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A Role for NF-κB Activation in Perforin Expression of NK Cells Upon IL-2 Receptor Signaling[1]

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Optimal NK cell development and activation as well as cytolytic activity involves IL-2Rβ signals that also up-regulate expression of the pore-forming effector molecule perforin. Although the Jak/Stat pathway and specifically Stat5 transcription factors are required to promote many of the respective downstream events, the role of additional signaling pathways and transcription factors remains to be clarified. This report investigates the role of NF-κB activation for perforin expression by NK cells. It is demonstrated that IL-2-induced up-regulation of perforin in primary NK cells and in a model cell line is blocked by two pharmacological agents known to inhibit NF-κB activation. Direct evidence for the activation of the NF-κB pathway by IL-2R signals in NK cells involves activation of the IKKα kinase, inhibitory protein κBα degradation, nuclear translocation of p50/p65 complexes, and ultimately, transcriptional activation of the perforin gene via an NF-κB binding element in its upstream enhancer. Taken together, these observations strongly suggest that IL-2R signals can activate a pathway leading to NF-κB activation in NK cells and that this pathway is involved in the control of perforin expression. ***The Journal of Immunology,*** **2002, 169: 1319–1325.**

Natural killer cells survey emerging tumors and can clear pathogen-bearing cells (1). This function involves the expression of several gene products, including the pore-forming effector molecule perforin (2–4). Interestingly, the development and activation of NK cells as well as their levels of perforin expression are controlled by IL-2Rβ signals (5–7). The IL-2Rβ chain (CD122) is an important component of the IL-2 and IL-15R (6, 8, 9). The regulation of perforin expression by IL-2Rβ signals has been attributed to two enhancers that reside 15 kb (10) and respectively, 1 kb upstream of the promoter (10, 11). The control of both enhancers involves STAT5 transcription factors also known to be essential for NK cell development (12). STAT5 is activated by the well-defined JAK/STAT pathway emerging from the IL-2R (6, 13, 14) and the similar IL-15R (14, 15). Given the profound effects that the IL-2Rβ, the IL-15Rα, as well as the STAT5 deficiency have on NK cells in vivo (7, 12, 16), it was of interest to address whether additional signaling pathways and transcription factors contributed to the IL-2R signaling-dependent up-regulation of perforin expression in NK cells.

Several complementary, but less well-appreciated, observations prompted us to investigate in this study the activation of NF-κB by IL-2R signaling and its role in perforin regulation in NK cells. Unlike the well-described JAK/STAT pathway (6, 15, 17), knowledge is limited of how NF-κB activation could be linked to IL-2 and/or IL-15R signaling, and of which genes could be targeted in NK cells. Nevertheless, it has been reported that DNA-binding activity of NF-κB can be induced by IL-2R signaling in T cells (18). Also, a pharmacological inhibitor of NF-κB impaired NK cell-mediated cytotoxicity (19), albeit this result has not been linked to the expression of specific genes. Last, the genetic absence of at least one of the NF-κB family members (RelB) has been associated with an intrinsic defect in the cytolytic activity of cytokine-induced NK cells in vitro (20).

The NF-κB transcription factor family consists of five members that function either as homodimers or heterodimers (21). Their activity is regulated by specific inhibitor proteins, the inhibitory protein κBs (IκBs)[4] that retain the transcription factor in a latent stage in the cytoplasm (21, 22). A broad range of stimuli leads to their phosphorylation by IκB kinase complexes that contain the related IKKα and IKKβ kinases as well as additional proteins (21, 23, 24). Upon phosphorylation, IκB proteins become ubiquitinated and degraded via the proteasome pathway, which results in the nuclear translocation, the DNA binding, and ultimately the transcriptional activation of target genes by the NF-κB components (21, 23). The following report demonstrates that IKKα is activated upon IL-2R signaling in an NK-model cell line and that this event is associated with the degradation of IκBα and the activation of dimeric p50/RelA components. Furthermore, NF-κB components bind to and activate the upstream enhancer of the perforin gene. These events may explain, at least in part, the also investigated sensitivity of perforin expression to two distinct classes of NF-κB inhibitors in the cell line model and in normal primary NK cells. Taken together, our data strongly suggest that IL-2R signaling activates an NF-κB signaling pathway in NK cells, and that this pathway is involved in the control of perforin expression in NK cells.

Materials and Methods

Materials

RPMI 1640 culture medium, the Superscript preamplification system and TRIzol were purchased from Life Technologies (Rockville, MD). FBS was obtained from Equitech-Bio (Kerrville, TX). Lymphocult-T (an IL-2 containing growth factor supplement) was obtained from Biotest Diagnostic (Denville, NJ). Human rIL-2 was kindly provided by Dr. C. W. Reynolds

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[4] Abbreviations used in this paper: IκB, inhibitory protein κB; PDTC, pyrrolidine dithiocarbamate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; β_2M, β_2-microglobulin; PI, propidium iodide.

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(Biological Resources Branch, National Cancer Institute, Frederick, MD). TACS annexin V kit was obtained from Trevigen (Gaithersburg, MD). IGEPAL CA-630, pyrrolidine dithiocarbamate (PDTC), and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-IκBα, IKKα, anti-p65, and anti-p50 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated IgG Abs including anti-CD4, anti-CD8, anti-CD19, anti-γδTcR (GL3), anti-class II MHC (I-A^b), anti-erythroid cell (TER-119), anti-granulocyte (Gr-1), and anti-FcRγ (2.4G2) were purchased from BD PharMingen (San Diego, CA). Biotinylated anti-macrophage (F4/80) was obtained from Caltag Laboratories (Burlingame, CA). RNase A was purchased from Boehringer Mannheim (Indianapolis, IN). *Taq* DNA polymerase, T4 polynucleotide kinase, DEAE-Dextran, and Dual Luciferase Assay System were obtained from Promega (Madison, WI). [α-^{32}P]UTP (~3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [γ-^{32}P]ATP (>4000 Ci/mmol) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). QuickChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). RNeasy Mini kit and Plasmid Midi kit were obtained from Qiagen (Valencia, CA). BigDyeTerminator Cycle Sequencing kit was purchased from Applied Biosystems (Foster City, CA).

