CRITICAL ROLE OF TNF-RECEPTOR 1 BUT NOT 2 IN HEPATIC STELLATE CELL-INDUCED EXTRACELLULAR MATRIX REMODELING AND IN VIVO LIVER FIBROSIS

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List of Abbreviations: α-SMA, alpha-smooth muscle actin; ECM, extracellular matrix; HSC, hepatic stellate cell; TIMP, tissue inhibitor of metalloproteinase; TNFR-DKO, TNF-R1 and TNF-R2 double knockout; MMP, matrix metalloproteinase.
ABSTRACT

Background & Aims: TNF has been implicated in the progression of many chronic liver diseases leading to fibrosis; however, the specific contribution of TNF or its receptors, TNF-R1 and TNF-R2, to HSC activation remains to be established.

Methods: We have isolated hepatic stellate cells (HSC) from wild-type, and TNF-Receptors 1 (TNFR1), 2 (TNFR2), and double (TNF R-DKO) knockout mice, and cultured on plastic. Proliferation was assessed by $[^3]$H-Thymidine incorporation to DNA. Expression of α-SMA was determined by western-blot. TGF-β, TIMP-1, pro-Colα1(Ι), MMP-2, and MMP-9 mRNA expression was determined by quantitative real-time PCR. Bile duct ligation was performed in wild-type and TNFR-DKO mice to analyze liver damage and fibrogenesis.

Results: Loss of both TNF receptors reduced pro-Colα1(I) expression, slowed down HSC proliferation, and are required for PDGF-induced pro-mitogenic signaling in HSC. Antagonism of TNFR1 by specific neutralizing antibodies reproduced the behavior of TNFR-DKO HSC accompanied with decreased p-AKT and proliferation in response to PDGF, due to the absence of TNF-R1. In addition, MMP-9 expression was dependent on TNF binding to TNF-R1 in primary mouse HSC. These results were validated in the human HSC cell line LX2 using neutralizing antibodies against TNF-R1 and TNF-R2. Moreover, in vivo liver damage and fibrosis were reduced after cholestasis induction in TNFR-DKO mice compared to wild-type mice.

Conclusions: TNF receptors regulate HSC-induced extracellular matrix remodeling in vitro and in early liver fibrosis.
INTRODUCTION

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 superfamily, namely TNF-R1 and TNF-R2. The cytoplasmic tail of TNF-R1 contains a death domain (DD), which is essential for induction of apoptosis. However, this motif is missing in TNF-R2 and the function of this latter receptor is poorly understood (Grell et al., 1995; Grell et al., 1999). In the liver, TNF functions as double-edged sword through TNF-R1 being required for normal hepatocyte proliferation during liver regeneration (Yamada 1998a, 1998b) and induction of NF-kappaB, which is essential to elicit antiapoptotic defense an 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 mediator in patients with alcoholic liver disease and nonalcoholic steatohepatitis or viral hepatitis (Bradham et al 1998, Schwabe et al 2006). 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 (Friedman 2008, Bataller and Brenner 2005). While the contribution of TNF to hepatocellular injury and inflammation has been widely studied (Bradham et al.; 1998, Wullaert et al., 2006; Schwabe and Brenner 2006; Han et al., 2009) its specific contribution to HSC activation and liver fibrosis remains controversial. In this sense, experimental studies performed with knockout mice after CCl₄ administration have shown that the
absence of either TNF-R1 (Sudo et al., 2005) or TNF-R1 and TNF-R2 (TNFR-DKO) (Simeonova et al., 2001) inhibit liver fibrosis accompanied by reduced expression of pro-collagen alpha 1(I) mRNA, without effect on hepatic injury. In contrast, a recent study shows that the TNFR-DKO exhibit reduced liver damage after CCl₄ challenge (de Meijer et al., 2010). Moreover, several reports using cultured HSC point to an anti-fibrogenic role of TNF via inhibition of the collagen alpha1(I) gene expression (Hernández et al., 2000, Hernández-Muñoz et al., 1997; Houglum et al. 1998; Solis-Herruzo et al., 1988) due in part via glutathione depletion (Varela-Rey et al., 2007).

