Death Receptor-Induced Signaling Pathways Are Differentially Regulated by Gamma Interferon Upstream of Caspase 8 Processing

Daniela Siegmund,¹ Andreas Wicovsky,¹ Ingo Schmitz,² Klaus Schulze-Osthoff,²
Sebastian Kreuz,³ Martin Leverkus,⁴ Oliver Dittrich-Breiholz,⁵
Michael Kracht,⁵ and Harald Wajant^{1*}

Department of Molecular Internal Medicine, Medical Polyclinic, University of Würzburg, Röntgenring 11, 97070 Würzburg, Germany¹; Institute of Molecular Medicine, University of Düsseldorf, Universitäts-Str. 1, 40225 Düsseldorf, Germany²; Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany³; Laboratory for Experimental Dermatology, Otto-von-Guericke-University Magdeburg, Leipzigerstr. 44, 39120 Magdeburg, Germany⁴; and Institute of Pharmacology, Medical School Hannover, Carl-Neuberg Strasse 1, 30625 Hannover, Germany⁵

Received 25 January 2005/Returned for modification 22 February 2005/Accepted 4 May 2005

FasL and gamma interferon (IFN- γ) are produced by activated T cells and NK cells and synergistically induce apoptosis. Although both cytokines can also elicit proinflammatory responses, a possible cross talk of these ligands with respect to nonapoptotic signaling has been poorly addressed. Here, we show that IFN- γ sensitizes KB cells for apoptosis induction by facilitating death-inducing signaling complex (DISC)-mediated caspase 8 processing. Moreover, after protection against death receptor-induced apoptosis by caspase inhibition or Bcl2 overexpression, IFN- γ also sensitized for Fas- and TRAIL death receptor-mediated NF- κ B activation leading to synergistic upregulation of a variety of proinflammatory genes. In contrast, Fas-mediated activation of JNK, p38, and p42/44 occurred essentially independent from IFN- γ sensitization, indicating that the apoptosis- and NF- κ B-related FasL-IFN- γ cross talk was not due to a simple global enhancement of Fas signaling. Overexpression of FLIP_L and FLIP_S inhibited Fas- as well as TRAIL-mediated NF- κ B activation and apoptosis induction in IFN- γ -primed cells suggesting that both responses are coregulated at the level of the DISC.

Fas (CD95/APO-1) is the prototypic representative of the death receptor subgroup of the tumor necrosis factor (TNF) receptor superfamily and has been implicated in a wide range of physiological and pathophysiological apoptosis-related processes, including T-cell-induced cytotoxicity, deletion of autoreactive T and B cells, activation-induced cell death, tumor surveillance, immune privilege, angiogenesis, autoimmunity, fulminant hepatitis, and neurodegeneration (25, 27, 41). However, Fas can also activate the NF- κ B, JNK, extracellular signal-regulated kinase (ERK), and p38 pathways and has been involved in nonapoptotic processes like inflammation, proliferation, liver regeneration, and neurite outgrowth (25, 41).

After ligation of preassembled Fas complexes the Fas-associated death domain protein (FADD) and procaspase 8 are rapidly recruited to form together with Fas the so-called death-inducing signaling complex (DISC) (22, 35). In the context of this complex, procaspase 8 gets activated by dimerization and converts to the processed heterotetrameric mature form of caspase 8, which is released into the cytoplasm (3, 9). Active caspase 8 cleaves a narrow range of substrates, including effector caspases and BID. In some cells (type I cells), the

caspase 8-mediated activation of effector caspases is sufficient for robust apoptosis induction, and BID cleavage, which can lead to apoptogenic activation of the mitochondrial pathway, attains no relevance for Fas-induced apoptosis. However, in another cell type (type II cells) BID cleavage and apoptogenic activation of the mitochondria contribute measurably to Fas-induced cell death (2).

Interferons are able to block viral replication and additionally induce a variety of other effects, including immune modulation, differentiation, apoptosis, and inhibition of proliferation and angiogenesis. While alpha interferon (IFN- α) and IFN- β are produced by most cells in response to virus infections and double-stranded RNA (dsRNA), IFN- γ is secreted from activated Th1 T cells and natural killer cells (7). IFN- γ alone can be sufficient to induce apoptosis in some cells, but often sensitizes cells for death receptor-induced apoptosis without being apoptotic per se (7). Induction of apoptosis by IFN- γ is slow (24 to 48 h), and IFN- γ -mediated sensitization requires pretreatment for 1 to 2 days, suggesting the involvement of IFN- γ -induced genes in both cases (7). In fact, several proapoptotic genes, including those encoding caspase 8, TRAIL, and FasL, have been identified as transcriptional targets of IFN- γ (31–34, 43, 44). Here, we show that IFN- γ not only sensitizes towards FasL and TRAIL-induced apoptosis but also enhances NF- κ B activation induced by these death ligands under conditions of impaired apoptosis signaling. We

* Corresponding author. Mailing address: Department of Molecular Internal Medicine, Medical Polyclinic, University of Würzburg, Röntgenring 11, 97070 Würzburg, Germany. Phone: 49 (931) 201 71010. Fax: 49 (931) 201 71070. E-mail: harald.wajant@mail.uni-wuerzburg.de.

FIG. 1. IFN- γ sensitizes KB cells for Fas-induced apoptosis upstream of caspase 8 and caspase 10 processing. (A) KB cells were seeded in 96-well plates (10×10^3 cells per well). The next day cells were incubated for 24 h with IFN- γ (20 ng/ml) or remained untreated. Cells were then challenged in triplicates with the indicated concentrations of soluble Flag-tagged FasL (Flag-FasL) complexed with the monoclonal anti-Flag antibody M2 (1 μ g/ml) in the presence or absence of z-VAD-fmk (20 μ M). After an additional 18 h, cell viability was determined by crystal violet staining. (B and C) Untreated and IFN- γ -prestimulated (20 ng/ml, 24 h) cells were challenged with 200 ng/ml of M2-cross-linked Flag-FasL for the indicated times. Cell protein lysates were prepared, and immunoblot analyses were performed with anti-caspase 8 (B) and anti-caspase 10 (C) antibodies. To control protein loads, filters were reprobed with antivinculin antibodies. α -, anti-. (D) IFN- γ -prestimulated (20 ng/ml, 24 h) KB and KB-GFP-Bcl2 cells were analyzed with respect to FasL-induced apoptosis (D) and caspase 8 processing (E) as described for panels A and B. (F) Triplicates of untreated and IFN- γ -stimulated (20 ng/ml, 24 h) KB cells were stained with FITC-labeled anti-Fas antibodies or the corresponding isotype control and analyzed by FACS (upper panel). Representative histograms of each group were shown in the lower panel. (G) Immunoblot analysis of triplicates of untreated and IFN- γ -stimulated (20 ng/ml, 24 h) cells with anti-caspase 8, anti-caspase 10, anti-FLIP, and anti-FADD antibodies. tub., tubulin.

give evidence that the cross talk of IFN- γ and FasL or TRAIL occurs at the level of the receptor signaling complex. In contrast, activation of JNK, p38, and ERK by FasL were not or only hardly affected by IFN- γ , demonstrating that the latter enhances certain but not all death receptor-induced signaling pathways.