Primary NK cell enrichment

Primary NK cells were enriched by negative selection from murine as previously described (25) and modified herein. Briefly, spleen cell suspensions were prepared by forcing the spleen through a wire mesh screen. RBCs were eliminated by Ficoll-Hypaque gradient centrifugation separation. The splenocytes were resuspended in PBS buffer containing 0.1% BSA, and exposed to biotinylated IgG Abs including anti-CD8, anti-CD4, anti-CD19, anti-γδTcR (GL3), anti-macrophage (F4/80), anti-class II MHC (I-A^b), anti-erythroid cell (TER-119), and anti-granulocyte (Gr-1) at 4°C for 15 min. Unlabeled anti-FcRγ (2.4G2) was added to block FcR on NK cell surface. The spleen cell suspension was washed twice with PBS and then treated with streptavidin microbeads (10/1 dilution) for 15 min at 4°C. The NK cells were then negatively enriched by an autoMACS cell separator system (Miltenyi Biotech, Auburn, CA). Total B cells, T cells, and macrophages were reduced to <1% of the eluted cells as determined by flow cytometry.

Cell lines and culture

NK3.3 and YT cell lines were kindly provided by Drs. J. Kornbluth (Department of Pathology, St. Louis University, St. Louis, MO) and J. Yodoi (Institute for Virus Research, Kyoto University, Kyoto, Japan), respectively. The NK3.3 cell line was cultured in complete RPMI 1640 medium containing 10% FCS, 15% Lymphocult-T, and 0.3 mg/ml L-glutamine. To minimize the effects of Lymphocult-T, the NK3.3 cells were starved overnight in most experiments in medium without Lymphocult-T before stimulation with 100 U/ml IL-2. YT cells were maintained in complete RPMI 1640 medium supplemented with 10% FBS, and do not require IL-2 for growth. Enriched NK cells were divided into unstimulated and IL-2-stimulated groups. The IL-2-treated groups were stimulated with 1000 U/ml IL-2 in complete RPMI 1640 medium supplemented with 10% FBS. All cells were cultured in a humidified incubator at 37°C and 5% CO_2.

Cell viability assay

The potential cytotoxicity of PDTC and TPCK toward murine primary splenic NK and NK3.3 cells was assessed using the TACS Annexin V kit. The cells were washed twice with PBS and then resuspended in 1 × binding buffer at a concentration of ~1×10^6 cells/ml. A total of 100 μl aliquots were incubated with propidium iodide (PI) and annexin V-FITC for 15 min at room temperature in the dark, and upon dilution were analyzed by flow cytometry on a BD Biosciences FACScan, using CellQuest 3.0.1 software (BD Biosciences, Mountain View, CA).

RT-PCR assay

Total RNA was extracted from NK3.3 cells using TRIzol, and from primary murine NK cells using RNeasy Mini kit. The first-strand cDNA was synthesized from total RNA of NK3.3 cells and primary murine NK cells, respectively, in a total volume of 20 μl, using random primer and Superscript preamplification system. One hundred fifty or 30 ng of cDNA template from NK3.3 cells and primary murine NK cells, respectively, were subjected to PCR amplification using the following synthetic oligonucleotide primer pairs. Human perforin: 5′-CAC ACA GCC GCA CGC AGA GTG C-3′ combined with 5′-GGG AGT GTG TAC CAC ATG GAA ACT G-3′ (26). Mouse perforin: 5′-AGC CCC TGC ACA CAT TAC TG-3′ combined with 5′-CCG GGG ATT GTT ATT GTT CC-3′. Amplified products are 349 and 491 bp, respectively. Amplification primers for human β_2-microglobulin (β_2m) and murine cyclophilin A were 5′-GCC TGC CGT GTG AAC CAC GTG AC-3′ combined with 5′-TAC CTG TGG AGC AAC CTG CTC AGA-3′ (26) and 5′-ATT TGG CTA TAA GGG TTC CTC-3′ combined with 5′-ACG CTC CAT GGC TTC CAC AAT-3′. The amplified products are 279 and 291 bp, respectively. These products were used as internal standards for quantification. PCR amplifications were performed using 0.25 μl/tube *Taq* DNA polymerase in 25-μl reaction assemblies. To determine the linear region of the PCR signal amplification, the amplified PCR products for mRNAs of human perforin, murine perforin, human β-microglobulin, and murine cyclophilin A were calculated from densitometric measurements of the ethidium bromide-stained agarose gels and plotted on logarithmic scale against the cycle number. The samples for human perforin/β_2m, murine perforin/cyclophilin A were amplified for 19 and 24 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min), respectively. The PCR cycles were predetermined in preliminary experiments to be in the optimal linear range for amplification. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide (0.5 μg/ml). Densitometric analysis was performed using NIH Image 1.54 software (Scion, Frederick, MD) for one-dimension gels. The PCR products were extracted and purified from the gel, and the specificity PCR products were sequenced by Department of Biochemistry, Washington State University (Pullman, WA), using BigDyeTerminator Cycle Sequencing kit.

