Sustained JNK Activation in Response to Tumor Necrosis Factor Is Mediated by Caspases in a Cell Type-specific Manner*

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In most cell types, tumor necrosis factor (TNF) induces a transient activation of the JNK pathway. However, in NFκB-inhibited cells, TNF stimulates also a second sustained phase of JNK activation, which has been implicated in cell death induction. In the present study, we have analyzed the relationship of cell death induction, caspase activity, JNK, and NFκB stimulation in the context of TNF signaling in four different cellular systems. In all cases, NFκB inhibition enhanced TNF-induced cell death and primed most, but not all, cells for sustained JNK activation. The caspase inhibitor Benzoylcarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-fmk) and overexpression of the antiapoptotic proteins FLIP-L and Bcl2 differentially blocked transient and sustained JNK activation in NFκB-inhibited KB and HaCaT cells, indicating that the two phases of TNF-induced JNK activation occur at least in these cellular models by different pathways. Although the broad range caspase inhibitor Z-VAD-fmk and the antioxidant butylated hydroxyanisole interfered with TNF-induced cell death to a varying extent in a cell type-specific manner, inhibition of JNK signaling had no or only a very moderate effect. Notably, the JNK inhibitory effect of neither Z-VAD-fmk nor butylated hydroxyanisole was strictly correlated with the capability of these compounds to rescue cells from TNF-induced cell death. Thus, sustained JNK activation by TNF has no obligate role in TNF-induced cell death and is mediated by caspases and reactive oxygen species in a cell type-specific manner.

Cell death induction by apoptosis and necrosis, NFκB activation, and stimulation of the JNK cascade are the most prominent cellular responses of TNFR1 signaling (1). Notably, the pathways that mediate these responses do not act independently in a parallel manner but are interconnected to a TNFR1-signaling network through various mechanisms. For example, apoptosis induction is inhibited by several target genes of the NFκB pathway, including those encoding cFLIP, cIAP2, TRAF1, Bcl2, BclXL, Bfl1, and XIAP (1). Although the latter four factors globally protect against apoptosis, cFLIP and a complex containing TRAF1 and cIAP2 specifically interfere with apoptosis triggered by death receptors and TNFR1, respectively. Conversely, NFκB activation is blocked by initiator as well as effector caspases that become activated during apoptosis (1). This inhibitory effect relies on caspase-mediated cleavage of various signaling intermediates of the NFκB pathway and typically results in the generation of dominant negative fragments of the corresponding intact proteins. Caspase targets of the NFκB pathway include proteins such as IκK2, p65, and IκBα, which are crucial mediators of NFκB signaling by a broad range of NFκB stimuli, but also proteins like RIP and TRAF1, which are specifically employed by TNFR1 and some related receptors (1). In recent years, there is growing evidence that the balance between NFκB activation and apoptosis induction in the context of TNFR1 signaling is regulated by the JNK pathway (2, 3). The role of the various JNKs and their major targets, the AP1/Jun family of transcription factors in the TNF-induced signaling network, is complex and depends on the cellular context. For example, a protective function of the JNK pathway in TNF-induced cell death has been deduced from studies with mouse embryonal fibroblasts (MEFs) of JNK1 and JunD knock-out mice, whereas JNK2-deficient MEFs have been found to be either sensitized or protected from TNF-induced apoptosis, depending on the genetic background (4, 5). Moreover, TRAF2-deficient MEFs are impaired in TNF-induced JNK activation, and display increased sensitivity toward TNF, despite showing an almost normal NFκB response (6). Because TRAF2 also has a crucial role in the recruitment of the antiapoptotic reactive oxygen species; Z-VAD-fmk, benzoylcarbonyl-Val-Ala-Asp(Ome)-fluoromethyl ketone; MEF, mouse embryonal fibroblast; PARP, poly(ADP)-ribose polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DN, dominant negative; NEMO, NFκB essential modifier.

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3 The abbreviations used are: JNK, c-Jun NH2-terminal kinase; TNF, tumor necrosis factor; BHA, butylated hydroxyanisole; CHX, cycloheximide; ROS, reactive oxygen species; Z-VAD-fmk, benzoylcarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone; MEF, mouse embryonal fibroblast; PARP, poly(ADP)-ribose polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DN, dominant negative; NEMO, NFκB essential modifier.
proteins cIAP1 and cIAP2 to TNFR1 (7, 8), it is currently unclear whether JNK activation and/or recruitment of cIAPs contribute to the antiapoptotic action of TRAF2 in TNF signaling.

