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TNFα is required for cholestasis-induced liver fibrosis in the mouse

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Abstract

TNFα, a mediator of hepatotoxicity in several animal models, is elevated in acute and chronic liver diseases. Therefore, we investigated whether hepatic injury and fibrosis due to bile duct ligation (BDL) would be reduced in TNFα knockout mice (TNFα−/−). Survival after BDL was 60% in wild-type mice (TNFα+/+) and 90% in TNFα−/− mice. Body weight loss and liver to body weight ratios were reduced in TNFα−/− mice compared to TNFα+/+ mice. Following BDL, serum alanine transaminases (ALT) levels were elevated in TNFα+/+ mice (268.6 ± 28.2 U/L) compared to TNFα−/− mice (105.9 U/L ± 24.4). TNFα −/− mice revealed lower hepatic collagen expression and less liver fibrosis in the histology. Further, α-smooth muscle actin, an indicator for activated myofibroblasts, and TGF-β mRNA, a profibrogenic cytokine, were markedly reduced in TNFα−/− mice compared to TNFα+/+ mice. Thus, our data indicate that TNFα induces hepatotoxicity and promotes fibrogenesis in the BDL model.

Keywords

TNFα; Tumor necrosis factor α; Knockout mice; Bile duct ligation; BDL; Cholestasis; Liver fibrosis; TGF-β

Chronic cholestatic liver disorders are a serious clinical problem and often require liver transplantation due to cirrhosis [1]. The most common cholestatic liver diseases are primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) [2]. Other conditions resulting in hepatic fibrosis and cirrhosis are alcohol consumption, viral infections, autoimmune and metabolic disorders, all leading to accumulation of extracellular matrix proteins, mainly collagen type I [3]. The hepatic stellate cell (HSC) is the main cell-type responsible for this hepatic collagen production [4].

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Tumor necrosis factor α (TNFα), a cytokine with diverse biological functions like cytotoxicity, inflammation, growth stimulation and immune modulation is elevated in most liver diseases [5]. For example, increased TNFα levels are found in patients with PBC [6], chronic viral hepatitis [7], fulminant hepatic failure [8], and negatively correlate with survival in patients with alcoholic liver disease [9]. In addition, TNFα levels are elevated in the serum of patients with alcoholic liver cirrhosis [10] and polymorphisms in the TNFα gene are associated with advanced fibrosis in alcoholic steatohepatitis [11]. Further, TNFα aggravates liver fibrosis induced by schistosomiasis in humans [12].

TNFα mediates liver injury in several animal models such as alcohol- or dimethylnitrosamine-induced liver injury [13,14] and plays an important role in perpetuation of HSC activation *in vitro* [15]. Further, TNFα induces liver failure or exacerbates liver injury following exposure to hepatotoxins such as carbon tetrachloride or amanitin [16,17]. On the other hand TNFα is required for liver regeneration after partial hepatectomy [18].

In experimental cholestasis induced by bile duct ligation (BDL) TNFα and IL-6 are elevated [19]. Further, hepatic macrophages from cholestatic livers exhibit high TNFα levels [20]. In a model of pulmonary fibrosis, TNFα receptor knockout mice were protected from the development of fibroproliferative lesions [21]. Also, hepatic injury following administration of the hepatotoxin carbon tetrachloride was inhibited in TNFα knockout mice [22]. This study was designed to investigate if hepatic injury and fibrosis due to BDL would be altered in TNFα−/− mice. Experimental cholestasis significantly decreased animal survival in wild-type mice compared to TNFα−/− mice. In addition, BDL caused liver injury and fibrosis in TNFα+/+ mice, whereas pathology was largely prevented in TNFα−/− mice, indicating that TNFα plays an important pathophysiological role in the development of cholestasis-induced liver fibrosis.

Materials and methods

Animals

A breeding colony of TNFα−/− mice, a kind gift of breeding pairs from Michael W. Marino Sloan-Kettering, New York [23] and TNFα+/+ mice (C57Bl/6) was established at the University of North Carolina at Chapel Hill. All animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care and received humane care in compliance with institutional guidelines. Animals were maintained under standard conditions.

