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Mechanisms of crosstalk between TNF-induced NF-κB and JNK activation in hepatocytes

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ABSTRACT

Hepatocyte cell death is a universal feature of inflammatory liver diseases. The observation that mice deficient in the activation of nuclear factor-κB (NF-κB) are not viable because of excessive hepatocyte apoptosis induced by tumor necrosis factor (TNF) made it crystal-clear that NF-κB plays a central role in protecting hepatocytes against TNF-induced cell death. Also during TNF-mediated liver injury, NF-κB was shown to have an essential anti-apoptotic effect, underscoring the therapeutic importance of understanding its underlying molecular mechanisms. For a long time, the ability of NF-κB to induce the expression of a variety of anti-apoptotic proteins was thought to be solely responsible for its cytoprotective effects. However, during the past few years it has become clear that NF-κB-mediated inhibition of cell death also involves attenuating TNF-induced activation of c-Jun activating kinase (JNK). Whereas transient activation of JNK upon TNF treatment is associated with cellular survival, prolonged JNK activation contributes to cell death. Several studies have shown that NF-κB activation inhibits the sustained phase of TNF-induced JNK activation and thus protects cells against TNF cytotoxicity. In this review, we will discuss the various mechanisms by which NF-κB activation blunts TNF-induced JNK activation, including the induction of JNK inhibitory proteins and controlling the levels of reactive oxygen species (ROS). Moreover, because the cytoprotective effects of NF-κB activation are particularly important in liver physiology, we will put each of these JNK-inhibitory mechanisms into a 'hepatic perspective' by discussing their role in various mouse models of TNF-mediated liver injury.

1. TNF-induced signaling pathways

The pro-inflammatory cytokine TNF plays a key role in a wide variety of physiological processes, including inflammation, proliferation and programmed cell death. These pleiotropic biological effects of TNF result from its ability to initiate different intracellular signaling pathways (see Fig. 1). Upon binding of TNF to TNF receptor 1 (TNF-R1), the signaling molecules TNF-R associated death domain (TRADD), TNF-R associated factor 2 (TRAF2), and receptor interacting protein 1 (RIP1) are recruited to form the so-called complex I [1]. This complex I initiates a signal transduction pathway leading to the activation of the transcription factor NF-κB. Briefly, TNF-induced NF-κB activation results from the activation of the inhibitor of κB kinase (IKK) complex, which phosphorylates the inhibitor of κB (IκB) family members that are normally bound to NF-κB and thereby retain it in the cytoplasm. This phosphorylation

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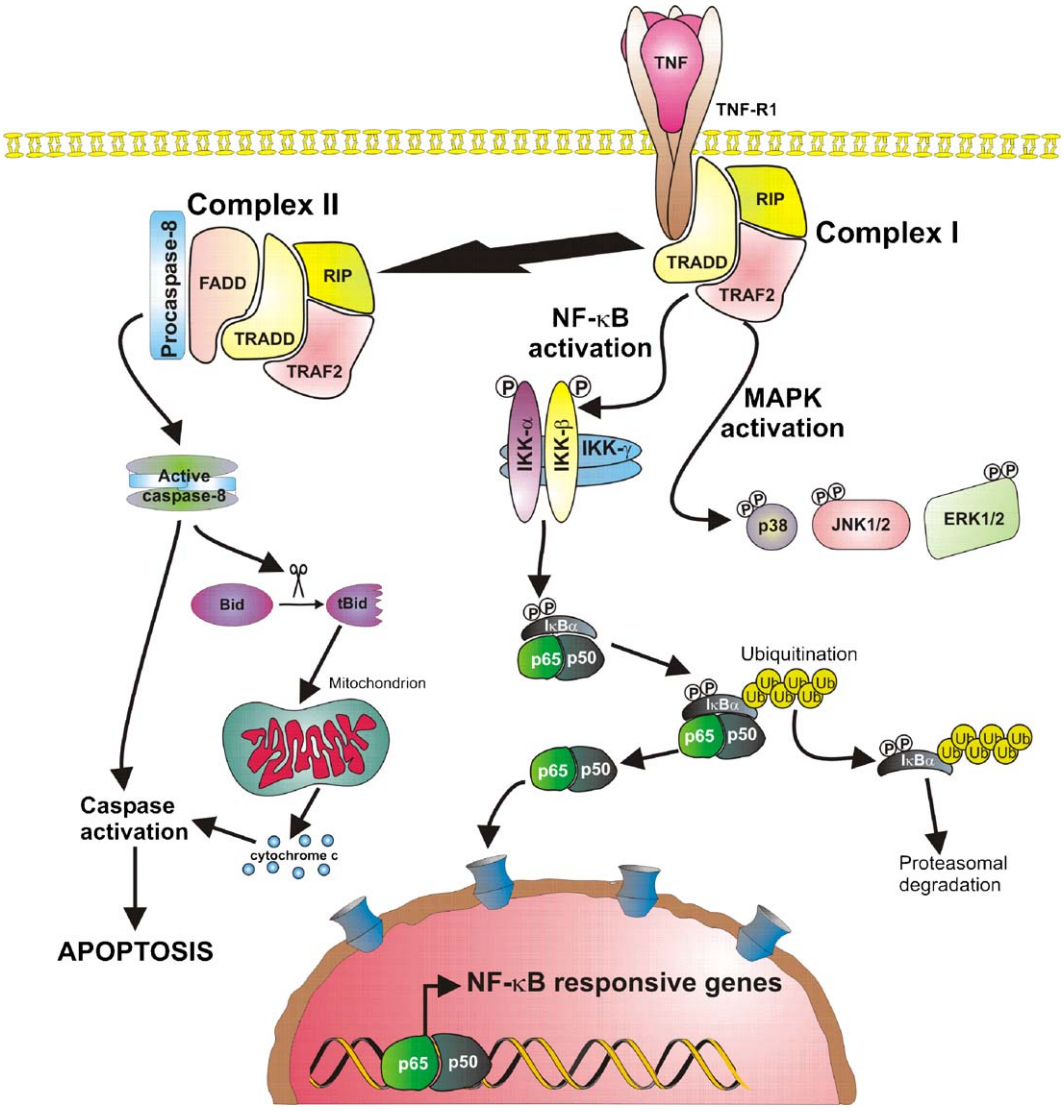