Hence, while TNF has been implicated in the progression of many chronic liver diseases leading to fibrosis the specific involvement of TNF or its receptors, TNF-R1 and TNF-R2, in HSC activation remains to be established. Given that the morphological and metabolic changes associated with HSC activation are reproduced by culturing isolated HSC on plastic (de Leeuw et al., 1984; Friedman et al., 1992), we have isolated HSC from wild-type, TNFR-DKO, TNF-R1 and TNF-R2 knockout mice to evaluate the impact of TNF signaling on HSC activation, and thus, its potential direct contribution to liver fibrosis.
MATERIAL AND METHODS

Animals and HSC isolation. Wild-type, TNF-R1 knockout mice, TNF-R2 knockout mice, TNFR-DKO mice (10-18 weeks old) (C57BL/6 strain) were obtained by propagation of homozygous pairs and housed in a barrier room with regulated temperature and humidity, and an alternating 12-hour light and dark cycle. 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 NIH. HSC were isolated by perfusion with collagenase and cultured as previously described (Moles et al., 2009).

Cell lines and culture. In addition to primary HSC, the human cell line of hepatic stellate cells LX2 was used. Cells were cultured in DMEM complemented with 10% FBS, and antibiotics at 37ºC in a humidified atmosphere of 95% air and 5% CO₂. Cells were serum starved at 0.5% FBS before using TNF, PDGF, IL-1 (Preprotech EC) or LPS (Sigma-Aldrich). Neutralizing antibodies against TNFR1 and TNFR2 (R&D Systems) were used in 0.5% FBS at a concentration of 10ng/ml.

Real time RT-PCR and primer sequences. Total RNA from HSC, mouse liver tissues, or LX2 cells was isolated with TRIzol reagent. Real-time RT-PCR was performed with iScript™ One-Step reverse transcription (RT)-PCR Kit with SYBR® Green following the manufacturer's instructions. The primers sequences were designed based on published sequences (Table 1).

[^3H] Thymidine incorporation. Proliferation was estimated as the amount of[^3H] thymidine incorporated into TCA-precipitable material as previously described (Moles et al., 2009).
**SDS-PAGE and immunoblot analysis.** Lysates were prepared in RIPA buffer (50 mmol/L Tris·HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 1mmol/L Na$_3$VO$_4$, 1 mmol/L EDTA, 0.25% Sodium deoxycholate) 0.1% SDS, 1% Triton X-100 plus proteinase inhibitors). Protein concentration was determined by Bradford assay, and samples containing 10 to 30 µg were separated by 6-12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes. After membranes were blocked in 8% nonfat milk or 5% BSA in 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% Tween-20 for 1 h at room temperature, to visualize specific proteins, membranes were incubated in rabbit anti-α-SMA 1:1000 (Sigma-Aldrich), anti phospho-Akt at 1:250 (Santa Cruz Biotechnology), anti-MMP-9 at 1:200 (Santa Cruz Biotechnology) or mouse anti-β-actin (Sigma-Aldrich) at 1:5000 followed by incubation in horseradish peroxidase-conjugated secondary antibody anti-rabbit at 1:20,000 or anti-mouse at 1:20,000 (Sigma-Aldrich). The membrane was developed by incubating in ECL Chemiluminescence Substrate (Pierce, Rockford, IL).

**Histochemical staining.** Liver tissue was fixed with 10% formalin in PBS. Formalin-fixed liver specimens were dehydrated in alcohols, incubated in xylene, and embedded in paraffin. 7-µm-thick tissue sections were cut and stained with either Hematoxylin-Eosin or Sirius Red, according to manufacturer's protocols.

**Gelatin Zymography.** Medium from cultured HSC was treated with sample buffer without 2-mercaptoethanol and loaded onto SDS 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 (50mmol/L Tris-HCl pH 7.4, 0.2mol/L NaCl, 10 mmol/L CaCl$_2$, 0.002% sodium azide) at 37°C.
Afterwards, the gel was stained with a solution containing 0.5% Comassie Brilliant Blue, 40% methanol and 7% acetic acid, and destained.

**In vivo liver fibrosis following bile duct ligation.** Bile duct ligation (BDL) was performed as previously described (Moles et al 2010). In brief, wild-type and TNFR-DKO mice were anesthetized by isofluorane inhalation. Under anesthesia, the peritoneal cavity was opened and the common bile duct was double-ligated with 5-0 sutures, and cut between the ligatures. Controls underwent the same surgery except bile duct ligation and section. Animals were sacrificed 4 days after BDL, collecting liver and serum samples for analysis.

