MOLECULAR AND CELLULAR BIOLOGY, Nov. 1997, p. 6746–6754 Vol. 17, No. 11
0270-7306/97/$04.00+0

Synergistic Activation of NF-κB by Tumor Necrosis Factor Alpha and Gamma Interferon via Enhanced IκBα Degradation and De Novo IκBβ Degradation

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Received 12 June 1997/Returned for modification 21 July 1997/Accepted 15 August 1997

Tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) are required for an effective immune response to bacterial infection and these cytokines synergize in a variety of biological responses, including the induction of cytokine, cell adhesion, and inducible nitrous oxide synthase gene expression. Typically, the synergistic effect on gene expression is due to the independent activation of nuclear factor κB (NF-κB) by TNF-α and of signal transducers and activators of transcription or IFN-regulatory factor 1 by IFNs, allowing these transcription factors to bind their unique promoter sites. However, since activation of NF-κB by TNF-α is often transient and would not activate long-term κB-dependent transcription effectively, we explored the effects of IFN-γ on TNF-α-induced NF-κB activity. IFN-γ, which typically does not activate NF-κB, synergistically enhanced TNF-α-induced NF-κB nuclear translocation via a mechanism that involves the induced degradation of IκBβ and that apparently requires tyrosine kinase activity in preneuronal cells but not in endothelial cells. Correspondingly, cotreatment of cells with TNF-α and IFN-γ leads to persistent activation of NF-κB and to potent activation of κB-dependent gene expression, which may explain, at least in part, the synergy observed between these cytokines, as well as their involvement in the generation of an effective immune response.

Tumor necrosis factor alpha (TNF-α) is produced primarily by macrophages and functions immunologically through transcriptional up-regulation of genes encoding cell adhesion molecules required for the recruitment of inflammatory cells and genes encoding important inflammatory cytokines such as gamma interferon (IFN-γ) (reviewed in references 8, 13, and 71). IFN-γ is produced primarily by T cells and functions immunologically by leading to transcriptional up-regulation of class I and class II major histocompatibility complex (MHC) genes in antigen-presenting cells, by stimulating macrophages, and by inducing TNF-α secretion (reviewed in references 29 and 60). Data from in vivo experiments demonstrate that both TNF-α and IFN-γ are required for effective resistance to bacterial infection (7, 17, 24, 31, 32, 38, 39, 47, 54, 55, 61, 63). Also, it has been shown that TNF-α and IFN-γ play crucial roles in the progression of the inflammatory response associated with lipopolysaccharide-induced septic shock (29). Clearly, these pleiotropic cytokines have significant physiologic importance in regulating immune and inflammation processes.

TNF-α regulates the expression of genes which play critical roles during immunologically mediated inflammation responses. Activation of the transcription factor nuclear factor kappa B (NF-κB) by TNF-α is essential to elicit an effective response, since many of the TNF-α-regulated genes contain binding sites for NF-κB (reviewed in reference 6). The family of mammalian NF-κB/Rel transcription factors consists of at least five distinct members: c-Rel, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), and RelB (reviewed in references 3, 6, 48, 52 and 72). Gene knockout studies reveal the importance of NF-κB in several immunological pathways (9, 10, 18, 45, 70, 79); however, functional overlap may mask other important roles for NF-κB. NF-κB is a homo- or heterodimer which consists of various combinations of subunits. Classic NF-κB exists as a p50/p65 heterodimer and typically resides in the cytoplasm in an inactive form, bound by its inhibitor proteins IκBα and IκBβ (reviewed in reference 6). Upon TNF-α treatment, signal transduction events cause phosphorylation, ubiquitination, and degradation of IκB (15, 16, 19, 30), and the subsequent release of NF-κB, which translocates into the nucleus and regulates gene expression. IκBα has been studied more extensively than IκBβ; however, both inhibitor molecules appear to interact with NF-κB through similar domains and both preferentially bind p65- and c-Rel-containing dimers (76). Recently, several differences between these two inhibitors have been documented. First, IκBα is involved in the transient activation of NF-κB while IκBβ is targeted during the persistent activation of NF-κB (43, 51, 75, 76). Second, the mitogens and cytokines that activate NF-κB may favor the targeting of one inhibitor protein over that of the other. For example, data indicate that TNF-α preferentially targets IκBα, although some reports indicate that IκBβ can be targeted by TNF-α in some cell types (43; see Discussion also). In contrast, interleukin 1 (IL-1) targets both IκB molecules (43, 76; see Discussion also).

Studies with a variety of human cell lines have demonstrated that TNF-α and IFNs coregulate the expression of inflammation-associated genes such as MHC class I, intracellular cell adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 in a synergistic manner (14, 26, 27, 41, 42, 57). Specifically, TNF-α activates NF-κB, which binds κB elements (reviewed in reference 6) while IFNs activate various IFN-responsive factors which bind gamma-activated sites or IFN-stimulated elements (reviewed in references 59 and 69) in the promoters of target genes. Therefore, the synergistic induction

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of genes such as ICAM-1, vascular cell adhesion molecule 1, and MHC class I is a result of the independent activation of distinct transcription factors which engage unique promoter elements (for example, see reference 57). Other reports of transcriptional synergy between TNF-α and IFN-γ include studies on the genes that code for inducible nitric oxide synthase (50, 74, 81), IL-8 (82), IL-6 (66), regulated on activation normal T-expressed and -secreted chemokine (49), and inflammatory protein 10 (58), which are NF-κB regulated. Although it is clear that the transcriptional synergy between TNF-α and IFN-γ involves NF-κB and IFN-induced factors, it is also likely that sustained nuclear accumulation of NF-κB is required for long-term transcriptional responses involving NF-κB-regulated genes.

In this report, we describe the effects of IFN-γ on the regulation of TNF-α-induced NF-κB activity. We demonstrate that IFN-γ, which on its own does not significantly induce NF-κB, potentiates TNF-α-induced NF-κB nuclear translocation in a vascular endothelium cell line (EA.hy926) (28) and a preneuron-like pheochromocytoma cell line (PC12) (34). The mechanism involves the de novo degradation of IκBβ, leading to persistent NF-κB activation and to a potent κB-dependent transcriptional response. In endothelial cells, the mechanism also involves enhanced degradation of IκBα. Interestingly, the combination of IFN-γ and TNF-α at concentrations that alone would not activate NF-κB, leads to significant nuclear accumulation of this transcription factor. The protein tyrosine kinase (PTK) inhibitor genistein can inhibit this synergy in PC12 cells, suggesting that PTK activity is required. However, inhibition of PTK activity in endothelial cells augments the response, which is consistent with a recent report that induced PTK activity can limit NF-κB activation in some cells (73). The synergistic activation of NF-κB by two critical proinflammatory cytokines represents a mechanism by which NF-κB-induced gene expression could be enhanced under inflammatory conditions and may explain a critical aspect of the synergy between TNF-α and IFN-γ during an effective immune response.