Fig. 1 – TNF-R1-induced signaling pathways via complexes I and II. TNF binding to TNF-R1 leads to the recruitment of TRADD, TRAF2 and RIP, forming complex I. Signaling from complex I leads to NF-κB activation via activation of the IKK complex. The IKKβ catalytic subunit of the IKK complex phosphorylates the NF-κB-bound IκBα, leading to its ubiquitination and subsequent proteasomal degradation. This allows NF-κB to translocate to the nucleus, where it induces the transcription of genes with an NF-κB consensus site in their promoter region. In addition, signaling from complex I activates the p38, ERK and JNK MAP kinases. Recruitment of FADD and procaspase-8 results in the formation of the cytosolic complex II, where caspase-8 is activated. Caspase-8 initiates the mitochondrial pathway by cleaving Bid to tBid, which induces mitochondrial permeabilization that results in the release of cytochrome c. This initiates an amplification loop that results in full-blown caspase activity and subsequent apoptosis.

of IκB leads to its subsequent ubiquitination and proteasomal degradation, thereby allowing NF-κB to translocate to the nucleus where it activates the transcription of NF-κB-responsive genes. In addition to NF-κB activation, TNF-induced signaling from complex I leads to the activation of different mitogen-activated protein kinase (MAPK) cascades, which ultimately result in the activation of p38 MAPK, extracellular regulated kinase (ERK) and c-Jun activating kinase (JNK) [2,3].

Alternatively, TNF-induced signaling can lead to the internalization of complex I, which then recruits Fas-associated death domain (FADD) and caspase-8, thus constituting complex II [1]. Here, caspase-8 is activated through auto-catalytic cleavage. Active caspase-8 sets the cell death machinery in motion by cleaving Bid. The resulting tBid fragment translocates to the mitochondria where it causes permeabilization of the mitochondrial outer membrane. This leads to the release of cytochrome c and other mitochondrial apoptogenic factors, which cause activation of other caspases, leading to a positive feedback amplification circuit that results in full-blown caspase activity and ultimately cell death [2].

2. Crucial role of NF-κB activation in balancing life and death in the liver

Despite its ability to induce cell death, TNF stimulation does not typically lead to cytotoxicity because of the activation of NF-κB. This cytoprotective function of NF-κB has been shown by numerous studies in various cell types [4]. In particular, the anti-apoptotic effect of NF-κB in hepatocytes has been very well documented. Mice lacking either the p65 subunit of NF-κB, as well as mice lacking the essential IKKβ or IKKγ components of the IKK complex, display lethal hepatocyte apoptosis during embryogenesis [5–8]. Further studies have shown that also in adult mice, NF-κB activation is crucial for protecting hepatocytes against TNF hepatotoxicity. For instance, mice with a hepatocyte-specific deletion of either IKKβ or IKKγ are more sensitive than wild type mice to the hepatotoxic effects of injection of Con A or TNF itself, respectively [9,10]. These genetic studies clearly demonstrate that because of its cytoprotective effect, NF-κB activation plays a crucial role in the fate of TNF-stimulated hepatocytes. Indeed, it is generally accepted that NF-κB activation protects hepatocytes from TNF-induced cell death during liver injury. As the levels of circulating as well as hepatic TNF are increased in many inflammatory liver diseases, including viral and alcoholic hepatitis, acute liver failure and ischemia/reperfusion-induced liver damage [11], this implicates that the activation status of NF-κB has a great impact on the pathogenesis of these liver diseases.

Besides playing an important beneficial role in protecting hepatocytes against TNF cytotoxicity, the pro-survival activities of NF-κB activation can also have detrimental effects. Inappropriate suppression of cell death by NF-κB activation can give rise to the development of tumors. Moreover, its cytoprotective effect can contribute to the resistance of tumor cells to chemotherapy and as such impair the removal of these malignant cells. Also this role of NF-κB has already become apparent in the liver, as mice deficient in NF-κB activation have been shown to more susceptible to hepatocarcinogenesis [12,13].

3. Mechanisms of the cytoprotective effect of NF-κB activation

Given the central role for NF-κB in the decision between survival and cell death, understanding the underlying molecular mechanisms of the cytoprotective effects of NF-κB is of considerable therapeutic interest. For instance, such knowledge can facilitate us to mimic these cytoprotective actions in order to protect hepatocytes from cell death during liver injury. Conversely, comprehending the cytoprotective actions of NF-κB can give us clues on how to specifically block these mechanisms in order to sensitize malignant cells to the cytotoxic effects of TNF or chemotherapeutics.

For a long time, NF-κB-induced cytoprotective factors were thought to be fully responsible for the cell death inhibiting effect of NF-κB activation. These anti-apoptotic proteins, such as the caspase-8 inhibitor c-FLIP(L), the Bcl-2 family members Bcl-xL and A1/Bfl-1, X-linked inhibitor of apoptosis (XIAP), and cellular inhibitor of apoptosis (c-IAP)1 and c-IAP2 [14–19], interfere with TNF-induced apoptosis at various levels in the signaling pathways leading to caspase activation. For instance, c-FLIP(L) competes with caspase-8 for binding to the TNF-R1 complex II, thus impairing the formation of the Death Inducing Signaling Complex (DISC) [1]. Bcl-xL and A1/Bfl-1 act at the mitochondria, where they prevent tBid-induced mitochondrial permeabilization, thus reducing the production of ROS and cytochrome c mediated caspase-9 activation [20]. XIAP as well as the c-IAPs act more downstream, as they directly bind distinct caspases and thereby inhibit their proteolytical activity [21,22]. However, the observation that many of these anti-apoptotic proteins show cell type specific effects or are unable to fully protect NF-κB deficient cells against apoptosis, indicated that other NF-κB-dependent events contribute to the resistance of hepatocytes to TNF-induced apoptosis. Indeed, in recent years it became clear that the outcome of TNF treatment is also determined by the balance between TNF-induced activation of NF-κB and TNF-induced activation of JNK. Because the contribution of the above-mentioned NF-κB-dependent proteins to protecting cells against cell death has been the subject of previous reviews [4,23], we will focus on the role of the crosstalk between TNF-induced activation of JNK and NF-κB, and on how the interplay between these pathways influences the cell's decision between life and death. Furthermore, because of the particular relevance of the cytoprotective effect of NF-κB activation in liver (patho)physiology, emphasis will be put on the proposed mechanisms of NF-κB/JNK-crosstalk in hepatocytes and their possible role in TNF-mediated liver diseases.