Operative procedure and experimental protocol

TNFα−/− and TNFα+/+ mice were allocated randomly to four experimental groups and either bile duct ligation (BDL) or sham operations were performed as described previously [24]. On the day of sacrifice, mice were anesthetized, livers were harvested and blood samples were collected.

Clinical chemistry

Serum was stored at −20 °C until alanine aminotransferase (ALT) was analyzed by a standard quantitative colorimetric assay, according to the manufacturers' protocol (Sigma, St. Louis, MO, USA).

Liver histology and histochemical detection of collagen

Liver sections were fixed in 10% formalin and embedded in paraffin; 10 μm sections were mounted on glass slides. Sections were deparaffinized and the slides were incubated for 30 min in a solution of saturated picric acid containing 0.1% Sirius red and 0.1% fast green [24]. Image analysis was performed to quantify sirius red positive areas, as described [25].

Immunohistochemical staining for α-smooth muscle actin

Ten micron sections were deparaffinized, rehydrated and placed in phosphate buffered saline with 1% Tween 20. Immunohistochemistry was performed with a monoclonal primary mouse anti-α-smooth muscle actin antibody (Dako, Carpinteria, CA) and the EnVision kit (Dako, Carpinteria, CA). The primary antibody was diluted 1:200 with 1% bovine albumin (Sigma, St. Louis, MO) in PBS. The procedure followed the instructions outlined by the manufacturer. α-Smooth muscle actin positive cells were counted in 10 non-overlapping high power fields (hpf; magnification 400×).

RNase protection assay

Total RNA was isolated from liver tissue using RNA STAT 60 (Tel-Test Inc., Friendswood, TX). RNase protection assays were performed using the Ribo Quant multiprobe assay system (BD Biosciences, San Jose, CA) as described previously [25].

Western blot

Protein extraction and Western blotting were performed as described [26]. After electrophoresis the proteins were transferred onto nitrocellulose membranes and stained with 0.5% Ponceau S to assess equal protein loading and transfer. Membranes were incubated with anti-collagen type I antibody (1:1000, Rockland, PA, USA) or anti-α-tubulin (1:1000, Santa Cruz, CA, USA), followed by incubation with the corresponding secondary antibodies. Immunodetected proteins were visualized using the enhanced chemiluminescent ECL assay kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Gelatin zymography

Twenty micrograms of whole liver cell extract were subjected to SDS–PAGE using a 0.1% gelatin containing polyacrylamide gel (Invitrogen, Carlsbad, CA). After electrophoresis the gel was exposed to renaturing buffer (2,5% Triton X-100) for 1 h, followed by developing buffer (50 mM Tris–HCl, pH 7.5 containing 0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3) for 24 h. Gels were stained with 0.1% Coomassie brilliant blue and destained with a solution of 10% methanol and 10% acetic acid. MMP activity was determined by densitometric scanning of the 62 and 68 kD proteolytic bands, corresponding to MMP activity.

Statistical analysis

Analysis of variance (ANOVA) or Tukey's post-hoc tests were used for the determination of statistical significance, if appropriate. The Mann–Whitney rank sum test was used for statistical analysis of survival. $P < 0.05$ was selected as the level of significance. Data are expressed as means ± SEM.

Results

Body weight, liver to body weight ratios, survival and serum chemistry

Sham operation resulted in a body weight loss of about 10% in mice of both groups (Fig. 1A). BDL caused a decrease in body weight in wild-type mice by about 20% in week one and 30% in week three. In contrast, body weight loss was blunted in TNFα−/− mice. However, there was only a statistical trend which did not reach the level of significance.

Liver to body weight ratios were 3.9% and 4.0% in control mice (Fig. 1B). BDL caused a liver enlargement to 6.9% in TNFα+/+ mice and 6.0% in TNFα−/− mice ($P = 0.017$).

