

## Molecular Pathways: The Complex Roles of Inflammation Pathways in the Development and Treatment of Liver Cancer

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### Abstract

Inflammatory signals from the surrounding microenvironment play important roles in tumor promotion. Key inflammatory mediators and pathways that induce and sustain tumorigenesis have recently been identified in many different cancers. Hepatocellular carcinoma is a paradigm for inflammation-induced cancer, as it most frequently develops in the setting of chronic hepatitis, consecutive cellular damage, and compensatory regeneration. Recent studies revealed that liver damage-mediated inflammation and carcinogenesis are triggered by a complex cross-talk between NF- $\kappa$ B, *c-jun*-NH<sub>2</sub>-kinase, and STAT3 signaling pathways. Molecular dissection of the mechanisms involved in the interplay between these pathways identified promising new targets for therapeutic intervention. Targeting different components of the signaling cascades may provide efficient means for blocking the apparently irreversible sequence of events initiated by chronic liver inflammation and culminating in liver cancer. *Clin Cancer Res*; 19(11); 2810–6. ©2013 AACR.

### Background

Most malignant tumors contain somatic mutations in genes involved directly or indirectly in the regulation of cell growth or cell death. Epidemiologic studies suggest that these mutations are likely generated by different external factors including radiation, exposure to environmental pollutants or infectious agents, tobacco use, or diet (1). Besides inducing directly uncontrolled proliferation, these factors also trigger the activation of several immune cell types, which infiltrate the affected tissue and foster tumor development. Although the link between inflammation and carcinogenesis was first raised by Virchow in the 19th century, mechanistic insights into the process were obtained only during the past decade.

Hepatocellular carcinoma (HCC), the third most common cause of cancer-related mortality worldwide (2), is the most extensively investigated inflammation-based carcinogenic process. More than 90% of HCC cases are associated with chronic inflammation, which arises from hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, hemochromatosis, and alcoholic or nonalcoholic steatosis (3). Recent studies on mouse models provided important mechanistic insights into the pathogenesis by revealing the role of multiple signaling pathways that link chronic

inflammation to HCC. These include the NF- $\kappa$ B, the stress-responsive mitogen-activated protein kinase (MAPK), and the STAT pathways. Importantly, it was shown that these signaling pathways are linked to each other in a highly regulated manner, forming a highly complex signaling network that allows quality control and extensive communication between inflammatory cells and hepatocytes.

### Interplay between NF- $\kappa$ B and JNK signaling pathway

The NF- $\kappa$ B family consists of 5 transcription factors—p50, p52, p65, cRel, and RelB—which share an N-terminal Rel-homology DNA-binding and dimerization domain (4). NF- $\kappa$ B homodimers and heterodimers are sequestered in the cytoplasm via noncovalent interactions with I $\kappa$ B proteins (Fig. 1, red symbols and arrows). Upon stimulation, I $\kappa$ B is phosphorylated by the inhibitor of I $\kappa$ B kinase (IKK) complex, which consists of the IKK- $\alpha$  and - $\beta$  catalytic and the IKK- $\gamma$ /NEMO regulatory subunit (4, 5). Phosphorylation of I $\kappa$ Bs results in their K48-linked ubiquitination and subsequent degradation by the 26S proteasome complex (6). The free NF- $\kappa$ B dimers can then translocate to the nucleus and activate the transcription of genes encoding cytokines, chemokines, and antiapoptotic factors that promote cell growth and survival (7). NF- $\kappa$ B is also a potent inducer of the caspase-8 homolog FLICE-interacting protein (cFLIP) a repressor of death receptor-induced apoptosis (Fig. 1, red arrows; ref. 8).

