ing that the transfected cells are sensitive to the lethal effects of TNF-α (Fig. 3). However, a dose-dependent increase in cell survival was seen when RelA was provided, with virtually complete protection at the highest dose (Fig. 3). Thus, expression of RelA prevents cell death by TNF-α in cells that developed in a $RelA^{-/-}$ animal.

Our previous studies have demonstrated an essential role for RelA in hepatocyte survival (*7*). Interestingly, detectable amounts of TNF-α are present by mouse embryonic day 9 (*19*). The results presented here suggest that TNF-α produced by cells of hematopoietic origin in the fetal liver may affect hepatocyte survival. Conversely, RelA may be required for protection from a cell-autonomous killing mechanism operating in hepatocytes.

Recent studies have identified genes that are involved in transducing TNF-α death signals (*16*, *20*–*22*). However, the cytotoxic effects of TNF-α on most cells are only evident if RNA or protein synthesis is inhibited, suggesting that de novo RNA or protein synthesis protects cells from TNF-α cytotoxicity, probably by induction of protective genes (*23*, *24*). It would appear that TNF-α transmits one signal eliciting cell death and another, dependent on RelA, that protects against death by induction of gene expression. Consistent with this hypothesis, we have found that one such anti-apoptotic gene, *A20* (*25*), is induced in $RelA^{+/+}$ 3T3 cells after TNF-α treatment but is not induced in $RelA^{-/-}$ 3T3 cells (*9*). However, transfection of *A20* into $RelA^{-/-}$ 3T3 cells is unable to prevent cell death (*9*), suggesting that other genes (or multiple genes) may be required to protect cells from TNF-α cytotoxicity.

The results presented here, and in accompanying papers (*26*, *27*), indicate an intriguing role for NF-κB in cell survival that may provide additional therapeutic approaches against both inflammatory and proliferative diseases. TNF-α produced by infiltrating leukocytes is a key modulator of inflammation (*28*, *29*). Interestingly, two widely used anti-inflammatory drugs, salicylates and glucocorticoids, are both inhibitors of NF-κB (*30*–*32*), although it is presently unclear whether NF-κB inhibition by these drugs results in killing of potentially inflammatory cells. Additionally, if tumor cell sensitivity to TNF-α is controlled by NF-κB, a combination of NF-κB inhibitors and TNF-α may result in a dramatic enhancement of killing over TNF-α alone.

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13. Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum. Approximately 200,000 $Rel^{+/+}$ or $Rel^{-/-}$ embryonic fibroblasts were plated on each well of a six-well plate. Twenty-four hours later mouse or human TNF-α (both from Genzyme) was added for periods indicated in the text, after which the cells were trypsinized and viable cells counted by trypan blue exclusion. Mouse TNF-α (10 ng/ml, specific activity 5×10^7 U/mg) or hTNF-α (50 ng/ml, specific activity 1×10^7 U/mg) was used.
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17. Macrophages were prepared essentially as described [R. Coico, Ed., *Current Protocols in Immunology* (Wiley, New York, 1994)], except that fetal liver instead of bone marrow was used. Livers from 14-day $Rel^{+/+}$ or $Rel^{-/-}$ embryos were disrupted by passage through a syringe and plated overnight in DMEM with 20% fetal bovine serum and 30% of total volume of L929 fibrosarcoma-conditioned media (containing macrophage growth factors) to allow attachment of adherent cells. Cells present in the supernatant were then plated on a six-well plate. Within 2 days macrophage colonies appeared. Cells obtained in this way are positive for the macrophage-specific marker Mac-1. TNF-α was added when the plates were 50% confluent, for periods indicated in the text. After TNF-α treatment, cells were treated with Dispase II (Boehringer Mannheim) and scraped from the plates. Viable cells were counted by trypan blue exclusion.
18. $Rel^{-/-}$ 3T3 cells were plated as described in (*13*). Twenty-four hours later they were incubated with a calcium phosphate–DNA precipitate solution for 3 hours, after which the cells were treated with glycerol (Hepes-buffered saline with 15% glycerol) and then fresh media added. Thirty-six hours later TNF-α was added to the cells for 24 hours, after which they were washed with phosphate-buffered saline (PBS), fixed, and then treated with an X-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) staining solution. Three hours later blue cells were counted in 10 independent views of the plates. The plasmid pON 405, in which LacZ expression is driven by the cytomegalovirus promoter, was used to mark transfected cells. The plasmid pGD-p65 [M. Scott *et al.*, *Genes Dev.* **7**, 1266 (1993)] was used for expression of RelA in fibroblasts. The parental pGD vector was used to ensure that the total amount of DNA used in all transfections was identical.
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TNF- and Cancer Therapy–Induced Apoptosis: Potentiation by Inhibition of NF-κB

