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Nuclear factor-kappa B plays a central role in tumour necrosis factor-mediated liver disease

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Abstract

Deregulation of the apoptotic program is considered an important cause in liver disease. It became clear that the cytokine tumour necrosis factor (TNF) is of specific interest in this context. Therefore, from a clinical point of view, therapeutic control of TNF-receptor signalling pathways is highly desirable. These TNF-initiated signalling pathways result in a direct apoptotic response as well as potent activation of proinflammatory gene expression *via* activation of the transcription factor nuclear factor-kappa B (NF-κB). Since the latter pathway contributes to a series of liver pathologies, inhibition of hepatic NF-κB activation was viewed as a potential therapy for liver injury. However, the more recent finding that NF-κB activation in hepatocytes is anti-apoptotic shows that NF-κB signalling represents a problematic therapeutic target. Here we review the role of TNF and NF-κB in liver pathophysiology, and the underlying mechanisms of hepatocyte sensitisation to TNF toxicity *in vivo*. Based on this knowledge, we suggest some potential strategies for the treatment of TNF-mediated liver disease.

Keywords: Apoptosis; Hepatitis; Liver failure; NF-κB; Signal transduction; TNF

Acute liver failure is a clinical syndrome that results from massive necrosis and apoptosis of liver cells, leading to hepatic encephalopathy and severe impairment of hepatic function. It is a symptom of different kinds of liver diseases, such as viral hepatitis (A, B, C, ...), alcoholic hepatitis, autoimmune hepatitis, etc. [1]. Many studies have shown that TNF is a critical regulator of hepatocyte physiology in a variety of pathophysiologic conditions [2–4]. At first glance, TNF signalling seems to be an attractive target for therapeutic intervention in liver failure, thus eventually decreasing the need of liver transplantation in patients. The most prominent effects of TNF are the induction of either a proliferative or an inflammatory response [5,6]. In most cases, this is mediated by the TNF-induced expression of a variety of genes, in which the transcription factor NF-κB plays a central role. In addition, TNF is an important mediator of apoptosis (programmed cell death) [7]. Hepatocytes are normally resistant to TNF-induced cytotoxicity, but undergo cell death from TNF in the setting of global transcriptional or translational arrest, or selective inhibition of the transcription factor NF-κB [8,9]. The normal induction of NF-κB by TNF presumably leads to the transcriptional upregulation of genes that somehow inhibit the cell death pathway. Understanding the cross-talk between the NF-κB and cell death signalling cascades may lead to the development of novel approaches for the treatment of liver disease.

1. Role of TNF in liver disease

TNF was originally identified by its capacity to induce haemorrhagic necrosis of tumours in mice. Attempts to use TNF for systemic anti-cancer therapy have failed due to the appearance of severe side effects before therapeutic doses could be reached. One of the side effects of TNF treatment was an elevation in serum levels of transaminases and bilirubin levels, indicating a direct cytotoxic effect of TNF on human hepatocytes. Subsequent studies have shown that TNF may be involved in viral hepatitis, alcoholic liver disease, and fulminant hepatic failure. TNF serum levels

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Abbreviations: ConA, concanavalin A; GalN, galactosamine; IKK, I kappa B-kinase; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; TNF, tumour necrosis factor; TNF-R, TNF-receptor.

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are clearly elevated in patients with fulminant hepatitis [3]. Elevated plasma TNF and TNF-receptor (TNF-R) levels are also frequently observed in viral hepatitis [2,10,11]. Furthermore, it has been reported that specific hepatitis C proteins interact with the TNF receptor or TNF receptor associated proteins, modulating the sensitivity of infected cells to TNF [12–15]. Recently, it has also been shown that an adenovirus encoding hepatitis C virus core and E1 proteins can reduce liver cell injury in models of TNF-induced hepatotoxicity in mice [16].

