Persistent Tumor Necrosis Factor Signaling in Normal Human Fibroblasts Prevents the Complete Resynthesis of IκB-α*

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Transcription factor NF-κB is normally sequestered in the cytoplasm, complexed with IκB inhibitory proteins. Tumor necrosis factor (TNF) and interleukin-1 induce IκB-α phosphorylation, leading to IκB-α degradation and translocation of NF-κB to the nucleus where it activates genes important in inflammatory and immune responses. TNF and interleukin-1 actions are typically terminated by desensitization, and IκB-α reappearance normally occurs within 30–60 min. We found that in normal human FS-4 fibroblasts maintained in the presence of TNF, IκB-α protein failed to return to base-line levels for up to 15 h. Removal of TNF at any time during the 15-h period resulted in complete IκB-α resynthesis, suggesting that IκB-α reappearance was prevented by continued TNF signaling. Long term exposure of FS-4 fibroblasts to TNF led to a persistent presence of IκB-α mRNA, sustained IκB kinase activation, continuous proteasome-mediated degradation of IκB-α, and sustained nuclear localization of NF-κB. Continuous exposure of FS-4 cells to TNF did not lead to a sustained activation of p38 or ERK mitogen-activated protein kinases, suggesting that not all TNF-induced signaling pathways are persistently activated. These findings challenge the notion that all cytokine-mediated signals are rapidly terminated by desensitization and illustrate the need to elucidate the process of deactivation of TNF-induced signaling.

The transcription factor NF-κB is important in the regulation of genes involved in the immune and inflammatory responses, including genes encoding inflammatory cytokines (e.g. TNF, IL-1, IL-6, and IL-8), cell adhesion molecules (e.g. ICAM-1 and E-selectin), acute phase proteins, and many other proteins participating in host defenses, *e.g.* inducible nitric oxide synthase, major histocompatibility complex class I, and major histocompatibility complex class II (reviewed in Refs. 1–3). The NF-κB family is comprised of several proteins, including p65/RelA, RelB, c-Rel, p50/p105, and p52/p100. These Rel family members share an ~300 N-terminal amino acid sequence (the Rel homology domain), involved in subunit dimerization, DNA binding, and in the interaction of NF-κB proteins with members of the inhibitor of B (IκB) family of proteins. NF-κB is normally sequestered within the cytoplasm due to its interaction with IκB proteins, which bind to the NF-κB Rel homology domain and mask its nuclear localization sequence. Proteins comprising the IκB family include IκB-α, IκB-β, IκB-γ, IκB-ε, and Bcl-3.

A variety of stimuli, including the inflammatory cytokines TNF and IL-1, and bacterial lipopolysaccharide cause the inducible phosphorylation of N-terminal serines in IκB (Ser-32 and Ser-36 in the IκB-α isofrom) leading to the subsequent ubiquitination of neighboring lysines and the proteasome-mediated degradation of IκB-α (4–6). TNF initiates a signaling cascade that leads to IκB degradation by binding to cell surface TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Subsequent signaling (reviewed in Refs. 7 and 8) occurs through the recruitment of cytosolic signaling proteins including TNF receptor-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor interacting protein (RIP), eventually leading to the activation of the IκB kinase complex (IKK). The 700–900-kDa IKK complex, comprising the IKKa, IKKβ, and IKKγ subunits, directly phosphorylates IκB (9–11). Unmasking of the nuclear localization sequence following proteasome-mediated degradation of IκB permits NF-κB translocation to the nucleus, leading to transcriptional activation of a variety of genes. One of the genes transcriptionally activated by NF-κB is the gene encoding IκB-α (12), which upon its translation in the cytoplasm returns to the nucleus to dissociate NF-κB-DNA ternary complexes (13), thereby turning off transcription of NF-κB-driven genes. In addition, newly synthesized IκB-α interacts with NF-κB dimers in the cytoplasm and prevents NF-κB translocation to the nucleus. This autoregulatory loop serves to terminate NF-κB activation so as to prevent the protracted expression of mediators of host defense and inflammation that are regulated by NF-κB.

