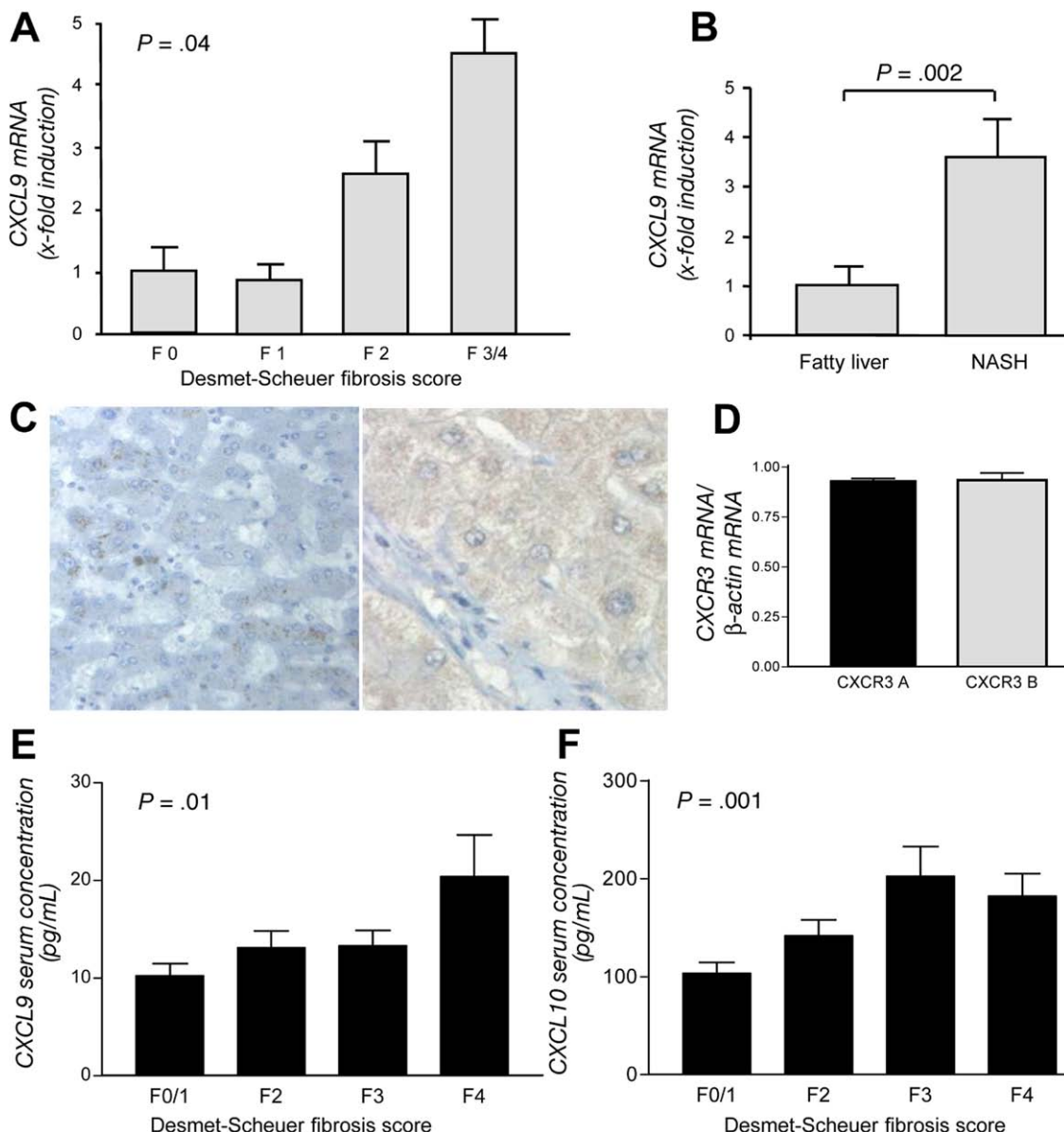
more prevalent in subjects with severe fibrosis (15.0%) compared with patients with mild fibrosis (6.4%,  $P = .001$ , Figure 2B). This haplotype is tagged by an SNP in the 3'-untranslated region of *CXCL9* (*rs3733236*). Genetic association of this *ht*SNP with the severity of liver fibrosis was evident in both investigated cohorts of HCV-infected patients (Supplementary Table 1). The association of the *CXCL9 ht*SNP with liver fibrosis held true after correction for sex and age in multiple logistic regression analysis. The duration of HCV infection was only available in a subgroup of patients ( $n = 243$ ). Despite the resulting loss of statistical power, progression of fibrosis was markedly higher in carriers of *rs3733236* T alleles (0.38 fibrosis stages/year) compared with individuals homozygous for the C allele (0.27 fibrosis stages/year). The association of *rs3733236* with liver fibrosis is not due to linkage with common coding variants in our patients upon sequencing of *CXCL9* exons (data not shown). Notably, when analyzing the serum concentrations of *CXCL9* in relation to *rs3733236* alleles, we found significantly reduced *CXCL9* levels in patients carrying the T allele (Figure 2C), suggesting a functional significance of the *ht*SNP. This analysis was performed in patients with only mild fibrosis to exclude independent effects of the fibrosis stage on *CXCL9* serum levels.