Survival was 100% in both groups of sham-operated mice whereas only 60% of wild-type mice survived the experimental period of 3 weeks. In contrast, 90% of TNFα−/− mice survived the experimental period (Fig. 1C).

The average ALT levels of wild-type and TNFα−/− mice were 54.9 ± 26.1 U/L and 70.1 ± 30.8 U/L after sham operation (Fig. 1D). BDL significantly increased serum ALT levels to 268.6 ± 28.2 U/L in TNFα+/+ mice but only 105.9 ± 24.4 U/L in TNFα−/− mice ($P < 0.004$).

Histological and immunohistochemical evaluation of fibrosis

Sirius red fast green staining and image analysis were performed to detect extracellular matrix proteins (ECM). No staining was observed in livers of sham-operated mice (Fig. 2A, panel I: 0.8 ± 0.3% sirius red positive area; panel III: 1.0 ± 0.2% sirius red positive area). In contrast, strong staining was found in bile duct ligated wild-type mice compared to control mice (Fig. 2A, panel II: 4.1 ± 0.5% sirius red positive area; $P < 0.001$). Fibrosis with bridging was predominantly present in periportal areas. In addition, necrotic foci were detected in bile duct ligated wild-type mice (data not shown). However, BDL did not cause significant fibrotic or necrotic changes in the livers of TNFα−/− mice (Fig. 2A, panel IV: 1.5 ± 0.3% sirius red positive area; $P < 0.001$).

α-Smooth muscle actin (α-SMA) is marker for activated HSC. α-SMA positive cells could not be detected in livers of sham operated mice (Fig. 2B, panel I; III), while α-SMA positive cells were identified in wild-type mice after BDL, mainly in necrotic foci and around portal areas (Fig. 2B, panel II: 47.7 ± 16.9 cells/hpf; $P < 0.001$). In contrast, positive staining was decreased in TNFα−/− mice after BDL (Fig. 2B, panel IV: 11.5 ± 6.2 cells/hpf; $P < 0.001$).

Evaluation of collagen expression

Collagen I protein expression was evaluated by Western blotting (Fig. 2C). In sham operated animals collagen I was not detectable. BDL resulted in a strong increase in the expression of

collagen I in TNFα+/+ mice, while collagen I protein was not detectable in TNFα−/− mice. α-Tubulin served as a control to ensure equal loading (Fig. 2C, lower panel).

TGF-β mRNA expression and MMP activity

TGF-β mRNA levels were analyzed by RNase protection assay. TGF-β mRNA was undetectable in livers of sham operated mice. BDL significantly increased TGF-β mRNA levels in both wild-type and TNFα−/− mice but the increase was less in TNFα−/− mice, indicating a decrease in the profibrogenic response in TNFα−/− mice after BDL (Fig. 2D).

To determine the activity of matrix metalloproteinase 2 and 9 (MMP-2, −9), gelatin zymography was performed (Fig. 2E). MMP activity was absent in mice after sham operation. MMP activity was clearly increased in TNFα+/+ and TNFα−/− mice after BDL, revealing a band at 68 kDa corresponding to MMP-2. No differences in MMP activity were found between both BDL groups.

Discussion

TNFα has many functions in hepatic injury. On the one hand, it plays an important role in liver regeneration [18], but it is also associated with liver injury in many experimental models [27] and human diseases [6,8,9].

Since experimental models showed that TNFα receptor knockout mice were protected from pulmonary fibrosis and carbon tetrachloride-induced liver injury [21,22,28] we wanted to test if liver injury and fibrosis caused by experimental cholestasis are attenuated in TNFα−/− mice. Indeed, we found that fibrosis and liver injury were reduced significantly in TNFα−/− mice following BDL-induced liver injury.

We chose the BDL model, because TNFα is elevated in the serum of jaundiced mice and concentrations of the soluble TNF receptor negatively correlate with mortality in this model [19,29]. In line with these data, our study showed a clear decrease of survival rates in TNFα +/+ mice, while survival was 90% in TNFα−/− mice, indicating a significant role of TNFα in the pathophysiological process in the BDL model. Moreover, body weight loss was less pronounced in absence of TNFα, confirming the cachectic effect of TNFα in the BDL model, previously attributed to TNFα as cachectin [30,31].

