NF-κB and Its Implication in Liver Health and Cancer Development

6

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Contents

Abstract

Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) belongs to one of the best described and most intensively studied transcription factors in biochemistry in the last 30 years. The NF-κB signaling cascade exists in two variants, the canonical and noncanonical pathway, and its transcription factors

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are key regulators of several biochemical processes like immune responses, inflammation, survival, and cellular development and growth.

Examination of various transgenic mouse models targeting NF-κB itself or signaling members discovered the implication of NF-κB in chronic inflammatory diseases and cancer development in different organs as in the skin, intestine, and liver.

In this review the focus lies on the central organ of metabolic and inflammatory processes: the liver. It seems that NF-κB is pivotal for the homeostasis in the different hepatic cell types concerning hepatic failure, fibrosis, and HCC progression. NF-κB has the ability to be a potential target in the attempt to circumvent or medicate liver fibrosis and HCC.

6.1   Introduction

Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) is one of the best and most intensive studied transcription factors in the field of biomedicine. It was discovered and first described 30 years ago by David Baltimore and colleagues [1]. Since that time many studies revealed the outstanding meaning of NF-κB in the development of inflammatory diseases such as arthritis and psoriasis, inflammatory bowel diseases, asthma, and neurodegenerative heart diseases and its contribution to cancer development [2–4]. It is expressed in most mammalian cell types and tissues and controls the transcription of genes involved in immune responses, cell survival, proliferation, and differentiation [5].

Next to *in vitro* approaches, examination of several transgenic mouse models with different NF-κB targets gave the opportunity to raise our understanding of the complex mechanisms behind inflammation-driven diseases *in vivo*. Moreover, the work with conditional murine knockout models in different organs uncovered the central role of NF-κB in mediating innate immune responses and cytokine expression in order to react on pathological outcomes of inflammation like chronical skin inflammation (e.g. psoriasis) and hepatocarcinogenesis [6–10]. The severe affection of organs or tissues during disbalanced NF-κB activation gains importance and constitutes a therapeutic challenge. Further investigations will support the development of clinical trials targeting certain molecules of the NF-κB signaling cascade.

6.2   Members of the NF-κB/Rel Family

The NF-κB signaling pathway is evolutionarily highly conserved and is, besides mammalians, also found in the fruit fly *Drosophila melanogaster*, cnidarians, porifera, viruses, and mollusks [11–14]. *Caenorhabditis elegans* and yeast are the main exceptions [15, 16].

Mammalian NF-κB itself is composed of different types of dimers, appearing as homo- or heterodimers. The single compounds of these dimers are p50 (*NFKB1*), p52 (*NFKB2*), c-Rel (*REL*), p65/RelA (*RELA*), and RelB (*RELB*). All

five transcription factors are characterized by an N-terminal Rel homology domain (RHD-NTD) which is needed to mediate DNA binding, homo- and heterodimerization, and nuclear translocation. In the nucleus NF-κB dimers bind at κB sites inside enhancer/promoter regions of target genes where they control transcription by recruiting coactivators and corepressors [17–19]. Furthermore, only p65, RelB, and c-Rel comprise a C-terminal transactivation domain (TAD) which is required for gene transcription. The other two members, p50 and p52, lacking TAD and are processed from the precursor proteins p105 (p50) and p100 (p52) (Fig. 6.1). Both are not directly involved in gene transcription except in combination with p65, RelB, c-Rel, or other proteins which are able to recruit coactivators. After successfully entering into the nucleus, it binds at the following consensus sequence 5′-GGGRNYYYCC-3′ (R, purine; Y, pyrimidine; N, any nucleotide) of DNA κB sites [19]. The TAD and RHD act in each case autonomously and underlay posttranscriptional modifications which might have an influence on NF-κB activation at the level of transcription and/or DNA binding [20].

The most abundant combination of NF-κB dimers are p50/p65 and p50/50, whereas, in contrast, the homodimer p50/p50 can act as a transcriptional repressor [21, 22].

6.3 The Negative Controllers of NF-κB: IκBs

NF-κB dimers are located in the cytoplasm, and translocation from the cytoplasm through the nucleus is regulated by another group of proteins named nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκBs) [15]. These proteins are tightly associated with the NF-κB dimers, preventing NF-κB activation by hindering NF-κB translocation through the nuclear membrane. The IκB family includes six members: IκBα (*NFKBIA*), IκBβ (*NFKBIB*), IκBε (*NFKBIE*), IκBγ (*NFKB1*), IκBζ (*NFKBIZ*), and Bcl-3 (*BCL3*) and the NF-κB precursors p100 and p105. All of them share several ankyrin repeat domains (ARD), which are necessary to interact with the RHD of NF-κB [23]. Crystal structure analysis of the IκBα/NF-κB (p50/p65) heterodimer and IκBβ/NF-κB (p50/p50) homodimer allowed a closer look through the binding conditions of each complex and revealed a binding ratio of 1:1. Inside the IκBα/NF-κB complex, the ankyrin repeat six and the C-terminal PEST sequence of IκBα are associated with the p65 RHD-NTD, impeding binding to the DNA κB site (Fig. 6.1). Additionally, p65 undergoes such a conformational change, which strongly supports the linkage to IκBα, holding NF-κB in its inactive state [24–26].

Every IκB member has its own favorite NF-κB dimer. IκBα/β/ε binds to NF-κB dimers, which contain a minimum of one p65 or c-Rel subunit. p100 and p102 are connected to all NF-κB subunits. IκBζ and Bcl-3 have a preference for p50 and p52 homodimers [27, 28].