4. NF-κB-dependent inhibition of JNK activation

Multiple studies have shown that crosstalk between TNF-induced NF-κB and JNK pathways is important for determining the biological outcome of TNF stimulation. As TNF-induced activation of JNK is not a simple on–off switch, both the level and the duration of JNK activity influence its biological effect. Several lines of evidence support the notion that transient and modest activation of JNK is associated with cellular survival, whereas prolonged and robust activation of JNK plays an important role in TNF-induced cell death. Moreover, the level of TNF-induced JNK activation seems to be controlled by NF-κB activation. In cells lacking p65 or IKKβ, or cells stably expressing an $I\kappa B\alpha^{s}$, JNK activation in response to TNF is sustained. This prolonged JNK activation contributes to subsequent cell death, as inhibition of JNK by pharmacological agents or dominant-negative kinase mutants effectively rescues these NF-κB-deficient cells from TNF-induced cytotoxicity [24–27]. These observations indicate that at least some of the protective effects of NF-κB activation in TNF-induced cytotoxicity are mediated by rapidly terminating TNF-induced JNK activation.

Also in hepatocytes, sustained activation of JNK is associated with enhanced TNF-induced apoptosis. In the RALA rat hepatocyte cell line, expression of an $I\kappa B\alpha^{s}$ was shown to prolong TNF-induced activation of JNK. As a dominant negative c-Jun mutant prevented subsequent TNF-induced apoptosis, a c-Jun-dependent pathway seems

to contribute to apoptosis in this cell line [28]. Although primary hepatocytes expressing an IκBα^{s} also display extended activation of JNK and concomitant apoptosis, a dominant negative c-Jun mutant failed to block cell death in these primary cells, suggesting that JNK directly regulated apoptosis independently of its activation of c-Jun [29]. The latter is in contrast with the results obtained in the RALA hepatocyte cell line, suggesting that the pro-apoptotic effects of c-Jun may differ between primary hepatocytes and hepatocyte cell lines. However, more importantly, inhibition of JNK activation not only protected NF-κB deficient primary hepatocytes from apoptosis induced by TNF as such, but also from TNF-induced apoptosis in the presence of ActD [29,30]. This indicates that in primary hepatocytes, JNK exerts its pro-apoptotic effects independently of transcription.

This pro-apoptotic effect of JNK activation in cultured hepatocytes was recently confirmed by genetic studies in NF-κB-deficient mouse embryos, showing that JNK also promotes TNF-induced cell death of hepatocytes *in vivo*. Whereas IKKβ-deficient fetuses display severe hepatocyte apoptosis at E14.5, fetal livers from IKKβ/JNK1 double knockout mice show no liver damage at this stage of embryogenesis. As a result, these double mutant fetuses survive four days longer than IKKβ-deficient fetuses [31]. As hepatotoxicity resulting from NF-κB deficiency is caused by TNF [32–34], these observations suggest that JNK1 plays a central role in TNF-induced apoptosis of embryonic hepatocytes.

Next to TNF hepatotoxicity during embryogenesis, JNK activation was also shown to participate in hepatotoxicity during TNF-mediated liver injury in adult mice. For instance, prolonged activation of JNK seems to contribute to reperfusion injury associated with liver transplantation, as specific JNK inhibitors prevent both hepatocyte apoptosis and necrosis in models of I/R-induced liver injury [35–37]. Furthermore, JNK activation appears to have a central role in the development of liver damage after administration of LPS/GalN. Indeed, pretreatment of mice with a JNK-inhibitory peptide was shown to prevent liver injury induced by LPS/GalN [31]. Similarly, TNF-induced activation of JNK correlates with liver damage after injection of Con A [38,39], and the hepatotoxicity induced by Con A can be blocked by a JNK-inhibitory peptide [31]. Moreover, in the Con A model it was shown that the anti-apoptotic effects of NF-κB activation during TNF-mediated liver injury are at least partially mediated by attenuating JNK activation. This was proven by the observations that hepatocyte-specific deletion of IKKβ enhances the activation of JNK by Con A and that suppression of this response by additional ablation of either JNK1 or JNK2 reduces Con A-induced liver injury [10]. In this study, binding of mTNF to TNF-R2 was suggested to be responsible for the increased Con A-induced JNK activation. In contrast with this suggestion, human TNF was shown to be capable of inducing prolonged JNK activation and subsequent apoptosis in mouse primary hepatocytes [29]. As human TNF only binds TNF-R1 on murine cells, this study suggests that TNF-R1 triggering is sufficient for inducing sustained activation of JNK.

The pro-apoptotic role of sustained JNK activation in hepatocytes during embryogenesis as well as during TNF-mediated liver injury in adult mice shows that the mechanisms controlling the level of JNK activity serve as key modulators of the biological outcome of hepatocyte responses to TNF. Therefore, elucidating how NF-κB activation regulates the strength and duration of TNF-induced JNK activation will be important to specifically interfere with distinct hepatocyte responses to TNF. Various mechanisms have been proposed by which NF-κB activation controls the length of TNF-induced JNK activation, either based on NF-κB-dependent genes that down-regulate TNF-induced JNK activation, or based on ROS-mediated regulation of JNK activation. An overview of NF-κB-dependent JNK-inhibitory mechanisms is given in Fig. 2.