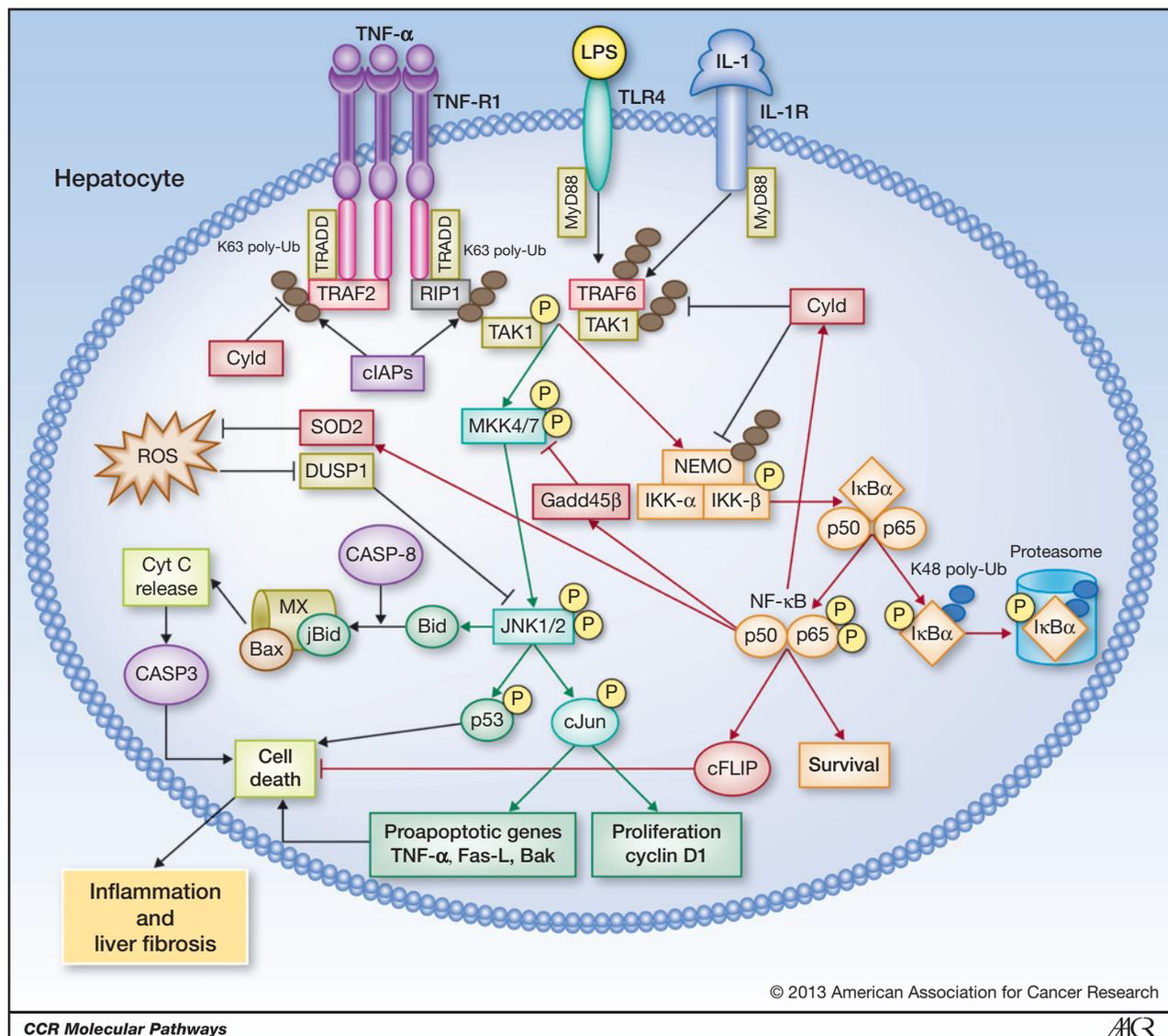
The NF- $\kappa$ B pathway is activated by various stimuli including lipopolysaccharide (LPS) and anti-inflammatory cytokines, such as TNF- $\alpha$  and interleukin (IL)-1, which elicit their effects through binding to Toll-like receptors (TLR) and to the TNF or IL-1 receptors (TNFR-1 or IL-1R), respectively (4, 9). These cytokines are produced by inflammatory cells, which accumulate in the liver upon virus infection—

