Role of NF-κB Activity in Apoptotic Response of Keratinocytes Mediated by Interferon-γ, Tumor Necrosis Factor-α, and Tumor-Necrosis-Factor-Related Apoptosis-Inducing Ligand

Jian-Zhong Qin, Patricia Bacon, Vijaya Chaturvedi, and Brian J. Nickoloff

Department of Pathology, Skin Cancer Research Program, Loyola University Medical Center, Cardinal Bernardin Cancer Center, Loyola University of Chicago, Chicago, Illinois, U.S.A.

An important step in tumorigenesis involves loss of sensitivity to various apoptotic signals by malignant cells, imbuing them with an enhanced survival phenotype. NF-κB also regulates epidermal thickness, susceptibility to apoptosis, and tumor formation in skin. Keratinocytes were examined for their susceptibility to apoptosis using cytokines produced during an immunologic response to tumor antigens, i.e., interferon-γ and/or tumor necrosis factor-α (TNF-α). The role for NF-κB in this response was examined using a retroviral vector containing a degradation-resistant form of IκBα. Whereas interferon-γ and TNF-α either alone or in combination did not induce apoptosis in keratinocytes, after infection with the retrovirus to block NF-κB activation they became susceptible to TNF-α but not Fas-induced apoptosis. Moreover, when keratinocytes with repressed NF-κB activity were simultaneously treated with interferon-γ, there was a synergistic induction of apoptosis by TNF-α that was dependent on FADD, tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), and caspase activation. Molecular abnormalities accompanying repressed NF-κB activity included failure to induce TNF-RII receptor together with enhanced levels of TRAIL death receptor 4. The ability of interferon-γ when combined with TNF-α to mediate keratinocyte apoptosis included induction of TRAIL coupled with diminished capacity of keratinocytes with repressed NF-κB activity to increase the TRAIL decoy receptor-1, as well as lower levels of several NF-κB-dependent antiapoptotic proteins accompanied by enhanced caspase 8 levels. These results indicate that interferon-γ and TNF-α synergistically induce keratinocyte apoptosis when concomitant induction of NF-κB is blocked. Participants in the apoptotic response mediated by NF-κB, besides cell-survival proteins, include modulation of TRAIL and both death and decoy receptors. Thus, not only does NF-κB signaling influence the intrinsic survival pathway for keratinocytes in normal skin, but it may also play a role in determining the apoptotic response to cytokines generated during an immune response via TRAIL produced by the keratinocytes themselves. ***Key words: cell death/cytokines/death receptors/skin cancer/transcription factor. J Invest Dermatol 117:898–907, 2001***

Not only does normal epidermal thickness require proper regulation of keratinocyte apoptosis, but an important step in cutaneous tumorigenesis involves loss of appropriate death signaling pathways in premalignant and malignant keratinocytes (Van Hogerlinden *et al*, 1999; Thompson, 1995; Ashkenazi and Dixit, 1998). There are several examples in which resistance of keratinocytes to apoptosis has been linked to development of skin cancer (Pena *et al*, 1998; Rodriguez-Villanueva *et al*, 1998; Duffey *et al*, 1999; Hill *et al*, 1999). An important family of gene regulatory proteins that can profoundly influence the induction of keratinocyte apoptosis involves NF-κB (Duffey *et al*, 1999; Hu *et al*, 1999; Li *et al*, 1999; Qin *et al*, 1999; Takeda *et al*, 1999; Seitz *et al*, 2000). NF-κB activation serves as a principal mediator for resistance to apoptosis by induction of numerous antiapoptotic factors such as TRAF-1, TRAF-2, c-IAP1, c-IAP2 (Wang *et al*, 1998), as well as enhancing levels of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ (Javelaud *et al*, 2000). The ability of tumor necrosis factor-α (TNF-α) to induce apoptosis in several cell types is potently suppressed, despite the engagement of death receptors (DR), because of the simultaneous activation of cell survival signals mediated via NF-κB (Beg and Baltimore, 1996; Van Antwerp and Verma, 1996; Wang *et al*, 1996). Indeed, enhanced antitumor effects have been achieved using TNF-α when accompanied by concomitant inhibition of NF-κB (Wang *et al*, 1999).

TNF-α can interact with two distinct receptors (TNF-RI, CD120a; or TNF-RII, CD120b), which are both expressed on

Manuscript received November 9, 2000; revised May 15, 2001; accepted for publication May 21, 2001.

Reprint requests to: Dr. Brian J. Nickoloff, Program Leader, Skin Cancer Research Laboratory, Building 112, Room 301, 2160 S. First Avenue, Maywood, IL 60153. Email: Bnickol@lumc.edu

Abbreviations: CHX, cycloheximide; DcR, decoy receptor; DN, dominant negative; DR, death receptor; PI, propidium iodide; RPA, RNase protection assay; TRAIL, tumor-necrosis-factor-related apoptosis-inducing ligand.

0022-202X/01/$15.00 · Copyright © 2001 by The Society for Investigative Dermatology, Inc.

many cell types (Tartaglia and Goeddel, 1992). Whereas TNF-RI contains a death domain and can trigger apoptosis (Feinstein *et al*, 1995), TNF-RII lacks a death domain and hence may be considered as a decoy receptor (DcR) (Park *et al*, 1999). Thus, these two TNF-α receptors are not homologous, and have distinct biologic roles that can influence apoptotic responses in either a positive or a negative fashion (Horie *et al*, 1999; Gaeta *et al*, 2000). To further explore the molecular basis determining susceptibility to apoptosis in keratinocytes, a dominant negative (DN) retroviral-based inhibitor of NF-κB activation was used, and the response to TNF-α alone and in combination with interferon γ (IFN-γ) was characterized.

IFN-γ and TNF-α represent primary cytokines implicated in the pathophysiology of benign and malignant skin diseases characterized by disordered epidermal kinetics and apoptotic responses (Nickoloff, 1991; Wrone-Smith *et al*, 1995; 1997; Kothny-Wilkes *et al*, 1998; Arnold *et al*, 1999). Moreover, IFN-γ when combined with TNF-α has shown clinical efficacy against some malignancies including cutaneous melanoma (Eggermont *et al*, 1996). Although we and others have previously utilized ultraviolet (UV) light as the stimulus to trigger keratinocyte apoptosis, it has become clear that this response involves many different DR and mediators of apoptosis (Schwarz *et al*, 1995; Brash *et al*, 1996; Henseleit *et al*, 1997; Rehemtulla *et al*, 1997; Aragane *et al*, 1998; Denning *et al*, 1998; Gutierrez-Steil *et al*, 1998; Sheikh *et al*, 1998; Chaturvedi *et al*, 1999; Leverkus *et al*, 2000; Qin *et al*, 2001). For example, UV-light-induced apoptotic signaling has been linked to the following molecules: TNF-α (Schwarz *et al*, 1995), Fas/Fas ligand (Rehemtulla *et al*, 1997; Aragane *et al*, 1998; Gutierrez-Steil *et al*, 1998; Leverkus *et al*, 1997), TNF-RI (Sheikh *et al*, 1998), caspases 3 and 8 (Chaturvedi *et al*, 1999), PKC-delta (Denning *et al*, 1998), as well as p53 (Brash *et al*, 1996; Henseleit *et al*, 1997; Tron *et al*, 1998). Thus, in contrast to these UV-light-related studies, this report will focus on more direct engagement of the TNF-α receptor family members by various ligands, and subsequent apoptotic signaling in keratinocytes.

There are a growing number of members of the TNF receptor family that can influence apoptotic responses (Dembic *et al*, 1990; Ashkenazi and Dixit, 1999). That so many different receptors are actually present on any one cell indicates that each type of receptor may participate with a distinctive role, and that complex regulation of apoptosis exists to respond to various stimuli. With respect to the TNF receptor family as mentioned earlier, one set of receptors that possess cytoplasmic death domains can initiate intracellular signaling events that lead to apoptosis, whereas another set of receptors – referred to as DcR – can bind various ligands but are not capable of triggering the caspase cascade due to either absent or truncated cytoplasmic death domains (Dembic *et al*, 1990; Lewis *et al*, 1991; Tartaglia *et al*, 1991; Pan *et al*, 1997; Sheridan *et al*, 1997). In this report, the receptors of interest include those that trigger apoptosis, such as TNF-RI, tumor-necrosis-factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) (DR4), TRAIL-R2 (DR5), and Fas, whereas the receptors not effective in killing are TNF-RII and TRAIL-R3 (DcR1, also referred to as TRID; Pan *et al*, 1997; Sheridan *et al*, 1997). The ligands for these receptors that are used in this study include TNF-α or TRAIL, which can induce apoptosis depending on the overall response of either normal proliferating keratinocytes or keratinocytes with repressed NF-κB activity. The novel observation that NF-κB-mediated signaling can influence levels of various DcR adds a new dimension to the regulation of apoptosis beyond death ligands and DR, which may participate in epidermal homeostasis, particularly as the apoptosis induced by IFN-γ plus TNF-α is partially mediated by endogenously produced TRAIL (Nickoloff and Denning, 2001).