There exist other mechanisms to prevent the deleterious effects that would likely result from persistent cytokine signaling. In view of the well known acute toxicity and chronic inflammatory disorders resulting from TNF overexpression (14–16), it is not surprising that upon their extended exposure to TNF cells often become desensitized to TNF action. The most common mechanism of desensitization involves the down-modulation of cell surface TNFR expression, which can occur by...
receptor-mediated endocytosis (17–19), by TNFR shedding (20–22), or by mechanisms that have not been fully characterized (23). Alternatively, TNF signaling may be blocked at some point along the intracellular signaling cascade by factors that inhibit the association or function of signaling intermediates in the TNF pathway, such as TRAF-interacting protein (TRIP (24)) and the recently identified silencer of death domain (SODD) protein (25).

In most cells, TNF induces IkBα degradation within 15 min, which is followed by IkBα resynthesis and complete reappearance of IkBα protein within approximately 30 min to 2 h (12, 26). Complete reappearance of IkBα protein commonly occurs even if cells are maintained in the continuous presence of TNF (27–29), and this has been ascribed to the previously mentioned autoregulatory NFκB loop and to cellular desensitization to TNF action. We show here that in normal human diploid FS-4 fibroblasts stimulated with TNF, IkBα is rapidly degraded but IkBα reappearance is incomplete because cells do not become desensitized to TNF signaling, and newly synthesized IkBα continues to be phosphorylated and degraded. Continued signaling by TNF in these cells is evidenced by persistent activation of the IKK complex, ongoing proteasome-mediated IkBα degradation, continued nuclear localization of p65/RelA, and the persistent presence of IkBα mRNA. We also demonstrate that not all TNF signaling pathways are persistently activated in FS-4 cells, as TNF did not produce a sustained activation of the ERK and p38 MAP kinases. Our results challenge the paradigm that TNF signaling (and cytokine signaling in general) is always rapidly terminated by desensitization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Normal human FS-4 diploid fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% heat-inactivated fetal bovine serum (FBS). For use in experiments cells at passage 14 were split into 10-cm plates and serum-starved for 2–5 days in DMEM containing 0.25% FBS. COS-1 African Green Monkey kidney cells were maintained in DMEM containing 10% FBS and were serum-starved for 1–2 days in DMEM, 0.5% FBS prior to stimulation.

**Materials**—DMEM was purchased from Life Technologies, Inc. Recombinant human TNF-α was a gift from Masafumi Tsujimoto of the Suntory Institute for Biomedical Research (Osaka, Japan). Recombinant human IL-1α was obtained from the NCI, National Institutes of Health, Bethesda, MD. The TNFR1-specific TNF mutein (Trp-32/Thr-32Pro) was purchased from Zymed Laboratories Inc. (South San Francisco, CA). Anti-panphospho-p38 (a rabbit polyclonal antibody that detects the phosphorylated Thr-180 and Tyr-182 residues in p38) and anti-phospho-p38 were used at a 1:1000 dilution, anti-phospho-IkBα was used at a dilution of 1:250, and anti-phospho-IkBβ was used at 1:1000. Antibodies against both p38 and phospho-p38 were used at 1:1000 dilution, anti-ERK was used at 1:500, and anti-phospho-ERK was used at 1:20,000. Detection was accomplished with the use of horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated donkey anti-rabbit IgG (Bio-Rad, both at 1:3000 in TBS, 5% milk for 1 h at room temperature. Bands were visualized using a chemiluminescence substrate kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

**Northern Blot Analysis**—Cells were lysed in denaturing solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, and 0.7% β-mercaptoethanol). RNA was extracted using phenol/chloroform as described previously (31). RNA pellets were dissolved in formamide, and 12–15 μg of RNA was electrophoretically separated and transferred via capillary action onto nylon membranes (Hybond-N, Amersham). Membranes were washed in 0.1% SDS/0.1% sodium citrate, and 12–15 × SSC, and then hybridized with the membranes for 1–3 h at 68 °C. Membranes were then washed in 2× SSC (0.3 M NaCl, 30 mM sodium citrate) with 0.1% SDS at room temperature for 30 min, followed by washes in 1× SSC, 0.1% SDS at room temperature, 1× SSC, 0.1% SDS at 65 °C, and finally 0.2× SSC, 0.1% SDS at 65 °C. Autoradiography was then performed.

