Critical Role of Tumor Necrosis Factor Receptor 1, but not 2, in Hepatic Stellate Cell Proliferation, Extracellular Matrix Remodeling, and Liver Fibrogenesis

Núria Tarrats,[1] Anna Moles,[1] Albert Morales,[1] Carmen García-Ruiz,[1] José C. Fernández-Checa,[1,2*] and Montserrat Marí[1*]

Tumor necrosis factor (TNF) has been implicated in the progression of many chronic liver diseases leading to fibrosis; however, the role of TNF in fibrogenesis is controversial and the specific contribution of TNF receptors to hepatic stellate cell (HSC) activation remains to be established. Using HSCs from wild-type, TNF-receptor-1 (TNFR1) knockout, TNF-receptor-2 (TNFR2) knockout, or TNFR1/R2 double-knockout (TNFR-DKO) mice, we show that loss of both TNF receptors reduced procollagen-α1(I) expression, slowed down HSC proliferation, and impaired platelet-derived growth factor (PDGF)-induced promitogenic signaling in HSCs. TNFR-DKO HSCs exhibited decreased AKT phosphorylation and *in vitro* proliferation in response to PDGF. These effects were reproduced in TNFR1 knockout, but not TNFR2 knockout, HSCs. In addition, matrix metalloproteinase 9 (MMP-9) expression was dependent on TNF binding to TNFR1 in primary mouse HSCs. These results were validated in the human HSC cell line, LX2, using neutralizing antibodies against TNFR1 and TNFR2. Moreover, *in vivo* liver damage and fibrogenesis after bile-duct ligation were reduced in TNFR-DKO and TNFR1 knockout mice, compared to wild-type or TNFR2 knockout mice. *Conclusion:* TNF regulates HSC biology through its binding to TNFR1, which is required for HSC proliferation and MMP-9 expression. These data indicate a regulatory role for TNF in extracellular matrix remodeling and liver fibrosis, suggesting that targeting TNFR1 may be of benefit to attenuate liver fibrogenesis. (HEPATOLOGY 2011;54:319-327)

Tumor necrosis factor (TNF) is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signaling events within cells. TNF exerts its biological functions by interactions with two members of the TNF receptor (TNFR) superfamily, namely TNFR1 and TNFR2. The cytoplasmic tail of TNFR1 contains a death

Abbreviations: α-SMA, alpha-smooth muscle actin; BDL, bile-duct ligation; CCl_4, carbon tetrachloride; DTT, dithiothreitol; ECL, enhanced chemiluminescense; EDTA, ethylenediamine tetraacetic acid; ECM, extracellular matrix; EGTA, ethylene glycol tetraacetic acid; ERK, extracellular signal-related protein kinase; FBS, fetal bovine serum; H&E, hematoxylin and eosin; HSC, hepatic stellate cells; HRP, horseradish peroxidase; IL-1α, interleukin-1 alpha; JAK, Janus kinase; LPS, lipopolysaccharide; mRNA, messenger RNA; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa light-chain enhancer of activated B cells; PDGF, platelet-derived growth factor; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, short interfering RNA; TCA, trichloroacetic acid; TGF-β, transforming growth factor beta; TIMP-1, tissue inhibitor of metalloproteinase-1; TNF, tumor necrosis factor; TNFR, TNF-receptor; TNFR-DKO, TNFR1/R2 double knockout.

From the [1]IDIBAPS, Liver Unit-Hospital Clínic, CIBEREHD, and Department of Cell Death and Proliferation, IIBB-CSIC, Barcelona, Spain; and the [2]Research Center for Alcoholic Liver and Pancreatic Diseases, Keck School of Medicine of the University of Southern California, Los Angeles, CA.

Received September 29, 2010; accepted April 13, 2011.

The work was supported by grants PI09/00056, PI10/02114 (Instituto de Salud Carlos III), 2008-02199, and 2009-11417 (Plan Nacional de I+D), Spain; and P50-AA-11999 (Research Center for Liver and Pancreatic Diseases, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health).

**These authors share senior authorship.*

Address reprint requests to: José C. Fernández-Checa, Ph.D., or Montserrat Marí, Ph.D., Liver Unit, Hospital Clinic, C/Villarroel 170, 08036 Barcelona, Spain. E-mail: checa229@yahoo.com or monmari@clinic.ub.es; fax: 34-93-363-8301.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.24388

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

domain, which is essential for the induction of apoptosis. However, this motif is missing in TNFR2 and the function of this latter receptor is poorly understood.[1,2] In the liver, TNF functions as a double-edged sword through TNFR1, being required for normal hepatocyte proliferation during liver regeneration[3,4] and induction of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB), which is essential to elicit antiapoptotic defense and in the control of the immune response. Yet, on the other hand, TNF is the mediator of hepatotoxicity and inflammation in many animal models and has also been implicated as an important pathogenic player in patients with alcoholic liver disease, nonalcoholic steatohepatitis, or viral hepatitis.[5,6] Human and animal studies suggest that hepatocellular injury, followed by inflammation and activation of the innate immune system, leads to early-stage liver fibrosis, ultimately resulting in hepatic stellate cell (HSC) activation and extracellular matrix (ECM) deposition.[7,8] Although the contribution of TNF to hepatocellular injury and inflammation has been widely studied,[5,6,9,10] its specific contribution to HSC activation and liver fibrogenesis remains controversial. In this sense, experimental studies performed with knockout mice after carbon tetrachloride (CCl_4) administration have shown that the absence of either TNFR1[11] or TNFR1/R2 double-knockout (TNFR-DKO)[12] mice inhibit liver fibrosis accompanied by reduced expression of procollagen-α1(I) messenger RNA (mRNA), without effect on hepatic injury, suggesting a profibrogenic role for TNF. In contrast, a recent study showed that the inhibition of TNF processing via TNF-alpha–converting enzyme attenuated liver injury and inflammation after CCl_4 administration, but increased collagen deposition, effects reproduced in the TNFR-DKO mice.[13] Moreover, several reports using cultured HSCs point to an antifibrogenic role of TNF via the inhibition of *procollagen-α1(I)* gene expression[14-17] due, in part, to glutathione depletion.[18]

Hence, although TNF has been implicated in the progression of many chronic liver diseases leading to fibrosis, the specific involvement of TNF or its receptors, TNFR1 and TNFR2, in HSC activation remains to be established. The morphological and metabolic changes associated with HSC activation, reproduced by culturing isolated HSCs on plastic,[19,20] were studied in HSCs from wild-type, TNFR-DKO, TNFR1, and TNFR2 knockout mice to evaluate the impact of TNF signaling and thus its potential direct contribution to liver fibrosis. The results, validated *in vitro* in human activated LX2 cells and *in vivo* using a bile-duct ligation (BDL) mice model, led us to underscore the contribution of TNFR1 in liver fibrosis, and suggest that the blockage of specific TNF receptors may be effective to reduce hepatic deterioration during fibrogenesis.