MATERIALS AND METHODS

Keratinocyte culture Normal human keratinocytes were isolated from neonatal foreskins and grown in a low calcium (0.07 mM), serum-free medium (Clonetics, San Diego, CA) on plastic dishes as previously described (Wrone-Smith *et al*, 1995). Primary keratinocyte cultures were passaged when reaching approximately 50% confluence, and could be maintained for 10–15 population doublings prior to the onset of spontaneous replicative senescence (Nickoloff *et al*, 2000). The DN IκBα cDNA (IκBαDN) was kindly provided by Dr. Tom Ellis (Loyola University Medical Center) and subcloned into the *BamHI* and *NotI* of LZRS and MGF-based retroviral expression vector as previously described (Qin *et al*, 1999). The LZRS vector containing enhanced green fluorescent protein (GFP) was kindly provided by Dr. Paul A. Khavari (Stanford University School of Medicine, Stanford, CA). The Phoenix-Ampo retroviral packaging cells were obtained from American Type Culture Collection (Manassas, VA) with permission from Dr. Gary P. Nolan (Stanford University Medical Center, Stanford, CA). The packaging cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) and transfected with LZRS–IκBαDN vector by using $CaCl_2$ and 2 × Hanks' balanced salt solution. After overnight incubation, the cells were fed with fresh medium and incubated at 32°C for an additional 24–48 h. The supernatants were collected for cell infection. Keratinocytes were seeded into six-well plates and infected with 300 μl of viral supernatant in the presence of 4 μg per ml hexadimethrine bromide (Polybrene Sigma J-9268) for 1 h at 32°C, and then the supernatant was removed and replaced with fresh medium, incubated at 37°C in 5% CO_2 overnight. Typically, the efficiency of viral infection as assessed by GFP analysis using flow cytometry of permeabilized cells is greater than 85%. The overexpression of the IκBαDN protein was detected by western blot analysis. For some experiments various caspase inhibitors were added 1 h before exposure to IFN-γ plus TNF-α. These inhibitors included ZVAD-fmk and DVED-fmk, which were purchased from Enzyme Systems Products (Livermore, CA) and used at a final concentration of 10 μM as previously described (Chaturvedi *et al*, 1999). In other experiments, a FADD DN retroviral construct was produced courtesy of Dr. Vishva Dixit (Genentech, South San Francisco, CA). Overexpression of FADD DN protein was confirmed by western blot analysis.

Materials Recombinant human TNF-α and IFN-γ were obtained from R&D Systems (Minneapolis, MN) and used at a final concentration of 500 U per ml for TNF-α and 10^3 U per ml for IFN-γ. For western blot analysis, keratinocytes were treated for 24 h, whereas for mRNA analysis keratinocytes were treated for 6 h in all experiments. LZ-TRAIL (100 ng per ml) was generated at Immunex (Seattle, WA) as previously described (Walczak *et al*, 1997; 1999). Basically, the production and purification of human TRAIL employs a plasmid containing the extracellular regions of the ligand (amino acids 95–281) and is linked to an exogenous, modified leucine zipper that drives trimerization and is expressed in Chinese hamster ovary cells (Qin *et al*, 2001). Antibodies used to detect IκBa (SC-371), p50 (SC-7178), p65 (SC-109), actin, TNF-RI (SC-7895), TNF-RII (SC-7862), TRAF1 (SC-983), TRAF2 (SC-877), TRADD (SC-7868), and $p21^{Waf1/Cip1}$ (SC-397) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to detect caspase 8 (66231 A, FADD (65751 A), intercellular adhesion molecule 1 (ICAM-1, CD54), and CD3 were purchased from PharMingen (San Diego, CA). Monoclonal antibodies to detect TRAIL-R1 (DR4), TRAIL-R2 (DR5), and TRAIL-R3 (DcR1), as well as cell surface detection of TRAIL (monoclonal antibody 181; Fanger *et al*, 1999), were obtained from Immunex, as were receptor-fusion proteins that could neutralize TNF-α (TF:Fc), and TRAIL (TR:Fc) with CTLA4:Ig used as a control fusion protein. A FasL:Fc reagent was purchased from Alexis Biochemicals (San Diego, CA). To determine if apoptosis could be induced via activation of Fas, an anti-Fas IgM antibody was used that triggers apoptosis in Jurkat cells when used at 100 ng per ml (CH11; Upstate Biotechnology, Lake Placid, NY). In some experiments, keratinocytes were pretreated for 2 h with 5 μg per ml cycloheximide (CHX, Sigma Chemical, St. Louis, MO) for 6 h prior to addition of anti-Fas antibody.

Western blot analysis Nuclear cell lysate and whole cell lysate were prepared to detect different proteins as described previously (Qin *et al*, 1999). In brief, for nuclear keratinocytes cells were harvested by scraping and washed with phosphate-buffered saline (PBS); the pellet was resuspended in buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 1.0 mM ethylenediamine tetraacetic acid (EDTA), 1.0 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.1 mM dithiothreitol (DTT)], incubated in ice for 20 min with 10% Nonidet P-40, and then microfuged, and the supernatant was discarded. The pellet was resuspended in buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) for 15 min on ice. Cells were vortexed and microfuged and the

supernatant was saved and frozen at –80°C. For the whole cell lysate, keratinocytes were washed with PBS and were incubated in ice for 15 min in CHAPS buffer (Chaturvedi *et al*, 1999). Cells were microfuged and supernatants were saved and frozen at –80°C. The protein concentration of each sample was determined using Bio-Rad protein assay as previously described (Qin *et al*, 1999). Thirty micrograms of protein were loaded on 8%–12.5% sodium dodecyl sulfide polyacrylamide gel, transferred to Immobilon-p (PVDF) membrane, and blocked in 5% nonfat powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaC1, 0.01% Tween-20). The membrane was incubated with the primary antibody in 2.5% powdered milk in TBST and was washed extensively with TBST, and then incubated with 1:1500 diluted antirabbit or mouse horseradish peroxidase (Amersham, IL). Proteins were visualized with enhanced chemiluminescence reagents (Amersham) according to the manufacturer's instruction. Loading of proteins to verify equivalent distribution of proteins in each well was confirmed by Ponceau-S staining and detection of actin levels.

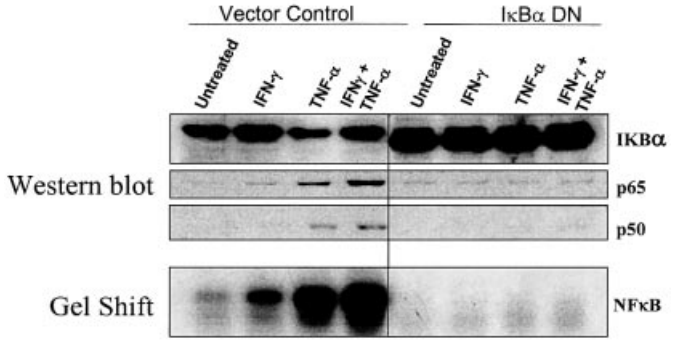

Figure 1. Overexpression of a degradation-resistant form of IκBα in keratinocytes prevents activation of NF-κB by IFN-γ and/or TNF-α. *Left side panel*: Keratinocytes infected with an empty vector (only linker). Constitutive and cytokine-induced levels of IκBα in the cytoplasm, as well as intranuclear levels of p50 and p65 subunits of NF-κB, are assessed (western blot) and compared to DNA binding activity (gel shift). Note that IFN-γ and/or TNF-α induced p65, and to a lesser extent p50 levels, with enhanced DNA binding. By contrast, keratinocytes infected with IκBαDN-containing retrovirus (*right side panel*) have enhanced constitutive levels of IκBα, and fail to induce either p50/p65 levels or DNA binding after IFN-γ and/or TNF-α exposure. Results portrayed are representative of three independent experiments.

RNase protection assay (RPA) Total cellular RNA was extracted using Trizol Reagent (Gibco BRL, Grand Island, NY) as previously described (Qin *et al*, 1999). The RPA was performed according to the supplier's instructions (PharMingen). Briefly, human apoptosis template set hAP0-5 was labeled with [α-^{32}P] uridine triphosphate. RNA (10 μg) and 8×10^5 cpm of labeled probes were used for hybridization, and after RNase treatment the protected probes were resolved on a 5% sequencing gel.

Electrophoretic mobility shift assays (EMSA) EMSA were performed as previously described (Qin *et al*, 1999). In brief, 1 μg of poly(dI-dC) (Pharmacia Biotech) and 10^4 cpm of ^{32}P-labeled double stranded oligonucleotide were incubated with 5 μg of nuclear protein on ice for 30 min. The reaction mixture was separated on 4% native polyacrylamide gel, dried, and autoradiographed. The NF-κB oligonucleotide had the following sequence: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′.