**Immunoprecipitation of IKK and Kinase Assays**—Whole cell lysates were prepared as described for immunoblotting. The IkB kinase (IKK) complex was immunoprecipitated with an antibody to IKKα (3 μg) for 1–2 h at 4°C, and immunocomplexes were collected with protein A/G-agarose (1–2 h, 4°C). The detergent-soluble protein fraction was then reprecipitated with [γ-32P]ATP/sample, and 5 μg of GST-IkBα/sample for 30 min at 30°C. The kinase reaction was terminated by boiling the samples in reducing sample buffer. Samples were separated on 12% SDS-PAGE, the gel was dried, and autoradiography was performed. To quantify their intensity, autoradiography bands were subjected to densitometric analysis (NIH Image 1.61).

**Pulse-Chase Metabolic Labeling Experiments**—Serum-starved FS-4 fibroblasts were plated on glass coverslips precoated with 0.1% gelatin (150,000 cells/coverslip) in 24-well plates. After the appropriate treatments cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with PBS, 0.2% Triton X-100 for 20 min at room temperature. The permeabilized cells were blocked in TBS, 5% bovine serum albumin (1–2 h, room temperature), and then incubated with anti-p65/RelA antibody (1 μg/ml final concentration) for 1–2 h at room temperature. Cells were then washed with PBS, 0.1% Triton X-100, and incubated with a Texas Red-conjugated goat anti-rabbit IgG secondary antibody (12 μg/ml final concentration) for 1–2 h at room temperature while shielded from light. Cells were next washed with PBS, 0.1% Triton X-100 and with PBS, counterstained with Hoechst nuclear dye (1 μg/ml) for 10–15 min, and washed with PBS. Coverslips were then mounted on microscope slides and examined by fluorescence microscopy.

**Pulse-Chase Metabolic Labeling Experiments**—Serum-starved FS-4 cells were either left untreated or stimulated with TNF as indicated. The cells were then washed and incubated for 1 or 2 h in amended DMEM (10% FBS, pH 7.4, 150 mM NaCl) with 5 mM 32P orthophosphate for 5 h, followed by washing in fresh amended DMEM with 50 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonl fluoride, and 100 mM sodium fluoride. Lysates were boiled for 5 min in denaturing sample buffer, separated via SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes (Millipore), blocked for 1 h at room temperature in Tris-buffered saline (TBS, 10 mM Tris, pH 7.4, 150 mM NaCl) with 5% milk, and incubated for 16 h with shaking with the appropriate antibody at 4°C. All antibody dilutions were prepared in TBS containing 0.02% Tween-20. Labeled IκBα was used at a dilution of 1:250, and anti-phospho-IκBα was used at 1:1000. Antibodies against both p38 and phospho-p38 were used at 1:1000 dilution, anti-ERK was used at 1:500, and anti-phospho-ERK was used at 1:20,000. Detection was accomplished with the use of horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated donkey anti-rabbit IgG (Bio-Rad, both at 1:3000 in TBS, 5% milk for 1 h at room temperature. Bands were visualized using a chemiluminescence substrate kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

**p65/RelA NF-κB Immunolocalization Studies**—Serum-starved FS-4 fibroblasts were plated on glass coverslips precoated with 0.1% gelatin (150,000 cells/coverslip) in 24-well plates. After the appropriate treatments cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with PBS, 0.2% Triton X-100 for 20 min at room temperature. The permeabilized cells were blocked in TBS, 5% bovine serum albumin (1–2 h, room temperature), and then incubated with anti-p65/RelA antibody (1 μg/ml final concentration) for 1–2 h at room temperature. Cells were then washed with PBS, 0.1% Triton X-100, and incubated with a Texas Red-conjugated goat anti-rabbit IgG secondary antibody (12 μg/ml final concentration) for 1–2 h at room temperature while shielded from light. Cells were next washed with PBS, 0.1% Triton X-100 and with PBS, counterstained with Hoechst nuclear dye (1 μg/ml) for 10–15 min, and washed with PBS. Coverslips were then mounted on microscope slides and examined by fluorescence microscopy.
Evidence for Persistent TNF Signaling: Steady-state IkB-α mRNA Levels and the Effect of Proteasome Inhibition on IkB-α Protein Levels—Our next series of experiments was designed to investigate the mechanism whereby complete IkB-α reappearance was curtailed in the continuous presence of TNF. In one group of experiments we used Northern blot analysis to study steady-state IkB-α mRNA levels in FS-4 cells incubated for periods ranging from 30 min to 18 h, either in the continuous presence of TNF or with TNF removed from the cultures following an initial 15-min stimulation. Peak steady-state IkB-α mRNA levels were reached at 1 h under both continuous and “pulse treatment” conditions (Fig. 4). In the continuous presence of TNF, IkB-α mRNA was sustained near this peak level for up to 18 h. In contrast, IkB-α mRNA levels decreased rapidly upon TNF removal from the culture medium. This result showed that the failure of IkB-α protein to return to control levels in the continuous presence of TNF was not due to a decreased availability of IkB-α mRNA. In fact, our observa-