Materials and Methods

Animals and HSC Isolation. Wild-type, TNFR1 knockout mice, TNFR2 knockout mice, and TNFR-DKO mice (10-18 weeks old) (C57BL/6 strain), a generous gift of Dr. Bluethmann (Discovery Technologies, Hoffmann-La Roche Ltd., Basel, Switzerland), were obtained by the propagation of homozygous pairs. The animals had free access to water and standard purified rodent diet throughout the study. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (Bethesda, MD). HSCs were isolated by perfusion with collagenase and cultured as described.[21]

Cell Lines and Culture. In addition to primary mouse HSCs, we used the human HSC cell line, LX2. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS), and antibiotics were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were serum-starved with 0.5% FBS before using TNF-α, PDGF-BB, interleukin-1 alpha (IL-1α), and IL-1β (PreproTech EC, London, UK) or lipopolysaccharide (LPS) (from *Escherichia coli* serotype 0128:B12; Sigma-Aldrich Quimica SA, Madrid, Spain). Neutralizing antibodies against human TNFR1 and TNFR2 (R&D Systems, Minneapolis, MN) were used at a concentration of 10 μg/mL. *In vitro* short interfering RNA (siRNA) transfection was performed using commercially available siRNA (Santa Cruz Biotechnology, Heidelberg, Germany) as previously described.[21] Unless otherwise stated, all reagents were from Sigma-Aldrich.

Real-time RT-PCR and Primer Sequences. Total RNA from HSCs, mouse tissue, or LX2 cells was isolated with TRIzol reagent (Invitrogen, Paisley, UK). Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed with the iScript One-Step reverse transcription (RT)-PCR kit with SYBR Green (Bio-Rad Laboratories SA, Madrid, Spain). Primer sequences were designed based on published sequences (Table 1).

[^{3}H] Thymidine Incorporation. Proliferation was estimated as the amount of [^{3}H]-thymidine incorporated into trichloroacetic acid (TCA)-precipitable material, as previously described.[21]

Table 1. Primers Used in Quantitative Real-Time RT-PCR

	5′ primer	3′ primer
Target gene (mouse)		
β-actin [NM_007393]	GACGGCCAGGTCATCACTAT	CGGATGTCAACGTCACACTT
α-SMA [NM_007392]	ACTACTGCCGAGCGTGAGAT	AAGGTAGACAGCGAAGCCAA
TIMP-1 [NM_001044384]	CATGGAAAGCCTCTGTGGAT	CTCAGAGTACGCCAGGGAAC
MMP-2 [NM_008610]	ACCTGAAGCTGGAGAACCAA	CACATCCTTCACCTGGTGTG
MMP-9 [NM_013599]	CAAATTCTTCTGGCGTGTGA	CGGTTGAAGCAAAGAAGGAG
TGF-β [NM_011577]	GTCAGACATTCGGGAAGCAG	GCGTATCAGTGGGGGTCA
Procollagen α1(I) [NM_007742]	GAGCGGAGAGTACTGGATCG	GTTCGGGCTGATGTACCAGT
TNF [NM_013693]	CTGAACTTCGGGGTGATCGGT	ACGTGGGCTACAGGCTTGTCA
Target gene (human)		
β-actin [NM_001101]	GGACTTCGAGCAAGAGATGG	AGGAAGGAAGGCTGGAAGAG
α-SMA [NM_001613]	CCGACCGAATGCAGAAGG	ACAGAGTATTTGCGCTCCGGA
TIMP-1 [NM_003254]	AGTGGCACTCATTGCTTGTG	GCAGGATTCAGGCTATCTGG
MMP-2 [NM_004530]	ACGACCGCGACAAGAAGTAT	ATTTGTTGCCCAGGAAAGTG
MMP-9 [NM_004994]	GACAAGCTCTTCGGCTTCTG	CTCGCTGGTACAGGTCGAGT

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immunoblot Analysis. Total cell lysates were incubated, after transferring to nitrocellulose membranes, with rabbit anti-phospho-Akt (1:250), anti-AKT (1:200), anti-MMP-9 (1:200; Santa Cruz Biotechnology), anti-phospho-ERK1/2, anti-ERK (extracellular signal-related protein kinase), anti-phospho-JAK2 (Janus kinase 2), anti-JAK2 (1:2,000; Cell Signaling Technology, Danvers, MA), or mouse anti-α-SMA (alpha-smooth muscle actin; 1:1000) and anti-β-actin (1:5000), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody anti-rabbit (1:20,000) or anti-mouse (1:20,000), and developed in enhanced chemiluminescent (ECL) substrate (Pierce, Rockford, IL).

Histochemical Staining. Liver tissue was fixed in 10% formalin/phosphate-buffered saline, dehydrated in alcohols, incubated in xylene, and embedded in paraffin. Then, 7-μm-thick tissue sections were cut and stained with hematoxylin and eosin (H&E), according to the manufacturer's protocols.

Gelatin Zymography. Medium from cultured HSCs was treated with sample buffer without 2-mercaptoethanol and loaded onto sodium dodecyl sulfate gel, containing 0.1% gelatin. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 15 minutes and incubated overnight in developing buffer (50 mmol/L Tris-HCl, pH 7.4, 0.2 mol/L NaCl, 10 mmol/L $CaCl_2$, and 0.002% sodium azide) at 37°C. After, the gel was stained with a solution containing 0.5% Coomassie Brilliant Blue, 40% methanol, and 7% acetic acid and destained. Bands were visualized using a Gel-Doc analyzer (Bio-Rad).

