

Loss of Discoidin Domain Receptor 2 Promotes Hepatic Fibrosis after Chronic Carbon Tetrachloride through Altered Paracrine Interactions between Hepatic Stellate Cells and Liver-Associated Macrophages

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Hepatic stellate cells (HSCs) interact with fibrillar collagen through the discoidin domain receptor 2 (DDR2) in acute hepatic injury, generating increased fibrosis. However, the contribution of DDR2 signaling to chronic liver fibrosis *in vivo* is unclear, despite its relevance to chronic human liver disease. We administered carbon tetrachloride (CCl₄) to *DDR2*^{+/+} and *DDR2*^{-/-} mice twice weekly, and liver tissues and isolated HSCs were analyzed. In contrast to changes seen in acute injury, after chronic CCl₄ administration, *DDR2*^{-/-} livers had increased collagen deposition, gelatinolytic activity, and HSC density. Increased basal gene expression of osteopontin, transforming growth factor- β 1, monocyte chemoattractant protein-1, and IL-10 and reduced basal gene expression of matrix metalloproteinase-2, matrix metalloproteinase-13, and collagen type I in quiescent *DDR2*^{-/-} HSCs were amplified further after chronic CCl₄. In concordance, *DDR2*^{-/-} HSCs isolated from chronically injured livers had enhanced *in vitro* migration and proliferation, but less extracellular matrix degradative activity. Macrophages from chronic CCl₄-treated *DDR2*^{-/-} livers showed stronger chemoattractive activity toward *DDR2*^{-/-} HSCs than *DDR2*^{+/+} macrophages, increased extracellular matrix degradation, and higher cytokine mRNA expression. In conclusion, loss of DDR2 promotes chronic liver fibrosis after CCl₄ injury. The fibrogenic sinusoidal milieu generated in chronic *DDR2*^{-/-} livers recruits more HSCs to injured regions, which enhances fibro-

sis. Together, these findings suggest that DDR2 normally orchestrates gene programs and paracrine interactions between HSCs and macrophages that together attenuate chronic hepatic fibrosis. (Am J Pathol 2011, 179:2894–2904; DOI: 10.1016/j.ajpath.2011.09.002)

Hepatic fibrosis is a wound-healing response to chronic injury characterized by failure of regeneration and inflammatory responses that provoke deposition of a fibrous collagen-rich extracellular matrix (ECM).^{1–3} Hepatic stellate cell (HSC) activation orchestrates the fibrogenic and inflammatory response to chronic liver injury. Activation of HSCs is initiated by paracrine stimulation from injured neighboring cells.^{4–6} In particular, soluble factors released by liver-associated macrophages within the fibrotic milieu render HSCs susceptible to a wide number of cytokines, chemokines, and growth factors that promote their migration and accumulation at the sites of tissue repair. Activated HSCs synthesize the collagenous scar tissue, and secrete both gelatinases that degrade the normal ECM matrix [matrix metalloproteinase (MMP)-2 and MMP-9], and collagenases that degrade fibrillar collagen (MMP-1, MMP-8, and MMP-13 in humans and MMP-13 in mice).^{7,8} They also contribute to the immunoregulatory response to injury through the release of chemokines and cytokines.⁹

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Chronic fibrosis develops as autocrine stimuli, perpetuating HSC synthesis of excessive collagenous ECM. As a result, signaling of ECM receptors in the HSCs is altered and contributes to a vicious cycle of inflammatory and fibrogenic stimulation.^{3,10} HSCs express two types of collagen receptors: integrins¹¹ and discoidin domain receptors (DDR).¹² DDR2 is an unusual receptor because, although it belongs to the tyrosine kinase family, it signals in response to fibrillar collagen instead of soluble growth factors.^{13,14} Increased DDR2 expression occurs in humans during development and in pathologic processes, such as osteoarthritis, cancer, and cirrhosis,^{15,16} and DDR2 expression also is induced in experimental alcoholic liver fibrosis.¹⁷

Based on DDR2's unique responsiveness to collagen ligands and potential role in transducing signals from the accumulating collagen in liver injury, we compared the responses of wild-type (*DDR2*^{+/+}) and DDR2-deficient (*DDR2*^{-/-}) mice with chronic liver injury by CCl₄. Although our earlier studies explored the contribution of DDR2 receptors to HSC responses after acute injury, their characterization in a chronic model of injury was essential to model human liver disease. Our results indicate that the absence of DDR2 in mice exacerbates injury, leading to increased liver fibrosis. The data also underscore the importance of ECM receptors in the interaction between HSCs, macrophages, and the ECM in the generation of a fibrotic response.

Materials and Methods

Animals

DDR2 knock-out mice (*DDR2*^{-/-}) were generated as described.¹⁸ Heterozygous DDR2 knock-out/LacZ knock-in mice were kindly donated by Regeneron Pharmaceuticals (Tarrytown, NY).

Model of Liver Injury

To induce liver injury, 6- to 8-week-old male *DDR2*^{+/+} and *DDR2*^{-/-} mice were administered biweekly doses of 0.5 μ L of CCl₄ per gram of body weight by gavage diluted in olive oil.¹² Control animals received olive oil. As in our prior studies,¹² we considered "acute" injured livers those submitted to a single gavage dose of 0.5 μ L of CCl₄ per gram. They were analyzed 3 days after intoxication.

Immunohistochemical Detection of DDR2

Liver sections (10 μ m thick) fixed in cold acetone were processed for H&E stain or reacted with 1:100 dilution of polyclonal antibody (pAb) R2-JM, raised against the juxtacrine domain of mouse DDR2 (amino acids 429–557), followed by the appropriate fluorescent secondary antibody.

For the detection of β -galactosidase activity, livers were fixed in 0.4% (v/v) glutaraldehyde, 2 mmol/L MgCl₂, and 5 mmol/L EGTA in a sodium phosphate buffer (77

mmol/L NaHPO₄/22 mmol/L NaH₂PO₄), washed three times for 15 minutes in a solution containing 2 mmol/L MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in sodium phosphate buffer, and stained for 2 to 4 hours at 30°C in the washing solution containing 1 mg/mL X-gal, 5 mmol/L potassium ferricyanide, and 5 mmol/L potassium ferrocyanide. Livers then were rinsed in phosphate-buffered saline (PBS) and cleared in a benzyl benzoate:benzyl-alcohol (2:1) solution.

Immunohistochemistry and Image Analysis

Picosirius red staining was performed as previously described.¹⁹ Liver sections (10 μ m thick) fixed in cold acetone were processed with 1:50 dilution of anti-human α -smooth muscle actin (α -SMA) monoclonal antibody (Sigma Chemical Co, St. Louis, MO), rabbit anti-mouse desmin polyclonal antibody (1:200; Dako, Glostrup, Denmark), followed by the appropriate fluorescent secondary antibodies. Irrelevant appropriate immunoglobulins were used as negative controls. Images were quantified using the AnalySIS V3.2 program (Olympus Software Imagine Solutions GMBH, Munster, Germany). Results were expressed as a percentage of specifically colored tissue area relative to the whole analyzed area.

