receptor for InlA, E-cadherin (*2*, *13*, *14*). Entry of *L. innocua* expressing *inlA* into Caco-2 cells was inhibited by nanomolar or micromolar concentrations of wortmannin or LY294002, respectively (9), indicating that PI-3 kinase activity is needed for efficient InlA-mediated entry. However, in Caco-2 cells we failed to detect an increase in ^{32}P-labeled PI(3,4)P_2 or PI(3,4,5)P_3 upon infection with *L. monocytogenes* (9), suggesting that InlA does not stimulate PI-3 kinase activity. Interestingly, uninfected Caco-2 cells contained PI(3,4)P_2 and PI(3,4,5)P_3 in amounts [about 1 and 4% the amount of PI(4,5)P_2, respectively] that were considerably greater than those in uninfected Vero cells. These results suggest that Caco-2 cells have higher basal PI-3 kinase activity than Vero cells and raise the possibility that InlA–E-cadherin–mediated entry requires that host PI 3-kinase be constitutively activated.

It is not known how activation of p85α-p110 affects bacterial invasion, but one possibility is through reorganization of the actin cytoskeleton. PI(3,4)P_2 and PI(3,4,5)P_3 (and D-4–phosphorylated phosphoinositides) uncap barbed actin filaments in permeabilized platelets (*15*), suggesting that bacterial stimulation of PI 3-kinase might drive local changes in the cytoskeleton by directly affecting actin polymerization. In addition, p85α-p110 can interact with proteins that regulate the actin cytoskeleton, such as pp125^{FAK} (*16*) or rho guanosine triphosphatases (GTPases) (*4*), and such interactions could participate in invasion. PI 3-kinase activity also appears to be involved in phagocytosis and some endocytic processes (*4*, *5*), and p85α can interact with the GTPase dynamin (*17*), an essential component of clathrin-dependent endocytosis. It is possible that dynamin or other components of endocytosis are also used by invasive pathogens to enter host cells. Understanding the function of PI 3-kinase in bacterial uptake might therefore help elucidate the roles of this lipid kinase in certain endocytic processes.

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12. Percent invasion values are the means ± SD of four experiments. Construction of CHO-Δp85α cells will be described elsewhere (*11*). CHO and CHO-Δp85α cells were grown as described (*10*) and used between passages 4 to 8 and 5 to 8, respectively. Hygromycin (150 ng/ml) was included in the medium for routine growth of CHO-Δp85α cells, except when seeded for invasion tests. Invasion assays were performed on 2-day-old monolayers in 24-well tissue culture plates (about 4 × 10^4 cells per well). Expression of Δp85α did not affect bacterial adhesion, or cell viability, as determined by Trypan blue staining.
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18. Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) with fetal calf serum (10%) (Gibco) and Non-Essential Amino Acids (Gibco). Caco-2 cells were grown as described (*13*) and used between passages 78 and 86. Subconfluent monolayers of cells for invasion tests were obtained by seeding 2 × 10^4 Vero or 4 × 10^4 Caco-2 cells per well in 24-well tissue culture plates and incubating at 37°C in 10% CO_2 for 2 days before the test. Strain EGD (*13*) was prepared for invasion tests as described (*2*), and a multiplicity of infection (MOI) of about 100 bacteria per cell was used. Vero or Caco-2 cells were treated with wortmannin (Sigma or Biomol) or LY294002 (Biomol) 20 min before infection, as well as during the 60-min infection. Inhibitors were added from 1000× solutions in DMSO, and therefore DMSO was added at a final concentration of 0.1% in the control (0 concentration). At the concentrations used, the inhibitors had no effect on cell or bacterial viability as assayed by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)] test (*19*) or titering of viable bacteria, respectively. The inhibitors also did not affect bacterial adhesion to Vero or Caco-2 cells.
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21. Supported by grants from the Ministere de l'Agriculture et la Peche (R94-35), DRET (93-109), INSERM (CRE93013), the Ministere de la Recherche (ACCSV6), and the Pasteur Institute. K.I. is a recipient of a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research. We thank P. Boquet for suggesting wortmannin, and Jerôme Mengaud for critically reading the manuscript.