MATERIALS AND METHODS

Materials and cell culture. The KB populations overexpressing green fluorescent protein (GFP)-Bcl2, FLIP_L-GFP, and FLIP_S-GFP have already been described and were cultured in RPMI medium with 10% heat-inactivated serum (23). Supernatants of Hek293 cells stably transfected with an expression plasmid encoding human Flag-tagged soluble FasL (amino acids 139 to 281) were collected, and the recombinant FasL protein was purified by affinity chromatography with anti-Flag M2 agarose beads (Sigma, Deisenhofen, Germany). An expression plasmid encoding the extracellular domain of FasL carboxy-terminally fused to human Fc was used for production of Fc-FasL and was a kind gift from Ottmar Janssen (Institute for Immunology, Medical Center Schleswig-Holstein, Schleswig-Holstein, Germany). Human recombinant TNF was obtained from Knoll AG (Ludwigshafen, Germany). z-VAD-fmk [benzyloxycarbonyl-Val-Ala-

Asp(OMe)-fluoromethylketone] was purchased from Bachem (Heidelberg, Germany). Cycloheximide (CHX), the anti-Flag mouse monoclonal antibody (MAb) M2, protein A and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) were from Sigma. The TRAILR1- and TRAILR2-specific agonistic rabbit antisera are described elsewhere (30). Fluorescein isothiocyanate (FITC)-conjugated anti-Fas and anti-IFN- γ antibodies were obtained from R&D Systems (Wiesbaden, Germany), anti-caspase 10 was obtained from MBL (Woburn, MA) and anti-FADD and anti-RIP were purchased from BD Pharmingen (Heidelberg, Germany). The antibodies specific for JNK, phospho-JNK, p42/44, phospho-p42/44, p38, and phospho-p38 were from Cell Signaling (Beverly, MA). Anti-I κ B α , anti-TRAF2, anti-Fas, and antivinculin were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLIP MAb NF6 was from Alexis, and antitubulin was from Dunn Labortechnik (Asbach, Germany).

Cell death assays. Cells (10×10^3 /well) were seeded in 96-well tissue culture plates. The next day, cells were treated with IFN- γ or remained untreated. The following day the cells were then incubated in triplicates as indicated for an additional day, and finally cell viability was determined by crystal violet staining (20% in methanol) for 20 min. After several washes with water plates were air dried and the plate-bound dye was dissolved in methanol (1 to 2 h) to measure the optical density at 595 nm.

Determination of IL-8 production. Cells (10×10^3 /well) were seeded in triplicates in 96-well tissue culture plates, cultured overnight, and incubated in the

FIG. 3. Transcriptional regulation of apoptosis-related genes by IFN- γ in KB cells. KB cells were incubated for 24 h with 20 ng/ml IFN- γ (I) or remained untreated in the absence and presence of 20 μ M z-VAD-fmk (Z). Total RNAs were isolated for RPA analyses, and 10 μ g of each RNA sample was analyzed with a variety of multiprobe template sets containing probes for the indicated mRNAs. GAPDH and L32 served as internal controls.

absence or presence of IFN- γ for an additional day. The following day medium was changed and cells were challenged in triplicates with the reagents of interest. For IFN- γ -pretreated cells the new medium was again supplemented with IFN- γ . After 6 h supernatants were collected and interleukin-8 (IL-8) was quantified by enzyme-linked immunosorbent assay (ELISA).

Western blotting. Cell lysates for Western blotting analysis with non-phosphoprotein-specific antibodies (e.g., I κ B α degradation, caspase processing) were prepared in radioimmunoprecipitation assay buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). After removal of cellular debris by centrifugation (10,000 $\times$ g, 10 min, 4°C), protein concentrations were determined using the Bradford assay. Proteins (100 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking nonspecific binding sites by incubation in Tris-buffered saline containing 0.05% Tween 20 and 3% (wt/vol) dry milk, Western blot analyses were performed with primary antibodies of the indicated specificity, horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako, Hamburg, Germany) or horseradish peroxidase-conjugated goat anti-mouse IgG (Dako) and the ECL Western blotting detection reagents and analysis system (Amersham Biosciences). To avoid dephosphorylation of phosphoproteins, cells were gently scraped with a rubber policeman into an excess of ice-cold phosphate-buffered saline, collected by centrifugation (2,000 rpm, 2 min, 4°C), and pellets were directly dissolved in 4 $\times$ sample buffer (8% SDS, 0.1 M dithiothreitol, 40% glycerol, 0.2 M Tris, pH 8.0) supplemented with phosphatase inhibitor cocktails I and II (Sigma). Phosphoprotein samples were then boiled for 5 min and processed as described above by SDS-PAGE and Western blotting with phospho-JNK, phospho-p42/44 and phospho-p38-specific antibodies. Endogenous expression of FLIP was too low to allow reliable determination by the above ECL protocol. To increase sensitivity Western blot detection of FLIP proteins was therefore performed with an Alexa Fluor 680-labeled secondary antibody and the ODYSSEY infrared imaging system (LI-COR Bioscience) according to the manufacturer's recommendations.

RPA. Cells were stimulated as indicated with the various reagents, and total RNAs were isolated with the peqGOLD RNAPure reagent (PeqLab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. Total RNAs were analyzed using customer Multi-Probe template sets (PharMingen, Hamburg, Germany) with respect to the expression of the indicated genes. L32 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) served as internal controls. Probe synthesis, hybridization, and RNase treatment were performed with the RiboQuant Multi-Probe RNase protection assay (RPA) system (BD PharMingen) according to the manufacturer's recommendations. Protected transcripts were separated by denaturing polyacrylamide gel electrophoresis on 5% acrylamide gels and analyzed on a PhosphorImager with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

DISC analysis. Immunoprecipitation of the DISC of Fas and TRAIL death receptors was performed with Flag-FasL, Fc-FasL, and Flag-TRAIL, respectively, as recently described (26). Briefly, one confluent 175-cm² flask of KB cells was used per condition. Cells were treated with 20 ng/ml IFN- γ for 24 h or left untreated. Cells were washed and stimulated with 5 ml of medium supplemented with either a mixture of 0.5 μ g/ml Flag-FasL or Flag-TRAIL and 2 μ g/ml anti-Flag (M2; Sigma) or with 0.5 μ g/ml Fc-FasL for the indicated time at 37°C or left untreated. Subsequently, cells were washed twice with ice-cold phosphate-buffered saline and were lysed with 1 ml lysis buffer (30 mM Tris-HCl, pH 7.5, 1% Triton X-100, 10% glycerol, 120 mM NaCl) supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) for 15 min on ice. After centrifugation (15 min, 14,000 $\times$ g) the DISC was precipitated from the cleared lysate with protein G Sepharose beads (20 μ l of a 50% slurry) overnight at 4°C. To the lysates from unstimulated cells a mixture of either 0.2 μ g Flag-FasL or Flag-TRAIL and 0.2 μ g M2 anti-Flag or 0.2 μ g/ml Fc-FasL was added together with the protein G beads. The precipitates were washed five times with ice-cold lysis buffer, and bound proteins were eluted by incubation at 70°C for 5 min in Laemmli sample buffer.

RESULTS

IFN- γ enhances Fas-mediated apoptosis induction and NF- κ B activation. IFN- γ showed a barely detectable apoptotic effect in KB cells. However, pretreatment for 24 h with IFN- γ enabled anti-Flag MAb cross-linked soluble Flag-FasL to trigger robustly apoptosis. Even high concentrations (500 ng/ml) of FasL induced apoptosis only in a minor fraction of KB cells, whereas a concentration of 10 ng/ml was sufficient to kill completely IFN- γ -pretreated cells (Fig. 1A). In previous studies we found that Fas-mediated apoptosis in KB cells requires down-regulation of the antiapoptotic FLIP proteins by CHX or FLIP-specific small interfering RNAs (siRNAs) (23, 37). No additional treatment with these compounds, however, was needed to reveal the apoptosis-enhancing effect of IFN- γ . As the FLIP proteins inhibit Fas-mediated apoptosis by blocking procaspase 8 conversion at the DISC, the most relevant effect of IFN- γ on Fas-induced apoptosis should occur in KB cells at the level of the DISC too. In agreement with this idea, we found no processing of caspase 8 and 10 in total cellular lysates after FasL stimulation without IFN- γ pretreatment, but a rapid and robust onset of these special hallmarks of death receptor-induced apoptosis upon treatment with IFN- γ (Fig. 1B, C). Of note, processing of caspase 8 occurred similarly in Bcl2-protected KB cells, indicating that cleavage of this initiator caspase was a direct consequence of DISC activation and not secondary to the activation of effector caspases triggered via the intrinsic mitochondrion-dependent pathway (Fig. 1D, E).