![Image 1](59x666 to 287x729)

![Image 2](320x625 to 542x729)
incubated with TNF for 1, 4, and 15 h, MG132 treatment selectively binds mutant, on the degradation and reappearance of IκBα. Serum-starved FS-4 fibroblasts were treated for the indicated times with TNF (20 ng/ml, A) or a TNF mutant protein that binds exclusively to TNFR1 (20 ng/ml, B). Where indicated by wash, the cells were washed twice with PBS, replenished with fresh serum-starvation medium (DMEM, 0.25% FBS), and incubated for the remaining time at 37°C. Cells were then lysed, and lysates were resolved on 10% SDS-PAGE and immunoblotted with an anti-IκBα antibody as described in Fig. 1. Ctrl, control.

Fig. 3. Effect of treatment of FS-4 cells with TNF, or a TNFR1-selective binding mutant, on the degradation and reappearance of IκBα. Serum-starved FS-4 cells were treated with TNF (20 ng/ml) for the indicated times. Where indicated, TNF was removed after a 15-min stimulation, and the cells were washed and replenished with fresh medium without TNF. At the end of the incubation period the cells were lysed, and RNA was extracted. RNA was electrophoretically separated on 1% agarose gels, transferred onto nylon membranes, the cells were lysed, and culture supernatants were separated on 10% SDS-PAGE and immunoblotted with anti-IκBα (upper panels), anti-phospho-IκBα (middle panels), or anti-ERK (lower panels) as a loading control. Bands were visualized as described in Fig. 1.

Fig. 4. Steady-state IκBα mRNA levels in FS-4 fibroblasts treated with TNF. Serum-starved FS-4 cells were treated with TNF (20 ng/ml) for the indicated times. Where indicated, TNF was removed after a 15-min stimulation, and the cells were washed and replenished with fresh medium without TNF. At the end of the incubation period the cells were lysed, and RNA was extracted. RNA was electrophoretically separated on 1% agarose gels, transferred onto nylon membranes, membranes were then washed and exposed to film. Membranes were then stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to control for equal RNA loading. Ctrl, control.

Steady-state IκBα mRNA levels were sustained in the continuous presence of TNF, but not when TNF was removed after 15 min, as the opposite of what is seen at the protein level (Fig. 3A). Increased IκBα mRNA levels in cells treated continually with TNF probably reflect persistent TNF signaling, with ongoing TNF-mediated NF-κB activation likely to be driving the transcription of IκBα under these conditions.

We then considered the possibility that ongoing proteasome-mediated degradation of IκBα in cells treated continually with TNF may explain the failure of complete IκBα reappearance, despite the presence of higher levels of IκBα mRNA in such cells. FS-4 cells were stimulated with TNF for periods ranging from 5 min to 15 h and treated with the proteasome inhibitor MG132 as indicated (Fig. 5). Cell lysates were subjected to immunoblot analysis with antibodies directed against IκBα and N-terminally phosphorylated IκBα. Pretreatment of cells with MG132 decreased TNF-induced IκBα degradation (Fig. 5, lanes 4 and 6, upper and middle panels). Importantly, in cells incubated with TNF for 1, 4, and 15 h, MG132 treatment resulted in the reappearance of the phosphorylated IκBα species as well as in greatly increased levels of IκBα. These findings indicate that proteasome-mediated degradation of inducibly phosphorylated IκBα is likely to be important in the failure of IκBα to return to control levels in cells maintained in the presence of TNF.