MATERIALS AND METHODS

Cell culture and treatments. The preneuron-like rat adrenal pheochromocytoma cell line PC12 (CRL 1721; American Type Culture Collection, Rockville, Md.) (34) was maintained in Dulbecco's modified Eagle's medium F12 supplemented with 10% fetal bovine serum and antibiotics. The human vascular endothelial cell line EA.hy926 (gift from Cora-Jean S. Edgell, University of North Carolina, Chapel Hill) (28) was maintained in Dulbecco's modified Eagle's medium H supplemented with 10% fetal bovine serum, 1× hypoxanthine-aminopterin-thymidine medium supplement (Boehringer Mannheim Biochemica, Indianapolis, Ind.), and antibiotics. Cells were incubated for the times indicated in Results with 0.025 to 10 ng of human recombinant TNF-α (Boehringer Mannheim) per ml, 50 to 100 U of human or rat recombinant IFN-γ (Life Technologies, Gaithersburg, Md.) per ml, 100 U of human recombinant IFN-αA/D (Biosource International, Camarillo, Calif.) per ml, 10 μg of cycloheximide (CHX; Sigma Chemical Company, St. Louis, Mo.) per ml, and 100 μM genistein (Sigma) or 50-ng/ml phorbol myristate acetate (PMA; Sigma).

Nuclear and cytoplasmic extracts. Cells were trypsinized, resuspended in complete medium, and plated in 100-mm-diameter tissue culture plates (10^7 PC12 cells/plate or 2×10^6 EA.hy926 cells/plate) 16 to 18 h before treatment. After treatment, nuclear and cytoplasmic extracts were made by using a modified version of a previously described procedure (12). Cells were washed twice with ice-cold phosphate-buffered saline, gently scraped from the plates, transferred to microcentrifuge tubes, and lysed on ice in 3 pellet volumes of cytoplasmic extraction buffer (10 mM HEPES [pH 7.6], 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg each of aprotinin, leupeptin, and pepstatin per ml). Nuclei were pelleted (200 × *g*, 4°C, 5 min), and cytoplasmic supernatants were transferred to fresh tubes and maintained on ice. Nuclei were washed gently with 100 μl of cytoplasmic extraction buffer without Nonidet P-40 and pelleted (200 × *g*, 4°C, 5 min), and the supernatants were discarded. Two pellet volumes of nuclear extraction buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, 2.5 μg each of aprotinin, leupeptin, and pepstatin per ml) were added, and the final salt concentration was adjusted to ~400 mM with 2.5 M NaCl. Nuclear pellets were resuspended by vortexing, and the nuclear lysates were maintained on ice for 10 min with occasional vortexing. All cytoplasmic and nuclear extracts were cleared (16,000 × *g*, 4°C, 10 min) and transferred to fresh tubes. Next, 100% glycerol was added to the cytoplasmic extracts to a final concentration of 20%, protein concentrations were determined by the Bradford assay with the Bio-Rad protein assay dye reagent (no. 500-0006; Bio-Rad Laboratories, Hercules, Calif.), and all extracts were stored at −70°C.

EMSAs. For electrophoretic mobility shift assays (EMSAs), equal amounts of nuclear extracts (5 or 10 μg of protein) were incubated for 15 min at room temperature with a ^{32}P-labeled probe containing a κB site from the class I MHC promoter (5′CAGGGCTG**GGGATTCCCC**ATCTCCACAGTTTCACTTC3′ [the NF-κB binding site is in bold face]) (4, 67) in binding buffer (10 mM Tris [pH 7.7], 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol) (37) plus 2 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia Biotech, Piscataway, N.J.). Complexes were separated in 5% polyacrylamide gels in high-ionic-strength Tris-glycine-EDTA buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA), dried, and autoradiographed. As a control in CHX experiments, nuclear extracts were incubated with a ^{32}P-labeled probe containing an early growth response protein 1 (EGR-1) binding site from the NF-κB1 (p50) promoter (5′TCGACTCCCGC CCCCGCTGCG3′) (21).

For supershift experiments, 1 to 5 μl of rabbit polyclonal antibodies against the p50 subunit (sc-114; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) or the p65 subunit (100-4165; Rockland, Gilbertsville, Pa.) of NF-κB was incubated with nuclear extracts for 15 min prior to the addition of poly(dI-dC) · poly(dI-dC) and a ^{32}P-labeled probe and then analyzed on 5% polyacrylamide gels.

Western blot analysis. Equal amounts of cytoplasmic extracts (25 or 50 μg of protein) were electrophoresed in 10% polyacrylamide–sodium dodecyl sulfate gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). The upper half of each membrane was probed with an antibody specific for IκBβ (sc-945; Santa Cruz), and the lower half was probed with an antibody specific for IκBα (100-4167C; Rockland). Specific proteins were visualized by enhanced chemiluminescence (Amersham Life Science Inc., Cleveland, Ohio).

Transfection conditions and luciferase assays. Transient transfection of PC12 cells was accomplished by using 20 μl of Lipofectamine reagent (Life Technologies) per ml and a total of 6 μg of DNA for each sample. Plasmid pGEM7Zf(+) was used to equalize the amount of DNA transfected in each set of samples. The MHC-NF-κBLuc plasmid contains three tandem repeats of the κB site from the MHC class I enhancer, and the MHCmut-NF-κBLuc plasmid contains three copies of the mutated enhancer sequence (5) cloned into a luciferase expression vector. (Luciferase expression vectors were a gift from Bill Sugden, University of Wisconsin, Madison.) The pCMVIκBα expression plasmid was described previously (11). The IκBα constitutive repressor expression plasmid IκBαS32/36A, in which serines 32 and 36 were mutated to alanines was a gift from Dean Ballard (Vanderbilt University, Nashville, Tenn.) (68). In general, 7×10^6 PC12 cells were plated in 60-mm-diameter tissue culture plates 16 to 18 h before transfection and fed with fresh, complete medium 1 to 2 h before transfection. Lipofectamine-DNA complexes were allowed to form for 30 min in serum-free medium before being added to the cells which were washed out of complete medium and into 2 ml of serum-free medium just prior to the addition of complexes. Cells were incubated with the complexes for 7 to 8 h, the medium was replaced with medium containing 0.5% serum, and 8 h of cytokine treatment began 36 h after the medium change. Cells were washed twice with ice-cold phosphate-buffered saline, collected, resuspended in 2 pellet volumes of 0.25 M Tris (pH 7.5), and subjected to three freeze-thaw cycles. Samples were cleared (16,000 × *g*, 4°C, 10 min), and protein concentrations were determined with the Bio-Rad protein assay dye reagent. Luciferase assays were performed in duplicate on equal amounts of protein (25 or 50 μg) with 200 μM D-luciferin as the substrate (Sigma), and relative light units were determined with an AutoLumat LB953 luminometer (Berthold Analytical Instruments, Inc., Nashua, N.H.).