Next, we checked the effect of IFN- γ on the expression levels of FasL, Fas, FADD, caspase 8, caspase 10, and FLIP proteins by fluorescence-activated cell sorter (FACS) analysis (Fig. 1F), RPA (see Fig. 3 below), and Western blotting (Fig. 1G). FasL was not detectable by any of the methods mentioned above (data not shown), and FADD as well as caspase 10 expression remained unaffected at the protein level (Fig. 1G). For caspase 8 we observed a slight upregulation at the mRNA level by IFN- γ , but this did not lead to a significant increase at the protein level (Fig. 1G and Fig. 3, second row, 5th panel). By FACS analysis we found an increase in Fas cell surface expression of 25 to 40% (Fig. 1F). There was a slight increase in FLIP mRNA by IFN- γ treatment, but Western blotting revealed that expression of FLIP_S as well as of FLIP_L was reduced at the protein level after 24 h of IFN- γ treatment by 60 and 80%, respectively (Fig. 1G). Thus, IFN- γ could favor Fas signaling by blocking expression of the inhibitory FLIP protein and a concomitant upregulation of Fas expression.

To rule out that there was a global defect in FasL-induced Fas signaling complex formation in the absence of IFN- γ , we performed DISC analyses. Anti-Flag MAb cross-linked Flag-

FasL induced recruitment of caspase 8, FADD, FLIP_L, and FLIP_S to the DISC within 30 min in otherwise untreated KB cells (Fig. 2A), indicating that early assembly of Fas signaling complex formation was not inhibited. The Flag-FasL-immunoprecipitated DISC contained mainly unprocessed caspase 8, which is characteristic for a FLIP_S-arrested Fas DISC (24), and only a small fraction of the coprecipitated caspase 8 corresponded to the p41 and p43 intermediates of procaspase 8 which typically appear in the FLIP_L-arrested Fas DISC (Fig. 2) (24). Remarkably, pretreatment with IFN- γ for 24 h enhanced Fas DISC formation and caspase 8 processing. Especially, there was a significant increase in DISC-associated p18 subunit of caspase 8, which is indicative for fully processed and active heterotetrameric caspase 8 (Fig. 2A). Although overall FLIP expression in the cytoplasm was significantly reduced (Fig. 1G), FLIP recruitment to Fas was increased in IFN- γ -treated cells. However, likewise we found more DISC-associated FADD and caspase 8 (Fig. 2). As expression of these molecules was not significantly changed at the protein level, this suggests that overall DISC formation was increased in IFN- γ -treated cells. We were not able to reliably detect Fas itself in Flag-FasL/anti-Flag immunoprecipitates due to cross-reactivities of the secondary antibodies used for Fas detection and the anti-Flag antibody M2 used for immunoprecipitation. To verify the idea of an overall increase in the formation of an immunoprecipitable Fas-DISC in IFN- γ -treated cells, we performed DISC analysis experiments using Fc-FasL and protein G beads (Fig. 2B). Again there was an increase in Fas-associated FADD and caspase 8 in IFN- γ -treated cells, which was even more pronounced as compared with Flag-FasL/anti-Flag immunoprecipitates. The latter may correspond to the fact that Fc-FasL has an approximately fourfold higher specific activity than cross-linked Flag-FasL (data not shown). Taken together the DISC analysis revealed that IFN- γ -pretreatment enhanced Fas DISC formation and DISC-mediated caspase 8 processing.

In systematic RPA analyses screening for IFN- γ -regulated apoptosis-related genes, we found transcriptional upregulation of proapoptotic molecules, e.g., Bak, but also induction of antiapoptotic proteins such as Mcl-1 (Fig. 3). As with the exception of Fas and FLIP all the IFN- γ -regulated molecules found in this screen act downstream of the DISC; they cannot be of immediate relevance for the strong increase in FasL-induced caspase 8 activation described above.

After characterization of the apoptotic IFN- γ -Fas cross talk, we next investigated whether IFN- γ is also able to regulate Fas-mediated NF- κ B activation. FasL induced hardly detectable NF- κ B activation in otherwise untreated KB cells in terms of I κ B α degradation and upregulation of NF- κ B target genes.

FIG. 4. IFN- γ sensitizes KB cells for FasL-induced NF- κ B activation under nonapoptotic conditions. (A) KB cells were cultured for 24 h with or without 20 ng/ml IFN- γ . To demonstrate I κ B α degradation, cells were stimulated the next day with M2-cross-linked Flag-FasL (200 ng/ml) or TNF (20 ng/ml) for the indicated times. Protein lysates were prepared and immunoblot analyses were performed with anti-I κ B α antibodies and anti-tubulin (tub.) α/β as a loading control. (B) Untreated cells and IFN- γ -primed cells were challenged with cross-linked Flag-FasL (200 ng/ml) for 6 h in the presence and absence of z-VAD-fmk (20 μ M). For the analysis of the transcription of the bona fide NF- κ B target genes coding for I κ B α and IL-8, total RNAs were isolated and assayed by RPA analysis. (C) Untreated cells and IFN- γ -pretreated cells were challenged in triplicates with the indicated combinations of cross-linked Flag-FasL (200 ng/ml) and z-VAD-fmk (20 μ M). After 6 h supernatants were removed, cleared by centrifugation, and analyzed for their IL-8 content by ELISA. (D) Immunoblot analysis of triplicates of untreated and IFN- γ -stimulated (20 ng/ml, 24 h) cells with anti-TRAF2 and anti-RIP antibodies.

FIG. 5. FLIP_L and FLIP_S inhibit Fas-mediated NF-κB activation and apoptosis induction. (A) Parental KB cells and derived transfectants stably expressing FLIP_L-GFP and FLIP_S-GFP were seeded in triplicates in 96-well plates (10×10^3 per well), incubated with or without IFN-γ (20 ng/ml) for 24 h, and challenged with the indicated concentrations of Flag-FasL complexed with the Flag-specific MAb M2 (1 μg/ml). After an additional 18 h, cell viability was determined by crystal violet staining. (B) Untreated and IFN-γ-pretreated cells (20 ng/ml, 24 h) stimulated with 200 ng/ml of M2-cross-linked Flag-FasL for the indicated times were analyzed with respect to caspase 8 processing by immunoblotting. (C) FasL-induced IκBα degradation in untreated and IFN-γ-primed (20 ng/ml, 24 h) cells. KB, KB-FLIP_L-GFP, and KB-FLIP_S-GFP cells were analyzed by immunoblot analyses of IκBα. Detection of tubulin α/β served as a loading control. (D) The various KB cells were seeded in triplicates in 96-well plates and treated or not with IFN-γ (20 ng/ml, 24 h). Then supernatants were changed and cells were challenged for 6 h with the indicated concentrations of M2-cross-linked Flag-FasL in the presence of z-VAD-fmk to prevent apoptosis. In the IFN-γ-primed groups IFN-γ was again added. Finally, supernatants were removed, and IL-8 concentrations were determined by ELISA. (E) KB, KB-FLIP_L-GFP, and KB-FLIP_S-GFP cells were primed with IFN-γ (20 ng/ml, 24 h) and challenged for 6 h with M2-cross-linked Flag-FasL (200 ng/ml), and total RNAs were assayed for transcription of the indicated genes.

FIG. 5. Continued.

However, in IFN- γ -sensitized cells Fas stimulation elicited a robust NF- κ B response indicated by I κ B α degradation and upregulation of the bona fide NF- κ B target genes I κ B α , IL-6, and IL-8 (Fig. 4A to C). Noteworthy, TNF-mediated NF- κ B activation occurred independent from IFN- γ priming (Fig. 4A). In IFN- γ -protected cells FasL induced a sustained reduction in the I κ B α protein and a transient reduction when cells were protected by addition of z-VAD-fmk (Fig. 4A). While I κ B α degradation was more obvious in apoptosis-prone cells, upregulation of NF κ B target genes was significantly higher in cells rescued from apoptosis by z-VAD-fmk treatment (Fig. 4B, C). I κ B α expression, which is reduced by degradation in the course of NF- κ B activation, can be rapidly restored by resynthesis due to increased transcription of its gene. Most NF- κ B stimuli (TNF, IL-1, lipopolysaccharide) therefore trigger normally only a transient reduction of I κ B α expression. However, in cells undergoing apoptosis overall protein synthesis is blocked. Consequently resynthesis of I κ B α protein expression is therefore delayed or even not detectable. Thus, as the upregulation of

the mRNA of I κ B α and other NF- κ B target genes is significantly stronger in IFN- γ -primed cells rescued from apoptosis, the higher FasL-induced reduction in I κ B α expression observed in IFN- γ -treated cells undergoing apoptosis is apparently related to apoptosis-dependent inhibition of I κ B α resynthesis and does not necessarily reflect a higher NF- κ B activation. Similar to our earlier studies where CHX or FLIP-specific siRNAs were used to sensitize for Fas-mediated NF- κ B activation (23, 39), it was therefore necessary to block concomitant induced apoptosis to observe a maximal NF- κ B response. Fas-induced NF- κ B activation occurred robustly only after IFN- γ pretreatment, whereas the Fas-related death receptor TNFR1 already signaled NF- κ B activation in otherwise untreated cells (Fig. 4A), suggesting that both receptors are linked with the NF- κ B pathway in different ways. TRAF2 and RIP have been implicated in Fas-induced NF- κ B signaling (19, 23, 39). The expression of these proteins was not changed after IFN- γ priming.

