The regulation of the JNK cascade and programmed cell death by NF-κB: mechanisms and functions

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Abstract

NF-κB is an evolutionarily conserved family of transcription factors that play a central role in immune and inflammatory responses. They also play a pivotal role in cell survival, whereby activation of NF-κB antagonizes programmed cell death (PCD) induced by tumour necrosis factor receptors (TNF-Rs) and other cell death signals. The pro-survival function of NF-κB has been implicated in a wide range of biological processes, including the development and homeostasis of the immune system and liver. It has also been implicated in the pathogenesis of numerous diseases, including cancer, chronic inflammation and certain hereditary disorders. The protective activity of NF-κB can also hamper tumour cell killing inflicted by radiation or chemotherapeutic drugs, thereby promoting resistance to anticancer treatments. This pro-survival activity of NF-κB involves the suppression of sustained Jun-N-terminal kinase (JNK) activation and of the accumulation of cytotoxic reactive oxygen species (ROS). NF-κB mediates this function by inducing the transcription of target genes, whose products inhibit the JNK signaling pathway and suppress ROS accumulation through their anti-oxidant functions. The development of specific inhibitors that target the critical downstream NF-κB-regulated genes that promote survival in cancer and other diseases potentially holds a key to developing specific, and effective therapeutic strategies to combat these disorders.

Keywords: NF-κB, programmed cell death, JNK, reactive oxygen species, Gadd45β, cancer, inflammation

1. Introduction

Programmed cell death (PCD) is a form of cellular suicide that plays a central role in various processes in animal physiology, including normal cell turnover and tissue homeostasis, as well as proper development and functioning of the immune system in order to eliminate defective or potentially dangerous cells [1-3]. Signaling pathways that govern cell death are of critical importance as inappropriate PCD (either too little or too much) is a major contributing factor in many human diseases [1-3]. Exaggerated cell death, for instance, is associated with neurodegenerative diseases, ischemic damage and immunodeficiency while at the other end of the spectrum, autoimmune diseases and cancer are linked to reduced cell death with concomitant increased cell survival [1-3]. Inducible regulation of gene expression allows organisms to adapt to environmental, mechanical, chemical and microbiological stresses which could potential lead to cell death. Since its discovery almost a quarter of a century ago, NF-κB has served as a model for inducible transcription factors [4]. NF-κB plays its most important and evolutionary conserved role in the innate and adaptive immune systems, however, NF-κB also acts broadly to influence gene expression events that impact cell survival, differentiation and proliferation [4-6]. The ability of NF-κB to suppress PCD is of major biomedical interest since the promotion of cell survival by NF-κB is a fundamental contributing factor in tumorigenesis, chemoresistance in cancer and autoimmune diseases [7-9]. Advances over the last decade in the undersatanding of how NF-κB controls PCD have led to the discovery that the NF-κB-mediated antagonism of PCD involves the suppression of the c-Jun-N-terminal (JNK) mitogen-activated protein kinase (MAPK) cascade and reactive oxygen species (ROS) [8-11]. This antagonism of PCD is mediated by a specialized subset of NF-κB target genes (discussed below). Currently, there is a great deal of interest in developing drugs that target the products of these NF-κB-induced genes, with the aim of allowing selective inhibition of the pro-survival action of NF-κB, without compromising the capacity of NF-κB to serve in immunity, inflammation and tissue development.

2. The NF-κB pathway

Transcription factors of the NF-κB family are best known for their central role in immunity, inflammation, and oncogenesis [4-6,12]. The mammalian NF-κB family consists of 5 members: RelA (p65), RelB, c-Rel, p50/NF-κB1 and p52/NF-κB2 [4-6,13]. All members of this family share a structurally

conserved Rel homology domain (RHD), 300 amino acids in length at their N-terminus, which mediates nuclear localization, DNA binding, dimerization with other NF-κB subunits, and interaction with inhibitors of NF-κB (IκBs) [4-6,13]. NF-κB family members are divided into two subfamilies based on divergence at their C-terminus. The RelA (p65), RelB and c-Rel proteins all contain carboxyl-terminal transactivation domains (TADs), which strongly activate transcription from NF-κB-binding sites within regulatory regions of target genes [4-6,13]. The p50 and p52 proteins, which are generated from proteolytic cleavage of the p105 and p100 precursor proteins, respectively, lack the TAD but are still capable of binding to NF-κB-consensus DNA sites [4-6,13]. Although NF-κB is composed of numerous heterodimers or homodimers of various members, the most abundant and ubiquitous dimer in cells is p50(NF-κB1)/RelA(p65). Inducible NF-κB activation depends on the phosphorylation-induced proteosomal degradation of IκBs (IκBα, -β, -γ, -ε), which sequester inactive NF-κB dimers in the cytoplasm of unstimulated cells [4-6,13]. The majority of NF-κB-inducing signaling pathways converge on the IκB kinase (IKK) complex, which is responsible for the IκB phosphorylation thereby allowing the liberated NF-κB to enter the nucleus and regulate transcription of a variety of target genes encoding many immunoregulatory, inflammatory mediators and inhibitors of apoptosis [4-6,13]. Through the induction of these distinct sets of target genes, NF-κB coordinates innate and adaptive immunity, inflammation, cell differentiation and cell survival [4-6,13].

There are two signaling pathways that are primarily responsible for NF-κB activation, and which have been described as the classical or canonical pathway, and the alterative or non-canonical pathway [4-6,13,14]. The IKK complex is composed of three subunits, the highly homologous catalytic subunits IKKα and IKKβ, and the non-enzymatic regulatory component, IKKγ/NEMO [4-6,13,14]. Despite the structural similarities between IKKα and IKKβ, most stimuli that lead to NF-κB activation, including TNF-R1, rely on IKKβ activity, and indeed IKKβ-deficient mice and cells exhibit defective TNFα-induced NF-κB activation [4-6,13,14]. The classical pathway is triggered in response to stress, microbial products and pro-inflammatory cytokines, resulting in the IKKβ-dependent nuclear translocation of p50/RelA and p50/c-Rel dimers, and is involved primarily in the acute responses to infection and injury [4-6,13,14]. The alternative pathway, which is generally activated by specific members of the TNF cytokine family such as lymphotoxin (LT)β, B-cell activating factor (BAFF) and the CD40 ligand, results in the specific activation of p52/RelB

dimers [4-6,13,14]. Unlike the classical pathway, which is dependent on IKK-β and IKK-γ activity, the alternative pathway depends on IKK-α activity [4-6,13,14], which induces the proteolytic processing of the p52 precursor, p100, and nuclear entry of p52/RelB heterodimers and is largely involved in developmental roles, including survival of premature B cells and development of secondary lymphoid organs [14]. These functions have been demonstrated in $RelB^{-/-}$ and $IKK\alpha^{-/-}$ knockout mice, which both suffer from defective secondary lymphoid organ development [12,15-19]. Although both pathways of NF-κB activation are capable of regulating cell survival and cell death, it is the classical NF-κB pathway that is primarily responsible for inhibition of PCD under most circumstances (*e.g.*, in the context of inflammation) [4-6,13]. A third pathway leading to NF-κB activation is triggered by genotoxic stresses that result in DNA damage through the atypical NF-κB activation pathway, which does not fall in the abovementioned canonical and non-canonical pathways [20]. An IKK-independent pathway for ultraviolet (UV)-induced NF-κB activation has also been characterized that involves casein kinase 2 (CK2) [21-23]. UV exposure activates CK2 via p38 MAP kinase, which acts as an allosteric CK2 regulator. Activated CK2 then directly phosphorylates IκBα triggering its degradation by the proteasome, resulting in NF-κB activation [22].

3. The pro-survival role of NF-κB in animal physiology

NF-κB transcription factors are central players in a system that allows cells to adapt and respond to environmental changes, a process pivotal for survival of organisms. The pro-survival function of NF-κB has been implicated in a wide range of biological processes, and NF-κB's role in PCD is of critical importance in the development and homeostasis of the immune system [25,29]. NF-κB guides the development of T and B lymphocytes, ensuring their survival at critical stages in their developmental program, thereby allowing progression to functional maturity. In the B-cell lineage, NF-κB is constitutively activated by the triggering of the TNF-R family receptors, TACI, BCMA and BAFF-R [24], and this constitutive NF-κB activity is necessary for differentiation and maintenance of mature IgM^{low}/IgD^{high} B lymphocytes [25-27]. NF-κB also plays an important protective role in the development of thymocytes, and is required in the periphery for the productive responses of mature B and T lymphocytes following exposure to antigen and costimulatory molecules such as CD40 ligand (CD40L) and B7-1, respectively [25,27-29]. The classical and alternative pathways of NF-κB activation antagonize PCD signaling triggered downstream of numerous receptors, such

as Toll-like receptors (TLRs) and death receptors (DRs) including Fas and TNFR1 [25,26], found on the surface of cells of the immune system. Various knockout mice models for NF-κB signaling components display defective lymphopoiesis and function due to an inability to protect developing and mature B and T lymphocytes from apoptosis induced by high levels of TNFα, owing to a lack of sufficient NF-κB activity to overcome cell death pathways [25,29].