Nuclear Extract Isolation. Briefly, 2×10^6 HSC or LX2 cells were scraped in Buffer A (10 mmol/L Hepes, 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.1 mmol/L ethylene glycol tetraacetic acid [EGTA], 1 mmol/L dithiothreitol [DTT], and 0.5 mmol/L phenylmethylsulfonyl fluoride [PMSF]), kept on ice for 15 minutes, and lysed by the addition of 1/20 (vol/vol) 10% Igepal and vortexed for 10 seconds. Nuclei were pelleted (12,000*g*, 30 seconds), resuspended in Buffer C (20 mmol/L Hepes, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF), and incubated for 15 minutes on ice with gentle mixing. After, nuclear extracts were obtained by centrifuging at 4°C, 12,000*g* for 5 minutes.

In Vivo Liver Fibrosis After BDL. BDL was performed as previously described.[22]

Statistical Analysis. Results were routinely expressed as mean ± standard deviation, with the number of individual experiments detailed in figure legends. Statistical significance of the mean values was established by the Student *t* test.

Results

Loss of TNFR1/TNFR2 Reduces Procollagen-α1(I) mRNA Expression. To evaluate the participation of TNF receptors on the activation of HSC, we isolated HSC from wild-type and TNFR-DKO mice and plated them on plastic with medium containing 10% FBS to allow their activation. As expected, though TNFR-DKO HSCs did not respond to TNF, as shown by the lack of NF-κB activation after TNF challenge, represented as the translocation of p65 to the nucleus, they were able to activate NF-κB in response to other stimuli, such as LPS addition (Fig. 1A). The expression of α-SMA was followed at different time points along the activation of HSCs. α-SMA protein expression increased during the time of culture

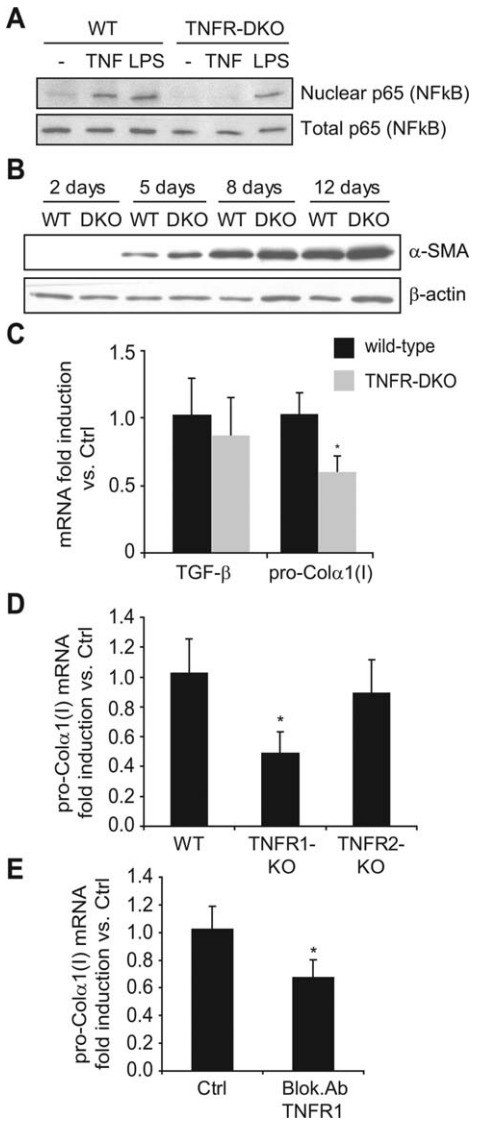

Fig. 1. Expression pattern of wild-type (WT) and TNFR-DKO HSC. (A) p65 subunit of NF-κB translocation to nuclei in HSC after TNF (10 ng/mL) or LPS (50 ng/mL) challenge for 30 minutes. (B) Time course of α-SMA protein expression by western blot. (C) TGF-β and procollagen-α1(I) mRNA expression. Procollagen-α1(I) mRNA expression in TNFR1-KO and TNFR2-KO HSCs (D) or in LX2 cells (E) after incubation with blocking antibody (Ab) anti-TNFR1 (10 μg/mL, 24 hours). Data are mean ± standard deviation; in (C,D,E), $n \geq 3$ and $^*P \leq 0.05$ versus WT HSC.

and its levels were similar among wild-type and TNFR-DKO HSCs (Fig. 1B). Moreover, paralleling the effects on α-SMA, transforming growth factor beta (TGF-β) mRNA levels were comparable in wild-type and TNFR-DKO HSCs, after 7 days of culture. However, procollagen-α1(I) mRNA levels were significantly decreased in TNFR-DKO HSCs during *in vitro* activation (Fig. 1C) and also in TNFR1-KO HSCs, but not in TNFR2-KO (Fig. 1D). In addition, LX2 cells incubated with neutralizing antibody against TNFR1 receptor displayed a significant decrease in procollagen-α1(I) mRNA expression (Fig. 1E), thus indicating that the expression of TNFR1 is necessary in HSCs for optimal expression of procollagen-α1(I).

TNFR1 Is Required for PDGF-Induced AKT Phosphorylation and HSC Proliferation. Next, we assessed whether a lack of TNF signaling would affect HSC proliferation. HSCs from TNFR-DKO displayed a reduced proliferation rate, compared to wild-type HSCs, during their transdifferentiation into myofibroblast-like cells (Fig. 2A). To further evaluate the potential mechanisms involved, we first addressed whether the decreased proliferation of HSCs was due to a reduced ability of TNF to stimulate proliferation. TNF itself did not stimulate the proliferation of HSCs (Fig. 2B). Moreover, because PDGF is a potent mitogenic stimulus for HSCs, we next examined whether TNF would potentiate PDGF signaling and stimulation of cell proliferation. Although PDGF stimulated wild-type HSC cell proliferation, this effect was not enhanced in the presence of TNF, thus discarding a direct role of TNF in HSC proliferation (Fig. 2B). Moreover, to examine whether TNF receptors were required for optimal PDGF signaling, we addressed the effect of PDGF in TNFR-DKO HSCs. As shown, the proliferating effect of PDGF was markedly reduced in TNFR-DKO HSCs (Fig. 2C) due to impaired AKT phosphorylation (Fig. 2D).