TNF serum levels are also increased in patients with alcoholic hepatitis, and the levels correlate inversely with patient survival. TNF concentrations are significantly higher in patients who do not survive an episode of acute alcoholic hepatitis [4]. Monocytes isolated from patients with alcoholic hepatitis spontaneously produce higher amounts of TNF compared with healthy controls, also in response to lipopolysaccharide (LPS). Several hypotheses have been raised to explain increased TNF levels in patients with chronic ethanol exposure. Chronic ethanol feeding increases the permeability of the gut to bacterial products such as LPS, potentially inducing TNF production in macrophages [17]. In addition, studies investigating the promoter polymorphism in patients with alcoholic steatohepatitis indicated that these patients had a mutation in the TNF promoter that increases its activity [18]. Thus environmental as well as genetic factors may be involved in the increased TNF production in patients with alcoholic hepatitis.

It is important to note that the effect of the sole administration of TNF on healthy hepatocytes, either *in vitro* or *in vivo*, is beneficial in many biological situations. For example, following partial hepatectomy in mice and in culture of primary hepatocytes, TNF potentiates hepatocyte proliferation [19]. In contrast, in compromised hepatocytes, for example, due to the administration of a transcription blocker or oxidative stress, TNF is a strong inducer of hepatocyte apoptosis [8]. In addition, the overall effect of TNF on hepatocytes is also influenced by the cytokine environment generated concomitantly with a toxic insult. For example, injection of interleukin-10 (IL-10) can protect murine liver from TNF-induced hepatocyte apoptosis [20].

2. Animal models for TNF-mediated liver disease

The role of TNF in liver injury has been studied in several animal models. Although in all these models TNF is crucially involved in the induction of liver injury, it is important to note that important molecular differences have been described. In the TNF/galactosamine (GalN) model, TNF is administered in combination with D-(+)-GalN, a hepatotoxin that selectively blocks transcription in hepatocytes by depleting uridine nucleotides [21], inducing activation of caspases and subsequent hepatocyte apoptosis, infiltration of leukocytes and macrophages, finally leading to lethality [7,22]. TNF-R1 knockout mice are resistant to TNF/GalN treatment, demonstrating the essential role of TNF-R1 in this apoptosis model [23]. The sensitising effect of GalN suggests that the transcriptional block induced by GalN directly inhibits synthesis of anti-apoptotic proteins, the nature of which is still unknown. Since sensitisation to TNF-induced liver failure is also observed by specific inhibition of NF-κB activation ([24], our own unpublished results), NF-κB-dependent gene expression probably plays an important role in the sensitising effect of GalN.

A second model for TNF-mediated liver injury is the LPS/GalN-model. Although LPS-induced liver failure is TNF-dependent [25], some important differences with the TNF/GalN model have been noted. For example, mice deficient in the Ron tyrosine kinase are not protected in the TNF/GalN model, but are completely resistant in the LPS/GalN model, despite the production of increased TNF levels [26]. Moreover, LPS/GalN-induced liver injury is dependent on inducible NO synthase (iNOS), whereas TNF/GalN-induced liver failure is iNOS-independent [27].

Another model that is frequently used to study TNF-mediated liver failure is the ConA model. This is a suitable *in vivo* model to study the molecular mechanisms of T cell-dependent hepatitis, in which the administration of the T cell mitogenic plant lectin concanavalin A (ConA) to mice results in fulminant liver injury and in the production of several cytokines, including IL-2, TNF, and interferon-γ (IFN-γ). ConA-induced liver injury is also crucially dependent on IFN-γ and TNF [28,29]. Moreover, ConA-induced liver injury is dependent on both TNF-Rs, whereas TNF/GalN-induced is solely mediated by TNF-R1 [23,30]. Like the LPS/GalN model, also the ConA model is dependent on iNOS [27]. Interestingly, and in contrast to the TNF/GalN model, FADD/caspase and NF-κB pathways are not of major relevance for the degree of liver injury after ConA administration. In contrast, JNK- and IRF-1-dependent pathways play an important role in ConA-induced liver failure [31].

It should be clear from the comparison of these three mouse models that TNF can activate different pathways contributing to liver injury, dependent on the primary stimulus and the cytokine environment. Besides the signalling proteins already mentioned above, many other proteins have been shown to play an essential role in the development of liver disease in one or more of these models, the discussion of which is beyond the scope of this review.