### *CXCL9 Increases With More Advanced Stages of Fibrosis in Humans*

We next assessed the intrahepatic expression of *CXCL9* at different stages of liver fibrosis. As shown in Figure 3A, the mRNA expression of *CXCL9* significantly increases with advanced stages of HCV-induced liver fibrosis ( $P = .04$ ). An increase in *CXCL9* mRNA levels with regard to fibrosis was confirmed in liver biopsy specimens from patients with nonalcoholic steatohepatitis with and without liver fibrosis ( $P = .002$ , Figure 3B) and by immunohistochemistry in advanced HCV-induced fibrosis (Figure 3C). We also assessed the mRNA expression of the splice variants A and B of the *CXCL9* receptor *CXCR3* in HCV-infected liver. As shown in Figure 3D, both splice variants were equally expressed. The elevated intrahepatic levels of *CXCL9* mRNA were also reflected by elevated serum concentrations at higher stages of liver fibrosis ( $P = .01$ , Figure 3E). This association is not specific for *CXCL9* but is also found for serum levels of *CXCL10*, another *CXCR3* agonist ( $P = .001$ , Figure 3F).

### *CXCL9 Exerts Antifibrotic Effects In Vitro*

We next assessed the functional relevance of *CXCL9* with regard to activated stellate cells. Isolated primary HSCs display low basal *CXCL9* mRNA expression, which significantly ( $P = .02$ ) increases after IFN- $\gamma$  stimulation of the cells (Figure 4A). *CXCL9* specifically binds to the chemokine receptor *CXCR3*, which is expressed on HSCs.<sup>22</sup> Interestingly, stellate cells express equal amounts of both splice variants of the receptor



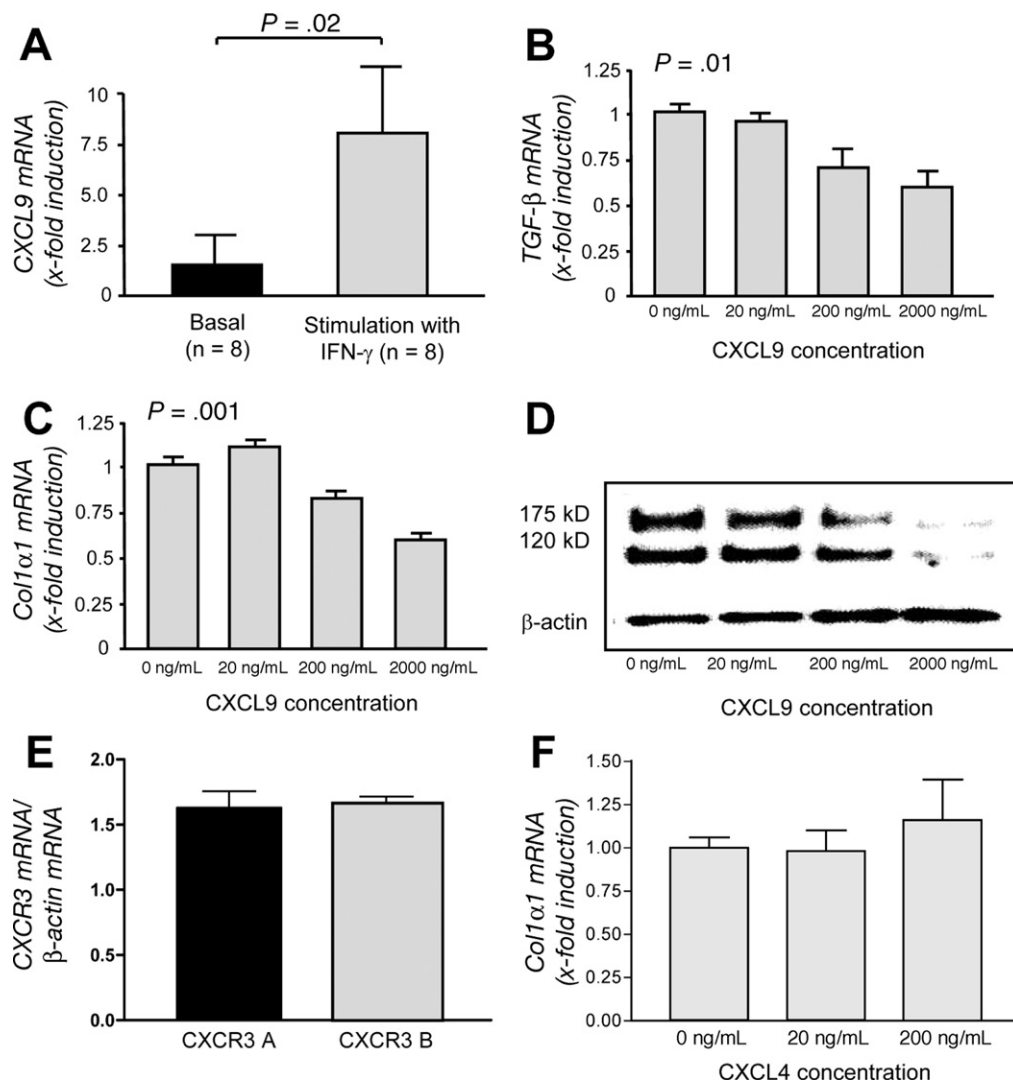
**Figure 3.** Analyses of CXCL9 in human liver fibrosis. (A) CXCL9 mRNA expression is significantly associated with the degree of liver fibrosis in HCV ( $P = .04$ ). (B) Association of CXCL9 mRNA expression with liver fibrosis is confirmed in patients with fibrosis due to nonalcoholic steatohepatitis ( $P = .002$ ). (C) Immunohistochemistry of CXCL9 confirms a strong up-regulation in chronic liver damage (normal liver, left; HCV-induced cirrhosis, right). (D) The mRNA of both splice variants of the CXCL9 receptor CXCR3 are present in HCV-infected liver. (E) CXCL9 and (F) CXCL10 serum concentrations are also associated with the severity of liver fibrosis in HCV infection ( $P = .01$  and  $P = .001$ , respectively).