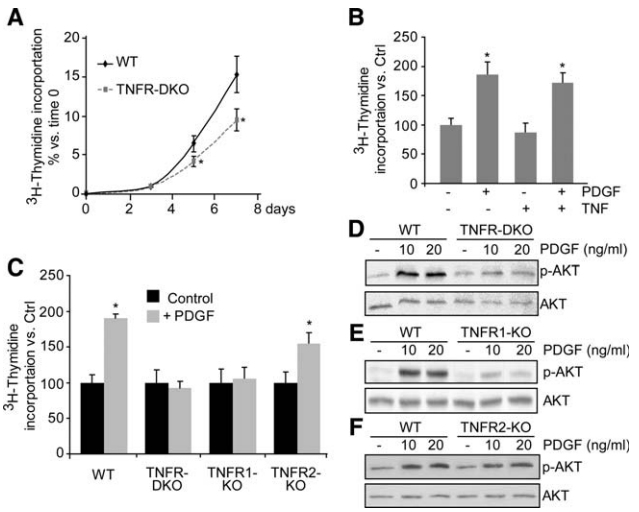

Fig. 2. Lack of TNF receptors affects HSC proliferation. (A) Wild-type and TNFR-DKO HSC proliferation. (B) LX2 proliferation after 24 hours of PDGF (20 ng/mL) and/or TNF (50 ng/mL) challenge. (C) Wild-type, TNFR-DKO, TNFR1-KO, and TNFR2-KO proliferation after 24 hours of PDGF (20 ng/mL) challenge. AKT phosphorylation induced by PDGF (10 or 20 ng/mL) for 15 minutes in wild-type versus TNFR-DKO (D), TNFR1-KO (E), or TNFR2-KO (F) HSCs. Data are mean ± standard deviation in (A) and (C), $n = 3$ and $^*P \leq 0.05$ versus wild-type HSCs; in (B), $^*P \leq 0.05$ versus PDGF untreated cells.

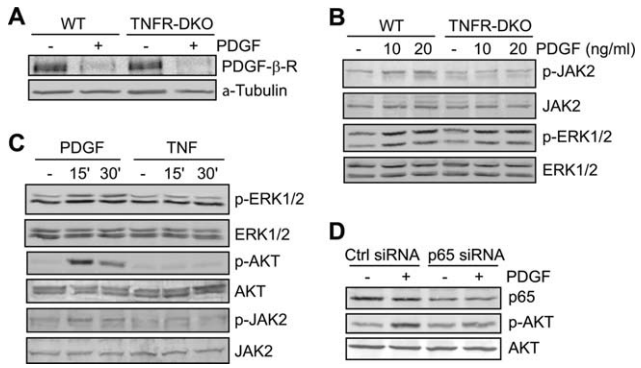

Fig. 3. PDGF-β-receptor signaling in TNF-DKO HSC. (A) PDGF-β-receptor degradation after PDGF challenge (20 ng/mL, 2 hours) and (B) JAK2 and ERK1/2 activation after PDGF challenge (15 minutes) in wild-type and TNFR-DKO HSCs. (C) ERK1/2, AKT, and JAK2 activation by PDGF (20 ng/mL) or TNF (50 ng/mL) in LX2 cells. (D) p65 expression and AKT activation by PDGF (15 minutes) in control and p65 siRNA-transfected LX2 cells.

Moreover, TNFR1-KO HSCs displayed a reduced phosphorylation of AKT in response to PDGF (Fig. 2E); however, TNFR2-KO HSCs (Fig. 2F) were able to phosphorylate AKT similarly to wild-type HSCs, thus suggesting an intricate interplay between TNFR1 and PDGF signaling. Consistent with these observations, cell proliferation in response to PDGF was impaired in TNFR1-KO, but not in TNFR2-KO, HSCs (Fig 2C).

Furthermore, we addressed downstream signaling pathways involved in the proliferation of HSCs induced by PDGF. First, we observed that PDGF receptor degradation stimulated by ligand binding was unimpaired in TNFR-DKO HSCs (Fig. 3A). Moreover, in addition to the requirement for TNFR1 for Akt phosphorylation in response to PDGF (Fig 2E), PDGF also induced the phosphorylation of ERK1/2 and JAK2 in wild-type HSC or LX2 cells (Fig. 3B,C). However, the phosphorylation of JAK2, but not ERK1/2, was impaired in TNFR-DKO HSC (Fig. 3B). Unlike ERK1/2, we did not observe p38 phosphorylation by PDGF in mouse HSC or human LX2 cells (not shown). In addition, AKT phosphorylation by PDGF was dependent on NF-κB activation, because p65 silencing by siRNA reduced PDGF-dependent AKT activation, compared to control-siRNA–transfected cells (Fig. 3D).

TNFR1 Controls MMP-9 Expression in HSCs and Human LX2 Cells. Because matrix remodeling is another critical facet of liver fibrosis and a consequence of HSC activation, we next examined the role of TNF receptors on MMP-9 expression. In the presence of 10% FBS, MMP-9 mRNA expression was reduced in TNFR-DKO HSCs (Fig. 4A). To validate the importance of TNF as a putative inducer of MMP-9, HSCs from wild-type and TNFR-DKO mice were depleted of serum up to 0.5% and incubated with TNF. This maneuver resulted in an induction of MMP-9 mRNA (Fig. 4B) and protein (Fig. 4C) in wild-type, but not in TNFR-DKO, HSCs. The induction of MMP-9 was mediated by TNFR1, as TNFR2-KO HSCs were able to activate MMP-9 mRNA (Fig. 4B). Of note, under conditions of serum limitation (0.5% FBS), the expression of MMP-9 mRNA in wild-type HSCs was similar to that of TNFR-DKO HSCs, indicating that the basal induction of MMP-9 is independent of TNF, but that its induction under growing conditions required TNF (Supporting Fig. 1). Moreover, the induction of MMP-9 by TNF in mouse HSCs was dependent on the time of activation being higher in 14-day, compared to 7-day, HSC cultures and similar to the levels observed with IL-1α or IL-1β (Fig. 4D). The participation of TNFR1 as the receptor responsible for MMP-9 induction was further validated in