FLIP_L and FLIP_S interfere with apoptosis induction and NF- κ B activation by Fas. The role of FLIP proteins, especially

FIG. 6. IFN- γ sensitizes KB cells for TRAILR1- and TRAILR2-induced apoptosis. (A to C) Untreated and IFN- γ -stimulated (20 ng/ml, 24 h) KB cells in a 96-well plate were treated in triplicates with the indicated concentrations of protein A-cross-linked anti-TRAILR1 (A) or anti-TRAILR2 antiserum (B) or Flag-TRAIL (C). After 18 h cell viability was determined by crystal violet staining (left panel). (D) KB cells were cultured in 175-cm² tissue culture flasks and treated with or without IFN- γ (20 ng/ml) for 24 h. Cells were then incubated with Flag-TRAIL (500 ng/ml) cross-linked with anti-Flag MAb M2 (2 μ g/ml) for the indicated times. Lysates isolated from unstimulated cells were supplemented with Flag-TRAIL and M2 and served as a negative control (C). The DISC was immunoprecipitated and analyzed by Western blotting with the indicated primary antibodies. α -, anti-; casp., caspase.

FLIP_L in Fas-mediated NF- κ B activation has been controversially discussed (for a review, see reference 40). Based on the finding that transiently overexpressed FLIP_L interacts with the adapter protein TRAF2 and activates the NF- κ B pathway, an NF- κ B-inducing role of FLIP in Fas signaling has been proposed (6, 15, 18, 19). However, other studies analyzing NF- κ B signaling after Fas triggering found an inhibitory effect of FLIP_L (16, 23, 39). In particular, we have already shown that FasL-induced NF- κ B activation is blocked in CHX-sensitized KB cells expressing FLIP_L-GFP or FLIP_S-GFP (23). As expected these transfectants were also protected against Fas-induced apoptosis after IFN- γ priming (Fig. 5A and B). More importantly, I κ B α degradation was almost completely blocked in KB cells expressing FLIP_L or FLIP_S and induction of the NF- κ B target genes IL-8 and TRAF1 was strongly reduced (Fig. 5C to E). The fact that expression of FLIP proteins efficiently rescued IFN- γ -primed cells from FasL-induced apoptosis and interfered with Fas-mediated NF- κ B activation emphasized again that other IFN- γ -induced changes in expression of proteins related to apoptosis or NF- κ B signaling (for exam-

ples, see Fig. 3) have at best a secondary role in the Fas-IFN- γ cross talk.

IFN- γ enhances apoptosis induction and NF- κ B activation by TRAILR1 and TRAILR2. Fas and the TRAIL death receptors induce apoptosis by very similar mechanisms and were also able to activate NF- κ B (38, 39). We therefore checked whether IFN- γ modulates TRAIL death receptor signaling in a similar way as Fas signaling. We showed already earlier in KB cells that both TRAIL death receptors induce to some extent apoptosis but only elicit a robust apoptotic response after sensitization with CHX or FLIP-specific siRNAs (37). Here, we found in addition that apoptosis induction by cross-linked soluble TRAIL as well as by TRAILR1- and TRAILR2-specific agonistic antisera were likewise enhanced by pretreatment with IFN- γ (Fig. 6A to D). Again, there was coincidence of IFN- γ -mediated enhancement of apoptosis induction with increased caspase 8 processing (data not shown) and accelerated DISC formation (Fig. 6D), identifying the TRAIL death receptor signaling complexes as another relevant target of the apoptotic IFN- γ -TRAIL cross talk. TRAIL-, TRAILR1-, and

FIG. 7. TRAILR1- and TRAILR2-induced NF- κ B signaling is enhanced by pretreatment with IFN- γ and inhibited by FLIP_L and FLIP_S. (A) KB, KB-FLIP_L-GFP, and KB-FLIP_S-GFP cells were primed with IFN- γ (20 ng/ml, 24 h) and challenged for the indicated time with protein A-cross-linked anti-TRAILR1 (1:500) or anti-TRAILR2 (1:500) antiserum, and I κ B α expression was assayed by Western blotting. Parental cells were analyzed in the presence of z-VAD-fmk to prevent apoptosis induction. (B) Cells were treated as described for panel A. IFN- γ -primed cells were treated for 6 h with protein A-cross-linked anti-TRAILR1 (1:500) or anti-TRAILR2 (1:500) antiserum, and total RNAs were isolated for RPA analyses of the indicated mRNAs. (C) KB, KB-FLIP_L-GFP, and KB-FLIP_S-GFP cells were seeded in triplicates in 96-well plates and treated for 6 h with the indicated concentrations of protein A-cross-linked anti-TRAILR1, anti-TRAILR2, or TNF. Parental KB cells were treated in the presence of z-VAD-fmk. Finally, supernatants were removed and IL-8 concentrations were determined by ELISA analysis. α -, anti-; tub., tubulin.

FIG. 8. IFN- γ priming is not required for FasL-induced activation of JNK, p38, and p42/44 ERK. (A) KB cells were primed with IFN- γ (20 ng/ml, 24 h) or remained untreated and were then challenged for the indicated time with M2-cross-linked Flag-FasL (200 ng/ml). Phospho-JNK, phospho-p38, phospho-p42/44, and the corresponding total proteins were assayed by Western blotting. (B) KB cells were again primed with IFN- γ (20 ng/ml, 24 h) or remained untreated and were challenged the next day for 4 h with M2-cross-linked Flag-FasL (200 ng/ml) in the absence and presence of 20 μ M z-VAD-fmk. The activity of JNK and p38 was again monitored by Western blotting.

TRAILR2-induced activation of the NF- κ B signaling pathway is significantly enhanced by CHX (data not shown) or IFN- γ priming (Fig. 7A, B), provided that concomitantly induced apoptosis was inhibited. Moreover, induction of NF- κ B target genes, such as I κ B α , IL-6, IL-8, and TRAF1, was blocked via both TRAIL death receptors in FLIP_L- and FLIP_S-expressing cells (Fig. 7A, B) suggesting that NF- κ B activation by Fas and the TRAIL death receptors is regulated by the same FLIP-dependent mechanism.

IFN- γ priming has only a minor effect on Fas-induced activation of MAP kinases. In order to investigate additional signaling pathways known to be activated by Fas, we next analyzed whether IFN- γ also regulates Fas-induced activation of JNK, p38 and ERK. Using antibodies specific for the phosphorylated and activated forms of these kinases, we found activation starting after 2 h and persisting for several hours. This is in sharp contrast to the very fast and transient activation of JNK, p38, and ERK observed after stimulation of the death receptor TNFR1 (41). Activation of the JNK and p38 pathway by FasL took place in the absence of sensitization and was only moderately affected by IFN- γ . FasL-induced ERK activation was unchanged or even slightly suppressed in IFN- γ -primed cells (Fig. 8A). To verify whether the increase in FasL-induced JNK and p38 activation was due to caspase activation, we analyzed these responses in the presence of z-VAD-fmk. In

accordance with the finding that the JNK and p38 stimulating MEKK1 kinase is activated by caspase-mediated cleavage (5), we found that FasL-induced JNK and p38 were strongly blocked in IFN- γ -primed cells rescued from apoptosis by addition of z-VAD-fmk. Surprisingly, FasL-induced activation of JNK and p38 was also inhibited by z-VAD-fmk in KB cells not treated with IFN- γ and thus not undergoing significant apoptosis. FasL-induced caspase-dependent JNK and p38 signaling therefore correlated with neither the amount of processed caspase 8 found in the cytoplasm (Fig. 1B) nor with the induction of apoptosis (Fig. 1A).