5. NF-κB-dependent inhibitors of JNK activation

The NF-κB-dependent genes that have been put forward as potential candidates to mediate the shut down of TNF-induced JNK activation include growth arrest DNA damage-inducible gene 45β (GADD45β), the already mentioned caspase inhibitor XIAP, and the zinc finger protein A20 [25,26,40].

5.1. *GADD45β-mediated inhibition of JNK activation*

GADD45β establishes a link between the TNF-induced anti-apoptotic NF-κB and pro-apoptotic JNK pathways because expression of GADD45β depends on NF-κB, and GADD45β inhibits TNF-induced JNK activation and subsequent apoptosis in NF-κB deficient cells [25]. This JNK inhibitory effect of GADD45β results from blocking the catalytic activity of the upstream JNK kinase MKK7 [41]. The latter was already implicated in TNF-induced cell death, as silencing of MKK7 expression significantly protects cells from the apoptotic effects of TNF [42]. Although these studies provide evidence for a cytoprotective effect of GADD45β-mediated JNK inhibition, GADD45β-deficient MEF cells do not display enhanced JNK activation upon TNF stimulation and are not hypersensitive to TNF-induced apoptosis [43]. Also in hepatocytes, a protective role for GADD45β in TNF-induced apoptosis remains to be shown. On the contrary, the observation that GADD45β expression is significantly down-regulated in hepatocellular carcinoma's is not in line with its proposed role as an anti-apoptotic protein [44,45]. On the other hand, studies in hepatic stellate cells do support a pro-survival role for GADD45β, as stellate cell apoptosis promoted by inhibition of NF-κB activation is associated with down-regulation of GADD45β along with increased JNK activation [46]. These contradictory observations suggest that the role of GADD45β in regulating TNF-induced JNK activation and subsequent apoptosis could be cell type specific, which might also explain the results obtained in GADD45β-deficient MEF cells.

5.2. *XIAP-mediated inhibition of JNK activation*

Besides GADD45β, also XIAP was suggested to protect from TNF-induced apoptosis by inhibiting JNK activation, as over-expression of XIAP diminishes JNK activation by TNF in p65 deficient cells [26]. In hepatocytes, RNAi-mediated knockdown of XIAP was recently shown to enhance TGFβ-induced JNK activation and subsequent apoptosis. In addition, a deletion mutant of XIAP that fails to inhibit caspases still blocked JNK

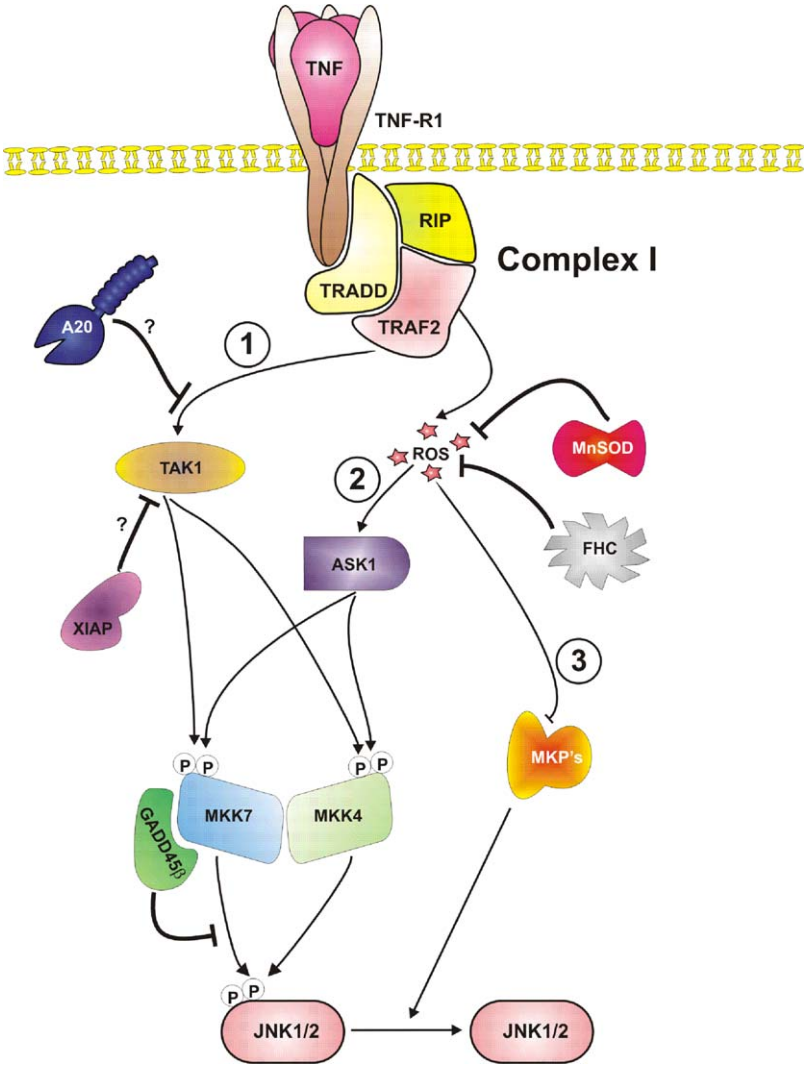

Fig. 2 – Regulation of TNF-induced JNK activation by NF-κB-dependent genes. TNF binding to TNF-R1 leads to the formation of complex I, which induces JNK activation via direct activation of TAK1 (1), which subsequently activates the upstream JNK kinases MKK4 and MKK7. In addition, accumulation of ROS contributes to JNK activation via activation of ASK1 (2), which then also activates MKK4 and MKK7. Accumulated ROS also contribute to JNK activity by inhibiting the MKPs (3), which normally dephosphorylate activated JNK. In the presence of NF-κB activation, the NF-κB-dependent JNK inhibitors GADD45β, XIAP and A20 prevent phosphorylation of JNK. GADD45β blocks JNK activation by binding to the upstream kinase MKK7, thereby blocking its catalytic activity. In contrast, the molecular mechanisms by which A20 and XIAP inhibit JNK activation are not clear. In addition, NF-κB activation leads to the expression of the anti-oxidants FHC and MnSOD, both of which prevent accumulation of ROS, thus inhibiting its JNK-stimulatory actions.