6 June 1996; accepted 4 September 1996

An Essential Role for NF-κB in Preventing TNF-α–Induced Cell Death

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Studies on mice deficient in nuclear factor kappa B (NF-κB) subunits have shown that this transcription factor is important for lymphocyte responses to antigens and cytokine-inducible gene expression. In particular, the RelA (p65) subunit is required for induction of tumor necrosis factor–α (TNF-α)–dependent genes. Treatment of RelA-deficient (RelA$^{-/-}$) mouse fibroblasts and macrophages with TNF-α resulted in a significant reduction in viability, whereas RelA$^{+/+}$ cells were unaffected. Cytotoxicity to both cell types was mediated by TNF receptor 1. Reintroduction of RelA into RelA$^{-/-}$ fibroblasts resulted in enhanced survival, demonstrating that the presence of RelA is required for protection from TNF-α. These results have implications for the treatment of inflammatory and proliferative diseases.

The transcription factor NF-κB is a critical regulator of cytokine-inducible gene expression (*1*). Pro-inflammatory cytokines, such as TNF-α and interleukin-1 (IL-1), allow rapid nuclear translocation of NF-κB through degradation of IκB inhibitory cytoplasmic retention proteins (*1*, *2*). Genes regulated by nuclear NF-κB include those involved in inflammatory responses such as hematopoietic growth factors, chemokines, and leukocyte adhesion molecules (*1*). Thus, NF-κB is a key mediator of TNF-α responses and an attractive target for therapeutic intervention against inflammation and inflammatory diseases such as rheumatoid arthritis.

The most common form of NF-κB is a heterodimer of a 50-kD protein (p50) and a 65-kD protein (RelA, also termed p65) (*3*–*6*). As expected, embryonic fibroblasts (EFs) derived from RelA$^{-/-}$ embryos are unable to transcriptionally activate certain TNF-α–inducible genes with NF-κB binding sites (*7*), although p50$^{-/-}$ EFs show normal activation (*8*, *9*). TNF-α is not only involved in inflammatory responses but also possesses considerable cytotoxic activity, especially against tumor cells (*10*–*12*). The inability of RelA$^{-/-}$ EFs to activate target genes prompted us to investigate whether long-term TNF-α treatment leads to alterations in cellular function. An 8-hour treatment of RelA$^{-/-}$ EFs with mouse TNF-α (mTNF-α) resulted in a dramatic decrease in viability (Fig. 1A) (*13*). Longer treat-

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ment did not result in additional cell deaths. In contrast, no substantial effect on viability of RelA$^{+/+}$ or p50$^{-/-}$ EFs was noticed at any time after TNF-α treatment (Fig. 1, A and B). Thus, the absence of RelA sensitizes fibroblasts to TNF-α, which suggests that RelA regulates a cellular protective mechanism against the cytotoxic effects of TNF-α.

Repeated passage of EFs resulted in establishment of immortalized cell lines (3T3 cells) from both RelA$^{+/+}$ and RelA$^{-/-}$ EFs. Treatment of RelA$^{-/-}$ 3T3 cells with mTNF-α also resulted in loss of viability (Fig. 2A), suggesting that this effect was not limited to primary cells. Additionally, there were residual surviving cells. It is unclear whether TNF-α is unable to deliver a sufficiently potent signal to kill these cells or whether they are protected by a cellular mechanism independent of RelA.