FasL and IFN- γ synergistically upregulate proinflammatory genes in nonapoptotic cells. NF- κ B-inducing cytokines such as TNF can cooperate with IFN- γ in inflammation. We therefore analyzed the regulation of inflammation-related genes by FasL in IFN- γ -pretreated cells comprehensively using a customized DNA oligonucleotide microarray developed in our laboratory (14, 23). In z-VAD-fmk-protected KB cells, several NF- κ B-related genes were significantly but only moderately expressed. This is in agreement with the requirement for sensitization to unravel a robust NF- κ B response by FasL in these cells. IFN- γ treatment upregulated a variety of known IFN- γ target genes, such as those coding for CCL20, IRF1, IRF2, gbp1, and XAF1, but showed no or only a moderate induction of NF- κ B target genes, such as those coding for IL-8 or IL-1 (Table 1). Most notably, there was a strong synergistic upregulation of most of the NF- κ B or IFN- γ target genes when Fas was stimulated in the presence of z-VAD-fmk in IFN- γ -pretreated cells (Table 1). The moderate upregulation of NF- κ B target genes by IFN- γ was presumably secondary to IFN- γ -induced production of NF- κ B stimulatory proteins, such as those coding for IL-1 α , IL-1 β , and TRAIL, and was not further investigated in this study. With respect to the synergistic action of FasL and IFN- γ three scenarios become immediately obvious. First, it could be possible that the low NF- κ B activation by FasL in nonsensitized KB cells is already sufficient to boost a parallel IFN- γ response. Second, the synergistic action of IFN- γ and FasL observed in IFN- γ -sensitized cells may depend on primary IFN- γ -mediated priming for robust Fas-induced NF- κ B activation and subsequent cooperation of the fully activated pathways engaged by each of the two cytokines. Third, FasL-induced mitogen-activated protein (MAP) kinase signaling, which robustly takes place independently from IFN- γ treatment, cooperates with IFN- γ in inflammatory signaling. To distinguish these possibilities we reanalyzed some typical IFN- γ and NF- κ B target genes in more detail by multitemplate RPA analysis. We analyzed the FasL-IFN- γ cross talk again after 24 h of IFN- γ -priming and in addition under costimulatory conditions. In the latter case the stimulation time was too short to enable IFN- γ to enhance Fas-mediated NF- κ B signaling. While RPA analyses confirmed the synergistic induction of typical NF- κ B target genes such as TRAF1 and IL-8 by FasL and IFN- γ when the latter was applied 24 h earlier, there was almost no synergistic effect under costimulatory conditions (Fig. 9). The lack of synergistic action of FasL and IFN- γ under costimulatory conditions was not due to a difference in the quality of immediate and delayed IFN- γ signaling, as the IFN- γ target genes coding for Fas, caspase 4, and caspase 7 were comparably induced under pre- and costimulatory conditions (Fig. 9). Thus, IFN- γ -mediated sensitization

TABLE 1. Identification of FasL and IFN- γ regulated genes

Gene name	Gene product description	Relative gene expression ^a		
		FasL	IFN- γ	FasL + IFN- γ
<i>cxcl8</i>	IL-8 precursor	3.7	1.0	9.0
<i>ccl20</i>	Chemokine (c-c motif) ligand 20	34.5	9.8	151.0
<i>ptgs2</i>	Prostaglandin-endoperoxide synthase 2 precursor	3.5	1.3	6.9
<i>birc3</i>	Baculoviral iap repeat-containing protein 3	3.2	1.3	8.7
<i>tnfa</i>	TNF- α	2.4	1.0	2.3
<i>Nfkb1</i>	Nuclear factor of kappa light polypeptide gene	2.5	1.4	4.4
<i>tnfaip3</i>	TNF- α -induced protein 3	3.1	2.0	10.1
<i>il1α</i>	IL-1 α proprotein	3.5	2.7	17.7
<i>gch1</i>	GTP cyclohydrolase 1 (dopa-responsive dystonia)	2.1	1.6	4.4
<i>ltb</i>	Lymphotoxin- β isoform	1.9	1.6	2.9
<i>ptx3</i>	Pentaxin-related gene, rapidly induced by IL-1 β	6.3	5.5	55.2
<i>il1b</i>	IL-1, β proprotein	1.3	1.2	5.4
<i>itgb8</i>	Integrin, β 8	2.4	2.3	5.4
<i>tnfrsf9</i>	TNF receptor superfamily, member 9	2.3	2.3	5.4
<i>icam1</i>	Intercellular adhesion molecule 1 precursor	1.9	2.0	6.5
<i>bcl2a1</i>	bcl2-related protein a1	1.2	1.4	4.9
<i>col7a1</i>	α 1-type vii collagen precursor	1.4	1.7	2.6
<i>saa1</i>	Serum amyloid a1	1.9	2.5	6.9
<i>birc2</i>	Baculoviral iap repeat-containing protein 2	1.6	2.2	7.0
<i>c3</i>	Complement component 3 precursor	1.2	1.7	2.8
<i>il6</i>	IL-6 6 (IFN- β 2)	2.6	3.7	12.5
<i>dscr1</i>	Down syndrome critical region protein 1	1.2	1.7	2.4
<i>vcam1</i>	Vascular cell adhesion molecule 1 isoform a precursor	1.4	2.2	3.7
<i>Fgf2</i>	Fibroblast growth factor 2	1.2	1.9	3.7
<i>sod2</i>	Superoxide dismutase 2, mitochondrial	2.0	3.3	7.6
<i>ripk2</i>	Receptor-interacting serine-threonine kinase 2	1.6	2.7	5.2
<i>ccr2a</i>	Chemokine (c-c motif) receptor 2 isoform α	1.3	2.3	4.7
<i>Lta4h</i>	Leukotriene a4 hydrolase	1.1	0.5	0.4
<i>tnfrsf5</i>	TNF receptor superfamily, member 5	1.2	2.7	4.7
<i>ccl5</i>	Small inducible cytokine 5 precursor	1.3	3.1	3.6
<i>tnfrsf10</i>	TNF ligand family, member 10	1.4	3.7	5.3
<i>irf7</i>	IFN regulatory factor 7 isoform α	1.9	5.2	6.8
<i>irf1</i>	IFN regulatory factor 1	1.8	5.1	9.4
<i>ifu1</i>	IFN-induced protein with tetratricopeptide	2.3	7.8	11.6
<i>bf</i>	Complement factor b preprotein	1.7	7.6	15.9
<i>tlr3</i>	Toll-like receptor 3	1.1	5.6	8.0
<i>tnfrsf13b</i>	TNF ligand family, member 13b	0.9	5.4	6.3
<i>cxcl10</i>	IFN- γ -induced precursor	4.6	27.4	111.2
<i>mda5</i>	Melanoma differentiation-associated protein 5	2.4	16.6	21.0
<i>hsxiapaf1</i>	XIAP-associated factor 1	1.0	8.5	9.3
<i>mx1</i>	Myxovirus resistance protein 1	2.3	21.4	25.0
<i>gbp2</i>	Guanylate binding protein 2, IFN inducible	1.1	12.0	16.8
<i>gbp1</i>	Guanylate binding protein 1, IFN inducible, 67 kDa	2.2	165.0	339.7

^a KB cells were treated for 24 h with IFN- γ or remained untreated and were then challenged with M2-cross-linked FasL in the presence of z-VAD-fmk. Thereafter total RNA was isolated and used to prepare double-stranded cDNA followed by cRNA synthesis and labeling with Cy3. DNA microarrays were performed with an MWG Biotech AG inflammation array containing amino-modified oligonucleotide probes, representing 136 genes relevant to inflammation as well as a number of housekeeping genes. Fluorescence intensities of bound cRNAs were recorded, normalized, and used to identify genes that were regulated by FasL and IFN- γ . Inducible expression of genes was determined as a ratio of relative gene expression compared to unstimulated cells. The average from two independent experiments is shown.

for FasL-induced NF- κ B activation can lead to a secondary enhancement of the initial IFN- γ response by FasL. However, we also found one IFN- γ -regulated gene, namely RANTES, which is already synergistically induced by IFN- γ and FasL under costimulatory conditions (Fig. 9). It is tempting to speculate that in this case the FasL-IFN- γ cross talk relies on cooperation of FasL-mediated MAP kinase activation, which in contrast to FasL-induced NF- κ B activation occurs independent from primary IFN- γ signaling.