3. NF-κB is a key regulator of TNF-signalling in hepatocytes

Understanding of the TNF signalling pathways is important in developing new therapeutic strategies for liver

disease. TNF interacts with two plasma membrane receptors, TNF-R1 and TNF-R2. In most cells, only TNF-R1 is able to induce apoptosis, but TNF-R2 may have a potentiating role by a still unknown mechanism. In this context, a role for parenchymal TNF-R2 in T cell-mediated hepatocyte apoptosis has recently been reported [32]. After ligand binding, TNF-R1 recruits the adapter protein TRADD to its intracellular domain, and this protein interacts with distinct adapter proteins allowing the activation of specific downstream signalling pathways. TNF may induce apoptosis through the caspase cascade *via* the FADD protein. The latter binds the cysteine protease caspase-8, leading to caspase-8 activation and initiation of the cell death pathway (Fig. 1). Through actions on Bcl-2 family members, activated caspase-8 induces mitochondrial release of proapoptotic factors such as cytochrome *c* that then lead to the activation of caspase-9. Directly, or through its effect on caspase-9, caspase-8 promotes the activation of downstream 'effector' caspase-3 and -7 that ultimately kill the cell [33]. In addition, other recent reports have suggested that alternative death pathways exist in hepatocytes. In this context, it has been shown that the caspase-mediated release of cathepsin B from lysosomes enhances mitochondrial release of cytochrome *c* and subsequent caspase activation in TNF-treated hepatocytes [34]. Moreover, deletion of the cathepsin B gene resulted in diminished liver injury and enhanced survival after treatment *in vivo* with TNF in sensitised mice [24]. Similarly, TNF-mediated hepatocellular apoptosis and liver damage is completely prevented in acidic sphingomyelinase knockout mice [35], further indicating an important function for acidic components in TNF-induced liver injury. Acidic sphingomyelinase was shown to contribute to TNF-induced hepatocellular apoptosis by promoting the mitochondrial localisation of glycosphingolipids and the permeabilisation of the mitochondrial membrane.

Hepatocytes are inherently resistant to TNF toxicity because of their ability to transcriptionally upregulate a cytoprotective pathway that blocks the TNF death signalling pathway. This protective pathway is mediated at least partially by the rapid TNF-induced activation of the transcription factor NF-κB. In this pathway, the TNF-R associated protein TRADD interacts with the adapter proteins TRAF2 and RIP, which eventually activate either JNK or NF-κB. In the case of NF-κB, this involves the activation of a kinase complex, consisting of the adapter protein I kappa B-kinase γ (IKKγ)/NEMO and the catalytic kinases IKKα and IKKβ, which phosphorylate the NF-κB inhibitor IκB. The latter retains NF-κB in the cytoplasm in an inactive dimeric form. Once phosphorylated, IκB is marked for ubiquitination and subsequent degradation by the proteasome, allowing the nuclear translocation of NF-κB and its binding to specific promoters [33]. The role of TNF-dependent NF-κB activation in the liver has been extensively studied in recent years. The first intimation that NF-κB activation may modulate hepatocyte responses relevant to liver injury was the finding that mice deficient for NF-κB RelA exhibit a surprisingly severe phenotype in

