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IL-17A Enhances the Expression of Profibrotic Genes through Upregulation of the TGF-β Receptor on Hepatic Stellate Cells in a JNK-Dependent Manner

Thomas Fabre,*† Hassen Kared,* Scott L. Friedman,‡ and Naglaa H. Shoukry*§

Activation of hepatic stellate cells (HSCs) is a key event in the initiation of liver fibrosis, characterized by enhanced extracellular matrix production and altered degradation. Activation of HSCs can be modulated by cytokines produced by immune cells. Recent reports have implicated the proinflammatory cytokine IL-17A in liver fibrosis progression. We hypothesized that IL-17A may enhance activation of HSCs and induction of the fibrogenic signals in these cells. The human HSC line LX2 and primary human HSCs were stimulated with increasing doses of IL-17A and compared with TGF-β– and PBS-treated cells as positive and negative controls, respectively. IL-17A alone did not induce activation of HSCs. However, IL-17A sensitized HSCs to the action of suboptimal doses of TGF-β as confirmed by strong induction of α-smooth muscle actin, collagen type I (COL1A1), and tissue inhibitor of matrix metalloproteinase I gene expression and protein production. IL-17A specifically upregulated the cell surface expression of TGF-βRII following stimulation. Pretreatment of HSCs with IL-17A enhanced signaling through TGF-βRII as observed by increased phosphorylation of SMAD2/3 in response to stimulation with suboptimal doses of TGF-β. This enhanced TGF-β–response of HSCs induced by IL-17A was JNK-dependent. Our results suggest a novel profibrotic function for IL-17A by enhancing the response of HSCs to TGF-β through activation of the JNK pathway. IL-17A acts through upregulation and stabilization of TGF-βRII leading to increased SMAD2/3 signaling. These findings represent a novel example of cooperative signaling between an immune cytokine and a fibrogenic receptor.


The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ACTA2, gene coding for α-smooth muscle actin; COL1A1, gene coding for collagen type I; ΔCp, change (delta) in crossing points; HBV, hepatitis B virus; HSC, hepatic stellate cell; MFI, mean fluorescence intensity; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; α-SMA, α-smooth muscle actin; TIMP, tissue inhibitor of matrix metalloproteinase; Treg, regulatory T cell.

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IL-17A ENHANCES THE FIBROGENIC EFFECT OF TGF-β ON HSCs

Peripheral blood (16, 17), IL-17–producing cells were linked to liver fibrosis progression in different liver pathologies, including alcoholic hepatitis and hepatitis B virus (HBV) infection (18, 19). IL-17A induces chemokine secretion by HSCs and hepatocytes, which include IL-8 and growth-regulated protein α that are important in the recruitment of profibrotic macrophages, monocytes, and neutrophils (18). Furthermore, signals induced by the IL-17 receptor complex IL-17RA/IL-17RC in different cell types, including HSCs, lead to activation of the intracellular factors NF-kB, JNK, MAPK, and STAT-3, which are all linked to inflammation and liver fibrosis progression (20–24). IL-17A also enhances liver fibrosis through activation of macrophages leading to production of profibrotic cytokines such as IL-6, TNF-α, TGF-β, and PDGF (8, 25). Using IL-17RA–/– knockout mice, it was demonstrated in vivo that specific depletion of IL-17RA on macrophages results in reduced liver fibrosis (25).

In this study, we have developed an in vitro fibrosis assay based on the quantification of profibrotic markers α-SMA, collagen type I, and TIMP-I produced by the hepatic stellate cell line (LX2) or primary human HSCs. This model was used to assess the molecular mechanisms and the direct fibrotic functions of IL-17A on HSCs. Using a combination of quantitative RT-PCR, Western blot, picro-Sirius red staining, and flow cytometry, we demonstrate that IL-17A has profibrotic properties. This cytokine enhances the response of HSCs to the major profibrotic cytokine TGF-β by upregulating the expression of the TGF-βRII on the surface of HSCs in a JNK-dependent manner.