TNFR1-induced JNK activation is generally very rapid and transient in viable cells, whereas in NFkB-inhibited cells, which are primed to undergo cell death after TNFR1 stimulation, JNKS are persistently activated (9). Based on in vitro experiments, it has been suggested that expression of the NFkB target genes A20, GADD45β, and X-linked IAP (XIAP) inhibit TNF-induced JNK activation and thus prevent prolonged JNK signaling after TNFR1 stimulation in normal cells (9). However, analyses of the corresponding knock-out mice failed to confirm a crucial role of one of these factors in NFkB-dependent inhibition in TNF-induced JNK signaling until now but leave open the possibility that these proteins act in a redundant fashion. Alternatively, sustained JNK activity in TNF signaling might be the result of reactive oxygen species (ROS)-mediated oxidative inhibition of JNK-inactivating phosphatases (10). In fact, the NFkB pathway not only blocks apoptosis but also ROS production and necrosis by inducing ROS-detoxifying enzymes such as manganese superoxide dismutase and the ferritin heavy chain (11, 12). Moreover, in murine embryonal fibroblasts, cell death-associated prolonged TNF-induced JNK activation is partly mediated by the JNK-stimulating MAP3K apoptosis signaling kinase 1 (ASK1), which is activated by ROS (13, 14). Notably, in MEFs, TNF induces apoptosis, necrosis, or a mixture of both, depending on the number of passages and nature of the sensitizing treatment (cycloheximide (CHX), NFkB inhibition) (15). Sakon et al. (16) have demonstrated that prolonged TNF-induced JNK activation is mediated by ROS in necrotic MEFs. It is therefore tempting to speculate that ROS generation, oxidative inhibition of phosphatases, and apoptosis signaling kinase activation are of special relevance for sustained JNK activation in cells undergoing TNF-induced necrosis. The cell death-promoting effects of persistent JNK activation in TNF signaling has been attributed to caspase-independent cleavage of the BH3-only protein BID, resulting in a fragment designated as JID and activation of the E3 ligase Itchy (17, 18). By unknown mechanisms, JID stimulates the selective release of SMAC/DIABLO from the mitochondria, which then abrogates the interaction of the caspase inhibitory IAP (inhibitor of apoptosis proteins) from interaction with effector caspases and TRAF2. Itch sensitizes for TNF-induced apoptosis by driving enhanced proteasomal degradation of FLIP-L (17, 18).

In this study, we show that TNF-induced persistent JNK activation in cells with inhibited NFkB signaling is cell type-dependently blocked by the pan-caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-fmk). Cells protected from TNF-induced apoptosis by overexpression of FLIP or Bcl2 displayed no sustained caspase-mediated JNK signaling but were not affected in transient JNK activation. This suggests that the caspase-mediated mode of TNF-induced sustained JNK activation is an indirect consequence of apoptosis induction rather than reflecting the lack of a direct JNK inhibitory effect of a NFkB target gene product.
TNF-induced Sustained JNK Activation

A

Jurkat

HaCaT

KB

MEF

B

Jurkat

Jurkat NEMO def.

HaCaT

HaCaT IKK2-DN

KB

KB IKK2-DN

C

Jurkat

Jurkat NEMO def.

HaCaT

HaCaT IKK2-DN

KB

KB IKK2-DN

TNF

Pellet

S/N

Pellet

S/N

Pellet

S/N

Pellet

S/N

cytochrom c

SMAC

tubulin

Hsp 60

cytochrom c

SMAC

ERK 42/44

Hsp 60

cytochrom c

SMAC

tubulin

Hsp 60
(ADP)-ribose polymerase (anti-PARP) were from BD Biosciences, and anti-tubulin was obtained from Dunn Labortechnik (Asbach, Germany). The p21WAF1/Cip1-specific antibody was obtained from Millipore (Billerica, MA).