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Many cells are resistant to stimuli that can induce apoptosis, but the mechanisms involved are not fully understood. The activation of the transcription factor nuclear factor–kappa B (NF-κB) by tumor necrosis factor (TNF), ionizing radiation, or daunorubicin (a cancer chemotherapeutic compound), was found to protect from cell killing. Inhibition of NF-κB nuclear translocation enhanced apoptotic killing by these reagents but not by apoptotic stimuli that do not activate NF-κB. These results provide a mechanism of cellular resistance to killing by some apoptotic reagents, offer insight into a new role for NF-κB, and have potential for improvement of the efficacy of cancer therapies.

Observations that NF-κB (*1*) is activated by certain apoptotic stimuli has led to the speculation that this transcription factor may mediate aspects of programmed cell death. An anti-apoptotic function of NF-κB is also suggested, however, because mice that lack the NF-κB p65/RelA gene die embryonically from extensive apoptosis within the liver (*2*). Many cells that respond to TNF, a strong activator of NF-κB, are also resistant to cell killing, which is enhanced in the presence of protein synthesis inhibitors (*3*). We investigated, therefore, whether the transcription factor NF-κB is protective against apoptotic killing

induced by TNF in a model cell system. We initiated our studies using the human fibrosarcoma cell line HT1080, which is relatively resistant to killing by TNF (*4*). To potentially block the activation of NF-κB in response to TNF stimulation, we established an HT1080 cell line (HT1080I) expressing a super-repressor form of the NF-κB inhibitor IκBα. The super-repressor IκBα contains serine-to-alanine mutations at residues 32 and 36, which inhibit signal-induced phosphorylation (*5*) and subsequent proteasome-mediated degradation of IκBα (*6*). This mutant IκBα protein acts as a super-repressor because it binds to NF-κB and inhibits DNA binding as well as nuclear translocation but is unable to respond to cellular signals such as those induced by TNF (*5*, *6*). A control line (HT1080V) was established that contained the empty vector and the hygromycin selectable marker. TNF-α–induced NF-κB activation, as measured by DNA binding of nuclear extracts, was effectively blocked by the super-repressor IκBα in HT1080I cells as compared with activation in the control cell line [(*4*) and below].

TNF-α is more effective at inducing apoptosis in the IκBα super-repressor–expressing cells (HT1080I) than in the control cell line (HT1080V) (Fig. 1A). Similar results were obtained with pooled clones of HT1080V or HT1080I cells (*4*), which indicates that the results we obtained were because of the overexpression of the super-repressor IκBα and were not due to clonal variation. That cells were killed by apoptosis was confirmed by the use of the deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, which measures DNA strand breaks and is diagnostic for cells undergoing apoptosis. Apoptosis was observed only in the HT1080I cells treated with TNF (Fig. 1B). Other cells were more sensitive to TNF-α killing when the IκBα super-repressor was expressed, which shows that the results were not unique to the HT1080 cells (*4*). Thus, expression of a super-repressor form of IκBα potently enhanced the ability of TNF to initiate apoptosis in a variety of cells that are normally resistant to this cytokine, which suggests that the activation of NF-κB by TNF is protective.