**Statistical analysis.** Statistical analyses were performed using Microsoft Excel software. The statistical significance of differences was performed using the unpaired, non-parametric Student’s $t$ test.
RESULTS

Loss of TNFR1 and TNFR2 reduces pro-Colα1(I) mRNA expression.

To evaluate the participation of TNF receptors on the so-called activation of HSC, we isolated HSC from wild-type and TNFR-DKO mice and plated them on plastic dishes with medium containing 10% FBS to allow their activation. As expected, while TNFR-DKO HSC did not respond to TNF, as shown by the lack of NF-κB activation after TNF challenge, represented as translocation of p65 to the nucleus, they were able to activate NF-κB in response to other stimuli such as LPS addition (Figure 1A). The expression of α-SMA protein was followed at different time-points along the activation of HSC. As shown in Figure 1B, α-SMA protein expression increased during the time of culture and its levels were similar among wild-type and TNFR-DKO HSC. However, while TGF-β mRNA expression levels were comparable in both groups, the expression of pro-collagen-α1(I) was significantly decreased in HSC from TNFR-DKO mice (Figure 1C), indicating that the expression of TNF receptors is necessary in HSC for optimal expression of Colα1(I) when cultured in plastic in the presence of growth factors. These data reveal a differential role of TNF receptors on the expression of fibrogenic targets in HSC.

TNF-R1 is required for PDGF-induced AKT phosphorylation and proliferation.

Next we assessed if lack of TNF signaling affected HSC proliferation. As shown in Figure 2A, HSC from TNFR-DKO displayed reduced proliferation rate compared to wild-type HSC during their transdifferentiation into myofibroblasts-like cells. To ascertain if either TNF itself or TNF-derived gene transcription was responsible for this decreased proliferation along the activation process in the
presence of growth factors (serum), and since PDGF is one of the most potent mitogenic stimuli for HSC, we next evaluated in one hand the effect of TNF on PDGF-induced proliferation; and on the other hand the effect of PDGF itself on TNFR-DKO HSC. As shown in Figures 2B and D, while PDGF promoted \[^{3}H\]-thymidine incorporation and AKT activation in wild-type HSC, TNF did not induced neither proliferation by itself nor enhanced the response elicited by PDGF thus discarding a direct role for TNF on HSC proliferation. On the other hand, TNFR-DKO HSC, displayed a reduced proliferation rate (Figure 2A) and their growth-rate was curtailed in response to PDGF (Figure 2C). Moreover, this deficiency in proliferation was accompanied by an almost undetectable phosphorylation of AKT after PDGF challenge (Figure 2D).

To deepen our knowledge into the mechanism responsible for this reduced proliferation of TNFR-DKO HSC, we analyzed these responses in TNF-R1 or TNF-R2 knockout HSC. As shown in Figure 2E while TNF-R1 displayed a reduced phosphorylation of AKT in response to PDGF, TNF-R2 knockout (Figure 2F) were able to phosphorylate AKT similarly to wild-type HSC, thus suggesting an intricate interplay between TNF-R1 and PDGF signaling. Consistent with these findings cell proliferation in response to PDGF was impaired in TNF-R1 knockout HSC but not in TNF-R2 knockout HSC (not shown). Thus these findings establish a critical role for TNF-R1 in the mitogenic role of PDGF in HSC.