**Retroviral Infection**—The pCFG5-IGZ retroviral vector containing a cDNA encoding kinase-dead IKK2 (IKK2DN) was used for infection of HaCaT cells as described elsewhere (21). Briefly, the amphotrophic producer cell line PNX was transfected with 10 μg of the retroviral vector by calcium phosphate precipitation. To select transfected producer cells, 1 μg/ml puromycin (Sigma) or 0.5 μg/ml zeocin were added to the culture medium for 7–14 days to obtain >95% green fluorescent protein-positive producer cells. Cell culture supernatant containing viral particles was generated by incubation of producer cells with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum overnight. Following filtration (45 μm), the culture supernatant was added to HaCaT cells seeded in 6-well plates 24 h earlier in the presence of 1 μg/ml polybrene. Cells were centrifuged for 3 h at 21 °C, and viral particle-containing supernatant was subsequently replaced by fresh medium. After 10–14 days recovery of bulk infected cultures, fluorescence-activated cell sorter analysis for green fluorescent protein expression and Western blot analysis for IKK2DN expression were performed on the expanded polyclonal cells to confirm ectopic expression of these molecules. For sequential transduc-
TNF-induced Sustained JNK Activation

with IKK2DN and FLIP-L-encoding constructs, cells were first infected with FLIP-L as previously described (22) and subsequently infected with either IKK2DN or the respective empty construct and selected as described above.

**Cell Death Assays**—Adherent cells (20 × 10^3) were seeded in 96-well plates. The following day, the cells were preincubated as indicated with CHX (2.5 μg/ml), Z-VAD (40 μM), BHA (200 μM), or SP600125 (20 μM) or left untreated. After 1 h, the cells were treated with recombinant TNF or Fc-CD95L, and cell viability was determined 16 h later by crystal violet staining. Cell viability of suspension cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining. Jurkat cells (60 × 10^3) were seeded in 96-well plates, and after the indicated treatments, they were incubated for 2 h with 10 μl of MTT solution. After overnight lysis in MTT lysis buffer (15% SDS, 50% N-N-dimethylformamide, pH 4.7, acetic acid), the optical density was measured at 570 nm.

**Western Blotting**—Cell lysates for Western blotting with phosphoprotein-specific antibodies were prepared in 4× sample buffer (2% SDS, 0.1 m dithiothreitol, 10% glycerol) supplemented with phosphatase inhibitor mixtures I and II (Sigma). Cells were harvested with a rubber policeman into ice-cold phosphate-buffered saline, collected by centrifugation, and then lysed in 4× sample buffer. After brief sonification, the protein samples were boiled for 5 min at 96 °C, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Cell lysates for Western blotting with non-phosphoprotein-specific antibodies were prepared in lysis buffer (30 mM Tris-HCl, pH 7.4, 120 mM NaCl, 10% glycerol, 1% Triton X-100) supplemented with a protease inhibitor mixture (Roche Applied Science). After removal of cellular debris by two centrifugation steps (5,000 × g, 5 min, 4 °C and 14,000 × g, 20 min, 4 °C), protein concentrations were determined by Bradford assay. Proteins of interest were detected with the appropriate primary antibodies and horseradish peroxidase-labeled secondary antibodies. Antigen-antibody complexes were visualized using the ECL detection system.

**Subcellular Fractionation**—For subcellular fractionation, cells were harvested, washed with ice-cold phosphate-buffered saline, resuspended at 5 × 10^6 cells/ml in buffer (100 mM KCl, 2.5 mM MgCl₂, 250 mM sucrose, 20 mM HEPES/KOH, pH 7.5, 1 mM dithiothreitol, 5 μg/ml cytochalasin B, and 50 μg/ml cycloheximide) containing 0.05% digitonin and complete protease inhibitors (Roche Applied Science) and incubated for 10 min on ice. After centrifugation (10,000 × g, 10 min, 4 °C), supernatant and pellet fractions were carefully separated and analyzed by SDS-PAGE and Western blot.