(Figure 4E). We tested the direct effects of CXCL9 on profibrogenic markers of LX-2 and primary stellate cells. As shown in Figure 4B and C, increasing concentrations of CXCL9 lead to dose-dependent down-regulation of transforming growth factor  $\beta 1$  ( $P = .01$ ) and collagen  $1\alpha 1$  mRNA ( $P = .001$ ). Importantly, collagen protein expression of stellate cells was strongly suppressed by CXCL9 (Figure 4D and Supplementary Figure 2). We also treated LX-2 cells with CXCL4, an agonist for CXCR3-B,<sup>18</sup> to determine specific effects of the CXCR3-B splice variant. As depicted in Figure 4F, CXCL4 had only minor effects on collagen mRNA expression in LX-2 cells. This

was also true for *TIMP-1* and *TGF- $\beta 1$*  mRNA (data not shown).

#### Genetic Deletion of the CXCL9 Receptor CXCR3 Leads to Increased Fibrosis In Vivo

Based on these results, we hypothesized that genetic deletion of the specific CXCL9 receptor CXCR3 should lead to increased liver damage in vivo. We thus challenged CXCR3<sup>-/-</sup> mice and wild-type littermates for 6 weeks with CCl<sub>4</sub>. Upon histologic examination, CXCR3<sup>-/-</sup> animals indeed displayed significantly higher stages of fibrosis (Figure 5A and C). This exaggerated



**Figure 4.** Functional analysis of CXCL9 in stellate cells. (A) CXCL9 mRNA is expressed in HSCs and is significantly increased ( $P = .02$ ) after treatment of the cells with IFN- $\gamma$ . (B and C) Stimulation of LX-2 cells with CXCL9 leads to a dose-dependent down-regulation of TGF $\beta$  ( $P = .01$ ) and COL1A1 mRNA expression ( $P = .001$ ), respectively. The columns represent mean values  $\pm$  SEM of 3 independent experiments. (D) The down-regulation of collagen by CXCL9 in HSCs is confirmed by Western blot with an antibody detecting the immature (175 kilodaltons) and mature (120 kilodaltons) chain of collagen. (E) Both splice variants of the CXCL9 receptor CXCR3 are present in LX-2 cells. (F) However, stimulation of the CXCR3-B isoform with CXCL4 leads only to a modest increase in COL1A1 mRNA.

fibrogenic response in CXCR3 $^{-/-}$  mice was confirmed by increased concentrations of the collagen-specific amino acid hydroxyproline in knockout animals ( $P = .03$ , Figure 5B). Next we extended the findings of an exaggerated fibrogenic response in CXCR3 $^{-/-}$  mice to a second fibrosis model, thioacetamide, which was chronically administered for 6 weeks. As shown in Figure 5D–F, lack of CXCR3 resulted in increased stages of fibrosis ( $P = .02$ ) and significantly higher hydroxyproline concentrations in knockout mice compared with their wild-type littermates ( $P = .004$ ), confirming the results in the CCl $_4$  model.