Nuclear run-on assay

NK3.3 cells were incubated with experimental agents for 2 h. Isolation of nuclei and the elongation reactions were conducted as previously described (27, 28). Briefly after cell membrane lysis, nuclei were collected by centrifugation and resuspended in 100 μl of glycerol buffer (50 mM Tris-HCl (pH 8.3), 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). The elongation reaction was performed by adding 100 μl of reaction buffer (10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 300 mM KCl, 1 mM each of ATP, CTP and GTP, and 100 μCi [^{32}P]UTP) into each sample for 60 min at 30°C. RNA was extracted with acid/guanidinium isothiocyanate/phenol/chloroform, and treated with 0.2 M NaOH for 10 min. The RNA solutions were diluted with 10 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10 mM EDTA, and 0.2% SDS. The filters were hybridized with the same number of cpm of ^{32}P-labeled RNA. After hybridization, the filters were washed with 2 × SSC in the presence of 10 μg/ml RNase A at 37°C for 30 min. The filters were washed again in 2 × SSC, and radioactivity levels were determined using a phosphoimager (Cyclone; Packard Bioscience, Meriden, CT). The denatured human perforin and β_2m cDNAs were slot blotted onto a nylon filter (Hybond N+; Amersham Pharmacia Biotech) and cross-linked with a UV Stratalinker 1800 (Stratagene).

Kinase assay

NK3.3 cells, stimulated in presence or absence of IL-2, were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, and 10 μg/ml PMSF. The cleared cell lysates were incubated with 2 μg anti-IKKα and 20 μl protein G-plus agarose for 4 h at 4°C. The immunoprecipitate was washed three times with 10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, and 0.1% Nonidet P-40, and then equilibrated in 17 μl kinase buffer (20 mM Tris-HCl (pH 7.4), 10 mM MgCl, 1 mM DTT, 10 μM ATP, 300 μM sodium orthovanadate, 10 mM β-glycerophosphate). The kinase reaction was initiated by the addition of 10 μCi [γ-^{32}P]ATP and 2 μg human rIκBα at 30°C for 30 min. Products were boiled for 5 min, analyzed on SDS-PAGE, and detected by autoradiography.

Western blotting assay

NK3.3 cells were treated with IL-2 in the presence or absence of 50 μM PDTC and 40 μM TPCK. Whole-cell lysates were prepared as described previously (27). Protein concentrations were determined using the Bio-Rad protein assay system according to the manufacturer's instructions. A total of 60 μg of protein were subjected to 10% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were immunoblotted overnight at 4°C with primary anti-IκBα or anti-actin Abs, using 1/1000 dilution. After three washes, the membranes were further incubated with HRP-conjugated anti-rabbit and anti-goat antisera, respectively, for 1 h at room temperature. After three washes, IκBα and actin proteins were detected by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL). Actin served as a loading control.

Nuclear extract and EMSA

Nuclear extracts were prepared as described previously (27). A total of 6 μg of nuclear protein from each sample was incubated with ^{32}P-labeled NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Promega), or ^{32}P-labeled perforin NF-κB and mutant perforin NF-κB oligonucleotides (5′-GCA AGAC ATG AGC CCC AAA GTG-3′ and 5′-GCA AGAC ATG AGC CCC AAA GTG-3′, respectively) in 10 μl of binding buffer (50 mM Tris-HCl (pH 7.5), 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 DTT, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC)) for 15 min at room temperature before loading onto 6% native polyacryamide gels and separation in 1 × Tris borate buffer (89 mM Tris-HCl (pH 8.0), 89 mM Boric acid, 2 mM EDTA).

To investigate the interaction between p50, one of NF-κB subunits, and the perforin upstream enhancer, ^{32}P-labeled 117 bp of perforin upstream enhancer (M31951) was generated using PCR (see *Footprinting analysis*). Wild-type p50 binding oligonucleotides were labeled with [γ-^{32}P]ATP (>4000 Ci/mmol, ICN Pharmaceuticals), using T4 polynucleotide kinase. Human recombinant p50 (0.5–3.0 gel shift units) were incubated with the ^{32}P-labeled perforin upstream enhancer DNA fragment or wild-type p50 binding oligonucleotides in 10 μl of binding buffer for 15 min.

For supershift assays, the nuclear protein reaction mixture was incubated with Abs against p50 and p65 for 30 min. For competition assays, 10- or 50-fold excess of unlabeled consensus NF-κB and AP1, as well as perforin NF-κB and mutant perforin NF-κB oligonucleotides were used to challenge binding of labeled consensus NF-κB, perforin NF-κB oligonucleotides, and labeled perforin upstream enhancer, as indicated in *Results and Discussion*. The binding mixtures were separated on 6% native polyacrylamide gels in 1 × Tris borate buffer. The gels were dried and exposed to radiographic film overnight at −70°C.

Footprinting analysis

A 117-bp perforin upstream enhancer DNA fragment was end-labeled, using a [γ-^{32}P]ATP (ICN Pharmaceuticals) kinased primer and PCR followed by gel purification (8% polyacrylamide gel). About 5×10^5 cpm of purified probe was incubated with and without eight gel shift units of human recombinant p50 for 1 h at room temperature, and subsequently digested with DNase I. The DNase I treatment was terminated with stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml yeast RNA). The samples were extracted once with phenol/chloroform, precipitated with ethanol, and dissolved in the sequencing loading buffer (0.1 M NaOH:formamide (1:2), 0.1% xylene cyanol, 0.1% bromophenol blue). Separation was conducted on an 8% polyacrylamide −8 M urea sequencing gels in 1 × Tris borate buffer. The A + G sequencing reaction of the labeled perforin upstream enhancer DNA fragments was performed by the Maxam-Gilbert sequencing method (29).

Site-directed mutagenesis, transient transfection, and luciferase assay

The human perforin upstream enhancer contained in an SV40 driven luciferase vector (10) was mutated using QuickChange site-directed mutagenesis kit and the following oligonucleotides: GGA CAT AAA CGC AAG **AC**A TGA GCC CCA AAG TGT GAC C (mutant primer 1), GAG CAG GGA CGG AAG CAC **TG**A CAT AAA CGC AAG G (mutant primer 2), and CGA GAA GAC ATA AGC **TG**C TGT TCC TGT AAG AGC AG (mutant primer 3). Underlined and bold residues represent mutations of the wild-type sequences (see Fig. 4).