The NF-κB-mediated control of PCD is also crucial to the physiology of organs outside the immune system. The first direct evidence that NF-κB inhibited PCD was provided by the observation that ablation of RelA in mice causes embryonic lethality due to massive apoptosis in the liver [30]. The role of NF-κB in embryonic hepatic cell survival was further underscored by the similar phenotypes of mice that lack IKKβ, IKKγ or RelA [31-33]. Notably, both liver damage and embryonic lethality in mutant mice were reversed by the compound mutation of TNF-R1, confirming that liver apoptosis depends on signaling via this receptor [34,35]. The hepatoprotective activity of NF-κB was also confirmed in adult animals [9,36,37], with the demonstration that NF-κB activity is essential for antagonism of TNF-R-mediated damage. The protective action of NF-κB against PCD has been implicated in several other processes, including epidermal homeostasis, hair follicle development, and the development and function of the central nervous system [31,38,39].

4. NF-κB-mediated inhibition of PCD in human disease

As a consequence of the extensive range of stimuli inducing NF-κB activation, and of the multitude of target genes NF-κB induces, deregulation of NF-κB activity has been implicated in numerous diseases. NF-κB controls cell survival through the antagonism of PCD, and this suppressive action on PCD is crucial in carcinogenesis. Genes encoding NF-κB/IκB-family members are frequently amplified, rearranged or mutated in certain human cancers, and most cellular and viral oncogene products, including Bcr-Abl, Her-2/Neu and oncogenic H-Ras and K-Ras, are capable of eliciting NF-κB activation [12,40-43]. Additionally, mutations that result in NF-κB activation in malignant cells occur in genes encoding for signaling proteins that feed into the IKK-NF-κB axis or cause NF-κB to be activated by exposure to proinflammatory cytokines in the tumour microenvironment [12,40-43]. Direct evidence from various *in vivo* and *in vitro* models now indicate that the NF-κB-mediated suppression of PCD is crucially involved in various aspects of cancer

biology including malignant transformation, tumour progression and resistance to anticancer therapy [12,40-45]. Indeed, constitutive NF-κB activity is required for the survival of several cancerous cells, such as those in diffuse B-cell lymphoma (DLBCL), acute lymphoblastic leukaemia (ALL), multiple myeloma (MM), Hodgkin's lymphoma (HL), breast cancer and many other haematological and solid tumours [12,40, 46-50].

Mutations that give rise to constitutive NF-κB are best characterized in B-cell malignancies. NF-κB is constitutively activated in activated B-cell like (ABC)-DLBCL but not in germinal centre B-cell-like (GCB)-DLBCL and results in increased B-cell proliferation and survival even after the initial antigenic stimulus has ceased [47]. Mutations in the *CARD11* gene were discovered in a subset of ABC-DLBCL, and result in a protein product that is a constitutive activator of the IKK-NF-κB pathway [48,51]. In other ABC-DLBCLs, mutations that modify the Toll-like receptor (TLR)-adaptor protein, *MyD88*, have been identified which also results in constitutive NF-κB activity [52]. Furthermore, mutations involving the NF-κB pathway are present in at least 20% of multiple myeloma tumours [49,50]. Although activated NFκB is a common feature of this plasma cell malignancy, no mutations in NF-κB or IκB encoding genes were discovered in this disease. Rather, a number of mutations in genes encoding upstream signaling molecules that lead to the stabilization and accumulating of NF-κB-inducing kinase (NIK), a member of the MAP3K family causing activation of both the classical and alternative NFκB signaling pathways, were identified [49,50,53].

Recent studies have shed new light on the basis for the pivotal role of NF-κB in carcinogenesis. One common epigenetic event in cancer is inflammation, and indeed, chronic infections and inflammation account for 15-20% of all cancer deaths in humans [12,54,55]. Hepatocellular carcinoma (HCC), for instance, often results from cirrhosis or chronic infection with HBV or HCV viruses [56]. Notably, it was shown that a crucial link between inflammation and carcinogenesis depends on NF-κB and that this is mediated by at least two mechanisms: firstly, within pre-cancerous/cancerous cells, NF-κB upregulates pro-survival genes that enable these cells to evade PCD and propagate their malignant phenotype. Secondly, in non-parenchymal cells, NF-κB induces pro-inflammatory cytokines (*e.g.*, TNFα, IL-6) that stimulate cancer cell growth [12,43,55]. Furthermore, NF-κB has recently been discovered to play an additional role in this link, via the regulation of the differentiation/function of so-called tumour-associated macrophages (TAMs), which infiltrate tumours to promote cancer growth, angiogenesis and metastasis formation [57,58]. NF-κB

activation was demonstrated to maintain the alternative immunosuppressive phenotype (M2 phenotype) of TAMs and negatively regulate macrophage tumoricidal activity (M1 phenotype) [59,60].

Various experimental models have highlighted NF-κB's importance in the link between cancer and inflammation. In a mouse model of colitis-associated cancer (CAC), cell-specific ablation of IKKβ within intestinal epithelial cells (*i.e.*, enterocytes) or myeloid cells demonstrated that IKKβ-driven NF-κB activity contributes to the development of CAC through two distinct cell-type-specific mechanisms [61]. Selective inactivation of the *Ikbkb* gene within enterocytes resulted in an 80% reduction in CAC tumour multiplicity [61]. However, tumour size was not affected in this animal model, indicating that IKKβ-mediated NF-κB activity in enterocytes contributes to tumour initiation and/or early tumour promotion, rather than to tumour progression, most likely through the activation of anti-apoptotic genes and consequent suppression of apoptotic elimination of pre-neoplastic cells [61]. Conversely, deletion of IKKβ in myeloid cells, but not in enterocytes, resulted in only a 50% reduction in tumour multiplicity, but tumour volume was significantly reduced. Hence, it was concluded that in myeloid cells NF-κB promotes the production of cytokines that act as growth factors for pre-malignant enterocytes. One of these growth factors was subsequently identified as being IL-6, which is encoded by a NF-κB target gene. Indeed, the inhibition of IL-6 signaling resembled IKKβ ablation in myeloid cells, as tumour growth was inhibited with little effect on tumour multiplicity [61,62]. Another mouse model of inflammation-driven carcinogenesis highlighting the role of NF-κB in the tumour microenvironment is the multidrug resistance 2 (Mdr-2)-knockout mouse, in which the absence of the MDR2 phospholipid transporter leads to the accumulation of bile acids and phospholipids within hepatocytes, resulting in low-grade hepatic inflammation which ultimately gives rise to HCC [63]. Inhibition of hepatocyte NF-κB through the expression of a non-degradable variant of IκBα blocked the development of HCC and enhanced apoptosis in premalignant hepatocytes [64]. Although the initial stimulus leading to NF-κB activation in *mdr2*$^{-/-}$ mice was not defined, it appears to be associated with a chronic inflammatory response to free bile acids that is propagated via paracrine TNF production. This is because the administration of a neutralizing anti-TNFα antibody was shown to inhibit NF-κB activation in hepatocytes and to diminish expression of NF-κB-dependent anti-apoptotic genes [64].

The role of NF-κB in cancer is not always pro-tumorigenic. In a study by Maeda *et al.* (2005) of diethylnitrosamine (DEN)-induced HCC requires NF-κB activation in myeloid cells. The specific ablation of IKKβ in Kupffer cells (resident liver macrophages) resulted in a down-regulation of inflammatory cytokines such as IL-6 and TNFα and hepato-mitogens needed for the growth and survival of tumour cells, with concomitant reduction in tumour load [65]. By contrast, hepatocyte-specific ablation of IKKβ sensitized DEN-treated premalignant hepatocytes to increased cell death. However, owing to the regenerative capacity of the liver, increased cell death led to compensatory proliferation of initiated cells and an enhancement of HCC [65]. Hence, in this model of HCC, NF-κB inactivation in the liver resulted in an increase of HCC incidence, owing to the activation of Kupffer cells and concomitant release of pro-inflammatory mediators following the detection of necrotic debris, thereby supporting hepatocyte re-growth and the transmission of oncogenic mutations [65]. This compensatory proliferation is required for fixation of oncogenic mutations that would otherwise be lost in the absence of cell proliferation. Subsequent work by He *et al.* (2010), utilizing an experimental system that involved the transplantation of DEN-initiated hepatocytes into MUP-uPA mouse liver, allowed the examination of factors and mechanisms that affect the progression of initiated, pre-neoplastic hepatocytes into full-blown HCC, demonstrating that hepatocyte IKKβ-driven NF-κB suppresses malignant progression. IKKβ-driven NF-κB was shown to reduce late tumour promotion and progression of initiated hepatoma cells in this model, by preventing the accumulation of reactive oxygen species (ROS) that resulted in activation of the oncogenic transcription factor, STAT3, critical for HCC development [66]. Other mouse models indicating a tumour suppressor function for NF-κB include studies on the role of NF-κB in the skin, whereby disrupting NF-κB activity in keratinocytes was shown to induce squamous cell carcinoma (SCC). RelA null and IKKβ null mice as well as transgenic mice constitutively expressing IκB-SR in the epidermis develop keratinocyte hyperplasia, followed by SCC development. SCC development in these mouse models is dependent on TNF-R1 signaling, as TNF-R1 blockade resulted in the restoration of a normal epidermis [67-69].