Assessment of apoptosis and cell surface detection of DR/DcR and TRAIL For cell cycle and apoptosis analyses, DNA content was measured by propidium iodide (PI) staining and flow cytometry (Qin *et al*, 1999). After treatments, keratinocytes were trypsinized, combined with floating cells, and counted, and 10^6 cells were washed once in 2 ml fluorescence-activated cell sorter (FACS) buffer (PBS, 5% fetal bovine serum, 0.02% sodium azide). The cell pellet was suspended in 100 μl fetal bovine serum on ice, and 600 μl ice-cold 100% ethanol was added with gentle vortexing. The cells were incubated for 30 min on ice and washed once with FACS buffer. The cells were suspended in 0.5 ml 10 μg per ml RNase in PBS and incubated at 37°C for 15 min. After 5 min at room temperature, 0.5 ml of 100 μg per ml PI in PBS was

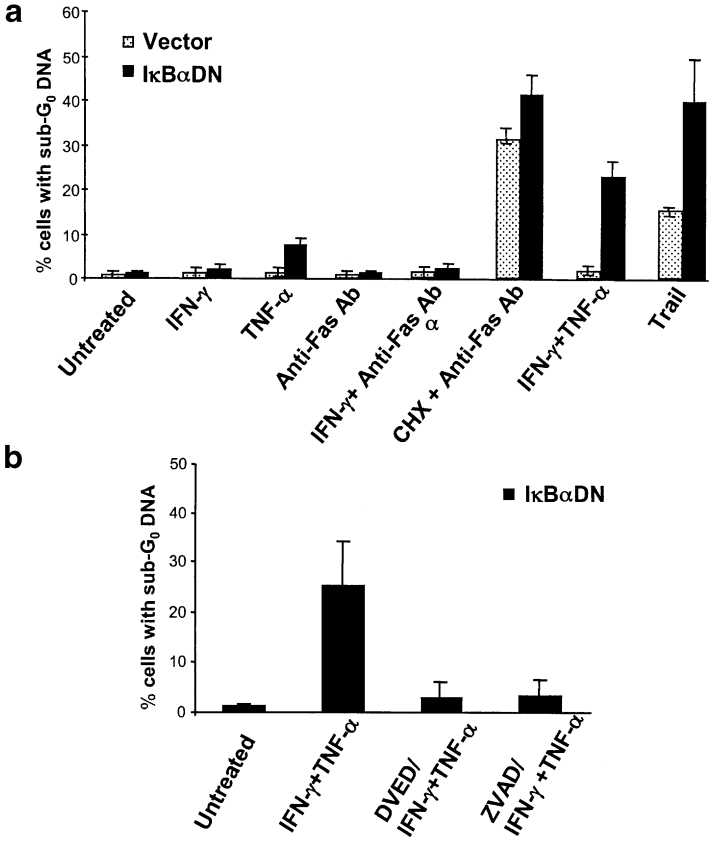

Figure 2. Enhanced susceptibility of keratinocytes with repressed NF-κB activity to apoptosis induced by TNF-α, but not by anti-Fas antibody; IFN-γ plus TNFα; and TRAIL. (*a*) Keratinocytes were infected with either empty retrovirus (vector) or IκBαDN-containing retroviruses and then exposed to various treatments including the following stimuli: IFN-γ (10^3 U per ml), TNF-α (500 U per ml), anti-Fas antibody (100 ng per ml), or TRAIL (100 ng per ml), for 18 h followed by PI staining and FACS analysis. As indicated, some cultures were exposed to IFN-γ plus anti-Fas antibody, CHX pretreatment (5 μg per ml, 2 h) followed by anti-Fas antibody, or IFN-γ plus TNF-α. (*b*) Dependence of IFN-γ plus TNF-α induced apoptosis on caspase activation demonstrated by using protease inhibitors, DVED-fmk and ZVAD-fmk. Results portrayed represent the relative mean number of apoptotic cells (% cells with sub-G_0 DNA content, *y* axis) ± SEM for three independent experiments.

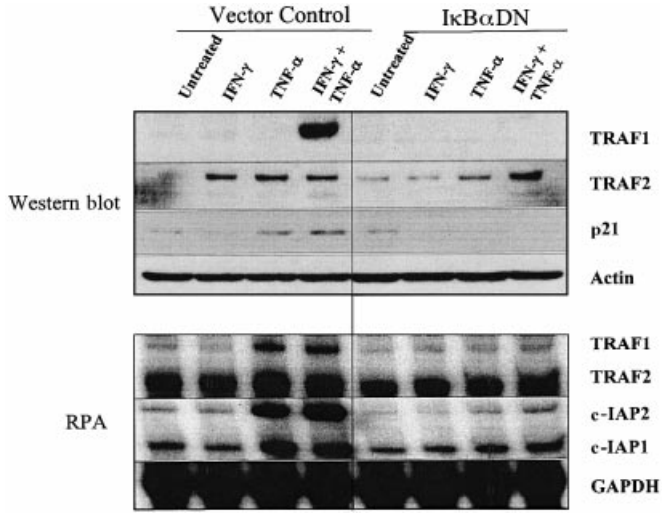

Figure 3. Inhibition of NF-κB signaling in keratinocytes results in diminished or absent induction by IFN-γ and/or TNF-α for molecular mediators involved in apoptosis. Keratinocytes with either normal (vector) or NF-κB-repressed (IκBαDN) activity differentially respond to cytokine stimulation as assessed by Western blot analysis (*upper panels*) and RPA (*lower panels*). Note that whereas normal keratinocytes (*left side panel*) upregulate protein and mRNA levels for cell survival mediators (i.e., TRAF1, c-IAP1, c-IAP2, and $p21^{Waf1/Cip1}$), keratinocytes with repressed NF-κB activity fail to induce these molecules to the same extent. Results portrayed are representative of one of two independent experiments. Equivalent loading was confirmed by using actin for immunoblots and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for RPA.

added to each sample, mixed gently, and incubated at 4°C for at least 1 h. PI staining was quantitated by running the samples on a Coulter Epics XL-MCL flow cytometer. Cells with DNA content less than the G_0/G_1 amount of untreated cells were considered apoptotic. In some assays, apoptosis was measured using Annexin-V/PI staining by following the manufacturer's instructions (Beckman-Coulter, Hialeah, FL), and then the cells analysed using the aforementioned flow cytometer. Results portrayed represent mean values for three independent experiments, and in the apoptotic assays results are provided plus/minus standard errors under the indicated conditions. To identify cell surface expression, keratinocytes were incubated with the indicated primary monoclonal antibodies, washed, and resuspended in PBS containing 2% fetal bovine serum. Fluorescein isothiocyanate (FITC) conjugated secondary antibody (Biosource International, Hopkinton, MA) was added, and after washing cells were analyzed by flow cytometry as previously described (Qin *et al*, 1999). Statistical analysis was performed and differences were considered significant using Student's *t* test when $p < 0.05$.

RESULTS

Overexpression of IκBαDN blocks cytokine-induced NF-κB activation As several different cytokines can activate NF-κB in various cell types, normal human keratinocytes were examined for the intranuclear levels of p50 and p65 subunits of NF-κB, as well as DNA binding, before and after exposure (60 min) to IFN-γ (10^3 U per ml) and/or TNF-α (500 U per ml). Keratinocytes infected with a vector control (retrovirus containing only linker) had detectable constitutive levels of IκBα, but there was no detectable p50, p65, or significant DNA binding (**Fig 1**, *left side*). Exposure to either IFN-γ alone or TNF-α alone, however, produced increased nuclear levels of p65 and p50, as well as enhanced DNA binding. Combining IFN-γ and TNF-α further enhanced these responses. By contrast, keratinocytes infected with the IκBαDN-containing retrovirus had higher constitutive levels of IκBα (reflecting the resistance to degradation), but there was no constitutive or inducible levels for either of the p65 and p50 subunits of NF-κB or DNA binding activity in these cultured keratinocytes with repressed NF-κB activity (**Fig 1**, *right side*). Taken together, these results confirm the efficacy of using this retroviral-based protocol to generate keratinocytes with dysfunctional NF-κB-mediated signaling. As such, these IκBαDN-infected keratinocytes can serve as model target cells to simulate the types of epidermal cells involved in cutaneous carcinogenesis (Van Hogerlinden *et al*, 1999).

Repression of NF-κB activation renders keratinocytes susceptible to apoptosis To extend earlier studies (Qin *et al*, 1999; Seitz *et al*, 2000) demonstrating an enhanced susceptibility of keratinocytes with repressed NF-κB activity to UV-induced apoptosis, the response to cytokines and death ligands was explored. Under normal culture conditions, addition of IFN-γ or TNF-α either alone or in combination only induces growth arrest, but not significant levels of apoptosis in proliferating keratinocytes (**Fig 2**). Cell cycle analysis revealed similar patterns of growth arrest for both normal keratinocytes and those with repressed NF-κB activity following exposure to IFN-γ or TNF-α. The G_1:*S* ratios for a representative experiment are as follows: control keratinocytes (linker only), untreated 2.9, after IFN-γ treatment 5.9, after TNF-α alone 4.6; IκBαDN-infected keratinocytes, untreated 2.6, after IFN-γ 5.4, after TNF-α treatment 4.6. There is less than 2% apoptosis amongst keratinocytes either before or 18 h after addition of these cytokines containing sub-G_0 DNA content after PI staining and flow cytometry. By phase contrast microscopy these keratinocyte cultures also displayed no visual evidence of any cytopathic effects following addition of IFN-γ and/or TNF-α (data not shown). In keratinocytes infected with the IκBαDN-containing retrovirus, however, whereas there is no spontaneous apoptosis, addition of TNF-α but not IFN-γ triggered apoptosis as the cells become rounded-up and detached from the surface with membrane blebbing (data not shown), and increased sub-G_0 DNA content after PI-FACS analysis (**Fig 2**). Eight percent of keratinocytes in the IκBαDN cultures become apoptotic after TNF-α exposure, which is an approximately 4-fold increase ($p < 0.05$) compared to untreated cells. Moreover, when IFN-γ is combined with TNF-α in these keratinocytes with deficient NF-κB signaling, there was clearly more cytopathic effect visible by phase contrast microscopy in the culture, and an approximately 12-fold increase ($p < 0.05$) in cells with sub-G_0 DNA content (mean value 24%).