activation and protected hepatocytes from TGFβ-induced apoptosis, indicating that XIAP exerts an anti-apoptotic activity in hepatocytes that not solely depends on caspase inhibition, but also involves attenuation of JNK activation. Moreover, this study revealed the molecular mechanism for XIAP-mediated inhibition of TGFβ-induced JNK activation, as XIAP induced ubiquitin-mediated proteasomal degradation of TAK1 [47]. Because TAK1 is critically involved in TNF-induced JNK activation [48,49], it is tempting to speculate that XIAP uses a similar mechanism of action in TNF signaling. However, definite proof for such a mechanism is still lacking. Moreover, studies in XIAP-deficient MEF cells did not support a non-redundant JNK-inhibitory role for this protein [50], suggesting that XIAP-mediated inhibition of JNK activation might be cell type or stimulus-specific and emphasizing the need for further studies to confirm a physiological JNK inhibitory function of XIAP.

5.3. *A20-mediated inhibition of JNK activation*

Finally, the zinc finger protein A20 is a possible contender to mediate NF-κB-dependent inhibition of TNF-induced JNK activation. Expression of A20 is upregulated by NF-κB in most cell types, including hepatocytes, and functions as a negative feedback regulator of NF-κB activation [40,51]. In addition, overexpression of A20 was shown to block TNF-induced JNK

activation and cell death [52]. Consistent with this observation, treatment of A20-deficient MEF cells with TNF leads to enhanced activation of JNK and increased cell death [40]. These data suggest that A20 exerts its anti-apoptotic effect through inhibition of JNK activation. Although a JNK inhibitory role for A20 in hepatocytes remains to be shown, its anti-apoptotic effects have already proven to be beneficial in TNF-mediated liver injury. Indeed, adenoviral gene transfer of A20 protects mice against LPS/GalN-induced liver failure by decreasing hepatocyte apoptosis [51]. Remarkably, A20 also appears to enhance hepatocyte proliferation, enabling it to protect mice from lethal radical hepatectomy [53]. Given the inhibitory function of A20 in NF-κB as well as JNK activation, two pathways that are essential for efficient liver regeneration, the proliferative effect of A20 in hepatocytes seems quite puzzling and suggests a complex role for A20 in the regulation of hepatocyte responses. Therefore, further studies will be essential to dissect the exact role of this protein in hepatocytes.

6. ROS-mediated regulation of JNK activation

Next to enhancing the expression of JNK inhibitory proteins, another mechanism by which NF-κB activation inhibits prolonged JNK activation is preventing the accumulation of ROS. Indeed, a growing body of evidence supports a role for ROS in sustaining TNF-induced JNK activation. It is already known for a long time that TNF signaling is associated with enhanced generation of ROS, which significantly contribute to TNF-induced cell death. Under normal conditions, the level of intracellular ROS is strictly regulated by enzymatic and non-enzymatic anti-oxidant effectors that can either eliminate or scavenge ROS. As such, oxidative damage to cellular components only occurs when the amount of ROS exceeds the cell's anti-oxidant capacity. The latter seems to be critically regulated by NF-κB activity, as impaired NF-κB activation results in excessive ROS generation. Moreover, TNF-induced ROS accumulation in NF-κB-deficient cells contributes to TNF-induced cell death by sustaining JNK activation. This was shown by pretreatment of NF-κB deficient cells with anti-oxidants, which abolished the extended JNK activation and subsequently protected against TNF-induced cell death [54]. This study establishes ROS as a crucial link between the TNF-induced anti-apoptotic NF-κB and pro-apoptotic JNK pathways and raises two important questions. First, how does NF-κB activation prevent ROS accumulation? And second, how does ROS accumulation lead to prolonged JNK activation?

6.1. *How does NF-κB activation prevent ROS accumulation?*

The anti-oxidant enzymes that eliminate ROS include superoxide dismutases (SODs), catalase, glutathione peroxidases (GPxs) and peroxiredoxins (PRxs). In general, SODs convert O_2^- to H_2O_2, which is subsequently eliminated by catalase, GPxs and PRxs [55]. Although some of these enzymes are inducible by NF-κB activation, no decrease in their expression levels could be detected in p65-or IKKβ-deficient cells [54,56], indicating that basal expression of these enzymes, irrespective of the amount of NF-κB activation, should be sufficient to dispose of intracellular ROS. Indeed, overexpression of manganese-dependent SOD (MnSOD) confers only little or even no protection against TNF-induced ROS accumulation and subsequent apoptosis in NF-κB-deficient MEF cells [54,57,58]. In fact, several other anti-oxidant enzymes fail to significantly protect NF-κB-deficient cells from TNF-induced cell death as well [54], indicating that other NF-κB-dependent molecules or mechanisms might be involved in preventing ROS-mediated cell death.