How does absence of TNFα protect the liver from hepatic injury and fibrosis due to BDL? Obstructive jaundice produces high levels of TNFα in macrophage-rich organs and peritoneal macrophages in response to LPS [32]. Hepatic macrophages, i. e. Kupffer cells (KC), largely contribute to liver fibrosis, since depletion of KC using $GdCl_3$ prevents HSC activation and the development of cirrhosis in the rat [33].

BDL also leads to accumulation of hydrophobic bile acids which cause oxidative stress in the liver [34,35]. Oxidative stress can induce TGF-β in several cell types [36,37]. In the BDL model oxidative stress can increase TNFα and IL-6 concentrations via NFκB activation in KC leading to enhanced TGFβ and collagen expression [20,38]. Conclusively, we showed decreased fibrosis and less hepatic collagen expression in mice lacking TNFα, while wild-type mice showed strong fibrotic changes after BDL. Since TNFα is able to

induce profibrogenic signals in KC, a possible mechanism could be that the absence of TNFα leads to a reduction of cytokines, such as TGFβ, from KC [20,38].

TGFβ is one of the main profibrogenic factors involved in collagen synthesis and widely used to analyze the fibrogenic response [39]. Here, we demonstrate that TGFβ mRNA levels were significantly diminished in TNFα−/− mice after BDL, previously described in carbon tetrachloride-induced liver fibrosis [22,28]. Since TGFβ is upregulated in liver fibrosis and known to cause HSC proliferation and production of collagen [40,41], TNFα leads to upregulation of TGFβ in the BDL model thereby amplifying fibrosis.

Hepatocytes contribute to the profibrogenic response, because signals from hepatocytes undergoing oxidative stress can increase proliferation and collagen synthesis in HSC [42]. TNFα has cytotoxic effects so that an effect of TNFα on hepatocytes cannot be ruled out [43]. Massive hepatocyte cell death and necrosis, followed by macrophage activation, subsequently leads to excessive repair and fibrosis, induced by stimuli from different hepatic cell-types [4]. Indeed, BDL significantly increased ALT values in serum of TNFα+/+ mice indicating parenchymal liver cell damage, while ALT release in TNFα−/− mice was attenuated after BDL. We conclude that the absence of TNFα results in decreased parenchymal cell damage and attenuation of liver fibrosis.

TNFα has direct effects on HSC. For example, TNFα can induce transcription factors only in activated, but not in quiescent HSC [15]. Moreover, TNFα activates HSC by stimulating MAP-kinase activity and increased AP-1 binding [44,45]. In the current study, HSC activation, shown by expression of α-SMA, was clearly decreased in TNFα−/− mice after BDL. Data supporting this showed that inhibition of TNFα-induced MAP kinase activation also reduced α-SMA expression in HSC [44]. Thus, TNFα influences HSC activation in the BDL model.

Since TGFβ and TNFα can upregulate TIMP expression, thereby enhancing liver fibrosis [46], we analyzed collagenase activity by gelantin zymography. However, no differences in MMP activity were found in the BDL groups, indicating that TNFα does not interfere with collagenase activity in this model.

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This work is dedicated to the late Prof. R. G. Thurman.