To determine if this enhanced susceptibility based on IFN-γ pretreatment was specific for the TNF-α-receptor mediated death pathway, keratinocyte cultures were also exposed to an agonistic anti-Fas antibody (100 ng per ml) or TRAIL (100 ng per ml). **Figure 2(*a*)** reveals that no increased apoptosis was triggered in either proliferating keratinocytes with linker or IκBαDN-infected keratinocytes to either anti-Fas antibody or IFN-γ (10^3 U per ml) with anti-Fas antibody. To verify the agonistic functional activity of the CH11 antibody, keratinocytes were pretreated with CHX (which by itself does not induce apoptosis – data not shown) which did enhance sensitivity to pro-apoptotic effects of anti-Fas antibody. By contrast to these anti-Fas results, 18% of normal keratinocytes underwent apoptosis after exposure to TRAIL, and keratinocytes with repressed NF-κB activity displayed an even greater enhanced apoptotic response ($p < 0.05$) to TRAIL (increased to 38% of keratinocytes with sub-G_0 DNA content).

To determine if caspase activation was necessary for induction of IFN-γ plus TNF-α mediated apoptosis under these conditions, two different protease inhibitors were used. Addition of either DVED-fmk or ZVAD-fmk blocked the ability of IFN-γ plus TNF-α to induce apoptosis in the NF-κB-repressed keratinocyte cultures (**Fig 2*b***).

Influence of NF-κB activity on cell survival pathway To begin to explore molecular mechanisms underlying the enhanced apoptotic susceptibility to IFN-γ plus TNF-α in NF-κB-repressed keratinocytes, the relative levels of proteins contributing to cell survival were analyzed. **Figure 3** depicts protein (western blot, *upper panel*) and mRNA (RPA, *lower panel*) levels in keratinocytes with intact (vector control) or repressed (IκBαDN) NF-κB

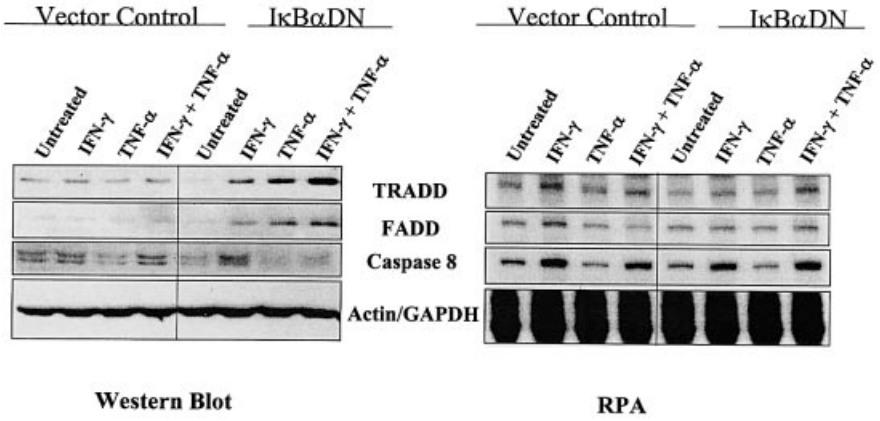

Figure 4. Modulation of death effector molecules in normal and NF-κB-repressed keratinocytes in response to IFN-γ and/or TNF-α. Analysis by both Western blot (*left side*) and RPA (*right side*) reveals that, in NF-κB-repressed keratinocytes, the indicated cytokines induced higher levels of pro-apoptotic mediators such as TRADD and FADD, but not caspase 8. Results portrayed are representative of two independent experiments. Equivalent loading was confirmed by using actin for immunoblots and GAPDH mRNA for RPA.

activity, before and after exposure to cytokines (24 h treatment for protein analysis, and 6 h treatment for mRNA analysis). The ability of IFN-γ and/or TNF-α to induce TRAF1 was completely blocked by IκBαDN-infected keratinocytes, with a lesser degree of inhibition of TRAF2. Similarly, there was almost complete inhibition of the IFN-γ and/or TNF-α induced levels of C-IAP2, and to a lesser extent C-IAP1. As TRAF1 and TRAF2 are both required for optimal NF-κB signaling, these reduced levels may contribute not only to the failure to activate NF-κB in response to the cytokines in **Fig 1**, but also to the absent or low levels of the antiapoptotic C-IAP proteins depicted in the lower portion of **Fig 3**. Levels of $p21^{Waf1/Cip1}$ were also examined because induction of $p21^{Waf1/Cip1}$ can protect against apoptosis, and is dependent on NF-κB activation (Javelaud *et al*, 2000). **Figure 3** (*upper panel*) reveals that, whereas $p21^{Waf1/Cip1}$ can be induced by cytokine treatment in vector-control-infected keratinocytes, there is complete inhibition of $p21^{Waf1/Cip1}$ induction in keratinocytes infected with the IκBαDN-containing retrovirus. In the next section we move from cell survival pathways to the death effector pathways.

Modulation of death effector and related molecules Many molecular mediators involved in apoptotic signaling have been discovered that are either directly/indirectly related to TNF receptor family members or participate in the apoptotic response. **Figure 4** represents an analysis (protein and mRNA levels) for some of these molecular mediators in normal keratinocytes (vector, *left side*) or NF-κB-repressed keratinocytes (IκBαDN, *right side*). IFN-γ and/or TNF-α slightly increased TRADD in control cultures, which was further enhanced in the NF-κB-repressed cultures at the protein level, with less change in mRNA levels. FADD protein levels were significantly increased in NF-κB-repressed cultures after exposure to IFN-γ and/or TNF-α. IFN-γ exposure consistently enhanced mRNA and protein levels of caspase 8. This induction of caspase 8 was not dependent on NF-κB activation, as the IκBαDN-infected cells had a fairly similar response to the empty-vector-infected cells. As the markedly enhanced apoptotic susceptibility of the NF-κB-repressed keratinocytes to IFN-γ and TNF-α may have involved an additional set of molecular mediators, attention was directed at the relative levels of DR expression by the keratinocytes.

NF-κB activity and cytokine exposure influences levels of TNF receptor family members As TNF-α exposure alone induced a greater apoptosis when NF-κB activation was blocked (**Fig 1**), the initial experiments examined the influence of cytokines on TNF-RI and TNF-RII levels, with particular focus on

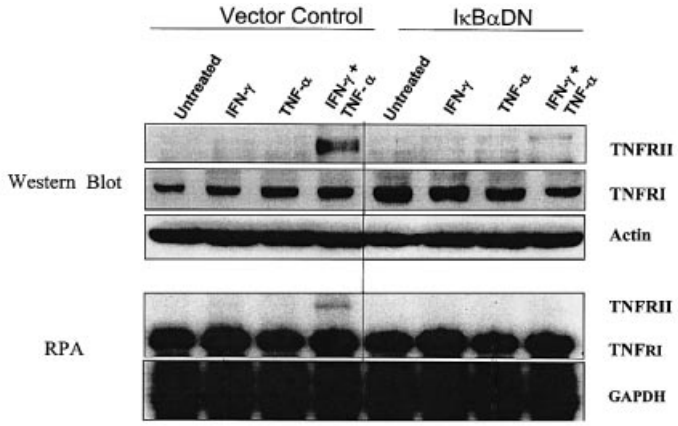

Figure 5. Differential regulation of TNF-RI *versus* TNF-RII expression in normal and NF-κB-repressed keratinocytes in response to IFN-γ and/or TNF-α. *Left side panels* portray keratinocytes infected with empty vector (control) virus, whereas the *right side panel* portrays keratinocytes infected with IκBαDN-containing retrovirus, before and after exposure to IFN-γ and/or TNF-α. Cells were analyzed by western blot (*upper panels*) and RPA (*lower panels*) to detect TNF-RII and TNF-RI protein and mRNA levels, revealing constitutive presence of TNF-RI but not TNF-RII. Note that NF-κB-repressed keratinocytes were unable to induce TNF-RII after exposure to IFN-γ plus TNF-α. Equivalent loading was confirmed by using actin for immunoblots and GAPDH mRNA for RPA.

comparisons/contrasts between normal and NF-κB-repressed keratinocytes. **Figure 5** reveals that TNF-RI levels were not significantly influenced by the cytokines in all keratinocytes examined, irrespective of their NF-κB status. TNF-RII (which lacks the capacity to trigger apoptosis and hence may serve as a DcR) was only induced in the normal keratinocytes by IFN-γ and TNF-α, however; such induction of TNF-RII was blocked in the NF-κB-repressed keratinocytes.

To more completely characterize other members of the TNF receptor family, as well as related DcR, additional studies were conducted. Western blot analysis revealed that TRAIL-R1 (DR4) was significantly induced only in the NF-κB-repressed keratinocytes by cytokine treatment, being particularly prominent after treatment with IFN-γ + TNF-α (**Fig 6**, *upper panel*). By contrast TRAIL-R2 (DR5) levels were constitutively prominent and did not change after cytokine exposure; and were not influenced by the

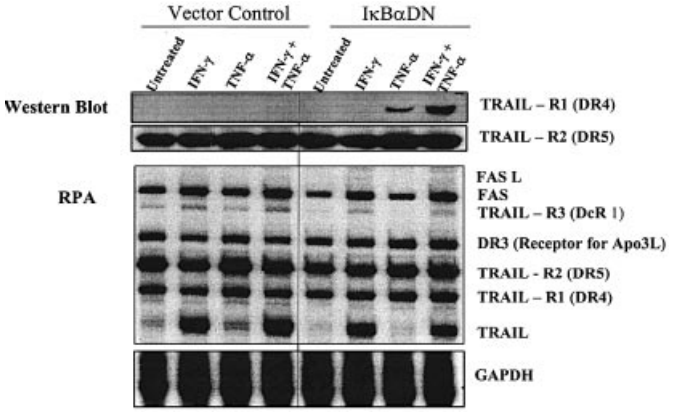

Figure 6. IFN-γ induces TRAIL mRNA levels independent of NF-κB activation, but NF-κB-repressed keratinocytes fail to constitutively express the TRAIL DcR1 but enhance their expression of TRAIL-R1 (DR4) after IFN-γ plus TNF-α exposure. *Left side panel* represents vector-control-infected keratinocytes, and *right side panel* represents keratinocytes infected with IκBαDN-containing retrovirus. Note increased protein levels for TRAIL-RI (DR4) in the NF-κB-repressed keratinocytes after exposure to IFN-γ plus TNF-α, which is not induced in normal keratinocytes. Also, RPA demonstrates the lower cytokine-inducible mRNA level for TRAIL-R3 (DcR1) in the NF-κB-repressed keratinocytes. Many other pro-apoptotic mediators such as Fas, DR3 (receptor for Apo 3 L), TRAIL-R1 (DR4), and TRAIL-R2 (DR5) are constitutively present and not significantly changed by cytokine exposure or status of NF-κB activity.