In contrast to the above-mentioned anti-oxidant enzymes, the amounts of GSH and NADPH are considerably diminished in p65-deficient cells versus wild-type cells [54]. As NADPH is required for converting oxidized glutathione disulfide (GSSG) to reduced GSH, which in turn is essential for maintaining the activities of GPxs, GSH and NADPH act in concert to detoxify ROS. Consequently, the observed accumulation of ROS in NF-κB deficient cells might result from a shortage of these non-enzymatic anti-oxidants. Interestingly, TNF-induced hepatocyte cell death has been shown to be associated with loss of GSH as well as NADPH. In addition, depletion of GSH strongly sensitizes hepatocytes to the cytotoxic effects of TNF [59,60]. The relevance of this observation is illustrated by the fact that GSH depletion is also caused by alcohol and as such contributes to the alcohol-induced sensitization of hepatocytes to TNF. Indeed, chronic ethanol intake sensitizes hepatocytes to TNF-induced cell death, and replenishment of GSH levels *in vivo* protects hepatocytes from ethanol-fed rats against this cytotoxicity [61,62]. Although the mechanism by which GSH depletion promotes TNF-induced cell death is not completely understood, profound GSH depletion already causes sustained activation of JNK in the absence of TNF stimulation. This activation of JNK is not further enhanced after TNF stimulation, but inhibition of JNK activation partially blocks sensitization to TNF-induced apoptosis [63]. This indicates that a decrease in GSH levels contributes to TNF-induced hepatocyte cell death at least partially via promoting JNK activation. Taken together, these observations suggest that maintaining normal GSH levels might be an important mechanism by which NF-κB activation prevents ROS accumulation and as such exerts its cytoprotective function. However, it remains an unresolved question how NF-κB activation prevents this drop in GSH levels upon TNF stimulation.

Another mechanism by which cells overcome ROS is limiting the availability of transition metals, such as iron. This metal catalyzes the generation of O_2^- in mitochondria and participates in the formation of $^{\bullet}OH$. Controlling iron levels is achieved in part through metal sequestration by ferritin, the major iron storage mechanism [64]. Recently, expression of the ferritin heavy chain (FHC) was shown to be NF-κB-dependent. Moreover, expression of FHC was able to prevent ROS accumulation in p65-deficient MEF cells and thereby blocked TNF-induced sustained JNK activation and subsequent cell death. Conversely, RNAi-mediated knockdown of FHC sensitized wild-type fibroblasts to TNF-induced apoptosis to the same extent as knockdown of p65, indicating that FHC is a dominant component of the anti-apoptotic NF-κB machinery in fibroblasts [58]. Although a role for FHC in protecting hepatocytes against TNF cytotoxicity remains to be shown, systemic iron depletion protects mice against TNF-mediated liver injury [65]. This observation reflects a detrimental role of iron in hepatocyte cell

death, suggesting that iron scavenging by FHC may take part in protecting the liver against cytotoxic insults.

Because many proteins are involved in the regulation of intracellular ROS concentration, it is rather unlikely that the above-mentioned correlations between the level of NF-κB activation and the expression of only a few anti-oxidantia can explain how NF-κB activation suppresses ROS accumulation. For instance, hepatic expression of several members of the family of cytochrome P450 (CYP) proteins is downregulated upon LPS or cytokine stimulation [66–68]. Several reports have suggested that NF-κB activation could be responsible for the dimished expression of these CYPs. Although the mechanisms are not clear yet, NF-κB mediated down-regulation of CYP expression has been suggested to result from impairing the recruitment of the aryl hydrocarbon receptor (AhR) to various CYP promoter regions [69]. Alternatively, the NF-κB mediated effect on CYP expression levels could be secondary to an inhibitory effect on the transcriptional activities of the glucocorticoid receptor (GR) and the constitutive androstane receptor (CAR) [70–72]. However, irrespective of these possible molecular mechanisms, an NF-κB mediated drop in CYP expression levels could partially account for the suppressive effect of NF-κB on ROS accumulation, since CYP proteins are capable of generating ROS. Unfortunately, convincing evidence for this notion is still lacking. In fact, the only member of the P450 cytochrome family for which an unequivocal correlation between NF-κB activation and its expression level has been established is CYP1B1, as the expression of CYP1B1 is significantly higher in IKKβ-deficient cells in comparison with wild-type cells [56]. This indicates that also CYP1B1 might contribute to ROS accumulation in the absence of NF-κB activity. However, neither the mechanism by which NF-κB represses the expression of CYP1B1, nor its relevance in TNF-mediated liver injury has been described yet.

6.2. *How does ROS accumulation lead to prolonged JNK activation?*

As ROS accumulation resulting from impaired NF-κB activation leads to prolonged TNF-induced JNK activation, another important issue is how ROS achieve this long-lasting JNK activity. Obviously, the magnitude and duration of JNK activity is determined by the balance between activating kinases and inhibitory phosphatases, establishing two possible strategies for ROS to enhance JNK activation: stimulating the activating kinases and/or blocking the inhibitory phosphatases.

Remarkably, different activating kinases appear to be involved in the initial TNF-induced JNK activation versus the prolonged stage of this response. Whereas, acute JNK activation by TNF involves the activation of TAK1, the sustained phase of JNK activation depends on another member of the MAP3K family, apoptosis signal-regulating kinase 1 (ASK1). While TAK1 is activated directly by TNF-R1-induced signaling, activation of ASK1 is secondary to the generation of ROS. Indeed, ASK1 is normally kept inactive by thioredoxin (Trx), a redox regulatory protein that is oxidized and thereby inactivated by ROS, resulting in the subsequent activation of ASK1 [73]. This ROS-dependent activation of ASK1 appears to be essential for TNF-induced cell death, as ASK1-deficient cells do not display prolonged JNK activation upon TNF treatment and are significantly protected against the apoptotic effects of TNF [74]. In addition to this proposed role for ROS in stimulating ASK1 activation, ROS have been suggested to establish extended JNK activation by inactivating MAP kinase phosphatases (MKPs), which are essential for dephosphorylating activated JNK. TNF-induced ROS were shown to oxidize critical Cys residues in the catalytic site of various MKPs, leading to their inactivation. Moreover, ROS-mediated oxidation of MKP-1 rapidly leads to its degradation by the ubiquitin-proteasome pathway [57]. Taken together, these studies suggest that TNF-induced ROS manipulate JNK activity by promoting the persistent activation of ASK1 and simultaneously blocking the inhibitory MKPs, thus shifting the balance toward prolonged TNF-induced JNK activation.