Materials and Methods

Antibodies

For flow cytometry, directly conjugated Abs against TGF-βRII-PE (R&D Systems, Minneapolis, MN) and CD217 (IL-17RA)–allophycocyanin (clone 424LTS) (eBioscience, San Diego, CA) were used. For Western blot, Abs against the following molecules were used: TIMP-I (clone 2E7.1) (Abcam, Cambridge, MA), MMP-2 (clone 6E3F8) (Abcam, Toronto, ON, Canada), α-SMA (clone 1A4) (Sigma-Aldrich, St. Louis, MO), SMAD3 (clone C6H7) (Cell Signaling Technology, Danvers, MA), phospho-SMAD2/3 (clone C25A9) (Cell Signaling Technology), and GAPDH (clone 6C5) (Santa Cruz Biotechnology, Santa Cruz, CA.).

Cell culture

The human HSC line LX2 and primary human HSCs were used as previously described (11). LX2 cells were cultured in DMEM (Wisent, St. Bruno, QC, Canada) supplemented with 10% FBS (HyClone, Nepean, ON, Canada) and GlutaMAX (Life Technologies, Burlington, ON, Canada). For all experiments, LX2 cells were seeded at 2 × 10^5 cells/well in a 48-well plate, 2 × 10^3 cells/well in a 6-well plate, or 1 × 10^3 cells/well in a 12-well plate. When cells reached 70% confluence, they were serum-starved in DMEM supplemented with GlutaMAX without FBS for 48 h prior to a 48-h stimulation in serum-free conditions.

Cytokines

Recombinant human IL-17A and TGF-β were obtained from R&D Systems.

Quantitative real-time RT-PCR

After stimulation, total RNA from the LX2 cells was extracted using a RealTime ready cell lysis kit (Roche). cDNAs were generated with a Transcripter Universal CDNA (Roche) kit with DNase I treatment, then diluted in ultrapure water (Roche) at a ratio of 1:5 and amplified using the LightCycler 480 SYBR Green I Master kit on a LightCycler 480 (Roche). Relative expression of the profibrotic genes ACTA2 (coding for α-SMA), COL1A1 (coding for collagen type I), TIMP-I, and TGF-B1 were measured and normalized to ribosomal 28S rRNA expression. Standard curves were generated for each gene to determine the reaction efficiency. We used the advanced relative quantification method from Roche comparing change (delta) in crossing points (ΔCp), according to the formula: efficacy of target gene = 2^(-ΔCp) / efficacy of housekeeping gene. Primer sequences are listed in Table I. TGF-β1 primers were purchased from Qiagen (Hs_TGFB1_1_SG QuantiTect Primer Assay, catalog no. QT0000726).

Western blotting

After stimulation, cells were lysed with RIPA buffer (NaCl, Nonidet P-40, SDS, Tris-HCl buffer [pH 8]) in the presence of protease and phosphatase inhibitors (Roche). For detection of α-SMA, MMP-2, and TIMP-1, 20 μg total proteins, quantified using a Bradford assay, was loaded on a 20% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). For detection of SMAD2/3 and phospho-SMAD2/3, only 10 μg total proteins was used. Blots were incubated with primary Ab overnight at 4˚C and then with secondary HRP-conjugated Ab (Cell Signaling Technology) at room temperature for 1 h. Blots were developed with the ECL prime Western blotting detection reagent (GE Healthcare, Buckinghamshire, UK.). GAPDH was used as a loading control.

Picro-Sirius red staining and immunofluorescence

LX2 cells (5 × 10^5) were cultured in an eight-chamber slide (BD Biosciences, San Jose, CA) to 70% confluence. Cells were then serum-starved for 48 h followed by another 48 h stimulation in serum-free conditions. Collagen type I production was measured by picro-Sirius red staining. Cells were fixed in an acetone bath at −20˚C for 15 min. Slides were incubated for 1 h in picro-Sirius red (Sigma-Aldrich) and washed twice in 0.05% glacial acetic acid. Sirius red quantification was performed on three different areas per condition using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). For immunofluorescence, cells were fixed in a 20% aceton/80% methanol bath at −20˚C for 15 min. Slides were blocked in PBS 1% BSA for 1 h and then incubated 1 h with anti–phospho-SMAD2/3 (clone C25A9) followed by a 1-h incubation with goat anti-rabbit Alexa Fluor 488 (Life Technologies). Slides were then mounted with ProLong Gold antifade reagent with DAPI (Life Technologies). Image acquisition and analysis were performed on a Zeiss Axio Imager M2 using Zen software (Carl Zeiss Canada, Toronto, ON, Canada).