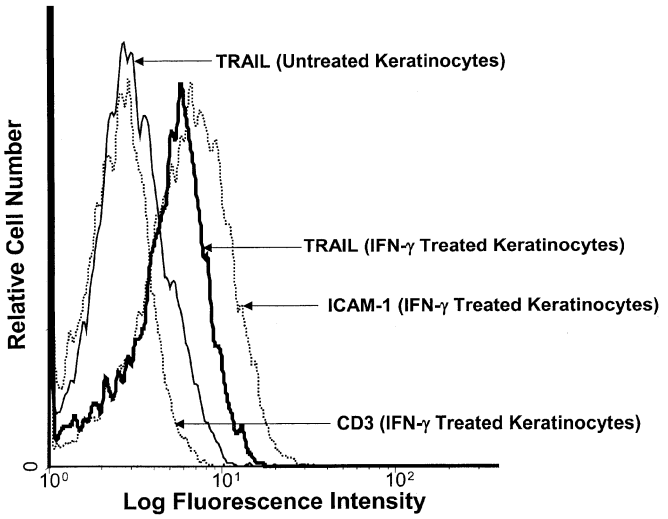

Figure 7. Flow cytometric analysis to detect cell surface levels of TRAIL. FACS analysis reveals low but detectable constitutive surface levels of TRAIL, which are increased 2-fold after keratinocytes are treated with IFN-γ (24 h, 10^3 U per ml). Note the lack of staining for CD3 (negative isotype control monoclonal antibody) and higher surface levels of ICAM-1 induced by IFN-γ treatment.

NF-κB activity in cultured keratinocytes. In addition to these protein levels, mRNA levels were assessed using RPA. Beginning with Fas (i.e., CD95), IFN-γ, but not TNF-α, induced CD95 mRNA levels, and the presence of the IκBαDN did not influence this induction (**Fig 6**, *lower panel*). Under no conditions was CD95L mRNA detected in the keratinocyte cultures. This, together with the lack of response of either normal or NF-κB-repressed keratinocytes to anti-Fas antibody (either with or without IFN-γ), indicates that it is unlikely for either CD95 or CD95L to participate in this experimental system (also see **Fig 9**).

The DR3 (receptor for Apo 3 L/TWEAK), TRAIL-R1 (DR4), and TRAIL-R2 (DR5) mRNA levels were constitutively present in both normal and NF-κB-repressed keratinocytes, but there was no significant change after exposure to the cytokines (**Fig 6**). The differences between the protein and mRNA levels for TRAIL-R1 (DR4) suggest that this DR expression is regulated primarily by post-transcriptional events. IFN-γ induced TRAIL mRNA, either with or without concomitant TNF-α, in both normal and NF-κB-repressed keratinocytes. Interestingly, whereas normal keratinocytes constitutively expressed TRAIL-R3 (DcR1), which is a DcR for TRAIL (i.e., TRID) that lacks an intracellular death domain, the NF-κB-repressed keratinocytes had no detectable transcripts using RPA (**Fig 6**). Moreover, whereas IFN-γ either with or without concomitant TNF-α could induce DcR1 in normal keratinocytes, only a slight induction of TRAIL-R3 (DcR1) was observed in the NF-κB-repressed keratinocytes following IFN-γ or IFN-γ plus TNF-α exposure. To explore the actual cell surface levels of TRAIL, keratinocytes before and then 24 h after exposure to IFN-γ (10^3 U per ml) were examined by flow cytometry (FACS). **Figure 7** reveals constitutive detection of TRAIL on approximately 30% of normal keratinocytes. After IFN-γ treatment, 75% of keratinocytes were positive for TRAIL, with a 2-fold increase in the mean channel fluorescence. The induction of ICAM-1 by IFN-γ served as a positive control, with CD3 staining a negative (isotype) control.

Determination of cell surface levels of TNF receptor family members By FACS analysis, both normal and NF-κB-repressed keratinocytes were found to express identical cell surface levels of TNF-RI, but no detectable TNF-RII. **Figure 8**(***A***) reveals a representative FACS profile for normal keratinocytes. **Figure 8**(***B***) illustrates the expression of various TRAIL-related DR and DcR as indicated for keratinocytes infected with empty retroviral vector (linker, *left side panels*) and for keratinocytes infected with IκBαDN retroviral vector (*right side panels*). Normal keratinocytes express both DR (TRAIL-R1, DR4, and TRAIL-R2, DR5) as previously described (Leverkus *et al*, 2000). Compared to control keratinocytes (linker), keratinocytes with repressed NF-κB activity display enhanced expression of TRAIL-R2 (DR4) levels, whereas the repressed NF-κB keratinocytes express either low or undetectable levels of DcR (DcR1, DcR2).

Delineation of functional role for FADD and TRAIL in IFN-γ plus TNF-α mediated apoptotic response in NF-κB-repressed keratinocytes To establish that the IFN-γ plus TNF-α mediated apoptotic response of NF-κB-repressed keratinocytes was mediated by specific cell surface DR, several experimental approaches were undertaken. First, keratinocytes initially infected with the IκBαDN retroviral vector were secondarily infected with an additional retroviral vector containing a FADD-DN-bearing construct. **Figure 9**(***A***) (*upper panel*) reveals a western blot analysis confirming the overproduction of the FADD DN protein in keratinocytes infected by the relevant retroviral construct. Whereas addition of IFN-γ plus TNF-α induced apoptosis in the NF-κB-repressed keratinocytes (IκBαDN, **Fig 9*A***, *lower panel*), infection by the FADD DN in either linker cells or cells previously infected with IκBαDN significantly reduced the apoptotic response from 30% to 8% for the double infected cells.

To further probe the specific DR involved, several receptor fusion proteins with inhibitory activity were utilized. **Figure 9**(***B***) (*left side*) reveals the ability of the TRAIL-DR5 receptor fusion protein (TR:Fc) to completely inhibit the apoptotic response to TRAIL in NF-κB-repressed keratinocytes, whereas no inhibition was seen using the TNF-RI receptor fusion protein (TF:Fc), or the FasL:Fc fusion protein, or the control CTLA4:Ig fusion protein. When NF-κB-repressed keratinocytes were preincubated for 2 h with either TR:Fc or TF:Fc, but not CTLA4:Ig, the apoptotic response as assessed at 24 h (*mid panel*) or 48 h (*right side*) was significantly reduced, as shown in **Fig 9**(***B***). Combining the TR:Fc and TF:Fc returned the IFN-γ plus TNF-α apoptotic response to