**TNF-R1 controls MMP-9 expression in HSC.**

Since matrix remodeling is another critical facet of liver fibrosis and hence of HSC activation, we next examined the role of TNF receptors on MMP-9 expression. As shown in Figure 3A, in the presence of 10% fetal bovine serum,
MMP-9 mRNA expression was reduced in TNFR-DKO; while for instance, the expression of MMP-2 was unaffected (Figure 3E). To validate the importance of TNF as a putative inducer of MMP-9, HSC from wild-type and TNFR-DKO were depleted of serum up to 0.5% and incubated with TNF. This maneuver resulted in an induction on MMP-9 mRNA (Figure 3B), and activity (Figure 3C) in wild-type HSC but not in TNFR-DKO. The induction of MMP-9 was mediated by TNF-R1 since TNF-R2 knockout HSC were able to activate MMP-9 mRNA, TNF-R1 knockout HSCs did not (Figure 3B). Of note, under condition of serum limitation (0.5% FBS) the expression of MMP-9 mRNA in wild-type HSC was similar to that of TNFR-DKO HSC, indicating that the basal induction of MMP-9 is independent of TNF but that its induction under growing conditions required TNF (Figure 3D). The participation of TNF-R1 as the receptor responsible for MMP-9 induction was further validated in LX2 cells. As shown in Figure 4A, LX2 responded to TNF by inducing MMP-9 mRNA (Figure 4A), and its activity could also be clearly detected in the extracellular media by zymography (Figure 4B). In addition, by using blocking antibodies against TNF-R1 and TNF-R2 we could confirm that TNF-R1 was the receptor responsible for MMP-9 induction by TNF at the mRNA (Figure 4C) or activity level (Figure 4D). Of note, neither in LX2 (Figure 4E) cells nor in wild-type HSC (Figure 3E) TNF was able to increase the expression of the other important matrix collagenase, MMP-2, thus discarding the participation of TNF in MMP-2 regulation. In contrast, while TNF induced TIMP-1 mRNA in wild-type HSC (Figure 3E), which required TNF-R1 (Figure 3F), it failed to do so in LX2 cells (Figure 4E). These findings indicate that TNF-R1 is selectively needed for the expression of matrix remodeling factors such as MMP-9 and TIMP-1 in primary HSC.
**TNF is a relevant inducer of MMP-9 in HSC.**

The participation of cytokines, such as IL-1 (Han et al., 2004) or TNF (Migita et al., 2006; Zhou et al., 2009), in their induction of MMP-9 has been already described in HSC. However, the relative contribution or comparison between several cytokines or effectors to the activation of MMP-9 has not been carefully addressed. To this aim we have challenged primary mouse HSC as well as human LX2 cells with TNF, IL-1α, IL-1β, or LPS to analyze the extent of MMP-9 activation at the mRNA and protein level. As displayed in Figure 5, TNF induced MMP-9 in 6-day old primary mouse HSC to a similar extent than IL-1 (Figure 5A); however, it was a more potent inducer of MMP-9 in the human LX2 cell (Figure 5B), as displayed also by the enhanced activity of MMP-9 in extracellular media after TNF challenge (Figure 5C). Consistent with these findings, LPS which did not significantly increased MMP-9 overexpression in HSC did so in the human LX2 cell line.

**TNF-receptor 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 an *in vivo* model of fibrogenesis. Wild-type and TNFR-DKO mice were subjected to common bile duct ligation (BDL). TNFR-DKO mice had 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 ALT levels (Figure 6A and B), despite of having similar cholestasis than their wild-type controls (bilirubin levels: 10.2±1.7mg/dL in wild-type vs. 9.2±1.4mg/dL in TNFR-DKO). Interestingly, TNFR-DKO mice displayed reduced levels of hepatic TNF mRNA
(Figure 6C) compared to wild-type animals. This correlated with decreased levels of MMP-9, TIMP-1 mRNA (Figure 6D), and Colα1(I) mRNA (Figure 6E). In contrast, MMP-2 mRNA expression (Figure 6E) was not apparently regulated by TNF. α-SMA was also reduced in TNFR-DKO livers compared to wild-type (Figure 6F), indicating decreased HSC activation in vivo. Similar findings were observed in the TNF-R1 knockout mice. Therefore, in vivo BDL model recapitulates the in vitro effects observed in HSC showing the dependence on TNF-receptor signaling to induce changes in ECM remodeling during early fibrogenesis.
DISCUSSION

While TNF has been implicated in the development of many chronic liver diseases that could give rise to hepatic fibrosis, its specific contribution to the apparition of scar tissue has been difficult to dilucidate. It is patent that without damage to the liver the apparition of fibrosis is rare, and thus since TNF is an important mediator to hepatocellular damage its participation in the activation of HSC and extracellular matrix deposition, hallmarks of liver fibrosis, has been clearly overlooked. To address this issue we have used primary mouse HSC from wild-type and mice deficient in TNF-receptors, to analyze its activation in culture. Although aware of the limitations of this strategy, since a conditional knockout of TNF in HSC would have been the method of choice if available, up to date no other approach allows us to discern the role of TNF-receptors in HSC, and thus in liver fibrosis, without the involvement of the other cell types present in the liver that are highly TNF-responsive, such as hepatocytes and for instance Kupffer cells.