#### Increased Fibrosis in CXCR3 $^{-/-}$ Mice Is Associated With a Blunted Intrahepatic IFN- $\gamma$ Response

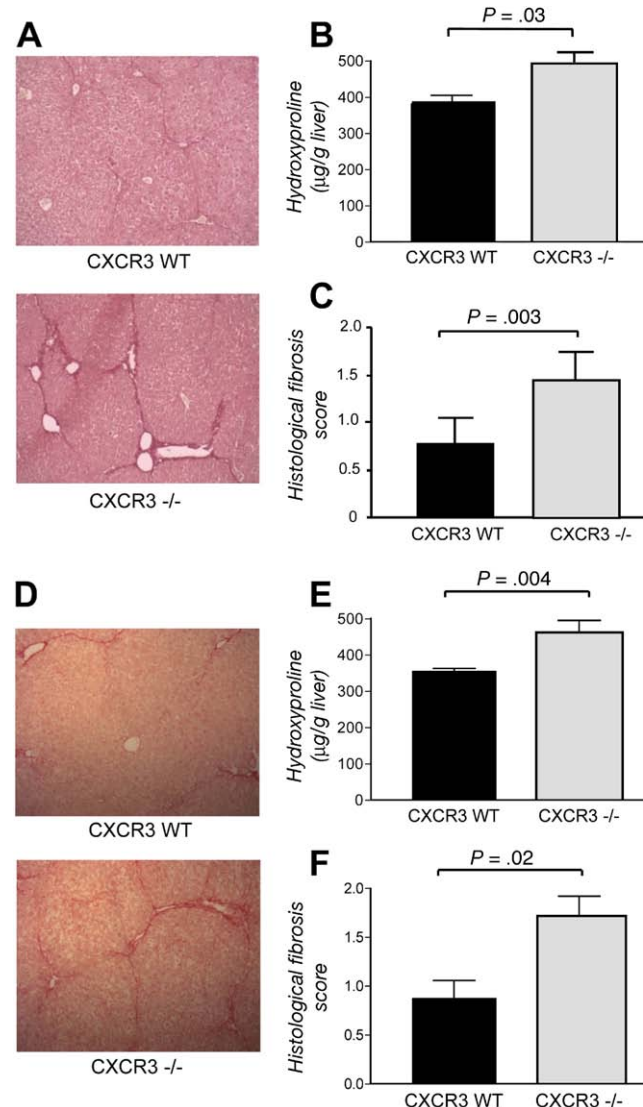
Because the CXCL9/CXCR3 system mediates the recruitment of immune cells upon liver injury, we next analyzed the hepatic mRNA expression of IFN- $\gamma$  at baseline and after treatment with CCl $_4$ . As depicted in Figure 6A, CXCR3 $^{-/-}$  mice had significantly decreased IFN- $\gamma$  mRNA levels compared with wild-type mice after induc-

tion of fibrosis ( $P = .04$ ). To validate these findings, we next performed fluorescence-activated cell sorter analysis for intrahepatic IFN- $\gamma$ -positive T cells before and after CCl $_4$  treatment. As shown in Figure 6B and C, the recruitment of IFN- $\gamma$ -positive T cells into the liver after CCl $_4$  challenge is reduced in CXCR3 $^{-/-}$  mice compared with their wild-type littermates ( $P = .01$ ). In contrast to the fibrotic features, the necroinflammatory response is not significantly altered in CXCR3 $^{-/-}$  mice as shown by histology (Figure 6D) and aspartate aminotransferase/alanine aminotransferase levels (Figure 6E).

#### Discussion

We here identify the CXCL9/CXCR3 system as an antifibrotic pathway of liver fibrosis in vivo and in vitro. The concordant results in murine models and the data obtained in patients with chronic liver diseases suggest that the CXCL9/CXCR3 pathway is conserved between species and might therefore be an attractive target for the evaluation of antifibrotic treatment strategies.





**Figure 5.** In vivo evidence for a role of CXCR3 for liver fibrosis. (A) Representative Sirius red stainings of wild-type and CXCR3<sup>-/-</sup> mice after challenge with CCl<sub>4</sub>. (B) Augmented fibrosis in CXCR3<sup>-/-</sup> mice is validated by increased concentrations of hydroxyproline ( $P = .03$ ) and (C) significantly higher semiquantitative fibrosis scores ( $P = .003$ ). (D) The exaggerated fibrogenic response in CXCR3<sup>-/-</sup> mice is confirmed in the thioacetamide model of liver fibrosis as shown by representative Sirius red stains. Increased deposition of collagen in CXCR3<sup>-/-</sup> animals is shown by (E) hydroxyproline concentrations ( $P = .004$ ) and (F) increased fibrosis scores ( $P = .02$ ).