Histologic Analysis of Macrophages

Liver sections (10 μ m thick) fixed in cold acetone were immunostained with F4/80 antibody (1:100), followed by the appropriate fluorescent secondary antibody. Stained cells were counted in 10 randomly selected high-power fields. In some studies, scar-associated macrophages were counted. A positive cell was included only if it was within or apposed to the scar.²⁰

DDR2 Immunodetection

Immunoprecipitation/Western blot analysis for DDR2 was performed as previously described¹² using pAb R2-JM. In brief, primary rat stellate cell cultures were lysed in radio immunoprecipitation assay buffer containing protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN), 2 mmol/L sodium orthovanadate, and 10 mmol/L sodium fluoride. Lysate (250 μ g) was incubated with R2-JM polyclonal anti-DDR2 Ab for 1 hour at 4°C. Protein A-Sepharose beads (Sigma Chemical Co) were added for 1 hour at 4°C, then precipitated by centrifugation, and washed three times with radio immunoprecipitation assay buffer. The protein A-Sepharose-bound proteins were extracted by 10-minute incubation in Laemmli buffer at 80°C. Protein aliquots (80 to 300 μ g) were separated through 8% SDS-PAGE electrophoresis and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). DDR2 was detected using the R2-JM anti-DDR2 Ab (1:1000) and horseradish-peroxidase-conjugated protein A (1:20,000). Phosphorylated DDR2 was detected using the antiphosphotyrosine monoclonal Ab 4G10 (1:20,000; Upstate Biotechnology, Inc, Lake Placid, NY) and

horseradish-peroxidase-conjugated goat anti-mouse IgG (1:20,000). The bands were visualized using the Super Signal Femto Substrate kit (Pierce Chemical Co, Rockford, IL).

Gelatinase and Collagenase Activities

Flash-frozen liver samples were homogenized in 100 mmol/L Tris, 200 mmol/L NaCl, 0.1% Triton X-100 (pH 7.4). The homogenates were centrifuged at $10,000 \times g$ for 20 minutes at 4°C. Gelatinolytic and collagenolytic activity in the supernatant of tissue homogenates and in supernatants from cell cultures were determined using biotinylated gelatinase or collagenase substrates and detected at OD 450 nm following the manufacturer's instructions (Chemicon International). To activate latent MMPs, conditioned media were incubated for 2 hours at 37°C with 2.5 mmol/L APMA (*p*-aminophenylmercuric acetate; Sigma Chemical Co.).²¹

Isolation and Primary Culture of HSCs and Liver Macrophages from $DDR2^{+/+}$ and $DDR2^{-/-}$ Mice

Quiescent hepatic sinusoidal cells from untreated mice and *in vivo* activated ones from mice treated for 10 days with CCl_4 were isolated as described.²² In brief, enzymatic perfusion was followed by isopycnic gradient centrifugation of dispersed liver cells placed on top of a triple layer of Percoll solutions (50%, 31%, and 24%). HSCs were collected from the 24%/31% Percoll solution interphase and macrophages from the 31%/50% interphase. All cells were centrifuged to remove cell debris and re-suspended in serum-free RPMI-1640 media at 1.5×10^6 cells/mL. HSCs were shed onto uncoated plastic wells and nonadhered cells, collected after 15 minutes. As a result, HSC cultures were 99% macrophage-free. Then, HSCs immediately were submitted to RNA extraction or were plated on culture wells coated with a 0.5-mm layer of collagen type I (Becton Dickinson). Media was changed and cells were maintained in RPMI-1640 media plus 5% fetal calf serum (FCS) for up to 48 hours. Plate efficiency was 81 ± 20 . For migration experiments, HSCs were placed on the upper compartment of a migration well.

We used HSCs from untreated mice as quiescent HSCs. They contained high amounts of large vitamin A droplets and of the succinate receptor (GPR91) mRNA, two markers of quiescent HSCs.²³ As previously reported,²³ we used HSCs isolated from livers treated for 10 days with CCl_4 as the source of *in vivo* activated HSCs. Finally, some quiescent HSCs were activated *in vitro* by culture on plastic surface in RPMI-1640 media plus 5% FCS for 7 days, detached with ice-cold EDTA solution in PBS, and plated onto migration wells for migration experiments. Both *in vivo* and *in vitro* activated HSCs contained small vitamin A droplets while expressing α -SMA, desmin, and very low amounts of GPR91 mRNA (data not shown). They did not display signs of apoptosis (data not shown). Western blot revealed that DDR2 expression and

phosphorylation were detectable in quiescent $DDR2^{+/+}$ HSCs after 16 hours of culture on collagen type I, and strongly expressed by the *in vivo* activated $DDR2^{+/+}$ HSCs (data not shown). Conditioned media was obtained on the second day of culture after 16 hours under serum-free conditions.

Macrophages from untreated and fibrotic livers were cultured for 16 to 24 hours at 1.5×10^5 cells/mL of RPMI-1640 media plus 5% FCS. On average, 86% of the cells expressed F4/80. Conditioned media was obtained on the second day of culture after 16 hours under serum-free conditions.

Cell Growth Assays

Cell proliferation was measured using the nontoxic alamarBlue assay (BioSource International, Camarillo, CA), according to the manufacturer's instructions. In brief, freshly isolated HSCs were cultured on top of a thin matrix of collagen type I in RPMI-1640 plus 1% FCS.¹² AlamarBlue was added, and absorbance at 595 nm and 575 nm was measured every 8 hours using a Perkin Elmer HTS700-Plus plate reader (Applied Biosystems, Foster City, CA). Results were expressed as a percentage of reduced alamarBlue.

Cell Migration Through Type I Collagen

Cell migration assays were performed as described previously¹² with minor modifications. Culture inserts with 8- μ m pores coated with 0.001% collagen type I (Becton Dickinson) were placed on top of 24-well plates. HSCs suspended in RPMI-1640 supplemented with 0.5% FCS were placed on the culture insert. Macrophages were isolated from $DDR2^{-/-}$ or $DDR2^{+/+}$ fibrotic livers, and their conditioned media was used as chemoattractants in the lower compartment of $DDR2^{-/-}$ or $DDR2^{+/+}$ HSC migration chambers. In other experiments, RPMI-1640 supplemented with 10% FCS was used as a chemoattractant. Cells were allowed to migrate for 20 hours. Non-migrated cells were removed with a cotton swab. Cells that migrated and adhered to the underside of the membrane were fixed in 100% methanol, stained with 0.2% crystal violet, and counted in 10 random, high-power fields per membrane.

Quantitative RT-PCR

Total RNA from whole liver lysates or freshly isolated cells was extracted using RNeasy columns (Qiagen GmbH, Hilden, Germany). RNA quantity and quality were analyzed with a capillary 2100 Bio-analyzer (Agilent Technologies, Palo Alto, CA). Quantitative RT-PCR was performed using the commercially available primer sets SYBR Green PCR Core Reagent kit and the Gene Amp 5700 sequence detection system (all from Applied Biosystems).