To exclude the possibility that the expression of the super-repressor form of IκBα leads to a function that is different from the inhibition of NF-κB, we confirmed the requirement for NF-κB in inhibition of TNF-induced apoptosis. The pretreatment of HT1080V cells with interleukin-1 (IL-1, an activator of NF-κB that does not initiate apoptosis) blocked the subsequent killing of these cells induced by combined cycloheximide (CHX) and TNF treatment (Fig. 2A). As a control and to determine that it was NF-κB that was responsible for the protection, we found that IL-1 had no protective effect on the HT1080I cell line, in which NF-κB activation is blocked (*4*) by the expression of the super-repressor IκBα (Fig. 2A). IL-1 is known to block TNF-mediated killing (*3*). We determined whether a proteasome inhibitor would enhance cell killing of HT1080 cells in response to TNF treatment, because the degradation of IκBα is controlled by the proteasome after inducible phosphorylation and subsequent ubiquitination (*6*). Proteasome inhibitors of the peptide aldehyde category are potent inhibitors of NF-κB activation (*6*). In a dose-dependent fashion, the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-H) strongly enhanced the killing of HT1080V cells in response to TNF (*4*).

If NF-κB inhibition was critical for making cells vulnerable to TNF killing, then overexpression of NF-κB subunits should restore protection against cell killing in the HT1080I model. We transfected vectors encoding the p50 and RelA/p65 subunits of NF-κB or the empty cytomegalovirus (CMV) vector control into the HT1080I cells and stimulated them with TNF. As expected, the vector alone did not provide protection against cell killing induced by TNF (Fig. 2B). However, expression of the NF-κB p50 and RelA/p65 subunits provided protection against TNF-induced apoptosis, indicating that it is NF-κB that is blocked by the super-repressor IκBα and that NF-κB expression blocks programmed cell death. Additional evidence that NF-κB is required for protection against cell killing induced by TNF is shown by the fact that embryonic fibroblasts from RelA/p65 null mice (*2*) are killed by TNF with a much higher frequency than are those from wild-type animals (*4*, *7*).

Many cancer therapies function to kill transformed cells through apoptotic mechanisms; resistance to apoptosis provides protection against cell killing initiated by these therapies (*8*). To determine if other apoptotic stimuli activate NF-κB and whether NF-κB is protective against these stimuli, we analyzed ionizing radiation–, daunorubicin-, and staurosporine-treated cells. Ionizing radiation is known to activate NF-κB in several cell types (*9*). We therefore investigated whether

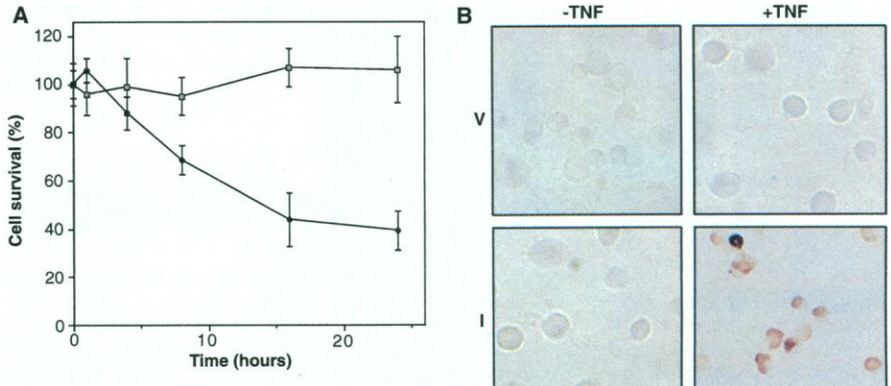

Fig. 1. Expression of the super-repressor IκBα overcomes the block to TNF-mediated apoptosis. (**A**) HT1080 fibrosarcoma cells were cotransfected with the pCMV empty vector or with same vector containing a cDNA encoding the super-repressor IκBα and with the pCEP4 vector for hygromycin B selection (400 μg/ml). Transfection was by the lipofectamine protocol (Gibco/BRL). HT1080I expresses the super-repressor IκBα, and HT1080V contains the empty expression vector. IκBα levels were determined by immunoblotting (ECL, Amersham) of equivalent amounts of protein from the different cells with an antibody to human IκBα (Rockland, Boyertown, Pennsylvania). Expression of the super-repressor IκBα in HT1080 cells efficiently blocked TNF-stimulated NF-κB nuclear translocation, as determined by electrophoretic mobility-shift assay (EMSA) (*4*). Either HT1080V cells (open squares) or HT1080I cells (solid diamonds) were treated with TNF-α (20 ng/ml) for varying times, and surviving cells were quantified by crystal violet assay (*19*). Data shown are the mean of three independent experiments ± SD, and the percentage cell survival was defined as the relative number of TNF-treated versus untreated cells. (**B**) Detection of TNF-induced apoptosis by TUNEL staining (*20*). HT1080V (V) or HT1080I (I) cells were either untreated (−TNF) or were stimulated (+TNF) with TNF-α (50 ng/ml) for 7 hours and then fixed with 4% paraformaldehyde. The staining was done according to the manufacturer's instructions (Boehringer Mannheim). Positive cells show the condensed morphology typical of apoptotic cells.

