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*J Immunol* 2000; 165:4927-4934; doi: 10.4049/jimmunol.165.9.4927
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We and others have reported that IFN-α potentiates the apoptotic effects of TNF through a mechanism that is not understood. Because the nuclear transcription factors NF-κB and AP-1 have recently been reported to mediate anti-apoptosis and cell survival, we hypothesized that IFN-α potentiates the cytotoxic effects of TNF by suppressing TNF-induced activation of NF-κB and AP-1. We tested this hypothesis by pretreating human Jurkat T cells with IFN-α, which blocked TNF-induced activation of NF-κB and AP-1 in a time- and dose-dependent manner as determined by EMSA. IFN-α blocked TNF-induced phosphorylation and degradation of the inhibitor subunit of NF-κB, and suppressed NF-κB and AP-1 activation induced by various other inflammatory stimuli. NF-κB-dependent reporter gene expression activated by TNF, TNFR1, TNF receptor-associated factor 2, and NF-κB-inducing kinase was also abrogated by IFN-α pretreatment. The suppression of NF-κB and AP-1 correlated with the potentiation of TNF-induced cytotoxicity and caspase activation. Overall our results suggest that IFN-α potentiates the apoptotic effects of TNF possibly by suppressing NF-κB and AP-1 activation. The Journal of Immunology, 2000, 165: 4927–4934.

Materials and Methods

Materials

Clinical grade IFN-α (Roferon A) was kindly supplied by Dr. Moshe Talpaz of the M.D. Anderson Cancer Center (Houston, TX). Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of $5 \times 10^7$ U/mg, was kindly provided by Genentech (South San Francisco, CA). Abs against IκBα and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Poly (ADP ribose) polymerase (PARP) Ab was purchased from PharMingen (San Diego, CA). Phospho-IκBα (Ser27) Ab was purchased from New England Biolabs (Beverly, MA). Expression plasmids encoding FLAG-tagged NIK (18) were kindly provided by Dr. D. Wallach (Weizmann Institute of Science, Rehovot, Israel). The expression plasmid encoding myc-tagged TRAF2, NIK, p65, and dominant-negative IκBα have been previously described (19).

Cell lines

In this study, we used Jurkat (T-cells) obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

NF-κB and AP-1 activation assays

To measure NF-κB and AP-1 activation, nuclear extracts from treated cells were subjected to EMSA essentially as described by our laboratory (20).

NF-κB-dependent reporter gene transcription

The effect of IFN-α on TNF-TRAF-2, NIK-, and p65 (transactivation subunit of NF-κB)-induced NF-κB-dependent reporter gene transcription was measured as previously described (19). Human Jurkat T cells were transiently transfected by the calcium phosphate method with 1 ml medium

Received for publication February 17, 2000. Accepted for publication August 3, 2000.

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This research was supported by The Clayton Foundation for Research.

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Abbreviations used in this paper: TRADD, TNF receptor-associated death domain; IκB, inhibitory subunit of NF-κB; TRAF, TNF receptor-associated factor; NIK, NF-κB-inducing kinase; IKK, IκBα kinase; PARP, poly (ADP ribose) polymerase; SEAP, secretory alkaline phosphatase.
containing plasmid DNAs for TRAF2, NIK, or p65 (1 µg each) along with 0.5 µg NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (SEAP) gene. The total final amount of DNA was maintained at 3 µg by the addition of the control plasmid pCMVFLAG1 DNA. Twenty-four hours after transfection, the conditioned medium was removed and assayed for SEAP activity essentially as described by the manufacturer (Clontech, Palo Alto, CA).

In brief, 25 µl medium was mixed with 30 µl of 5× buffer (500 mM Tris (pH 9) and 0.5% BSA) in a total volume of 100 µl in a 96-well plate and incubated at 65°C for 30 min. The plate was chilled on ice for 2 min. Then 50 µl of 1 mM 4-methylumbelliferyl phosphate was added to each well and incubated at 37°C for 2 h. The activity of SEAP was assayed on a 96-well fluorescent plate reader (Fluoroscan II, Lab Systems, Needham Heights, MA) with excitation set at 360 nm and emission at 460 nm. The average number (± SD) of relative fluorescent light units for each transfection was then determined and reported as fold activation with respect to TNF for 30 min. The specificity was established of this reporter system by the fact that assays was shown to be activated by TNF and by overexpression of TNFR.