In addition to the roles of NF-κB in tumorigenesis, the protective activity of NF-κB can hamper tumour cell killing inflicted by radiation or chemotherapeutic drugs and in doing so promote cancer resistance to anticancer treatments. NF-κB regulates several genes, including those encoding bcl-2, bcl-xL,

XIAP, survivin and AKT, whose expression has been reported to mediate chemoresistance and radioresistance in various tumour cells [44,45]. Ionizing radiation has been reported to activate NF-κB in both *in vitro* and *in vivo* models, while several chemotherapeutic agents including paclitaxel, vinblastine, vincristine, doxorubicin, daunomycin, 5-fluorouracil, cisplatin, tamoxifen, and bortezomib have been reported to induce NF-κB activation in different cell-types [44,45]. Accordingly, the development of several classes of NF-κB inhibitors for use in conjunction with chemotherapy and radiotherapy could be of enormous benefit in terms of blocking the various steps leading to NF-κB activation, and thereby sensitize tumour cells to the beneficial effects of chemotherapeutic drugs and radiation.

In addition to cancer, the NF-κB protective activity is critically involved in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel disease whereby inflammation is sustained by a positive feedback loop between TNFα and NF-κB [70-73]. Compelling genetic, biochemical and clinical evidence now demonstrates that inflammation is a key ethio-pathogenetic factor also in the development of metabolic diseases as well as of the complications that emerge from these pathologies, particularly in the context of obesity, insulin resistance, type-2 diabetes and atherosclerosis [74,75]. Consistent with its roles in immunity and inflammation, several studies have highlighted the key role that the IKK/NF-κB signaling pathway plays in the development of inflammation-associated metabolic diseases. Consequently, the NF-κB-mediated inhibition of PCD could play an important contributing role in the onset of metabolic disorders [74,75].

Certain inherited conditions are also characterized by constitutive activation of NF-κB, including familial expansile osteolysis (FEO) and familial Paget disease of bone (PDB), both caused by hypermorphic mutations of RANK and an exaggerated survival and function of osteoclasts [76]. Conversely, an impairment in cell survival is a key pathogenetic element in incontinentia pigmenti (IP) – a rare X-linked disorder usually caused by rearrangements of the IKKγ/NEMO gene – and other genetic illnesses characterized by various defects in the NF-κB activation pathway, including primary immunodeficiencies (ID) and anhidrotic ectodermal dysplasia (AED) syndromes [76,77].

5. TNF-R1 induced pathways of PCD

The pro-survival action of NF-κB was first discovered and is best understood in the context of the ligation and activation of the prototypical member of the "death receptors", TNF-R1, by TNFα [78-80]. TNFα is a pleiotropic cytokine that plays an important role in inflammation, immunity, cell growth, differentiation and the induction of PCD [79]. There are two receptors for TNFα, namely, TNF-R1 and TNF-R2. TNF-R1 is ubiquitously expressed, whereas TNF-R2 is mainly expressed in immune cells [79,80]. Although both receptors bind to TNFα, the main receptor mediating the cellular effects of TNFα in most cell types is TNF-R1. TNFα signaling through its receptors can trigger cell death [79,80]. Normally, however, despite this well documented ability to induce cell death, stimulation of TNF-R1 by TNFα does not result in cell death, unless NF-κB activation or new RNA/protein synthesis is blocked [81]. Hence, depending on the cell type, cell activation state and microenvironment factors, TNFα may result in cell survival, apoptosis or necroptosis. This functional trichotomy of TNF-R1 reflects the existence of an intricate network of signals that operate downstream of TNF-R1 and which can 'switch' between different patterns of response. Indeed, the biological outcome to the ligation of TNF-R1 depends on the sequential assembly and activation of multi-proteic complexes [78,80,82] (see Figure 1).

TNF receptors lack intrinsic enzymatic activity, thus, signaling by TNF-R1 and TNR-R2 is achieved by the recruitment of adaptor proteins that bind to their cytosolic region [78,80,82]. Upon binding of a TNFα homotrimer to trimerized TNF-R1, the intracellular portion of TNF-R1 recruits the adaptor protein TNFR-associated death domain (TRADD) through homotypic interaction between their death domains (a conserved protein-protein interaction motif of 80 amino acids contained in all DRs) [78,80,82]. TRADD serves as a platform to recruit downstream adaptor proteins and leads to the formation of a membrane-proximal supramolecular structure, that includes receptor-interacting protein 1 (RIP1), TNF-R associated factor 2 (TRAF2), TRAF5, cellular inhibitor of apoptosis (cIAP)-1 and cIAP2, termed *complex I* [78,80,82]. The assembly of this complex triggers cellular pathways that lead to the activation of NF-κB activation, as well as the JNK cascade [78,80,82]. Once this core complex is assembled, several ubiquitylation events occur the promote full assembly and function of *complex I*. Briefly, the term ubiquitylation refers to the covalent attachment of the small protein, ubiquitin, to target proteins. Ubiquitylation is a posttranslational modification that serves as recognition signals and can regulate, activate or inactivate proteins within signal

transduction cascades [83,84]. The ubiquitylation reaction involves the action of an ubiquitin-activating enzyme (E1) and an ubiquitin-conjugating enzyme (E2), which in the presence of an ubiquitin ligase (E3), mediate the attachment of ubiquitin to lysine residues or the N terminus of a target protein [83,84]. The E3 ubiquitin ligases, cIAPs, which were previously known as apoptosis inhibitors due to their functions in caspase inactivation [85], are recruited to *complex I* by TRAF2, which stabilizes them by preventing their polyubiquitylation [86]. cIAPs catalyze the attachment of Lys63-linked polyubiquitin chains to RIP1 [87], which allows the stabilization and recruitment of the IKK and the transforming growth factor β-activated kinase 1 (TAK1)-TAK1 binding protein (TAB) complexes [88]. Recent studies have introduced further levels of complexity in terms of TNFR-signaling. The E3 ligase activity of cIAPs have also demonstrated to be required for the efficient recruitment of an E3 ligase complex, known as linear ubiquitin chain assembly complex (LUBAC) [89]. The LUBAC complex, consisting of the HOIL-1, HOIP and Sharpin proteins, generates linear ubiquitin chains through the α-amino group of the N-terminal methionine residue of the ubiquitin molecule [83,84]. LUBAC binds linear ubiquitin chains to RIP1 and NEMO (IKKγ) leading to an overall stabilization and extension of the half-life of *complex I*, thereby allowing enhanced IKK activation [90]. The activated IKK complex leads to phosphorylation of IκBα, primarily mediated by IKKβ, with subsequent ubiquitylation and degradation by the proteasome, allowing the liberated NF-κB to translocate to the nucleus and activate transcription of an array of target genes that, ultimately, promote cell survival. Indeed, reduction of the various components of LUBAC (HOIL-1, HOIP and Sharpin) through genetic ablation demonstrates that absence of LUBAC results in reduced NF-κB activation, and increased TNFα-induced cell death [83,84,90].

In addition to NF-κB, TNF-R1 signaling leads to the activation of the JNK MAP kinase-signaling cascade (see Figure 1). RIP1 is also required for efficient JNK activation [91] and a number of MAP3 kinases have been implicated in the activation of JNK downstream of TNF-R1 based on the induction of their activity by TNFα and the phenotypes of various knockout model systems, including MEKK1, ASK1, and TAK1 [92-94]. MEKK1 and TAK1 have both been proposed to be essential for TNFα-induced JNK activation, based on signaling in deficient embryonic fibroblasts [95,96]. *Complex I* recruits and phosphorylates MAP3Ks, which in turn activate MKK4 and MKK7, leading to activation of JNK [78-80].

Despite the initial controversy in the role of JNK activation in TNF-induced cell death, it is now clear that in the majority of cases sustained JNK activation is pro-apoptotic.

The internalization of the TNF-R1 complex and modification of its conformation ensure the formation of a cytosolic, death-inducing signaling complex (DISC), better known as *complex II*, which leads to the promotion of either apoptosis or necroptosis [82]. The key event in the apoptotic signaling pathway of cell death is the activation of caspase proteases, which are cysteine proteases that cleave specific cellular substrates leading to cell death [97]. Normally these events result in cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation, plasma membrane blebbing, and the formation of membrane-bounded bodies containing the cellular structures and organelles [97]. *Complex II* formation occurs upon deubiquitylation of RIP1 by the enzyme cylindromatosis (CYLD), leading to the dissociation of the TRADD-RIP1-TRAF2 complex from TNF-R1 [98]. Caspase activation during TNFα-mediated apoptosis is achieved through the recruitment of Fas-associated death domain (FADD) and RIP3 (cognate kinase to RIP1) to this secondary complex [97,99]. FADD recruits and causes autocatalytic activation of the initiator caspases, caspase 8 and caspase 10 [97,99]. In *complex II*, caspase 8/10 cleaves and inactivates RIP1 and RIP3 kinases to preclude necroptosis, and initiates the pro-apoptotic caspase activation cascade [100-102]. In certain cells, activation of this extrinsic death receptor pathway is insufficient to induce cell death, and requires a mitochondrial amplification loop (the intrinsic apoptotic pathway). Mitochondrial function is an important checkpoint in PCD and mitochondrial integrity is necessary for maintenance of cellular homeostasis. Loss of mitochondrial integrity and the release of mitochondrial apoptogenic proteins, such as cytochrome c and second mitochondrial activator of caspases (SMAC), as a result of increased mitochondrial outer membrane permeabilization (MOMP), is a trigger for apoptosis [97,99,102]. Pro-apoptotic members of the Bcl-2 family of proteins, such as Bid (in its truncated tBid form following proteolytic cleavage by caspase 8), Bax and Bak, positively regulate cytochrome c release in response to death stimuli by acting directly on the outer mitochondrial membrane [97,99,102]. The subsequent disruption of mitochondrial membrane potential, results in the release of cytochrome c and Smac/DIABLO from the mitochondrial inter-membrane space into the cytoplasm [97,99]. Newly released cytochrome c, along with dATP, binds the adaptor protein apoptotic protease-activating factor 1 (APAF1), which leads to the formation of the so-called

apoptosome complex that recruits and activates the initiator caspase 9. Activated caspase 9 in turn activates effector caspases 3 and 7, leading to the cleavage of protein substrates and apoptotic cell death [97,99].