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ionizing radiation, the chemotherapeutic compound daunorubicin, and staurosporine activated NF-κB in the HT1080V cells and in the HT1080I cells. Both daunorubicin and ionizing radiation activated NF-κB (Fig. 3A). HT1080I cells were blocked by these two stimuli in their ability to activate NF-κB (Fig. 3A), which is consistent with the expression of the super-repressor IκBα. In contrast, staurosporine was not effective at NF-κB activation (Fig. 3A). We then tested whether ionizing radiation–, daunorubicin-, and staurosporine-induced cell killing can be enhanced by the inhibition of NF-κB activity. For these experiments, we used the HT1080V control fibrosarcoma cells and the HT1080I derivative that expresses the super-repressor form of IκBα. Apoptotic stimuli that induce NF-κB, namely daunorubicin (Fig. 3B) and ionizing radiation (Fig. 3C), are enhanced in their ability to kill the HT1080I cells. However, apoptosis induced by staurosporine is not enhanced by the expression of IκBα (*4*), which is consistent with the observation that staurosporine does not effectively activate NF-κB (Fig. 3A). Thus, the activation of NF-κB is part of the cellular response to a variety of genotoxic agents, and under stress-induced conditions, this transcription factor provides significant protection against apoptosis.

Our data indicate that the activation of NF-κB by TNF, ionizing radiation, and daunorubicin provides protection against apoptotic cell killing induced by these stimuli. Distinct signaling pathways initiated by TNF engagement of its receptor lead to activation of both apoptosis and NF-κB, and NF-κB does not play a positive role in the induction of apoptosis (*10*). In the case of ionizing radiation and daunorubicin, the activation of apoptosis appears to be initiated by ceramide production (*11*) and the cytotoxic effects of TNF have been reported to require ceramide activation (*12*). Ceramide alone has been shown to lead to apoptosis (*13*), but the details of this apoptotic pathway are not fully understood. In each of these three cases, the apoptotic stimulus also leads to an inhibition of apoptosis through the activation of NF-κB. It should be noted that several groups have suggested that NF-κB may function pro-apoptotically under some conditions and in certain cell lines (*14*). The mechanism whereby NF-κB protects cells against apoptosis is presently unclear. Because cell killing by TNF and other apoptotic agents is enhanced by the protein synthesis inhibitor CHX, the activation of NF-κB probably functions to transcriptionally up-regulate a gene or group of genes encoding proteins involved in protection against cell killing.

Growing evidence indicates that a variety of anticancer agents kill through programmed cell death. Resistance to anticancer therapies appears to be mediated by resistance to apoptosis (*8*). Our data show that several anticancer agents may be less effective at inducing programmed cell death because of their concomitant activation of NF-κB. Another cancer therapy, etoposide, activates NF-κB (*15*) and our preliminary data indicate that cell killing by vincristine is augmented by the inhibition of NF-κB (*4*). Therefore, approaches that inhibit nuclear translocation of NF-κB, including gene therapy delivery of the super-repressor IκBα or the use of a variety of agents that block NF-κB function (such as proteasome inhibitors), may prove to be highly beneficial in the treatment of tumors when combined with standard anticancer therapies. In fact, glucocorticoids, which are widely used as immune and inflammatory suppressants and inhibit NF-κB (*16*), are used as part of a therapy for certain hematological malignancies (*17*). It may be, therefore, that the function of glucocorticoids in these therapies is to inhibit NF-κB, potentiating killing by the other chemotherapeutic compounds. Thus, combined therapy that inhibits NF-κB func-