In vivo, the involvement of ROS-mediated JNK activation in TNF-mediated liver injury is suggested by the observation that an anti-oxidant diet protects mice against Con A-induced liver failure. Moreover, the protection afforded by this anti-oxidant diet was not only associated with the expected prevention of ROS accumulation but also with inhibition of prolonged JNK activation [57]. Although the mechanism by which ROS lead to prolonged JNK activation in hepatocytes remains to be demonstrated, both inhibition of ASK1 and overexpression of Trx have been shown to prevent hepatocyte apoptosis *in vitro* as well as *in vivo* [75–77]. In addition, overexpression of SOCS1, a ubiquitin ligase capable of inducing proteasomal degradation of ASK1, protects primary hepatocytes against TNF-induced apoptosis [78,79]. These observations demonstrate a pro-and anti-apoptotic role for ASK1 and Trx in hepatocytes, respectively, which would be in line with inactivation of Trx followed by activation of ASK1 as a mechanism by which ROS contribute to prolonged JNK activation and subsequent TNF-mediated liver injury.

7. How does prolonged JNK activation contribute to cell death?

The above observations show that the mechanisms by which NF-κB activation controls the level of JNK activation, either directly via NF-κB-dependent genes or indirectly via limiting ROS generation, are not completely understood and probably are very cell type specific. Nevertheless, sustained activation of JNK appears to be a general effector of TNF-induced cell death *in vitro* as well as *in vivo* and as such contributes to TNF-mediated liver injury.

Therefore, it is important to understand the mechanisms by which this prolonged activation of JNK contributes to TNF-induced cell death. The specific contributions of the two different JNK isoforms, JNK1 and JNK2, to TNF-induced cell death are highly controversial. Studies in mouse embryonic fibroblasts deficient in either JNK1 or JNK2 have provided evidence supporting the involvement of both of these isoforms in TNF cytotoxicity [80,81]. Also in TNF-mediated liver injury, the distinct contributions of JNK1 and JNK2 are not very clear. Since JNK1- as well as JNK2-deficient mice are protected against the hepatotoxic effects of Con A injection, activation of both of these JNK isoforms seems to contribute to the development of Con A-induced liver damage [10,31]. In the LPS/GalN-induced model of liver injury, a study by Chang et al.

showed that JNK1-deficient mice are more resistant to LPS/GalN-induced hepatotoxicity than wild type mice [31]. In contrast to this study, Wang et al. could not observe any difference between the response of wild type and JNK1-deficient mice to LPS/GalN. Instead, these authors show that JNK2-deficient mice are significantly protected against this model of liver injury [82]. Thus far, there is no clear explanation for the contrasting observations made in these studies.

Despite the discrepancy between the studies of Chang et al. and Wang et al. concerning which of the JNK isoforms is involved in liver damage, both support a role of JNK activation very upstream in the TNF-induced signaling pathway to cell death. Indeed, JNK1- as well as JNK2-deficient mice fail to activate caspase-8 after Con A or LPS/GalN treatment, respectively [31,82]. In addition, primary hepatocytes from JNK1-deficient mice did not activate caspase-8 after stimulation with TNF/cycloheximide (CHX) [31]. Moreover, the study by Chang et al. provides a molecular explanation for this observation (Fig. 3). TNF was found to induce proteasomal degradation of the anti-apoptotic protein $cFLIP_L$ in wild type but not in JNK1-deficient cells. The link between JNK1 and this degradation of $cFLIP_L$ was provided by the ubiquitin ligase Itch. Prolonged activation of JNK1 leads to phosphorylation of Itch, thereby activating its ubiquitin ligase activity, which results in polyubiquitination and subsequent degradation of $cFLIP_L$. Because $cFLIP_L$ impairs the recruitment of procaspase-8 to complex II and thus is a potent inhibitor of caspase-8, JNK1-initiated signaling toward its degradation allows full activity of caspase-8 and results in cell death. The crucial role of Itch in this process was demonstrated by Itch mutant mice, which do not display $cFLIP_L$ degradation during liver injury and thus are protected against hepatotoxicity induced by Con A, LPS/GalN as well as TNF/GalN [31]. A role of JNK activation upstream of caspase-8 activation was also suggested by the observation that prolonged JNK activation results in the production of jBid, a proteolytic fragment of Bid that specifically induces the release of mitochondrial Smac. This apoptogenic protein displaces c-IAP1 from the TNF-R1 complex and as such allows the activation of caspase-8 and the initiation of apoptosis [42]. In all of these studies, interference of JNK with caspase-8 activation prevents the subsequent cleavage of Bid, pointing to a role of JNK in promoting the initiation of the mitochondrial pathway of cell death.

Alternatively, the pro-apoptotic effects of JNK activation might be situated more downstream in this mitochondrial cell death pathway. Activated JNK has been shown to phosphorylate Bcl-2 and Bcl-xL, thereby inactivating their anti-apoptotic