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**Figure 1.** Regulation of cell death and proliferation by the NF- $\kappa$ B and JNK pathways. Complexes assembled on ligand-bound TNF or TLR and IL-1 receptors mediate TAK1 activation, whose activity is modulated by Cyld. TAK1 phosphorylates IKK- $\beta$ , which in turn phosphorylates I $\kappa$ B, leading to its ubiquitin-proteasome-mediated degradation and release of NF- $\kappa$ B (p50-p65) factors, which translocate to the nucleus to activate the transcription of pro-survival genes. NF- $\kappa$ B also induces the expression of cFLIP and SOD2, which inhibit apoptosis and reactive oxygen species (ROS) accumulation. TAK1 also phosphorylates MKK4/7, which activates JNK1/2. Short-term JNK activation induces the expression of genes involved in proliferation control, via the phosphorylation-mediated activation of JUN transcription factors. Sustained JNK activation induces the expression of proapoptotic genes, and stabilizes p53 and the Bax/Bak-dependent apoptotic pathway. NF- $\kappa$ B feedback regulates its own activation via modulating Cyld expression and the activation of JNK via activating Gadd45 $\beta$  expression, which inhibits MKK4/7. JNK activation is also modulated by ROS accumulation through dual-specificity phosphatase 1 (DUSP1) phosphatase activity. CASP-3/8, caspase-3/8.

induced hepatitis or through the action of other causative agents of inflammation. Upon stimulation by the corresponding ligands, rapid assembly of complexes containing TNFR-associated death domain (TRADD), TNFR-associated factor (TRAF), and receptor-interacting protein 1 (RIP1) proteins occur at the TLR/IL-R or TNFR, which recruit and activate TGF- $\beta$ -activated kinase 1 (TAK1) through TRAF6 or TRAF2, respectively. TAK1 subsequently phosphorylates IKK- $\beta$  and MAPK kinase 4/7 (MKK4/7), which in turn activate NF- $\kappa$ B and *c-jun*-NH<sub>2</sub>-kinase (JNK), respectively (refs. 10–12; Fig. 1). The assembly step of activated receptor

complexes involves TRAF2 and cellular inhibitor of apoptosis protein 1/2 (cIAP1/2)-mediated K63-linked ubiquitination of several of the components, including TRAF2, TRAF6, TAK1, RIP1, and NF- $\kappa$ B essential modulator (NEMO), which facilitates protein-protein interactions and the assembly of the signaling complexes (10–14). The main enzyme that removes polyubiquitin chains from the above proteins is the cylindromatosis tumor suppressor cylindromatosis deubiquitinase (Cyld; refs. 15–20). As a result of Cyld-mediated deubiquitination of TAK1 and other components of the complex, NF- $\kappa$ B signaling is

inhibited. Importantly, the *Cyld* gene is transcriptionally activated by NF- $\kappa$ B, which provides a negative feedback regulatory loop that could function in balancing activated NF- $\kappa$ B levels (ref. 21; Fig. 1).

Although NF- $\kappa$ B activation has prosurvival, antiapoptotic effects, JNK signaling (Fig. 1, green symbols and arrows) has been implicated in the induction of either cell proliferation or apoptosis (22–24). JNK can phosphorylate various substrates, including c-Jun, JunB, JunD, ATF2, p53, Bcl2, Bcl-xL, Bid, Bad, and Bax proteins, which regulate cell growth and death (25). One mechanism by which JNK contributes to cell survival involves JunD phosphorylation, which transcriptionally activates antiapoptotic genes (Fig. 1). For example, the potent apoptosis repressor gene *cIAP2* contains a composite promoter with tandem AP1 and NF- $\kappa$ B-binding sites, through which JunD/Fos and NF- $\kappa$ B dimers cooperate and activate transcription in a synergistic manner (26). This generates a positive feedback regulatory circuit: NF- $\kappa$ B- and JNK-activated JunD induces *cIAP* expression, which promotes K63-linked polyubiquitination of upstream signaling molecules, leading to TAK1 activation. TAK1 in turn phosphorylates IKK- $\beta$  and MKK4/7 to activate NF- $\kappa$ B and JNK (Fig. 1).

Although the initial TNFR1-mediated JNK activation is transient and promotes cell survival and proliferation, the opposite effect is seen when JNK activation is sustained for a prolonged period. Chronic JNK activation induces a Bax/Bak-dependent apoptotic pathway, via mitochondrial release of cytochrome *c*, which in turn activates Apaf1 and various caspases (Fig. 1; refs. 27, 28). The proapoptotic effect of JNK is also exerted through transcriptional activation of apoptosis-inducing genes such as *TNF- $\alpha$* , *Fas-L*, or *Bak*, or by phosphorylation of the tumor suppressor p53 and via phosphorylation of the E3 ubiquitin ligase Itch homolog (29–32). Itch facilitates cell death by promoting degradation of the NF- $\kappa$ B-induced antiapoptotic caspase-8 inhibitor cFLIP (32).