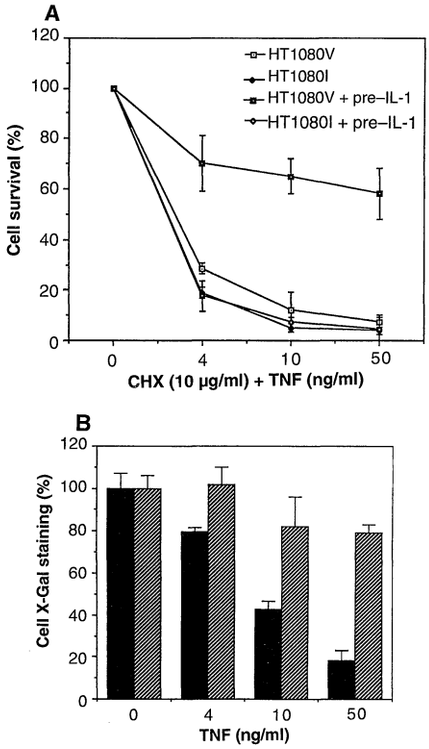


Fig. 2. NF-κB is a suppressor of TNF-induced apoptosis. (**A**) IL-1 pretreatment inhibits TNF + CHX–induced apoptosis in HT1080V cells. HT1080V or HT1080I cells were preincubated with IL-1β (10 ng/ml) (R&D Systems) for 5 hours as indicated (+pre–IL-1) or were left untreated. After the incubation, cells were treated with TNF-α at different concentrations (as indicated) and with CHX (10 μg/ml). Surviving cells were quantified by the crystal violet assay described above. (**B**) Expression of the p50 and RelA/p65 NF-κB subunits restores cell resistance to TNF killing. HT1080I cells (expressing the super-repressor IκBα) were either cotransfected with pcDNA3-lacZ (Invitrogen) and pCMV-p65 (2 μg) and pCMV-p50 (2 μg) (hatched bars) or with lacZ and empty vectors (solid bars) by the lipofectamine protocol. After 40 hours, cells were treated with different concentrations of TNF-α for an additional 24 hours. The results are from the mean ± SD of two experiments. X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside.

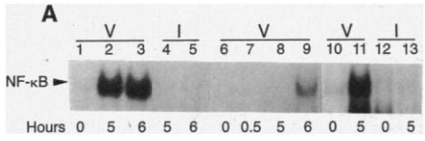
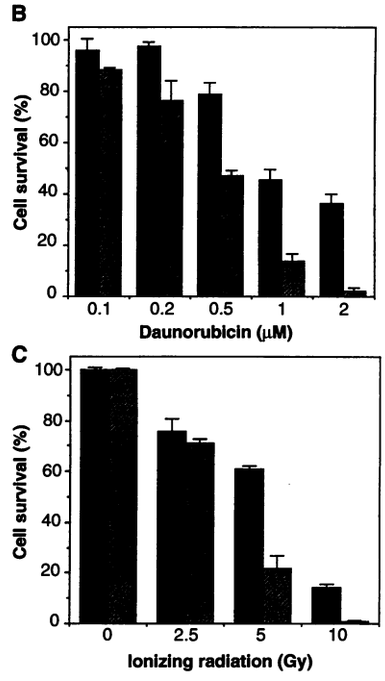


Fig. 3. Activation of NF-κB by ionizing radiation or daunorubicin protects against apoptosis induced by these cancer therapies. (**A**) Daunorubicin and ionizing radiation induce nuclear translocation of NF-κB. Either HT1080V (V) or HT1080I (I) cells were treated with 1 μM daunorubicin (Sigma) or 50 nM staurosporine (Sigma) or were irradiated [at 5 grays (Gy)] for the indicated times. EMSA was performed as previously described (*21*). Lanes 1 through 5, daunorubicin; lanes 6 through 9, staurosporine; and lanes 10 through 13, ionizing radiation. (**B** and **C**) The overexpression of super-repressor IκBα enhanced cell killing by daunorubicin and ionizing radiation. (B) HT1080V cells (solid bars) or HT1080I cells (hatched bars) were treated with the indicated concentration of daunorubicin for 24 hours. Cell survival was assayed as described in Fig. 1. Data are from the mean of four separate experiments. (C) Five hundred HT1080V or HT1080I cells were plated in six-well plates, and 24 hours later the cells were exposed to ionizing radiation at the indicated doses. Cell clones were counted after 14 days. Each experimental group was performed in triplicate. The results shown here represent three independent experiments and are expressed as the mean ± SD.