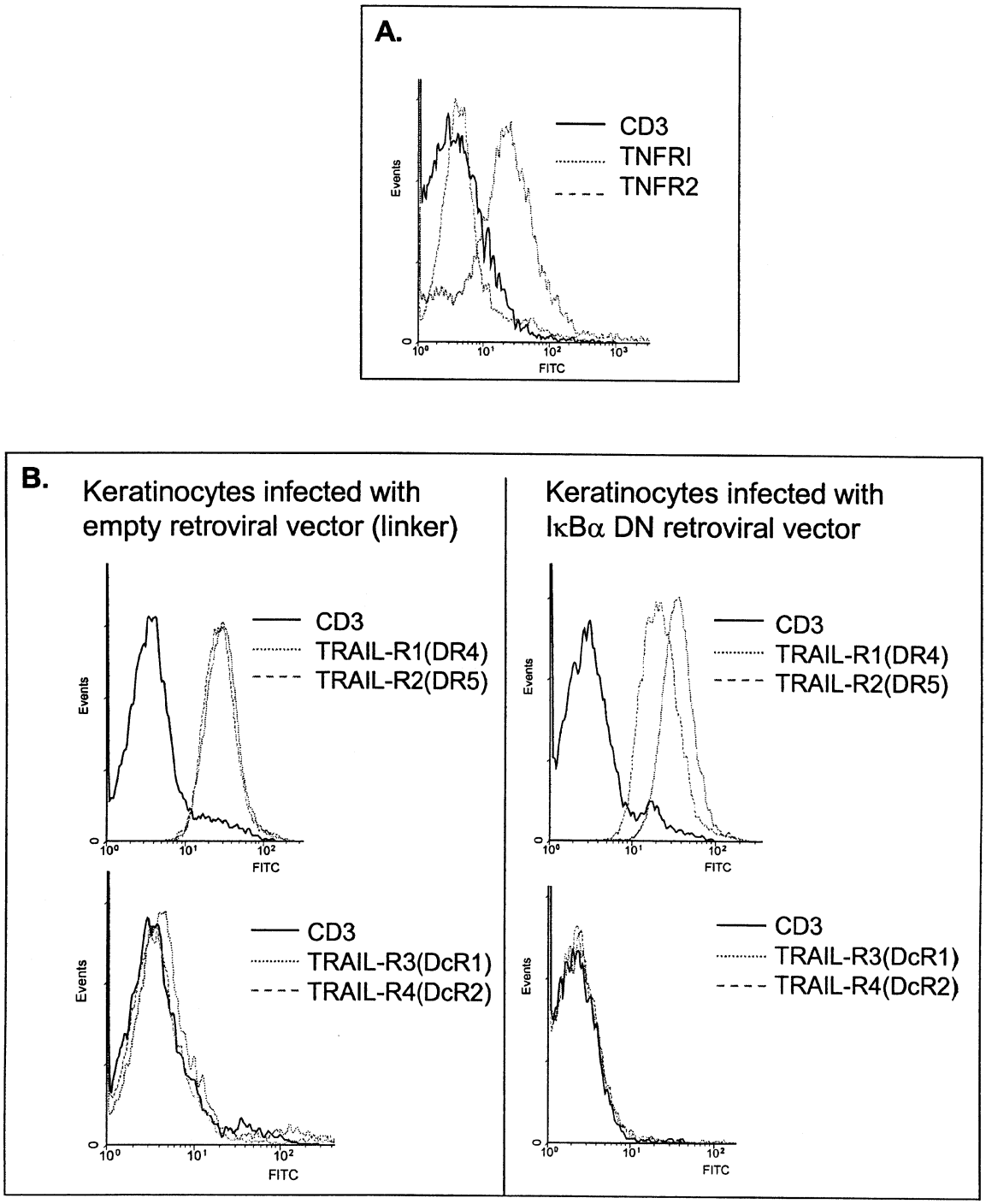

Figure 8. Flow cytometric analysis to detect cell surface levels of TNF family members. (*A*) *Upper panel* reveals prominent surface expression for TNF-RI but not TNF-RII on cultured keratinocytes (CD3 represents isotype control staining). (*B*) *Lower panel* reveals surface staining profile for keratinocytes infected with empty retroviral vector (linker) (*left side*) compared to keratinocytes infected with IκBαDN retroviral vector (*right side*). Note the enhanced expression of TRAIL-R1 (DR4) and absent DcR1 and DcR2 on the NF-κB-repressed keratinocytes compared to normal keratinocytes.

near baseline levels. Taken together, these functional results indicate that the apoptosis induced by IFN-γ plus TNF-α in NF-κB-repressed keratinocytes is related to the TRAIL and TNF-α mediated death pathways.

DISCUSSION

Many members of the TNF receptor family (Ashkenazi and Dixit, 1998; 1999) are involved in regulating the apoptotic response of keratinocytes to various stimuli (reviewed in Kothny-Wilkes *et al*, 1999; Teraki and Shiohara, 1999; Wehrli *et al*, 2000). In this study, we focused on the ability of two different primary cytokines implicated in many benign and malignant skin diseases (i.e., IFN-γ and TNF-α) to trigger apoptosis. This experimental system featured normal proliferating keratinocytes, as well as proliferating keratinocytes with dysfunctional NF-κB signaling created by a mutant IκBα molecule that could resist degradation and hence sequester p65 and p50 in the cytoplasm, thereby preventing

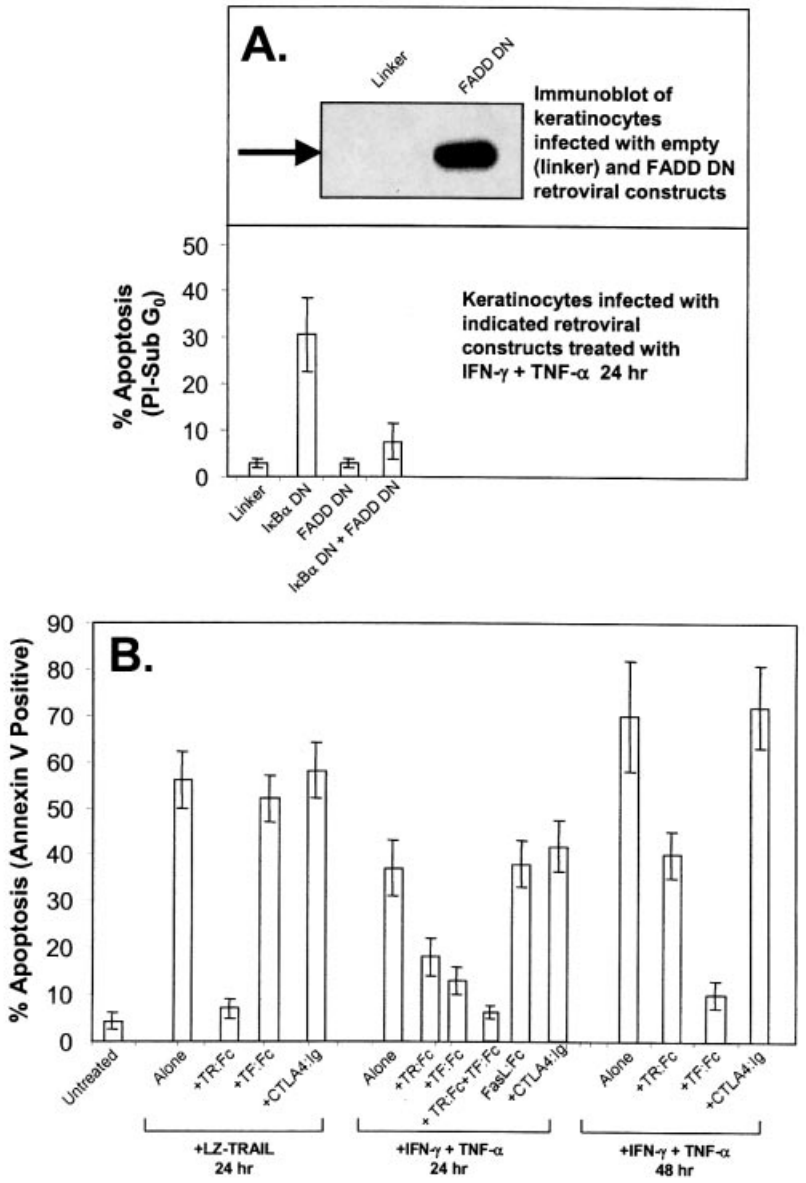

Figure 9. Immunoblot analysis and functional studies demonstrating FADD and TRAIL dependence of the apoptotic response of NF-κB-repressed keratinocytes to IFN-γ plus TNF-α. (*A*) *Upper panel* reveals western blot demonstrating enhanced expression of FADD in keratinocytes infected with FADD-DN-containing retrovirus compared to linker control. (*A*) *Lower panel* reveals functional apoptosis assay (PI/sub-G_0) in which the IFN-γ plus TNF-α induced apoptosis of NF-κB-repressed keratinocytes is dependent on intact FADD. (*B*) Functional studies (Annexin/PI) in which NF-κB-repressed keratinocytes are pretreated with medium alone, TR:Fc, TF:Fc, TR:Fc plus TF:Fc, FasL:Fc, or CTLA4:Ig for 2 h at 5 μg per ml, followed by addition of LZ TRAIL (*left side*), or IFN-γ plus TNF-α for 24 h (*middle*), or IFN-γ plus TNF-α for 48 h (*right side*).

translocation to the nucleus (**Fig 1**). The most important functional result in this report is the enhanced susceptibility of keratinocytes with repressed NF-κB activity to induction of apoptosis by TNF-α (**Fig 2**). Moreover, when IFN-γ, which by itself did not induce apoptosis, was combined with TNF-α, only the NF-κB-repressed keratinocytes were synergistically triggered to rapidly become apoptotic (within 18 h; **Fig 2**). Similarly, the NF-κB-repressed keratinocytes were also more susceptible to TRAIL-induced apoptosis compared to normal keratinocytes (**Fig 2**). By using receptor fusion proteins, we could demonstrate that the IFN-γ plus TNF-α mediated apoptosis in NF-κB-repressed keratinocytes was dependent on endogenously produced TRAIL with no participation of the Fas/FasL apoptotic pathway (**Fig 9**).

These results confirm and extend earlier studies in which keratinocytes that were either pharmacologically or genetically altered to repress NF-κB activity had increased sensitivity to UV-light-induced apoptosis (Qin *et al*, 1999), as had immortalized HaCaT cells with disrupted NF-κB signaling (Chaturvedi *et al*, 2001). An important role for caspase participation was demonstrated by use of various caspase inhibitors (**Fig 2**), and by the ability of IFN-γ to increase caspase 8 levels (**Fig 4**). Recently, an independent group also observed that IFN-γ can enhance caspase 8 levels and thereby sensitize tumor cells to a DR apoptotic program (Ruiz-Ruiz *et al*, 2000). The inability of IFN-γ by itself to induce apoptosis in normal keratinocytes despite increasing TRAIL may reflect the concomitant expression of DcR.