**RESULTS**

**Inhibition of the NFκB Pathway Sensitizes for TNF-induced Apoptosis**—To analyze the relationship of NFκB activation, apoptosis induction, and JNK stimulation in TNF signaling, we investigated four cell models in which the NFκB pathway is inhibited, Jurkat cells with a defect in NEMO/IKK2 expression, MEFs derived from NEMO knock-out mice, and KB and HaCaT cells stably expressing a dominant negative mutant of IKK2. Although in Jurkat cells and MEFs deficient in NEMO expression as well as in the IKK2DN-overexpressing HaCaT cells, phosphorylation and degradation of IκBα after TNF stimulation were fully blocked, the IKK2DN KB cells also showed no IκBα degradation but residual phosphorylation of IκBα. (Fig. 1). In all of the investigated cell models, inhibition of the NFκB pathway was associated with a strong enhancement of TNF-induced cell death (Fig. 2A). However, in KB cells and in MEFs, robust cell death induction by TNF required co-treatment with cycloheximide. In the human cell lines, we also analyzed the processing of BID, PARP, and caspase-3, -8, and -9. Enhancement of TNF-induced cell death in NFκB-inhibited HaCaT and Jurkat cells was reflected by a strong increase in the processing of caspases and their substrates (Fig. 2B). In accordance with the sensitivity of CHX-sensitized KB cells for TNF-induced apoptosis, cleavage of caspases and BID and PARP1 was already prominently induced in these cells by TNF despite an intact NFκB-signaling pathway (Fig. 2B). The additional enhancement of TNF-induced cell death in KB cells by NFκB inhibition, nevertheless, resulted in acceleration of caspase activation (Fig. 2B). In accordance with the sensitizing effect of NFκB inhibition and CHX treatment on TNF-induced cell death, we observed release of SMAC and cytochrome c into the cytosol 6 h past TNF treatment (Fig. 2C). TNF-induced cell death was completely blocked in KB cells by the pan-caspase inhibitor Z-VAD-fmk, indicating that it occurred only by apoptosis (Fig. 2D). In the other cell types, Z-VAD-fmk rescued TNF-stimulated cells incompletely to a varying extent despite efficiently.
blocking cleavage of the caspase substrates PARP1 and BID, suggesting that, in these cells, TNF also triggers caspase-independent cell death, e.g. necrosis (Fig. 2E). Notably, cell death triggered by the TNFR1-related death receptor CD95 was bar-ley-enhanced in the NFκB-compromised cells (Fig. 2F). CD95 and TNFR1 signal apoptosis downstream of the initiator caspase caspase-8 by a common pathway (23). The differential modulation of CD95- and TNFR1-induced apoptosis implicates that one or more NFκB-regulated factors interfere with TNFR1-mediated apoptosis upstream of caspase-8 activation and/or that TNFR1 (but not CD95) prevents apoptosis induction by concomitant activation of NFκB.

FIGURE 4. Sustained (but not early) JNK activation by TNF is blocked in apoptosis-resistant transfectants derived from Bcl2-overexpressing KB and FLIP-L/IKK2DN-overexpressing HaCaT cells. A and B, KB cells overexpressing Bcl2 and HaCaT cells overexpressing I KK2DN and FLIP-L, respectively, were seeded in triplicates in 96-well plates and were challenged the next day with the indicated concentrations of TNF over-night. KB cells were incubated in the presence of cycloheximide (2.5 μg/ml). Cell viability was finally determined by crystal violet staining. C and D, cells were incubated for the indicated times with TNF (20 ng/ml) and lysed. KB cells were pretreated with 2.5 μg/ml CHX. Activation of the JNK pathway was monitored by immuno blotting with anti-phospho-JNK antibodies. Detection of total cellular JNK served as the loading control. E, CHX-sensitized (2.5 μg/ml) KB and KB-Bcl2 cells were stimulated for the indicated times with TNF (20 ng/ml), and cytosolic extracts were analyzed by Western blotting with respect to processing of the indicated proteins. Tubulin served as a load control.

