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IL-17A Plays a Critical Role in the Pathogenesis of Liver Fibrosis through Hepatic Stellate Cell Activation

Zhongming Tan,*†§∥1 Xiaofeng Qian,*†∥ Runqiu Jiang,*† Qianghui Liu,*† Youjing Wang,*† Chen Chen,*†∥ Xuehao Wang,*† Bernhard Ryffel,∥§∥ and Beicheng Sun*†∥

Liver fibrosis is a severe, life-threatening clinical condition resulting from nonresolving hepatitis of different origins. IL-17A is critical in inflammation, but its relation to liver fibrosis remains elusive. We find increased IL-17A expression in fibrotic livers from HBV-infected patients undergoing partial hepatectomy because of cirrhosis-related early-stage hepatocellular carcinoma in comparison with control nonfibrotic livers from uninfected patients with hepatic hemangioma. In fibrotic livers, IL-17A immunoreactivity localizes to the inflammatory infiltrate. In experimental carbon tetrachloride–induced liver fibrosis of IL-17A–deficient mice, we observe reduced neutrophil influx, proinflammatory cytokines, hepatocellular necrosis, inflammation, and fibrosis as compared with control C57BL/6 mice. IL-17A is produced by neutrophils and T lymphocytes expressing the Th17 lineage–specific transcription factor RORα–related orphan receptor γt. Furthermore, hepatic stellate cells (HSCs) isolated from naïve C57BL/6 mice respond to IL-17A with increased IL-6, α-smooth muscle actin, collagen, and TGF-β mRNA expression, suggesting an IL-17A–driven fibrotic process. Pharmacologic ERK1/2 or p38 inhibition significantly attenuated IL-17A–induced HSC activation and collagen expression. In conclusion, IL-17A– Retinoic acid receptor–related orphan receptor γt neutrophils and T cells are recruited into the injured liver driving a chronic, fibrotic hepatitis. IL-17A–dependent HSC activation may be critical for liver fibrosis. Thus, blockade of IL-17A could potentially benefit patients with chronic hepatitis and liver fibrosis. The Journal of Immunology, 2013, 191: 1835–1844.

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iver fibrosis is a common outcome of chronic virus-induced hepatitis, autoimmune liver disease, drug abuse, and alcoholic liver disease, and it is a significant cause of liver failure, as well as the principal cause of morbidity and mortality (1). Accumulating evidence indicates that immune dysregulations occur in liver fibrosis, leading to unbalanced extracellular matrix synthesis and degradation (2).

Hepatic stellate cells (HSCs) are located in the spaces of Disse in close contact with hepatocytes, sinusoidal endothelial cells, and autonomic nerve fibers (3). A critical role of activated HSCs in collagen and extracellular matrix production has been previously observed. HSC activation is characterized as a shift from the quiescent “storing” phenotype to the highly proliferative “myofibroblast-like” phenotype (4) and leads to increased fibrillar extracellular matrix production with reduced capabilities for degradation and remodeling (3). Several proinflammatory mediators are secreted from the activated HSCs, leading to further amplification of the inflammatory process (4, 5).

Numerous mechanisms contribute to the activation of HSCs, including matrix stiffness, altered E- to N-cadherin expression and inflammatory mediators (4, 5). IL-17A is produced by specific CD4+ Th cells (Th17) with proinflammatory properties that differ from Th1 and Th2 cells in development and function. Differentiation of Th17 cells requires the combined action of IL-1β, TGF-β, IL-6, and IL-23 (6, 7). These cytokines, which are induced by activated Kupffer and other cells, promote the expression of the lineage-specific transcription factor orphan nuclear receptor retinoic acid receptor retinoic acid receptor γt (RORγt; in mice) or RORγt (in humans), which is necessary and sufficient for the development of Th17 cells. IL-17A plays a critical role in neutrophil recruitment, angiogenesis, inflammation, and autoimmune disease that has been previously described extensively (6), including in pulmonary and cardiac fibrosis via IL-17RA and MAPK signaling (8, 9). However, very few studies have investigated the role of IL-17A in liver fibrosis (10).

In this study, using IL-17A KO mice, we report that upregulated expression of IL-17A by RORγt neutrophils is crucial for carbon tetrachloride (CCL4)–induced liver injury leading to inflammation and fibrosis. Furthermore, IL-17A–dependent HSC...
activation and collagen production depend on phosphorylation of MAPK, especially ERK1/2 and p38. Therefore, we conclude that IL-17A–dependent HSC activation is critical in CCl4-induced liver injury and fibrosis. These findings provide a potential novel treatment target that could benefit patients by reducing liver fibrosis and leading to an improved prognosis.