From a mechanistic viewpoint, several results revealed a potential role for the modulation of various DR and DcR, as well as NF-κB-dependent cell survival gene products, in this experimental system. Beginning with NF-κB-dependent anti-apoptotic proteins, it was clearly demonstrated by using the degradation-resistant IκBα-containing retroviral vector, that keratinocytes with repressed NF-κB activity failed to induce their C-IAP2 levels after IFN-γ plus TNF-α exposure (**Fig 3**). In addition, the inability of NF-κB-repressed keratinocytes to induce $p21^{Waf1/Cip1}$ is similar to an earlier report demonstrating the importance of $p21^{Waf1/Cip1}$ in antagonizing the apoptotic effect of TNF-α in an NF-κB-dependent fashion (Javelaud *et al*, 2000). Furthermore, the inability of the NF-κB-repressed keratinocytes to significantly enhance their TRAF1 levels could create a vicious cycle as optimal NF-κB activation via TNF-RI requires TRAF1 and TRAF2 (Wang *et al*, 1998).

Whereas IFN-γ plus TNF-α strongly induced DcR TNF-RII in normal keratinocytes but not in NF-κB-repressed keratinocytes (**Fig 5**), we were unable to detect any surface expression for TNF-RII (despite easily detected TNF-RI) on these cultured keratinocytes (**Fig 7**). Several other previous investigators have also failed to detect TNF-RII on the surface of keratinocytes (Kristensen *et al*, 1993; Trefzer *et al*, 1993; Tobin *et al*, 1998). Thus, the functional significance of the presumed induction of intracellular levels is unknown at this time, although Gaeta *et al* (2000) have observed in endothelial cells that intracellular TNF receptors may influence receptor desensitization. Given the presence of an NF-κB binding site in the TNF-RII promoter (Santee and Owen-Schaub, 1996), our findings using keratinocytes (**Fig 5**) were not entirely unexpected. In a different experimental system it was previously observed that IFN-γ could rescue a malignant cell line from TNF-α-induced apoptosis by upregulation of TNF-RII (Horie *et al*, 1999).

Another contributing molecular mechanism to be considered is the TRAIL-mediated pathway, rather than the TNF-α-mediated pathway. As seen in **Fig 6**, IFN-γ could induced markedly elevated levels of TRAIL mRNA in a non-NF-κB dependent fashion, but the NF-κB-repressed keratinocytes were distinguished from normal keratinocytes by their failure to constitutively express TRAIL DcR1. Indeed, the levels of TRAIL-R3 (DcR1) mRNA 6 h after exposure to IFN-γ plus TNF-α in the NF-κB-repressed keratinocytes revealed that they barely reached the constitutive levels in normal keratinocytes. By flow cytometry, the NF-κB-repressed keratinocytes were characterized by relatively higher levels of DR, without detectable concomitant DcR (**Fig 8**), confirming and extending the RPA and western blot analyses (**Fig 6**). The ability of IFN-γ to enhance cell surface levels of TRAIL was observed by flow cytometry (**Fig 7**). Studies are under way to characterize the biologic properties of TRAIL produced by keratinocytes (i.e., in the absence and presence of TNF-α), because we recently observed highly distinctive apoptotic responses of keratinocytes to various preparations of TRAIL (Qin *et al*, 2001). Another group has also demonstrated that TRAIL expressed by epidermal keratinocytes is biologically active and can trigger apoptosis of indicator cell lines (Bachmann *et al*, 2001).

Taken together, these quantitative RNA and protein levels combined with functional studies suggest the following scenario. When normal keratinocytes are infected by a retrovirus containing mutant IκBα cDNA, the infected keratinocytes with repressed NF-κB activity undergo several phenotypic and molecular changes, including the following. (i) Constitutive and cytokine-inducible NF-κB levels are decreased, resulting in decreased antiapoptotic gene products. (ii) A vicious cycle is created such that subsequent NF-κB signaling is further disrupted by inadequate levels of TRAF-1/TRAF-2. (iii) Important TRAIL DcR such as DcR-1 and DcR-2 disappear, whereas TRAIL DR are upregulated (Ashkenazi and Dixit, 1999). Hence, when various pro-apoptotic stimuli are present, such as delivered by IFN-γ (which can enhance TRAIL) coupled with TNF-α, the repressed NF-κB cells are unable to mobilize the antiapoptotic constituents and succumb to the pro-apoptotic signals delivered through the DR, including TNF-RI and TRAIL DR4/DR5, in the setting of enhanced FADD and caspase 8 levels (Kuang *et al*, 2000; Sprick *et al*, 2000). The enhanced susceptibility induced by IFN-γ did not appear to be a generalized phenomenon, because pretreatment with IFN-γ did not create any apoptotic response triggered through the Fas receptor.

Based on these results, it may be possible to devise new treatments for skin cancer to take advantage of the enhanced susceptibility of NF-κB-repressed keratinocytes to apoptosis induced by IFN-γ plus TNF-α. For example, if delivery of agents that could interfere with NF-κB signaling could be introduced into tumor cells (Duffey *et al*, 1999), the local immune response that features production of IFN-γ and TNF-α could be more effective at triggering apoptosis in the tumor cells. Such an approach has been successfully developed in animal models using TNF-α to overcome the NF-κB activation mediated resistance to apoptosis following conventional chemotherapy (Wang *et al*, 1999).

Progress in this area may be impeded by the complexity of the system. In normal keratinocytes, activation of NF-κB is associated with growth arrest; thus keratinocytes with impaired NF-κB signaling would be expected to display an enhanced proliferative response, but at the same time be rendered more susceptible to apoptosis (Seitz *et al*, 1998; 2000). It has been demonstrated that transgenic mice engineered to have selective inhibition of NF-κB signaling (using a mutant IκBα cDNA) in epidermal keratinocytes not only have the predicted enhanced susceptibility of keratinocytes to apoptosis, but surprisingly are prone to develop squamous cell carcinoma (Van Hogerlinden *et al*, 1999). Of interest, whereas TNF-α levels were also unexpectedly elevated in the skin of these mice, Van Hogerlinden *et al* (1999) suggest that there may be a concomitant impaired immune response. It will be intriguing to determine if such an altered immune response includes defective IFN-γ levels, which could explain why the tumor cells with repressed NF-κB activity could flourish, rather than be susceptible to the synergistic pro-apoptotic response of the combination of IFN-γ plus TNF-α as documented in our report. In any event, these *in vivo* results warrant caution in extrapolating *in vitro* data, and there may be other surprises that emerge during attempts to unravel the molecular mechanisms that underlie the role of apoptotic signaling during cutaneous carcinogenesis, and the use of TRAIL or IFN-γ plus TNF-α in therapeutic settings to combat skin cancer.

The authors thank Immunex Corporation for providing LZ-TRAIL and other reagents, and Crystal Tabor, Heide Bauer, and Brian Bonish for preparation of figures and manuscript.

REFERENCES

Aragane Y, Kulms D, Metze D, Wilkes G, Poppelmann B, Luger TA, Schwarz T: Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. *J Cell Biol* 140:171–182, 1998

Arnold R, Seifert M, Asadullah K, Volk HD: Crosstalk between keratinocytes and T lymphocytes via Fas/Fas ligand interaction: modulation by cytokines. *J Immunol* 162:7140–7147, 1999

Ashkenazi A, Dixit VM: Death receptors signaling and modulation. *Science* 281:1305–1308, 1998

Ashkenazi A, Dixit VM: Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* 11:255–260, 1999

Bachmann F, Buechner SA, Wernli M, Strebel S, Erb P: Ultraviolet light downregulates CD95 ligand and TRAIL receptor expression facilitating actinic keratosis and squamous cell carcinoma formation. *J Invest Dermatol* 117:59–66, 2001

Beg AA, Baltimore D: An essential role for NF-κB in preventing TNF-α-induced cell death. *Science* 274:784–787, 1996

Brash DE, Ziegler A, Jonason AS, Simon JA, Kunala S, Leffel DJ: Sunlight and sunburn in human skin cancer: p53, apoptosis and tumor promotion. *J Invest Dermatol Symp Proc* 1:136–142, 1996

Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ: Apoptosis in proliferating, senescent, and immortalized keratinocytes. *J Biol Chem* 274:23358–23367, 1999

Chaturvedi V, Qin J-Z, Denning MF, Choubey D, Diaz MO, Nickoloff BJ: Abnormal NF-κB signaling pathway with enhanced susceptibility to apoptosis in immortalized keratinocytes. *J Dermatol Sci* 26:67–78, 2001

Dembic Z, Loetscher H, Gubler U, *et al*: Two human TNF receptors have similar extracellular, but distinct intracellular domain sequences. *Cytokine* 2:231–239, 1990

Denning MF, Wang Y, Nickoloff BJ, Wrone-Smith T: Protein kinase C delta is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. *J Biol Chem* 273:29995–30002, 1998

Duffey DC, Chen Z, Dong G, *et al*: Expression of a dominant-negative mutant inhibitor –κBα of nuclear factor –κB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth *in vivo*. *Cancer Res* 59:3468–3474, 1999

Eggermont A, Manusama E, Tenhagen TLM: Regional application of TNF-α in the treatment of cancer: a preclinical–clinical interactive program. *J Inflamm* 47:104–113, 1996

Fanger NA, Maliszewski CR, Schooley K, Griffith TS: Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Exp Med* 190:1155–1164, 1999

Feinstein E, Kimchi A, Wallach D, Boldin M, Varfolomeev E: The death domain: a

module shared by proteins with diverse cellular functions (letter). *Trends Biochem Sci* 20:342–344, 1995

Gaeta ML, Johnson DR, Kluger MS, Pober JS: The death domain of tumor necrosis factor receptor 1 is necessary but not sufficient for Golgi retention of the receptor and mediates receptor desensitization. *Laboratory Invest* 80:1185–1194, 2000