Flow cytometry analysis

LX2 cells were detached using Versene (Life Technologies), washed twice in FACS buffer (PBS 1×, 1% FBS, 0.02% sodium azide), and incubated for 30 min with the Abs. Cells were then washed in FACS buffer and fixed with fixation buffer (PBS 1×, 1% formaldehyde). Data acquisition was performed on a BD LSR II equipped with blue (488 nm), red (633 nm), and violet (405 nm) lasers using FACSFlow software (version 5.0.3) (BD Biosciences). FACS analysis was performed on FlowJo software (version 9.4.11) for Macintosh (Tree Star, Ashland, OR).

Statistical analysis

All data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA), and differences between the means for each condition were evaluated by a one-way ANOVA followed by a Tukey post hoc test.

Results

IL-17A synergizes with TGF-β to induce hepatic stellate cell activation

To evaluate the effect of IL-17A on HSCs, we stimulated LX2 cells for 48 h with a low dose of 1 ng/ml and a high nonphysiological dose of 40 ng/ml IL-17A (referred to hereafter as IL-17Alo and IL-17Ahi, respectively). Cells stimulated with TGF-β were treated with a dose of 40 ng/ml TGF-β1hi (referred to hereafter as TGF-β1hi, respectively). IL-17Ahi induced a 5-fold increase in the expression of COLA1A1, ACTA2 (α-SMA), and TIMP-I genes as compared with the PBS-treated LX2 cells (n = 3, p < 0.0001) (Fig. 1A). Increased production of α-SMA and TIMP-I proteins was also observed by Western blotting (Fig. 1B). Similarly, picro-Sirius red staining showed a 4-fold increase in collagen type I production in the TGF-β1hi–treated as compared with the PBS-treated LX2 cells (n = 3, p < 0.05) (Fig. 1D, 1F). We also used a suboptimal dose of...
FIGURE 1. IL-17A enhances the fibrogenic process in LX2 and primary human HSCs in response to TGF-β. (A) Relative expression of the profibrotic genes COL1A1, ACTA2, and TIMP-I by LX2 cells after 48 h stimulation with IL-17A with and without TGF-β was determined by quantitative PCR, and results are shown as fold change compared PBS- versus cytokine-treated LX2 cells. Black asterisks represent a significant increase compared with PBS-treated cells, whereas white asterisks represent a significant increase compared with cells treated with TGF-β at low dose. Protein expression of α-SMA, TIMP-I, and MMP-2 after 48 h stimulation with IL-17A with and without TGF-β was evaluated in LX2 (B) and primary human HSCs (C) by Western blot. Collagen type I secretion was measured by picro-Sirius red staining of LX2 (D) and primary human HSCs (E) after stimulation with IL-17A with and without TGF-β, and relative quantification of picro-Sirius red staining was performed (F). Figure is representative of three independent experiments. One-way ANOVA was used followed by a Tukey post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.
TGF-β (0.5 ng/ml), referred to as TGF-β<sub>lo</sub>, that induces intermediate activation of HSCs with a 2-fold increase in COL1A1 (n = 3, p = 0.0001) and TIMP-1 (n = 3, p < 0.05) mRNA. This dose also induced low levels of α-SMA protein as measured by Western blot and weak picro-Sirius red staining. IL-17A alone, at both low and high doses, was insufficient to activate LX2 cells. No significant increase in profibrotic markers was observed as compared with PBS-treated cells at either the mRNA (Fig. 1A) or the protein level (Fig. 1B, 1D, 1F). IL-17A at both doses induced strong expression of the TGF-β1 gene (Supplemental Fig. 1), as previously described (22, 26). Similar results were observed with shorter stimulation time (data not shown). However, we observed strong activation of LX2 cells, similar to TGF-β<sub>hi</sub>, when IL-17A was combined with a suboptimal TGF-β<sub>lo</sub> dose. A 5-fold increase in COL1A1 (n = 3, p < 0.0001), ACTA2 (n = 3, p < 0.0001), and TIMP-1 (n = 3, p < 0.0001) expression was measured by quantitative RT-PCR (Table I) when compared with PBS-treated cells. Furthermore, an increase of >2-fold (n = 3, p < 0.001, represented by white asterisks) was observed when compared with TGF-β<sub>lo</sub> alone (Fig. 1A). These transcription profiles were validated at the protein level. A strong production of α-SMA and TIMP-1 was observed by Western blot in LX2 cells treated with IL-17A and TGF-β<sub>lo</sub> (Fig. 1B). Finally, collagen type I production was also higher (n = 3, p < 0.05) when compared with both TGF-β<sub>lo</sub> and PBS-treated LX2 cells (Fig. 1D, 1F). Collectively, these results suggest that IL-17A alone is not sufficient to prime LX2 cell activation. However, this cytokine can act in synergy with TGF-β to induce the fibrogenic process.