Sustained Activation of JNK by TNF Is Cell type-dependently Blocked by Z- VAD-fmk—In parental Jurkat cells, TNF stimulation induced a moderate JNK activation that was observed at late time points, but this response was much stronger in the NEMO-deficient counterparts (Fig. 3). In some experiments, very weak transient phosphorylation of JNK was observed in both Jurkat cell lines, suggesting that this early phase of JNK activation was too weak to allow regular detection (data not shown). Sustained JNK activation was also observed in wild-type and, to lower extent, in NEMO-deficient MEFs (Fig. 3). Likewise, there was robust and persistent activation of JNK in I KK2DN-expressing HaCaT cells but not in the corresponding control cells (Fig. 3). Early activation of JNK occurred in both HaCaT variants but was hardly detectable. Thus, in these cell types (Jurkat, HaCaT, MEF), TNF-induced persistent activation of JNK coincided with NFκB inhibition and cell death induction. Notably, this correlation was not found in KB cells. Sustained TNF-induced JNK activation occurred both in control and I KK2DN-expressing cells and furthermore required sensitization with CHX (Fig. 3). Moreover, in KB cells, which were protected against TNF-induced apoptosis by overexpression of Bcl2, sustained JNK activity and effector caspase activation were also absent (Fig. 4, A, C, and E) despite NFκB activation (data not shown). Thus, sustained JNK activation correlated in KB cells with TNF-induced apoptosis and caspase activation rather than with NFκB inhibition. Likewise, in HaCaT-I KK2DN cells, overexpression of FLIP-L prevented both cell death induction and sustained JNK signaling, arguing again for a relationship between apoptosis/necrosis and JNK signaling (Fig. 4, B and D). Many reports have shown that prolonged activation of JNK by the TNFR1-related death receptor CD95 is mediated by caspases (24). We have therefore next analyzed TNF-induced JNK activation in the presence of Z- VAD-fmk. Although TNF-induced persistent JNK activation was almost completely blocked in NFκB-compromised Jurkat, KB, and HaCaT cells, it was not significantly affected in both wild-type and NEMO-deficient MEFs (Fig. 5A). Early transient activation of JNK by
TNF, which is observed similarly in control and NFκB-compromised KB and HaCaT cells, was also not affected by Z-VAD-fmk (Fig. 5A). Thus, at least in these cells, transient and sustained JNK activation by TNF are mediated by different caspase-independent and -dependent pathways. Our data thus argue against a model in which sustained JNK activation is the result of non-inhibited transient JNK activation.

It has been shown that, during Fas-, detachment-, and paclitaxel-induced apoptosis, caspase-mediated cleavage of MEKK1 and p21WAF1/Cip1 results in the generation of fragments of these proteins with high JNK-stimulating activity (26–28). Notably, we observed similar cleavage of MEKK1 and p21WAF1/Cip1 in cells primed by CHX or NFκB inhibition for TNF-induced sustained JNK signaling (Fig. 5B).

Sustained Activation of JNKs by TNF Is Dispensable for Cell Death Induction—JNK activation can mediate not only proapoptotic but also antiapoptotic effects in death receptor signaling, especially after TNFR1 stimulation (see Introduction). To elucidate the functional consequences of prolonged TNF-induced JNK stimulation in the cellular models investigated in this study, we blocked JNK activity with the pharmacological JNK inhibitor SP600125 (25). Although TNF-induced phosphorylation of the JNK substrate c-Jun was nearly completely blocked by SP600125 in NFKB-inhibited cells, cell death induction by TNF was not (NEMO-deficient Jurkat, KB-IKK2DN, HaCaT-IKK2DN) or only moderately (NEMO-deficient MEFs) inhibited by this drug (Fig. 6). SP600125 treatment also strongly inhibited JNK phosphorylation (Fig. 6A). This could be caused by inhibition of the known JNK autophosphorylating activity or by blockage of a feed-forward JNK activation loop (26).

ROS have been identified as major mediators of prolonged TNF-induced JNK activation in MEFs. We have therefore next analyzed the effect of the antioxidant BHA on JNK activation and cell death induction by TNF. In NEMO-deficient Jurkat cells and MEFs, BHA displayed a strong inhibitory effect on prolonged JNK activation, whereas in HaCaT and KB cells, there was only moderate inhibition of TNF-induced JNK activation (Fig. 7A). Further, BHA showed a cell type-specific inhibitory effect on TNF-induced cell death. Although BHA treatment showed no effect on TNF-induced cell death in NFκB-inhibited Jurkat and KB cells and only a minor protective effect on HaCaT-IKK2DN cells, it did almost completely protect NEMO-deficient MEFs from TNF-induced killing (Fig. 7B). The inhibition of JNK by BHA was not associated with protection from TNF-induced cell death in NEMO-deficient Jurkat cells but correlated with protection in NEMO-deficient MEFs (Fig. 7B). However, inhibition of prolonged JNK activation by BHA can be only partially responsible for the observed protective effect, as SP600125 blocked JNK activity in TNF-treated NEMO-deficient MEFs as efficiently as BHA but showed only a moderate protective effect (Fig. 6). Thus, ROS also mediate cytotoxic effects independent from JNK activation.