Persistent TNF Signaling: Activation of the IκB Kinase Complex and Nuclear Localization of p65/RelA—Seeking further evidence for persistent TNF signaling, we examined the activity of the IKK complex. The IKK complex includes the IKKα and IKKβ subunits and is responsible for the inducible phosphorylation of IκBα (3, 9–11). FS-4 cells were incubated for periods ranging from 15 min to 15 h, either in the continuous presence of TNF or with TNF present only during the initial 15 min. The IKK complex was immunoprecipitated, incubated with GST-IκBα in an in vitro kinase reaction, then resolved on SDS-PAGE, and subjected to autoradiography (Fig. 6). Results from this experiment indicated that IKK was very strongly activated at 15 min after TNF stimulation. In cells maintained in the continuous presence of TNF, IKK activity decreased gradually at later times but was still detectable after 15 h. Densitometric analysis revealed that the strength of the phosphorylated band at 15 h was roughly 5% of the level observed after a 15-min stimulation. IKK-mediated IκBα phosphorylation decreased faster when TNF was removed after a 15-min stimulation, with no kinase activity detectable by 15 h.

To investigate further the effect of TNF stimulation on NF-κB activation, we examined the subcellular localization of p65/RelA in TNF-treated cells (Fig. 7). Cells were treated with TNF for the times shown, and where indicated TNF was removed after an initial 20-min stimulation. Fixed and permeabilized cells were incubated with an antibody against p65/RelA and a Texas Red-conjugated secondary antibody and were examined by fluorescence microscopy. Whereas p65/RelA was present in the cytoplasm of untreated cells, cells treated with TNF for 20 min, 1 h, or 15 h showed p65/RelA exclusively in the nucleus, consistent with a persistent activation of NF-κB. Removal of TNF following an initial 20-min stimulation produced a more heterogeneous p65/RelA staining pattern by 15 h, with p65/RelA detectable in both the nucleus and cytoplasm.

Stability of IκBα Protein in Unstimulated and TNF-stimulated Cells—To analyze the stability of the IκBα protein in unstimulated cells, as well as the half-life of IκBα protein in the presence or absence of TNF, we conducted pulse-chase metabolic labeling experiments. In control, unstimulated FS-4 cells we determined the half-life of basal IκBα to be 3.5 h (Fig. 8A, panel 1). Two other groups of cells were initially treated with TNF for 15 min in order to deplete cells of the IκBα protein. Cells in these groups were then metabolically labeled with [35S]methionine and -cysteine in the presence (Fig. 8A, panel 2) or absence (Fig. 8A, panel 3) of TNF and “chased” with

![Fig. 3](image3.png)

![Fig. 4](image4.png)

![Fig. 5](image5.png)
Cytokines are potent intercellular signaling molecules that serve important functions in host defenses. To prevent toxic side effects cytokines are generally produced only for limited periods. The continuous presence of TNF can lead to persistent activation of all TNF-responsive signaling pathways in FS-4 cells, as indicated (lanes 1 and 2) or chased in cytokine and methionine-containing medium in the absence or presence of TNF for 1 h as indicated (lanes 3 and 4). At the times indicated cells were lysed. IκB-α was immunoprecipitated from the lysates, and immunoblots were collected by centrifugation with protein A-Sepharose beads. Samples were resolved by SDS-PAGE and subjected to autoradiography. A fraction of the immunoprecipitate was retained and immunoblotted with antibody to IκB-α to control for equal loading (not shown). Curves were fitted to the data points and used to estimate the half-life of IκB-α protein under different treatment conditions (panels B1–3). Ctrl, control.

FIG. 6. Persistence of IKK complex activation in TNF-treated FS-4 fibroblasts. Serum-starved FS-4 fibroblasts were treated with TNF (20 ng/ml) for the indicated times. Where noted, cells were stimulated with TNF for 15 min, washed, and incubated without TNF for the remainder of the experiment. IKK complex was immunoprecipitated (IP) with anti-IKKα, and immunoprecipitates were then assayed for kinase activity using GST-IκB-α as a substrate. Samples were then boiled, separated on 12% SDS-PAGE, and the dried gel subjected to autoradiography (upper panel). Half of the immunoprecipitated fraction was separated by SDS-PAGE and blotted with anti-IKKα to control for equal amounts of IKK complex (lower panel). Ctrl, control.