As a first approach to identify novel mediators of fibrogenesis, we performed extensive genetic analysis in inbred mice and patients with HCV infection. In both species, we identified variants in the genetic region of IFN-inducible chemokines that are statistically associated with the degree of liver fibrosis. In mice, the associated gene variants were fine mapped within the distal part of the *Cxcl* chemokine cluster, a region that has been linked to experimental fibrosis in an earlier study.<sup>11</sup> However, identification of new genes and related pathways has been hampered by the lack of reproducibility and scarce

functional data. To circumvent these limitations, the Complex Trait Consortium<sup>23</sup> has proposed that new candidate genes identified in experimental crosses should be validated by functional analyses.

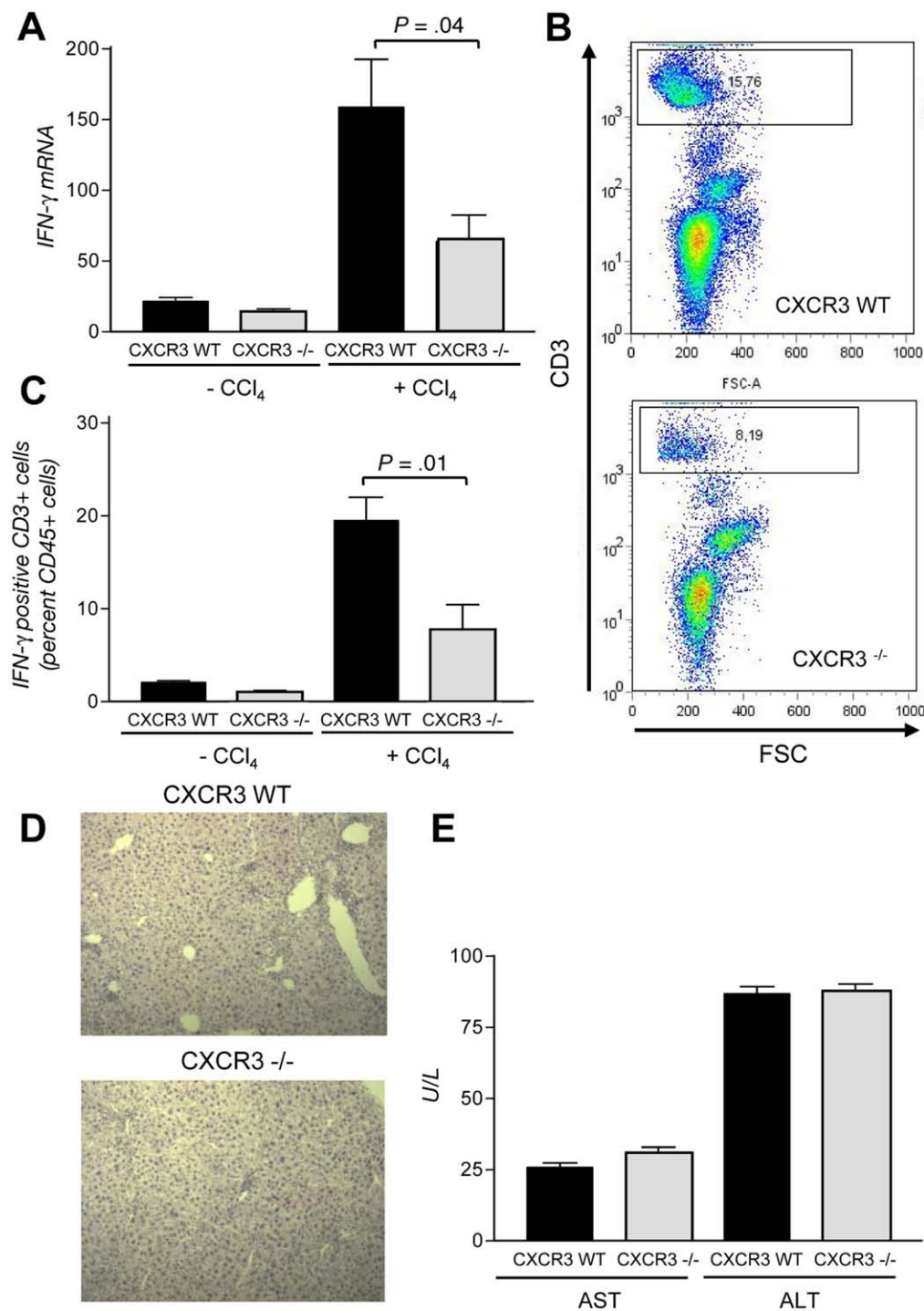
We therefore performed expression analysis in the parental strains of our experimental cross and found that *Cxcl9* is differentially expressed on the mRNA and protein level between BALB/cJ and A/J mice. Interestingly, protein and mRNA expression of *Cxcl9* was higher in the resistant strain, suggesting a potential antifibrotic action of this chemokine, which is expressed by hepatocytes,<sup>24</sup> endothelial cells,<sup>25</sup> and HSCs.<sup>26</sup> In infectious murine models, *Cxcl9* is strongly up-regulated in the liver during viral infection, thereby enhancing T-cell infiltration.<sup>27</sup> Notably, in accordance with our results, *Cxcl9* has already been shown to have protective roles in viral-induced encephalitis and hepatitis.<sup>28,29</sup>