DISCUSSION

TNF elicits a wide variety of cellular effects dependent on cell type and environmental cues. The pleiotropic cellular effects of TNF are, in part, the consequence of intense and multifold cross-talk between the major TNF-induced intracellular signaling pathways especially those leading to apoptosis or activation.
of NFκB and JNK. Although the molecular basis of the antagonizing actions of NFκB and apoptosis in the context of TNF signaling is comparably well understood, the role of JNK is still poorly defined. In fact, a variety of studies in recent years revealed that the JNK pathway can tip the balance between cell death induction and survival signaling in TNF-stimulated cells in both directions (2, 3). TNF-induced JNK activation appears typically with rapid and transient kinetics. The cell death-promoting effect of JNK in TNF signaling, however, has been attributed to a second sustained phase of JNK activation that largely or completely inhibited by overexpression of dominant negative IKK2 or by NEMO deficiency. In all cases, NFκB inhibition was associated with TNF-induced cell death and prolonged JNK activation, although in KB cells and MEFs, these effects were only robustly apparent upon additional sensitizing with the protein synthesis inhibitor CHX (Fig. 2A). Moreover, TNF-induced apoptosis and prolonged JNK activation were also detectable in cells with an intact NFκB-signaling pathway when sensitized with CHX (KB cells and several other cell lines) (Fig. 3) (data not shown). Notably, the CHX concentrations

follows on the early phase of JNK activation, especially in cells sensitized for apoptosis by CHX treatment or inhibition of NFκB. Several mechanisms by which JNK activity enhances TNF-induced cell death have been identified and include caspase-independent cleavage of BID, proteasomal degradation of FLIP-L, and ROS production (17, 18). Whether TNF-induced cell death signaling is also involved in the modulation of JNK signaling is largely unknown. In particular, the relationship of TNF-induced apoptotic caspases and prolonged JNK signaling is unclear. It has been shown that caspases activate JNK-inducing kinases. Moreover, the TNFR1-related death receptor CD95 induces prolonged JNK activation in apoptotic cells by caspase-dependent mechanisms. In fact, caspase-dependent activation of JNK can be achieved by cleavage and activation of MEKK1 or by cleavage and inactivation of JNK1-associated p21(WAF1/CIP1) (26–28). Although TNF-induced prolonged JNK activation occurs in a caspase-independent manner in necrotic MEFs (16), it seems possible that there is interplay between JNK and caspases in other cell types, where apoptosis induction by TNF is more prominent.

We therefore analyzed four different cellular systems with respect to the relationship of cell death induction, caspase activation, and JNK and NFκB stimulation in the context of TNF signaling. As described in detail in the Introduction, prolonged JNK activation by TNF has been ascribed to the relief from inhibitory actions of the NFκB pathway. We therefore analyzed cell lines in which the latter has been largely or completely inhibited by overexpression of dominant negative IKK2 or by NEMO deficiency. In all cases, NFκB inhibition was associated with TNF-induced cell death and prolonged JNK activation, although in KB cells and MEFs, these effects were only robustly apparent upon additional sensitizing with the protein synthesis inhibitor CHX (Fig. 2A). Moreover, TNF-induced apoptosis and prolonged JNK activation were also detectable in cells with an intact NFκB-signaling pathway when sensitized with CHX (KB cells and several other cell lines) (Fig. 3) (data not shown). Notably, the CHX concentrations
used did partly reduce protein synthesis (data not shown) and still allowed production of NFκB-regulated proteins. Thus, TNF-induced sustained JNK activation was highly correlated with apoptosis induction and not stringently with NFκB inhibition. Moreover, when we prevented NFκB-inhibited cells from going into TNF-induced apoptosis by overexpression of FLIP-L, this was accompanied with a blockage of the sustained phase of TNF-triggered JNK activity (Fig. 4, B and D). Thus, our data point out a tight connection of sustained JNK activation and apoptosis induction. Especially our data open the possibility that, in the NFκB-inhibited cellular models analyzed in this study, sustained JNK activation is not caused by a relief from the direct JNK inhibitory action of a NFκB target gene but is rather an indirect consequence of the lack of NFκB-mediated apoptosis inhibition.