In addition to apoptosis, TNFα is capable of activating programmed necroptosis, a form of caspase-independent cell death whose molecular regulation is poorly understood [82,102]. Cellular swelling, organelle dysfunction, extensive mitochondrial damage and extensive plasma membrane rupture characterize necroptosis [82,102]. Failure of energy metabolism and massive generation of ROS are each thought to promote necroptosis [82,102]. Indeed, ROS can play a role in both apoptosis and necroptosis, whereby moderate oxidative stress generally enhances apoptosis execution, whereas high exposure to ROS results in necroptosis [103]. An important physiological consequence between the decision to undergo apoptosis or necroptosis, is that whereas necrotic cells release factors such as chromatin-associated HMG1 and heat shock protein 70 (HSP70), which serve as potent stimuli for inflammation [104], apoptotic cells suppress immunity and are rapidly engulfed by phagocytes with little inflammation, indicating a potential physiological roles for programmed necroptosis in inflammation and antiviral host defense [105]. Recent studies have revealed that following caspase 8 inhibition (by pharmacological inhibition or under certain biological circumstances like cellular stress), *complex II* is unable to trigger apoptosis [106,107]. Under these conditions the activation of TNF-R1 leads to the formation of a unique pro-necrotic *complex II* (necroptosome), which leads to necroptosis in a cell-type specific manner (see Figure 1). Although TRADD and FADD seem to be present in this multiprotein complex [106], various studies have now shown that mutual phosphorylation of RIP1 and RIP3 to be the crucial step in the stable assembly of this complex and in the induction of programmed necroptosis [100,105]. Unlike its role in the activation of NF-κB and JNK, the pro-necrotic role of RIP1 requires its kinase activity [102]. Indeed, Degterev *et al.* (2008) identified the small molecules necrostatin 1 and necrostatin 2 as specific inhibitors of RIP1 kinase activity [108]. In doing so, these molecules inhibited necroptosis, but the RIP1-mediated activation of NF-κB and JNK was unaffected [108]. The distinct molecular mechanism by which the RIP1/RIP3-assembled necroptosome contributes to the execution of TNF-R1-iniated programmed necroptosis is presently unclear. However, most studies are focusing on its potential link to mitochondrial disruption and exacerbation of ROS release. The first link between mitochondrial energy metabolism and the execution of necroptosis was established in the early 1990s, when

Schulze-Osthoff *et al.* (1992) demonstrated that ROS production by mitochondrial respiratory complex I is essential for the necrotic response of L929 cells to TNFα [109]. Although ROS production is not essential in all cases for TNF-induced necroptosis, the kinase activity of RIP3 may potentially link TNF-R1 signaling, mitochondrial energy metabolism and ROS overproduction. Although the downstream effects of the RIP3 kinase activity are uncertain, RIP3 has been demonstrated to physically interact with and activate several enzymes within metabolic pathways, including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase (GLUD1), leading to an increase energy metabolism-associated ROS production. Indeed, RNAi-mediated knockdown of any of these enzymes attenuates TNFα-mediated ROS production and necroptosis [110].

6. The JNK MAPK cascade

The JNK pathway, also formerly known as the stress activated protein (SAP) kinase pathway, is one of the major mitogen activated protein kinase (MAPK) cascades mediating the intracellular transduction of signals. Like the other two main MAPK cascades – the p38 and the extracellular-regulated kinases (ERK) cascades – the JNK cascade transduces signals triggered by diverse stimuli through the sequential phosphorylation of hierarchically arranged, kinase modules in order to elicit an appropriate cellular response [111-113]. The JNK signaling pathway is activated through a 3-tier kinase cascade that includes multiple MAP3Ks, two MAP2Ks (MKK4 and MKK7), and three JNKs depending on the tissue, stimulus and biological context [111-113]. In mammals, 3 genetic loci encode the terminal enzymatic modules of this cascade − JNK1, JNK2 and JNK3 − each of which has 2 to 4 isoforms that results from the alternative splicing of the corresponding pre-mRNAs [111-113]. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is expressed predominantly in the brain and to a lesser extent, in the heart and testis [111-113]. JNK kinases are primarily activated upon exposure to pro-inflammatory cytokines such as TNFα and IL-1β or stress stimuli such as UV radiation and genotoxic, osmotic, oxidative and hypoxic stress [111-113]. Induction of the JNK cascade by these stimuli has been linked to different biological responses, including growth and differentiation. However, a large body of evidence exists that suggests that the activation of JNK is in most cases a trigger for cell death and inflammation.

Evidence for the involvement of JNK in PCD signaling originated from studies using mouse embryonic fibroblasts (MEFs) derived from *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ knockout animals. MEFs lacking both JNK1 and JNK2 demonstrated resistance to apoptosis in response to UV irradiation [114]. This defect in apoptosis was correlated with a lack of cytochrome c release from mitochondria and subsequent caspase activation. Moreover, thymocytes and peripheral T cells from either *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice are seemingly protected against anti-CD3-induced death and ACID, respectively [111,115]. Consistent with this pro-apoptotic role of JNK is the observation that T cells deprived of either JNK1 or the JNK kinase, MKK7/JNKK2, mount an exaggerated proliferative reaction to antigen stimulation [111,115-117]. The role of JNK3 in apoptosis was also established using *jnk3*$^{-/-}$ mice. These mice were observed to be resistant to excitotoxic agent (glutamate, kainite)-induced apoptosis of their hippocampal neurons compared to wild-type control animals [118].

Despite this large body of work indicating a pro-apoptotic role for JNK, some initial studies had suggested that JNK activation by TNFα either enhanced or did not affect cell survival [119,120]. This issue has now been clarified by the analyses of JNK and NF-κB-deficient models. The use of these models has brought about a general agreement that JNK signaling plays an obligatory role in TNF-R inflicted killing in many systems, and that the targeting of JNK signaling is an important means by which NF-κB suppresses PCD [8,9,121]. Normally, cells survive TNFα treatment due to the fact that TNFα triggers potent, but only transient activation of the JNK cascade, with basal levels rapidly re-established generally within 30 to 60 minutes [122-124]. Blocking NF-κB activity, however, by either the knockout deletion of RelA or IKKβ, or ectopic expression of IκBαM, a degradation-resistant variant of IκBα, markedly impairs the normal shutdown of TNFα-induced JNK signaling, thereby unveiling an additional sustained phase of JNK signaling [122-124]. Indeed, it is this prolonged phase of JNK induction by TNFα that has been implicated in the activation of PCD [122-124]. Therefore, in the presence of NF-κB, JNK activity is elevated by TNFα only transiently, which explains why this transient activation occurs without significant cytotoxicity. In NF-κB-deficient cells, however, JNK induction by TNFα remains sustained even after anti-apoptotic treatment with the caspase blocker, Z-VAD$_{fmk}$ [121,124]. Hence the inhibitory effects of NF-κB on the JNK pathway do not appear to be an indirect consequence of suppressing the activation of caspases, despite the fact that these

proteases are potentially capable of activating MAP3Ks [97]. This ability of NF-κB to control the sustained activation of JNK signaling is a pivotal protective mechanism against TNF-R-induced cytotoxicity.

Consistent with this concept, suppression of JNK signaling by either pharmacological means or expression of dominant-negative kinase mutants effectively rescues NF-κB-deficient cells from TNFα-induced cell death [122-124]. Likewise, either compound deletion of JNK1 and JNK2 or the silencing MKK7 expression virtually abrogates TNFα-induced death in *relA*$^{-/-}$, *ikkβ*$^{-/-}$ or IκBαM-expressing cells [125-127]. Further evidence highlighting the biological relevance of this antagonist crosstalk between the NF-κB and JNK pathways in promoting cell survival was demonstrated *in vivo*. Analyses of IKKβ/JNK1 double mutants showed that the absence of JNK1 − one of the two major JNK isoforms expressed in the liver − delayed embryonic mice lethality due to compromised NF-κB activation, suggesting that JNK signaling is an important mediator of apoptosis in the liver during embryogenesis and that the suppression of JNK signaling is a key mechanism by which NF-κB promotes survival during liver development [128]. In a mouse model of TNFα-mediated liver injury caused by the systemic administration of concavalin A (ConA), NF-κB induced anti-apoptotic effects by attenuating JNK activation [129]. This was demonstrated by the observations that hepatocyte-specific deletion of IKKβ enhances the activation of JNK and that suppression of this response by the additional ablation of either JNK1 or JNK2 reduces ConA-induced liver injury [129]. Generation of double mutants mice harboring specific inhibition of both IKKβ and JNK1 (IKKβ$^{\Delta hep}$/*jnk1*$^{-/-}$) provided additional evidence for crosstalk between the NF-κB and JNK pathways in promoting cell survival. Indeed, JNK1 deletion reversed increased hepatocyte death and the susceptibility of mice to HCC development in the absence of IKKβ [130]. Hence, the available evidence indicates that an abrogation of sustained activation of the JNK cascade is crucial for control of TNFα-induced PCD, and furthermore, that this abrogation is critically dependent on NF-κB.