tion in the presence of apoptotic stimuli may lower the anti-apoptotic threshold of tumors to provide a more effective treatment against resistant forms of cancer. Additionally, the inhibition of NF-κB function in association with TNF treatment may broaden the limited ability of this cytokine to function in an anti-tumor manner.

Note added in proof. Wu *et al.* (*18*) recently demonstrated that NF-κB blocks apoptosis in B cells.

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Suppression of TNF-α–Induced Apoptosis by NF-κB

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Tumor necrosis factor α (TNF-α) signaling gives rise to a number of events, including activation of transcription factor NF-κB and programmed cell death (apoptosis). Previous studies of TNF-α signaling have suggested that these two events occur independently. The sensitivity and kinetics of TNF-α–induced apoptosis are shown to be enhanced in a number of cell types expressing a dominant-negative IκBα (IκBαM). These findings suggest that a negative feedback mechanism results from TNF-α signaling in which NF-κB activation suppresses the signals for cell death.

The relation between TNF-α signals for NF-κB activation and apoptosis suggests that the two pathways are independent, diverging early in the TNF-α signaling cascade (*1*). Because TNF-α–induced apoptosis is enhanced in the absence of de novo RNA or protein synthesis (*2*), and NF-κB rapidly activates target gene transcription upon TNF-α stimulation, we investigated whether the absence of NF-κB–induced genes alone might enhance TNF-α–induced apoptosis. To test this hypothesis on various cell types, we generated a transdominant-negative mutant of IκBα (*3*). Many signal transduction pathways resulting in NF-κB activation culminate in a serine phosphorylation of IκBα on residues 32 and 36 (*4*). Phosphorylation of the COOH-terminal PEST sequence has been implicated in constitutive turnover of IκBα (*5*). We combined the NH_2- and COOH-terminal phosphorylation mutants into a single cDNA (IκBαM) and examined its ability to inhibit NF-κB signaling. We then generated stable transformants expressing IκBαM (*6*) in primary mouse and human fibroblasts, a human lymphoma cell line (Jurkat), and a well-characterized TNF-α–resistant cell line (T24, human bladder carcinoma) (*7*).

Infection with IκBαM retrovirus resulted in a loss of NF-κB inducibility (Figs. 1 and 2). Each cell line represented pools of infected cells to avoid artifacts arising from clonal analysis. Human embryo fibroblasts (HEF) either alone or expressing IκBαM were stimulated for various time periods to examine NF-κB inducibility (Fig. 1). Protein immunoblotting (*8*) (Fig. 1A) shows the expression of murine IκBαM, which migrated faster on SDS–polyacrylamide gel electrophoresis (PAGE) than the endogenous human IκBα (*4*). The expression of IκBαM was only modestly higher than that of endogenous IκBα, demonstrating the abilityof IκBαM to inhibit NF-κB. After TNF-α stimulation in both control and IκBαM cells, endogenous IκBα was phosphorylated and degraded with similar kinetics, demonstrating that in IκBαM-expressing cell lines, the signal transduction pathway upstream of NF-κB activation was not blocked (Fig. 1A). IκBαM was not degraded, presumably because it was not phosphorylated. Because the IκBα gene is induced

Table 1. Annexin V–FITC flow cytometric analysis of Jurkat cells stably transduced with IκBαM. Normal or IκBαM Jurkat cells were treated with TNF-α (100 ng/ml) for the indicated times and stained with FITC-labeled annexin V. The cells were then analyzed by flow cytometry as described (*14*). Five thousand cells were analyzed under each condition.

Time (hours)	Percent annexin V binding	
	Control	IκBαM
0	10.4	12.3
3	13.7	26.6
7	24.1	39.9
24	28.2	62.3
48	30.1	86.3

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