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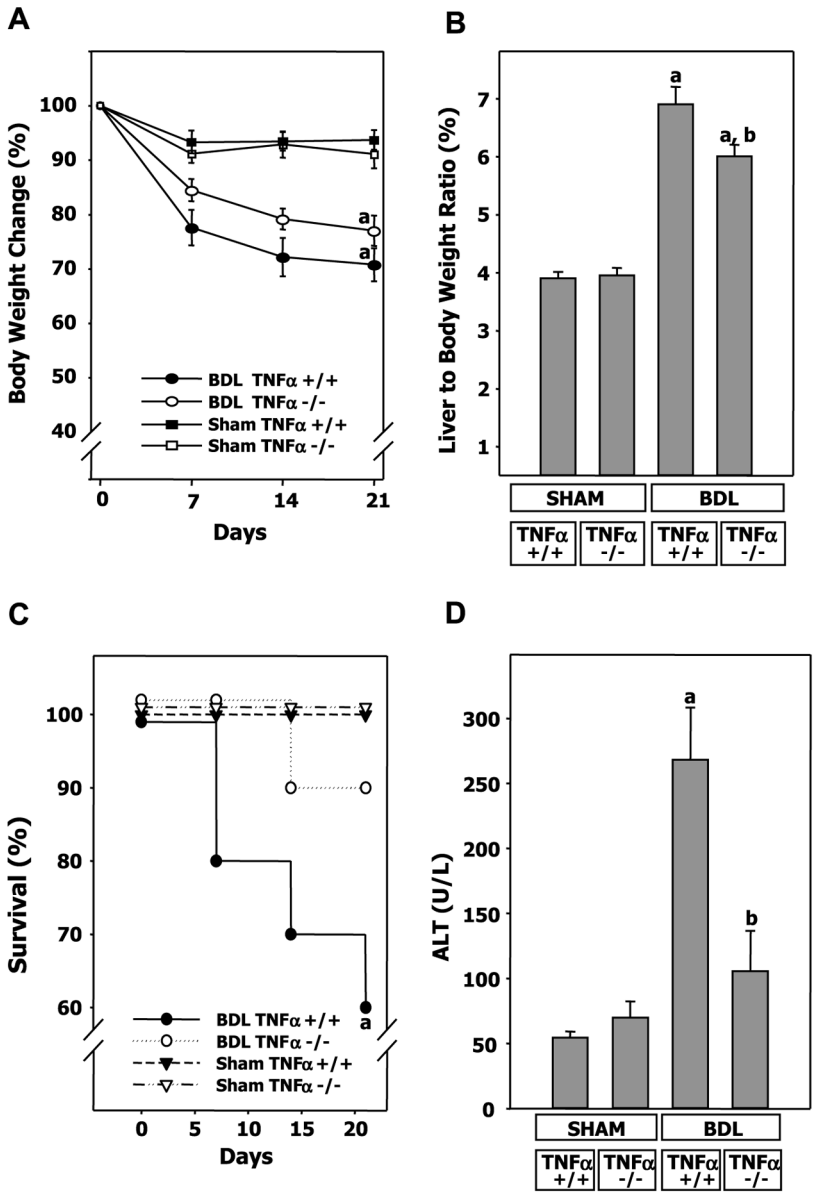

Fig. 1.

Effect of BDL on body weight, liver to body weight ratios, survival and serum chemistry in TNFα+/+ or TNFα−/− mice. (A) Wild-type (black symbols) or TNFα−/− (white symbols) mice underwent BDL (circles) or sham operation (squares) as described. Body weights (n = 6–8) were measured weekly and normalized to percent of initial weight. Data represent mean ± SEM. [a]$P < 0.05$ compared with mice receiving sham operation by two-way ANOVA. (B) Body and liver weights (n = 6–8) were determined at sacrifice. Data are presented as mean ± SEM. [a]$P < 0.05$ compared with mice receiving sham operation. [b]$P < 0.05$ compared with BDL wild-type mice by two-way ANOVA with Tukey's post-hoc test. (C) Survival was determined after 3 weeks (n = 6–8). [a]$P < 0.05$ compared with mice receiving sham operation by Mann–Whitney rank sum test. (D) Alanine aminotransferase (ALT) levels (n = 5–8) were determined as described in methods. Data presented are mean ± SEM. Two-way ANOVA

with Tukey's post-hoc test was used for determination of statistical differences. [a]$P < 0.05$ compared with mice receiving sham operation. [b]$P < 0.05$ compared with TNFα+/+ mice receiving BDL.