Materials and Methods

Patients

For this study, we enrolled patients who presented from December 2011 to January 2012 at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). Informed consent for patient sample analysis was obtained from each subject before surgery; the study was approved by the appropriate Institutional Ethics Committee. Ten fibrotic livers were collected after partial hepatectomy due to early-stage hepatocellular carcinoma which followed hepatitis B virus–related cirrhosis; five control livers are normal tissues obtained from hepatectomy for hepatic hemangioma. Clinical characteristics are described in Table I.

Animals

All mouse strains, bred or purchased, consisted of C57BL/6. IL-17RA–deficient mice (11) and RORγt-enhanced GFP (EGFP) reporter mice (12) including control C57BL/6 were bred at the Immunologie et Neurogenétique Expérimentales et Moléculaires, Orleans, France. The 6- to 10-wk-old male mice were maintained in sterile, isolated, ventilated cages with controlled temperature (21–23˚C) and light (12-h light/12-h dark cycle) conditions and ad libitum access to food and water. All experimental procedures were performed according to the local Animal Care Committee following guidelines that comply with those of the French and Chinese governments, and the experimental protocol was approved by the local ethics committee.

Animal model of acute liver injury and liver fibrosis

Mice received an i.p. injection of CCl4 (0.5 g/kg; catalog no. 488488; Sigma-Aldrich, St. Louis, MO) in a volume of corn oil equivalent to 200 μl/mouse to create an acute liver injury model. Mice were sacrificed 24 h after the challenge. For the chronic hepatitis and fibrosis model, mice received a dose of CCl4 (0.5 g/kg) in a volume of corn oil equivalent to 200 μl/mouse with i.p. injections performed every 3 or 21 d. Twenty-four hours after the last challenge, all mice were sacrificed. All control mice were injected with corn oil only. All procedures were performed under sterile conditions.

Immunohistochemistry

IL-17A immunohistochemistry was performed using 5-μm paraffin sections. The sections were deparaffinized in xylene and rehydrated in alcohol and distilled water. Ag retrieval was performed by heating sections in solution (10 mM citrate buffer, pH 6.0) in a pressure cooker. Three percent H2O2 was used to eliminate endogenous peroxidase. Slides were washed with TBS-Triton three times and one time with TBS for 5 min. Non-specific binding was blocked using 5% goat serum for 30 min. The blocking buffer was then removed and sections were incubated with a polyclonal anti–IL-17A Ab (ab79056; Abcam, Cambridge, U.K.). Control experiments were performed without rmIL-17A restimulation. The reaction was stopped adding 100 μlH2O2 0.05%. After 15-min incubation at 37˚C, the media was then removed, and the HSCs were fixed with 4% buffered formaldehyde. Then cells were rehydrated in alcohol and rehydrated in alcohol and dehydrated in xylene before mounting in xylene. The slides were then mounted in Prolong Gold Antifade Mountant (Invitrogen) and imaged using a DM4000 B Upright Research Microscope (200× magnification).

Cytokine analysis

Supernatants of liver homogenate samples were collected from mice injected with 0.5 g/kg CCl4 or vehicle; cell media were obtained from 0, 1, 10, 30, or 100 ng/ml rmIL-17A reactivation for 24 h or from 30 ng/ml rmIL-17A reactivation for 0, 1, 3, 12, and 24 h (1 × 105 cells/ml at 6-well plate). The collagen concentration was measured using the Sircol assay according to the manufacturer’s recommended procedure (BioColor S1000; BioColor, Carrickfergus, U.K.). Note that collagen levels induced by different concentrations of rmIL-17A were normalized by cell numbers and are represented by percentage changes with respect to those of the HSCs without rmIL-17A reactivation.

Liver homogenization and myeloperoxidase activity

Myeloperoxidase (MPO) activity assay was used as indirect index of the neutrophil presence in lung, liver, and intestine (16). The 200-mg fresh samples in 1 ml PBS were homogenized and resuspended in PBS (1 ml) containing 0.5% hexadecyltrimethyl ammonium bromide and 5 mM EDTA. After centrifugation, aliquots of 50 μl of supernatants were placed in test tubes containing 2 ml HBSS, 100 μl o-dianisidine dihydrochloride (1.25 mg/ml), and 100 μl H2O2 0.05%. After 15-min incubation at 37˚C with shaking, the reaction was stopped adding 100 μl NaNO2 1%. Absorbance was determined at 460 nm using a microplate reader.