**IL-17A profibrotic function is validated in primary human HSCs**

Next, we validated our results using primary human HSCs. Similar to previous reports, TIMP-1 and MMP-2 expression were higher by primary HSCs than LX2 cells (27). IL-17A at both doses did not induce α-SMA, collagen type I, MMP-2, or TIMP-1. However, the addition of IL-17A to suboptimal TGF-β<sub>lo</sub> doses led to strong induction of α-SMA and TIMP-1 (Fig. 1C) observed by Western blot, as compared with PBS- or TGF-β<sub>lo</sub>-treated HSCs. Picro-Sirius red staining demonstrated a similar effect on collagen type I production by primary HSCs (Fig. 1E). However, these cells were more sensitive to TGF-β than were LX2 cells, as we observed a 3-fold increase in picro-Sirius red stain with TGF-β<sub>hi</sub> alone. The addition of IL-17A to TGF-β<sub>lo</sub> still induced a 5-fold increase in picro-Sirius red stain (Fig. 1F) and was significantly higher than TGF-β<sub>lo</sub> alone (p < 0.05). Thus, our observations underscore again a synergetic effect between IL-17A and TGF-β, which validated our results obtained with the LX2 cell line.

**Blockade of TGF-β receptor I– and II–associated kinase abrogates the enhanced activation of HSCs by IL-17A and TGF-β**

Next, we sought to determine whether the IL-17A profibrotic effect is mediated through an increased response of HSCs to TGF-β. We used Ly2109761, a specific inhibitor of kinases associated to both the TGF-βRI and TGF-βRII subunits (28). This reagent interferes with TGF-β signaling by decreasing the phosphorylation of SMAD2/3 and has no effect on IL-17A signaling (Supplemental Fig. 2). We determined the optimal concentration of Ly2109761 to be 100 μM (data not shown). The addition of Ly2109761 abrogated the transcription of COL1A1, ACTA2, and TIMP-1 (n = 3, p < 0.0001), which is normally observed with the combination of IL-17A with TGF-β<sub>lo</sub> stimulation (Fig. 2A). Furthermore, COL1A1, ACTA2, and TIMP-1 transcription was lower in the presence of the inhibitor than in PBS-treated cells. Inhibition of profibrotic gene expression induced by Ly2109761 was >95% (data not shown). These results were validated at the protein level by Western blot. Upregulation of TIMP-1 and α-SMA induced by IL-17A at both doses in combination with TGF-β<sub>lo</sub> was lost by the addition of Ly2109761 (Fig. 2B). Altogether, these results prove that TGF-β signaling is required for the enhancement of HSC activation observed in LX2 cells stimulated with IL-17A and suboptimal doses of TGF-β.