In most conditions, an inverse correlation exists between activation of the NF- $\kappa$ B and JNK signaling axes, which is at odds with the finding that both pathways are induced by the same upstream kinase, TAK1. The negative cross-regulation is mainly achieved by NF- $\kappa$ B-dependent transcriptional activation of the *Gadd45 $\beta$*  gene, which represses MKK4/7-mediated phosphorylation of JNK1/2 (Fig. 1; refs. 33, 34). In conditions when ROS accumulate, the opposite regulation of the 2 pathways is elicited by a complex negative feedback circuit, which involves NF- $\kappa$ B-mediated induction of the ROS-metabolizing enzyme, superoxide dismutase 2 (SOD2; ref. 35). This regulatory axis prevents ROS accumulation, which otherwise causes chronic JNK activation via inhibiting JNK phosphatases (Fig. 1; ref. 36).

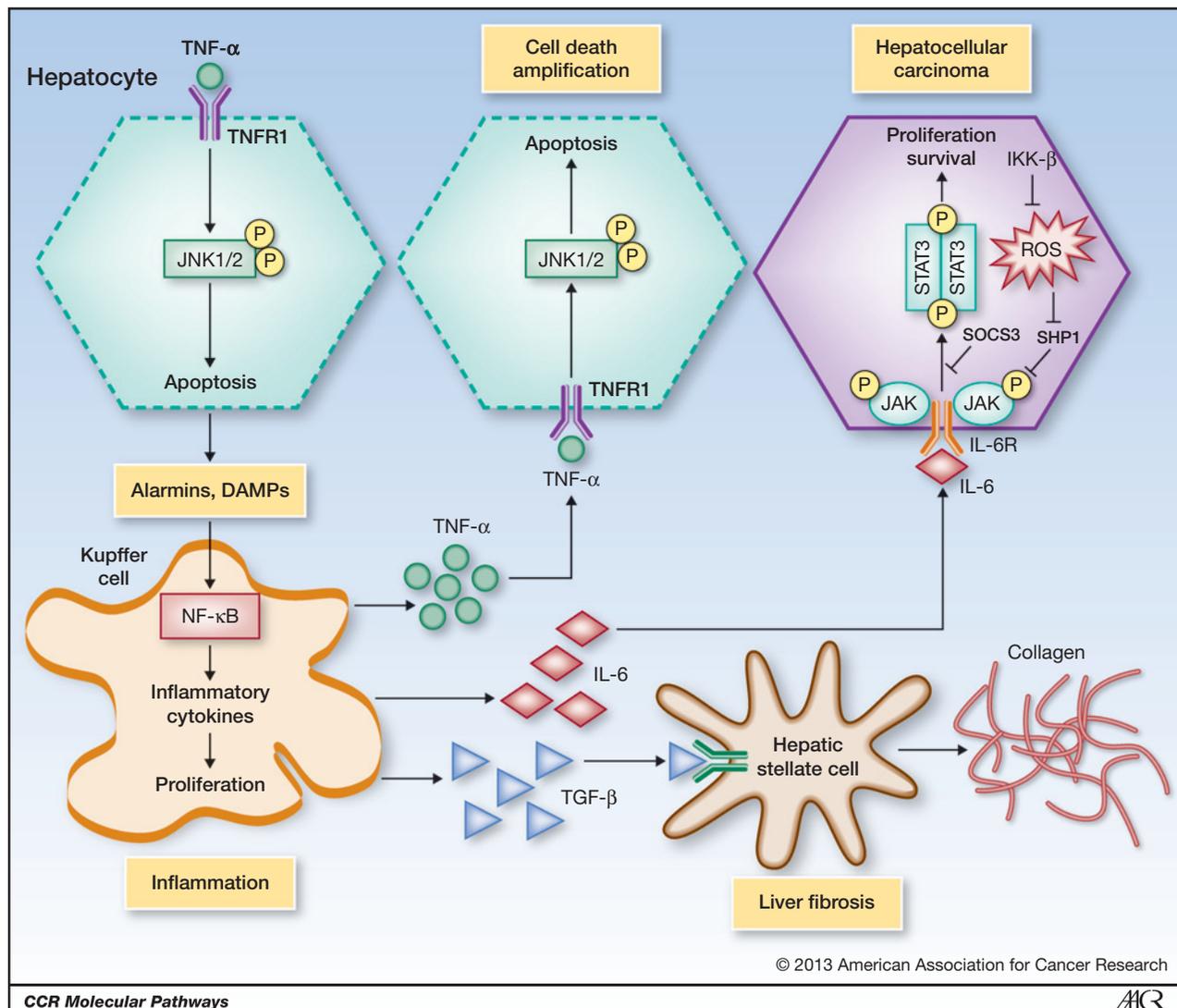
### Signal transduction pathways establish communication between different cell types during hepatocarcinogenesis