Microscopic investigation and TUNEL assay

Fragments of liver tissues were fixed in 4% buffered formaldehyde. Sections (3 μm) of liver were cut and stained with H&E. Detection of apoptosis by TUNEL assay was according to the protocol (Millipore S7100; Billerica, MA) (17). Liver H&E sections were graded for CCl4 injury under Leica DM4000 B Upright Research Microscope (200× and 400×) using the system devised by Suzuki et al. (18) and as described previously. TUNEL+ cells were counted at ×200 fields. All of the microscopic figures were acquired by Leica Microsystems software (Leica, Germany).

Cytokine analysis

The homogenized tissue was centrifuged at 10,000 rpm for 15 min, and the supernatant was recovered and stored at −80˚C until analyses. The levels
of cytokines were measured by ELISA using commercial kits (R&D, Abingdon, U.K.) according to manufacturer’s instructions.

**Western blot**

Proteins were extracted from cultured cells and quantified using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Protein samples pretreated with protease and phosphatase inhibitors were fractionated by SDS-PAGE, and upon electrophoresis, the proteins were transferred onto nitrocellulose membranes and blotted overnight as described previously (19). The anti-α-smooth muscle actin (α-SMA; ab5694; Abcam), anti-ERK1 (sc-93), anti-p-ERK1/2 (sc-16962R), The p(Thr183) anti-p38 (sc-728), anti-p-p38 (sc-7975R, Tyr180), JNK (sc-571), anti-p-JNK1 (sc-6254), and anti–IL-17RA (ab134086) were used (Santa Cruz Biotechnology, Dallas, TX; Abcam; or Cell Signal Technology, Beverly, MA). Moreover, for computer-assisted semiquantitative analysis of MAPK phosphorylation and α-SMA expression, ImageJ software (http://rsbweb.nih.gov/ij/) was used according to the manufacturer’s instructions.

**Measurement of plasma alanine aminotransferase activity**

Blood was obtained by cardiac puncture at the time of sacrifice for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnostic kit (Sigma-Aldrich).

**Liver mononuclear cell isolation and flow cytometry analysis**

Liver mononuclear cells were isolated from mice 24 h after the CCl4 challenge as described previously (20). In brief, liver mononuclear cells were collected and plated at 1 × 10^7/ml and restimulated at 6 h in vitro with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (750 ng/ml) in complete medium (IMDM supplemented with 5% [v/v] FCS, L-2-ME [50 nM], all from Invitrogen). PBMCs were isolated from the clinic samples by Ficoll gradient and stimulated at 37˚C for 6 h. The Abs used for FACS analysis, FITC–anti-CD3e (clone 145-2C11), PerCP-Cy5.5–anti–IL-17A (clone TC11-18H10), Pacific blue–anti-CD4 (clone RMA-2-ME [50 nM], all from Invitrogen). PBMCs were isolated from the clinic samples by Ficoll gradient and stimulated at 37˚C for 6 h. The Abs used for FACS analysis, FITC–anti-CD3e (clone 145-2C11), PerCP-Cy5.5–anti–IL-17A (clone TC11-18H10), Pacific blue–anti-CD4 (clone RMA-
classifiers were purchased from BD Pharmingen (San Diego, CA). Rabbit anti-mouse GFP IgG fraction and Alexa Fluor 488–labeled goat anti-rabbit IgG were purchased from Invitrogen (Shanghai). PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

**Statistical analysis**

Data are expressed as mean ± SEM. Comparisons between groups were performed by using a two-tailed unpaired Student t test or one-way ANOVA with Newman–Keuls post hoc for individual subgroup comparison. The 4.0 version (2005) of the GraphPad Prism software was used for this purpose. A p value <0.05 indicates a significant result.

**Table I. Clinical characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Fibrotic Liver</th>
<th>Control Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Sex</td>
<td>10 male</td>
<td>5 male</td>
</tr>
<tr>
<td>Mean age (minimum–maximum), y</td>
<td>55.1 (43–61)</td>
<td>46.6 (36–51)</td>
</tr>
<tr>
<td>HBV infected, n</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Mean ALT level (minimum–maximum), IU/l</td>
<td>42.4 (15–120)</td>
<td>21.1 (13–68)</td>
</tr>
<tr>
<td>Mean AST level (minimum–maximum), IU/l</td>
<td>38.8 (14–112)</td>
<td>18.6 (11–44)</td>
</tr>
<tr>
<td>Mean total bilirubin level (minimum–maximum)</td>
<td>16.5 (4–25)</td>
<td>12.7 (5–19)</td>
</tr>
<tr>
<td>Mean serum albumin (minimum–maximum), g/l</td>
<td>31 (26–36)</td>
<td>34 (31–36)</td>
</tr>
<tr>
<td>Mean primary mass size (minimum–maximum), cm</td>
<td>5.1 (3.8–9.1)</td>
<td>8.5 (6.7–12)</td>
</tr>
</tbody>
</table>