Here, IκBα is investigated at best and is a central regulatory factor in the canonical NF-κB signaling pathway as described in the next chapter.
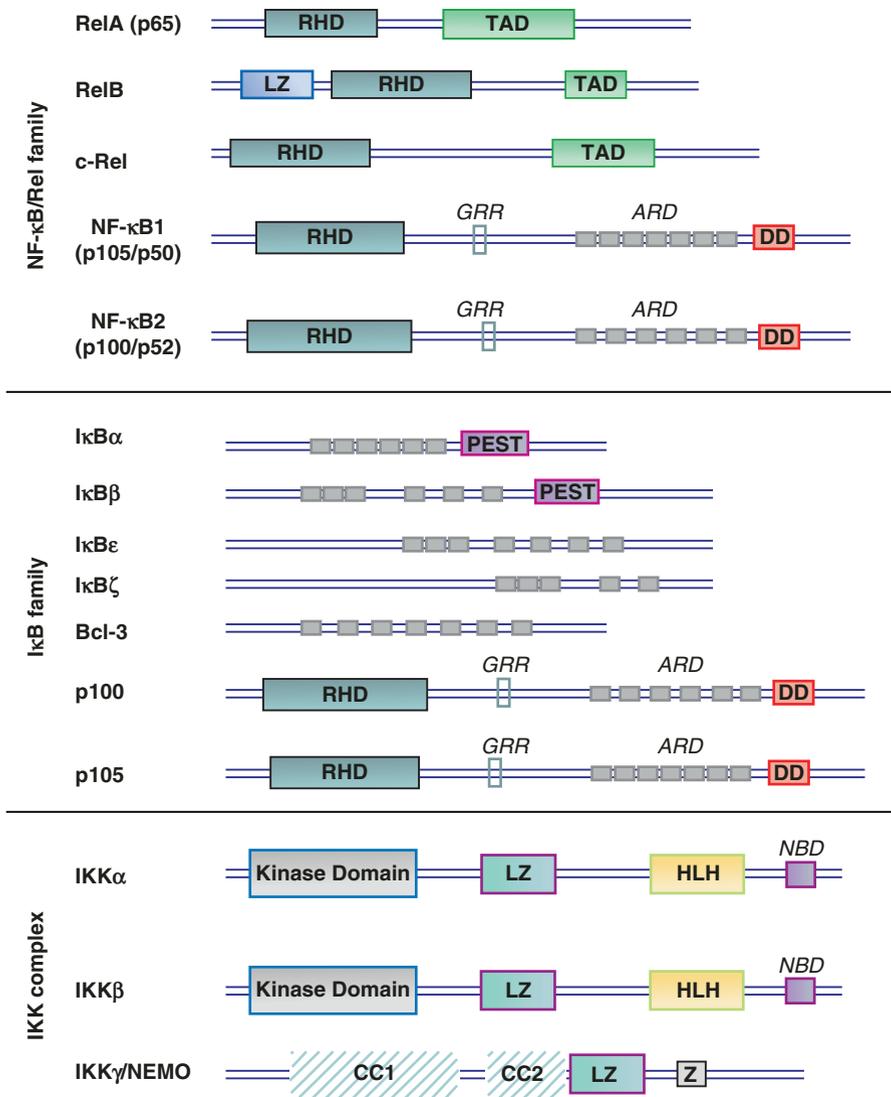
Fig. 6.1 Schematic diagram of the domain structures of each individual NF-κB, IκB, and IKK complex protein family members. All three family groups have a typical domain structure as the Rel homology domain (RHD) for the NF-κB family members, the ankyrin repeat domains (*ARD*) for the IκB family members, and the leucine-zipper (*LZ*) motif for the IKK complex members. On the basis of their function, p100 and p105 are also associated with the NF-κB and IκB family. *CC* coiled coil, *DD* death domain, *GRR* glycine-rich region, *HLH* helix-loop-helix, *NBD* NEMO-binding domain, *PEST* proline-, glutamic acid-, serine-, and threonine-rich region, *TAD* transactivation domain. Adapted from Oeckinghaus et al.: The NF-κB Family of Transcription Factors and its Regulation, Cold Spring Harb Perspect Biol. 2009, 1(4): 1–14

6.4   The Canonical NF-κB Pathway

Various cellular stress stimuli and certain endogenous and exogenous ligands lead to NF-κB activation. The stimulus decides if the canonical or noncanonical NF-κB pathway is activated. Both cascades lead to NF-κB nuclear translocation but are regulated by different checkpoints within the cascade. In the last few years, extensive studies have been performed to characterize both variants of NF-κB activation and to identify important key players. It was shown that the canonical pathway is primarily activated during physiological stress conditions like inflammation, exposure to bacterial products like lipopolysaccharide (LPS), and oxidative stress [29, 30]. The canonical NF-κB signaling cascade is generally activated by the following receptors: tumor necrosis factor receptor (TNFR), interleukin 1 receptor (IL-1R), Toll-like receptor (TLR), B-cell receptor (BCR), and T-cell receptor (TCR) [31]. Receptor ligation leads to the recruitment of certain adapter proteins to TNF receptor-associated factor (TRAF) and receptor-interacting protein kinase 1 (RIPK1). RIPK1 is connected to the TGFβ-activated kinase 1 (*TAK1*)-binding protein (TAB2-TAB3-TAK1) complex and the NF-κB essential modulator (NEMO) via ubiquitin chains, bringing TAK1 into close vicinity to NEMO (IKKγ), the regulatory subunit of the IκB kinase (IKK) complex. This complex is composed of two more members, the catalytic subunits IKKα (IKK1) and IKKβ (IKK2), and represents the crucial step in NF-κB nuclear translocation by controlling proteasomal degradation of IκBα. More precisely, phosphorylation of TAK1 at Thr178 and Thr184 permits a direct phosphorylation of IKKβ inside its activation loop at Ser177 and Ser181 leading straightly to IκBα phosphorylation at Ser32 and Ser36. Phosphorylation of IκBα follows activation of the IKK complex, the second essential regulatory step in NF-κB activation, because IκBα undergoes K48-linked polyubiquitination by the SCF^{βTrCP} ubiquitin ligase complex, which induces its fast degradation by the 26S proteasome. Finally, IκBα degradation exposes the nuclear localization site (NLS) of NF-κB, which is needed for nuclear access, DNA binding, and transcription of target genes (Fig. 6.2) [32–37].