RESULTS

TNF-α and IFN-γ synergistically activate NF-κB nuclear translocation. Synergy between TNF-α and IFN-γ has been reported in numerous cell types; therefore, we chose two cell lines for our studies. PC12 cells are rat preneuron-like pheochromocytoma cells (34) that exhibit rapid and transient activation of NF-κB and provide a model for the potential involvement of NF-κB in neurological disorders (35, 44). EA.hy926 cells are immortalized human endothelial cells which exhibit many normal endothelial responses (28). These cells are particularly useful for studies on the role of NF-κB during immunologically induced inflammation. To determine the effect of IFN-γ on TNF-α-induced NF-κB DNA binding activity in vitro, the cell lines were treated with each cytokine alone or with both cytokines simultaneously and nuclear extracts were analyzed by EMSA (Fig. 1). As expected, there was very little binding to a consensus κB site with nuclear extracts from

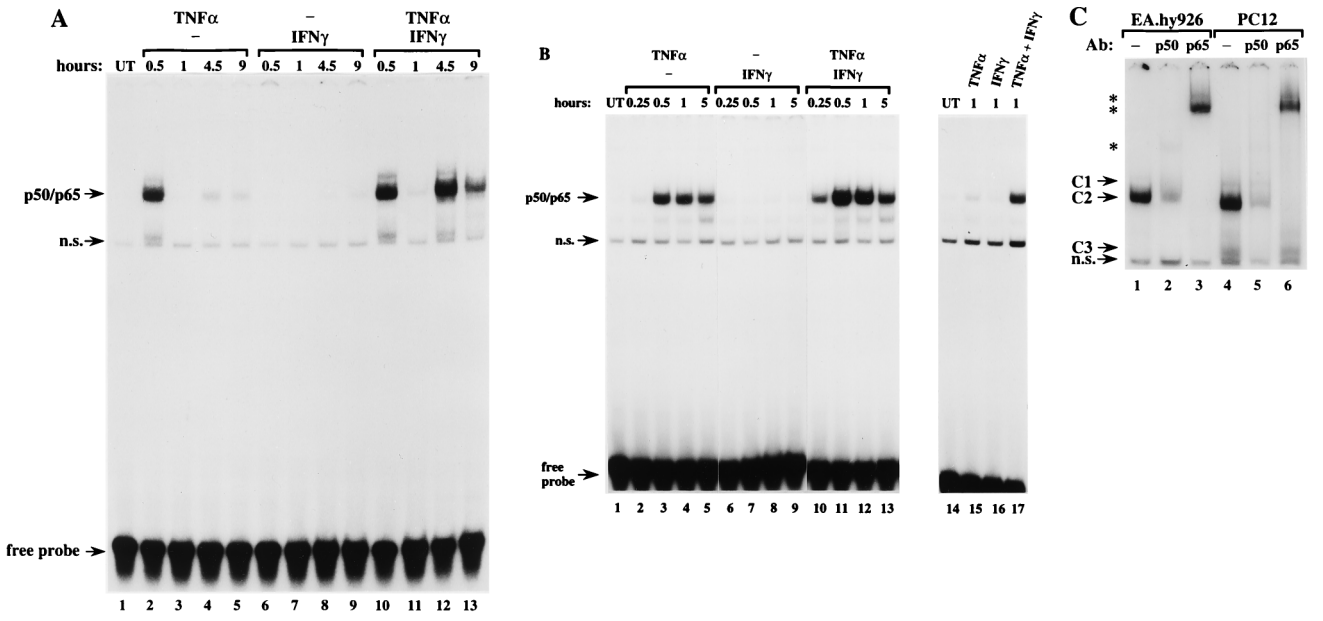

FIG. 1. Synergistic activation of NF-κB by cotreatment with TNF-α and IFN-γ. (A) EMSA of equal amounts of protein from PC12 cell nuclear extracts using a probe containing a consensus NF-κB binding site. The time and treatment are indicated above each lane (UT is untreated). The TNF-α and IFN-γ concentrations were 10 ng/ml and 100 U/ml, respectively. Arrows indicate the major NF-κB-specific band (p50/p65), a nonspecific band (n.s.), and the free probe. (B) EMSA of equal amounts of protein from EA.hy926 cell nuclear extracts. The probe used and labeling of lanes are the same as in panel A. The TNF-α and IFN-γ concentrations were 0.1 ng/ml and 100 U/ml, respectively, for lanes 1 to 13 and 25 pg/ml and 100 U/ml, respectively, for lanes 14 to 17. (C) Identification of NF-κB complexes. Nuclear extracts were incubated with antibodies specific for individual NF-κB subunits, and complexes were resolved by EMSA. Cells were left untreated or were treated for 0.5 h with TNF-α at 0.1 (EA.hy926 cells) or 10 (PC12 cells) ng/ml. Antibodies (Ab) are indicated at the top of each lane, and arrows indicate all NF-κB-specific bands (C1, C2, and C3) and a nonspecific band. Supershifted complexes are indicated by asterisks.