AST, aspartate aminotransferase; HBV, hepatitis B virus.
Inflammatory cytokine expression levels after acute liver injury are dependent on IL-17RA signaling

Although IL-17RA deficiency largely protected the mice from CCl4-induced acute liver injury, the exact mechanisms underlying this protective capacity remain unclear. Therefore, we investigated the inflammatory cytokine expression profiles in C57BL/6 and IL-17RA−/− mice injected CCl4 or corn oil. IL-1β is the principal proinflammatory cytokine that activates several cytokines and chemokines leading to inflammatory cell recruitment and cell damage (22). IL-1β expression in the liver from IL-17RA–deficient mice was measured by real-time PCR in fibrotic or normal liver separately. Data are representative of 10 liver specimens and expressed as mean ± SEM (n = 10; *p < 0.05, **p < 0.01, ***p < 0.001).

FIGURE 1. IL-17A is increased in clinical liver fibrosis. Fibrotic and normal liver tissues were collected from patients undergoing partial hepatectomy for early-stage hepatocellular carcinoma or hepatic hemangioma, respectively. (A) Collagen concentration was measured in liver homogenate (0.1 g) in fibrotic or normal livers. (B) α-SMA RNA was assayed by real-time PCR. (C) Collagen deposition in liver using Masson's trichrome stain (original magnification ×200). (D) Immunostaining of liver with anti–human IL-17A Ab (×400 original magnification is shown). One representative result is shown from 10 specimens. Negative controls are shown in Supplemental Fig. 1. (E and F) The transcription of IL-1β and IL-17A genes was measured by real-time PCR in fibrotic or normal liver separately. Data are representative of 10 liver specimens and expressed as mean ± SEM (n = 10; *p < 0.05, **p < 0.01, ***p < 0.001).

FIGURE 2. IL-17RA deficiency protects from CCl4-induced acute liver injury. C57BL/6 and IL-17RA–deficient mice were subjected to a single dose of 2 g/ml CCl4 or vehicle by i.p. administration, and the livers were analyzed 24 h after administration. (A) IL-17A in liver homogenates (ELISA) from C57BL/6 or IL-17RA−/− mice. (B) Microscopic sections showing inflammation and necrosis (H&E, original magnification ×200 and ×400). (C) Representative TUNEL-stained sections showing apoptosis (original magnification ×200 and ×400), (D) Suzuki score estimating the liver lesion and (E) TUNEL-positive cells were counted from 20 random microscope fields and the mean values ± SEM were presented. (F) Serum ALT levels. (G) Hepatic MPO activity from CCl4-injected C57BL/6 and IL-17RA−/− mice compared with mice after vehicle administration. Data represent the mean values ± SEM (n = 6–8 mice per group) and are representative of three independent experiments (***p < 0.01, ***p < 0.001).
mice was significantly lower after the CCl4 challenge as compared with C57BL/6 mice (Fig. 3A). Moreover, IL-6, an important cytokine for Th17 differentiation, was attenuated in IL-17RA−/− mice (Fig. 3B), suggesting that IL-6 may contribute to the response in accordance with previous reports (7, 23). CXCL1/KC and TNF-α upregulation in CCl4 injected in C57BL/6 mice was significantly attenuated in IL-17RA−/− mice, indicating that IL-17RA deficiency largely limits inflammation in tissue (Fig. 3C, 3D). Levels of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinase 1 (TIMP-1), which reflect matrix remodeling enzymes, were increased in C57BL/6 mice and were IL-17RA signaling dependent (9) (Fig. 3E, 3F). Therefore, IL-1β and several key proinflammatory mediators were significantly diminished in the absence of IL-17RA signaling.

Neutrophils are the principal IL-17A–producing cells after CCl4-induced liver injury