6.5   The Noncanonical or Alternative NF-κB Pathway

Next to the extensively studied canonical pathway, an alternative NF-κB activation exists. The so-called noncanonical NF-κB pathway activates NF-κB not by degradation of IκBα/β/ε and p105, but via processing the inactive p100/RelB NF-κB heterodimer through the active p52/RelB heterodimer [38, 39]. This pathway seems to be crucial in lymphoid organogenesis, B-cell maturation and survival, dendritic cell activation, and osteoclastogenesis. The major ligands which initiate these variants of NF-κB activation are the lymphotoxin β receptor (LTβR), B-cell-activating factor belonging to the TNF family receptor (BAFF-R), CD40, and receptor activator of
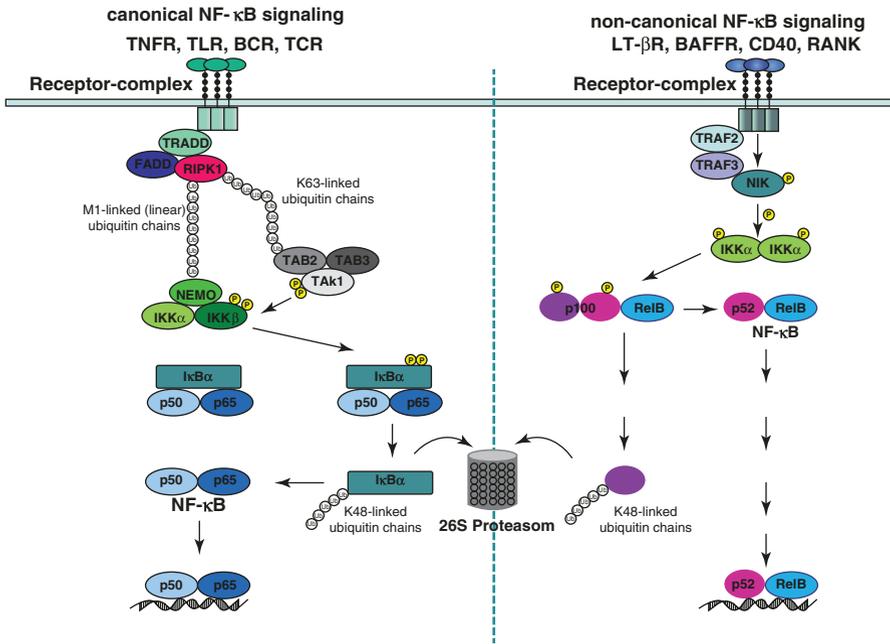
Fig. 6.2 The canonical and noncanonical NF-κB signaling cascade. After activation of the respective receptors, which are able to induce the canonical NF-κB pathway, the TAB-TAK1 complex and NEMO, the regulatory subunit of the IKK complex, get ubiquitinated by RIPK1. These ubiquitin chains bring both complexes into closer vicinity to each other, whereby TAK1 phosphorylates IKKβ, one of the catalytic subunits of the IKK complex. The activated IKK complex phosphorylates two serine residues of the NF-κB inhibitor IκBα, thereby initiating its proteasomal degradation. After IκBα degradation, NF-κB translocates through the nucleus to induce gene expression. The noncanonical NF-κB signaling cascade is controlled at the upper part by NIK. NIK mediates phosphorylation leading to the activation of the homodimer IKKα, another catalytic subunit of the IKK complex. IKKα cleaves the inactive precursor p100 into the active NF-κB subunit p52. In combination with RelB, it forms a heterodimeric NF-κB molecule which enters the nucleus to induce target gene expression. *BCR* B-cell receptor, *BAFF-R* B-cell-activating factor receptor, *FADD* Fas-associated death domain, *IκBα* inhibitor of NF-κB, *IKKα/β* IκB kinase α/β, *LT-βR* lymphotoxin-β receptor, *NEMO* NF-κB essential modulator, *NIK* NF-κB-inducing kinase, *RANK* receptor activator of NF-κB, *RIPK1* receptor-interacting protein 1, *TAB2/3* TGFβ-activated kinase 1 (*TAK1*)-binding protein, *TAK1* TGFβ-activated kinase 1, *TCR* T-cell receptor, *TLR* Toll-like receptor, *TRADD* TNF receptor-associated death domain, *TRAF2/3* TNF receptor-associated factor 2/3, *TNFR* tumor necrosis factor receptor. Adapted from Luedde et al.: The role of NF-κB in hepatic disease models, Translational Research in Chronic Liver Disease, Falk Workshop, Shaker Verlag Aachen 2009: 57–89

NF-κB (RANK). Moreover, uncontrolled stimulation of the noncanonical NF-κB signaling cascade leads to severe diseases such as B-cell lymphomas, ulcerative colitis, and rheumatoid arthritis [40].

Processing of p100 depends on the NF-κB-inducing kinase (NIK), IKKα homodimers, and βTrCP, a subunit of the SCF^βTrCP ubiquitin ligase. NIK mediates phosphorylation of p100 at Ser866 and Ser870 in its NIK-responsive domain (NRD)

and IKKα activation. Activated IKKα phosphorylates p100 at Ser99, Ser108, Ser115, Ser123, and Ser872 which is needed for the recruitment of βTrCP. The SCF^{βTrCP} ubiquitin ligase complex facilitates ubiquitination of p100 and thereby its 26S proteasomal degradation to p52. Interestingly, canonical and noncanonical pathways exhibit some similarities regarding regulatory mechanisms as shown by equivalent phosphorylation sites of p100 and IκBα or ubiquitin-mediated 26S proteasomal degradation of NF-κB inhibitors (Fig. 6.2) [41–43]. In contrast to the canonical pathway, the noncanonical cascade is characterized by a slow and persistent signaling and protein synthesis [44, 45].

Next to ubiquitin, SUMOylation is another regulatory mechanism to shape NF-κB signaling in both pathways. SUMOylation induces posttranslational modifications like ubiquitination and phosphorylation and influences protein-protein interaction and gene transcription [46]. It was shown in different studies that the interplay of SUMO, SUMO proteases, and NF-κB signaling members like NEMO, IκBα, or p100 represent another important level of signal transduction [47, 48].

6.6   NF-κB-Associated Human Diseases and Genetic Mouse Models

Due to the immense effort taken in examining NF-κB and its biological function, the crucial role of NF-κB in cellular homeostasis was uncovered. Several human diseases like psoriasis, colitis ulcerosa, Crohn's disease, rheumatoid arthritis, or cancer development are a result of dysregulated NF-κB activation [2–4].

For a better understanding of the mechanisms behind NF-κB dysregulation *in vivo*, numerous mouse models were developed and examined. These comprise constitutive, tissue-specific conditional knockouts using the cre/loxP technology, gene knock-ins, and reporter mice of single or more IKK subunits or in combination with other members of the NF-κB signaling cascade [6–8, 49–53].

Caused by the tremendous information on the different genetic mouse models and their implication in broadening our understanding of NF-κB-associated human diseases, the next paragraph will focus particularly on one organ which has been extensively studied over the last few years and is a prime example for the importance of NF-κB homeostasis: the liver.

6.7   NF-κB and Its Critical Role for Liver Homeostasis

The liver is the biggest organ in the human body with a weight of 1.2–1.8 kg and makes approximately 2–3% of the whole body weight. The liver is of great importance because it is responsible for different metabolic processes like synthesis of vitally essential proteins (e.g. albumin, blood coagulation factors, hormones), utilization of food residues (e.g. conversion of glucose to amylum), detoxification of intermediate catabolic metabolites (e.g. from medicine), and bile production. Additionally, it is a storage organ for important macromolecules such as hormones

or amylum. Besides the wide metabolic functions, the liver has the capability of reacting immunologically as well [54]. During all these biochemical and immuno-logical processes, the different cell types forming the liver are faced with degrada-tion products which might be harmful, such as oxygen radicals, or are attacked by bacterial components like lipopolysaccharides (LPS) or viruses, such as hepatitis viruses. NF-κB activation protects the cells against apoptosis and supports pro-inflammatory responses. Hepatocytes are the dominant hepatic cell type and stress factors, such as cytokines like TNFα or IL-1, and initiate the NF-κB signaling cas-cade to protect them against cell death [31].