Statistics

Results refer to mean \pm SEM. Comparisons among groups were performed with the Student's *t*-test, and *P* values less than 0.5 were considered significant. Each experiment was performed in duplicate or triplicate and repeated at least two times with independent samples derived from at least two mouse livers or two independent primary cultures.

Results

Chronic CCl₄ Injury Increases Fibrogenic and ECM Remodeling Responses and Alters Cell Recruitment to the Fibrotic Scars in DDR2-Deficient Livers

Based on the role of DDR2 in ECM signaling, we sought to assess the impact of DDR2 deficiency on the hepatic response to sustained liver injury. *DDR2*^{+/+} and *DDR2*^{-/-} mice were injected chronically with CCl₄ for up to 30 days. Control animals received olive oil. By quantitative RT-PCR analysis of whole liver lysates, collagen α 1(I) mRNA expression was equal between *DDR2*^{+/+} and *DDR2*^{-/-} mice that were oil-treated and at day 4, after a single acute CCl₄, and slightly but not significantly increased after a 10-day CCl₄ administration in *DDR2*^{-/-} livers compared to *DDR2*^{+/+} fibrotic livers. CCl₄ treatment for an additional 20 days rendered the relative amount of collagen type I mRNA in *DDR2*^{-/-} fibrotic livers approximately twofold higher than in *DDR2*^{+/+} mice (Figure 1A). Morphometric quantitation of Picrosirius red staining confirmed an equal accumulation of fibrillar collagen in the fibrotic tissue of all mice after 10 days of CCl₄ administration, whereas it was 2.5-fold greater in *DDR2*^{-/-} mice at day 30 (Figure 1, B–D). In contrast, there was no difference in the extent of injury. Specifically, at both days 3 and 30 of CCl₄ administration, hepatocyte damage was similar in both *DDR2*^{+/+} and *DDR2*^{-/-} livers based on similar levels of centrilobular necrosis, and serum alanine aminotransferase (270 and 258 mL/L), aspartate aminotransferase (170 and 160 IU/L), and albumin levels (20 and 24 g/dL).

ECM remodeling is another key feature of liver fibrosis. Gelatinolytic and collagenolytic (G/C) activities were below detection levels in untreated and oil-treated livers (data not shown). Liver tissue extracts after acute injury contained low G/C activities (data not shown) that reached measurable levels after 10 days of treatment, a time point at which *DDR2*^{-/-} extracts contained 50% less activity than *DDR2*^{+/+} (Figure 2A). As expected, further treatment with CCl₄ increased G/C activity in all of the livers. G/C activity in *DDR2*^{+/+} mice was augmented by 60%. Interestingly, the increase in the gelatinolytic activity in the *DDR2*^{-/-} livers was 2.8-fold higher than in the *DDR2*^{+/+} livers, whereas the increase in collagenolytic activity was not significantly different (Figure 2B).

MMPs are the main enzymes responsible for G/C activities in the fibrotic liver.²⁴ MMP-2 and MMP-9 are the main mouse liver gelatinases, and MMP-13 is the main

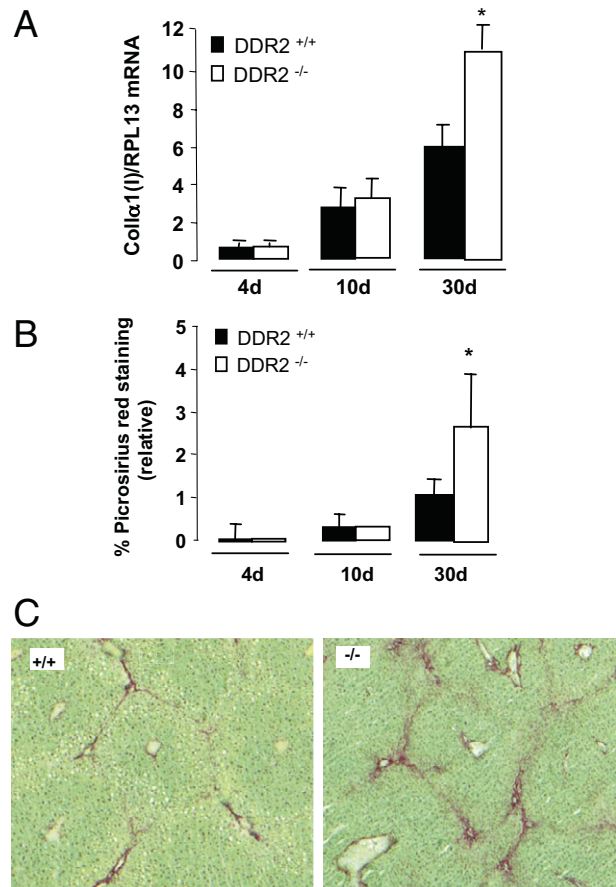


Figure 1. Collagen expression in *DDR2*^{-/-} and *DDR2*^{+/+} livers after chronic CCl₄. **A:** Real-time quantitative analyses of collagen type I (I) mRNA after 10 and 30 days of treatment with CCl₄. Results are expressed as a ratio of collagen type I mRNA versus RPL13 mRNA. **B:** Computer-assisted semi-quantitation of picrosirius red-stained livers after 10 and 30 days of treatment with CCl₄. Results are expressed as the percentage of region of interest. **P* < 0.05, significantly different compared with *DDR2*^{+/+} group. **C:** Picrosirius red staining of collagen deposits in *DDR2*^{+/+} and *DDR2*^{-/-} livers treated for 30 days with CCl₄.

collagenase. Mouse DDR2 signaling is associated with MMP-2 and MMP-10 activities, whereas MMP-9 activity is DDR2-independent.^{25,26} Figure 2C documents significantly lower levels of MMP-2 and MMP-13 mRNAs, and equal levels of MMP-9 in fibrotic *DDR2*^{-/-} livers after a 10-day treatment, compared with *DDR2*^{+/+} livers. As observed for G/C activities, chronic treatment induced MMP mRNA expression. However, *DDR2*^{-/-} livers expressed 2.1-fold greater MMP-2 mRNA and 30% more MMP-13 mRNA, which correlated with their augmented collagenolytic activity (Figure 2D). These results indicate that during persistent injury, DDR2 expression inversely correlates with hepatic gelatinase, probably resulting from reduced MMP-2 activity.