Hepatic-infiltrating mononuclear cells were obtained from mice at 24 h after CCl4 administration (2 g/kg i.p.), and IL-17A–producing cells were analyzed by flow cytometry. First, among CD3+ cells, proportion of IL-17A+ cells is presented in CD3+ populations (Fig. 4A). CD4+ T cells and CD8+ T cells were analyzed based on CD3+ gate (Fig. 4B). Using RORγt–EGFP mice, we investigated the IL-17A–related transcription factor expressed in CD4+ and CD8+ subpopulations (Fig. 4C). IL-17A expression in Gr-1hi neutrophils (Fig. 4D). FACS analysis indicating that IL-17A expression in Gr-1hi neutrophils is RORγt–EGFP dependent (Fig. 4E). Total cell counts of CD4+, CD8+, or Gr-1hi subpopulations expressing IL-17A of liver-infiltrating mononuclear cells from vehicle or CCl4 challenge. Data are presented as mean value ± SEM (**p < 0.01, ***p < 0.001). Results from flow cytometry are from one representative experiment of six independent studies.
IL-17A expression did not increase (Fig. 4A). Using the CD3+ gate, we investigated IL-17A expression in CD4+ and CD8+ T cells. We found a slight increase of IL-17A expression in CD4+ T cells that typically express IL-17A (24) from 4.11 to 5.56%, but also in CD8+ T cells (from 3.43 to 5.78%) after CCl4 administration (Fig. 4B). Furthermore, we asked whether the Th17 lineage marker is expressed in CD4+ and CD8+ T cells. We induced acute liver injury in RORγt–EGFP reporter mice and found that the recruited CD4+ and CD8+ T cells expressed RORγt (Fig. 4C). Because we established abundant neutrophil recruitment in IL-17A-deficient CCl4-injury-induced acute liver inflammation, we asked whether neutrophils may also be a source of IL-17A in acute liver injury (25, 26). Therefore, we labeled IL-17A+ cells with the Gr-1 (Ly-6G) Ab and found a dramatic increase of Gr-1hi in CCl4-challenged mice that was attenuated in IL-17RA–deficient mice (Fig. 4D). Furthermore, the Gr-1hi IL-17A+ neutrophils exhibited the Th17 lineage transcription factor RORγt (Fig. 4E). Based on the different IL-17A+ proportions of cells and of total infiltrating cell counts (Table II), we calculated the numbers of IL-17A producing cells in each population (Fig. 4F). These absolute numbers reveal a major contribution by neutrophils and, to a lesser extent, by CD4+ and CD8+ T cells to IL-17A production. Therefore, in addition to a direct effect of neutrophils on hepatocytes and nonparenchymal hepatic cells, neutrophils at the injury site potentially augment the inflammation and liver injury through the IL-17A production. The fact that Gr-1hi neutrophils express the RORγt lineage marker and represent the major source of IL-17A is biologically significant, suggesting an amplification of the inflammatory response by neutrophils.

**CCl4-induced chronic inflammation and liver fibrosis is IL-17RA dependent**

Because IL-17RA deficiency largely protected mice from CCl4-associated liver injury, and upregulated MMP-9 and TIMP-1 were also attenuated in IL-17RA KO mice (28), we used IL-17RA–deficient mice to investigate the contribution of IL-17A signaling to CCl4-induced fibrosis (9). In brief, CCl4 was injected into C57BL/6 or IL-17RA KO mice at 0.5 g/kg every 3 d. After 21 d, mice were sacrificed. The microscopic liver sections revealed atrophy of central areas, and some parenchymal nodules were completely surrounded by fibrosis, indicating early cirrhosis, which was drastically reduced in IL-17RA KO mice (Fig. 5B). Liver injury, measured by serum ALT activity, exhibited a significant increase in CCl4-challenged mice that was attenuated in IL-17RA–deficient mice (Fig. 5D). Collagen concentrations in liver homogenate were also diminished in IL-17RA KO mice, which is consistent with the reduced Masson staining results (Fig. 5E). TGF-β, the typical fibrosis-associated cytokine, is IL-17RA dependent (Fig. 5F), suggesting that the mechanism underlying the protection of the liver from CCl4-induced fibrosis in IL-17RA–deficient mice might be partly due to downregulation of TGF-β.

**Activation of HSCs and production of collagen are IL-17A dependent**

We then asked whether HSCs contribute to IL-17A–dependent liver fibrosis. First, we confirmed that isolated HSCs express IL-17RA and respond to rIL-17A stimulation (Supplemental Fig. 1B). We found that in vitro activation of HSCs is IL-17A dependent. rIL-17A was used to activate isolated quiescent HSCs for 24 h at 0, 1, 10, 30, or 100 ng/ml. We demonstrated that IL-6 and α-SMA gene transcripts were dose dependently increased by rmIL-17A stimulation, whereas TGF-β gene expression exhibited significant increase only upon 100 ng/ml rmIL-17A restimulation (Fig. 6A). Moreover, collagen production in the medium was upregulated by the increasing concentration of rmIL-17A stimulation (Fig. 6B, Supplemental Fig. 1C). Increased collagen production was observed already 1 h after the culture and increased during the 48-h culture period at 30 ng/ml rmIL-17A in quiescent HSCs (Fig. 6C), which supports the notion that IL-17A activates HSCs to produce collagen. We further investigated the mechanisms underlying IL-17A promotion of liver fibrosis. We found that the phosphorylation of MAPK members, such as ERK1/2, JNK, and p38, are IL-17A signaling dependent in HSCs, and that the phosphorylation was distinct at 30 min and plateaued for p-ERK1/2 between 6 and 24 h (Fig. 6D). We further used nontoxic concentrations of MAPK inhibitors to investigate whether IL-17A–induced collagen production is MAPK signaling dependent and which pathways are potentially involved. Isolated HSCs were cultured in complete medium with JNK inhibitor SP600125 (25 µM), ERK/MEK1 inhibitor PD98059 (20 µM), or p38 inhibitor SB203580 (10 µM) for 1 h. The cells were stimulated with rmIL-17A for 24 h and absence of a cytotoxic effect of the pharmacologic inhibitors was confirmed by MTT viability assay (Supplemental Fig. 1D). We found that SB203580 or PD98059 significantly reduced collagen production in IL-17A–stimulated or quiescent HSCs, whereas the inhibitory effects of SP600125 were observed only in quiescent HSCs (Fig. 6E). Moreover, α-SMA production appears to be ERK1/2 and p38 dependent (Fig. 6F). These results strongly suggest that IL-17A activate HSCs in vitro independently, whereas IL-17A–dependent collagen expression is largely dependent on ERK1/2 and p38 pathway.