To uncover a possible causal relationship between caspase activation and thus apoptosis induction and JNK signaling, we next analyzed the effect of pharmacological inhibitors of caspases and JNK on TNF-induced cell death and JNK activation. Caspase activation was practically fully blocked by Z-VAD-fmk in the various cell lines studied (Fig. 2E). Although this compound completely rescued CHX-sensitized KB/KB-IKK2DN cells from TNF-induced cell death, it protected the other NFκB-inhibited cells partly, ranging from ~23% (NEMO-deficient Jurkat) to 60% (NEMO-deficient MEFs), induced prolonged JNK activation was cell type-dependently mediated by caspases and had no essential role in cell death induction by TNF in the cellular models investigated in this study. However, JNK-mediated phosphorylation of c-Jun (Fig. 6A, right panels), which is involved in transcriptional up-regulation of the death ligands TRAIL and FasL/CD95L, may allow the autocrine production of these death ligands in cells where the apoptotic machinery is insufficiently activated (30).

ROS have been implicated in JNK activation as well as in cell death induction (apoptosis, necrosis) by TNF. We have therefore also analyzed the impact of the antioxidant BHA in cells undergoing TNF-induced cell death. In NEMO-deficient MEFs and Jurkat cells, BHA strongly inhibited prolonged JNK activation by TNF, whereas it showed no or only a minor effect in IKK2DN-overexpressing HaCaT and KB cells (Fig. 7A). In NEMO-deficient MEF cells, BHA also blocked TNF-induced cell death, whereas in the other cell lines, there was no or only a minor effect (Fig. 7B). Thus, ROS might have a crucial role in TNF-induced killing of NEMO-deficient MEFs but are not sufficient for triggering cell death in NFκB-inhibited Jurkat cells, although in the latter case, ROS might contribute to JNK activation. The latter is also blocked by the caspase inhibitor Z-VAD-fmk (Fig. 5), opening the possibility that ROS and caspases act together in a feed-forward loop (Fig. 2D). Incomplete protection from TNF-induced cell death by caspase inhibition has already been observed earlier in Jurkat cells and MEFs and has been ascribed to caspase-independent necrotic cell death (29). Z-VAD-fmk also showed varying effects on TNF-induced prolonged JNK activation. In Jurkat and KB cells, Z-VAD-fmk fully blocked prolonged TNF-induced JNK activation, whereas in HaCaT cells, there was partial inhibition and in MEFs no inhibition at all (Fig. 5A). Notably, there was no strict correlation of caspase-mediated cell death (apoptosis) and caspase-mediated JNK activation. This was most obvious in Jurkat cells where Z-VAD-fmk had only a minor effect on cell death induction by TNF but completely abrogated prolonged JNK signaling upon TNF treatment (Figs. 2D and 5A). Vice versa, Z-VAD-fmk significantly protected NEMO-deficient MEFs from TNF-induced cell death but showed no effect on JNK activation (Figs. 2B and 5). Further, the pharmacological JNK inhibitor SP600125 efficiently blocked JNK activity but had no or only a moderate effect on TNF-induced cell death (6). Thus, TNF-induced Sustained JNK Activation
to stimulate JNK in TNF-treated, NFκB-inhibited Jurkat cells.

The cell type-specific inhibitory effects of the caspase inhibitor Z-VAD-fmk and the ROS scavenger BHA on sustained JNK activation and the differential regulation of rapid transient and late sustained JNK activation by TNF observed in this study is reflected by the fact that apoptosis, necrosis, and NFκB-regulated factors that stimulate/regulate JNK activity by different means (necrosis, e.g., ROS production or ceramide release; apoptosis, e.g., caspase-mediated activation of JNK-inducing kinases; NFκB, indirectly, e.g., by induction of caspase inhibitors and ROS scavengers) are connected by positive and negative feedback loops including among others (i) caspase-mediated inactivating cleavage of RIP, which is essentially involved in TNF-induced necrosis but also in TNF-induced NFκB activation (29, 31, 32); (ii) caspase-mediated inhibition of NFκB signaling (1); (iii) caspase-mediated cleavage of PARP-1, which shifts the balance from apoptosis to necrosis by ATP depletion (33), and (iv) inhibition of apoptosis and ROS production by NFκB-regulated genes (1, 11, 12). Thus, the exceptional high degree of cross-talk between the above-mentioned pathways makes TNF signaling, in general, and JNK activation, in particular, highly receptive for modulation by a variety of cellular and extracellular insults and casually explains the observed cell type dependence in the mechanisms of TNF-induced JNK activation.

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