Despite the bad reputation of inflammatory processes, it plays in the liver a cen-tral role for wound healing induced by injury processes, such as alcohol abuse or medication intake, which bear a high risk for liver fibrosis and cirrhosis progression and, finally, hepatocellular carcinoma (HCC) development. However, also viral infections such as hepatitis B virus (HBV) and HCV are potent inducers of liver fibrosis and HCC development. During fibrosis, the liver tissue undergoes a peren-nial process of inflammation, apoptotic and necroptotic events, and compensatory renewal. These chronical processes cause severe side effects, such as the production of highly reactive molecules like reactive oxygen species (ROS), chromosomal aberrations, and possibly malignant alterations of proliferating hepatocytes. Other diseases and reasons which can also trigger HCC formation are nonalcoholic steato-hepatitis (NASH), obesity, diabetes, aflatoxin-contaminated nutrients, exposure to toxic compounds like vinyl chloride, and genetic predisposition such as hemochro-matosis [55, 56].

6.8   A Deeper View on TNF and NF-κB

Only two possibilities remain if a cell is faced with strong stress: survival or death. TNFα and IL-1 are classical stress inducers and belong to the TNF superfamily. Next to these prominent members, Fas (Apo-1) and TRAIL are also well known and are currently intensively studied proteins. These signaling cascades simultaneously induce expression of pro-survival and proapoptotic proteins and, according to the stimulation strength, determine if the cell dies or survives [57–59].

The balance between life and death is absolutely essential to keep the liver in a healthy status. Apoptosis and necroptosis are different ways of cellular death but are strictly coordinated processes with common molecular characteristics, but with dif-ferent cellular fragmentation processes into several small pieces [60].

TNFα acts on different biological processes facilitated by TNF-R1 and TNF-R2. Soluble TNFα initiates TNF-R1 signaling, whereas activation of TNF-R2 signaling needs binding of membrane-bound TNFα [61]. Activation of the TNF-R1 by TNFα leads to its trimerization and initiates recruitment of different adaptor proteins build-ing the membrane-bound TNFR-complex I. The complex I comprises TRADD, RIPK1, cIAP1/2, and TRAF2/5. All seven TRAF family members have a C-terminal coiled coil domain which supports protein-protein interaction. Only TRAF2-7 exhib-its an N-terminal RING domain which transfers K63-linked ubiquitin to target

proteins, acting as E3 ligases. Nevertheless, it is not clarified till now if TRAF proteins mainly act as E3 ligases or as adaptors [20, 62]. It was shown that TRAFs are involved in both, canonical and noncanonical NF-κB pathway, and those TRAFs are also needed for activation of other signaling cascades, therefore acting as a distributor platform for several pathways. RIPK1 is, differently to the TRAFs, exclusively engaged in the canonical NF-κB pathway. RIPK1 and TRAF2/5 seem to interact with each other by TRAF2/5-mediated K63-linked polyubiquitination of RIPK1 (Lys377) [63]. Alternatively, it is also discussed that cIAP1/2 is responsible for RIPK1 polyubiquitination and that TRAF2 only recruits them to the receptor complex. Despite intensive research the exact function of TRAF2/5 and cIAP1/2 concerning RIPK1 ubiquitination could not have been solved satisfactorily to date [64]. Besides K63-linked ubiquitination, RIPK1 undergoes a second, linear Met1-linked ubiquitination simultaneously. This is mediated by the linear ubiquitin chain assembly complex (LUBAC), another E3 ligase complex [65, 66]. RIPK1 is linked with the linear chain to NEMO and with the K63-linked chain to the TAB2/3-TAK1 complex and brings the IKK complex and TAK1 in closer vicinity to each other, leading to the phosphorylation of IKKβ and finally NF-κB activation as described above.

6.9   NF-κB and Its Function in Hepatogenesis

A deeper understanding of NF-κB importance and its components was achieved by using genetically modified mouse models. These models include a setup of diverse genetic approaches such as constitutive knockout models, dominant-negative expression or overexpression of single or double IKK subunits or IκB proteins, tissue-specific conditional knockouts by using cre/loxP recombination, reporter systems, and gene knock-ins [50].

Knockout mice deficient in *Rela* (*p65*) die in the uterus between embryonic days 15 (E15) and E16 because of hepatocyte apoptosis. The primary cause of this event is the failure of a TNFα-mediated IκBα induction and granulocyte/macrophage colony-stimulating factor (GM-CSF) as shown in murine embryonic fibroblasts (MEFs), revealing that RelA has a protective function against TNFα [67, 68]. These findings were approved by generating a double knockout of *Tnf* and *Rela*, which led to a normal embryonic development and a full rescue from lethality [69]. However, another study reported similar results, but these double knockout mice died 10 days after birth from acute hepatitis and neutrophil infiltration [70]. Both studies indicate that RelA and TNF-R1 are not essential for liver development in mice, but seem to sensitize these animals to infections leading to death within a very short time frame. Further investigations of other NF-κB subunits showed that the genetic loss of both *c-Rel* (*Rel*) and *Rela* causes liver failure as well [71]. Moreover, genetic ablation of both transcription factors triggers impaired maturation of B cells, T cells, and macrophages, denoting important roles in controlling genes relevant for immune responses. Genetic manipulation of murine livers by using adenoviral technique allowed transcription of an IκB superrepressor (Ad5IkappaB) which abolished NF-κB linkage to the respective DNA binding sites. After partial hepatectomy the

adenoviral infected livers displayed increased apoptosis rates of hepatocytes, proving the importance of NF-κB [72]. For the remaining NF-κB subunits RelB, NF-κB1, and NF-κB2, they were shown to play important roles in the differentiation and proper function of hemopoietic cells [73].

Next to the studies on NF-κB subunits, some studies carried out to define the functional impact of the single IKK members. Constitutive deletion of *Ikkβ* (*Ikk2*$^{-/-}$) in mice leads to embryonic lethality at day E12.5 as a cause of enhanced liver damage. Cell culture experiments of primarily isolated MEFs from these animals revealed impaired NF-κB activation in response to TNFα and IL-1. These results revealed a central function for IKKβ in controlling liver development and NF-κB activity, whereas loss of IKKβ cannot be fully compensated by IKKα (IKK1) [74–76]. Ablation of the catalytic subunit *Nemo* (*Ikkγ*$^{-/-}$) has a similar phenotypical effect as the embryos die between day E12.5 and E13.0 from massive apoptotic liver failure, and experiments with isolated *Ikkγ*$^{-/-}$ MEFs also showed disturbed NF-κB activity after treatment with TNFα, IL-1, LPS, and Poly(I:C), leading to high susceptibility to apoptosis [77]. As stated above, IKKα does not seem to have an impact on liver development and NF-κB induction triggered by pro-inflammatory substances. *Ikkα*$^{-/-}$ mice die perinatally and develop severe skeletal and skin-related defects during embryogenesis [78, 79].