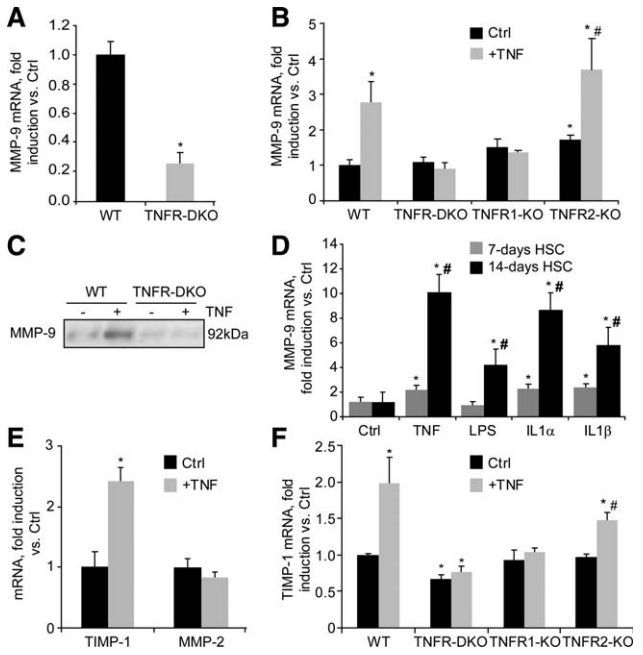

Fig. 4. TNF controls MMP-9 expression in HSCs. (A) Basal MMP-9 mRNA expression in wild-type and TNFR-DKO HSC and (B) after TNF challenge (50 ng/mL) for 24 hours in wild-type, TNFR-DKO, TNFR1-KO, and TNFR2-KO HSCs. (C) Effect of TNF (50 ng/mL, 24 hours) on MMP-9 protein expression. (D) MMP-9 mRNA expression in primary 7- and 14-day-old HSCs incubated with an equivalent concentration (50 ng/mL) of TNF, LPS, IL-1α, and IL-1β for 24 hours. (E) MMP-2 and TIMP-1 mRNA expression in wild-type HSC after TNF challenge (50 ng/mL, 24 hours). (F) TIMP-1 mRNA expression after TNF challenge (50 ng/mL) for 24 hours in wild-type (WT), TNFR-DKO, TNFR1-KO, and TNFR2-KO HSCs. Data are mean $\pm$ standard deviation; $n = 4$, $^*P \leq 0.05$ versus WT or untreated HSCs; in (B) and (F), $\#P \leq 0.05$ versus TNFR2-KO Ctrl HSCs; in (D), $\#P \leq 0.05$ versus 7-day-old treated HSCs.

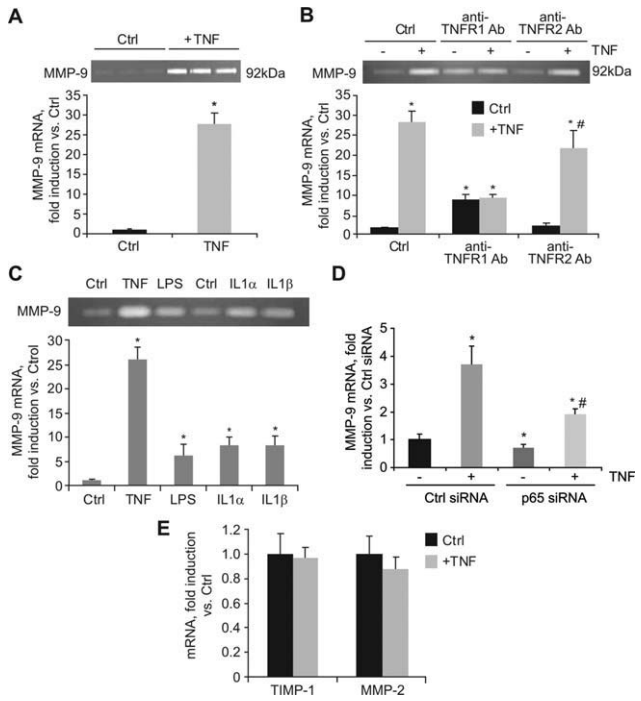

Fig. 5. TNF controls MMP-9 expression in LX2. (A) Effect of TNF (50 ng/mL, 24 hours) on MMP-9 mRNA expression and activity by zymography. (B) MMP-9 mRNA and activity after 24-hour challenge with TNF (50 ng/mL) in LX2 cells in the presence of TNFR1- or TNFR2-blocking antibodies (preincubation at 10 μg/mL, 30 minutes). (C) MMP-9 mRNA expression and activity in LX2 cells incubated with equivalent concentration (50 ng/mL) of TNF, LPS, IL-1α, and IL-1β for 24 hours. (D) MMP-9 mRNA expression in control and p65 siRNA-transfected LX2 cells after TNF challenge (50 ng/mL, 24 hours). (E) TIMP-1 and MMP-2 mRNA expression after TNF challenge (50 ng/mL, 24 hours) in LX2 cells. Data are mean $\pm$ standard deviation, $n = 3$, $^*P \leq 0.05$ versus Ctrl LX2 cells; in (B), $\#P \leq 0.05$ versus anti-TNFR2-treated control LX2 cells; in (D), $\#P \leq 0.05$ versus p65 siRNA-untreated LX2 cells.

LX2 cells. LX2 responded to TNF by inducing MMP-9 mRNA, and its activity could be clearly detected in extracellular media by zymography (Fig. 5A). In addition, by using blocking antibodies against TNFR1 and TNFR2, we could confirm that TNFR1 was the receptor responsible for MMP-9 induction by TNF at the mRNA or activity level (Fig. 5B). Intriguingly, MMP-9 expression by TNF in LX2 cells was higher than that caused by LPS or IL-1α/IL-1β (Fig. 5C), correlating with the nuclear translocation of p65 (not shown). Of note, neither in LX2 cells (Fig. 5E) nor in wild-type HSCs (Fig. 4E) was TNF able to increase the expression of another important matrix collagenase, MMP-2, thus discarding the participation of TNF signaling in MMP-2 regulation. In contrast, although TNF induced TIMP-1 mRNA in wild-type HSCs (Fig. 4E), which required TNFR1 (Fig. 4F), it failed to do so in LX2 cells (Fig. 5E). Despite the divergence observed in TIMP-1 regulation, results obtained in activated human LX2 cells emphasize the specific requirement for TNFR1-dependent signaling in the expression of matrix-remodeling factors, such as MMP-9 in HSCs.