To further dissect the functional role of *Cxcl9*, we performed experiments in CXCR3<sup>-/-</sup> mice in which the specific receptor for *Cxcl9* is deleted.<sup>12</sup> Consistent with our genetic and expression results, these mice displayed pronounced liver fibrosis in 2 independent models of chronic liver injury. Enhanced fibrosis in CXCR3<sup>-/-</sup> mice was associated with reduced mRNA expression levels of *Ifn-γ* and a reduction of *Ifn-γ*-positive intrahepatic T cells. Importantly, such a Th2-dominated cytokine environment has already also been associated with enhanced pulmonary and renal fibrosis in CXCR3<sup>-/-</sup> mice.<sup>30,31</sup>

Interestingly, we were able to validate the genetic findings in mice by haplotype analysis in patients with HCV infection. In this cohort, an *ht*SNP in the 3'-untranslated region of *CXCL9* was associated with severe fibrosis with odds ratios that are expected for genetically complex diseases.<sup>32</sup> We did not analyze mRNA stability, but the risk allele of this SNP was functionally associated with reduced serum levels of *CXCL9* in our patients. Because the risk allele is overrepresented in subjects with severe fibrosis, the reduced serum concentrations of the IFN-inducible *CXCL9* are a first hint for a potential antifibrotic role of this chemokine in humans as well. Whether genetic variation in the *CXCL9* receptor CXCR3 also contributes to the findings in our cohort needs to be investigated, but only one SNP has yet been identified in CXCR3 in white subjects ([www.hapmap.org](http://www.hapmap.org)). Nevertheless, while the antifibrotic effects of IFN and related pathways are well proven in mice,<sup>33</sup> the evidence is less convincing in humans. In fact, the largest intervention trial with IFN- $\gamma$  for reversion of HCV-induced liver fibrosis did not show an overall beneficial effect of this cytokine.<sup>34</sup>

However, subgroup analysis indicated that individuals with a strong up-regulation of IFN-inducible chemokines do benefit from IFN- $\gamma$ .<sup>34</sup> We therefore further investigated the role of *CXCL9* in humans by measuring intrahepatic and peripheral concentrations. In line with recent studies,<sup>35,36</sup> intrahepatic *CXCL9* mRNA and protein in-





**Figure 6.** Immunologic characterization of CXCR3 $^{-/-}$  mice. (A) Deletion of CXCR3 significantly alters the intrahepatic *Ifn- $\gamma$*  mRNA expression after induction of fibrosis ( $P = .04$ ). (B) Representative fluorescence-activated cell sorter analysis of intracellular *Ifn- $\gamma$* -positive T cells in fibrotic livers of CXCR3 $^{-/-}$  mice and wild-type littermates. (C) Abundance of CXCR3 leads to a significant reduction of *Ifn- $\gamma$* -positive T cells after CCl<sub>4</sub> administration for 6 weeks ( $P = .01$ ). Despite changes in fibrotic features, the necroinflammatory response is not significantly altered in CXCR3 $^{-/-}$  mice as demonstrated by (D) histology and (E) aspartate aminotransferase/alanine aminotransferase levels.

creased with higher stages of fibrosis. We extend these results by showing higher CXCL9 mRNA levels in non-alcoholic steatohepatitis and a correlation between CXCL9 serum levels and liver fibrosis. At this stage, it is difficult to explain why a potentially antifibrotic chemokine is elevated at higher stages of fibrosis. This might be due to differences in IFN responsiveness between species and the known differences in CXCR3 isoform expression between mice and humans,<sup>18,37</sup> but more experiments are clearly warranted to mechanistically resolve these aspects.