6.10   NF-κB and Its Pivotal Role for Liver Integrity

Generation of tissue-specific knockouts by using the cre/loxP recombination system gives the opportunity of a much more detailed view on NF-κB in particular with regard to protection against cytokine-induced hepatitis. Another great advantage of the cre/loxP technology is the time-dependent loss of target genes determined by activation of the respective promoter during later embryonic developmental stages or postnatally, circumventing embryonic lethality effects. This was demonstrated by a conditional cre-driven hepatocyte knockout of *Rela/p65*. These mice are viable, and isolated primary hepatocytes treated with TNFα were highly sensitive to apoptosis with concurrently enhanced c-Jun N-terminal kinase (JNK) expression and degradation of the anti-apoptotic protein cellular FLICE inhibitory protein long (c-FLIP$_L$) [80]. Investigation of hepatocyte-specific *Ikkβ* deletion in adult mice revealed an unexpected slight sensitivity in response to TNFα or LPS administration contrary to the murine embryonical state [52, 80, 81]. However, treatment with concanavalin A (ConA) promotes severe liver failure in adult *Ikkβ*-deficient animals, which is mainly supported by increased activation of JNK, a key mediator of ConA-induced liver failure. Next to JNK activation, ConA is also a potent activator of T cells, indicating that the anti-apoptotic function of IKKβ is the prevention of T cell-mediated cell death associated with decreased JNK activity [81].

Metabolic diseases represent another potential risk factor in mediating inflammatory processes like type 2 diabetes and obesity. Liver-specific *Ikkβ* ablation abolished insulin sensitivity, while these animals showed insulin resistance in muscle and fat induced by a high-fat diet and upon aging [82].

Double knockout of *Ikkα* and *Ikkβ* (IKKα/β[LPC-KO]) in liver parenchymal cells (hepatocytes and cholangiocytes) supported increased susceptibility of hepatocytes to LPS *in vivo*, which was not detected in the single knockout conditions, claiming for a more redundant function for both IKKs in canonical NF-κB activation. Of note, simultaneous deficiency of IKKα and IKKβ or the combined ablation of *Ikkα* and *Nemo*, but not *Nemo* alone, led to spontaneous development of cholangitis with disturbed portal bile ducts accompanied by severe jaundice, revealing the importance of both catalytic NF-κB subunits in controlling liver immunology and bile duct integrity [83]. Additional pivotal discoveries concerning the physiological impact of the IKK complex were achieved by examining adult mouse livers lacking the regulatory subunit NEMO. These livers are highly sensitive against TNF- and LPS-mediated inflammation and subsequently cell death *in vivo* and *in vitro* [8, 52].

Further studies regarding genetic ablation of other NF-κB signaling members alone or in combination with different IKKs revealed a great impact on cellular homeostasis as well. Hepatocyte-specific deletion of the mitogen-activated kinase kinase kinase (MAP3K) *TGF-β-activated kinase 1* (TAK1[LPC-KO]; TAK1[Δhep]) leads to a comparable phenotype as seen for IKKα/β[LPC-KO] with considerable cholangitis, early HCC development, and lethal jaundice at younger age [6, 51]. Deletion of death receptor-associated adaptor proteins like Fas-associated protein with death domain (*Fadd*) or *Tnfr1* in combination with *Nemo* (NEMO/FADD[LPC-KO]) or *Tak1* (TAK1/TNFR1[Δhep]) showed strongly reduced signs of inflammation, fibrosis, and cell death, raising evidence for a pro-apoptotic trigger driving these phenotypes [8, 51]. A look more downstream of the death receptor pathways, regarding caspase-8, highlighted a rescue of Caspase8/NEMO[Δhep] mice from steatosis and HCC development, but these animals developed a severe spontaneous phenotype of liver necrosis, cholestasis, and biliary lesions, most likely caused by a FasR-induced RIPK1-RIPK3-mediated necroptosis [84]. As stated above, the hepatoprotective function of NF-κB is also influenced by a sustained reduced expression level of JNK upon TNFα administration [85–87]. JNK belongs to the MAPK family and is activated via TRAF2, RIPK1, and MKK4/7. JNK is a major mediator of cell death, triggered not only by pro-inflammatory cytokines such as TNFα and IL-1β but also by cellular stressors such as UV radiation, osmotic, oxidative, hypoxic, and genotoxic events [88, 89]. The connective bridge between NF-κB and JNK is TAK1, which is able to phosphorylate IKKβ and MKK4/7, depending on stimulus strength. Next to JNK, TAK1 is also able to phosphorylate MKK3/6, which are activators of p38, another MAPK involved in cell proliferation, differentiation, and cell death [90, 91]. Studies with p38α/IKK2[LPC-KO] mice showed increased hepatocyte sensitivity against TNFα and LPS administration *in vivo*, which was not the case for the single knockout conditions. Moreover, significantly increased JNK expression levels could be detected, but were not strong enough to induce liver failure [92]. The results revealed impressively that the NF-κB signaling cascade is not an isolated pathway and is embedded in a broad network of stress-related signaling pathways which are tightly regulated in protecting the liver against harmful events.

6.11   NF-κB and Its Implication for HCC Development

Chronic inflammatory liver diseases belong to the main preconditions for generation and progression of liver cirrhosis and subsequently HCC development, affecting 80–90% of patients with liver cirrhosis [55]. The formation of cancer is generally characterized by a disbalance between cell death and survival/proliferation [93]. In most HCCs, NF-κB is constantly active, which drives a continuous burst of pro-inflammatory and anti-apoptotic signals [94, 95]. In the last two decades, murine genetic studies had a great impact on the understanding of molecular mechanisms driving HCC development [6–9, 96]. It has been shown that tumor formation is a process in which different liver cell types are involved and where NF-κB activation is time-dependent altered, particularly at early and late stages of cancer development.

One of the first studies in this direction was done on *Multidrug resistance protein 2* (*Mdr2*$^{-/-}$) mice (human homologue *MDR3*), lacking a permeability (P)-glycoprotein which is located in the bile canalicular membrane of hepatocytes with a function as a phospholipid export pump. Disruption of the functionality of the pump causes a spontaneous phenotype described by cholangitis with a dysfunctional biliary delivery, ending in HCC development at 4–6 months of age [96]. In this phenotype, enhanced NF-κB activity leads to a higher TNFα expression, and impairment of NF-κB activity with a hepatocyte-specific inducible IκB superrepressor transgene negatively affected tumor progression and hepatocyte cell death at later stages of tumor development, whereas NF-κB blockage at the initial stage of tumor formation has no inhibitory effect [97]. Besides this tumor-promoting effect of NF-κB, other studies argue for a tumor-suppressive function. Hepatocyte-specific knockout of *Nemo* promotes hepatitis in these animals at 2 months of age and spontaneous HCC development 12 months after birth, triggered by cytokines and a constant low intrinsic dosage of LPS coming from the commensal gut bacteria [8]. Further studies with different combined hepatocyte-specific knockouts of *Nemo* and *Tnfr1*, *Trail-r*, or *Fas* or quadruple knockout showed no rescue or improvement of this phenotype. Still the combination of NEMO/FADD^{LPC-KO} caused a much milder progress of liver failure and inflammation and abolished HCC development. Examination of liver sections from 1-year-old NEMO^{LPC-KO}/Tnf$^{-/-}$ mice with developed liver tumors revealed that TNF is not important for the development of spontaneous cell death, hepatitis, and HCC in NEMO^{LPC-KO} livers. Neither depletion of natural killer cells nor an intercrossing with *Rag-1* ablated mice (NEMO^{LPC-KO}/Rag1$^{-/-}$) prevented liver damage [49]. All these results demonstrated that the spontaneously developed hepatocyte-specific NEMO phenotype is neither the result of death receptor-mediated signaling cascades nor an immune response triggered once, and additional studies are needed to identify the mechanism behind. Interestingly, double knockout of *Nemo* and *Tak1* in hepatocytes rescued the massive phenotype detected in the *Tak1* single knockout condition (as stated above), claiming for a NF-κB-independent tumor formation in the TAK1^{LPC-KO} livers [6, 8]. Generation of knock-in mice