The importance of NF- $\kappa$ B and JNK signaling in the development of inflammation-associated HCC has been shown by recent studies using relevant animal models. NF-

$\kappa$ B seems to have a protumorigenic effect in *Mdr2*<sup>-/-</sup> mice, which exhibit low-grade chronic inflammation and spontaneous development of HCC (37). Inhibition of the NF- $\kappa$ B pathway by the expression of nondegradable I $\kappa$ B mutant prevented HCC formation and increased apoptosis of pre-malignant hepatocytes (38). Similar protective effects were observed after the administration of anti-inflammatory or anti-TNF drugs to *Mdr2*-deficient mice. Transgenic mice expressing lymphotoxins  $\alpha$  and  $\beta$  (L $\alpha\beta$ ) in the liver display inflammation and fibrosis, and develop HCC (39). In this model, the occurrence of inflammation and HCC depends on IKK- $\beta$  expression in hepatocytes but is independent of TNFR1 function (39).

In sharp contrast to the above protumorigenic effects, NF- $\kappa$ B signaling exhibits a tumor suppressor function in situations when liver inflammation is mainly driven by hepatocyte damage. For example, mice lacking IKK- $\beta$  in hepatocytes exhibit a marked increase in hepatocarcinogenesis after diethylnitrosamine (DEN) treatment (40–42). In this experimental condition, hepatocyte-specific IKK- $\beta$  depletion enhanced ROS production and induced JNK activation and hepatocyte death, which augmented compensatory proliferation of surviving hepatocytes. Hepatocyte death-mediated accumulation of inflammatory cells, including the activation of resident macrophages (Kupffer cells), was necessary for the carcinogenesis process (40). This requirement was revealed by the observation of decreased hepatocarcinogenesis in mice in which IKK- $\beta$  was deleted in both hepatocytes and Kupffer cells (40). Because Kupffer cell activation could not take place in the absence of NF- $\kappa$ B, the above results suggest that the NF- $\kappa$ B pathway coordinates the inflammatory cross-talk between hepatocytes and Kupffer cells (Fig. 2). Hepatocyte-specific inactivation of NEMO (IKK- $\gamma$ ) or TAK1, the upstream activators of NF- $\kappa$ B, resulted in spontaneous hepatocyte death, liver inflammation, and fibrosis, as well as the development of HCC (43–45). Interestingly, constitutive hyperactivation of TAK1 in *Cyld*-deficient hepatocytes displayed similar effects (46). Taken together, the above studies reinforced the established view that NF- $\kappa$ B signaling plays a central role in linking chronic inflammation to tumorigenesis.

A common mechanistic feature observed in all of the above models is the chronic activation of JNK, which triggers hepatocyte death. JNK activation in the NF- $\kappa$ B-deficient models is elicited by decreased growth-arrest DNA damage-45 protein  $\beta$  (*Gadd45 $\beta$* )-mediated inhibition of MKK4/7 and by ROS-mediated inactivation of JNK phosphatases (Fig. 1; refs. 33, 34, 36). In the case of *Cyld* deficiency, JNK activation is mediated by the constitutively active TAK1 (46). In all cases, prolonged activation of JNK induces hepatocyte death, which facilitates the activation of Kupffer cells and other inflammatory cells (Fig. 2). Upon activation, Kupffer cells produce various cytokines including TGF- $\beta$ , TNF- $\alpha$ , and IL-6 (47). In response to TGF- $\beta$ , hepatic stellate cells proliferate and transdifferentiate to myofibroblasts, producing a network of extracellular matrix, the hallmark of the fibrotic scar (48). Elevated local