untreated PC12 cells (Fig. 1A, lane 1) or untreated EA.hy926 cells (Fig. 1B, lane 1). Upon treatment for 30 min with TNF-α alone, one major complex (p50/p65; see below) formed in both cell lines. In PC12 cells, this complex disappeared by 1 h and reappeared at extremely low levels by 4.5 h (Fig. 1A, lanes 2 to 5). In EA.hy926 cells, the binding of this complex slowly decreased over time (Fig. 1B, lanes 2 to 5) but did not return to basal levels until greater than 12 h after treatment (data not shown). As anticipated, treatment with IFN-γ alone did not induce binding to the NF-κB-specific probe in either cell line (Fig. 1A and B, lanes 6 to 9). Cotreatment with TNF-α and IFN-γ elicited a striking cooperative effect on κB-specific binding activity in both cell lines. In PC12 cells, the major NF-κB complex exhibited a large increase in binding by 4.5 h of cotreatment which remained elevated for at least another 4.5 h (Fig. 1A, lanes 10 to 13). This second peak of binding activity was consistently greater than the binding observed after 30 min of cotreatment and significantly greater than the binding observed after 4.5 h of treatment with TNF-α alone (compare lanes 4, 10 and 12). In EA.hy926 cells, cotreatment with TNF-α and IFN-γ caused the major DNA binding complex to form earlier (within 15 min) and produced greater binding activity than did treatment with TNF-α alone (Fig. 1B, compare lanes 2 through 5 to lanes 10 through 13). The binding activity was at least fourfold higher and remained elevated longer in cotreated EA.hy926 cells than in cells treated with TNF-α alone. Although the NF-κB activation profiles of PC12 and EA.hy926 cells differ in regard to the timing and amount of binding, it is clear that TNF-α and IFN-γ exert a synergistic, not an additive, effect on the induction of binding to a κB-specific probe. The concentration of TNF-α was critical for the synergy between TNF-α and IFN-γ in EA.hy926 cells. For example, at TNF-α concentrations greater than 1 ng/ml, the synergy produced by cotreatment was obscured because the NF-κB binding activity was already maximal due to TNF-α alone. Of great interest relative to issues of inflammation and immune responses was the finding that a low concentration of TNF-α, which does not significantly activate NF-κB by itself, can act synergistically with IFN-γ to produce strong binding activity (Fig. 1B, lanes 14 to 17).

To verify that the induced complexes contained NF-κB and to identify the subunits in the complexes, antibody supershift experiments were performed on nuclear extracts from PC12 and EA.hy926 cells treated for 30 min with TNF-α by using antibodies specific for the p50 and p65 NF-κB subunits (Fig. 1C). In EA.hy926 cells, the p50-specific antibody shifted complex C2 (lane 2) and the p65 antibody completely shifted complexes C1 and C2 (lane 3). In PC12 cells, the p50-specific antibody repressed the formation of complex C2 and completely shifted complex C3 (lane 5) while the p65-specific antibody completely shifted complexes C1 and C2 (lane 6). These data indicate that the major induced complex in both cell lines is the p50/p65 heterodimer (C2) and the minor induced complexes are most likely p50/p50 homodimers (C3) and either p65/c-Rel or p65/p52 heterodimers (C1). Since c-Rel is a component of activated NF-κB in some cells, we tested whether the mobilization of c-Rel changes during TNF-α–IFN-γ costimulation. Our data indicate that c-Rel activation in response to TNF-α–IFN-γ cotreatment is not appreciably different from that caused by TNF-α alone in our two experimental cell lines (20a).

Synergistic activation of NF-κB by TNF-α and IFN-γ involves enhanced IκBα degradation and induced IκBβ degradation. Normally, NF-κB is sequestered in the cytoplasm by its inhibitory proteins, which are collectively referred to as IκBs. A variety of stimuli promote the degradation of specific IκB proteins, which results in the release and nuclear translocation of NF-κB (reviewed in reference 6). To elucidate the mecha-

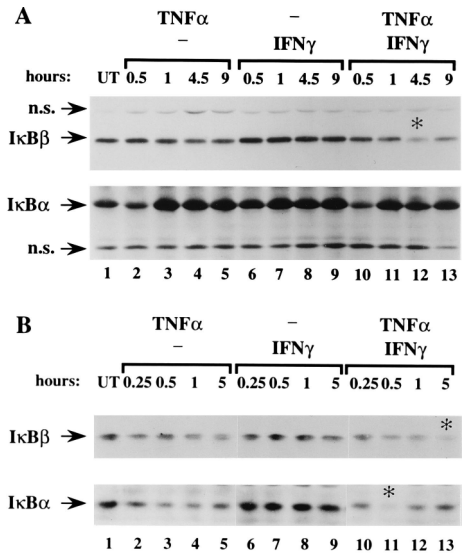

FIG. 2. Cotreatment with TNF-α and IFN-γ enhances IκBα degradation and induces IκBβ degradation. (A) Western analyses of equal amounts of protein from PC12 cell cytoplasmic extracts using polyclonal antibodies specific for IκBα or IκBβ. The time and treatment are indicated above each lane (UT is untreated). The TNF-α and IFN-γ concentrations were 10 ng/ml and 100 U/ml, respectively. Arrows indicate each specific IκB protein and nonspecific bands (n.s.). (B) Western analyses of equal amounts of protein from EA.hy926 cell cytoplasmic extracts. The antibodies used and labeling of lanes are the same as in panel A. The TNF-α and IFN-γ concentrations were 0.1 ng/ml and 100 U/ml, respectively. Asterisks highlight the synergistic effects of TNF-α–IFN-γ costimulation.

nism by which TNF-α and IFN-γ costimulate NF-κB, we investigated the effects of these cytokines on IκBα and IκBβ degradation. Western blot analyses examining the levels of the IκBα and IκBβ proteins were performed on cytoplasmic extracts collected at the same time as the nuclear extracts that were analyzed for Fig. 1. In PC12 cells, 30 min of TNF-α treatment reduced IκBα protein levels but did not reduce IκBβ (Fig. 2A, compare lanes 1 and 2). IFN-γ treatment alone did not cause degradation of either IκB protein (lanes 6 to 9). Interestingly, TNF-α–IFN-γ cotreatment caused extensive degradation of IκBβ by 4.5 h (lane 12). Similarly, in EA.hy926 cells, 15 to 30 min of TNF-α treatment reduced IκBα protein levels but did not reduce IκBβ levels (Fig. 2B, compare lanes 1 through 3) and IFN-γ treatment alone did not decrease the level of either IκB protein (lanes 6 through 9). TNF-α–IFN-γ cotreatment caused a greater loss of IκBα protein than did TNF-α treatment alone (compare lanes 3 and 11) and extensive degradation of IκBβ within 1 h (lanes 12 and 13). In both cell lines, IκBα was resynthesized and returned to basal levels after TNF-α treatment alone or TNF-α–IFN-γ cotreatment (Fig. 2A and B, lanes 2 to 5 and 10 to 13); however, IκBβ protein levels remained reduced for at least 5 h (Fig. 2A and B, lane 13). The expression of IκBα is transcriptionally regulated by NF-κB (reviewed in reference 6); however, the expression of IκBβ is not (76). This may explain the differential reappearance of these two inhibitors. Peptide competitions verified that the bands detected by immunoblotting were the proteins of interest (data not shown). In summary, TNF-α–IFN-γ cotreatment enhanced the degradation of IκBα compared to TNF-α treatment alone and induced the degradation of IκBβ.