endogenously expressing catalytically inactive RIPK1 D138N (*Ripk1D138N/D138N*) are alive after birth unlike mice conditionally lacking *Ripk1*. Moreover, these mice are protected against TNFα treatment and poly (I:C)-induced necroptosis *in vitro* and TNFα administration *in vivo*, indicating that the kinase activity of RIPK1 is not a prerequisite for cell survival but is crucial for TNFα-induced necroptosis [53]. Moreover, this result is supported by the finding that hepatocyte death and HCC development in NEMO[LPC-KO] mice is triggered by RIPK1's kinase activity, independent of NF-κB activity and RIPK1's scaffolding function. A complete NF-κB blockage induced by hepatocyte-specific single or combined knockout of *Rela*, *c-rel*, or *Relb* did not affect the liver, whereas constitutively active IKKβ prevented hepatocarcinogenesis in NEMO[LPC-KO] animals. These results revealed a NEMO protective function against HCC development. Hepatocyte-specific ablation of RIPK1 activated a TRADD-related apoptosis and HCC development, showing two different functions of RIPK1 [98].

Hepatocarcinogenesis chemically induced by a single injection of diethylnitrosamine (DEN) in 15-day-old IKKβ[Δhep] mice causes in 2-month-old mice massive liver tumor development which is not seen in untreated IKKβ[Δhep] livers [9, 81]. Compared to the untreated mice, DEN treatment supports enhanced ROS production correlating with increased JNK expression levels, hepatocyte death, and compensatory hepatocyte proliferation, which is similar to the results of the ConA-treated IKKβ[Δhep] mice [81]. Moreover, experiments with IKKβ^{−/−} fibroblasts showed that antioxidants like manganese superoxide dismutase (MnSOD) or MAPK phosphatases are needed to avoid ROS-mediated sustained JNK activity [99]. Besides ROS, nitric oxide (NO•) is another kind of agent radically synthesized by inducible nitric oxide synthase (iNOS), which is able to induce chronic inflammation and might influence tumor formation by controlling cell proliferation, angiogenesis, survival, medical resistance, and DNA repair [100–102]. Examination of *iNos* knockout mice showed significantly reduced NF-κB activities, and a higher concentration of iNOS is related to tumor proliferation, genomic instability microvascularization, and worse diagnosis for HCC patients. Treatment with iNOS inhibitors, like aminoguanidine, has a negative effect on HCC progression and NF-κB activity and a positive influence on apoptosis *in vivo* and *in vitro*. Moreover, interruption of the NF-κB cascade with sulfasalazine or siRNA led to decreased iNOS expression in different HCC cell lines [103].

Next to the components of the canonical NF-κB pathway, HCC development could also be triggered via the LTβR, one of the activators of the noncanonical NF-κB pathway. Enhanced levels of LTα, LTβ, and LTβR were detected in human HBV-/HCV-infected livers and in HCC. Examination of the transgenic mouse models of LTα and LTβ showed that hepatocyte-specific overexpression promotes fibrosis and HCC development. Ablation of IKKβ, specifically in hepatocytes, rescued mice from HCC development [7].

These results indicate a carcinogenesis promoting function of NF-κB, which is strongly supported by pro-inflammatory mediators.

6.12   NF-κBs Contribution on Liver Fibrosis and Cirrhosis

Liver fibrosis is a disease that develops as a consequence of recurrent "out-of-control" wound healing processes. Persistent removal of damaged and/or inflamed tissue with compensatory renewal leads over time to composition of fibrillar collagen in the affected areas, ending up in scarring liver tissue and loss of metabolic functional areas. Hepatic stellate cells (HSCs) are mainly involved in this process, whereas after certain stimuli this cell population is activated and undergoes a transformation to hepatic myofibroblasts which are built by decomposition of extracellular matrix proteins the collagen scars in the tissue. HBV and HCV infections, autoimmune hepatitis, alcohol abuse, NASH, and cholestasis trigger liver fibrosis and finally cirrhosis. For a long period, it was assumed that activated HSCs/hepatic myofibroblasts are the main source of liver fibrosis, but it seems to be a multifaceted process in which different signaling pathways and other liver cell types such as hepatocytes and Kupffer cells might be of importance [104]. Examination of intercrossed mice with a constitutively active human IKKβ (CAIKK2) allele in postnatal livers, controlled by a tetracycline promoter system, revealed modest liver injury, infiltration of immune cells, enhanced hepatocyte proliferation, and spontaneous liver fibrosis progression. Deeper analysis detected significantly enhanced levels of chemokines and certain chemokine receptors, while interruption of CAIKK2 expression led to declined expression levels in hepatocytes. Moreover, disruption of CAIKK2 expression for few weeks also reduced HSC activation but without significant improvement of fibrosis reduction. Only macrophage removal with liposomal clodronate positively affected liver fibrosis development caused by a reduced NF-κB activation. This indicates that next to transformed HSCs, recruitment of pro-inflammatory immune cells such as macrophages, mediated by prolonged hepatocellular NF-κB activation, are involved in promoting liver fibrosis [105]. Previous studies detected in activated HSCs enhanced levels of NF-κB, correlating not only with increased expression of pro-inflammatory and adhesion molecules, but also with upregulation of anti-apoptotic proteins like TRAF1/2, cIAP1/2, Bcl-X_L, and GADD45β [106]. Moreover, it seems that the CD95/Fas pathway is responsible for HSC death, whereas TGF-β and TNFα induce pro-survival signaling. Disruption of NF-κB activity by overexpression of an IκB superrepressor led to decreased expression levels of anti-apoptotic proteins like Bcl-X_L and enhanced proapoptotic proteins as caspase-3 during TGF-β and TNFα stimulation [107, 108]. Another study revealed a constitutive phosphorylation of p65 at Ser536 mediated by IKKβ and the autocrine renin-angiotensin system in human hepatic myofibroblasts. IKKβ-mediated phosphorylation of p65 allows nuclear translocation, whereas angiotensin II promotes myofibroblast survival in an autocrine and paracrine way [109]. Besides increased NF-κB activation, enhanced p-JNK levels could be detected in human- and murine-activated HSCs, indicating that JNK has an important function in liver fibrosis progression [110].