Interestingly, HSCs are known to express CXCR3.<sup>22</sup> Thus, we assessed the direct effects of CXCL9 on HSCs and LX-2 cells<sup>17</sup> in vitro, revealing reproducible repression of collagen mRNA and protein. However, the antifibrotic action of CXCL9 on HSCs could be counterbalanced by a greater inflammatory infiltrate associated with this chemokine, leading to increased fibrosis, despite the direct effects of CXCL9 on stellate cells. We also acknowledge that the antifibrotic action of CXCL9 on stellate cells might not be strong enough to ultimately cause a

reduction in fibrosis in human diseases. Notably, HSCs expressed both splice variants of CXCR3, which can mediate opposite effects.<sup>38</sup> We therefore also stimulated LX-2 cells with CXCL4, a selective ligand for CXCR3-B.<sup>18</sup> As shown in Figure 4F, CXCL4 had only minor effects on collagen mRNA expression. Therefore, the observed effects of CXCL9 on stellate cells do not seem to be mediated by the CXCR3-B isoform. However, CXCL4 has been shown to interact with other chemokines and might thus mediate effects via other receptors on HSCs, for example, by interacting with the CCR5 ligand CCL5.<sup>39</sup>

In summary, we here identify CXCL9 and its receptor CXCR3 as regulators of liver fibrosis in mice and provide first data for a role of this chemokine system in patients with HCV infection. These findings reinforce the involvement of mediators classically associated with the innate immune system in liver regeneration and chronic injury<sup>40</sup> and set the stage for further evaluation of this chemokine system as a potential antifibrotic target.

## Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2009.03.053.

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#### *Conflicts of interest*

The authors disclose no conflicts.

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**Supplementary Table 1.** Primers and Probes for Murine SNP Analysis

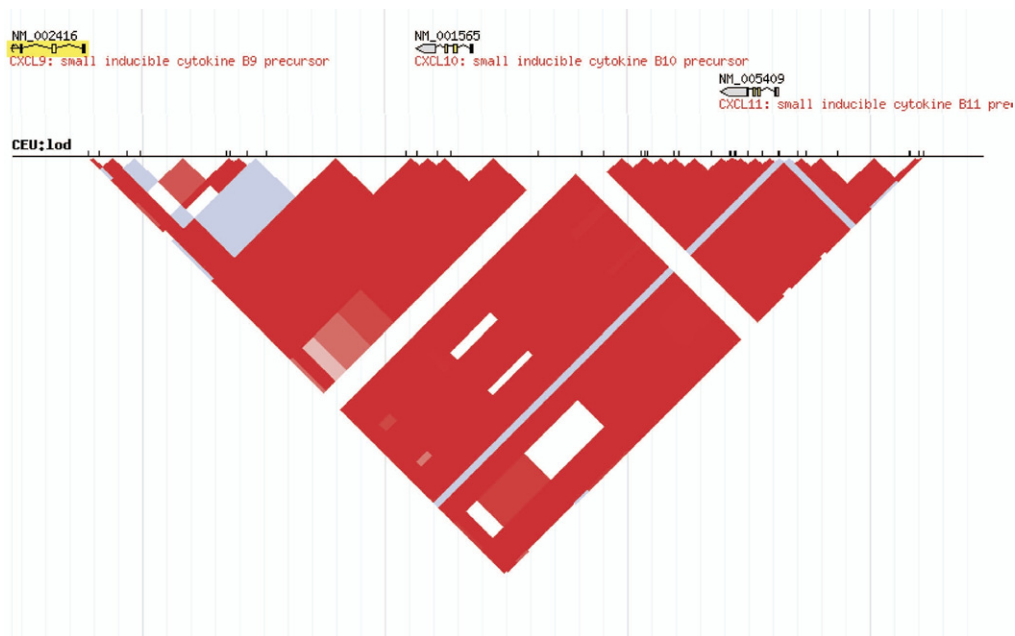
1. rs4225368
forward primer cagtgtttctgcagtccacatg
reverse primer acagacctcagcagacatactct
VIC actccaaagtgtatacaag
FAM ctccaaagtgtatacaag
2. rs4225374
forward primer cgggtccaggttctccttgag
reverse primer tctgaacgagaaaaggaaggttctg
VIC ccgtgcatcctca
FAM ccgtgcgtcctca
3. rs3023048
forward primer caaaagtaactgccgaagcaagaat
reverse primer gacagccatcccagcca
VIC ctgagtcctcactcaag
FAM tgagtcctcgtcaag

**Supplementary Table 2.** Demographic Data of Study Cohorts in Haplotype Analysis

Parameter	HCV-infected patients with mild fibrosis (n = 237)	HCV-infected patients with severe fibrosis (n = 167)	Controls (n = 232)
Age (y) <sup>a</sup>	44 ± 17	50 ± 11	56 ± 18
Sex (M/F)	150/87	105/52	142/90
Alcohol consumption (g/day)	<20	<20	NA
Hepatitis B surface antigen positive	0	0	0
Diabetes (%)	13.4	15	12.5

NA, not available.

<sup>a</sup>Data are given as mean ± SD.



Supplementary Figure 1. Heatmap of Linkage equilibrium in the genotype CXC cluster.