Comparing the NF-κB activation profiles shown in Fig. 1A and B to the degradation patterns of IκBα and IκBβ shown in Fig. 2A and B indicates that the TNF-α–IFN-γ-induced synergistic activation of NF-κB corresponds precisely to the enhanced degradation of IκBα and de novo degradation of IκBβ. Therefore, it is likely that the increased amount of NF-κB detected in nuclear extracts from cells cotreated with TNF-α and IFN-γ, compared with those treated with TNF-α alone, was due to the release of NF-κB molecules from both inhibitor proteins as they were degraded.

IFN-α also potentiates TNF-α-induced NF-κB activation. There are three distinct classes of IFNs which fall into two different categories based on their receptors and biological activities. IFN-α and IFN-β are type I interferons, whereas IFN-γ is a type II interferon (29, 60). To determine if the synergy between TNF-α and IFN-γ during NF-κB activation is unique to IFN-γ or if this synergy is common to both types of IFNs, we repeated the experiments described in Fig. 1B and 2B with EA.hy926 cells, comparing IFN-α to IFN-γ. Upon treatment with TNF-α alone, one major NF-κB complex formed (Fig. 3, lanes 2 to 5) and, as expected, treatment with IFN-γ or IFN-α alone did not activate NF-κB (data not shown). Cotreatment with TNF-α and IFN-α elicited the same striking cooperative effect on κB-specific binding as that obtained by cotreatment with TNF-α and IFN-γ (compare lanes 6 through 9 to lanes 10 through 13). The results were indistinguishable in that both IFNs synergized with TNF-α to induce NF-κB to similar levels in a similar time frame. The effects on IκB protein levels were analogous also. The degradation of IκBα was enhanced compared to that caused by TNF-α treatment alone, and de novo degradation of IκBβ occurred regardless of whether IFN-α or IFN-γ was used in combination with TNF-α (data not shown). As described earlier for TNF-α–IFN-γ cotreatment, the NF-κB activation profile observed with TNF-α–IFN-α cotreatment corresponds precisely to the degradation of IκBα and IκBβ.

Protein synthesis is not required for the synergistic activation of NF-κB by TNF-α and IFN-γ. To rule out the possibility that the TNF-α–IFN-γ synergy depends on an increase in TNF-α or IFN-γ receptor synthesis and to determine if the signal transduction molecules required for coactivation of NF-κB pre-exist in cells, PC12 cells were pretreated with the protein synthesis inhibitor CHX and then treated with TNF-α and/or IFN-γ. As previously documented, the induction of NF-κB DNA binding activity by TNF-α alone does not require protein synthesis (3, 48, 52, 72) (Fig. 4, compare lanes 3 and 4). Figure 4, lanes 7 to 10, indicates that CHX did not inhibit synergistic activation of NF-κB but actually enhanced its bind-

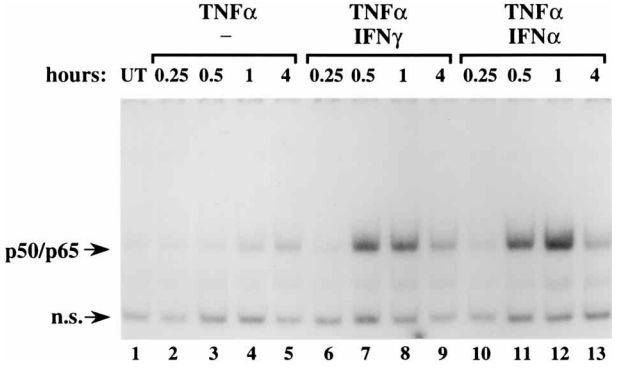

FIG. 3. Synergistic activation of NF-κB by cotreatment with TNF-α and IFN-α. EMSA of equal amounts of protein from EA.hy926 cell nuclear extracts using a probe containing a consensus NF-κB binding site. The time and treatment are indicated above each lane (UT is untreated). The TNF-α, IFN-γ, and IFN-α concentrations were 0.05 ng/ml, 100 U/ml, and 100 U/ml, respectively. Arrows indicate the major NF-κB-specific band (p50/p65) and a nonspecific band (n.s.).

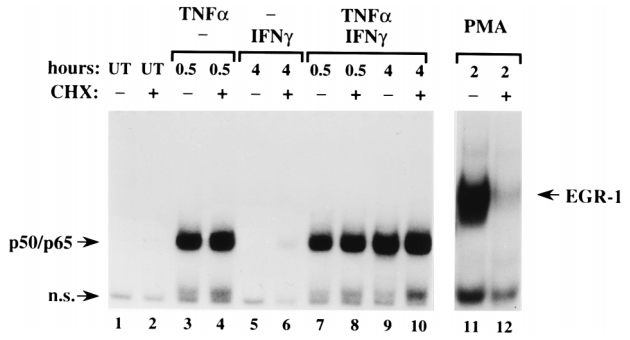

FIG. 4. Protein synthesis is not required for synergistic activation of NF-κB by TNF-α and IFN-γ. EMSA of nuclear extracts from PC12 cells pretreated for 1 h with 10-μg/ml of CHX. The time and treatment are indicated above each lane (UT is untreated). The TNF-α, IFN-γ, and PMA concentrations were 10 ng/ml, 100 U/ml, and 50 ng/ml, respectively. Arrows indicate the major NF-κB-specific band (p50/p65), the EGR-1-specific band, and a nonspecific band (n.s.).

ing slightly. This enhanced binding (over synergistic levels) was most likely due to the lack of IκBα resynthesis in the presence of CHX (data not shown). Western analyses also demonstrated that IκBβ was degraded upon cotreatment in the presence or absence of CHX (data not shown); therefore, the preservation of the synergistic activation of NF-κB in the presence of CHX was a result of the degradation of IκBβ and was not due to the lack of IκBα resynthesis alone. To verify that CHX inhibited protein synthesis adequately in these experiments, PMA activation of EGR-1 was used as a positive control since this transcription factor requires protein synthesis for activation (33). Pretreatment with CHX inhibited the binding of EGR-1 to its consensus site, which clearly demonstrated the integrity of the CHX reagent (Fig. 4, lanes 11 and 12). Similar experiments with CHX pretreatment of EA.hy926 cells followed by cotreatment with TNF-α and IFN-γ yielded results comparable to those obtained with PC12 cells (data not shown). In summary, our data show that protein synthesis was not required for synergistic activation of NF-κB by TNF-α–IFN-γ cotreatment.