We observed IFN- γ -induced upregulation of Fas and reduction in FLIP expression also in HT29 cells (Fig. 10A, B). Moreover, after IFN- γ priming FasL induced in these cells cell death as well as synergistic upregulation of NF- κ B target genes such as IL-8 and IP10 (Fig. 10C, D), suggesting that the proin-

flammatory FasL-IFN- γ cross talk commonly occurs and is not a specialized feature of KB cells.

DISCUSSION

A variety of studies reported enhancement of Fas-induced apoptosis by IFN- γ (7). However, a possible cross talk of the latter with nonapoptotic Fas signaling pathways has been poorly addressed. We have recently observed that FasL induces strong NF- κ B activation in KB cells via a FLIP-regulated pathway, including FADD, caspase 8, and RIP (23). Now, we analyzed these cells in respect to a possible cross talk of Fas and IFN- γ signaling. NF- κ B activation as well as apoptosis induction by FasL were both barely detectable in KB cells but

FIG. 9. Temporal order of FasL and IFN- γ stimulation determines the quality of the FasL-IFN- γ cross talk. KB cells were stimulated with M2-cross-linked Flag-FasL (200 ng/ml) in the presence of z-VAD-fmk for 6 h with or without IFN- γ (20 ng/ml) or after 24 h of priming with IFN- γ . Total RNAs were assayed for transcription of the indicated genes by multitemplate RPA. casp., caspase.

become robustly apparent after priming with IFN- γ for 24 h. In accordance with an apoptosis-associated inhibition of translation, FasL-induced upregulation of NF- κ B target genes was severalfold enhanced upon NF- κ B activation, when apoptosis was blocked using the broad-range caspase inhibitor z-VAD-fmk (Fig. 4). FasL-induced apoptosis and NF- κ B activation bifurcate at the level of the DISC and are both blocked by FLIP proteins in KB cells (23). No additional means interfering with FLIP expression were necessary for making the FasL-IFN- γ cross talk apparent; the Fas signaling complex itself therefore appeared as a possible major target of IFN- γ signaling in KB cells. In accordance with this idea, we found that the amount of processed caspase 8 observed in the cytosol was strongly increased in IFN- γ -primed cells (Fig. 1B). Moreover, we found a slight increase in Fas cell surface expression and a significant reduction in the expression of the FLIP proteins leading to an increase in the Fas/FLIP ratio by severalfold (Fig. 1). In accordance with other studies (17, 31, 44), caspase 8 was also upregulated by IFN- γ at the mRNA level (Fig. 3 and 9). However, the increase in caspase 8 mRNA was very low and reached no significance at the protein level (Fig. 1G). Taking into consideration the stoichiometric mode of action of FLIP_S and FLIP_L, it is conceivable that the IFN- γ -induced increase in the Fas/FLIP ratio exceeds a critical threshold where the number of FLIP molecules is not longer able to prevent DISC-mediated caspase 8 activation. In fact, in Fas immunoprecipitates of IFN- γ -primed cells the amount of DISC-associated FADD, caspase 8, and FLIP was increased (Fig. 2). As the expression of these molecules was not significantly changed at the protein level (FADD, caspase 8) or was even be reduced after IFN- γ treatment, this suggested that more DISC was formed in IFN- γ -treated cells. In fact, DISC analysis using Fc-FasL revealed an increase of precipitated Fas in IFN- γ -treated cells (Fig. 2B). Remarkably, the increase of DISC-associated Fas observed in IFN- γ -treated cells regularly exceeded the overall IFN- γ -induced increase in Fas cell surface expression. Thus, IFN- γ -induced changes in the ratio of DISC

components lead to enhanced DISC formation in a nonlinear manner. In line with the idea that not only the amount but also the ratio of DISC-associated proteins, such as FLIP and caspase 8, determines the amount of the immunoprecipitable DISC, two recent publications have shown that mechanisms associated with the cytosolic domain of Fas secondarily stabilize Fas cluster formation (13, 36). However, we cannot rule out that aside from the observed changes in Fas and FLIP expression other yet unknown mechanisms (additional DISC components, functional relevant modifications of DISC components) might contribute to the enhanced DISC formation in IFN- γ -primed KB cells.

It has been shown that heterodimers of caspase 8 and FLIP_L are enzymatically active (4, 28, 38). The well documented anti-apoptotic effect of FLIP_L is therefore apparently related to the fact that dimers of FLIP_L and caspase 8 stay tightly bound in the DISC and prevent repeated cycles of caspase 8 maturation. In supramolecular Fas clusters containing a mix of procaspase 8 dimers and heterodimers of procaspase 8 and FLIP_L, the latter may catalyze the maturation and DISC release of neighboring procaspase 8 dimers. Thus, dependent on the FLIP_L/caspase 8 ratio in the DISC, FLIP_L may act as an inhibitor or a promoter of caspase 8 processing. Such a switch depending on the FLIP/procaspase 8 ratio could explain that already minor IFN- γ -induced changes in Fas and FLIP expression lead to major alterations in Fas signaling.

In accordance with another study FLIP expression was increased at the mRNA level after IFN- γ treatment (11) (Fig. 3, second row, last panel). As Western blotting revealed, however, that expression of FLIP_S as well as of FLIP_L was reduced at the protein level (Fig. 1G), transcriptional upregulation of the FLIP gene has to be overcompensated for by posttranscriptional mechanisms. In this regard, it has been shown that the turnover of FLIP proteins is regulated by proteasomal degradation (10, 21). Notably, IFN- γ is able to stimulate the proteolytic activity of the proteasome, e.g., by inducing the proteasome regulators PA28 α and PA28 β (12). It is therefore

FIG. 10. IFN- γ modulates Fas and FLIP expression in HT29 cells and sensitizes for cell death induction and NF- κ B activation by Fas. (A) Untreated and IFN- γ -stimulated (20 ng/ml, 24 h) HT29 cells were stained with FITC-labeled anti-Fas antibodies or the corresponding isotype control and analyzed by FACS. (B) Immunoblot analysis of triplicates of untreated and IFN- γ -stimulated (20 ng/ml, 24 h) HT29 cells with anti-FLIP and antivinculin antibodies. (C) HT29 cells were seeded in 96-well plates (10×10^3 cells per well). The next day cells were incubated for 24 h with IFN- γ (20 ng/ml) or remained untreated. Cells were then challenged in triplicates with the indicated concentrations of soluble Flag-tagged FasL (Flag-FasL) complexed with the monoclonal anti-Flag antibody M2 (1 μ g/ml). After a further additional 18 h, cell viability was determined by crystal violet staining. (D) HT29 cells were then primed or not with IFN- γ (20 ng/ml, 24 h) and challenged for 6 h with the indicated combinations of M2-cross-linked Flag-FasL (200 ng/ml) and z-VAD-fmk (20 μ M). Finally, total RNAs were assayed for transcription of the indicated genes. α -, anti-.

tempting to speculate that IFN- γ -mediated inhibition of FLIP expression observed in our study was due to an increase of its proteasomal degradation. The balance between IFN- γ -induced upregulation of FLIP transcription and FLIP degradation may therefore be a determinant contributing to the cell type-specific effects of IFN- γ on Fas signaling.