Gutierrez-Steil C, Wrone-Smith T, Sun X, Krueger JG, Coven T, Nickoloff BJ: Sunlight-induced basal cell carcinoma tumor cells and ultraviolet-B irradiated psoriatic plaques express Fas ligand. *J Clin Invest* 101:33–39, 1998

Henseleit U, Zhang J, Wanner R, Hasse I, Kolde G, Rosenbach T: Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *J Invest Dermatol* 109:722–727, 1997

Hill LL, Ouhtit A, Loughlin SM, Kripke ML, Ancathaswamey HN, Owen-Schaub LB: Fas ligand: a sensor for DNA damage critical in skin cancer etiology. *Science* 285:898–900, 1999

Horie T, Dobanshi K, Iizuka K, Yoshii A, Shimzu Y, Nakazawa T, Mori M: Interferon-γ rescues TNF-α-induced apoptosis mediated by up-regulation of TNFR2 on EoL-1 cells. *Exp Hematol* 27:512–519, 1999

Hu Y, Baud V, Delhase M, *et al*: Abnormal morphogenesis but intact IKK activation in mice lacking the IKKα subunit of IκB kinase. *Science* 284:316–320, 1999

Javelaud D, Wietzerbin J, Delattre O, Besancon F: Induction of $p21^{Waf1/Cip1}$ by TNFα requires NF-κB activity and antagonizes apoptosis in Ewing tumor cells. *Oncogene* 19:61–68, 2000

Kothny-Wilkes G, Kulms D, Poppelmann B, Luger TA, Kubin M, Schwarz T: Interleukin-1 protects transformed keratinocytes from tumor necrosis factor-related apoptosis-inducing ligand. *J Biol Chem* 273:29247–29253, 1998

Kothny-Wilkes G, Kulms D, Luger TA, Kubin M, Schwarz T: Interleukin-1 protects transformed keratinocytes from tumor necrosis factor-related apoptosis-inducing ligand- and CD95-induced apoptosis but not from ultraviolet radiation-induced apoptosis. *J Biol Chem* 274:28916–28921, 1999

Kristensen M, Chu CQ, Eedy DJ, Feldmann M, Brennan FM, Breathnach SM: Localization of tumor necrosis factor alpha (TNF-alpha) and its receptors in normal and psoriatic skin: epidermal cells express the 55-kD but not the 75-kD TNF receptor. *Clin Exp Immunol* 94:354–362, 1993

Kuang AA, Diehl GE, Zhang J, Winoto A: FADD is required for DR4- and DR5-mediated apoptosis. *J Biol Chem* 275:25065–25068, 2000

Leverkus M, Yaar M, Gilchrest BA: Fas/Fas ligand interactions contribute to UV-induced apoptosis in human keratinocytes. *Exp Cell Res* 232:255–262, 1997

Leverkus M, Neumann M, Mengling T, Ranch CT, Brocker E-B, Krammer PH, Walczak H: Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. *Cancer Res* 60:553–559, 2000

Lewis M, Tartaglia LA, Lee A, et al: Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc Natl Acad Sci* 88:2830–2836, 1991

Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, Verma IM: IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev* 13:1322–1328, 1999

Nickoloff BJ: Cytokine network in psoriasis. *Arch Dermatol* 127:871–874, 1991

Nickoloff BJ, Chaturvedi V, Bacon PE, Qin J-Z, Denning MF, Diaz MO: Id-1 delays senescence but does not immortalize keratinocytes. *J Biol Chem* 275:27501–27504, 2000

Nickoloff BJ, Denning M: Life and death signaling in epidermis following a planned cell death pathway involving a TRAIL that does not lead to skin cancer. *J Invest Dermatol* 117:1–2, 2001

Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM: An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 277:815–818, 1997

Park YC, Burkitt V, Villa AR, Tong L, Wu H: Structural basis for self-association and receptor recognition of human TRAF2. *Nature* 398:533–538, 1999

Pena JC, Rudin CM, Thompson CB: A Bcl-x_L transgene promotes malignant conversion of chemically initiated skin papillomas. *Cancer Res* 58:2111–2116, 1998

Qin J-Z, Chaturvedi V, Denning MF, Choubey D, Diaz MO, Nickoloff BJ: Role of NF-kappa B in the apoptotic-resistant phenotype of keratinocytes. *J Biol Chem* 27:37957–37964, 1999

Qin J-Z, Chaturvedi V, Bonish B, Nickoloff BJ: Avoiding premature apoptosis of normal epidermal cells. *Nat Med* 7:385–386, 2001

Rehemtulla A, Hamilton CA, Chinnigan AM, Dixit VM: Ultraviolet radiation-induced apoptosis is mediated by activation of CD95 (Fas/APO-1). *J Biol Chem* 272:25783–25786, 1997

Rodriguez-Villanueva J, Greenhalgh D, Wang XJ, *et al*: Human keratin-1.bcl-2 transgenic mice aberrantly express keratin 6, exhibit reduced sensitivity to keratinocyte cell death induction, and are susceptible to skin tumor formation. *Oncogene* 16:853–863, 1998

Ruiz-Ruiz C, Munoz-Pinedo C, Lopez-Rivas A: Interferon-γ 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, 2000

Santee SM, Owen-Schaub LB: Human tumor necrosis factor receptor p75/p80 (CD120b) gene structure and promoter characterization. *J Biol Chem* 271:21151–21159, 1996

Schwarz A, Bhardwaj R, Aragane Y, *et al*: Ultraviolet-B induced apoptosis of keratinocytes: evidence for partial involvement of tumor necrosis factor-α in the formation of sunburn cells. *J Invest Dermatol* 104:922–927, 1995

Seitz CS, Lin Q, Deng H, Khavari PA: Alterations in NF-κB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-κB. *Proc Natl Acad Sci USA* 95:2307–2312, 1998

Seitz CS, Freiberg RA, Hinata K, Khavari PA: NF-κB determines localization and features of cell death in epidermis. *J Clin Invest* 105:253–260, 2000

Sheikh MS, Antinore MJ, Huang Y, Formace AJ Jr: Ultraviolet-irradiation-induced apoptosis is mediated via ligand independent activation of tumor necrosis factor receptor 1. *Oncogene* 17:2555, 1998

Sheridan JP, Marsters SA, Pitti RM, *et al*: Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 277:818–821, 1997

Sprick MR, Weigand MA, Rieser E, et al: FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* 12:599–609, 2000

Takeda K, Takeuchi O, Tsujimura T, *et al*: Limb and skin abnormalities in mice lacking IKKα. *Science* 284:383–316, 1999

Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MJ, Goeddel DV: The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci USA* 88:9292–9296, 1991

Tartaglia LA, Goeddel DV: Two TNF receptors. *Immunol Today* 13:151–153, 1992

Teraki Y, Shiohara Y: Apoptosis and the skin. *Eur J Dermatol* 9:413–426, 1999

Thompson CF: Apoptosis in the pathogenesis and treatment of disease. *Science* 267:1456–1462, 1995

Tobin D, van Hogerlinden M, Toftgard R: UVB-induced activation of tumor necrosis factor (TNF) receptor 1/TNF receptor-associated factor-2 mediates activation of Rel proteins. *Proc Natl Acad Sci USA* 95:565–569, 1998

Trefzer U, Brockhaus M, Lotscher H, *et al*: The 55-kD tumor necrosis factor receptor on human keratinocytes is regulated by tumor necrosis factor-alpha and by ultraviolet B radiation. *J Clin Invest* 92:462–470, 1993

Tron VA, Trotter MJ, Tang L, Krajewska M, Reed JC, Ho VC, Li G: p53-regulated apoptosis is differentiation dependent in ultraviolet B-irradiated mouse keratinocytes. *Am J Pathol* 153:579–585, 1998

Van Antwerp DJ, Verma IM: Signal-induced degradation of IκBα: association with NF-κB and the PEST sequence in IκBα are not required. *Science* 274:787–789, 1996

Van Hogerlinden M, Rozell BL, Ahrlund-Richter L, Toftgard R: Squamous cell carcinomas and increased apoptosis in skin with inhibited rel/nuclear factor –κB signaling. *Cancer Res* 59:3259–3303, 1999

Walczak H, Degli-Esposti MA, Johnson RS, *et al*: TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J* 16:5386–5397, 1997

Walczak H, Miller RE, Ariail K, et al: Tumoricidal activity of tumor necrosis factor related apoptosis-inducing ligand *in vivo*. *Nat Med* 5:157–163, 1999

Wang CY, Mayo MW, Baldwin AS Jr: TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science* 274:784–787, 1996

Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr: NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 281:1680–1683, 1998

Wang CY, Cusack JC Jr, Liu R, Baldwin AS Jr: Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. *Nature Med* 5:412–417, 1999

Wehrli P, Viard I, Bullani R, Tschopp J, French LE: Death receptors in cutaneous biology and disease. *J Invest Dermatol* 115:141–148, 2000

Wrone-Smith T, Johnson T, Nelson B, Boise LH, Thompson CG, Nunez G, Nickoloff BJ: Discordant expression of Bcl-x and Bcl-2 by keratinocytes *in vitro* and psoriatic keratinocytes *in vivo*. *Am J Pathol* 146:1079–1088, 1995

Wrone-Smith T, Mitra RS, Thompson CB, Jasty R, Castle VP, Nickoloff BJ: Keratinocytes derived from psoriatic plaques are resistant to apoptosis compared with normal skin. *Am J Path* 151:1321–1329, 1997