All untreated and control livers contained similar levels of desmin/ α -SMA-stained cells. As previously described,²⁷ desmin/ α -SMA-expressing spindle-shaped cells increased significantly in sinusoidal segments around centrilobular veins after chronic CCl₄ treatment. This occurred regardless of *DDR2* gene expression. However, we observed a higher density of desmin and α -SMA-expressing cells at bridging fibrotic tissue in

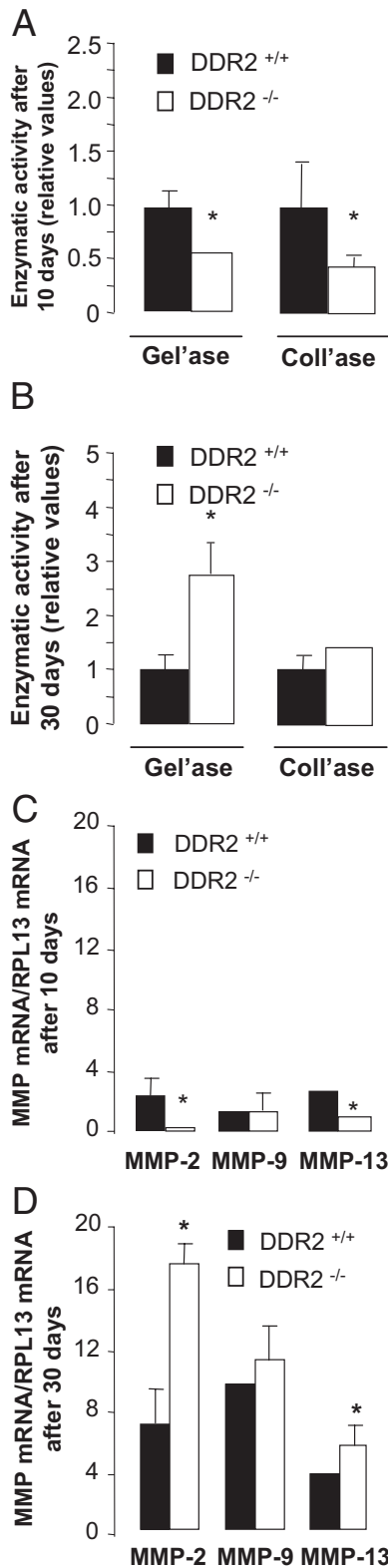


Figure 2. Gelatinase and collagenase activities in livers of *DDR2*^{-/-} and *DDR2*^{+/+} mice after CCl₄ administration. Relative gelatinase (A) and collagenase (B) activities in tissue extracts from *DDR2*^{-/-} and *DDR2*^{+/+} livers after 10 or 30 days of treatment with CCl₄. For each time point, enzymatic activity in *DDR2*^{+/+} livers was considered 1. Real-time quantitative analyses on MMP-2, MMP-9, and MMP-13 mRNA induction in *DDR2*^{-/-} and *DDR2*^{+/+} livers after 10 days (C) and 30 days (D) of treatment with CCl₄. Results are expressed as a ratio versus RPL13 mRNA. **P* < 0.05, significantly different compared with *DDR2*^{+/+} group.

DDR2^{-/-} mice that became significantly different at day 10, and reached a threefold difference at day 30 (Figure 3, A and B). These data correlate inversely with DDR2 expression in recruitment of activated HSCs to fibrotic scars of livers after chronic CCl₄.

Immunostaining of fibrotic livers revealed increased numbers of F4/80 macrophages as fibrosis developed, both within the sinusoids and opposite the collagenous septa surrounding the periportal regions.^{20,28–30} This effect was observed irrespective of the DDR2 status (data not shown). Despite this, Figure 3C shows that recruitment of F4/80 macrophages to the scar tissue of *DDR2*^{-/-} livers was 40% lower by day 10 and remained so at day 30. Both untreated and control livers contained similar levels of F4/80-stained sinusoids regardless of DDR2 expression, whereas acute treatment showed 18% reduced staining in *DDR2*^{-/-} tissue (data not shown).

These results indicated that chronic CCl₄ intoxication induced a more vigorous fibrogenic response associated with altered cell recruitment in *DDR2*^{-/-} mice. Although we previously described DDR2 mRNA and protein in isolated rat HSCs 48 hours after an acute dose of CCl₄ or after culture activation,¹² the contribution of DDR2 to chronic liver fibrosis was not established. Therefore, we next analyzed *in situ* hepatic DDR2 expression after CCl₄ administration.

DDR2 Expression Is Enhanced in HSCs of Livers Treated Chronically with CCl₄

Immunohistochemical analysis using a polyclonal antibody against murine DDR2 (see *Materials and Methods*) displayed minimal DDR2 protein expression in control livers (Figure 4A). Ten days of CCl₄ administration stimulated DDR2 expression in spindle-shaped cells within fibrotic scars (Figure 4B), and fibrotic lesions after 30 days contained a greater density of DDR2-expressing cells (Figure 4C). To further clarify hepatic expression of DDR2 *in situ*, heterozygous *DDR2* knock-out/LacZ knock-in mice were treated for 30 days with CCl₄, and β -galactosidase expression was analyzed as a surrogate marker for DDR2. As shown in Figure 4D, β -galactosidase was barely detectable in untreated livers, whereas it appeared in fibroblast-like cells of injured livers (Figure 4E), mainly located in the fibrotic bands, as revealed by Masson staining (Figure 4F).

Immunoprecipitation followed by Western blot analysis of DDR2 expression in freshly isolated HSCs showed enhanced expression after CCl₄ treatment of a 116-kDa protein corresponding to DDR2.¹² Antiphosphotyrosine antibody revealed a significant increase of phosphorylated DDR2 in HSCs from chronically treated livers compared with HSCs from untreated mice (Figure 4G).

These data pinpoint HSCs as the major DDR2-expressing cells in the fibrotic mouse liver after chronic injury with CCl₄, which correlates with their role as the main cell type responsible for pericentral fibrosis after persistent CCl₄ administration. HSC transdifferentiation from a quiescent cell to a migratory and fibrogenic phenotype reflects active changes in gene expression. Thus,