NK3.3 and YT cells were transiently transfected with 5 μg of the reporter gene vectors using DEAE-Dextran preparation, according to the manufacturer's instructions. The promoterless parental vector was used as control and standard. pRL-CMV (0.02 μg) was cotransfected with the reporter gene vectors above for normalization. Transfected NK3.3 and YT cells were incubated with RPMI 1640 medium in the presence or absence of 500 U/ml IL-2 for 48 h. The harvested cells were lysed in passive reporter lysis buffer. Firefly and *Renilla* luciferase activities were determined for 15 s using the Dual Luciferase Assay System and a luminometer (Microlumat LB 96P; EG & G Berthold, Bad Wildbad, Germany).

Statistics

In Fig. 2*B*, the density of the PCR bands from three different experiments is expressed as the mean ± SE. Statistical comparison of the means was conducted by repeated measures one-way analysis of variance. The Student-Newman-Keul test was used to compare the difference between groups. Differences were considered significant at $p < 0.05$.

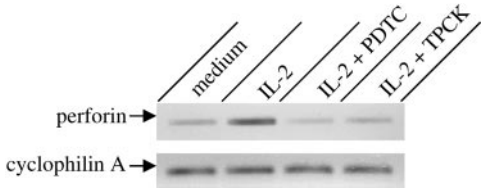

FIGURE 1. Perforin gene regulation in primary NK cells. Purified murine NK cells were stimulated with 100 U/ml IL-2 for 4 h in the presence or absence of 100 nM PDTC or 20 μM TPCK. RNA was extracted and analyzed by RT-PCR for perforin and cyclophilin A as an internal standard.

Results and Discussion

Perforin gene regulation by IL-2 in primary NK cells and the NK3.3 model

Based on the observations described in the introduction, we explored whether IL-2R signaling could involve an NF-κB-dependent component for the regulation of the perforin gene in primary NK cells. A semiquantitative RT-PCR analysis indicated that IL-2R signals up-regulate perforin mRNA of freshly enriched murine NK cells after a 4-h culture in the presence vs absence of cytokine (Fig. 1). These results are similar to those obtained from short-term lines of human NK cells (5). Interestingly, this up-regulation was inhibited by two distinct pharmacological agents known to block NF-κB activation (Fig. 1). The antioxidant, PDTC, prevents de novo phosphorylation of IκBα as well as its subsequent degradation via unknown primary cellular targets (30–32). The protease inhibitor, TPCK, prevents the degradation of IκB proteins (33, 34). Both agents have been applied in numerous cell types to study NF-κB-dependent events, suggesting that the observed block in the up-regulation of perforin in primary NK cells (Fig. 1) relates to the inhibition of an NF-κB-dependent event. Importantly, the inhibitors did not affect the viability of the cells used to prepare the mRNA for the blot in Fig. 1 as assessed by annexin V and PI staining.

To address in more detail the potential role of IL-2R signaling for NF-κB activation and the latter's role in the regulation of the perforin gene in NK cells, we set up a model using the IL-2-dependent NK3.3 cell line (35, 36). Briefly, washed cells were maintained overnight in the absence of cytokine and subsequently stimulated with 100 U/ml IL-2. These conditions did not affect survival in comparison to cells maintained in IL-2 (Fig. 2*A*). IL-2

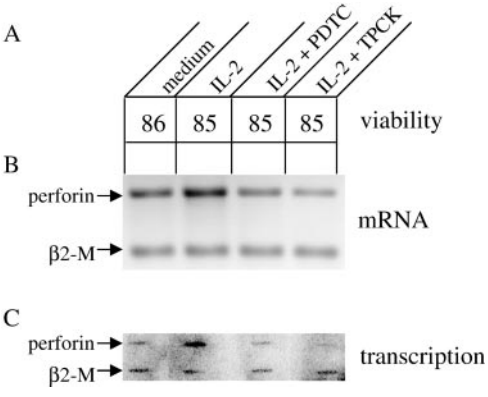

FIGURE 2. Regulation of perforin expression in NK3.3 cells stimulated with IL-2 in the presence or absence of 50 μM PDTC or 40 μM TPCK. *A*, The cell viability was assessed by the annexin V and PI staining after 5 h. *B*, RT-PCR analysis of perforin and β_2m mRNA levels after 5 h. *C*, Nuclear run-on analysis of the perforin gene locus and β_2m gene locus after 2 h of stimulation.

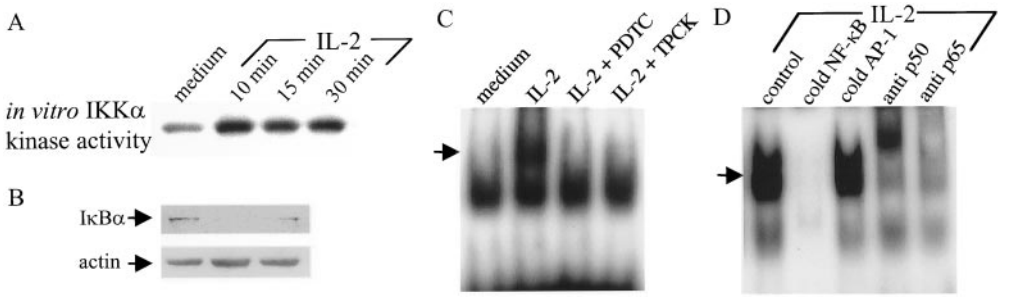

FIGURE 3. IL-2 stimulation of NK3.3 cells activates an NF-κB signaling pathway. *A*, Kinase assay analysis for IL-2-induced IKKα activity. NK3.3 cells were stimulated for the indicated times with IL-2, and IKKα immunoprecipitates were applied to in vitro kinase assays using rIκBα as substrate. The shown autorad is representative of two different experiments. *B*, IL-2-induced degradation of IκBα as determined by Western blot analysis of NK3.3 cells stimulated with IL-2. The result shown is representative of three independent experiments. *C*, EMSA of NF-κB DNA binding activity induced by IL-2 in NK3.3 cell nuclear extracts. NK3.3 cells treated with IL-2 for 4 h in the presence or absence of either 50 μM PDTC or 40 μM TPCK. The gel shift shown is representative of two different experiments. *D*, DNA binding specificities and identities of the IL-2-induced NF-κB-like binding activity. Cold competition EMSA and supershift assays with indicated probes and Abs were used to investigate the IL-2-induced NF-κB binding complex. The result shown is representative of three independent experiments.