**Figure 2.** Mechanism of cell death–mediated inflammation, fibrosis, and carcinogenesis. Sustained JNK activation causes hepatocyte death. Dying hepatocytes release alarmins/damage-associated molecular patterns (DAMP), which leads to the recruitment of Kupffer cells. NF- $\kappa$ B pathway activation in Kupffer cells leads to the expression and secretion of TGF- $\beta$ , TNF- $\alpha$ , and IL-6 cytokines. TGF- $\beta$  activates hepatic stellate cells, resulting in fibrogenesis. TNF- $\alpha$  induces apoptosis in the neighboring hepatocytes. Hepatocytes that escape or do not complete cell death may respond to IL-6 via activation of STAT3, which induces compensatory hepatocyte proliferation. IL-6R, IL-6 receptor.

levels of TNF- $\alpha$  cause activation of death receptor signaling in neighboring hepatocytes, which initiates a vicious circle of intercellular signaling between hepatocytes and Kupffer cells, leading to the amplification of hepatocyte death (ref. 46; Fig. 2). Hepatocytes that escape TNF-mediated death may respond to IL-6 and related cytokines via activation of STAT3 signaling (42). Binding of IL-6 to its receptor displaces Janus-activated kinase (JAK), which phosphorylates STAT3 (Fig. 2, blue symbols). Phospho-STAT3 translocates to the nucleus and activates numerous oncogenic genes, resulting in enhanced hepatocyte proliferation (Fig. 2; refs. 42, 49). In human HCC samples, an inverse correlation exists between NF- $\kappa$ B and STAT3 signaling (42, 50, 51). The underlying mechanism involves feedback inhibition of STAT3 activation via tyrosine phos-

phatases, such as src homology–containing phosphatase 1/2 (SHP1/2) and suppressor of cytokine signaling 3 (SOCS3). In this pathway, elevated ROS levels generated by NF- $\kappa$ B inhibition oxidize SHP1/2. Oxidized SHP1/2 lose their enzymatic activity toward JAK2 substrate, which leads to constitutive activation of the JAK–STAT3 pathway (ref. 52; Fig. 2).

Collectively, the above findings establish the view of a complex interplay between different signaling pathways that regulate distinct phases during the pathogenesis of inflammation-associated HCC. This "interpathway cross-talk" is accomplished through various feed-forward, feedback, and autoregulatory loops that operate not only within individual cells but also between inflammatory cells and hepatocytes.

### Clinical-Translational Advances

Because of the impaired liver function of patients with HCC, classic anticancer chemotherapeutics are toxic and ineffective. The recently introduced sorafenib is a tyrosine kinase inhibitor, targeting multiple molecular pathways. Although its superior efficacy over conventional chemotherapy has been established by 2 large-scale clinical trials, its overall value is considered low, as it improves median life expectancy by only 3 months over placebo (53, 54). Although a combination of sorafenib with cytotoxic (e.g., doxorubicin) or antiangiogenic (e.g., VEGF inhibitors) agents is being evaluated, to date no evidence has come to light for the potential of the currently used therapeutics to shrink cancerous lesions or to prevent cancer formation. This fact lends emphasis to the urgent demand for alternative approaches.

The main translational benefit of studies that established an unambiguous connection between chronic inflammation and HCC is the recognition of an increased repertoire of promising new targets for the development of effective systemic therapies for HCC. An important preventive approach is treatment with antiviral drugs against HBV and HCV, which would eliminate the main circumstance in which HCC develops. Unfortunately, despite extensive efforts using antiviral therapies, it is currently not possible to cure chronic viral hepatitis. The use of other anti-inflammatory drugs (e.g., nonsteroidal compounds, such as aspirin) has proven effective in other cancer types but has not been evaluated in HCC.