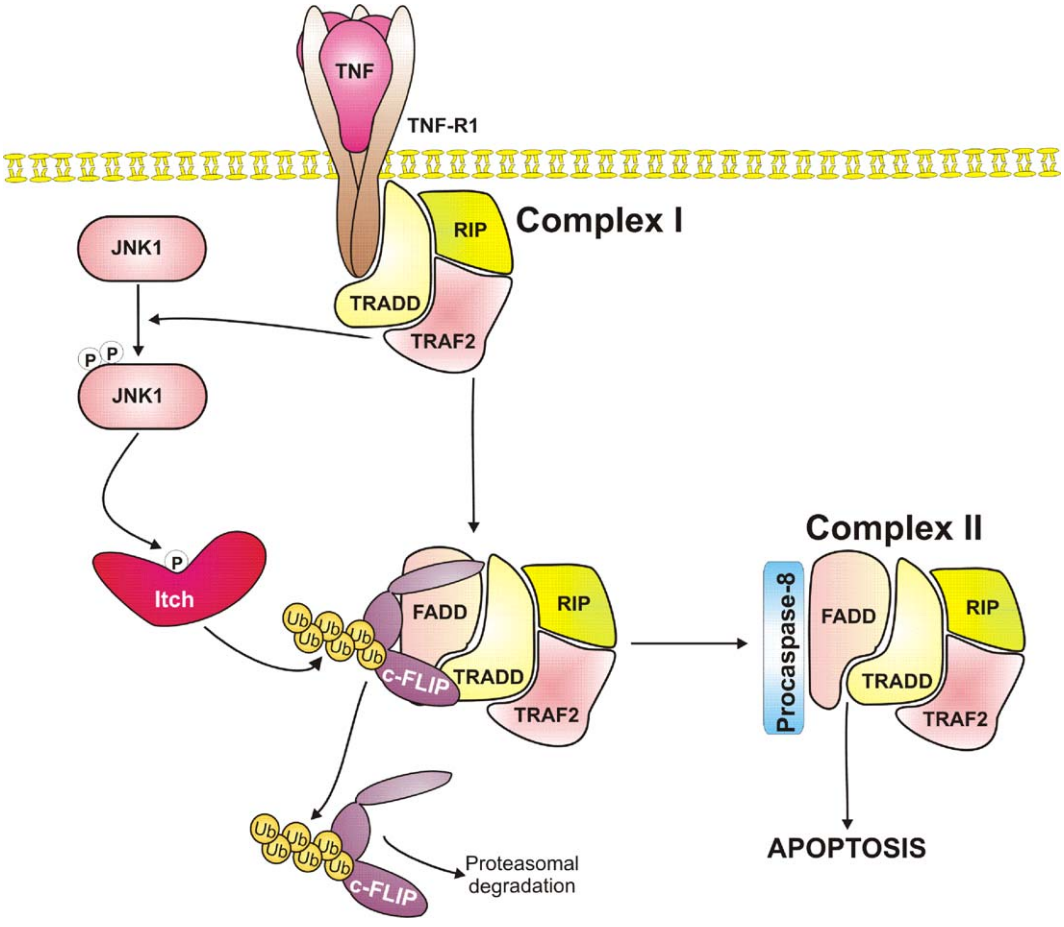

Fig. 3 – JNK1 promotes TNF-induced apoptosis by promoting Itch-mediated degradation of c-FLIP. TNF binding to TNF-R1 leads to activation of JNK1, which phosphorylates the ubiquitin ligase Itch. Activated Itch then ubiquitinates c-FLIP, which is bound to FADD and thereby prevents recruitment of procaspase-8 to complex II. This leads to proteasomal degradation of c-FLIP, allowing the proper assembly of complex II and subsequent signaling to apoptosis.

functions [83–85]. In addition, JNK-mediated phosphorylation of Bim and Bmf, two pro-apoptotic members of the Bcl-2 protein family, as well as the adapter protein 14-3-3, have been suggested to play a role in activating Bax and Bak [86,87]. In support of a role for JNK in this Bax/Bak-activating pathway, constitutively active JNK induces apoptosis in wild-type cells, but not in cells lacking Bax and Bak [88]. Although all of these studies suggest a role for prolonged JNK activation in shifting the Bcl-2 balance toward mitochondrial dysfunction, which is essential in hepatocyte cell death, these effects of JNK on Bcl-2 proteins have not yet been shown in hepatocytes.

Another possible mechanism by which JNK activation can promote cytotoxicity is by increasing intracellular oxidative stress, as is the case in necrotic cell death. Indeed, whereas, low amounts of oxidative stress contribute to and augment hepatocyte apoptosis, severe oxidative stress can result in hepatocyte necrosis. Interestingly, in cells lacking both JNK1 and JNK2, TNF-induced accumulation of ROS is abolished [89]. This observation is in contrast with the notion that ROS cause prolonged activation of JNK, and thus suggests that the coupling of ROS and JNK signaling is bidirectional. This could establish a positive feedback cycle in which TNF-stimulated ROS generation causes increased JNK activity that in turn leads to a further increase in ROS production. This enhanced production of ROS leads to necrosis, suggesting that the molecular ordering of JNK and ROS might determine the amount of ROS produced after TNF stimulation, and thus decides on the type of cell death. From a hepatic point of view, both apoptosis and necrosis almost always occur together during liver injury. Moreover, ROS are not only generated in increased amounts in hepatocytes, but also originate from Kupffer cells and infiltrating inflammatory cells such as neutrophils [90,91]. Also these extra-hepatocyte sources of ROS can contribute to hepatocyte necrosis. Therefore, it is very important to mention that despite the fact that JNK activation can result in apoptosis as well as necrosis, NF-κB activation prevents both types of cell death equally well [54,89].

8. Concluding remarks

Already a few years after its discovery, it became clear that NF-κB is a pivotal factor in the pathogenesis of many inflammatory diseases. Since then, NF-κB has become a favorite target for the development of drugs that aim to limit inflammation. However, one of the major drawbacks of using NF-κB inhibitors in therapy is their inevitable cytotoxic effect, as NF-κB is an essential pro-survival factor in most cell types. These potential hazards resulting from NF-κB inhibition call for accompanying measures that prevent cell death. Therefore, elucidating the mechanisms of the anti-apoptotic effects of NF-κB activation is very important, as such knowledge might enable us to substitute for the loss of these effects when using NF-κB inhibitors in therapy.

Recent advances in our understanding of the anti-apoptotic mechanisms of NF-κB made it clear that NF-κB does not only inhibit cell death by inducing transcription of proteins capable of blocking essential cell death molecules, but also by inhibiting the sustained phase of TNF-induced JNK activation. The finding that attenuating JNK activation is one of the mechanisms by which NF-κB prevents TNF-induced cell death indicates that synthetic inhibitors of JNK activation might be useful for preventing the cytotoxic side-effects of NF-κB inhibitors in TNF-mediated inflammatory diseases. Moreover, also anti-oxidants could be used for this purpose, as ROS seem to be involved in prolonging the activation of JNK. As JNK inhibitory and/or anti-oxidant adjuvants could thus help to render NF-κB inhibitory treatments more secure, this novel field of research in the twilight zone of NF-κB and JNK activation holds promising possibilities for future anti-inflammatory therapeutic strategies.

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