**Discussion**

Liver fibrosis is a serious medical condition that occurs upon chronic hepatitis virus infection, drug abuse, alcoholic liver disease, and autoimmune hepatitis, and potentially leads to portal hypertension, hepatic failure, and liver cancer (1). It has been widely reported that activated HSCs are the principal source of the extracellular matrix and collagen production (29), and upon activation they are instrumental in liver fibrosis and cirrhosis. We used

<table>
<thead>
<tr>
<th>Population Names</th>
<th>Vehicle (×10^6)</th>
<th>CCl4^a (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mononuclear cells, mean ± SD</td>
<td>1.38 ± 0.282</td>
<td>3.64 ± 0.46^a</td>
</tr>
<tr>
<td>Neutrophils, mean ± SD</td>
<td>0.0352 ± 0.031</td>
<td>0.6443 ± 0.081^b</td>
</tr>
<tr>
<td>CD4^+ T cells, mean ± SD</td>
<td>0.1808 ± 0.037</td>
<td>0.5913 ± 0.074^a</td>
</tr>
<tr>
<td>CD8^+ T cells, mean ± SD</td>
<td>0.0931 ± 0.019</td>
<td>0.3662 ± 0.046^b</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 10).

^a p < 0.01 or ^b p < 0.001, significantly different from control or CCl4 group, respectively, using Student’s t test.
gene-deficient mice and evaluated the role of IL-17A in CCl4-induced acute and chronic injury and HSC activation.

IL-17A/IL-17RA signaling plays a critical role in autoimmune diseases such as asthma, arthritis, ulcerative colitis, and Crohn’s disease (30–33). IL-17A is involved in neutrophil recruitment and angiogenesis (34), and IL-17A expression is partially dependent on IL-1 and IL-23 signaling (8, 35). However, the relationship between IL-17A and liver fibrosis remains elusive and further investigation is warranted. In this study, we used clinical samples and found that IL-17A was upregulated in fibrotic liver tissue. This finding is consistent with the elevated collagen and α-SMA levels that were found in the liver. Furthermore, we evaluated the contribution of IL-17A signaling in experimental CCl4-induced acute liver injury via genetic analysis. Compared with the wild type C57BL6 mice, IL-17RA–deficient mice exhibited a protective effect. Hepatic necrosis and inflammation, serum ALT levels, hepatic MMP-9, TIMP-1 and collagen content, and MPO activity were attenuated in IL-17RA KO mice, indicating the CCl4-induced liver injury and inflammation is likely IL-17A signaling dependent. IL-17A levels were not significantly reduced in IL-17RA KO mice upon 2-g/kg CCl4 challenge compared with WT mice. However, in the absence of IL-17RA, signaling of CCl4-induced liver injury and inflammation is attenuated, supporting the notion of an IL-17–dependent effect.