stimulation resulted in an ~2.5-fold increase in the perforin mRNA levels in NK3.3 cells as determined by semiquantitative RT-PCR (representative shown in Fig. 2*B*). Densitometry of three independent experiments indicated that the induction as well as inhibition by PDTC and TPCK was statistically significant ($p < 0.05$). A nuclear run-on analysis of NK3.3 indicated that perforin mRNA up-regulation by IL-2 involved increased levels of transcription, and this transcriptional response was severely impaired in the presence of PDTC and TPCK (Fig. 2*C*). Collectively, these results support the hypothesis that IL-2R signaling activates the NF-κB pathway in NK cells and that this activation may directly or indirectly involve the transcription of the perforin gene. These two inhibitors profoundly inhibit IL-2-induced perforin expression, and PDTC may not be a selective NF-κB inhibitor (37, 38). Thus, it is also possible that these inhibitors inhibit other signaling pathways such as the STAT pathway.

Activation of the NF-κB signaling pathway by IL-2 in NK3.3 cells

To support the concept that the NF-κB pathway is activated by IL-2R signaling in NK cells, we addressed known upstream events of NF-κB activation. Using rIκBα as a substrate in vitro, we show in Fig. 3*A* that IKKα kinase activity is induced upon IL-2R stimulation of NK3.3 cells. Also, Western blot analysis of whole-cell extracts indicated that IκBα was degraded (Fig. 3*B*). Presumably, this led to IL-2 inducible NF-κB DNA binding activity as detected by EMSA (Fig. 3*C*). Also, detected was a constitutive complex whose formation was unspecific (see band below arrow in Fig.

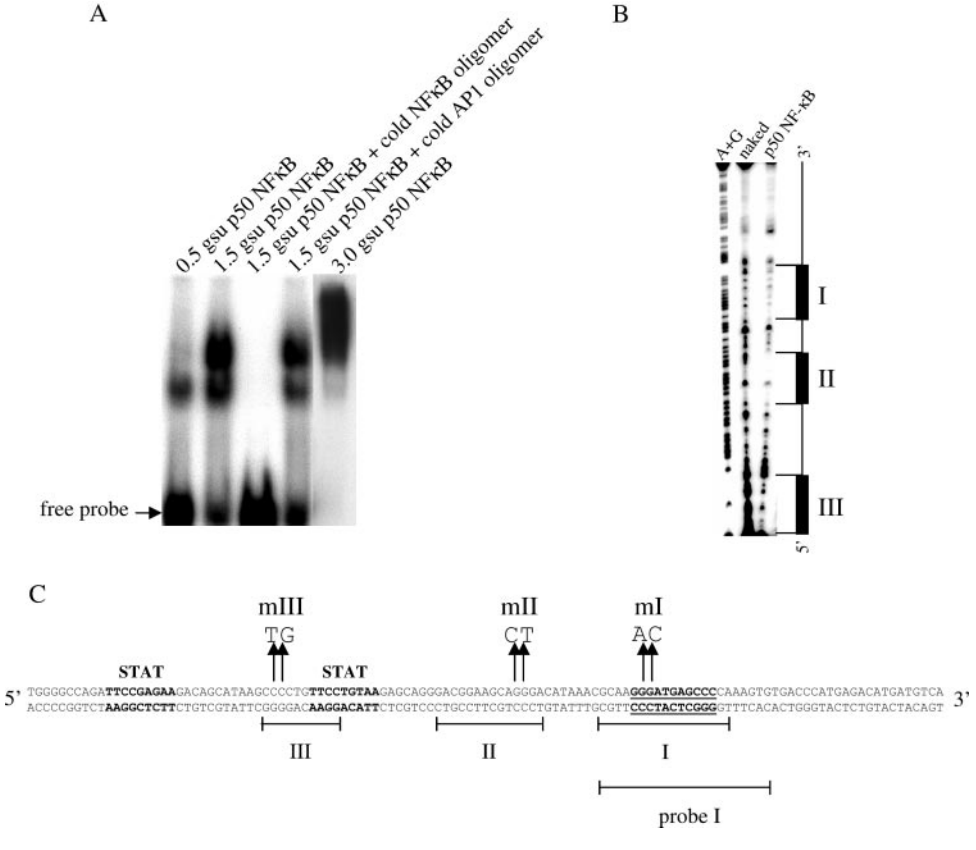

FIGURE 4. NF-κB p50 binds to the upstream enhancer of the perforin gene. *A*, EMSA of the full-length perforin upstream enhancer and human recombinant p50 NF-κB. The binding reactions were conducted in the presence of the indicated amounts of NF-κB and the radiolabeled probe in the presence or absence of the indicated cold competitors. *B*, In vitro DNase I footprinting analysis of the perforin upstream enhancer for NF-κB p50 binding sites. A ^{32}P 5′ end-labeled 117-bp DNA fragment containing the human perforin upstream enhancer was incubated with human recombinant p50, and subjected to DNase I footprinting analysis. *C*, Summary of the footprinting analysis of *B*. The sequence of upstream enhancer, three footprints of *B* (I, II, and III), three 2-bp mutations analyzed in Fig. 5, and the previously characterized tandem STAT element (10) are indicated. Probe I depicts the sequence used for EMSA in Fig. 6.