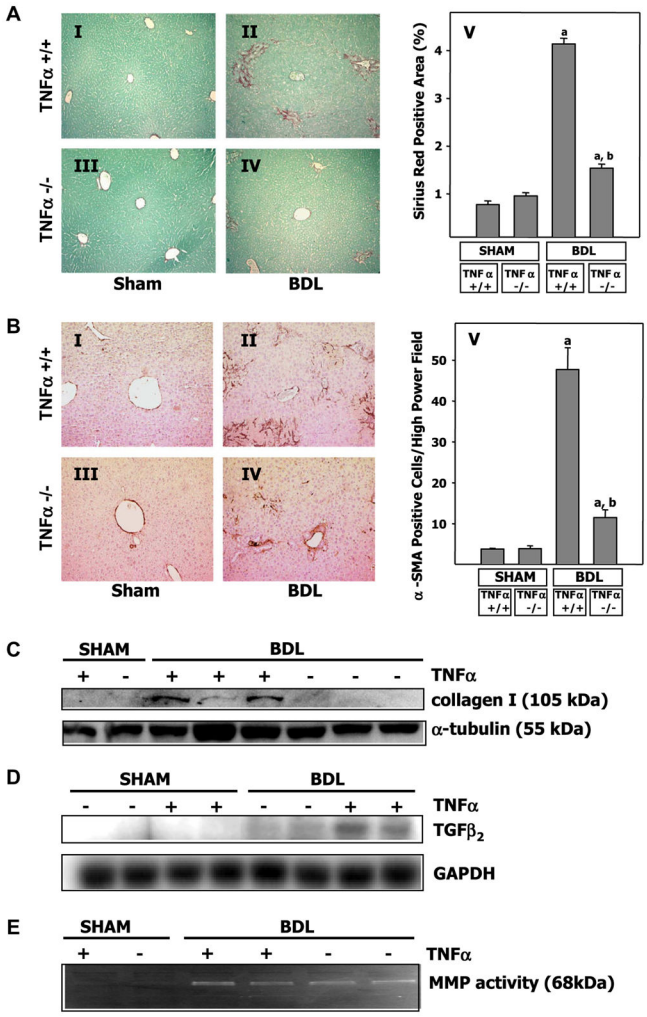

Fig. 2.

Evaluation of hepatic fibrosis and collagen expression after BDL in TNFα+/+ and TNFα−/− mice. (A) Sirius red fast green staining of mouse livers following 3 weeks of BDL. Sham operated TNFα+/+ (panel I) and TNFα−/− mice (panel III). BDL in TNFα+/+ (panel II) and TNFα−/− mice (panel IV). Magnification 100×. Representative micrographs are shown. Quantification of sirius red positive areas using image analysis (panel V). [a]$P < 0.05$ compared with mice receiving sham operation. [b] $p < 0.05$ compared with TNFα+/+ mice receiving BDL. (B) Immunohistochemical staining for α-smooth muscle actin (α-SMA) after 3 weeks of BDL. Representative micrographs. α-SMA staining after sham operation in TNFα+/+ (panel I) and TNFα−/− mice (panel III); effect of BDL on α-SMA staining in TNFα+/+ (panel II) and TNFα−/− mice (panel IV). Quantification of α-SMA positive cells (panel V). [a]$P < 0.05$ compared with mice receiving sham operation. [b]$P < 0.05$ compared with TNFα+/+ mice receiving BDL. (C) Expression of collagen I protein (105 kDa, upper panel) and α-tubulin (55 kDa, lower panel) in control mice (lane 1-2), TNFα+/+ mice (lane 3–5) and TNFα−/− mice (lane 6–8) after BDL using Western blot analysis. (D) TGF-β mRNA levels by RNase protection assay in TNFα+/+ and TNFα−/− mice after sham operation (lane 1–4) or BDL (lane 5–8). Representative autoradiogram. (E) Collagenase

activity assessed by gelatin zymography in TNFα+/+ and TNFα−/− mice after sham operation (lane 1 and 2) or BDL (lane 3–6). Representative zymogram.