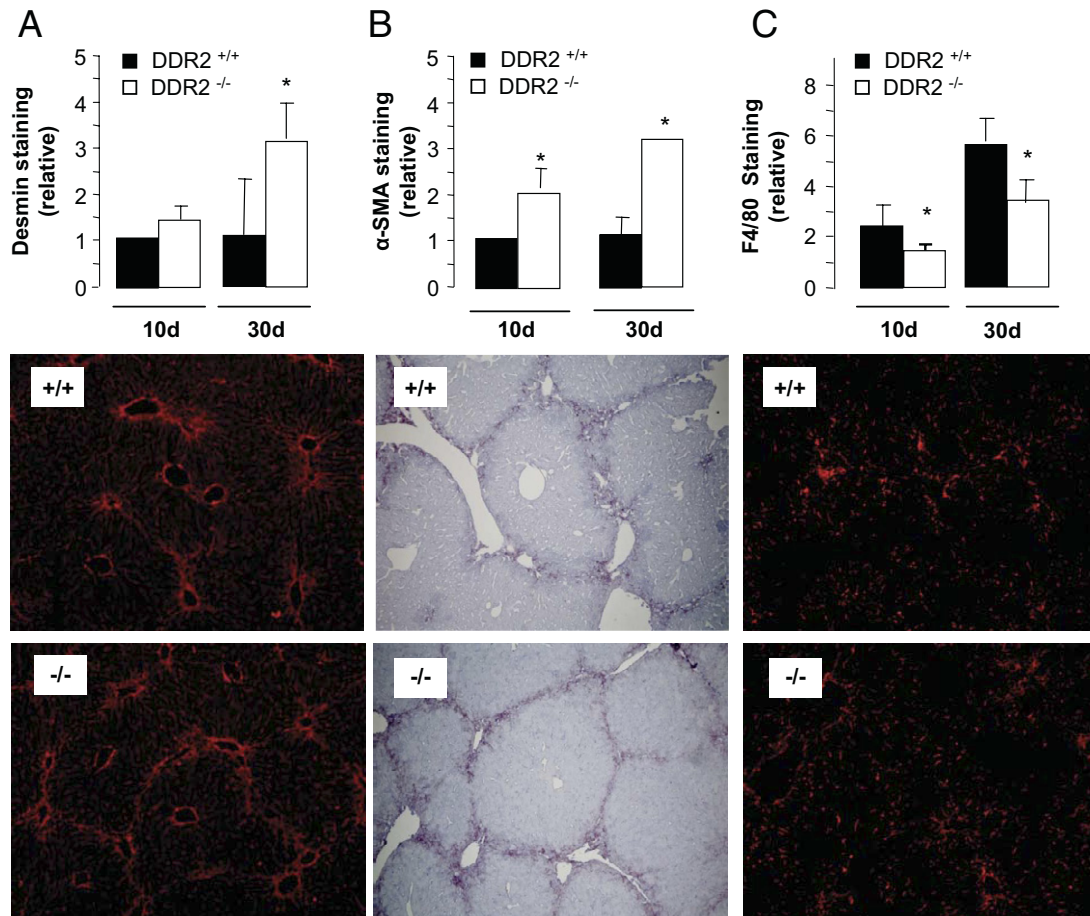


Figure 3. Immunohistochemical analysis of HSCs and macrophage recruitment after CCl₄ administration. Liver sections from *DDR2*^{-/-} and *DDR2*^{+/+} mice treated with CCl₄ for 10 or 30 days were stained with anti-desmin, anti-α-SMA, or anti-F4/80 antibodies. **A** and **B**: Computer-assisted semiquantitation of stained cells within the fibrotic scar in microphotographed labeled sections. Data are expressed as the percentage of region of interest, which was restricted to scarring tissue. **C**: Semiquantitation of anti-F4/80-stained macrophages associated with the scar. Only F4/80-positive cells located within or opposed the scar tracts were counted. **P* < 0.05, significantly different compared with the *DDR2*^{+/+} group. **Lower panels:** *DDR2*^{+/+} group. **Lower panels:** Microphotographs of liver sections from *DDR2*^{+/+} and *DDR2*^{-/-} mice treated with CCl₄ for 30 days and stained with anti-desmin (red; **A**), anti-α-SMA (blue; **B**) and anti-F4/80 (red; **C**) antibodies.

our next goal was to analyze mRNA expression of *DDR2*-dependent genes in HSCs after chronic CCl₄.

To do so, we built on our previous studies of gene expression signatures obtained by comparative DNA microarray analysis of freshly isolated quiescent *DDR2*^{+/+} and *DDR2*^{-/-} HSCs (I. Badiola, E. Olaso, O. Crende, F.L. Friedman, F. Vidal-Vanaclocha, unpublished data).

Amplified DDR2-Dependent Expression Levels of Immunomodulatory and Fibrogenic Genes in HSCs from Chronic CCl₄-Injured DDR2^{-/-} Livers

First, we selected several *DDR2*-dependent genes that are both differentially expressed in quiescent *DDR2*^{+/+} HSCs and *DDR2*^{-/-} HSCs (I. Badiola, E. Olaso, O. Crende, F.L. Friedman, F. Vidal-Vanaclocha, unpublished data), and also have been associated strongly with fibrogenic and immunomodulatory responses to chronic liver injury. After their validation by quantitative RT-PCR analysis, we further compared their expression in acti-

vated HSCs from chronically injured *DDR2*^{+/+} and *DDR2*^{-/-} livers.

Basal osteopontin and transforming growth factor (TGF)-β mRNA levels were significantly higher in isolated *DDR2*^{-/-} HSCs (Table 1). Moreover, the expected up-regulation of osteopontin and TGF-β mRNA levels in *DDR2*^{+/+} HSCs after chronic CCl₄ was increased twofold in HSCs from the CCl₄-treated *DDR2*^{-/-} livers. Furthermore, basal mRNA expression of the immunosuppressive cytokine IL-10 was doubled in quiescent *DDR2*^{-/-} HSCs, whereas chronic CCl₄ exacerbated an IL-10 mRNA decrease in *DDR2*^{-/-} HSCs. Quantitative RT-PCR analysis validated reduced expression of three key genes that regulate fibrogenesis. Collagen type I, MMP-2, and MMP-13 mRNA expression were reduced significantly in quiescent *DDR2*^{-/-} HSCs. More importantly, their mRNA expression remained reduced in HSCs from chronic fibrotic *DDR2*^{-/-} livers. Thus, quantitative RT-PCR analysis confirmed that *DDR2* deficiency remarkably altered HSC expression of important genes whose immunomodulatory and fibrogenic activities can modulate fibrosis. It also indicates that mRNA expression driven by