For instance, although the individual participation of IL-1[23] or TNF[24,25] in the induction of MMP-9 has been already described in HSCs, their relative contribution to the activation of MMP-9 has not been carefully addressed, nor has the comparison of their stimulating effect on MMP-9 expression between primary mouse and human HSCs. To this aim, we challenged primary mouse HSCs as well as human LX2 cells in parallel with TNF, IL-1α, IL-1β, or LPS to analyze the extent of MMP-9 activation at the mRNA and protein level. TNF induced MMP-9 in 7-day-old primary mouse HSCs to a similar extent as IL-1, and the extent of MMP-9 expression changed with activation, because, in 14-day-old HSCs, MMP-9 expression by TNF and IL-1 was greatly enhanced, and, at this stage, cells were also responsive to LPS (Fig. 4D). In addition, TNF was a more potent inducer of MMP-9 in the human LX2 cell, as displayed also by the enhanced activity of MMP-9 in extracellular media after TNF challenge (Fig. 5C). Thus, in comparison to other known MMP-9 inducers, TNF is a relevant trigger of MMP-9 expression in primary mouse and human HSCs, and this induction is mediated by NF-κB activation, as p65 silencing in LX2 cells reduced the expression of MMP-9 induced by TNF, compared to control-siRNA transfected LX2 cells (Fig. 5D).

TNFR Deletion Reduces Early Fibrogenesis in a Mouse Model of BDL. To evaluate the causal relationship between liver damage and fibrogenesis, we examined, in parallel, the injury and fibrosis in mice with impaired TNF signaling *in vivo* using the BDL model of liver fibrogenesis. TNFR1-KO mice displayed ameliorated tissue damage, compared with that of the wild-type controls, as indicated by the reduced volume of biliary infarcts in H&E staining and serum transaminase levels (Fig. 6A,B), despite similar bilirubin levels (8.78 $\pm$ 1.25 mg/dL in wild-type versus 8.64 $\pm$ 0.96 mg/dL in TNFR1-KO mice), indicative of comparable cholestasis in both wild-type and TNFR1-KO mice. Interestingly, after BDL, TNFR1-KO mice displayed reduced levels of hepatic TNF mRNA (Fig. 6C), compared to wild-type animals. This correlated with decreased levels of MMP-9, TIMP-1 mRNA (Fig. 6D), and procollagen-α1(I) mRNA (Fig. 6E). In contrast, MMP-2 mRNA expression (Fig. 6E) was not, apparently, regulated by TNF. α-SMA was also reduced in TNFR1-KO livers, compared to the wild type (Fig. 6F), indicating decreased HSC activation *in vivo*.

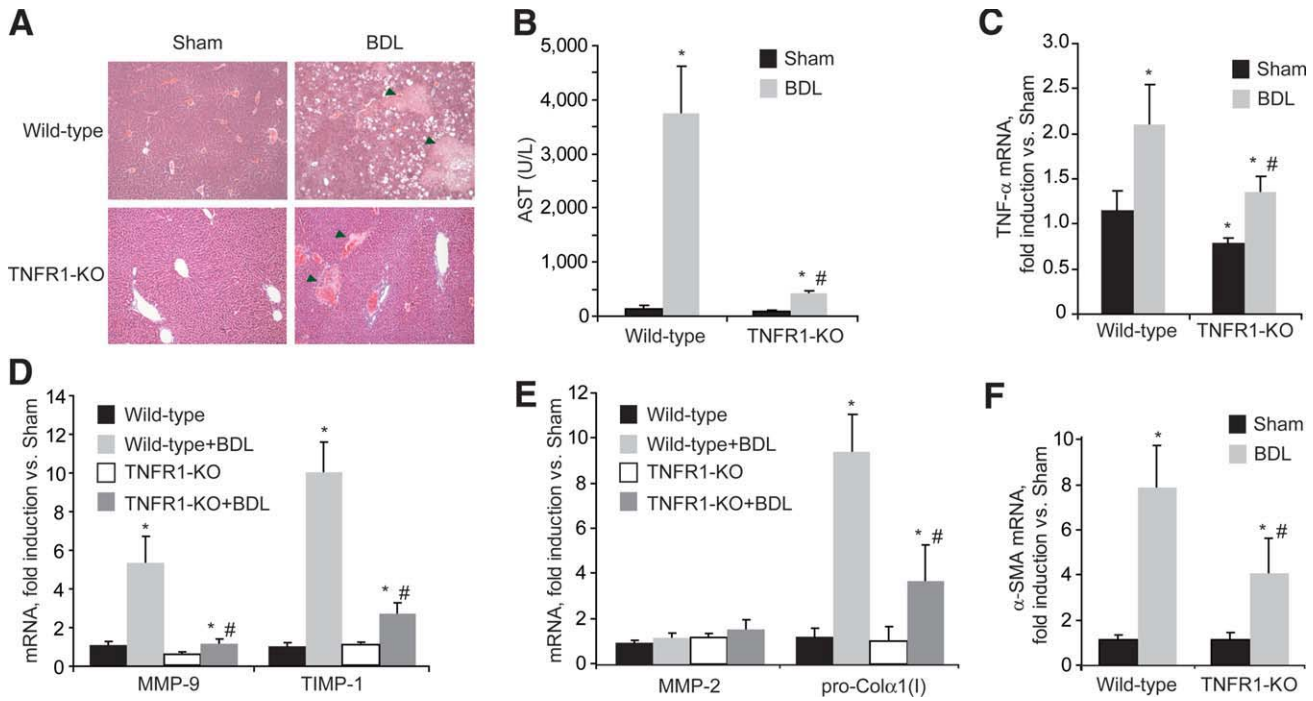

Fig. 6. Reduced liver damage and fibrosis in TNFR1-KO mice after BDL. H&E staining of liver sections (A) and serum transaminase levels (B) in wild-type and TNFR1-KO mice after BDL. TNF-α (C), MMP-9 and TIMP-1 (D), MMP-2 and pro-Colα1(I) (E), and α-SMA (F) mRNA expression in wild-type and TNFR1-KO livers after BDL. In (A), arrowheads signal bile infarcts. Data are mean ± standard error of the mean; $n = 4$-5 animals/group, $*P \leq 0.05$ versus wild-type Sham, $\#P \leq 0.05$ versus wild-type BDL.

Similar findings were observed in the TNFR-DKO mice, whereas TNFR2-KO mice behaved similarly to wild-type animals (Supporting Fig. 2). Therefore, the *in vivo* BDL model recapitulates the *in vitro* effects observed in HSCs, showing the dependence on TNFR1 signaling to induce changes in ECM remodeling during early fibrogenesis.