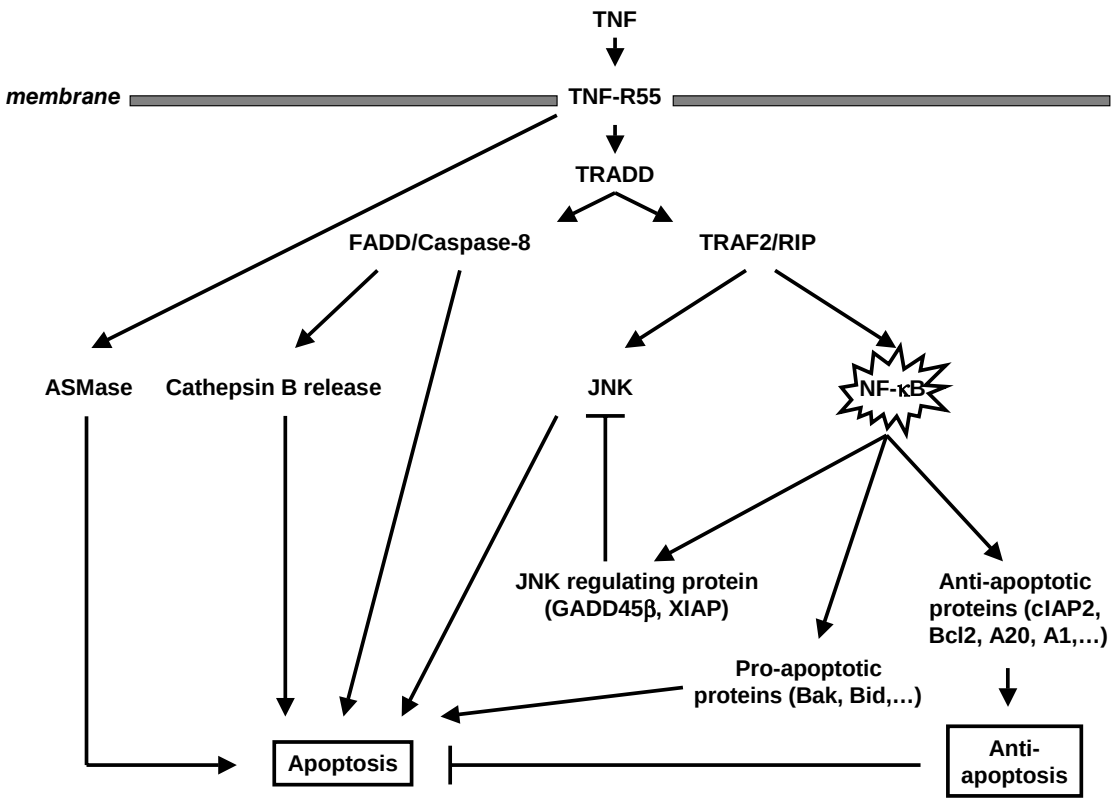

Fig. 1. Schematic representation of the cross-talk between different TNF-induced signaling pathways controlling TNF-induced apoptosis in hepatocytes.

that they die between 14.5 and 16 days of embryogenesis (E14.5–16) as a result of massive hepatocyte apoptosis [36]. Interestingly, elimination of TNF-R1 or TNF rescues the embryonic lethality of *RelA*$^{-/-}$ mice [37,38], suggesting that the pathology of apoptotic liver degeneration can be attributed to TNF signalling. The antiapoptotic role of NF-κB in the liver is further demonstrated by the phenotype of several other knockouts of genes that are related to NF-κB signalling, especially *IKKβ*- [39] and *IKKγ/NEMO*-deficient mice [40], all of which showed foetal liver apoptosis. As was true for *RelA*$^{-/-}$ mice, deletion of TNF-R1 rescues the embryonic lethality and liver apoptosis associated with IKKβ deficiency [41]. In addition to these knockout mice, it has been reported that *T2K*- (also called TBK1 or NAK, an IKKβ-related kinase) [42], *GSK-3β*- [43], *Raf-1*- [44], *SEK1/MKK4*- [45], *TAB2*- [46], and c-*Jun*-deficient embryos [47] show a similar liver degeneration during embryonic development. While the critical involvement of RelA, IKKβ and IKKγ in the TNF-R-mediated signalling pathway has clearly been shown, it remains to be elucidated whether T2K, GSK-3β, MKK4, TAB2, and c-*Jun* are important for the signalling *via* the TNF-R. For example, whereas GSK-3β deficient MEFs undergo apoptosis in response to TNF, T2K-deficient MEFs do not. On the other hand, liver apoptosis of GSK-3β- and T2K-deficient mice can be circumvented by the administration of anti-TNF antibodies or the generation of double mutant mice with TNF-R1. Moreover, it has been shown that TNF-induced NF-κB transcriptional activity was reduced in GSK-3β- and T2K-deficient MEFs. These findings suggest that these molecules are involved in TNF signalling by unknown mechanisms. In contrast, TAB2-deficient MEFs showed a normal response to TNF in terms of protection against apoptosis, IL-6 production, and NF-κB DNA-binding. In addition, double-deficient mice of TAB2 and TNF were still embryonically lethal. In this regard, it is noteworthy that Raf-1-, MKK4-, and c-*Jun*-deficient embryos died at almost the same stage as TAB2-deficient embryos. This suggests that an unknown factor and its receptor, which play an essential role in foetal liver maturation and homeostasis, might use these molecules to transmit the survival signal by a mechanism that is independent of TNF-mediated NF-κB activation. In contrast to the above-described essential role of NF-κB in foetal liver development, it was reported that inducible transgenic expression of IκB results in obstruction of NF-κB activation, yet produces no signs of liver dysfunction in adult mice [48].