3*C*). PDTC and TPCK inhibited the inducible gel shift (Fig. 3*C*), suggesting that these agents affected the activation of NF-κB in our experiments. Cold competition with the NF-κB consensus probe, and an unrelated AP-1 probe indicated that the inducible DNA binding was specific for the NF-κB probe. Moreover, supershift experiments indicated that the inducible complex contained p50 and p65 components of NF-κB (Fig. 3*D*). Results so far indicated that IL-2 stimulation of NK3.3 cells leads to a PDTC and TPCK sensitive activation of the NF-κB signaling pathway, including IKKα activation, IκBα degradation, and the formation of heterodimeric p50/p65 complexes, i.e., transcriptionally active NF-κB molecules (21–23).

Control of perforin transcription by NF-κB

To address whether the activation of the NF-κB signaling pathway could involve a direct transcriptional activation of the perforin gene, we investigated the two known enhancers of the perforin gene because they, and not the promoter, can mediate IL-2 responses (10). Computer-assisted binding site analysis as well as preliminary EMSA experiments of the full-length enhancers suggested that the upstream enhancer, but not the far-upstream enhancer, contain NF-κB binding sites. In fact, the EMSA analysis using recombinant NF-κB p50 protein and the upstream enhancer resulted in the sequential appearance of at least three complexes (Fig. 4*A*). This observation suggested the in vitro DNA binding involved several p50 DNA binding sites. To localize the respective elements, we performed DNase I footprint analysis of the enhancer. Recombinant p50 protected three areas of the enhancer sequence (Fig. 4, *B* and *C*). Footprint I displays a highly conserved NF-κB consensus (GGGATGAGCCC), while the areas of footprints II and III contain GGGA, which are known core motifs for p50 binding (22, 39).

To address whether any of these three potential binding sites for NF-κB components is functionally relevant for the IL-2-dependent transcriptional activity of the enhancer, we analyzed mutations of each potential element within the context of the full-length enhancer (Fig. 4*C*) in transient transfection studies. The normalized transcriptional activity of the enhancer was dependent on the integrity of the consensus NF-κB site because it was impaired by its mutation, (Fig. 5, *A* and *C*, mI), but not by mutations affecting the other two potential sites (Fig. 5*A*, mII and mIII). Importantly, an even more pronounced effect was noted in a second NK model, the NK-like YT lymphoma (Fig. 5, *B* and *C*), which does not depend on IL-2 for its growth or survival (40).

To formally address whether the functionally relevant NF-κB motif of the upstream enhancer of the perforin gene (indicated as the probe in Fig. 4*B*) binds NF-κB endogenously induced by IL-2, we analyzed nuclear extracts of NK3.3 cells by EMSA. We observed an IL-2-dependent gel-shift with the perforin probe, which was absent in nuclear extracts of PDTC- and TPCK-treated cells (Fig. 6, *left panel*). As expected, this gel-shift did not occur when the probe contained the mutation functionally analyzed above (Fig. 6, *middle panel*). Lastly, this IL-2-dependent gel-shift could not be competed out by the cold mutant probe, nor by an unrelated AP-1 probe; however, it was sensitive to cold competition with itself and an NF-κB consensus probe (Fig. 6, *right panel*). As seen before, the second gel-shift that occurred independently of IL-2 stimulation was nonspecific because it could not be competed out by the unlabeled probe (Fig. 6, *right panel*). Taken together with the reporter studies, these observations strongly suggest that the bold and underlined sequences in Fig. 4*C* comprise a functional NF-κB element of the upstream enhancer of the perforin gene. Furthermore, at least one outcome of the pharmacological inhibition of NF-κB activation, the impairment of perforin expression, involves an impairment of NF-κB binding to this element.

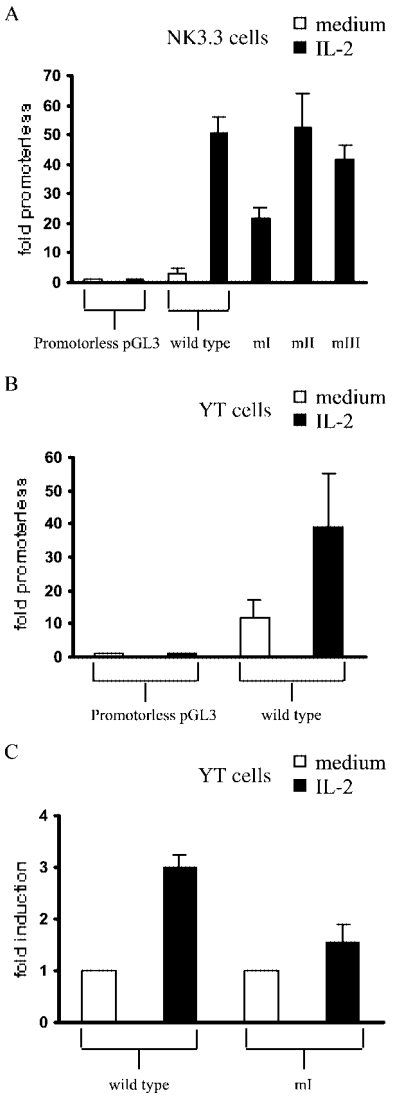

FIGURE 5. An NF-κB-like element of the perforin upstream enhancer is required for full enhancer activation by IL-2R signaling. *A*, Luciferase activity of the perforin upstream enhancer and three mutants (see Fig. 4) within the context of the SV40 promoter was determined upon cotransfection with pRL-CMV and standardization according to its activity in presence or absence of 500 IU/ml IL-2 in NK3.3 cells ($n = 3$). The first two bars represent the activity of the promotorless pGL3 vector in the absence and presence of IL-2. *B*, IL-2 induction (500 IU/ml) of the perforin upstream enhancer and the promotorless pGL3 vector in YT cells. The YT cells were transfected and analyzed as described for *A* ($n = 3$). The first two bars represent the activity of the promotorless pGL3 vector in the absence and presence of IL-2. *C*, Luciferase activity of the perforin upstream enhancer and mutant I (see Fig. 4) within the context of the SV40 promoter was determined upon cotransfection with pRL-CMV and standardization according to its activity in presence or absence of 500 IU/ml IL-2 in YT cells ($n = 3$).