**IL-17A induces the upregulation of TGF-βRII expression on the surface of LX2 cells**

We then explored the mechanisms by which IL-17A enhances the response to TGF-β. First, we evaluated the cell surface expression of TGF-βRII and IL-17RA upon cytokine stimulation. Flow cytometry analysis was performed on PBS-, IL-17A–, and/or TGF-β–treated cells for 48 h. The summary of five independent experiments is presented as average fold change in the mean fluorescence intensity (MFI) between PBS- versus cytokine-stimulated cells. The cell surface expression of TGF-βRII was significantly altered (n = 5, p = 0.0002) (Fig. 3A, 3B), but no significant variation in IL-17A RA expression was observed (n = 5, p = 0.243) (Fig. 3C). TGF-β stimulation induced a decrease in TGF-βRII cell surface expression that was significant at high but not at low doses (n = 5, p < 0.05) (Fig. 3A, 3B). This indicates that LX2 cells respond to TGF-β, and through feedback mechanisms they downregulate the cell surface expression of its receptor. IL-17A upregulated the cell surface expression of TGF-βRII when compared with PBS-treated HSCs (n = 5, p < 0.01) (Fig. 3A, 3B). LPS-induced upregulation of TGF-βRII was tested as a positive control and was similar to that of IL-17A (data not shown) (29). We also determined whether IL-17A could prevent the downregulation of the TGF-βRII expression induced by TGF-β. Therefore, we performed LX2 cell stimulation with TGF-β<sub>hi</sub> and TGF-β<sub>lo</sub> in combination with IL-17A. After 48 h of stimulation, IL-17A significantly prevented downregulation of the TGF-βRII induced by both doses of TGF-β (n = 5, p < 0.05) (Fig. 3B). These results indicate that IL-17A can partially prevent TGF-βRII downregulation induced by TGF-β and might lead to increased or sustained signaling.

### Table I: Primer sequences for quantitative RT-PCR

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<th>Gene</th>
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<th>Accession No.</th>
<th>Sequence (5’ → 3’)</th>
<th>Amplicon Size (bp)</th>
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<tr>
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</table>
IL-17A increases the phosphorylation of SMAD2/3 in response to suboptimal doses of TGF-β

To determine whether the IL-17A–mediated upregulation of TGF-βRII could enhance the response of HSCs to TGF-β, we evaluated whether IL-17A could modulate the activation of the SMAD2/3 pathway. LX2 cells were stimulated for 24 h with or without IL-17A at both doses followed by 15 min stimulation with suboptimal TGF-βlo. The phosphorylation of SMAD2/3 at Ser423/425 was evaluated by Western blot and compared with TGF-βhi. No phosphorylation of SMAD2/3 was observed when cells were treated with IL-17A alone. A low, almost undetectable, level of phosphorylation was induced by TGF-βlo as compared with TGF-βhi (Fig. 3D). However, similar to TGF-βhi, robust phosphorylation of SMAD2/3 was observed in LX2 cells stimulated with IL-17A at both doses prior to exposure with TGF-βlo (Fig. 3D). The level of total SMAD2/3 was increased in LX2 cells treated with IL-17A alone, which was not observed with TGF-βlo (Fig. 3D, right panel). We validated our results by confocal microscopy and observed increased phosphorylation of SMAD2/3 in TGF-βlo and IL-17A–prestimulated cells. However, the localization of SMAD2/3 was different with higher nuclear localization in IL-17A–stimulated cells (Fig. 3E, Supplemental Fig. 3A). These data correlate with the synergistic effect observed between IL-17A and TGF-βlo (Fig. 1), as well as the upregulation of TGF-βRII cell surface expression induced by IL-17A (Fig. 3A, 3B).

IL-17A enhances response of LX2 cells to TGF-β in a JNK-dependent manner

The increased nuclear localization of SMAD2/3 when HSCs were stimulated with IL-17A led us to investigate the activation of the JNK pathway. It was previously demonstrated that the activation of JNK enhances phosphorylation and nuclear translocation of SMAD2/3, leading to increased fibrosis (30, 31). We confirmed by Western blot that IL-17A alone induces phosphorylation of JNK (Fig. 4A). We next proceeded to inhibit the phosphorylation of JNK using the chemical inhibitor SP600125, which was previously shown to inhibit liver fibrosis (21, 32–34). We determined the optimal concentration of SP600125 to be 10 μM (data not shown). Blockade of the JNK pathway prevented the induction of profibrotic gene expression (data not shown) as well as protein production in response to IL-17A with suboptimal TGF-βlo (Fig. 4B). We also observed no significant variation in the gene expression profiles of COL1A1, ACTA2, and TIMP-I in LX2 cells stimulated with TGF-β alone in the presence or absence of SP600125 (data not shown). This suggests that SP600125 had no to little direct effect on the response to TGF-β itself. Moreover, inhibition of JNK blocked the upregulation of TGF-βRII induced by IL-17A (Fig. 4C, 4D). This observation correlated with a loss of increased phosphorylation of SMAD2/3, observed by Western blot and immunofluorescence staining, after stimulation with IL-17A and suboptimal TGF-βlo (Fig. 4E, 4F, Supplemental Fig. 3). Interestingly, we observed by confocal microscopy a low level of...
phosphorylated SMAD2/3 in IL-17A/TGF-β–stimulated HSCs, which was exclusively localized to the cytoplasm. This suggests that IL-17A enhanced the response of HSCs to TGF-β by the activation of the JNK pathway.