Discussion

TNF has been implicated in the development of many chronic liver diseases, and hepatic fibrosis is a hallmark of disease progression. Unlike its involvement in hepatocellular apoptosis and liver diseases, the role of TNF in liver fibrosis remains unclear, particularly whether TNF and its binding to specific TNFR1 or TNFR2 regulates HSC biology. Using genetic and pharmacological approaches, we show a profibrogenic role for TNF, specifically via binding to receptor R1. In particular, we used primary mouse HSCs from wild-type and mice deficient in TNFRs to analyze the role of TNF and its receptors in HSC activation and proliferation, which are critical steps in the cascade of events leading to fibrosis. Moreover, the findings were extended to human HSCs, in which TNF receptors were individually antagonized by specific neutralizing antibodies.

Our results indicate that although TNF does not directly participate in some fundamental traits of HSC transdifferentiation into a myofibroblast phenotype, such as increase in α-SMA or TGF-β expression, TNF, through TNFR1, has an important role in other important features, such as proliferation as well as MMP-9 and TIMP-1 expression. A significant difference between primary mouse and human HSCs was found in the participation of TNF in TIMP-1 induction. Although primary mouse HSCs augment TIMP-1 expression in response to TNF, we failed to observe any increase of TIMP-1 in LX2 cells under the same experimental conditions. Several conceivable possibilities could explain this differential behavior, including that TIMP-1 regulation may fundamentally differ between mouse and human HSCs. Another explanation could be the fact that LX2 cells display an almost negligible expression of TIMP-1, as compared to primary HSCs or to the parental cell line, LX1, implying that TIMP-1 expression may have been lost during its selection under low serum conditions (2% FBS).[26]

A striking finding was the decrease in proliferation observed in TNFR-DKO HSCs compared to wild-type HSCs. Mechanistically, the decreased proliferation was mediated by a defective PI3K/AKT pathway in TNFR-DKO HSC that was reproduced in TNFR1-KO, but not in TNFR2-KO, HSCs. Indeed, both TNFR1-KO

and TNFR-DKO HSCs display reduced AKT phosphorylation and proliferation in response to PDGF, a potent mitogen of HSCs, despite correct ligand binding and subsequent receptor degradation. These observations suggest that proteins or mediators necessary for PDGF signaling located upstream of AKT rely on NF-κB–dependent TNFR1 signaling, indicating a cross-talk between PDGF and TNFR1 receptors. In line with our observations, previous findings in vascular smooth muscle cells indicated a similar overlapping between TNF and PDGF necessary for cell migration and proliferation.[27] However, the identification of the NF-κB–dependent targets responsible for the reduced proliferation in response to PDGF in TNFR-DKO and TNFR1 KO HSCs remains unknown and requires further work.

In contrast to TGF-β expression, we observed a decreased basal level of procollagen-α1(I) in activated HSCs from TNFR-DKO and TNFR1-KO, compared to wild-type mice, findings that were reproduced in LX2 cells using anti-TNFR1-blocking antibody. Consistent with previous studies,[17,18] TNF addition to HSC cultures did not induce procollagen-α1(I) mRNA (data not shown), thus discarding a direct effect of TNF on procollagen-α1(I) regulation. However, the known ability of MMP-9 to activate latent TGF-β to its active form,[28] which plays an essential role in earlier stages of liver fibrogenesis when collagen production of HSCs is stimulated by TGF-β,[29] could explain why TNFR-DKO and TNFR1-KO HSCs show decreased basal levels of procollagen-α1(I) mRNA expression. In agreement, with impaired MMP-9 expression in TNFR-DKO HSCs, TGF-β would be normally produced, but not activated, by MMP-9, thus resulting in a deficient procollagen-α1(I) induction.

Unlike procollagen-α1(I), interestingly, we observed a differential role of TNF receptors in the regulation of MMPs in HSCs, in particular, the requirement of TNFR1 in the expression of MMP-9, but not MMP-2. In relation to MMP-9, it has been described, in the thioacetamide model of liver injury and fibrosis,[30] that MMP-9 colocalizes predominantly to desmin-positive cells, suggesting that HSCs are the source of MMP-9 cells *in vivo*. The importance of MMP-9 is highlighted by the observation that MMP-9–deficient mice are partially protected from liver injury and HSC activation.[30] In contrast to MMP-9, although associative studies and cell-culture findings suggest that MMP-2, a type IV collagenase up-regulated in chronic liver diseases and considered a profibrogenic mediator, promotes hepatic fibrogenesis, no *in vivo* model has definitively established a pathologic role for MMP-2 in the development and progression of liver fibrosis. In fact, recent findings, using MMP-2–deficient mice, suggest a protective, rather than pathogenic, role for MMP-2.[31]

Because the above findings indicated a selective requirement for TNFR1 in specific steps of HSC activation and proliferation, we next addressed the *in vivo* relevance for liver fibrogenesis. The data, using the BDL model of liver fibrosis, although limited in interpretation because the TNFR1-KO and TNFR-DKO mice displayed both reduced liver damage and decreased matrix deposition, suggest a correlation between TNF and MMP-9, TIMP-1, and procollagen-α1(I) mRNA expression. In contrast to the BDL model shown here, previous reports using the chronic administration of CCl_4 reported a controversial role of TNFR1 in liver fibrosis. For instance, the lack of TNFR1 inhibited procollagen-α1(I) expression and liver fibrosis after CCl_4 treatment without effect on liver injury.[11,12] However, interestingly, de Meijer et al.[13] recently reported decreased liver injury and inflammation, but increased collagen deposition, in the CCl_4 model by blocking TNF production through the inhibition of its processing via TNF-alpha-converting enzyme, as well as in TNFR-DKO mice.

Taken together, our observations in *in vitro* HSC culture and *in vivo* point to TNF not only as an inducer of hepatocellular damage, but also as a profibrogenic factor in the liver, and hence targeting TNF or its receptor, TNFR1, could be of benefit toward preserving hepatocellular integrity and prevent HSC proliferation and liver fibrosis.

Acknowledgment: The technical assistance of Susana Núñez is greatly appreciated. The authors thank Dr. Horst Bluethmann (Hoffmann-La Roche Ltd., Basel, Switzerland) for providing the knockout mice involved in this study. The work was carried out, in part, at the Esther Koplowitz Center, Barcelona, Spain.