In conclusion, our results suggest that IL-2R stimulation of NK cells results in the activation of the NF-κB signaling pathway, and that this pathway communicates with the upstream enhancer of the perforin gene in NK cells. Our findings raise several questions to be addressed in the future. Does the described IL-2R NF-κB signaling pathway similarly regulate other molecules of the granule exocytosis pathway? Does this signaling pathway of NK cells operate also in CTL? Yet another question to be addressed relates to

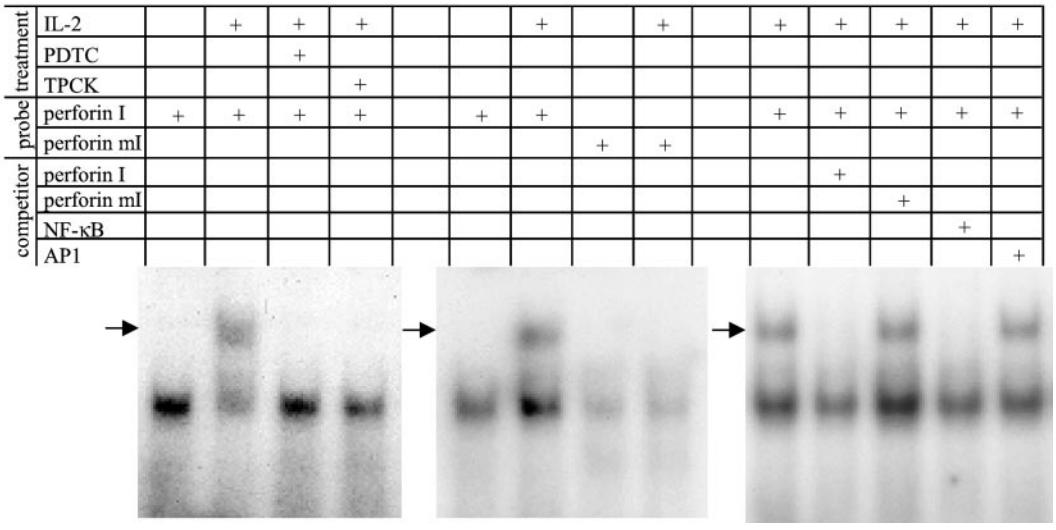

FIGURE 6. IL-2-dependent and PDTC and TPCK sensitive NF-κB binding to the NF-κB consensus element of the perforin upstream enhancer (EMSA). *Left panel*, Effects of IL-2, PDTC, and TPCK on the binding activity of NK3.3 cell nuclear extracts to the perforin NF-κB element. *Middle panel*, Binding specificity of the IL-2-induced complex. The indicated wild-type or mutant NF-κB elements are used as probe. *Right panel*, The IL-2 induced perforin NF-κB consensus binding activity is specific in cold competition analyses.

the exact downstream events of IL-2R signaling leading to NF-κB activation. In this regard, TNFR-associated factors are known to couple the proinflammatory cytokine receptors, such as IL-1 and TNF-α, to downstream events (41–43), yet they do not participate in IL-2R signaling (6). The missing link from the IL-2R to NF-κB activation could entail activation of mitogen-activated protein kinases, which have been linked to IL-2R signaling (6). At least one of them, mitogen-activated protein kinase kinase 1, is known to stimulate the IκB-kinase complex (44); and therefore, could link the IL-2R to NF-κB activation. Regardless, this or a similar pathway appear to operate in primary NK cells and NK cell line to control perforin expression (Figs. 1 and 2) and transcription (Fig. 5).