Because IL-17A plays an important role in the regulation of inflammation, we aimed to verify the sources of IL-17A in experimental liver lesions. Previous reports suggested that CD4+ T cells, NKT cells, and γδ T cells produce IL-17A (26, 36, 37). Specifically, CD8+ cells produce IL-17A in patients with chronic obstructive pulmonary disease (38). For innate immune cells, IL-17A expressed by Paneth cells has been implicated in the systemic inflammatory response (39); neutrophils also represent an important source of IL-17A in inflamed synovium (40) and renal ischemia/reperfusion (41). In liver, Kupffer cells were reported to produce IL-17A, which could potentially promote liver fibrosis in the bile duct ligation and CCl4 model (42). Our findings indicated that CD4+ and CD8+ T cells both exhibited increased IL-17A expression. However, Gr-1hi neutrophils represent the major source of IL-17A among the cells infiltrating the liver 24 h after CCl4 injection. The upregulation of transcription factor RORγt in neutrophils suggests that neutrophils express the Th17 transcription factor, and hence enabling those cells to produce IL-17A, which is consistent with our previous findings in hepatic ischemia/reperfusion injury (20). Therefore, IL-17A–expressing neutrophils potentially exhibit an autoamplification effect. T cells as the typical IL-17A–producing cells also present and contribute to IL-17A production, whereas NK and NKT cells did not exhibit upregulation of IL-17A or RORγt–EGFP (data not shown).

The IL-17RA–deficient mice exhibited dampened MMP-9 and TIMP-1 expression levels, which are essential to extracellular remodeling, indicating that IL-17A potentially plays a profibrotic role. Thus, by injecting mice with CCl4 at indicated intervals, we successfully induced liver fibrosis in C57BL/6 and IL-17RA–deficient mice. We demonstrated that IL-17RA deficiency largely protects mice from repeated challenges that cause liver injury, profibrotic cytokine expression, and fibrosis with collagen deposition. TGF-β, the hallmark of fibrotic processes, enhances collagen and matrix deposition by activation of HSCs through the Smad2 and Smad3 pathways. This is further supported by the fact that neutralization of the biological activity of TGF-β ameliorates experimental liver fibrosis (2). IL-17A was reported to exhibit the ability to promote pulmonary fibrosis via TGF-β (43). In this

![FIGURE 5](http://www.jimmunol.org/)

CCl4-induced liver fibrosis is IL-17RA dependent. C57BL/6 and IL-17RA–deficient mice were administered i.p. 0.5 g/kg CCl4 (diluted in corn oil) or oil only twice a week for 21 d, and mice were sacrificed at 24 h after the last challenge. (A) Liver sections from C57BL/6 and IL-17RA–deficient mice injected with CCl4 or oil were stained with H&E (original magnification ×200, ×400). (B) Collagen deposition in liver sections (Masson trichrome, original magnification ×100). (C) Microscopic liver lesions were graded by Suzuki scores. (D) Serum ALT levels indicating liver injury. (E) Sircol assay was performed to measure the collagen in the liver homogenate supernatant. (F) KC in liver homogenates (ELISA). Data are mean ± SEM (n = 6–8 mice/group) and are representative of three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).
study, we revealed IL-17RA–dependent TGF-β upregulation in CCl₄-induced liver fibrosis. Because TGF-β is the key cytokine that promotes IL-17A⁺ cell differentiation (7), further investigation of the signaling that drives the positive feedback of TGF-β and IL-17A is warranted.

In chronic liver disease, HSC differentiates into a myofibroblast-like phenotype characterized by proliferation and the deposition of abnormal matrix (4). HSCs that express multiple receptors are the primary targets of fibrogenic stimuli in the diseased liver. Among these, IL-17RA is the newly discovered receptor expressed in HSC-mediated alcoholic liver fibrosis (24). We also demonstrated that HSCs from C57BL/6 mice constitutively express IL-17RA (Supplemental Fig. 1B) and responded to rIL-17A restimulation by expressing IL-6, α-SMA, and TGF-β mRNA.

MAPKs are activated within the protein kinase family, which respond to extracellular stimuli (mitogens, heat shock, or proinflammatory cytokines) and modulate cellular activities, such as gene expression, proliferation, differentiation, mitosis, cell survival, and apoptosis (44). By using GFAP²αS–/– mice (glial fibrillary acidic protein, an HSC-specific marker), Meng et al. found that Stat3 are critical in IL-17A–induced HSC activation and collagen production, and rmIL-17A could promote the expression of α-SMA in isolated HSCs (42). Because Stat3 could be phosphorylated by activated ERK1/2, JNK, or p38 (at Ser727), we further investigated which MAPK pathway may be involved. We observed that ERK1/2, p38, and JNK exhibit time-dependent phosphorylation in HSCs upon IL-17A restimulation, and their response to rmIL-17A restimulation is rapid (within 30 min), like previously observed in human airway smooth muscle cells (45, 46). Furthermore, pharmacological inhibition of ERK1/2 or p38 decreased collagen and α-SMA expression in rmIL-17A–challenged or quiescent HSCs. This is a novel finding that may partially explain how IL-17A signaling induced profibrotic protein expression. JNK phosphorylation appeared to be rmIL-17A dependent, but JNK inhibition by the selective inhibitor SP600125 could reduce collagen and α-SMA production only in inactivated HSCs, but not on rmIL-17A–induced collagen deposition and α-SMA production. The disparity between JNK and ERK1/2 or p38 was consistent with the similar findings on rat HSCs (47). The conflicting effects of JNK on collagen production in activated and