7. How does JNK activation execute cell death?

A mechanistic site of action for JNK in apoptosis appears to be in the mitochondria. The most conclusive evidence for this role comes from a study where *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ primary MEFs were found to be resistant to UV radiation due to protection against cytochrome c release and mitochondrial depolarization [114]. NF-κB activation inhibits the ability of TNF-RI to induce apoptosis by maintaining high levels of

cFLIP, a specific inhibitor of caspase 8/10 activation [128]. Chang *et al.* (2006) demonstrated a link between JNK activity and cFLIP, whereby TNFα was found to induce proteosomal degradation of the anti-apoptotic protein cFLIP in wild-type but not JNK1-deficient cells [128]. JNK1, however, does not phosphorylate cFLIP directly and instead leads to the phosphorylation-dependent activation of the ubiquitin ligase Itch. Itch specifically interacts with the long isoform of cFLIP, $cFLIP_L$, promoting its polyubiquitylation and subsequent proteosomal degradation [128]. Since $cFLIP_L$ impairs the recruitment of pro-caspase 8 to *complex II*, the JNK1-induced degradation of $cFLIP_L$ allows for full activation of caspase 8, resulting in cell death [128]. Evidence also indicates that prolonged JNK activation promotes the processing of the BH3-domain protein Bid to the pro-apoptotic protein jBid (akin to caspase 8-cleaved Bid, tBid) [131]. This proteolytic fragment, jBid, specifically induces the release of mitochondrial Smac/DIABLO, an apoptotic protein that displaces c-IAP1 from the TNF-R complex and consequently allows caspase 8 activation and apoptosis initiation [131]. JNK has also been observed to modulate the activities of other pro-apoptotic BH3-domain containing members of the Bcl-2 family, such as Bim and Bmf [132]. Bim and Bmf are released from sequestering complexes following their phosphorylation by JNK, and once released phosphorylated Bim and Bmf play a role in activating the mitochondrial proteins Bax and Bak, thereby stimulating the release of cytochrome c from the mitochondrial inner membrane, leading to caspase-dependent apoptosis [132,133]. 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 deficient in Bax or Bak [134]. In terms of the nuclear signaling of JNK in the regulation of apoptosis, phosphorylation and nuclear translocation of JNK results in the transactivation of c-Jun. Phosphorylation of c-Jun leads to the formation of activator protein 1 (AP-1), a transcription factor complex composed of homodimers and/or heterodimers of Jun and Fos proteins [111,112]. Early *in vitro* studies indicated that increased AP-1 activity could lead to apoptosis in specific cell types. It has been noted that the JNK-AP-1 pathway is involved in the increased expression of pro-apoptotic genes such as Bim, Fas ligand, and TNFα [111,112].

8. Downstream effectors of NF-κB-mediated control of JNK signaling

The primary mechanism through which NF-κB secures an effective shutdown of the JNK pathway following the triggering of TNF-Rs involves the activation of a select subset of NF-κB target genes. These

NF-κB-regulated genes have been shown to suppress JNK signaling via different mechanisms, as discussed below (see also Figure 2):

8.1 Gadd45β

Following the implementation of a screen for cDNAs capable of blocking TNFα-induced PCD in *relA*$^{-/-}$ fibroblasts, Gadd45β/Myd118 was identified as a bone fide inhibitor of the JNK pathway [135]. Gadd45β is a member of the growth arrest and DNA-damage inducible (Gadd) 45 family of factors, which also includes Gadd45α and Gadd45γ [136]. Gadd45 genes encode for small (18 kDa), evolutionarily conserved proteins that are highly homologous to each other (55–57% overall identity at the amino acid level), are acidic, and are primarily, but not exclusively, localized within the cell nucleus [136]. Gadd45 proteins are induced under a wide variety of stress conditions, including cell cycle arrest, DNA repair, cell survival or apoptosis and modulation of immune response [136].

Gadd45β is induced rapidly by TNFα and other inflammatory stimuli through a transcriptional mechanism that is dependent on NF-κB [135]. The expression of Gadd45β is dependent on the binding of RelA-containing NF-κB complexes to three κB elements within the promoter region of its gene [137], and this may explain in part why *relA*$^{-/-}$ cells have been shown to be highly sensitive to TNFα-induced cell death and apoptosis induced by other DNA-damaging agents [135,137]. Indeed, the ectopic expression of Gadd45β rescued NF-κB null cells from TNFα-induced killing [135]. Most importantly, the inactivation of endogenous Gadd45β by either expression of antisense mRNAs or targeted deletion of the Gadd45β gene impairs cell survival and the downmodulation of JNK activity downstream of TNF-Rs, suggesting that in some tissues Gadd45β is required for cell survival through a NF-κB-mediated antagonism of JNK activation and PCD [135].

The Gadd45β-mediated inhibition of this JNK activation involves a direct blockade of the JNK kinase MKK7/JNKK2, and this interaction between Gadd45β and MKK7 represents a crucial link between the NF-κB and JNK pathways [138]. A model analysis of the Gadd45β/MKK7 complex predicts that Gadd45β binds to crucial residues in the catalytic pocket of MKK7, including the ATP binding residue, Lysine 149, thereby obstructing its access to ATP [138,139]. MKK7 is a selective activator of JNK, and its ablation in fibroblasts abolishes JNK induction by TNFα [140]. Thus, blocking this MAP2K is seemingly

sufficient alone to account for the specific and effective inhibition of the JNK cascade by Gadd45β downstream of TNF-Rs. Although Gadd45β has also been shown to interact with other constituents of MAPK pathways, such as the MAP3Ks, MEKK4/MTK1 and ASK1/MEKK5, it appears that these other interactions of Gadd45β are not involved in the control of JNK activation in the context of TNFα signalling [138]. With regard to the *in vivo* role of Gadd45β in cytoprotection, Gadd45β was demonstrated to have an essential role in the protection against JNK-induced hepatocyte death in a mouse model of liver regeneration after partial hepatectomy (PH) [141]. Gadd45β is consistently upregulated during the priming phase of liver regeneration following PH, a response that is coordinated by TNF-R1 through NF-κB/JNK crosstalk [141]. It was reported that whereas wild-type mice generally recover after PH, the survival rate of *gadd45β*$^{-/-}$ mice is dramatically reduced [141]. These mice exhibited impaired hepatocyte proliferation and increased PCD during liver regeneration [141]. Significantly, JNK and MKK7 activities were increased and sustained in the livers of *gadd45β*$^{-/-}$ mice compared with control animals after PH [141]. Indeed, *JNK2* double mutant mice harbouring the compound deletion of *gadd45β* (*gadd45β*$^{-/-}$/*jnk2*$^{-/-}$) exhibited reduced hepatocyte death and increased proliferation, thereby corroborating the role of Gadd45β in promoting cell survival through the modulation of JNK-mediated PCD [141]. Interestingly, Svensson *et al.* (2009) recently demonstrated that Gadd45β deficiency contributes to an enhanced MKK7 and JNK responses a mouse model of rheumatoid arthritis (RA), leading to increased expression of matrix metalloproteinases (MMPs) in the RA synovial tissue, and an exacerbation of joint destruction [142].

This body of work provides strong evidence that the protective activity of Gadd45β against TNFα-induced PCD involves suppression of the JNK cascade by means of MKK7 inactivation. Further evidence indicates that this Gadd45β-afforded suppression of JNK is crucial to the pro-survival activity of NF-κB. Gadd45β expression in MEF cells and 3DO T cells was elevated rapidly by TNFα through a NF-κB-dependent mechanism, which is essential for the antagonism of TNFα killing [143,144]. This anti-apoptotic role of Gadd45β was further supported by work from other groups, which demonstrated that overexpression of Gadd45β suppresses IL-1β induced apoptosis in insulin-producing cells, and that Gadd45β alleviated UV-induced apoptosis in hematopoietic cells by suppressing the JNK cascade [143,144]. However, this anti-apoptotic mechanism is subject to challenge by the findings of Engelmann *et al.* (2008), who demonstrated

that high Gadd45β protein levels does not protect tumour cells against UV or γ-irradiation induced cell apoptosis, but rather confers a strong and specific survival advantage to serum withdrawal [145]. Thus, it is possible that the magnitude and direction of Gadd45β regulation of cell fate varies depending on the cell type as well as on the properties of the stimuli. This degree of complexity in terms the biological function was further elucidated by *in vivo* studies investigating the role of Gadd45β in the modulation of the immune response. In a study by Liu *et al.* (2005), Gadd45β was demonstrated to regulate the homeostasis of $CD4^+$ T cells. T cells lacking Gadd45β proliferated faster than wild-type controls and demonstrated increased resistance to activation-induced cell death (AICD) *in vitro* [146]. Furthermore, *gadd45β*$^{-/-}$ mice displayed an exacerbated form of experimental autoimmune encephalomyelitis (EAE) compared to wild-type controls, likely owing to the Gadd45β anti-proliferative and pro-apoptotic functions in T cell homeostasis [146].