References

1. Grell M, Douni E, Wajant H, Löhden M, Clauss M, Maxeiner B, et al. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 1995;83:793-802.
2. Grell M, Wajant H, Zimmermann G, Scheurich P. The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. Proc Natl Acad Sci U S A 1998;95:570-575.
3. Yamada Y, Fausto N. Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1 but not type 2 tumor necrosis factor receptor. Am J Pathol 1998;152:1577-1589.
4. Yamada Y, Webber EM, Kirillova I, Peschon JJ, Fausto N. Analysis of liver regeneration in mice lacking type 1 or type tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor. HEPATOLOGY 1998;28:959-970.

5. Bradham CA, Plümpe J, Manns MP, Brenner DA, Trautwein C. Mechanisms of hepatic toxicity. I. TNF-induced liver injury. Am J Physiol 1998;275:G387-G392.
6. Schwabe RF, Brenner DA. Mechanisms of liver injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways. Am J Physiol Gastrointest Liver Physiol 2006;290:G583-G589.
7. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209-218.
8. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008;134:1655-1669.
9. Wullaert A, Heyninck K, Beyaert R. Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. Biochem Pharmacol 2006;72:1090-1101.
10. Han D, Ybanez MD, Ahmadi S, Yeh K, Kaplowitz N. Redox regulation of tumor necrosis factor signaling. Antioxid Redox Signal 2009; 11:2245-2263.
11. Sudo K, Yamada Y, Moriwaki H, Saito K, Seishima M. Lack of tumor necrosis factor receptor type 1 inhibits liver fibrosis induced by carbon tetrachloride in mice. Cytokine 2005;29:236-244.
12. Simeonova PP, Gallucci RM, Hulderman T, Wilson R, Kommineni C, Rao M, et al. The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. Toxicol Appl Pharmacol 2001;177:112-120.
13. de Meijer VE, Sverdlov DY, Popov Y, Le HD, Meisel JA, Nosé V, et al. Broad-spectrum matrix metalloproteinase inhibition curbs inflammation and liver injury but aggravates experimental liver fibrosis in mice. PLoS One 2010;5:e11256.
14. Hernández I, de la Torre P, Rey-Campos J, Garcia I, Sánchez JA, Muñoz R, et al. Collagen alpha1(I) gene contains an element responsive to tumor necrosis factor-alpha located in the 5′ untranslated region of its first exon. DNA Cell Biol 2000;19:341-352.
15. Hernández-Muñoz I, de la Torre P, Sánchez-Alcázar JA, García I, Santiago E, Muñoz-Yagüe MT, et al. Tumor necrosis factor alpha inhibits collagen alpha 1(I) gene expression in rat hepatic stellate cells through a G protein. Gastroenterology 1997;113:625-640.
16. Houglum K, Buck M, Kim DJ, Chojkier M. TNF-alpha inhibits liver collagen-alpha 1(I) gene expression through a tissue-specific regulatory region. Am J Physiol 1998;274:G840-G847.
17. Solis-Herruzo JA, Brenner DA, Chojkier M. Tumor necrosis factor alpha inhibits collagen gene transcription and collagen synthesis in cultured human fibroblasts. J Biol Chem 1988;263:5841-5845.
18. Varela-Rey M, Fontán-Gabás L, Blanco P, López-Zabalza MJ, Iraburu MJ. Glutathione depletion is involved in the inhibition of procollagen alpha1(I) mRNA levels caused by TNF-alpha on hepatic stellate cells. Cytokine 2007;37:212-217.
19. de Leeuw AM, McCarthy SP, Geerts A, Knook DL. Purified rat liver fat-storing cells in culture divide and contain collagen. Hepatology 1984;4:392-403.
20. Friedman SL, Rockey DC, McGuire RF, Maher JJ, Boyles JK, Yamasaki G. Isolated hepatic lipocytes and Kupffer cells from normal human liver: morphological and functional characteristics in primary culture. Hepatology 1992;15:234-243.
21. Moles A, Tarrats N, Fernández-Checa JC, Marí M. Cathepsins B and D drive hepatic stellate cell proliferation and promote their fibrogenic potential. Hepatology 2009,49:1297-1307.
22. Moles A, Tarrats N, Morales A, Domínguez M, Bataller R, Caballería J, et al. Acidic sphingomyelinase controls hepatic stellate cell activation and *in vivo* liver fibrogenesis. Am J Pathol 2010;177:1214-1224.
23. Han YP, Zhou L, Wang J, Xiong S, Garner WL, French SW, et al. Essential role of matrix metalloproteinases in interleukin-1-induced myofibroblastic activation of hepatic stellate cell in collagen. J Biol Chem 2004;279:4820-4828.
24. Migita K, Maeda Y, Abiru S, Nakamura M, Komori A, Yokoyama T, et al. Immunosuppressant FK506 inhibits matrix metalloproteinase-9 induction in TNF-alpha-stimulated human hepatic stellate cells. Life Sci 2006;78:2510-2515.
25. Zhou L, Yan C, Gieling RG, Kida Y, Garner W, Li W, et al. Tumor necrosis factor-alpha induced expression of matrix metalloproteinase-9 through p21-activated kinase-1. BMC Immunol 2009;10:15.
26. Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. Gut 2005;54:142-151.
27. Peppel K, Zhang L, Orman ES, Hagen PO, Amalfitano A, Brian L, et al. Activation of vascular smooth muscle cells by TNF and PDGF: overlapping and complementary signal transduction mechanisms. Cardiovasc Res 2005;65:674-682.
28. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000;14:163-176.
29. Cao Q, Mak KM, Lieber CS. Dilinoleoylphosphatidylcholine decreases acetaldehyde-induced TNF-alpha generation in Kupffer cells of ethanol-fed rats. Biochem Biophys Res Commun 2002;299:459-464.
30. Gieling RG, Wallace K, Han YP. Interleukin-1 participates in the progression from liver injury to fibrosis. Am J Physiol Gastrointest Liver Physiol 2009;296:G1324-G1331.
31. Radbill BD, Gupta R, Ramirez MC, Difeo A, Martignetti JA, Alvarez CE, et al. Loss of matrix metalloproteinase-2 amplifies murine toxin-induced liver fibrosis by upregulating collagen I expression. Dig Dis Sci 2011;56:406-416.