In addition to the death receptor family, two members of the Toll-like receptor (TLR) family, TLR4 and TLR9, were uncovered to be involved in mediating NF-κB activation during liver fibrosis progression as their activation could be a consequence of gut bacterial components [111, 112].

6.13   NF-κB in HVB/HCV Infections

Approximately 3% of the total world population have been infected with HCV, whereas more than 170 million people are suffering from chronic hepatitis, cirrhosis, and HCC. The annual rate of HCC development is strongly increased in cirrhotic livers [113]. The HCV core, the envelop protein E2, and HCV subgenomic replicons have been shown to enhance p38 and extracellular signal-regulated kinase (ERK) phosphorylation and initiate NF-κB [114, 115]. Moreover, it seems that oxidative stress (ROS) and TGF-β1 are important mediators of fibrogenesis in HCV infection. Silencing of p38, JNK, ERK1/2, and NF-κB (p65) in hepatocytes *in vitro* revealed their implication in enhanced TGF-β1 expression [116]. Experiments using different inhibitors for ROS (diphenyliodonium [DPI]), JNK (SP600125), IRE1 (Irestatin 9389), and NF-κB (6-amino-4-(4-phenoxyphenylethylamino [AQ]), have blocked significantly HCV-induced NF-κB and TGF-β1-mediated SMAD signaling. Silencing of JNK and IRE1 using siRNA inhibited efficiently ER stress, ROS, NF-κB, and TGF-β1 activity [117]. Moreover, it was shown *in vitro* that the hepatitis B virus X (HBx) protein, in addition to HCV infection, maintains enhanced NF-κB and AP-1 activation which might indicate a supportive role of HBx in HCC development [118]. Patients suffering from HBV have a much higher potential to develop HCC confirmed by studies which detected in nearly all examined HCCs of HBV patients chromosomally integrated viral DNA. Next to the HBx protein, the HBV surface antigen PreS2 is able to activate NF-κB and AP-1 via PKC-mediated induction of the c-Raf-1/MAPK signaling pathway, which further leads to increased hepatocyte proliferation, indicating a tumor-promoting function for PreS2 [119].

Stimulation of HCV core protein infected HeLa and HuH-7 cells with TNFα or LTα1/β2 mediates increased or sustained IκBβ degradation, whereas degradation of IκBα was only detected in LTα1/β2-stimulated HeLa cells. Besides cytokine treatment, higher levels of NF-κB activity were also detected in untreated HeLa and HuH-7 cells only harboring the HCV core protein. This finding shows that the HCV core protein is able to positively alter NF-κB initiation and, in combination with cytokine treatment, markedly increased this effect, which might have a direct influence on a stable and continuous NF-κB activation in HCV-infected cells [120].

After viral infection, the immune system reacts with the production of different NF-κB-related cytokines, especially IFNs to defend the infection [121–123]. NF-κB and IFN regulatory factor-3 (IRF-3) initiate alone or together the antiviral Janus kinases/signal transducer and activator (JAK-STAT) signaling cascade [124]. Despite the protective host defense response to eliminate viral infection accompanied by removing and restoring damaged tissue, continuous NF-κB activation also has negative side effects, such as activation of quiescent HSCs or proliferation of hepatocytes with oncogenic mutations, leading to liver fibrogenesis and HCC development. Therefore, therapeutics are needed which keep virus-induced oxidative stress or mediators of fibrosis at a minimum [125, 126].

6.14 NF-κB and Its Meaning for Obesity

Overweight and metabolic diseases have strongly increased all over the world, ranging from childhood to adult stage. The WHO estimated for 2015 that around 2.3 billion adults would be overweighted and approximately 700 million people would be obese [127]. Obesity is a gate opener not only for several diseases, such as insulin resistance, type 2 diabetes, cardiovascular disease, and atherosclerosis, but also for dementia, airway disease, and cancer [128, 129]. Genetic examination of murine and human adipose tissue, as well as murine hepatic tissue, uncovered strongly increased activation of an inflammatory and immune response gene network triggered by a multifaceted genetic loci and environmental issues [130–132]. However, the molecular mechanism of how obesity influences macrophage response is not yet fully understood. Currently, different theories are trying to explain how macrophages and obesity are connected to each other, e.g. by TLR4 induction through a high content of saturated fats, by inflammation of the central nervous system, or by the commensal gut microbiota [133–136].

Adipocytes are the cellular components of the adipose tissue and next to lipid storage they are also able to generate and release pro-inflammatory cytokines and adipokines which attract monocytes and T cells to infiltrate the adipose tissue, where monocytes differentiate to M1 macrophages. It is discussed that one major source triggering this process is metabolic stress caused by overnutrition, which leads to high levels of non-metabolized free fatty acids and ER stress inducing inflammatory responses. However, also removal of apoptotic adipocytes is another potent inducer of macrophage recruitment [128]. Besides macrophage recruitment, activation of TLRs is considered to initiate NF-κB-mediated generation of pro-inflammatory molecules. The NF-κB target IKKε was detected to be needed for high-fat diet (HFD)-induced obesity. Depletion of IKKε revealed a positive influence on high-fat diet fed mice, because these animals were protected from insulin resistance and hepatic steatosis, and they did not show any sign of chronic inflammation in liver or adipose tissue or induction of inflammatory pathways [137]. Similar results were obtained by HFD-treated mice with IKKβ deficiency in hepatocytes (*Ikbkb*$^\Delta$*hep*) or in myeloid (*Ikbkb*$^\Delta$*mye*) cells. These animals showed liver-specific insulin sensitivity, but revealed insulin resistance in muscle and adipose tissue, which was also examined in obese or aged mice. The *Ikbkb*$^\Delta$*mye* mice had preserved total insulin sensitivity with protection against insulin resistance [82]. Constitutively expressed IKKβ in hepatocytes caused increased NF-κB activity comparable to values detected in HFD-treated or obese mice. These animals also developed type 2 diabetes (T2D) with characteristic features such as hyperglycemia, severe hepatic insulin resistance and mild systemic insulin resistance, also affecting muscle tissue. Liver-specific expression of the IκBα superrepressor rescued this phenotype [138]. These results indicate a potential NF-κB function in obesity-related inflammation and T2D locally in the liver and myeloid cells, which seem to be crucial for improving systemic insulin resistance. Sustained excessive food intake also leads to NF-κB initiation via different pro-inflammatory pathways as in adipocytes and macrophages; TLR4 was discovered to be activated by free