Two TNF receptors have been identified, TNFR1 and TNFR2 (*14*, *15*), but only the former contains the "death domain," an intracellular region linked to cell death (*16*). Human TNF-α (hTNF-α) binds exclusively to mouse TNFR1 (*14*). To test whether TNF-α cytotoxicity in RelA$^{-/-}$ cells was mediated solely by TNFR1, we treated RelA$^{-/-}$ 3T3 cells with hTNF-α (Fig. 2B). Extensive loss of viability was evident, although with moderately delayed kinetics, indicating that TNF-α cytotoxicity is mediated largely or entirely by TNFR1. Macrophages are not only major producers of TNF-α but they are also key effector cells for this cytokine. Treatment of fetal liver–derived hematopoietic precursors with L929 cell–conditioned medium (containing macrophage growth factors) resulted in the appearance of macrophage colonies within 2 to 3 days of culture (*17*). Treatment of RelA$^{-/-}$ macrophages with mTNF-α or hTNF-α resulted in a dramatic loss of viability, whereas RelA$^{+/+}$ macrophages were unaffected (Fig. 2C). Thus, two key TNF-α responder cell types, fibroblasts and macrophages, require RelA for survival in the presence of this cytokine.

These experiments do not discriminate between an "active" protective role for RelA in the presence of TNF-α compared with a "developmental" effect that predisposes RelA$^{-/-}$ cells to die in the presence of this cytokine. The two possibilities can be discriminated by providing RelA directly to the RelA$^{-/-}$ cells (*18*). Either a mouse RelA expression vector or the pGD parental vector was therefore transfected into RelA$^{-/-}$ 3T3 cells along with a *lacZ* expression vector to mark the transfected cells. Mouse TNF-α was added 36 hours later for a 24-hour incubation, after which viable transfected cells were identified by X-Gal staining (Fig. 3). In the absence of transfected RelA, there was 20% survival, show-

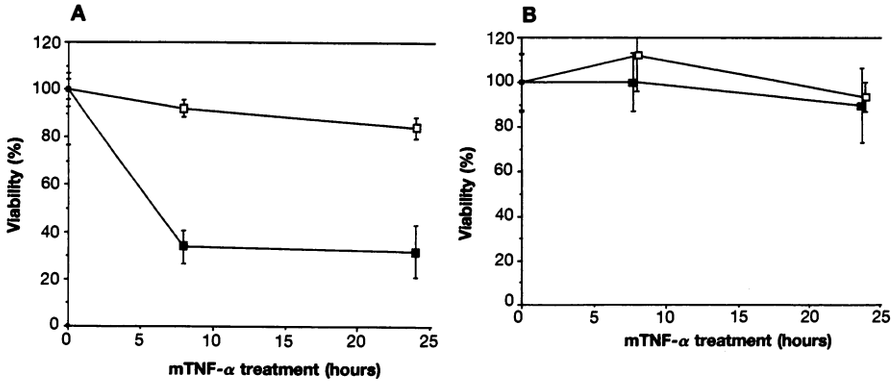

Fig. 1. TNF-α cytotoxicity of Rel$^{+/+}$ and Rel$^{-/-}$ EFs. (**A**) Rel$^{+/+}$ (□) or Rel$^{-/-}$ (■) EFs were treated with mTNF-α (10 ng/ml) for 8 or 24 hours. Viable cells remaining after treatment with mTNF-α are shown as a percentage of viable untreated cells. Standard deviations were calculated from three independent readings within a single experiment. Identical results were obtained with multiple independent isolates of EFs. (**B**) p50$^{+/+}$ (□) or p50$^{-/-}$ (■) EFs were treated with mTNF-α for 8 or 24 hours, after which viable cells were counted as described in (A).