FIG. 7. Subcellular localization of p65/RelA NF-κB in TNF-treated FS-4 fibroblasts. Serum-starved FS-4 cells were plated on gelatin-coated coverslips and treated with TNF (20 ng/ml) for the indicated times. Where indicated by wash, the cells were treated with TNF for 20 min, washed, and incubated with TNF-free medium for the remainder of the experiment. Cells were washed, fixed, permeabilized, and incubated with antibody to p65/RelA Texas Red-conjugated goat anti-rabbit IgG secondary antibody was used for detection. Cells were washed, and the coverslips were mounted on microscope slides and examined by fluorescence microscopy.

Continuous TNF Signaling Does Not Lead to Persistent ERK and p38 MAP Kinase Activation.—We have provided evidence that persistent signaling prevents the complete reappearance of IκB-α in cells treated continuously with TNF for up to 15 h. It was of interest to determine whether other TNF-elicited signaling pathways were persistently active in TNF-treated FS-4 cells, especially in comparison to IL-1-induced responses. Toward this end we treated FS-4 fibroblasts with TNF or IL-1 for periods ranging from 15 min to 15 h, and we examined cell lysates for the presence of phosphorylated (i.e., activated) forms of the ERK and p38 MAP kinases by immunoblot analysis (Fig. 9). Both TNF and IL-1 induced a marked increase in the phosphorylation of the p44 and p42 ERK and the p38 MAP kinases at 15 and 30 min, which was no longer observed after 1 h. Desensitization of cells to the MAP kinase-inducing activity of TNF and IL-1 was ligand-specific, because restimulation of FS-4 cells treated with TNF or IL-1 for 15 h with the heterologous cytokine produced a rapid (within 15 min) and full activation of p38 MAP kinase (data not shown). These data indicate that neither TNF nor IL-1 induced a persistent activation of the ERK and p38 MAP kinases and suggest that continuous treatment with TNF does not induce a generalized and persistent activation of all TNF-responsive signaling pathways in FS-4 fibroblasts.

DISCUSSION

Cytokines are potent intercellular signaling molecules that serve important functions in host defenses. To prevent toxic side effects cytokines are generally produced only for limited
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Fig. 9. Transient activation of ERK and p38 MAP kinases in TNF- or IL-1-treated FS-4 cells. Serum-starved FS-4 fibroblasts were treated for the indicated times with TNF (20 ng/ml) or IL-1 (4 ng/ml). Thereafter the cells were lysed, and lysates were processed as described in the legend for Fig. 1. Membranes were incubated with anti-phospho-ERK (pp44 and pp42), anti-ERK (p44 and p42), anti-phospho-p38 (pp38), or anti-p38 (p38). Blots were then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase and detected by chemiluminescence.

periods, usually in response to infection or trauma. Another feature that helps to guard against their deleterious side effects is that cytokines characteristically generate signals that are of short duration. Thus, long term exposure to a cytokine often leads to a selective desensitization of cells, which terminates signal propagation generated by this cytokine. One example of such desensitization is the resistance to TNF and IL-1 action that develops in cells chronically exposed to these cytokines. In most cells, TNF and IL-1 induce IκB-α degradation within 15 min, which is rapidly followed by IκB-α resynthesis and reappearance of the IκB-α protein (12, 26). Such complete IκB-α reappearance is observed even if the cells are maintained in the continuous presence of TNF or IL-1 (28, 29). In contrast, our present data show that in normal human FS-4 fibroblasts stimulated with TNF, IκB-α is rapidly degraded but IκB-α reappearance is incomplete. Analysis of the causes of incomplete IκB-α reappearance revealed that human FS-4 fibroblasts do not become completely desensitized to TNF signaling, such that newly synthesized IκB-α continues to be inducibly phosphorylated and degraded as a result of TNF action.