Our results indicate that although TNF does not participate in fundamental features of HSC transdifferentiation such as increase in α-SMA or TGF-β expression, it has an important role in other important features as Colα1(I), MMP-9, and TIMP-1 expression. These differential features among wild-type and TNFR-DKO HSC were mediated, as expected, by TNF-R1 as validated not only in mouse deficient in this receptor, but also in the human LX2 cell line using specific neutralizing antibodies against both receptors. A significant difference arise in the participation of TNF in TIMP-1 induction, while primary HSC 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. Two
conceivable possibilities could explain this discordance; one would be that TIMP-1 regulation differs between mouse and human in HSC. Another explanation could be the fact that LX2 cells display almost negligible expression of TIMP-1, at least as compared to primary HSC or to its parental cell line, the LX1 cells, that was lost during its selection under low serum condition (2% FBS) (Xu et al. 2005), and consequently, we are not able to evaluate its regulation by TNF. A striking finding was the decrease in proliferation observed in TNFR-DKO HSC compared to wild-type HSC. Mechanistically, this decrease proliferation was mediated by a defective PI3K/AKT pathway in TNFR-DKO HSC, in specific due to the lack of TNF-R1, since both TNFR1-KO and TNFR-DKO HSC display reduced AKT phosphorylation and proliferation in response to PDGF. Our observations probably indicate that proteins or mediators necessary for PDGF signaling located upstream of AKT depend on correct TNF-R1 signaling. Analogous overlapping pathways between TNF and PDGF, leading to migration and proliferation, have also been described in vascular smooth muscle cells (Peppel et al., 2005). However, the identification of the putative or putatives targets responsible for the reduced proliferation in response to PDGF in TNFR-DKO HSC is beyond the scope of our study.

As regards to the decreased basal levels of pro-Colα1(I) observed in the TNFR-DKO HSC, TNF addition to HSC cultures did not induce pro-Colα1(I) (data not shown), thus discarding a direct participation of TNF on its regulation. However, the known ability of MMP-9 to activate latent TGF-β (Yu et al., 2000) to its active form, of importance at earlier stages of liver fibrogenesis when collagen production of HSC is stimulated by TGF-β (Cao et al., 2002), could explain why
TNFR-DKO HSC show decrease basal levels of pro-Col\(\alpha\)1(I) mRNA expression. In TNFR-DKO HSC, TGF-\(\beta\) is normally produced but is not activated by MMP-9, thus resulting in a deficient pro-Col\(\alpha\)1(I) induction.

In relation to MMP-9, it has been described in the thioacetamide model of liver injury and fibrosis (Gieling et al, 2009) that MMP-9 colocalizes predominantly to desmin-positive cells, suggesting that HSC are the MMP-9 producing cells in vivo. The importance of MMP-9 is also highlighted by the observation that MMP-9-deficient mice are partially protected from liver injury and HSC activation (Gieling et al, 2009). In contrast to MMP-9, although associative studies and cell culture findings suggest that MMP-2, a type IV collagenase upregulated in chronic liver diseases and considered a profibrogenic mediator, promotes hepatic fibrogenesis, no \textit{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 (Radbill BD et al., 2010).

Regarding our \textit{in vivo} study using the bile duct ligation model, although limited in interpretation since the TNFR-DKO mice display both reduced liver damage and decreased matrix deposition, suggest a correlation among TNF and MMP-9, TIMP-1, and pro-Col\(\alpha\)1(I) mRNA expressions. Other studies already performed with chronic administration of CCl\(_4\) in animals lacking TNF-R1, despite differences in the extent of liver damage between wild-type and TNF-R1 knockout mice, also found reduced fibrogenesis, thus supporting the involvement of TNF-signaling in CCl\(_4\)-mediated hepatotoxicity (Simeonova et al., 2001; Sudo et al., 2005; de Meijer et al., 2010).
Taken together our observations in cell culture and \textit{in vivo} point to TNF not only as an inducer of hepatocellular damage, but also as a pro-fibrogenic factor in the liver.
ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

**Figure 1.** Expression pattern of WT and TNFR-DKO HSC. (A) p65 subunit of NF-κB translocation to nuclei in HSC after TNF (10ng/ml) or LPS (50ng/ml) challenge for 30 minutes. (B) Time-course of α-SMA protein expression by western blot. (C) TGF-β and pro-Colα1(I) mRNA expression. Data are mean±SD; in C, n=3 and *p≤0.05 vs WT HSC.