**Discussion**

We have examined the profibrogenic effect of IL-17A on both the LX2 cell line and primary human HSCs. We demonstrate that IL-17A alone was not sufficient to induce α-SMA, collagen type I, and TIMP-I production. However, IL-17A enhanced stellate cell responses to TGF-β by increasing cell surface expression of its receptor, which led to increased phosphorylation and nuclear translocation of the transcription factors SMAD2/3 in response to suboptimal doses of TGF-β. Furthermore, we demonstrated that this phenotype was dependent on the activation of the JNK pathway by IL-17A.
We also investigated whether IL-17A can directly induce profibrotic genes within HSCs. We demonstrated that IL-17 could not induce COL1A1, ACTA2, and TIMP-I gene or protein expression in LX2 cells or primary human HSCs. These results differ from recent reports suggesting that IL-17A can directly enhance the transcription of profibrotic genes in HSCs (22, 24). However, these two studies attributed this function to two different pathways (MAPK or STAT3). These discrepancies could also be explained by differences in the experimental conditions. In those two studies, the authors stimulated HSCs for 2–8 h, which in our hands did not show any effect (data not shown). This is why we chose to stimulate our cells for 48 h based on a previous report showing

**FIGURE 4.** IL-17A enhances TGF-β response of hepatic stellate cells in a JNK-dependent manner. (A) Phosphorylation of JNK was evaluated by Western blot after 15–30 min stimulation with IL-17A. (B) Protein expression of α-SMA and TIMP-I after 48 h stimulation with TGF-β or TGF-β plus IL-17A in the presence or absence of the JNK inhibitor, SP600125, was evaluated by Western blot. (C) Cell surface expression of TGF-βRII was measured by flow cytometry after 48 h stimulation with IL-17A and/or TGF-β in the absence (left panel) or presence of the JNK inhibitor (right panel). Representative histogram of TGF-βRII cell surface expression (n = 3) after IL-17A and/or TGF-β stimulation is shown. (D) Results are displayed as fold increase of MFI (n = 3). Original magnification ×40. (E) Phosphorylation of SMAD2/3 at Ser423/425 was evaluated by Western blot after 15 min stimulation with TGF-β in presence of JNK inhibitor. (F) Two-color immunofluorescence confocal microscopy was used to observe phosphorylated SMAD2/3 (green, Alexa Fluor 488) and nucleus (blue, DAPI). *p < 0.05, **p < 0.01, ***p < 0.001.
that efficient IL-17A signaling is observed after a minimum of 24 h (35). Furthermore, we performed all stimulations on serum-starved cells to eliminate the presence of TGF-β found in FBS. Alternatively, our results are concordant with other groups that demonstrated that IL-17A did not induce the expression of profibrotic genes but rather the expression of proinflammatory cytokines and chemokines involved in the recruitment of macrophages, neutrophils, and monocytes (18, 25, 36). Dermal fibroblasts were also reported to produce profibrotic cytokines and chemokines in response to IL-17A without producing profibrotic molecules such as collagen type I (37).

IL-17A can also enhance fibrosis indirectly by inducing expression of TGF-β1 (22, 26). We also observed that IL-17A induces TGF-β1 expression in a dose-dependent manner in LX2 cells, which suggests a complementary effect of these two cytokines. In mice, specific knockdown of IL-17RA on macrophages strongly reduced liver fibrosis, whereas modest reduction was observed when knockdown was performed on liver-resident cells (25). Indeed, IL-17A can induce the production of the two major profibrotic cytokines PDGF and TGF-β with other inflammatory cytokines IL-1β, IL-6, and TNF-α by macrophages, neutrophils, and monocytes (8, 18, 36). Furthermore, inflammatory cytokines during hepatitis lead to activation of dendritic cells and monocytes. Activated dendritic cells and monocytes then secrete key cytokines involved in the differentiation and activation of Th17 cells. Finally, liver inflammation leads not only to cytokine production but also to increased chemokine production by activated dendritic cells, monocytes, and hepatocytes, such as CCL20, CCL22, and CCL17, which are involved in the recruitment of Th17 cells to the liver (38). This suggests a possible interaction between IL-17A and TGF-β-producing cells and requires further investigation in in vivo models of fibrosis.