FIGURE 6. HSC activation is IL-17A dependent. Mouse HSCs were isolated from naive C57BL/6 mice and stimulated as previously described. (A) IL-6, TGF-β, and α-SMA RNA expression in HSCs was activated in vitro by rmIL-17A; qPCR expressed as fold RNA expression over GAPDH. (B) Collagen released into the supernatant upon 0, 1, 10, 30, and 100 ng/ml rmIL-17A exhibited dose dependency. Results were normalized to the cell viability and presented by percentage changes with respect to those of the cells without rmIL-17A restimulation. (C) Collagen released into the supernatant upon 30 ng/ml rmIL-17A restimulation was time dependent. (D) Western blot analysis at 0 min, 5 min, 10 min, 30 min, 1 h, 3 h, 12 h, and 24 h after 30 ng/ml rm-IL-17A stimulation to investigate whether the activation of HSCs is relying on ERK/MAPK or JNK phosphorylation. Quiescent HSCs (0 min) were taken as negative control. ImageJ was used for computer-assisted semiquantitative analysis of ERK1/2, JNK, or p38 phosphorylation. (E) HSCs were cultured with JNK inhibitor SP600125, ERK1/2 inhibitor PD98059, or p38 inhibitor SB203580 for 1 h, and stimulated with 30 ng/ml rmIL-17A or those inhibitors for another 24 h. The supernatant and cell proteins were collected, respectively. (F) α-SMA expression was examined by Western blot. ImageJ was used for computer-assisted semiquantitative analysis of α-SMA expression. Data represent mean ± SEM. Experiments were repeated three times; comparisons between subgroups were performed by one-way ANOVA followed by the Newman–Keuls posttest: *p < 0.05, **p < 0.01, ***p < 0.001 (compared with medium cultured cells); #p < 0.05, ##p < 0.01 (compared with rmIL-17A–treated cells).
quiescent HSCs need further investigation. The regulation of α5β1 and CD45 expression, which upon rmIL-17A stimulation seems to be multifactorial, involves the ERK1/2 pathway and p38, but not JNK. Although Valente et al. suggested that IL-17A stimulates MAPK through negative regulation of MAPK phosphatase-1 (48), the exact relationship between IL-17A and MAPK activation remains elusive.

In conclusion, our data emphasize the critical role of IL-17A in the pathogenesis of liver fibrosis. CCL4-induced liver injury and liver fibrosis is IL-17RA signaling dependent. IL-17A produced by neutrophils and CD4+ T and CD8+ cells promoted proinflammatory cytokine expression, neutrophil influx, liver injury, inflammation, and fibrosis, all of which were attenuated in IL-17RA KO mice. Because HSCs are the principal target cells of profibrotic mediators, our in vitro data on IL-17A–dependent HSC activation and collagen expression through ERK1/2 and p38 signaling provide a mechanism of fibrogenesis. Therefore, IL-17A neutralization could potentially benefit human patients with chronic hepatitis and liver fibrosis.

Disclosures

The authors have no financial conflicts of interest.

References


47. Schnabl, B., C. A. Bradham, B. L. Bennett, A. M. Manning, B. Stefanovic, and D. A. Brenner. 2001. TAK1/JNK and p38 have opposite effects on rat hepatic stellate cells. Hepatology 34: 953–963.

(A) Fibrotic liver tissues were collected from partial hepatectomy due to the early-stage hepatocellular carcinoma which base on cirrhosis; control livers are normal tissues obtained from hepatectomy for hepatic hemangioma. The liver sections were prepared and stained with rabbit serum replacing of anti-IL-17A antibody as negative control (400x magnification). One representative result is shown from ten specimen.

(B) Whole liver homogenate, recombinant IL-17A, isolated quiescent HSC and HSC followed 24 h restimulation of 30 ng/ml rmIL-17A and MAPK inhibitors were used to restimulate the quiescent HSC and measure cells viability via MTT reduction assay as previous described. Western Blot were performed as previous described. Western Blot were performed to check IL-17RA expression. (c) and (d) Different concentration of DMEM (solvent), mili-17A and MAPK inhibitors were used to restimulate the quiescent HSC and measure cells viability via MTT reduction assay as previous described. The data represent mean ± SEM. Experiments repeated three times. Comparisons between subgroups were performed by using a One-Way ANOVA of variance with Newman-Keuls post-hoc (* p<0.05, ** p<0.01, *** p<0.001).
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*Supplementary Table 1: Primers used*