TNF-dependent activation of NF-κB is also crucial for liver homeostasis. Inhibition of NF-κB activation after partial hepatectomy was shown to interfere with hepatocyte proliferation as well as to induce apoptosis, indicating that this part of the TNF-R1 signalling cascade controls cell proliferation and antiapoptotic pathways in hepatocytes [19,49]. Apoptosis may have resulted from a cell cycle block or from sensitisation to TNF that is produced following partial hepatectomy. An essential role for NF-κB activation during hepatocyte proliferation is supported by the finding that NF-κB inhibition did convert the hepatocellular response of rat hepatocytes to the mitogenic stimulus of TNF from proliferation to one of apoptosis [9]. Similarly, adenoviral delivery of IκB superrepressor (that retains NF-κB in the cytoplasm since it contains mutations at the phosphorylation sites that target it for ubiquitination and degradation) sensitises mice to TNF-induced hepatic failure ([24], our own unpublished results). Furthermore, pretreatment of mice with NF-κB activating agents such as TNF and IL-1 confers protection to the lethal effect of TNF/GalN [50]. Since this protection is not observed in mice compromised in NF-κB activation [51], NF-κB-dependent genes are likely to be responsible for this desensitising effect. However, these protective proteins remain to be identified. Possible candidate genes are the antiapoptotic proteins c-IAP1 and 2, Bcl-2, the Bcl-2 homologues A1/Bfl-1 and Bcl-xL, IEX-1L, and A20 (reviewed in [52]). More recently, the protective effect of NF-κB has been proposed to result from the inhibition of JNK signalling by an NF-κB responsive gene product [53,54]. NF-κB inhibition was shown to sensitise hepatocytes to TNF-induced apoptosis through a sustained activation of JNK and c-*Jun*, and increased AP-1 transcriptional activity [55]. Inhibition of the function of the JNK substrate and AP-1 subunit c-*Jun* in a rat hepatocyte cell line blocked cell death induced by NF-κB inactivation and TNF. The function of AP-1 in the TNF death pathway is presumably mediated through the upregulation of (a) proapoptotic gene(s). The latter was shown to function at a point upstream of the mitochondrial changes that result in the release of cytochrome *c* [55]. Studies in embryonic fibroblasts identified two genes, gadd45β and XIAP, as putative NF-κB-dependent genes that negatively regulate JNK activation [53,54], but it remains unclear whether these genes mediate TNF resistance in hepatocytes. Also in the ConA model, there is a direct correlation of liver injury and JNK activation [56]. Although the above observations demonstrate a proapoptotic function of JNK, another group showed that JNK exerts an antiapoptotic function in a c-*Jun* independent manner [57], indicating that other molecular JNK targets are essential to confer this mechanism. These authors used a dominant-negative TAK1-mutant to specifically block JNK, resulting in an earlier and stronger induction of apoptosis in hepatoma cells. The reason for these controversial results on the role of JNK is not clear, but the effect may depend on the cells used and the magnitude of activation.