8.2 XIAP

The inhibitory effects of NF-κB on the JNK cascade have also been associated with the upregulation of the caspase inhibitor, XIAP, a member of the inhibitor of apoptosis protein (IAP) family [147]. IAPs are classed in accordance with the existence of one to three baculovirus IAP repeat (BIR) domains within their structure [147]. XIAP contains three BIRs that can bind to and inhibit the activation of caspase 3, 7 and pro-caspase 9 [148]. XIAP has also been reported to be a downstream target of NF-κB, and its induction by TNFα is partially reduced in *relA*$^{-/-}$ MEFs [148]. Overexpression of XIAP was shown to inhibit TNFα-induced cytotoxicity and diminish activation of JNK in NF-κB-deficient cells [148]. Furthermore, thymocytes from XIAP-transgenic mice are resistant to apoptosis induced by various triggers [149]. Apart from its ability to block caspases, XIAP can suppress the JNK cascade, because when overexpressed in RelA null cells, it is capable of interfering with both the caspase-dependent and caspase-independent phases of JNK activation by TNFα [121,148]. However, the significance of XIAP to NF-κB-dependent survival is uncertain as *xiap*$^{-/-}$ mice exhibit no obvious apoptotic phenotype [150] and XIAP ablation in MEFs does not affect the kinetics of JNK induction by TNFα [151]. The precise mechanisms by which XIAP blocks JNK activation and ultimately PCD are still a matter of debate. The function of XIAP in death-receptor signaling has classically been associated with the presence of BIR domains and the direct inhibitory interaction with effector caspases [152]. However, many cellular IAPs contain a second class of domain, a C_3HC_4 RING

finger motif that has recently been shown to possess E3 ligase activity, and is capable of targeting signaling proteins for ubiquitylation [153]. In a study by Kaur *et al.* (2005) the RING domain of XIAP was shown to mediate the polyubiquitylation of the MAP3K, TAK1, triggering the targeted proteosomal degradation of this kinase [154]. Subsequent downregulation of TAK1 protein expression resulted in the inhibition of TGF-β1-mediated activation of JNK and apoptosis in murine hepatocytes [154]. This represents a novel mechanism by which XIAP could potentially suppress JNK activation and confer resistance to PCD, through the ubiquitin-mediated proteosomal degradation of crucial components of the JNK signaling pathway.

8.3 A20

Another target of NF-κB that is seemingly involved in mediating the inhibitory activity of NF-κB on the JNK pathway is the zinc finger protein, A20. Interestingly, initial studies indicated that the NF-κB target, A20, downregulated its own expression [155], indicating that A20 participated in a negative feedback loop to block NF-κB activation in response to pro-inflammatory signals, in order to attenuate and control inflammatory responses. Several models have validated this early hypothesis [156]. The most robust model involved the generation of A20 null mice, which succumb perinatally to systemic inflammation and multi-organ failure as a result of unchecked NF-κB activity [157]. Recently, substantial progress has been made in understanding the biochemical and molecular mechanisms by which A20 constitutes a negative feedback loop to terminate NF-κB activation. A20 possesses dual ubiquitin editing functions to RIP1, a critical signaling molecule in TNFα-mediated NF-κB activation [158]. In brief, ubiquitin editing can be broadly conceptualized as the removal of modifications that promote signaling complex assembly and activation, such as Lys63-linked poly-ubiquitylation, linear ubiquitin chains or even ligation with small ubiquitin-like modifier (SUMO), followed by the addition of modifications that promote substrate degradation, such as Lys11 or Lys48 polyubiquitylation, resulting in the attenuation of signalling [158]. A20 is capable of deubiquitinating Lys63-linked polyubiquitin chains of RIP1 by its N-terminal ovarian tumour (OTU) domain and adding Lys48-linked polyubiquitin chains by the C-terminal zinc-finger (ZnF) containing domain [159-161]. In addition to RIP1, A20 has been shown to target other ubiquitinated proteins in the NF-κB signaling pathway, such as TRAF6 and CIAP1 [158,162,163].

Given the pro-survival function of NF-κB and the ability of A20 to terminate NF-κB activity, A20 is expected to possess a potent pro-apoptotic function. This pro-apoptotic effect contributes to the tumour-suppressor function of A20 in lymphomas, where the re-introduction of wild-type A20 in A20-inactivated lymphoma cells promotes cell death [164,165]. However, this effect seems to be cell type-specific and context-specific, since accumulating evidence suggests the opposite, whereby A20 acts mainly as an anti-apoptotic protein, as it protects most cells from TNFα-induced cell death. Accordingly, *a20-/-* MEFs exhibit persistent JNK activation and exaggerated PCD following exposure to TNFα [157,162]. Additional A20 null cells, such as splenocytes and enterocytes, display enhanced sensitivity to TNFα-induced apoptosis [157,160]. In terms of the precise mechanism(s) by which A20 blunts activation of JNK cascade, RIP1 does not participate in the induction of JNK by TNF-Rs, therefore the A20-mediated suppression of the JNK pathway is likely to be mediated through a mechanism that does not involve the A20 ubiquitin-mediated inactivation of RIP1 and TNFR1 signaling complex formation. Indeed, in a recent study by Won *et al.* (2010), A20 was shown to blunt TNFα-induced JNK activation in cells by reducing the stability and promoting the degradation of ASK1, an important MAP2K in the JNK signaling pathway, through its ubiquitin editing activity [167]. Additional studies are required in order to further elucidate the functional characteristics of A20 in animal patho-physiology.

9. NF-κB mediated survival involves a suppression of ROS

Recent studies have unveiled another mechanism by which NF-κB blocks cell death triggered by TNFα-induced JNK cascade activation that involves the suppression of the accumulation of ROS [126,168,169]. ROS, which are produced by all types of mammalian cells, is a collective term that includes not only oxygen radicals (superoxide ($O_2^{\bullet -}$) and hydroxyl (OH•)) but also some non-radical derivatives of molecular oxygen (O_2), such as hydrogen peroxide (H_2O_2) [11,102,170]. Although ROS production is mostly thought to occur under pathological conditions, ROS are continuously generated during normal aerobic metabolism primarily by the mitochondrial electron-transport chain and peroxisomes [11,102,170,171]. Electron leakage from the electron transport chain results in the formation of the superoxide radical, a moderately reactive species that can generate H_2O_2, which in turn generates highly reactive hydroxyl radicals. Although, the mitochondria are by far the greatest source of cellular ROS, extra-mitochondrial

sources of ROS also exist. NAPDH oxidases are a family of enzymes that are dedicated to ROS production [172]. Activated cells of the innate immune system, such as macrophages and neutrophils, activate the phagocytic form of NAPDH oxidase, NOX2 (formerly known as gp91phox), in order to produce superoxide for defence against invading microbial pathogens. Various types of non-phagocytic cells, including endothelial cells, vascular smooth muscle cells, fibroblasts and cardiac myocytes are also known to produce ROS via NADPH oxidases [172]. Additional ROS-generating enzymes include lipoxygenases and cyclooxygenases, involved in inflammatory pathways [11,102,170]. Peroxisomes are essential cellular organelles that perform important metabolic functions in organisms. Mammalian peroxisomes are densely populated by enzymes that produce ROS, the majority of which are FAD-dependent oxidases − which generate H_2O_2 as a by-product [171]. Some other observations indicate the presence of the $O_2\bullet^-$-producing enzyme xanthine oxidoreductase [171]. Owing to their high reactivity, ROS represent a serious hazard for the cell, as they can oxidize macromolecules, thus damaging proteins, lipids and DNA. ROS accumulation, referred to as oxidative stress, results in cytotoxicity [11,102,170]. Hence, cell viability depends on diverse antioxidant systems for ROS detoxification. Under normal conditions, low levels of intracellular ROS are strictly maintained by systems of anti-oxidant enzymes and their substrates, such as the glutathione and thioredoxin systems, superoxide dismutases, catalase, and peroxiredoxins, as well as other non-enzymatic anti-oxidants, which collectively scavenge ROS [11,102,170]. Although ROS are rapidly eliminated and kept at very low levels, certain extracellular stimuli enhance ROS production, in which case ROS may serve as second messengers in downstream signaling pathways such as the TNFα signaling pathway. Indeed, TNFα has been reported to induce ROS accumulation in a variety of cell types and these ROS have been shown to be important mediators of PCD [11,102,170].

ROS are potent inducers of JNK in response to TNFα and other stimulus. Indeed, a substantial body of evidence indicates that NF-κB inhibits JNK activation, hence PCD, by controlling the accumulation of ROS induced downstream of TNF-Rs and other receptors [126,168,169]. ROS are key mediators in the death signaling pathways initiated by the triggering of TNF-R1. This physiological role of NF-κB in the restraint of ROS accumulation was initially suggested in studies utilizing NF-κB-deficient cells. Unlike what is seen in normal cells, treatment with TNFα leads to exaggerated accumulation of ROS in NF-κB-deficient cells,

indicating that these cells are defective in their ability to maintain redox homeostasis [126,168,169]. Consistently, exposure to anti-oxidant agents such as N-acetyl-cysteine (NAC), butylated hydroxyanisole (BHA) or pyrrolidine dithiocarbamate (PDTC) virtually abrogated TNFα-inflicted killing in NF-κB-deficient cells [126,168,169]. In terms of how ROS accumulation induces PCD, evidence suggests ROS inflicted cell death is mediated in part by a prolonged activation of the JNK cascade. In *relA*$^{-/-}$ fibroblasts and other NF-κB-deficient cells, sustained activation of JNK signaling downstream of TNF-Rs is abolished by treatment with the ROS neutralizing agents, NAC and BHA [126,168,169]. Several studies have established ROS as a link between the TNFα-induced anti-apoptotic NF-κB and pro-apoptotic JNK pathway both *in vitro* and *in vivo*, whereby, the attenuation of ROS represents an alternative indirect mechanism through which NF-κB exerts a restraint on sustained activation of the JNK cascade and subsequent induction of PCD. In the mouse model of DEN-induced HCC, specific deletion of IKKβ in hepatocytes resulted in enhanced oxidative stress ROS accumulation after DEN administration, resulting in JNK activation and compensatory proliferation of hepatocytes, with resultant HCC development [65]. Administration of the anti-oxidant BHA reversed the elevation in HCC load caused by IKKβ loss due to the blockade of prolonged JNK activation and compensatory proliferation. BHA was also shown to protect IKKβ$^{\Delta hep}$ mice from ConA-induced liver failure by elevating ROS accumulation and sustained JNK activation [126]. Two important questions that are raised from this study are firstly, how does ROS accumulation lead to prolonged JNK activation? Secondly, how does NF-κB activation prevent ROS accumulation?