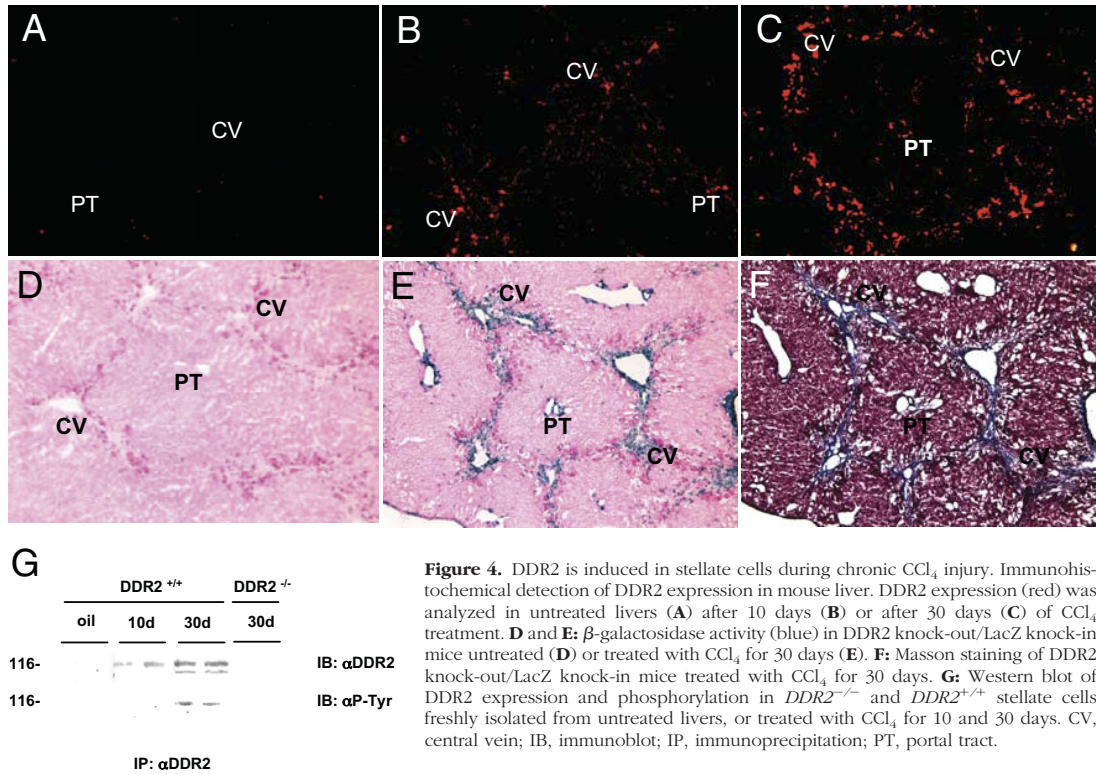


Figure 4. DDR2 is induced in stellate cells during chronic CCl₄ injury. Immunohistochemical detection of DDR2 expression in mouse liver. DDR2 expression (red) was analyzed in untreated livers (A) after 10 days (B) or after 30 days (C) of CCl₄ treatment. D and E: β-galactosidase activity (blue) in DDR2 knock-out/LacZ knock-in mice untreated (D) or treated with CCl₄ for 30 days (E). F: Masson staining of DDR2 knock-out/LacZ knock-in mice treated with CCl₄ for 30 days. G: Western blot of DDR2 expression and phosphorylation in DDR2^{-/-} and DDR2^{+/+} stellate cells freshly isolated from untreated livers, or treated with CCl₄ for 10 and 30 days. CV, central vein; IB, immunoblot; IP, immunoprecipitation; PT, portal tract.

DDR2 signaling that characterizes normal liver function in quiescent HSCs is exacerbated in activated HSCs after chronic CCl₄ administration.

Next, to further analyze our *in vivo* data on altered ECM remodeling and HSC recruitment, we isolated HSCs from chronically injured DDR2^{-/-} and DDR2^{+/+} livers, and analyzed *in vitro* their migration and proliferation rates, and their ECM degrading activities.

Enhanced in Vitro Migration and Proliferation and Reduced Matrix-Degrading Activities in HSCs from Chronically Injured DDR2^{-/-} Livers

Amplification of HSCs within fibrotic areas is the net result of migration and proliferation. Therefore, we next assessed the

impact of DDR2 on migration capacity of isolated HSCs using a Boyden chamber migration assay. The number of quiescent DDR2^{-/-} HSCs that migrated across the collagen-coated membrane in response to a FCS gradient was half that of quiescent DDR2^{+/+} cells, whereas the number of migrated DDR2^{-/-} HSCs from fibrotic livers was twice that of the *in vivo* activated DDR2^{+/+} cells (Figure 5A). We used alamarBlue, a nontoxic dye, to measure proliferation of activated HSCs (Figure 5B). The proliferation rate was 70% lower in quiescent DDR2^{-/-} HSCs than in quiescent DDR2^{+/+} ones, whereas it was 50% more in HSCs from fibrotic DDR2^{-/-} mice than from fibrotic DDR2^{+/+} mice. No signs of apoptosis or cell detachment were observed under any of the assayed conditions (data not shown). Quiescent DDR2^{-/-} HSCs activated by *in vitro* culture (see *Materials and Methods*) migrated and prolifer-

Table 1. Real-Time PCR Quantification of mRNA Levels in HSCs Isolated from Control and CCl₄-Treated DDR2^{-/-} and DDR2^{+/+} Livers

mRNA	Oil		CCl ₄	
	+/+	-/-	+/+	-/-
Osteopontin	1.0 ± 0.025	5.7 ± 0.003*	8.1 ± 0.001*	15.4 ± 0.012*†
TGF-β	1.0 ± 0.003	4.8 ± 0.030*	2.7 ± 0.025*	12.5 ± 0.020*†
IL-10	1.0 ± 0.050	1.9 ± 0.014*	0.8 ± 0.004	0.4 ± 0.003*†
Collagen I α1	1.0 ± 0.040	0.5 ± 0.125	3.5 ± 0.213*	2.1 ± 0.198*†
MMP-2	1.0 ± 0.210	0.7 ± 0.050*	10.6 ± 2.050*	3.2 ± 0.512*†
MMP-13	1.0 ± 0.060	0.8 ± 0.090	3.1 ± 0.048	1.5 ± 0.290*†

N = 16 mice from 2 independent treatments, 2 mice per group. Relative mRNA expression by HSCs from control (oil-treated) DDR2^{+/+} livers compared to RPL13 mRNA expression: osteopontin: 1.29 ± 0.01; TGF-β: 0.18 ± 0.01; IL-10: 0.71 ± 0.004; collagen I α1: 1.0 ± 0.024; MMP-2: 0.04 ± 0.008; MMP-13: 0.01 ± 0.000.

*P < 0.05 compared to HSCs from control DDR2^{+/+} livers.
 †P < 0.05 compared to HSCs from DDR2^{+/+} livers after CCl₄ administration.

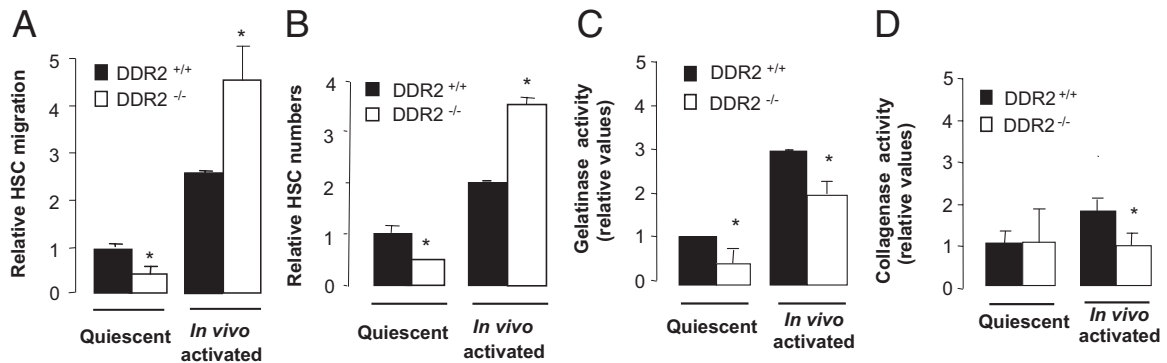


Figure 5. Enhanced chemotactic migration and proliferation and reduced ECM remodeling in *DDR2*^{-/-} HSCs after chronic liver injury. Quiescent and activated *DDR2*^{-/-} and *DDR2*^{+/+} HSCs were isolated from control and chronic fibrotic livers, respectively. **A:** HSCs were allowed to migrate for 16 hours through collagen type I-coated porous upper wells of migration chambers in response to a fetal calf serum gradient. **B:** HSC proliferation rates using the alamarBlue assay. Colorimetric-based assay of gelatinase (**C**) and collagenase (**D**) activities in HSCs isolated from *DDR2*^{-/-} and *DDR2*^{+/+} fibrotic livers after 16 hours culture on top of thin collagen type I-coated wells. **P* < 0.05, significantly different compared with *DDR2*^{+/+} group.

ated significantly less than quiescent *DDR2*^{+/+} HSCs cultured in the same conditions (data not shown).