**Figure 2.** Lack of TNF receptors affects HSC proliferation. (A) WT and TNFR-DKO proliferation. (B) LX2 proliferation after 24 hours of PDGF (20ng/ml) and/or TNF (50ng/ml) challenge. (C) WT and TNFR-DKO proliferation after 24 hours of PDGF (20ng/ml) challenge. AKT phosphorylation induced by PDGF (10 or 20 ng/ml) in WT vs TNFR-DKO (D), TNFR1-KO (E), or TNFR2-KO (F) HSC. Data are mean±SD in A, C n=3 and *p≤0.05 vs WT HSC; in B *p≤0.05 vs PDGF untreated cells.

**Figure 3.** TNF controls MMP-9 expression in HSC. (A) Basal MMP-9 mRNA expression in WT and TNFR-DKO HSC, and (B) after TNF challenge (50ng/ml) for 24 hours in WT, TNFR-DKO, TNFR1-KO, and TNFR2-KO HSC. (C) Effect of TNF (50ng/ml, 24 hours) on MMP-9 protein expression. (D) MMP-9 mRNA expression in WT and TNFR-DKO HSC in the presence of 10% or 0.5% of FBS in the media. (E) MMP-2 and TIMP-1 mRNA expression in WT HSC after TNF challenge (50ng/ml, 24 hours). (F) TIMP-1 mRNA expression after TNF challenge (50ng/ml) for 24 hours in WT, TNFR-DKO, TNF-R1-KO, and TNF-R2-KO HSC. Data are mean±SD, * p≤0.05 vs WT HSC, #p≤0.05 vs TNF-R2 KO Ctrl HSC.
**Figure 4.** TNF controls MMP-9 expression in LX2. Effect of TNF (50ng/ml, 24 hours) on MMP-9 mRNA expression (A) and activity by zymography (B). MMP-9 mRNA (C) and activity (D) after 24 hours challenge with TNF (50ng/ml) in LX2 cells in the presence of TNF-R1 or TNF-R2 blocking antibodies (pre-incubation at 10 µg/ml, 30min). (E) TIMP-1 and MMP-2 mRNA expression after TNF challenge (50ng/ml, 24 hours) in LX2 cells. Data are mean±SD, * p≤0.05 vs Ctrl LX2 cells., #p≤0.05 vs anti-TNF-R2-treated control LX2 cells.

**Figure 5.** Comparison of MMP-9 induction by several factors. Primary 6-day old HSC (A) or LX2 cells (B) were incubated with equivalent concentration (50ng/ml) of TNF, LPS, IL-1α, and IL-1β for 24 to determine MMP-9 mRNA expression. Activity in the extracellular media by gelatin zymography in LX2 cells (C). Data are mean±SD, * p≤0.05 vs Ctrl LX2 cells.

**Figure 6.** Reduced liver damage and fibrosis in TNFR-DKO mice after bile duct ligation. H&E staining of liver sections (A) and serum transaminase levels (B) in wild-type and TNFR-DKO after BDL. TNF-α (C), MMP-9 and TIMP-1 (D), MMP-2 and Colα1(I) (E), and α-SMA (F) mRNA expression in wild-type and TNFR-DKO livers after BDL. In A, Arrowheads signal bile infarcts. Data are mean±SEM, * p≤0.05 vs wild-type Sham, #p≤0.05 vs wild-type BDL.
Tarrats et al., Figure 1

A

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Nuclear p65 (NFκB)

Total p65 (NFκB)

B

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α-SMA

β-actina

C

mRNA fold induction

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Tarrats et al., Figure 2

A

% vs. time 0

WT
TNFR-DKO

3H-Thymidine incorporation

B

2H-Thymidine incorporation vs. Ctrl

C

Control
PDGF

3H-Thymidine incorporation vs. Ctrl

WT
TNFR-DKO

D

WT
TNFR-DKO

PDGF (ng/ml)

p-AKT
AKT

E

WT
TNFR1-KO

PDGF (ng/ml)
p-AKT
AKT

F

WT
TNFR2-KO

PDGF (ng/ml)
p-AKT
AKT
Tarrats et al., Figure 4

A

MMP-9 mRNA fold induction vs Ctrl

B

MMP-9 Ctrl TNF 92kDa

C

MMP-9 mRNA fold induction vs Ctrl

D

MMP-9 Ctrl TNF 92kDa

E

mRNA fold induction vs Ctrl

TIMP-1 MMP-2