Finally and to make things even more complex, NF-κB activation is also strongly involved in the cytokine-induced expression of proapoptotic Bak and Bid in hepatocytes [58]. Since NF-κB inhibition tips the balance towards apoptosis, the upregulation of some proapoptotic genes seems surprising. However, from the apoptosis-sensitising effect of NF-κB inhibition, one may conclude that the NF-κB-mediated upregulation of the antiapoptotic genes is dominant over the NF-κB-dependent activation of

proapoptotic genes. An overview of several signalling pathways contributing to TNF-induced hepatocyte apoptosis and the regulatory role of NF-κB is given in Fig. 1.

4. NF-κB: a problematic therapeutic target in the liver, or not?

The possibility that NF-κB activation in hepatocytes is protective following liver injury points to the complexity of events following global activation of NF-κB in all cell types in the liver. After a toxic stimulus, it is known that activation of NF-κB in hepatic macrophages results in the production of injurious products such as cytokines and reactive oxygen intermediates. Inhibition of hepatic NF-κB activation was therefore viewed as a potential therapy for liver injury. However, other studies have shown that hepatocyte cell lines and primary mouse hepatocytes can both be sensitised to TNF cytotoxicity by inhibiting NF-κB activation. These findings provide a possible explanation for the hepatocyte injury caused by TNF in toxin-induced liver injury. Hepatotoxins invariantly interfere with macromolecular synthesis, and could therefore block the TNF-induced, NF-κB-dependent transcriptional upregulation of genes that mediate hepatocyte resistance to TNF-induced apoptosis. Although the *in vivo* role of NF-κB-dependent genes in TNF-mediated liver injury remains unproven, it seems safe to conclude that NF-κB signalling represents a problematic therapeutic target since blanket inhibition of hepatic NF-κB activation may lead to both beneficial and detrimental effects. Cell-type specific targeting of NF-κB (e.g. in non-parenchymal cells) might be a future approach. Alternatively, the efficacy of TNF inhibition in liver disease would depend on which TNF pathways and which molecules are targeted. Targeting of molecules that are specifically involved in the death pathway might be safe. In this context, the observation that adenoviral delivery of a dominant-negative FADD-mutant protected mice in a model of TNF-mediated liver failure [59], illustrates that interfering with a specific protein–protein interaction in the death pathway (e.g. by small molecules) is a potential strategy. However, strategies that specifically target the death pathway will not interfere with the transcription-dependent proinflammatory effect of TNF in the liver, and might therefore only be partially effective. Because of its antiapoptotic function in the liver, targeting the proinflammatory NF-κB pathway has been excluded. However, tools that allow to inhibit the death pathway as well as the NF-κB pathway might still be relevant, assuming that the antiapoptotic effect is dominant. In this context, the use of the zinc finger protein A20, which prevents TNF-induced cell death despite its strong inhibitory effect on TNF-induced NF-κB activation [60,61], is a nice example. In fact, adenoviral delivery of A20 has already been shown to protect against liver injury in LPS/GalN [62] and TNF/GalN mouse models (our own unpublished observations). Similarly, adenoviral gene transfer of the A20-binding inhibitor of NF-κB activation protein ABIN-1 provides complete protection against TNF/GalN-induced liver failure, whereas transfer of an IκB-α superrepressor does not (our own unpublished observations). The protection with ABIN-1 is remarkable, because like A20 and the IκB-α superrepressor, ABIN-1 acts as a cellular inhibitor of TNF-induced NF-κB activation [61,63]. However, in contrast to A20, up to now no direct antiapoptotic effect could be demonstrated for this protein. Also a combination of NF-κB and JNK inhibitors might be worthwhile testing in view of the observation that NF-κB sensitises to apoptosis through the JNK pathway [55]. Finally, it is possible that TNF can activate different molecular forms of NF-κB dimers, and that dependent on the subunit composition and the duration of NF-κB activation, different target genes are activated. Therefore, it might be possible to specifically modulate the proinflammatory NF-κB pathway or the antiapoptotic NF-κB pathway. These interesting aspects of NF-κB signalling, if properly explored, may assist us in devising strategies to modulate the impact of NF-κB activation in TNF-mediated liver failure as well as other biological effects.

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