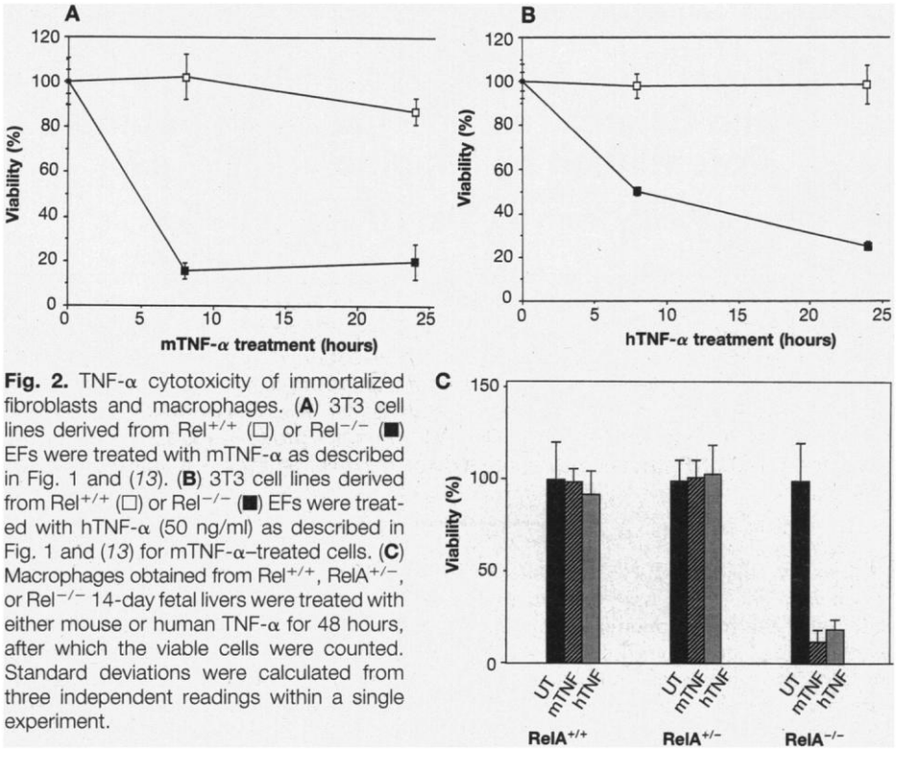

Fig. 2. TNF-α cytotoxicity of immortalized fibroblasts and macrophages. (**A**) 3T3 cell lines derived from Rel$^{+/+}$ (□) or Rel$^{-/-}$ (■) EFs were treated with mTNF-α as described in Fig. 1 and (*13*). (**B**) 3T3 cell lines derived from Rel$^{+/+}$ (□) or Rel$^{-/-}$ (■) EFs were treated with hTNF-α (50 ng/ml) as described in Fig. 1 and (*13*) for mTNF-α–treated cells. (**C**) Macrophages obtained from Rel$^{+/+}$, RelA$^{+/-}$, or Rel$^{-/-}$ 14-day fetal livers were treated with either mouse or human TNF-α for 48 hours, after which the viable cells were counted. Standard deviations were calculated from three independent readings within a single experiment.

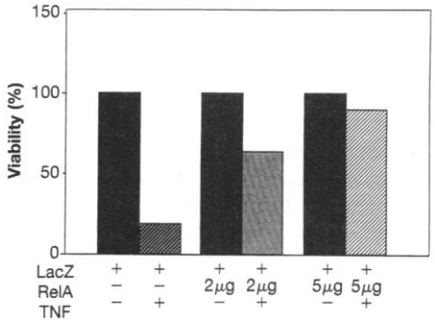

Fig. 3. Presence of RelA is required for protection from TNF-α cytotoxicity. Rel$^{-/-}$ 3T3 cells were calcium phosphate transfected with 1 μg of the LacZ-expressing vector pON 405 and the RelA-expressing vector pGD-65 in the amounts indicated. Mouse TNF-α was added (+) 36 hours later for 24 hours as indicated. After X-Gal staining, the number of blue cells was counted. Viable blue cells remaining after mTNF-α treatment are shown as a percentage of untreated viable blue cells. Similar results were obtained in multiple transfection experiments with these plasmids.

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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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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6 August 1996; accepted 2 October 1996

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