We demonstrated that IL-17A enhances the response of both LX2 cells and primary human HSCs to suboptimal doses of TGF-β. We observed robust production of profibrotic genes after stimulation of HSCs with IL-17A in combination with TGF-β, suggesting a synergistic effect between these two cytokines. Blockade of TGF-βRI-associated kinase prevented the induction of profibrotic genes during IL-17A/TGF-β stimulation, which indicates that this phenotype is dependent on TGF-β signaling. The flow cytometry analysis of IL-17RA and TGF-βRII showed that both receptors were expressed by HSCs. Cytokine stimulation did not affect the cell surface expression of IL-17RA. However, after IL-17A stimulation we observed increased cell surface expression of TGF-βRII as well as prevention of its downregulation induced by TGF-β. It was also recently demonstrated that IL-17A increases PDGFR expression in gut smooth muscle cells and may suggest that IL-17A can modulate expression of different growth factor receptors (39). Additionally, we showed in IL-17A–stimulated HSCs increased phosphorylation and nuclear translocation of SMAD2/3 in response to suboptimal TGF-β. Thus, IL-17A is a profibrotic cytokine that contributes to the regulation of the fibrogenic process by increasing the TGF-β response at several cellular levels in HSCs. Similar collaborations between two distinct signaling pathways were reported to enhance liver fibrosis and activation of HSCs (40). Collectively, these findings suggest that many interactions between fibrotic pathways are yet to be discovered.

The JNK pathway is one of the key fibrogenic and inflammatory pathways in the liver and can be activated by several cytokines, including IL-17A (20, 21, 34, 41). JNK-deficient mice exhibit reduced fibrosis after CCL4 treatment, and purified HSCs from these mice are less responsive to activation signals (42). JNK plays an important role in regulating the response to TGF-β through modulation of the phosphorylation and nuclear translocation of SMAD2/3 (30, 31). Furthermore, TGF-β itself induces late activation of JNK, leading to a second round of activation of SMAD2/3, which correlates with an increase in fibrosis (31). We observed activation of JNK and increased nuclear translocation of SMAD2/3 in IL-17A–stimulated HSCs after only 15 min stimulation with TGF-β, suggesting that IL-17A was responsible for this phenotype. Blocking JNK with a chemical inhibitor abrogated induction of profibrotic genes, upregulation of the TGF-βRII, and increased phosphorylation of SMAD2/3 after IL-17A with suboptimal TGF-β stimulation. Thus, IL-17A enhanced the response of HSCs to TGF-β in a JNK-dependent manner that may lead to increased fibrosis.

In conclusion, we have demonstrated that IL-17A is a profibrotic cytokine with no direct fibrogenic effect on HSCs. However, IL-17A enhances the response of HSCs to TGF-β, leading to increased production of collagen type I, α-SMA, and TIMP-1. IL-17A may also enhance liver fibrosis indirectly via other mechanisms. First, it induces the recruitment of proinflammatory macrophages by increasing chemokine secretion by HSCs (18). IL-17A then activates these newly recruited macrophages to produce profibrotic cytokines, including TGF-β (22, 25). IL-17A also induces TGF-β by fibrotic cells, including HSCs (22, 26). Finally, IL-17A enhances the response of HSCs to TGF-β by upregulating its receptor expression at the cell surface in a JNK-dependent manner, leading to increased fibrosis. Further studies should determine the interactions and cellular pathways involved in the crosstalk between TGF-β–producing cells, HSCs, and IL-17A–producing cells, such as Th17, NK, and NKT cells, during chronic hepatitis. Finally, functional characterization of intrahepatic versus peripheral blood Th17 during liver injury and inflammation should be studied to validate our in vitro data.

Disclosures
The authors have no financial conflicts of interest.

References