Several mechanisms are known to limit the duration and extent of cytokine signaling. The duration of NF-κB activation is limited by an autoregulatory process, in which NF-κB drives the transcription of its own inhibitor, IκB-α (27). Newly synthesized IκB-α retains NF-κB in the cytoplasm and, in addition, enters the nucleus to dissociate NF-κB bound to DNA in ternary complexes (13, 34, 35). TNF signaling is known to be curtailed by a variety of mechanisms, including the shedding of soluble TNF receptors (19–22), a regulated process that can be mediated by the action of the TNF-α-converting enzyme whose primary function is to proteolytically process the transmembrane precursor form of TNF to its soluble form (36). TNFR shedding decreases the availability of cell surface TNF receptors. In addition, soluble TNFR generated by this process may inhibit TNF signaling by competing with cell surface TNFR for ligand binding (37, 38). TNF signaling has also been shown to be regulated by the ligand-induced down-regulation of cell surface receptors via receptor-mediated endocytosis (17–19). Finally, cytokine signal duration may be limited by interactions between TNF signaling intermediates and intracellular inhibitors. Such signaling inhibitors include TRIP and I-TRAF, shown to prevent TRAF2-mediated NF-κB activation (24, 39), SODD that associates with TNFR1 and in the absence of TNF stimulation blocks TRADD and TRAF2 recruitment to the receptor death domain (25), and A20 which inhibits TNF and IL-1 signaling (40).

Our results show that despite the existence of numerous safeguards against persistent TNF signal transduction, signal- ing is not completely blunted in normal human fibroblasts exposed chronically to TNF, as evidenced by the failure of a complete reappearance of IκB-α for up to 15 h (Fig. 1), persistence of IκB-α mRNA levels (Fig. 4), persistent proteasome-mediated IκB-α degradation (Fig. 5), long term activation of the IKK complex (Fig. 6), and persistence of p65/RelA in the nucleus (Fig. 7). In addition, metabolic labeling experiments showed that in FS-4 cells exposed to TNF for 15 h, TNF continued to drive increased synthesis of IκB-α protein as well as its degradation (Fig. 8B). Earlier results obtained by electrophoretic mobility shift analysis also indicated that TNF-induced NF-κB activation in FS-4 cells persisted for at least 9 h (41). Preliminary evidence suggests that the nature and degree of persistent signaling depend upon the cytokine and cell type involved. When COS-1 cells were employed in an experiment similar to that shown in Fig. 1, complete reappearance of IκB-α protein was observed by 2 h in the presence of either TNF or IL-1 (data not shown). In addition, different signaling pathways activated by a single cytokine can differ in the degree of desensitization, as evidenced by the finding that both TNF- and IL-1-induced ERK and p38 MAP kinase activation in FS-4 cells were of a short duration (Fig. 9).

The molecular mechanisms that underlie the degree of desensitization to TNF and the level of persistent signaling have not yet been addressed. It is possible that these processes are affected by endogenous TNF signaling inhibitors, such as SODD (25). Human fibroblasts may express low levels of SODD, or failure of an efficient SODD-TNFR1 interaction in these cells may permit persistent TNF signaling. Similarly, the level or functional properties of other signaling inhibitory molecules, such as TRIP (24) or A20 (40), may affect the duration of TNF signaling. In addition, it is possible that TNF induces lower levels of receptor internalization or shedding in FS-4 fibroblasts compared with other cells, thus permitting longer signal duration. Diminished TNFR shedding has recently been described in hereditary inflammatory disorders involving mutations in TNFR1 that apparently limit the release of soluble TNFR and increase cellular responsiveness to TNF (42). One unanswered question is why TNF produces persistent NF-κB activation but only transient activation of members of the MAP kinase family (Fig. 9). Whereas RIP has been shown to play an essential role in TNF-mediated NF-κB activation and TRAF2 is required for TNF-driven JNK and p38 MAP kinase activation, both RIP and TRAF2 associate with TRADD (43, 44). The bifurcation in these two TNF-signaling pathways that begins at TRADD might determine the relative persistence of signaling via the NF-κB and the MAP kinase pathways.

Our results challenge the long held view that cytokine actions are terminated by rapid desensitization. The findings are biologically relevant because in many chronic inflammatory diseases persistent TNF signaling may lead to protracted NF-κB activation that induces the expression of inflammatory mediators, perpetuating a cycle of cellular activation, leukocyte migration, and coincident inflammation and tissue damage. Persistent signaling by TNF and other cytokines may be relevant in the context of inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and heart failure (15, 16). In addition, since TNF exerts a mitogenic effect on normal fibroblasts (45), persistent TNF signaling might also play a role in certain beneficial host responses, such as wound healing. Mechanisms responsible for the termination of cytokine signaling (and especially the failure
thereof) have not been extensively studied. Our data show that much remains to be learned about these processes.

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