To determine the effect of IFN-α on IkBa phosphorylation, cytoplasmic extracts were prepared from cells (2 × 10^6 cells/ml) treated with 100 U/ml IFN-α for 72 h and then treated with 0.1 nM TNF for different times. The extracts were then resolved on 10% SDS-PAGE and analyzed by Western blot using Abs against either IkBa or phosphorylated IkBa (21). After electrophoresis, the proteins were detected by chemiluminescence (Amer sham, Arlington Heights, IL).

IKK assay

The IKK assay was performed by a method described (22). Briefly, IKK signalosomes were precipitated by treating 300 µg cytoplasmic extracts with 1 µg anti-IKKα Ab overnight at 4°C (Imgenex, San Diego, CA; catalog no. IMG-136), followed by treatment with 20 µl protein A/G-Sepharose (Pierce, Rockford, IL). After 2 h, the beads were washed three times with lysis buffer and three times with the kinase assay buffer and then resuspended in 20 µl of kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl2, 2 mM DTT, 20 µCi γ-ATP, 10 µM unlabeled ATP, and 2 µg of substrate GST-IκBα (amino acid residues 1–54). After incubation at 30°C for 30 min, reaction was terminated by boiling with 5 µl of 5× SDS sample buffer for 5 min. Finally, the protein was resolved on 10% polyacrylamide gel under reducing conditions, the gel was dried, and the radioactive bands were visualized by PhosphorImager. To determine the total amounts of IKKα and IKKβ in each sample, 60 µg of the cytoplasmic protein were resolved on 7.5% acrylamide gels, electrotransferred to a nitrocellulose membrane, blocked on the membrane with 5% nonfat milk protein for 1 h, and then incubated with either anti-IKKα or anti-IKKβ (IMG-129) Abs (at 1:500 dilution) for 1 h. The membrane was then washed and reacted with HRP-conjugated secondary anti-mouse IgG.
Ab, and finally detected by chemiluminescence (Amersham Pharmacia Biotechnology, Arlington Heights, IL).

Cytotoxicity assay
The TNF-induced cytotoxicity was measured by the MTT assay (21). Briefly, cells (5,000 cells/well) were incubated in the presence or absence of the indicated test sample in a final volume of 0.1 ml for 72 h at 37°C. Thereafter, cell viability was examined by the MTT method. The ODs were measured at 590 nm using the extraction buffer as a blank.

Immunoblot analysis of PARP degradation
TNF-induced apoptosis was examined by proteolytic cleavage of PARP (21). Apoptosis was represented by the cleavage of 116-kDa PARP into an 85-kDa product.

Results and Discussion
In this report we examined the effect of IFN-α on TNF-induced cellular responses. For most studies, Jurkat T cells were used because these cells express both types of TNF receptor, and cellular responses to TNF are well characterized in our laboratory (23). For most studies IFN-α was used; similar results were obtained with IFN-β as well.

IFN-α suppresses TNF-induced NF-κB activation
Jurkat cells were treated with 1, 10, and 100 U/ml of IFN-α for 24, 48, and 72 h, and then activated for NF-κB by 0.1 nM TNF for 30 min and examined for NF-κB activation by EMSA. As shown in Fig. 1, TNF activated NF-κB by 5-fold, and IFN-α pretreatment diminished TNF-induced NF-κB activation in a time- and dose-dependent manner. Maximum suppression was observed when cells were treated with 100 U/ml IFN-α for 72 h. IFN-α alone did not activate NF-κB (Fig. 1). We also noted suppression of TNF-induced NF-κB activation by IFN-β (data not shown), which is
not too surprising considering that the two IFNs share receptor subunits (24).

Activated NF-κB that is inhibited by IFN-α consists of p50 and p65 subunits

Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA (14). To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-activated cells with Ab to either the p50 (NF-κB1) or p65 (Rel A) subunits and then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher m.w. (Fig. 2A), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor such irrelevant Abs as anti-cRel or anti-cyclin D1 affected the mobility of NF-κB. Excess unlabeled NF-κB (100-fold) caused complete disappearance of the band but mutant oligo did not, indicating the specificity of NF-κB.