Targeting the NF- $\kappa$ B pathway emerges as an alternative concept for curing HCC, given its central position in the regulation of inflammatory processes. Several observations in different mouse models that can be recapitulated in human HCCs point to the feasibility of pharmacologic targeting of NF- $\kappa$ B and NF- $\kappa$ B-linked signaling pathways. In the majority of human subjects, in whom HCC was preceded by chronic HBV- or HCV-mediated inflammation, the mechanism of disease progression resembles that described in *Mdr2* knockout (KO) mice or *L $\alpha$ / $\beta$*  transgenic mice, in which NF- $\kappa$ B function is absolutely required (37–39). In these cases blocking the NF- $\kappa$ B pathway (e.g., by IKK- $\beta$  inhibitors) may have beneficial effects. In cases in which HCC develops as a result of hepatocyte damage (e.g., in alcoholic or nonalcoholic steatohepatitis or after chronic toxic assaults), the situation is more complex. Interpreting data from the animal models where HCC is initiated by hepatocyte death raises arguments against the feasibility of using IKK- $\beta$  inhibitors for treatment, because inactivation of different components in the pathway (IKK- $\beta$ , NEMO, or TAK1) actually promotes carcinogenesis (see above). Importantly, however, HCC development is halted when IKK- $\beta$  is simultaneously ablated in hepatocytes and Kupffer cells, a situation that is more likely mimicked by systemic inhibitor treatment (40). On this conceptual basis, IKK- $\beta$  inhibitors can be considered good candidates for HCC treatment and definitely warrant additional studies. In this regard, we note that the extent of inhibition of the

individual components should be taken into account, as they may greatly affect the final outcome. In genetic models, the inhibition of the pathway is nearly complete, whereas in the case of treatment with pharmacologic inhibitors the extent of blockage is partial and in most cases adjustable.

Because of the dichotomous nature of their function, other players in the pathway, such as TAK1 or Cyld, cannot be considered promising targets. Either inactivation or hyperactivation of TAK1 leads to hepatocyte death, inflammation, fibrosis, and HCC development, suggesting that balanced levels of TAK1 activity are necessary for the maintenance of physiologic liver homeostasis (46). Additional studies on its ubiquitination-mediated regulation would be important to learn more about this enzyme, as, in addition to NF- $\kappa$ B, it can also activate the JNK pathway, which seems to represent a highly promising target for anticancer therapy.

The role of sustained JNK activation in hepatocyte death and subsequent inflammation and carcinogenesis is recapitulated in most of the mouse genetic models (IKK- $\beta$ -KO, NEMO-KO, TAK1-KO, and Cyld-KO) developing HCC (40–46). In addition, mice expressing HCV core protein activate JNK through ROS production (55). Importantly, JNK1 is phosphorylated in human HCC samples (56). Direct evidence for the idea that JNK could be a promising drug target was provided by the findings that administration of the JNK inhibitor SP600125 to Cyld-deficient mice or DEN-treated rats blocked the development of HCC (46, 57). SP600125 has also been shown to sensitize tumor cells, but not normal hepatocytes, to TRAIL, a major mediator of acquired immune tumor surveillance (58). Thus, JNK inhibitors that also sensitize TRAIL could be used in combination with TRAIL-targeting drugs to increase therapeutic efficiency.

The other potential target presented in this review is STAT3. In mice, prevention of STAT3 activation via inhibition of its upstream kinase JAK2 was found to be effective in blocking HCC development (42). STAT3 was detected in its activated form in more than 60% of human HCC samples, and phosphorylated STAT3 levels correlated with the aggressiveness of the tumors (42). Importantly, STAT3 deletion does not affect the survival of differentiated cells, but efficiently blocks cell proliferation, suggesting that STAT3 may be a safe target for cancer therapeutics.

Taken together, studies on mouse models have revealed a complex cross-talk between NF- $\kappa$ B, JNK, and STAT3 signaling pathways in inflammation-associated HCC. These studies have provided novel insights into the temporal order of regulated steps during pathogenesis, which raise the possibility of developing novel means to block the sequence of events that lead to HCC. Successful translation of the knowledge gained on NF- $\kappa$ B, JNK, and STAT3 signaling will depend on appropriate human studies that would motivate the development of safer and more effective cancer therapies.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

**Authors' Contributions**

**Writing, review, and/or revision of the manuscript:** K. Nikolaou, M. Sarris, I. Talianidis

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