Genistein inhibits TNF-α–IFN-γ-induced NF-κB activation in PC12 cells and enhances activation in EA.hy926 cells. IFNs are known to induce the activation of signal transducers and activators of transcription through the PTK activity of the

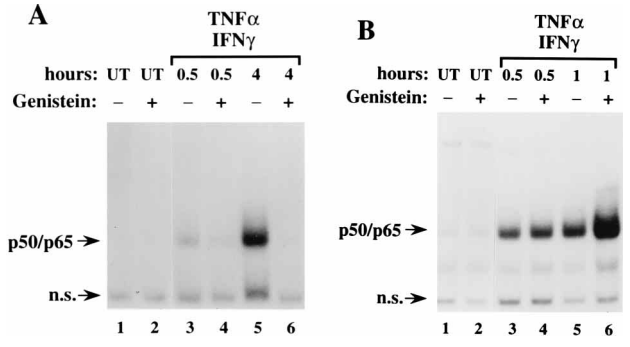

FIG. 5. The PTK inhibitor genistein affects TNF-α–IFN-γ-induced NF-κB activation. (A) EMSA of nuclear extracts from PC12 cells pretreated for 1 h with 100 μM genistein. The time and treatment are indicated above each lane (UT is untreated). The TNF-α and IFN-γ concentrations were 1 ng/ml and 100 U/ml, respectively. Arrows indicate the major NF-κB-specific band (p50/p65) and a nonspecific band (n.s.). (B) EMSA of nuclear extracts from EA.hy926 cells pretreated for 1 h with 100 μM genistein. The time and treatment are indicated above each lane. The TNF-α and IFN-γ concentrations were 0.05 ng/ml and 100 U/ml, respectively.

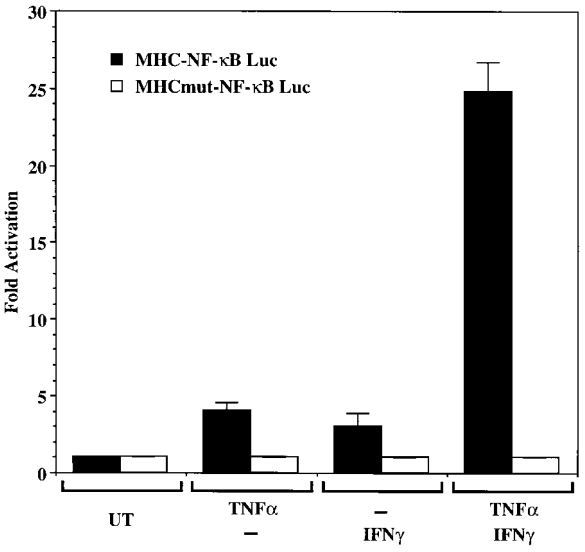

FIG. 6. Synergistic activation of κB-dependent reporter gene expression by TNF-α–IFN-γ cotreatment. PC12 cells transfected with equal amounts of a κB-dependent luciferase reporter construct (MHC-NF-κBLuc) or its mutated counterpart (MHCmut-NF-κBLuc) were treated with TNF-α at 5 ng/ml, IFN-γ at 50 U/ml, or a combination of both for 8 h. Lysates were assayed in duplicate for luciferase activity, and fold activation was determined by dividing the number of relative light units from treated samples by the number of relative light units from untreated (UT) samples. The data shown are averages of three independent experiments, and the standard errors of the means are indicated by error bars.

Janus family of protein kinases (JAKs) (25, 40, 69); therefore, we tested whether PTK activity is required for TNF-α–IFN-γ synergy in NF-κB activation. PC12 and EA.hy926 cells were pretreated with the PTK inhibitor genistein for 1 h before TNF-α–IFN-γ cotreatment, and then nuclear extracts were analyzed by EMSA. Figure 5A illustrates that in PC12 cells, the coactivation of NF-κB is completely blocked by genistein, indicating that PTK activity is required for this synergistic response (compare lane 3 to lane 4 and lane 5 to lane 6). In PC12 cells, the PTK activity is most likely controlled by IFN-γ since NF-κB binding that is attributed to activation by TNF-α alone is not blocked by genistein (data not shown). Conversely, in EA.hy926 cells, genistein enhances NF-κB synergistic activation, as revealed by the increase in NF-κB binding in the presence of this PTK inhibitor (Fig. 5B, compare lane 3 to lane 4 and lane 5 to lane 6). Genistein alone had no effect on the basal level of NF-κB activity in either cell line (Fig. 5A and B, compare lanes 1 and 2). These data indicate that regulatory mechanisms involved in the synergy of TNF-α and IFN-γ relative to NF-κB activation are likely to involve distinct mechanisms in PC12 and endothelial cells (see Discussion).

TNF-α and IFN-γ synergistically activate κB-dependent transcription. To test whether the synergistic activation of NF-κB leads to enhanced NF-κB-dependent transcriptional responses, we examined the ability of TNF-α and IFN-γ to costimulate κB-dependent reporter gene expression. PC12 cells were transiently transfected with a luciferase reporter construct containing three κB sites cloned in tandem in front of the minimal luciferase promoter (MHC-NF-κBLuc) or with a similar construct in which the κB sites were mutated (MHCmut-NF-κBLuc). The cells were treated with TNF-α and/or IFN-γ for 8 h, and lysates were assayed for luciferase activity. The wild-type κB sites conferred a 4-fold induction upon treatment with TNF-α, a 3-fold induction upon treat-

ment with IFN-γ, and a potent 25-fold induction upon TNF-α–IFN-γ cotreatment compared to the basal activity of this expression construct in untreated cells (Fig. 6).