IFN- γ not only regulates changes in DISC-associated proteins in KB cells but also modulates the transcription of several other apoptosis-related factors (Fig. 3). It is possible that changes in the expression of such apoptosis-related proteins secondarily enhance the increased DISC activity of FasL-trig-

gered IFN- γ -pretreated cells. Notably, we also observed IFN- γ -induced upregulation of TRAIL at the mRNA level. As IFN- γ treatment alone induced no or only modest apoptosis, this upregulation of TRAIL is apparently not sufficient to elicit a relevant apoptotic response. Nevertheless, as an autocrine interaction of TRAIL and TRAIL death receptors may reduce the amount of FLIP available for other secondary stimulated death receptors, it is possible that TRAIL upregulation sensitizes for apoptosis induction by other external death ligands, such as FasL.

TRAIL, which can activate two death receptors (TRAILR1

and TRAILR2) acting by mechanisms closely related to those of Fas, already induced cell death in KB cells without IFN- γ priming, predominantly by activation of TRAILR1 (Fig. 6A to C). As FLIP acts stoichiometrically to block Fas-, TRAILR1-, or TRAILR2-mediated caspase 8 activation, this could mean that the expression levels of TRAILR1 and TRAILR2 exceed FLIP expression in KB cells, so that TRAIL-induced caspase 8 activation cannot be completely prevented. A further increase in the death receptor/FLIP ratio in response to IFN- γ could then lead to accelerated caspase 8 activation in the DISC as is evident from the DISC analysis in Fig. 6D (please compare 30-min data points). However, it is also possible that other IFN- γ -dependent mechanisms play a role in the enhancement of apoptosis induction and NF- κ B activation by TRAIL death receptors.

Fas-mediated activation of JNK and p38 was blocked by z-VAD-fmk, suggesting that this is a caspase-dependent process. At first glance this does not fit the observation that no processed caspase 8 was found in lysates of FasL-stimulated cells (Fig. 1B). However, as discussed above FLIP_L and procaspase 8 can form enzymatically active heteromers in the DISC without release of mature caspase 8 into the cytoplasm (4, 28, 38). In accordance with a DISC-associated caspase activity in KB cells not undergoing apoptosis, we found in Fas immunoprecipitates of FasL-stimulated cells predominantly a FLIP_L fragment of 43 kDa (Fig. 2), which corresponds in size to a published caspase 8-derived cleavage product of FLIP_L (24). Moreover, especially in the DISC immunoprecipitated with Fc-FasL (Fig. 2B), we also found a p43/41 caspase 8 intermediate, which is generated by the first cleavage step during caspase 8 autoprocessing (24). Death receptor-induced caspase-mediated activation of JNK or p38 in the absence of apoptosis as described here and in some other studies might play a role in the recently described proliferation-promoting action of caspases (1, 8, 20, 29, 42).

Strikingly, FasL-induced activation of JNK, p38, and ERK already significantly took place in cells not primed with IFN- γ , whereas robust apoptosis induction and NF- κ B activation were highly dependent on such a treatment. Thus, IFN- γ -mediated enhancement of Fas-dependent apoptosis induction and NF- κ B activation as described above are not simply the result of a global enhancement of Fas signaling by IFN- γ but rather reflect a specific cross talk with certain but not all Fas-triggered pathways.

IFN- γ is coexpressed in activated T cells and NK T cells with FasL and TRAIL, respectively. The cross talk mechanisms described in this study therefore could have particular relevance in the biology related to these types of cells. IFN- γ -mediated enhancement of death receptor-induced apoptosis would be in good agreement with the cytotoxic functions of T and NK T cells. However, on target cells resistant to death receptor-induced apoptosis, IFN- γ may prompt a death receptor-mediated inflammatory response. This might be of special relevance for the proinflammatory effects of FasL observed in various models of FasL-mediated tumor rejection (40). Fas- and TRAIL death receptor-induced nonapoptotic signaling and its enhancement by IFN- γ may therefore get special relevance under pathophysiological conditions where apoptosis is blocked: e.g., by overexpression of antiapoptotic Bcl2 family members, viral or caspase inhibitors such as CrmA and IAPs,

or when crucial components of the apoptotic pathway are mutated.

ACKNOWLEDGMENTS

We thank Daniel Scholtysik (University of Düsseldorf), Heike Schneider (University of Hannover), and Evi Horn (University of Würzburg) for expert technical assistance.

H.W. was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 487, project B7) and Deutsche Krebshilfe (grant 10-1751-Wa 3). M.L. was supported by Deutsche Krebshilfe (grant 10-1951-Le1) and Wilhelm-Sander-Stiftung (grant 2000.092.2).

REFERENCES

1. Alam, A., L. Y. Cohen, S. Aouad, and R. P. Sekaly. 1999. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J. Exp. Med.* **190**:1879–1890.
2. Barnhart, B. C., E. C. Alappat, and M. E. Peter. 2003. The CD95 type I/type II model. *Semin. Immunol.* **15**:185–193.
3. Boatright, K. M., M. Renatus, F. L. Scott, S. Sperandio, H. Shin, I. M. Pedersen, J. E. Ricci, W. A. Edris, D. P. Sutherlin, D. R. Green, and G. S. Salvesen. 2003. A unified model for apical caspase activation. *Mol. Cell* **11**:529–541.
4. Boatright, K. M., C. Deis, J. B. Denault, D. P. Sutherlin, and G. S. Salvesen. 2004. Activation of caspases-8 and -10 by FLIP(L). *Biochem. J.* **382**:651–657.
5. Cardone, M. H., G. S. Salvesen, C. Widmann, G. Johnson, and S. M. Frisch. 1997. The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. *Cell* **90**:315–323.
6. Chaudhary, P. M., M. T. Eby, A. Jasmin, A. Kumar, L. Liu, and L. Hood. 2000. Activation of the NF-kappaB pathway by caspase 8 and its homologs. *Oncogene* **19**:4451–4460.
7. Chawla-Sarkar, M., D. J. Lindner, Y. F. Liu, B. R. Williams, G. C. Sen, R. H. Silverman, and E. C. Borden. 2003. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* **8**:237–249.
8. Choi, C., O. Kutsch, J. Park, T. Zhou, D. W. Seol, and E. N. Benveniste. 2002. Tumor necrosis factor-related apoptosis-inducing ligand induces caspase-dependent interleukin-8 expression and apoptosis in human astrogloma cells. *Mol. Cell. Biol.* **22**:724–736.
9. Donepudi, M., A. Mac Sweeney, C. Briand, and M. G. Grutter. 2003. Insights into the regulatory mechanism for caspase-8 activation. *Mol. Cell* **11**:543–549.
10. Fukazawa, T., T. Fujiwara, F. Uno, F. Teraishi, Y. Kadowaki, T. Itoshima, Y. Takata, S. Kagawa, J. A. Roth, J. Tschopp, and N. Tanaka. 2001. Accelerated degradation of cellular FLIP protein through the ubiquitin-proteasome pathway in p53-mediated apoptosis of human cancer cells. *Oncogene* **20**:5225–5231.
11. Grassi, F., A. Piacentini, S. Cristino, S. Toneguzzi, A. Facchini, and G. Lisignoli. 2004. Inhibition of CD95 apoptotic signaling by interferon-gamma in human osteoarthritic chondrocytes is associated with increased expression of FLICE inhibitory protein. *Arthritis Rheum.* **50**:498–506.
12. Groettrup, M., M. van den Broek, K. Schwarz, A. Macagno, S. Khan, R. de Giud, and G. Schmidtke. 2001. Structural plasticity of the proteasome and its function in antigen processing. *Crit. Rev. Immunol.* **21**:339–358.
13. Henkler, F., E. Behrle, K. M. Dennehy, A. Wicovsky, N. Peters, C. Warnke, K. Pfizenmaier, and H. Wajant. 2005. The extracellular domains of FasL and Fas are sufficient for the formation of supramolecular FasL-Fas clusters of high stability. *J. Cell Biol.* **168**:1087–1098.
14. Holzberg, D., C. G. Knight, O. Dittich-Breiholz, H. Schneider, A. Dorrie, E. Hoffmann, K. Resch, and M. Kracht. 2003. Disruption of the c-JUN-JNK complex by a cell-permeable peptide containing the c-JUN delta domain induces apoptosis and affects a distinct set of interleukin-1-induced inflammatory genes. *J. Biol. Chem.* **278**:40213–40223.
15. Hu, W. H., H. Johnson, and H. B. Shu. 2000. Activation of NF-kappaB by FADD, Casper, and caspase-8. *J. Biol. Chem.* **275**:10838–10844.
16. Imamura, R., K. Konaka, N. Matsumoto, M. Hasegawa, M. Fukui, N. Mukaida, T. Kinoshita, and T. Suda. 2004. Fas ligand induces cell-autonomous NF-kappaB activation and interleukin-8 production by a mechanism distinct from that of tumor necrosis factor-alpha. *J. Biol. Chem.* **279**:46415–46423.
17. Inaba, H., M. Glibetic, S. Buck, Y. Ravindranath, and J. Kaplan. 2004. Interferon-gamma sensitizes osteosarcoma cells to Fas-induced apoptosis by up-regulating Fas receptors and caspase-8. *Pediatr. Blood Cancer* **43**:729–736.
18. Kataoka, T., and J. Tschopp. 2004. N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kB signaling pathway. *Mol. Cell. Biol.* **24**:2627–2636.
19. Kataoka, T., R. C. Budd, N. Holler, M. Thome, F. Martinon, M. Irmeler, K. Burns, M. Hahne, N. Kennedy, N. Kovacsics, and J. Tschopp. 2000. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr. Biol.* **10**:640–648.
20. Kennedy, N. J., T. Kataoka, J. Tschopp, and R. C. Budd. 1999. Caspase activation is required for T cell proliferation. *J. Exp. Med.* **190**:1891–1896.