10. Mechanisms of ROS-mediated activation of JNK

In terms of elucidating how ROS accumulation results in prolonged JNK activation, a problem arises with defining ROS targets due to the fact that ROS have a mixture of roles in cell death. ROS may directly oxidize cellular proteins, lipids or nucleic acids and therefore cause general cellular damage, or ROS can initiate cell death through acting as either initiators or second messengers in various signaling pathways [11,102,170]. Another problem with defining the role of ROS is that they may function at multiple points within a given signaling pathway. Generally, the magnitude and duration of JNK activity is determined by a balance between activating kinases and inhibitory phosphatases, establishing two possible strategies for ROS to enhance JNK activation [11, 102,170]. In one mechanism, JNK activity is enhanced by the ROS-mediated

inactivation of JNK phosphatases [126]. A study by Kamata *et al.* (2005) demonstrated that phosphatases of the MAP kinase phosphatase (MKP) family (MKP-1, MKP-3, MKP-5 and MKP-7) − which are known to be involved in down modulation of MAPK activation − are targets of ROS in the TNFα-induced pathway of JNK activation and PCD [126]. TNFα-induced ROS was shown to oxidize critical cysteine residues in the catalytic site of various MKPs, leading to their inactivation and rapid degradation by the ubiquitin-proteasome pathway [126]. This ROS-mediated inactivation of MKPs impairs the shutdown of JNK signaling following exposure to TNFα, thus prompting persistent activation of JNK, and ultimately cell death [126]. Additionally, studies undertaken by Matsuzawa *et al.* (2005) indicate that ROS also trigger the activation of ASK1, a TRAF2-associating MAP3K that is essential for activation of JNK (and p38) downstream of TNF-R1 [173]. The reduced form of thioredoxin has been reported to bind to ASK1, thereby preventing its kinase activity. Following its ROS-induced oxidation, thioredoxin is released and ASK1 is activated [174]. Since *ask1*$^{-/-}$ MEFs are substantially resistant to the sustained JNK activity and apoptosis initiated by ROS such as H_2O_2 [175], the ROS-thioredoxin-ASK1 axis is believed to be an important molecular switch that may mediate ROS-dependent signaling to JNK, resulting in JNK activation. Taken together, these studies suggest that TNFα-induced ROS manipulate JNK activity by promoting the persistent activation of ASK1 [173-175] and simultaneously blocking the inhibitory MKPs, thus shifting the balance toward prolonged TNFα-induced JNK activation [126]. The relative importance of each mechanism for control of TNFα-induced JNK activity is likely to depend on the cell-type and biological context. There is now a general consensus that more often than not ROS lie upstream of JNK in the TNF-R-induced pathways of PCD.

11. The cellular origin of TNFα-induced ROS

The source of ROS produced downstream of TNF-Rs is still currently a subject of debate. TNFα-stimulated ROS has traditionally been proposed to come from downstream events involving the mitochondria [11,102,170]. However, more recently TNFα has been shown to cause the production of superoxide through the activation of NAPDH oxidases. Several lines of evidence have pointed to an involvement of mitochondrial-derived ROS in TNFα-induced PCD. Initial studies showed that treatment with TNFα results in damage and dysfunction of the mitochondrial electron transport chain thereby leading

to ROS production [11,102,170]. Furthermore, butylated hydroxyanisole, an anti-oxidant that accumulates in the mitochondria, protects cells from TNFα-induced PCD [126]. Evidence for an extra-mitochondrial, source of signal-transducing ROS induced in response to TNFα stimulation was presented when ectopic expression of the mitochondrial anti-oxidant enzyme, manganese superoxide dismutase (Mn-SOD), was shown to afford only limited protection against TNF-R-mediated cytotoxicity in NF-κB null cells [168,169]. NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. Most NOX enzymes primarily consist of two NADPH oxidase subunits, NOX2 and p22phox. They form a heterodimeric flavocytochrome b558 ('cyt b558') that constitutes the catalytic core of the enzyme, but exists in a dormant state in the absence of the other subunits. These additional subunits play mostly regulatory roles ensuring activity and membrane translocation, and are located in the cytosol during the resting state. They include the multi domain proteins p67phox, p47phox and NOXO1, as well as the small GTPase Rac, which is a member of the Rho family of small GTPases [170,172]. Numerous reports have demonstrated that TNFα signaling increases transcription of various NADPH oxidase components, resulting in TNFα-induced oxidase activity. TNFα has also been recently demonstrated to be a direct activator of NAPDH oxidases in various cell types. In L929 cells, Nox1, NOXO1 and Rac1 form a complex with TNF-R signaling components in a TNF-dependent manner, leading to cell death [176]. One mechanism suggested as a means of Nox1 activation involves the interaction between the TNF receptor complex adaptor proteins RIP1 and TRADD with NOXO1. Expression of a dominant negative TRADD with a mutation in its polyproline eliminated NOXO1 binding and diminished superoxide formation and cell death in TNFα-treated L929 cells [176]. RIP1 was also shown to be involved in the recruitment of Nox1 and Rac1 to the TNF-R signaling complex [176]. Based on the NOXO1 affinities for RIP1 and TRADD, it has been proposed that RIP1 recruits NOXO1 and the other signaling components (Nox1/NOXA1/Rac1) to the TNF-R complex, where the TRADD-NOXO1 interaction promotes oxidase activation [176]. Further studies will be required to determine the precise mechanisms involved in ROS production in response to TNFα and other inflammatory stimuli.

12. The NF-κB mediated targeting of ROS controls the JNK pathway

One of the most significant ways in which NF-κB activity has been shown to influence ROS accumulation is via increased expression of target genes encoding anti-oxidant proteins, as discussed below (see Figure 2):

12.1 FHC

Through the utilization of a gene array-based screen, ferritin heavy chain (FHC) was shown to be a critical mediator of the antioxidant and anti-apoptotic activities of NF-κB downstream of TNF-Rs [169,177]. Ferritin is a highly conserved and ubiquitously expressed iron storage protein that consists of two subunits, FHC and ferritin light chain, which sequester excess free iron molecules to minimize the generation of iron-catalyzed ROS [178,179]. FHC possesses a ferroxidase activity, which converts toxic Fe^{2+} into nontoxic Fe^{3+}, whereas FLC has no ferroxidase activity but is likely to contribute to stabilization of assembled ferritin proteins for long-term iron storage [178,179]. In eukaryotes, iron is required both for iron-dependent reactions and processes such as erythropoiesis, as well as for production of mitochondrial $\bullet O_2^-$ and for generating highly reactive •OH radicals from H_2O_2 (Fenton reaction) [178,179]. Therefore limiting the availability of this metal, through an upregulation of ferritin, represents a mechanism by which cellular ROS can be controlled. It should be noted however, that although large amounts of free iron is toxic, a small amount of free cytosolic iron – the so-called labile iron pool (LIP) – is tolerated by the cell [169,178,181]. Consequently, overexpression of FHC results in the down-regulation of LIP and lower levels of reactive oxygen species [169,180].

FHC is induced by TNFα through a mechanism dependent upon NF-κB and is required for antagonism of TNFα-induced killing [169]. FHC overexpression was also demonstrated to counter TNFα-induced apoptosis in NF-κB-deficient cells [169]. The protective action of FHC against TNFα-inflicted PCD is mediated through iron sequestration, which prevents ROS accumulation and the subsequent sustained activation of JNK downstream of TNF-Rs. The relevance of FHC to the NF-κB-activated mechanism for restraint of TNF-R-induced JNK signaling and PCD has been validated in FHC-deficient cells *in vitro* and *in vivo* systems [169,177,181]. Indeed, knockdown of FHC in fibroblasts results in persistent activation of JNK by TNFα and hypersensitivity to TNF-R-induced cytotoxicity. The induction of FHC appears to be a critical element for control of the TNFα-induced fluctuations of the intracellular labile iron pool [169,177,181,182].

Significantly, systemic administration of the iron chelator, deferoxamine (DFO), has been reported to protect mice against TNF-R-mediated lethality and tissue damage, thereby suggesting the relevance of this mechanism *in vivo* [181,182].