This synergistic induction of gene expression was completely abolished in transfections using the reporter construct containing mutated κB sites (Fig. 6), and cotransfections with a wild-type IκBα (11) or an IκBα constitutive repressor expression vector (68) inhibited the induction of MHC-NF-κBLuc by at least 50 and 95%, respectively (data not shown). Additionally, we have made two PC12 cell lines, one which stably expresses MHC-NF-κBLuc and one which stably expresses MHCmut-NF-κBLuc. The stable cell line expressing the wild-type plasmid exhibits a 29-fold induction of luciferase activity by TNF-α treatment alone, a 3-fold induction by IFN-γ alone, and a synergistic 68-fold induction by TNF-α–IFN-γ cotreatment (data not shown). The stable cell line expressing the plasmid containing mutated κB sites does not exhibit an increase in luciferase activity upon cytokine treatment (data not shown). Transfection experiments have not been repeated with EA.hy926 cells due to their loss of viability during transfection procedures.

The synergistic activation of NF-κB by TNF-α and IFN-γ also enhances the expression of endogenous genes that contain κB sites in their promoters. Northern analysis of PC12 cells indicates a synergistic increase in the abundance of IκBα mRNA upon cotreatment with TNF-α and IFN-γ compared with treatment with TNF-α alone (data not shown). In summary, our data indicate that the synergistic activation of NF-κB by TNF-α and IFN-γ leads to a potent synergistic effect on NF-κB-dependent transcription.

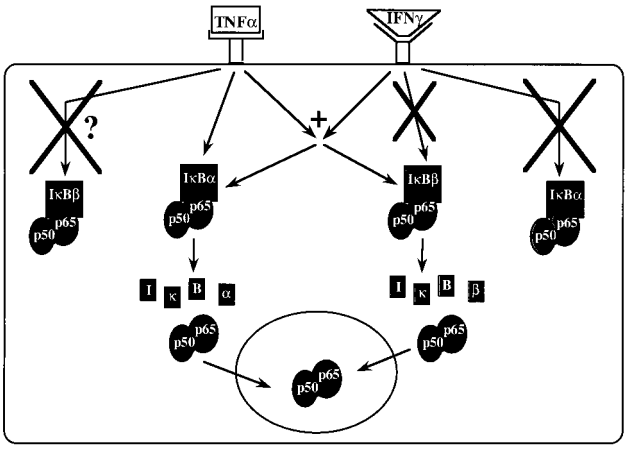

FIG. 7. Model of the synergistic activation of NF-κB by TNF-α and IFN-γ. NF-κB is sequestered in the cytoplasm by IκBα and IκBβ. Upon treatment with TNF-α alone, IκBα is modified and degraded, releasing NF-κB, which is free to translocate into the nucleus and bind to κB sites in target genes. Upon treatment with IFN-γ alone, neither IκB molecule is affected and therefore no NF-κB is released. Upon cotreatment with TNF-α and IFN-γ, IκBα degradation is enhanced and de novo IκBβ degradation occurs. This leads to persistent activation of NF-κB (74) and to a synergistic increase in κB-dependent gene expression. The question mark indicates that the relationship between treatment with TNF-α alone and IκBβ degradation has not been established definitively. This is discussed in the text.

DISCUSSION

The cytokines TNF-α and IFN-γ function cooperatively during many biological responses. For example, they have synergistic cytotoxic effects on tumor cells, they synergistically coactivate gene expression, they function together to elicit an effective immune response, they increase the activation of lymphocytes, and they induce the differentiation of neuroblastoma cells (reviewed in references 29 and 71). Furthermore, TNF-α and IFN-γ are intimately involved in the inflammation response during septic shock (29). There are several possible mechanisms by which these cytokines may collaborate. In some instances, their cooperativity may be explained by the IFN-γ-induced up-regulation of TNF-α receptors (1, 64, 78) or vice versa (46, 65). Also, TNF-α–IFN-γ synergy may be explained by the independent activation of distinct transcription factors (e.g., NF-κB, IFN-regulatory factor 1, and signal transducers and activators of transcription) which bind to unique promoter sites and synergistically regulate genes containing TNF-α- and IFN-responsive elements in their promoters. In addition, Drew et al. showed that NF-κB and IFN-regulatory factor 1 can physically interact (27). The transcription factor NF-κB is clearly important during an immune response, since it controls transcription of a variety of genes required for an effective response (3, 6, 48, 52, 72). Since NF-κB activation can be relatively transient in response to TNF-α stimulation, we have examined the potential for IFNs to enhance TNF-α-induced NF-κB activation. We investigated two distinct cell types to understand the potentially ubiquitous nature of the effects of TNF-α–IFN-γ cotreatment on the activation of NF-κB. We chose EA.hy926 cells since endothelial cells play a critical role during immunologically induced inflammatory responses by up-regulating the expression of κB-dependent cell adhesion molecules and by producing cytokines and growth factors (22, 23, 62). We selected PC12 cells since it has been proposed that activation of NF-κB in the nervous system may play a role in the progression of neurodegenerative diseases such as Alzheimer's disease, Down's syndrome, Parkinson's disease, and amyotrophic lateral sclerosis (44).

Our data clearly show that IFNs can strongly potentiate the ability of TNF-α to induce NF-κB nuclear translocation and stimulate κB-dependent transcription (Fig. 1, 3, and 6). A major component of this synergistic activation is a mechanistic switch from the degradation of IκBα to the targeted degradation of IκBβ, which leads to persistent activation of NF-κB. In our model (Fig. 7), TNF-α and IFN-γ bind to their respective receptors and activate individual signal transduction pathways. TNF-α, by itself, targets IκBα for degradation, allowing nuclear translocation of NF-κB. TNF-α, in the presence of signals originating from the interaction of IFN-γ with its receptor, targets both IκBα and IκBβ for degradation. This increases the amount of NF-κB that is free to translocate and therefore synergistically increases the amount of κB-dependent gene expression. Recently, Harhaj et al. demonstrated that CD28 and PMA can costimulate NF-κB activity during T-cell activation by inducing the rapid degradation of both IκBα and IκBβ (36). Although our model for TNF-α–IFN-γ costimulation is similar to these findings, there is a major fundamental difference. In our model, one stimulus (IFN-γ) does not activate NF-κB by itself while the other stimulus (TNF-α) does. In the T-cell activation system, each stimulus (CD28 or PMA) activates NF-κB by itself.