These results indicate that under basal conditions, DDR2 signaling induces migration and proliferation of quiescent HSCs, as shown by the reduced migration and proliferation of quiescent *DDR2*^{-/-} HSCs. In this regard, overexpression of nonsignaling DDR2 reduced migration of HSC-T6 cells,¹² a cell line derived from normal rat liver HSCs.³¹ On the other hand, *in vitro* data on HSCs from fibrotic *DDR2*^{-/-} livers correlate with histologic data showing larger numbers of activated HSCs recruited to the fibrotic scars after chronic CCl₄ (Figure 2). These apparent contradictory responses of *DDR2*^{-/-} HSCs suggest that a specific DDR2-dependent conditioning of the liver occurs that in turn affects HSC responses after chronic injury. These responses underscore the importance of DDR2 in orchestrating activation signals sent by the profibrogenic milieu generated during chronic liver injury.

Next, we challenged HSCs with collagen type I, the preferred ligand of DDR2 (see *Materials and Methods*), and their cellular supernatants were assayed for G/C activities. G/C activities were confined to the medium, and none were found in cell lysates (not shown). These activities accounted for approximately 50% of those present in the corresponding APMA-activated conditioned media, indicating that 50% of G/Cs were in their active form, regardless of the DDR2 status of the cells. Supernatants from quiescent *DDR2*^{-/-} HSCs contained approximately 54% of gelatinase activity, and supernatants from activated *DDR2*^{-/-} HSCs contained significantly (~30%) less (Figure 5C). Collagenase activity in response to collagen type I was similar in all quiescent cells but 45% lower in activated *DDR2*^{-/-} HSCs (Figure 5D). These data, in concert with the results on quantitative RT-PCR (Table 1), indicate that DDR2 induces MMP-2/MMP-13 expression and associated G/C activity in HSCs under both normal conditions and after chronic CCl₄ administration. It also indicates that other cellular sources of DDR2-dependent MMP activity exist in the fibrotic livers.

Thus, we next explored how DDR2 may contribute to the profound changes in the cellular milieu characteristic of chronic fibrosis. Although we found no evidence of DDR2 expression by macrophages (data not shown),

increasing data indicate a direct role of macrophages (Kupffer cells and monocyte-derived macrophages) in liver fibrosis by release of MMPs and fibrosis-related cytokines,³⁰ and through HSC recruitment to the fibrotic tissue.³² Furthermore, addition of Kupffer cell-conditioned medium during culture activation of HSCs leads to a closer recapitulation of *in vivo* gene expression that resembles changes seen after chronic CCl₄.²³

Thus, to analyze the role of DDR2 in the chronic responses of the fibrogenic liver microenvironment, we next analyzed the chemotactic and ECM-degrading activities of macrophages from livers after chronic CCl₄. We correlated these findings with quantitative RT-PCR analysis for a set of critical chemotactic and profibrogenic cytokine mRNAs secreted by macrophages during hepatic fibrosis that are altered in whole *DDR2*^{-/-} liver extracts.

Paracrine DDR2-Mediated Modulation of Macrophages Mediates the Enhanced Migration of HSCs in Chronically Injured DDR2^{-/-} *Livers*

Next, we studied HSC migration in response to soluble factors derived from liver macrophages. Quiescent HSCs and macrophages were isolated from oil-treated livers, and activated HSCs and macrophages were isolated from CCl₄-treated livers. HSCs were placed in the upper chamber of a migration device, and supernatants from macrophage cultures were added to the lower compartment. Under this condition, quiescent *DDR2*^{-/-} HSCs migrated approximately twofold more than quiescent *DDR2*^{+/+} HSCs (Figure 6A). Maximal migration was displayed by activated *DDR2*^{-/-} HSCs cultured with media from macrophages isolated from *DDR2*^{-/-} livers. The promigratory effect of macrophages from CCl₄-treated *DDR2*^{-/-} livers was stronger toward *DDR2*^{-/-} HSCs than toward *DDR2*^{+/+} HSCs. This migratory enhancement was not observed using media conditioned by macrophages from untreated or oil-treated mice (data not shown), which suggests an inhibitory paracrine effect of DDR2 in the secretion of chemotactic factors for HSCs by macrophages from fibrotic livers.