An elevation of ROS is often required for oncogene-driven transformation [179,183]. On the other hand, this elevation can also enhance the propensity of certain malignant cells to undergo cell death. On balance, accumulating evidence suggest that FHC plays an important role in cancer. For instance, an increased expression of FHC has been observed in colon cancer cell line that showed a highly malignant phenotype i*n vivo* [184]. Increased ferritin concentration in tumour versus normal tissue has also been shown in several human malignancies, such as colon cancer [185], breast cancer [186], seminoma [187] and renal cell carcinoma [188]. In a recent study on cutaneous T-cell lymphoma (CTCL), inhibition of the NF-κB pathway by specific inhibitors caused an iron- and ROS-dependent, but caspase- and cathepsin-independent, cell death of CTCL cell lines and of primary cells from patients with Sézary syndrome − a leukemic variant of CTCL [177]. Interestingly, the induction of oxidative stress and cell death following the inhibition of NF-κB in these cells was ablated by DFO treatment [177]. Moreover, the utilization of a murine T-cell lymphoma model demonstrated that NF-κB inhibition significantly delays tumour growth *in vivo* by down-regulating FHC [177]. Collectively, this evidence suggests that the upregulation of *FHC* represents a means by which NF-κB promotes oncogenesis and cancer progression [177, 184-188].

12.2 Mn-SOD

The NF-κB antioxidant activity has also been associated with the upregulation of the ROS scavenger, Mn-SOD [8,11,183,189]. Mn-SOD is a key antioxidant enzyme located in the mitochondrial matrix that protects cells from oxidative stress by catalyzing the dismutation of $\bullet O_2^-$ to H_2O_2 [190]. Mn-SOD is a TNFα-inducible NF-κB target, and when overexpressed, has been demonstrated to attenuate TNFα-mediated cytotoxicity in certain systems [169,183,189]. Furthermore, mice lacking Mn-SOD die perinatally after birth due to massive oxidative stress [191]. However, Mn-SOD significance to the pro-survival action of NF-κB remains uncertain owing to the observations that in various NF-κB-deficient systems, ectopic expression of Mn-SOD affords little or no protection against TNFα-induced PCD [168,169,192,194]. Nevertheless, Mn-SOD may be a critical effector of the antioxidant and protective activities in specific

biological contexts. Indeed, Mn-SOD was shown to be essential for radiation resistance in cancer cells [193]. Sun *et al.* (1998) demonstrated that Mn-SOD overexpression protects Chinese hamster ovarian (CHO) cell lines from cell death induced by ionizing radiation [195]. Furthermore, Josson *et al.* (2005) found that RelB nuclear localization and Mn-SOD promoter binding is increased following ionizing radiation of the aggressive PC-3 prostate cancer cell line. Inhibition of RelB activity in PC-3 cells by RNAi of overexpression of dominant negative p100 mutant results in a decrease in MN-SOD expression and an increase in radiation sensitivity [196].

It is possible that for an effective control of ROS levels, the synergistic activities of FHC and Mn-SOD are potentially crucial. While induction of Mn-SOD promotes dismutation of $\bullet O_2^-$ into H_2O_2, FHC-mediated iron sequestration may facilitate disposal of H_2O_2 by peroxidases and catalases [179,190]. In NF-κB-deficient cells, FHC levels are usually low [169], and as a consequence, free iron remains available to catalyze the Fenton reaction reducing H_2O_2 into highly reactive •OH radicals [178,179]. This could explain the inability of ectopic Mn-SOD to inhibit, alone, TNFα-induced cytotoxicity in NF-κB null cells. Further studies will be required to clarify this issue [168,169,189].

13. Concluding remarks

In recent years, the search for anticancer therapies has focused on targeting specific signaling pathways that drive inappropriate cell growth and survival, thereby offering the promise of greater specificity coupled with reduced systemic toxicity. The pivotal importance of the NF-κB pathway in human diseases has resulted in a surge in interest in developing specific inhibitors of the NF-κB pathway for treating cancer and other disorders. However, NF-κB-targeting drugs have failed to produce the intended results in patients. Proteasome inhibitors (*e.g.*, bortezomib), for instance, which block the proteolysis of IκBs and have indication in multiple myeloma, are dose-limiting and ineffective in achieving long-lasting disease remission in cancer patients, due to low therapeutic indices and adverse side-effects owing to the pleiotropic functions of NF-κB and the proteasome [45]. Serious misgivings also exist for IKK inhibitors, which have yet to be clinically approved due to safety concerns [12,45,197]. These safety concerns arise from the pronounced ability of IKK inhibitors to enhance the production of IL-1β and related cytokines [197]. A preferable therapeutic approach would be to develop drugs that target the critical downstream NF-κB-regulated genes

that promote survival in cancer and link this with inflammation rather than NF-κB itself. The discovery that the suppression of ROS and JNK signaling is a key protective mechanism mediated by NF-κB now affords an opportunity to develop such drugs. Small molecular inhibitors that block Gadd45β binding to MKK7 or inhibit the anti-oxidant proteins induced by NF-κB potentially could result in an elevation of JNK and ROS cytotoxic signaling, thereby blocking cell proliferation and inducing cell death in cancerous and pro-inflammatory cells. Such strategies could potentially enable the selective blockade of the pro-survival action of NF-κB in cancer or specific inflammatory cells, without significantly compromising the ability of NF-κB to serve in immunity and other functions. Additionally, since NF-κB, JNK and ROS activities are likely to be predominantly upregulated in inflamed and cancerous tissues, therapeutic strategies aimed at inferring with the interplay between these activities are likely to have inherent specificity for diseased tissues. Such selective blockade could help limit the inherent toxicity of drugs that target core components of the NF-κB pathway.

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Abbreviations

AP, activator protein; ASK, apoptosis signal-regulating kinase; ATF, activating transcription factor; BHA, butylated hydroxyanisole; cIAP, cellular inhibitor of apoptosis; DD, death domain; deferoxamine, DFO; DEN, diethylnitrosamine; DISC, death-inducing signaling complex; DR, death receptor; FADD, Fas-associated death domain; FHC, ferritin heavy chain; FLIP, FLICE-inhibitory protein; GADD, growth arrest and DNA damage-inducible gene; IκB, inhibitors of nuclear factor κB; IKK, IκB kinase; IL, interleukin; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MAP2K, mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MKP, MAPK phosphatase; MnSOD, manganese superoxide dismutase; NAC, N-acetyl-cysteine; NF-κB, nuclear factor κB; PCD, programmed cell death; PDTC, pyrrolidine dithiocarbamate; RHD, Rel homology domain; RIP, receptor-interacting protein; ROS, reactive oxygen species; TGF, transforming growth factor; TAK, transforming growth factor β-activated kinase; TAB, TAK1 binding protein; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR1, type 1 TNF-α receptor; TRADD, TNF receptor-associated death domain; TRAF, TNFR-associated factor; XIAP, X chromosome–linked inhibitor of apoptosis.

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Figure Legends

Figure 1: Tumour necrosis factor receptor 1 (TNF-R1) induced pathways modulating cell death and survival.

Ligation of TNF-R1 by TNFα promotes the intracellular assembly of *complex I*, which includes TNF receptor-associated death domain (TRADD), receptor-interacting protein 1 (RIP1), TNFR-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis protein-1 (cIAP-1) and cIAP-2. cIAP-mediated Lys63-ubiquitylation of RIP1 allows the recruitment of the IKK and the transforming growth factor β-activated kinase (TAK1)-TAK1 binding protein (TAB) complexes. *Complex I* can also bind the LUBAC complex that induces ubiquitin modifications resulting in the further stabilization of *complex I*, thereby allowing enhanced IKK activation. The activated IKK complex phosphorylates IκBα with subsequent ubiquitylation and degradation by the proteasome, allowing the nuclear translocation and activation of NF-κB, which comprises of the subunits p50 and RelA. NF-κB activates the transcription of an array of target genes that ultimately lead to cell survival. *Complex I* can also activate the JNK signaling pathway through the recruitment and phosphorylation of MAP3Ks (TAK1 and apoptosis signal-regulating kinase (ASK1)), which in turn activate MAP2Ks (MKK7 and MKK4), leading to activation of JNK. JNK phosphorylates Itch that targets c-FLIP for ubiquitylation and degradation, thereby promoting caspase 8-dependent apoptosis. JNK can also induce mitochondrial-dependent apoptosis through the activation of pro-apoptotic proteins jBid, Bim and Bax. Deubiquitylation of RIP1 by cylindromatosis (CYLD) results in the internalization of the TNF-R1 complex and modification of its binding partners that leads to the cytosolic assembly of *complex II*. *Complex II* comprises TRADD, FAS-associated death domain (FADD), RIP1, RIP3 and caspase 8. Caspase 8 cleaves and inactivates RIP1 and RIP3 kinases to preclude necroptosis, and initiates apoptosis by activating the classical caspase cascade. Under certain conditions in which caspase 8 activation is inhibited, RIP1 and RIP3 are phosphorylated and a unique signaling complex termed the necroptosome is formed, which leads to cell death through a caspase-independent process known as necroptosis. MAP2K, mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MKP, MAPK phosphatase.

Figure 2: The downstream effectors of NF-κB-mediated suppression of JNK and ROS activities and their proposed mechanisms of action.

The main protective activity of NF-κB against TNF-R1-induced killing involves the activation of NF-κB-inducible target genes, which act to induce an effective shutdown of prolonged JNK cascade activation following the triggering of TNF-Rs. One subset of NF-κB-inducible target genes, which includes Gadd45β, XIAP and A20, act directly on the JNK cascade. Another subset of target genes, including FHC and Mn-SOD, block prolonged JNK signaling by suppressing the accumulation of ROS derived from various sources including the mitochondria, peroxisomes, NAPDH oxidases and free iron.