References

1. Herberman, R. B., C. W. Reynolds, and J. R. Ortaldo. 1986. Mechanism of cytotoxicity by natural killer (NK) cells. *Annu. Rev. Immunol. 4:651.*
2. Podack, E. R., H. Hengartner, and M. G. Lichtenheld. 1991. A central role of perforin in cytolysis. *Annu. Rev. Immunol. 9:129.*
3. Stepp, S. E., P. A. Mathew, M. Bennett, G. de Saint Basile, and V. Kumar. 2000. Perforin: more than just an effector molecule. *Immunol. Today 21:254.*
4. Shi, L., C. M. Kam, J. C. Powers, R. Aebersold, and A. H. Greenberg. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J. Exp. Med. 176:1521.*
5. Salcedo, T. W., L. Azzoni, S. F. Wolf, and B. Perussia. 1993. Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *J. Immunol. 151:2511.*
6. Nelson, B. H., and D. M. Willerford. 1998. Biology of the interleukin-2 receptor. *Adv. Immunol. 70:1.*
7. Suzuki, H., G. S. Duncan, H. Takimoto, and T. W. Mak. 1997. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor β chain. *J. Exp. Med. 185:499.*
8. Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med. 180:1395.*
9. Grabstein, K. H., J. Eisenman, K. Shanebeck, C. Rauch, S. Srinivasan, V. Fung, C. Beers, J. Richardson, M. A. Schoenborn, M. Ahdieh, et al. 1994. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. *Science 264:965.*
10. Zhang, J., I. Scordi, M. J. Smyth, and M. G. Lichtenheld. 1999. Interleukin 2 receptor signaling regulates the perforin gene through signal transducer and activator of transcription (Stat) 5 activation of two enhancers. *J. Exp. Med. 190: 1297.*
11. Yu, C.-R., J. R. Ortaldo, R. E. Curiel, H. A. Young, S. K. Anderson, and P. Gosselin. 1999. Role of a STAT binding site in the regulation of the human perforin promotor. *J. Immunol. 162:2785.*
12. Moriggle, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, et al. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity 10:249.*
13. Beadling, C., D. Guschin, B. A. Witthuhn, A. Ziemiecki, J. N. Ihle, I. M. Kerr, and D. A. Cantrell. 1994. Activation of JAK kinases and STAT proteins by interleukin-2 and interferon α, but not the T cell antigen receptor, in human T lymphocytes. *EMBO J. 13:5605.*
14. Lin, J. X., T. S. Migone, M. Tsang, M. Friedmann, J. A. Weatherbee, L. Zhou, A. Yamauchi, E. T. Bloom, J. Mietz, S. John, and W. J. Leonard. 1995. The role of shared receptor motifs and common Stat proteins in the regulation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity 2:331.*
15. Johnson, J. A., C. M. Bacon, D. S. Finbloom, R. C. Rees, D. Kaplan, K. Shibuya, J. R. Ortaldo, S. Gupta, Y. Q. Chen, J. D. Giri, and J. J. O'Shea. 1995. Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukin 2 and 15. *Proc. Natl. Acad. Sci. USA 92:8705.*
16. Lodolce, J. P., D. L. Boone, S. Chai, R. E. Swain, T. Dassopoulos, S. Trettin, and A. Ma. 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity 9:669.*
17. Yu, C. C., H. A. Young, and J. R. Ortaldo. 1998. Characterization of cytokine differential induction of STAT complexes in primary human T and NK cells. *J. Leukocyte Biol. 64:245.*
18. Brach, M. A., H. J. Gruss, D. Riedel, R. Mertelsmann, and F. Herrmann. 1992. Activation of NF-κB by interleukin 2 in human blood monocytes. *Cell Growth Differ. 3:421.*
19. Blazquez, M. V., I. Luque, E. Collantes, E. Aranda, R. Solana, J. Pena, and E. Munoz. 1997. Cellular redox status influences both cytotoxic and NF-κB activation in natural killer cells. *Immunology 90:455.*
20. Caamaño, J., J. Alexander, L. Craig, R. Bravo, and C. A. Hunter. 1999. The NF-κB family member RelB is required for innate and adaptive immunity to *Toxoplasma gondii. J. Immunol. 163:4453.*
21. Baldwin, Jr., A. S. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol. 14:649.*
22. Grimm, S., and A. Baeuerle. 1993. The inducible transcription factor NF-κB: structure-function relationship of its protein subunits. *Biochem. J. 290:297.*
23. Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.* 12:141.
24. Ip, Y. T., and R. J. Davis. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)-from inflammation to development. *Curr. Opin. Cell Biol. 10:205.*
25. Blank, S. E., L. J. Pfister, R. M. Gallucci, and G. G. Meadows. 1993. Ethanol-induced changes in peripheral blood and splenic NK cells. *Alcohol Clin. Exp. Res. 17:561.*
26. Lu, P., J. A. Garcia-Sanz, M. G. Lichtenheld, and E. R. Podack. 1992. Perforin expression in human peripheral blood mononuclear cells: definition of an IL-2 independent pathway of perforin induction in $CD8^+$ T cells. *J. Immunol. 148: 3354.*
27. Zhou, J., A. D. Struthers, and G. A. Lyles. 1999. Differential effects of some cell signalling inhibitors upon nitric oxide synthase expression and nuclear factor-κB activation induced by lipopolysaccharide in rat aortic smooth muscle cells. *Pharmacol. Res. 39:365.*
28. Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. *Nature 311:433.*

29. Maxam, A. M., and W. Gilbert. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA 74:560.*
30. Schreck, R., B. Meier, D. N. Männel, W. Dröge, and P. A. Baeuerle. 1992. Dicthiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med. 175:1181.*
31. Meyer, M., R. Schreck, and P. A. Baeuerle. 1993. H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J. 12:2005.*
32. Traenckner, E. B., S. Wilk, and P. A. Baeuerle. 1994. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of Iκ-α that is still bound to NF-κB. *EMBO J. 13:5433.*
33. Henkel, T., T. A. I. Machleldt, M. Krönke, Y. Ben-Neriah, and P. A. Baeuerle. 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. *Nature 365:182.*
34. Mellits, K. H., R. T. Hay, and S. Goodbourn. 1993. Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. *Nucleic Acids Res. 21:5059.*
35. Kornbluth, J., N. Flomenberg, and B. Dupont. 1982. Cell surface phenotype of a cloned line of human natural killer cells. *J. Immunol. 129:2831.*
36. Kornbluth, J., B. Spear, S. S. Raab, and D. B. Wilson. 1985. Evidence for the role of class I and class II HLA antigens in the lytic function of a cloned line of human natural killer cells. *J. Immunol. 134:728.*
37. Li, J. J., C. Westergaard, P. Ghosh, and N. H. Colburn. 1997. Inhibitors of both nuclear factor-κB and activator protein-1 activation block the neoplastic transformation response. *Cancer Res. 57:3569.*
38. Martinez-Martinez, S., P. Gomez del Arco, A. L. Armesilla, J. Aramburu, C. Luo, and A. Rao. 1997. Blockade of T-cell activation by dithiocarbamates involves novel mechanism of inhibition of nuclear factor of activated T cells. *Mol. Cell. Biol. 17:6437.*
39. Urban, M. B., R. Schreck, and P. A. Baeuerle. 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J. 10:1817.*
40. Wano, Y., T. Uchiyama, K. Fukui, M. Maeda, H. Uchino, and J. Yodoi. 1984. Characterization of human interleukin 2 receptor (Tac antigen) in normal and leukemic T cells: co-expression of normal and aberrant receptors on Hut-102 cells. *J. Immunol. 132:3005.*
41. Bradley, J. R., and J. S. Pober. 2001. Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene 20:6482.*
42. Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko, and M. P. Boldin. 1999. Tumor necrosis factor receptor and Fas signaling mechanism. *Annu. Rev. Immunol. 17:331.*
43. Malinin, N. L., M. P. Boldin, A. V. Kovalenko, and D. Wallach. 1997. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature 385:540.*
44. Lee, F. S., R. T. Peters, L. C. Dang, and T. Maniatis. 1998. MEKK1 activates both IκB kinase α and IκB kinase β. *Proc. Natl. Acad. Sci. USA 95:9319.*