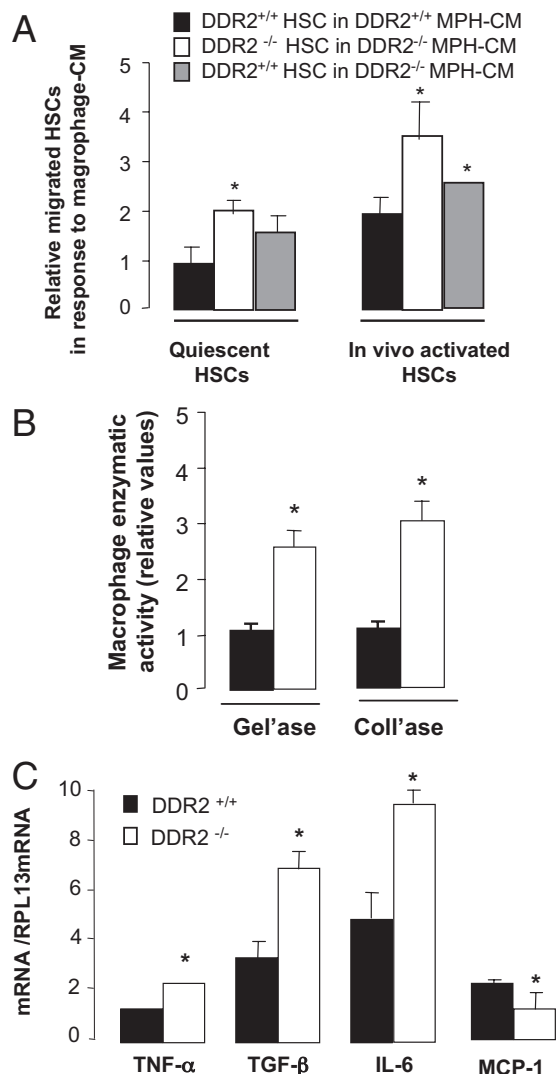


Figure 6. Profibrotic role of macrophages from chronically injured *DDR2*^{-/-} livers. **A:** Quiescent HSCs from oil-treated and activated HSCs from CCl₄-intoxicated livers were allowed to migrate for 16 hours through collagen type I-coated porous upper wells of migration chambers in response to media conditioned by macrophages from fibrotic livers (MPH-CM). **B:** Gelatinase and collagenase activities in supernatants from macrophages isolated from fibrotic livers and cultured for 16 hours. **C:** Differential mRNA expression pattern of fibrosis-related genes in macrophages isolated from fibrotic livers. (*n* = 16 mice from 2 independent treatments, 2 mice per group). Relative mRNA expression in macrophages from control *DDR2*^{+/+} livers compared to RPL13 mRNA expression: TGF-β: 0.06 ± 0.007; tumor necrosis factor-α (TNF-α): 1.61 ± 0.254; IL-6: 0.08 ± 0.002; monocyte chemoattractant protein-1 (MCP-1): 0.31 ± 0.034. **P* < 0.05 relative to *DDR2*^{+/+} HSC in *DDR2*^{+/+} MPH-CM (**A**) or control *DDR2*^{+/+} livers (**B** and **C**).

Altered MMP Activity and Cytokine mRNA Expression in Macrophages from Chronically Injured *DDR2*^{-/-} Livers

Next, macrophages were isolated from fibrotic livers and analyzed for MMP activity and expression of genes implicated in the paracrine modulation of HSC migration and fibrogenic responses. Figure 6B shows that supernatants of macrophages from fibrotic *DDR2*^{-/-} livers contained 3.3-fold higher gelatinolytic activity and two-fold higher collagenolytic activity than cells from *DDR2*^{+/+} livers. These results suggest a combined

DDR2-dependent role for HSCs and macrophages in ECM remodeling during chronic liver fibrosis.

Scar-associated macrophages during injury are associated with a high expression of fibrogenic TGF-β1 in the scars and high levels of liver tumor necrosis factor-α and IL-6 mRNAs.^{20,30} Figure 6C indicates that macrophages from fibrotic *DDR2*^{-/-} livers displayed a greater induction of TGF-β, tumor necrosis factor-α, and IL-6 mRNAs and a reduced expression of chemotactic monocyte chemoattractant protein-1 mRNA. Quiescent macrophages from untreated *DDR2*^{-/-} showed no significant differences in gene expression compared with macrophages from untreated *DDR2*^{+/+} livers, indicating that the effect of *DDR2* removal on macrophages occurs only after persistent liver injury. These results are in correlation with gene expression analysis of whole liver extracts (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

Discussion

In this work, we show enhanced liver fibrosis after CCl₄ administration in *DDR2*-deficient mice, suggesting that in normal liver, *DDR2* suppresses fibrosis in chronic injury. Mechanistically, *DDR2* deficiency attenuates the function of quiescent HSCs, as shown by their enhanced basal mRNA expression of fibrogenic and inflammatory factors, and their impaired migratory, proliferative, and gelatinolytic activities. CCl₄ administration within this altered, *DDR2*-deficient sinusoidal milieu affects secretion by macrophages of fibrogenic and chemotactic factors. Ultimately, chronic liver fibrosis in *DDR2*-deficient mice yields larger collagenous scar tissue that is populated by greater numbers of activated HSCs.

DDR2 expression has been associated with inflammatory and fibrogenic reactions such as wound healing, arthritis, fibrosis, and cancer. We previously reported enhanced *DDR2* mRNA expression in HSCs after acute CCl₄ administration.¹² In this work, we show that *DDR2* expression increases after chronic CCl₄ treatment, and that HSCs that initiated their activation program in response to liver injury are the main cellular source of *DDR2*.

Regulation of gene expression by *DDR2* has been described primarily *in vitro* using cells overexpressing *DDR2*^{12,33-35} and studies have been restricted to ECM-related genes. Using primary cells isolated from *DDR2*-deficient mice, we recently described *DDR2*-dependent basal gene expression in murine HSCs and skin fibroblasts.²⁶ In this work, we analyzed for the first time the regulatory role of *DDR2* in the expression of genes related to immunomodulation and fibrogenesis in HSCs activated by persistent injury. To support the possible role of *DDR2* as a suppressor of chronic fibrosis, quantitative RT-PCR showed that expression of TGF-β1, a pluripotent immunomodulatory and fibrogenic factor that largely regulates HSC transition to myofibroblast-like cells,³⁶ of immunosuppressive cytokine IL-10 and of chemokine-like protein osteopontin³⁷ are partially *DDR2*-dependent in HSCs. The relevance of *DDR2* signaling in chronic injury is reinforced by the augmented *DDR2*-dependent altered gene expression in HSCs from fibrotic

livers, a situation in which intense DDR2 phosphorylation is taking place.

We also found that DDR2 modulation of collagen type I, MMP-2, and MMP-13 occurs in HSCs irrespective of their activation status. Surprisingly, although collagen type I directly promoted DDR2 expression and augmented MMP activity in quiescent HSCs, chronic liver injury in DDR2-deficient mice led to larger collagen deposits, MMP activity, and HSC migration. Thus, the positive loop created between ECM composition, DDR2 signaling, and ECM remodeling in HSCs under normal liver function is altered in chronic liver injury.

Experimental models of liver fibrosis highlight the importance of macrophages in perpetuating an inflammatory phase, resulting in the massive release of proinflammatory cytokines including tumor necrosis factor- α , IL-6, TGF- β , and chemokines, as well as activation of HSCs.³² Hepatic macrophages do not express DDR2, but results indicate that *DDR2* gene deletion in HSCs alters macrophage responses to chronic injury. It is tempting to speculate that changes in the liver microenvironment generated by chronic DDR2 signaling in activated HSCs influence macrophage profibrogenic activities. For example, HSC expression of DDR2-dependent cytokines monocyte chemoattractant protein-1 and osteopontin modulate macrophage recruitment and macrophage release of MMPs³⁸ and TGF- β production.³⁷

Our results also may indicate that enhanced fibrosis after chronic liver injury in the absence of DDR2 in HSCs result from the disruption of the fine balance between secretion of pro-activation factors by both activated macrophages and activated HSCs, to enhance recruitment of activated HSCs to the fibrotic scar, and secretion of antimigratory factors for macrophages by scar-recruited activated HSCs.

In summary, our work underscores the role of altered receptor-ECM interactions in HSCs within a fibrogenic microenvironment. Our findings support a model in which DDR2 signaling in HSCs down-regulates gene expression of fibrosis-related cytokines and up-regulates ECM-related genes such as fibrillar collagen type I, basal membrane degrading MMP-2, and collagenolytic MMP-13. Chronic injury induces an HSC activation program that includes enhanced DDR2 expression and signaling, leading to downstream modulation of DDR2-dependent genes. Furthermore, the altered fibrogenic sinusoidal milieu resulting from tonic DDR2 signaling leads to paracrine inhibition of profibrogenic activities by scar-associated macrophages.

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