Supplementary Table 3. Haplotype Analysis of the Distal CXC Cluster in Healthy Individuals

SNP/haplotype	rs2869461 C>T	rs3733236 C>T	rs2276886 C>T	rs867562 C>T	rs7670789 G>A	rs4859583 A>T	rs4512021 A>G	rs4129781 C>G	Frequency (%)
dCXC_1	C	C	C	C	<b>G</b>	A	A	G	41.1
dCXC_2	C	C	<b>T</b>	C	A	A	G	C	23.5
dCXC_3	C	C	C	<b>T</b>	A	A	G	C	15.7
dCXC_4	T	<b>T</b>	C	C	A	A	G	C	7.5
dCXC_5	C	C	C	C	A	A	G	C	5.2

NOTE. Haplotypes of the distal part of the CXC chemokine cluster were determined in 232 healthy individuals (2n = 464) without liver fibrosis. Haplotypes were reconstructed by the PHASE 2.0 algorithm. htSNPs were identified by the BEST algorithm.

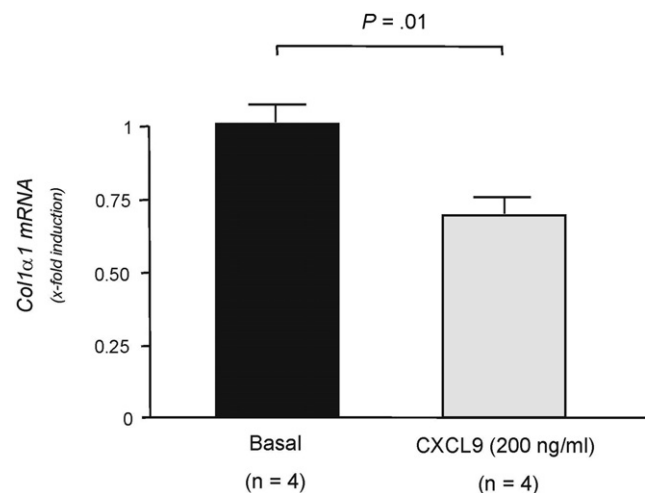
**Supplementary Table 4.** Genotype Analysis of rs3733236 in all HCV-Infected Patients

Aachen/Bonn/Regensburg					
CXCL9 <i>ht</i> SNP	No. (frequency) of alleles/genotypes		Tests for association <sup>a</sup>		
	Mild fibrosis (2n = 292)	Severe fibrosis (2n = 158)	Odds ratio	Confidence interval	P
rs3733236 T	275 (94.2)	138 (87.3)	2.34	1.19–4.62	.018
rs3733236 C	17 (5.8)	20 (12.7)			
rs3733236 CC	129 (88.4)	61 (77.2)	3.35		.012
rs3733236 CT	17 (11.6)	16 (20.3)			
rs3733236 TT	0 (0)	2 (2.5)			
Berlin					
CXCL9 <i>ht</i> SNP	No. (frequency) of alleles/genotypes		Tests for association <sup>a</sup>		
	Mild fibrosis (2n = 232)	Severe fibrosis (2n = 200)	Odds ratio	Confidence interval	P
rs3733236 C	221 (95.3)	180 (90.0)	2.23	1.36–4.18	.040
rs3733236 T	11 (4.7)	20 (10.0)			
rs3733236 CC	105 (90.5)	80 (80.0)	2.39		.027
rs3733236 CT	11 (9.5)	20 (20.0)			
rs3733236 TT	0 (0)	0 (0)			

<sup>a</sup>Tests for association were performed by 2-sided Fisher exact test for comparison of allele frequencies and with Cochran–Armitage trend test (<http://ihg.gsf.de>) for comparison of genotypes, respectively. The odds ratio is calculated in the direction of having a severe liver fibrosis in carriers of the T allele or the CT or TT genotype.

**Supplementary Table 5.** Primer Sequences and Annealing Temperatures for Polymerase Chain Reaction Amplifications of Coding Regions of CXCL9

Amplified region	Sequence (5' → 3')	Annealing temperature (°C)
Exon 1		
F	TGTGCCAAAGGCTATCAGTG	62
R	GCAGGCAGAACATTCACCTT	
Exon 2		
F	GCAGGCAGAACATTCACCTT	62
R	GCAAGCACTCCAAGGTAAA	
Exon 3		
F	CAACCAGTGACTCATCTTGTC	48
R	TGAAAAGCAGATTCTTTGTCTTCT	
Exon 4		
F	GCCCCTCTGAGAGTGAAGTG	51
R	TGGTGGGTGGTAGAAGAACA	

**Supplementary Figure 2.** Stimulation of primary human microfibroblasts with rh CXCL9. Col 1  $\alpha$  1 mRNA was determined by RT-PCR (see Materials and Methods). Experiments were performed in microfibroblasts from four different donors in triplicates.