TNF-α is a well-known initiator of NF-κB nuclear translocation controlled through the targeted phosphorylation and subsequent degradation of IκBα. The distinct regulatory components of the signal transduction pathway(s) involved in this response have not been defined clearly. The induced phosphorylation of IκBα on serines 32 and 36 leads to ubiquitination and subsequent degradation via a proteasome-mediated mechanism (15, 16, 19). IκBβ is presumed to be regulated by phosphorylation on two serines in analogous positions; however, IκBβ is more resistant to targeting by TNF-α (76, 80).

Additionally, when IκBβ is targeted, it is usually degraded with slower kinetics than IκBα (76). The basis of these phenomena is not understood; thus, it is not known whether IκBβ is regulated by an inducible kinase that is distinct from the kinase that regulates IκBα (20).

Although there is a report that IFN-γ may cause low levels of NF-κB activity in peritoneal macrophages after several hours of treatment (56), IFNs do not generally activate NF-κB. In our experiments, IFN-γ by itself did not significantly activate NF-κB. How does TNF-α–IFN-γ cotreatment lead to enhanced activation of NF-κB? First, we show that the synergistic induction of NF-κB involves a switch from targeting IκBα for degradation to targeting IκBβ. Although both PC12 and EA.hy926 cells exhibit enhanced activation of NF-κB and both show the IκBα-to-IκBβ switch mechanism, there are cell type-specific differences in response to TNF-α–IFN-γ cotreatment. In EA.hy926 cells, cytokine cotreatment enhances IκBα degradation and targets IκBβ for degradation, which amplifies NF-κB activation. The earlier and stronger degradation of IκBα which leads to enhanced activation of NF-κB in EA.hy926 cells was not observed in PC12 cells. Second, we show that the synergistic activation of NF-κB is independent of protein synthesis; therefore, this activation cannot be explained by increased synthesis of TNF-α or IFN-γ receptors. This suggests that the activation of signal transduction pathways initiated by IFN-γ control the response. Third, we implicate PTK activity in the synergistic activation of NF-κB by TNF-α–IFN-γ cotreatment in PC12 cells.

Of particular interest were the different cellular responses to the PTK inhibitor genistein. Genistein was originally characterized and is commonly used as a PTK inhibitor (2), although it has other biological activities (reviewed in reference 77). Genistein blocked the IκBβ-dependent mechanism in PC12 cells and, as predicted, blocked the second wave of the NF-κB biphasic response but did not block the early IκBα-controlled response. This implies that a genistein-sensitive PTK is not involved in the ability of TNF-α to lead to the phosphorylation of IκBα but may be required for IFN-γ to induce IκBβ phosphorylation. Although there are several possible mechanisms, it is conceivable that a member of the IFN-sensitive JAKs (which is sensitive to genistein) could be a component of the signaling pathway in PC12 cells. Curiously, the TNF-α–IFN-γ-induced NF-κB activity in EA.hy926 cells was not blocked by genistein but was enhanced under these conditions. Consistent with our data, Tiisala et al. demonstrated that genistein enhances the TNF-α-induced cell surface expression of the κB-dependent gene for ICAM-1 in EA.hy926 cells (77). This indicates a different mechanism of control in these cells, in which PTK activity may normally inhibit the synergistic induction process. Recently, Singh and coworkers showed that tyrosine phosphorylation of IκBα functions negatively to feed back and abrogate the TNF-α activation of NF-κB; therefore, this mechanism may explain the results obtained with EA.hy926 cells (73). Alternatively, the signal transduction cascade initiated by IFN-γ may be negatively controlled by tyrosine kinase activity. In both of these scenarios, IFN-γ may potentiate the activity of a single IκB kinase to induce phosphorylation of IκBα and IκBβ. This would support a model whereby the "strength" of the signal directed to a single kinase determines whether IκBβ is targeted for degradation. The strength of the signal could depend on the concentration or specific activity of the inducer(s), as well as the cell type or particular inducer(s) used. For instance, the initial characterization of NF-κB inducers which target IκBβ demonstrated that neither PMA treatment of 70Z/3 pre-B cells nor TNF-α treatment of Jurkat T cells induced IκBβ degradation (76). However, more recent studies show that both PMA and TNF-α target IκBβ in human umbilical vein endothelial cells (43). Thus, cell type-specific responses may control whether or not IκBβ is targeted. In an alternate model, a distinct IκBβ kinase may exist and, by an unknown mechanism, IFN-γ may lead to the activation of this kinase, but only in the presence of signals initiated by TNF-α. These questions can be answered only when more is revealed about the nature and identity of the IκB kinase(s). In either model, the targeting of IκBβ leads to persistent activation of NF-κB. Suyang and colleagues have described a mechanism in which IκBβ can function either as an inhibitor (under nonstimulated conditions) or as a chaperonelike protein (under stimulated conditions) which protects NF-κB from the inhibitory properties of IκBα (75). The latter mechanism is explained by the differential phosphorylation of IκBβ, leading to inhibitory or protective functions.

The biological implications for TNF-α–IFN-γ synergies are extensive and may underlie many important aspects of immune function and inflammatory diseases. Monocytes and macrophages are the primary sources of TNF-α under conditions of inflammation (reviewed in reference 71), and T cells are the primary source of IFN-γ in response to immunological stimuli (reviewed in reference 29). Due to the interactions between these cell types, there is extensive opportunity for TNF-α–IFN-γ synergy to occur. Under these conditions, enhanced NF-κB activation would be important in regulating a number of immunologically significant genes, such as those encoding cell adhesion molecules or cytokines. Since the genes coding for TNF-α and IFN-γ may be regulated by NF-κB (reviewed in reference 6), there is the opportunity for enhanced production of both cytokines under these synergistic conditions. Of particular interest is the observation (Fig. 1B) that suboptimal levels of TNF-α can activate NF-κB in the presence of IFN-γ, indicating that IFN-γ can make cells more susceptible to the stimulatory effects of TNF-α. Additionally, IFN-γ may sensitize cells to other inducers of NF-κB. Consistent with this idea, Murphy et al. have reported that IFN-γ augments the ability of lipopolysaccharide to activate NF-κB and to induce IL-12 p40 gene expression (53). Understanding the mechanisms whereby IFNs signal to modulate NF-κB functional activity is essential for understanding the basic components of immunological and inflammatory processes. The ability to repress such a pathway would have important implications for the inhibition of chronic inflammatory diseases.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI35098 (A.S.B.) and by NIH/NRSA fellowship 1 F32 AG05745-01 (J.L.C.).

We thank William E. Miller for critical reading of the manuscript.

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