21. Kim, Y., N. Suh, M. Sporn, and J. C. Reed. 2002. An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J. Biol. Chem.* **277**:22320–22329.
22. Kischkel, F. C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P. H. Krammer, and M. E. Peter. 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.* **14**:5579–5588.
23. Kreuz, S., D. Siegmund, J. J. Rumpf, D. Samel, M. Leverkus, O. Janssen, G. Hacker, O. Dittrich-Breiholz, M. Kracht, P. Scheurich, and H. Wajant. 2004. NF κ B activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *J. Cell Biol.* **166**:369–380.
24. Krueger, A., S. Baumann, P. H. Krammer, and S. Kirchhoff. 2001. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol. Cell. Biol.* **21**:8247–8254.
25. Lambert, C., A. M. Landau, and J. Desbarats. 2003. Fas-beyond death: a regenerative role for Fas in the nervous system. *Apoptosis* **8**:551–562.
26. Leverkus, M., M. R. Sprick, T. Wachter, T. Mengling, B. Baumann, E. Serfling, E. B. Brocker, M. Goebeler, M. Neumann, and H. Walczak. 2003. Proteasome inhibition results in TRAIL sensitization of primary keratinocytes by removing the resistance-mediating block of effector caspase maturation. *Mol. Cell. Biol.* **23**:777–790.
27. Li-Weber, M., and P. H. Krammer. 2003. Function and regulation of the CD95 (APO-1/Fas) ligand in the immune system. *Semin. Immunol.* **15**:145–157.
28. Micheau, O., M. Thome, P. Schneider, N. Holler, J. Tschopp, D.W. Nicholson, C. Briand, and M. G. Grutter. 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J. Biol. Chem.* **277**:45162–45171.
29. Muhlenbeck, F., E. Haas, R. Schwenzer, G. Schubert, M. Grell, C. Smith, P. Scheurich, and H. Wajant. 1998. TRAIL/Apo2L activates c-Jun NH2-terminal kinase (JNK) via caspase-dependent and caspase-independent pathways. *J. Biol. Chem.* **273**:33091–33098.
30. Muhlenbeck, F., P. Schneider, J. L. Bodmer, R. Schwenzer, A. Hauser, G. Schubert, P. Scheurich, D. Moosmayer, J. Tschopp, and H. Wajant. 2000. The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. *J. Biol. Chem.* **275**:32208–32213.
31. Ruiz-Ruiz, C., C. Munoz-Pinedo, and A. Lopez-Rivas. 2000. Interferon-gamma treatment elevates caspase-8 expression and sensitizes human breast tumor cells to a death receptor-induced mitochondria-operated apoptotic program. *Cancer Res.* **60**:5673–5680.
32. Sedger, L. M., D. M. Shows, R. A. Blanton, J. J. Peschon, R. G. Goodwin, D. Cosman, and S. R. Wiley. 1999. IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. *J. Immunol.* **163**:920–926.
33. Shin, E. C., J. M. Ahn, C. H. Kim, Y. Choi, Y. S. Ahn, H. Kim, S. J. Kim, and J. H. Park. 2001. IFN-gamma induces cell death in human hepatoma cells through a TRAIL/death receptor-mediated apoptotic pathway. *Int. J. Cancer* **93**:262–268.
34. Shustov, A., P. Nguyen, F. Finkelman, K. B. Elkon, and C. S. Via. 1998. Differential expression of Fas and Fas ligand in acute and chronic graft-versus-host disease: up-regulation of Fas and Fas ligand requires CD8+ T cell activation and IFN-gamma production. *J. Immunol.* **161**:2848–2855.
35. Siegel, R. M., J. K. Frederiksen, D. A. Zacharias, F. K. Chan, M. Johnson, D. Lynch, R. Y. Tsien, and M. J. Lenardo. 2000. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science* **288**:2354–2357.
36. Siegel, R. M., J. R. Muppidi, M. Sarker, A. Lobito, M. Jen, D. Martin, S. E. Straus, and M. J. Lenardo. 2004. SPOTS: signaling protein oligomeric transduction structures are early mediators of death receptor-induced apoptosis at the plasma membrane. *J. Cell Biol.* **167**:735–744.
37. Siegmund, D., P. Hadwiger, K. Pfizenmaier, H. P. Vornlocher, and H. Wajant. 2002. Selective inhibition of FLICE-like inhibitory protein expression with small interfering RNA oligonucleotides is sufficient to sensitize tumor cells for TRAIL-induced apoptosis. *Mol. Med.* **8**:725–732.
38. Wachter, T., M. Sprick, D. Hausmann, A. Kerstan, K. McPherson, G. Stassi, E. B. Brocker, H. Walczak, and M. Leverkus. 2004. cFLIPL inhibits tumor necrosis factor-related apoptosis-inducing ligand-mediated NF- κ B activation at the death-inducing signaling complex in human keratinocytes. *J. Biol. Chem.* **279**:52824–52834.
39. Wajant, H., E. Haas, R. Schwenzer, F. Muhlenbeck, S. Kreuz, G. Schubert, M. Grell, C. Smith, and P. Scheurich. 2000. Inhibition of death receptor-mediated gene induction by a cycloheximide-sensitive factor occurs at the level of or upstream of Fas-associated death domain protein (FADD). *J. Biol. Chem.* **275**:24357–24366.
40. Wajant, H., K. Pfizenmaier, and P. Scheurich. 2003. Non-apoptotic Fas signaling. *Cytokine Growth Factor Rev.* **14**:53–66.
41. Wajant, H., K. Pfizenmaier, and P. Scheurich. 2003. Tumor necrosis factor signaling. *Cell Death Differ.* **10**:45–65.
42. Wilhelm, S., H. Wagner, and G. Hacker. 1998. Activation of caspase-3-like enzymes in non-apoptotic T cells. *Eur. J. Immunol.* **28**:891–900.
43. Xu, X., X. Y. Fu, J. Plate, and A. S. Chong. 1998. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res.* **58**:2832–2837.
44. Yang, X., M. S. Merchant, M. E. Romero, M. Tsokos, L. H. Wexler, U. Kontny, C. L. Mackall, and C. J. Thiele. 2003. Induction of caspase 8 by interferon gamma renders some neuroblastoma (NB) cells sensitive to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) but reveals that a lack of membrane TR1/TR2 also contributes to TRAIL resistance in NB. *Cancer Res.* **63**:1122–1129.