fatty acids. Ablation of TLR4 protected these mice from insulin resistance in muscle tissue, disturbed glucose metabolism, and inflammatory signaling in liver and adipose tissue [139]. Additional studies showed that loss of function mutation in TLR4 also hindered the development of insulin resistance in adipocytes and in diet-initiated obesity [135, 140, 141]. Next to TLR4, TLR2 seems to have a comparable role in mediating NF-κB activation during HFD-induced obesity and the formation of insulin resistance [142, 143]. Metabolic stress signals attract monocytes to remove apoptotic pancreatic β-cells, thereby secreting TNFα, IL-6, and IL-1 to support β-cell dying. Increased TNFα levels in blood and peripheral tissues were detected in insulin-resistant rodents as well, and HFD promotes enhanced secretion of TNFα, IL-6, and IL-1 in hepatocytes and adipocytes. Inactivation of TNFα in different genetic rodent models mediated a significant peripheral glucose uptake during insulin secretion. Moreover, hepatocyte deficiency of IL-6 receptor (IL-6Rα^{L-KO}) impaired obesity-related insulin resistance and glucose tolerance. Depletion of IL-6Rα induced a massive inflammatory response indicated by enhanced levels of TNFα, IL-6, and IL-10 and IκBα phosphorylation. Glucose tolerance in these animals was reinstated by TNFα neutralization or Kupffer cell deficiency [144–146]. All these results imply a critical role for NF-κB in obesity-mediated insulin resistance and T2D progression and that a controlled release of inflammatory inducers is necessary for a healthy hepatic metabolism.

6.15 NF-κB and Its Function in Hepatic Ischemia/Reperfusion (I/R) Injury

During and after liver transplantation or hepatic resection, ischemia/reperfusion (I/R) is a main reason for liver failure caused by hypoxia or anoxia if oxygen supply and tissue pH are reinstated after clamping of the hepatic blood flow. The pathobiochemical mechanisms behind I/R are versatile and affect all liver cell types [147, 148].

Several studies were done to clarify if I/R induces apoptosis or necroptosis [149–154]. During I/R, hepatocytes express damage-associated molecular patterns (DAMPs)/pathogen-associated molecular patterns (PAMPs) on their surface, and macrophages, Kupffer cells, and dendritic cells bind these DAMPs/PAMPS by TLRs, thereby initiating the immune system. Adaptive and innate immune cells are recruited to the affected tissue sections where they secrete additional inflammatory mediators as TNFα, IL-1, IL-12, and INFγ which fuel these processes [148, 155]. Hepatic adenoviral overexpression of an IκB superrepressor in rats impeded NF-κB activation by decreased TNFα expression, preventing negative effects of hepatic I/R [156]. Furthermore, it was shown that heat shock preconditioning of rat livers significantly impaired I/R-induced NF-κB activation and expression of several inflammatory mediators [157]. Conditional ablation of *Ikkβ* in murine hepatocytes led to impaired liver necrosis and inflammation during I/R compared to the wild-type condition. Also administration of the chemical IKKβ inhibitor AS602868 failed to induce I/R-mediated liver failure without induction of pro-apoptotic side effects,

making this inhibitor potentially useful for therapy [52]. A20 is another critical NF-κB target gene activated during inflammatory processes in hepatocytes and is involved in blocking apoptosis, but is also part of a negative feedback loop to control NF-κB activation [158–160]. Murine hepatic recombinant adenoviral overexpression of A20 revealed a significantly increased survival rate after I/R in these animals, indicated by considerably reduced bilirubin and transaminase levels, reduced hemorrhagic necrosis and steatosis, and enhanced hepatocyte proliferation. Moreover, A20 induced the release of peroxisome proliferator-activated receptor alpha (PPARα), an important controller of lipid homeostasis and oxidative damage, which prevented oxidative induced necrosis [161].

These results revealed that interference of IKKβ activation by pharmacological treatment is a putative instrument to protect livers from I/R injury. A20, another crucial NF-κB target, seems to be an additional promising candidate which might have the capacity as a therapeutic target which efficiently blocks I/R [86, 161]. In contrast, mice given recombinant receptor activator of NF-κB ligand (RANKL) before or during hepatic I/R showed enhanced NF-κB activation accompanied by less liver damage [162].

6.16 NF-κB as Therapeutic Target

Liver diseases are characterized by multifaceted biochemical processes, whereas the balance between apoptotic and survival signaling pathways is of great importance for liver homeostasis. Impaired homeostasis leads to chronic inflammation and compensatory proliferation ending up in liver cirrhosis and HCC development. Tremendous work was done in these fields, and particularly the use of genetic modified mouse models showed that NF-κB is one of the most important key players in preserving liver integrity. However, the function of NF-κB in the development of liver diseases is not black and white nor is it equal for all liver cell types. This makes it extremely challenging to design cell type and disease-specific drugs which only affect the cells of interest and not interfering with NF-κB activity or other signaling cascades in healthy cells.

Several biological and chemical compounds have been tested to modify NF-κB activity as steroids, selective estrogen receptor modulators, antioxidants, proteasome inhibitors, and IKK inhibitors [126].

The administration of hormones is critical to assess because of the increased risk of negative side effect induction in the liver or other organs or their prospective impact on the respective gender [126, 163]. Phytochemicals from tea or other plants have a great antioxidant potential which seems to have a significant positive influence on liver health, but further investigations are needed for refining substance purification and antioxidant composition [164–167]. The development of chemical compounds, such as proteasome inhibitors like Bortezomib, mediates reduced NF-κB activation. Treatment of human hepatoma cells with MG132, another protease inhibitor, resulted in apoptosis induction but affected the β-catenin pathway. Currently, the use of proteasome inhibitors as a therapeutic target needs more

scientific research because of the diversity of bad side effects induced next to decreased NF-κB activation [168–171]. Regarding specific NF-κB blockage, the IKK complex member IKKβ seems to be a putative target. It has been shown that aspirin and sulfasalazine are able to impair the catalytic activity of the IKK complex. Furthermore, the development of a new class of chemical compounds, referred to as "small molecules," is able to impede NF-κB activation by binding to the ATP-binding pocket of IKKβ, thereby inducing conformational changes. Despite the higher binding specificity of small molecules for their targets and effectiveness compared to other compounds, a potential risk remains for the development of side effects, such as induction of inflammatory responses [126, 172, 173].

In addition to biological and chemical compounds, therapeutics on the DNA/RNA level have also been developed. Different studies were done with small interfering RNAs (siRNAs), microRNAs (miRNAs), and antisense oligodeoxynucleotides (ODNs), placing emphasis on liver fibrosis. Several studies have obtained promising results, but more genetic and clinical trial studies are needed to gain a better mechanistic understanding and to improve application effectiveness of small RNA and DNA products to enhance therapeutic success in the treatment of liver diseases [174].

In summary, further scientific and clinical studies are needed to develop effective therapeutics that are fine-tuned for the control of hepatic NF-κB activation to prevent progression of liver diseases, but without inducing harmful side effects.

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