Almost 24- to 72-h treatment of cells was required to suppress TNF-induced NF-κB activation. Whether IFN-α suppresses TNF-induced NF-κB activation through suppression of synthesis of NF-κB proteins, was examined. As shown in Fig. 2B, treatment of cells with different concentrations of IFN-α for 72 h had no effect on the levels of p50, p65, or IκBα. These results suggest that IFN-α modulates TNF signaling leading to NF-κB activation. However, whether protein synthesis is required for modulation of TNF signaling by IFN-α could not be ascertained from these experiments.

Inhibition of NF-κB activation by IFN-α is not cell type specific

All of the effects of IFN-α described in this report were observed in human Jurkat T cells. Therefore, we also studied whether IFN-α could also block TNF-induced NF-κB activation in myeloid (U-937) cells, epithelial (HeLa) cells, and embryonic kidney (293) and glioma (H4) cells. In these experiments (Fig. 3), IFN-α inhibited TNF-induced NF-κB activation in all cell types, suggesting that this effect is not restricted to T cells.

IFN-α inhibits TNF-dependent phosphorylation and degradation of IκBα

To determine whether inhibition of TNF-induced NF-κB activation was due to inhibition of IκBα degradation, cells were pretreated with 100 U/ml IFN-α for 72 h, exposed to 0.1 nM TNF for different times, and then examined for NF-κB in the nucleus by EMSA and for IκBα in the cytoplasm by Western blot. As shown in Fig. 4A, TNF activated NF-κB in the control cells in a time-dependent manner but had no effect in IFN-α-pretreated cells. TNF induced IκBα degradation in control cells to a maximum reached at 15 min, but in IFN-α-treated cells TNF-induced IκBα degradation was completely suppressed (Fig. 4B). We also examined the hyperphosphorylated form of IκBα by Western blot using Abs that detect only the serine-phosphorylated form of IκBα. The results in Fig. 4C clearly show that TNF induced the phosphorylation of IκBα which could be seen as early as 5 min, and IFN-α completely
suppressed IκBα phosphorylation. Because TNF-induced phosphorylation of IκBα is mediated through IKK-β (25), these results suggest that IFN-α must inhibit IKK-β activation. Therefore, we investigated the effect of IFN-α on TNF-induced IKK-β activation. As shown in Fig. 4D (upper panel), in the immunocomplex kinase assays TNF-activated IKK-β and IFN-α treatment completely suppressed the activation. Under these conditions, IFN-α had no effect on the IKK-α and IKK-β protein levels (middle and lower panels).

IFN-α represses TNF-induced NF-κB-dependent reporter gene expression

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor TNFR1 with TRADD, TRAF2, NIK, and IKK-β, resulting in phosphorylation of IκBα (16, 17). To delineate the site of action of IFN-α in the TNF-signaling pathway leading to NF-κB activation, cells were transfected with TNFR1, TRAF2, NIK, and p65 plasmids, and then NF-κB-dependent SEAP expression was monitored in IFN-α-untreated and -treated cells. As shown in Fig. 5, TNFR1, TRAF2, NIK, and p65 plasmids induced gene expression, and IFN-α suppressed TNFR1-, TRAF2-, and NIK-induced expression but had little effect on p65-induced NF-κB reporter expression. Receptor activator of NF-κB, another NF-κB-inducing receptor that is a member of the TNFR1 family (19), was minimally affected by IFN-α, indicating the specificity. The lack of effect of IFN-α on the NF-κB activation induced by p65 and receptor activator of NF-κB plasmids also indicates that IFN-α treatment does not affect the transfection efficiency. Specificity of the assay results are also indicated by suppression of TNF-induced NF-κB reporter activity by IκBα-dominant negative mutant plasmid. Thus IFN-α must act at a step downstream from NIK. Because NIK is known to activate IKK-β, which in turn phosphorylates IκBα, it appears that IFN-α blocked the
activity of IKK-β, a kinase that phosphorylates IκBα directly. This is in agreement with our results indicated in the previous section that IFN-α inhibits TNF-induced IKK-β activation.

**IFN-α blocks LPS- and ceramide-mediated activation of NF-κB**

NF-κB is also activated by a wide variety of other agents besides TNF, including phorbol ester, LPS, okadaic acid, ceramide, and HIV-tat. However, the signal transduction pathway induced by various activators may differ (26, 27). Therefore, we examined the effect of IFN-α on the activation of NF-κB by these various agents. The results shown in Fig. 6 indicate that IFN-α suppressed the activation of NF-κB induced by LPS and ceramide but had minimal effect on NF-κB activation induced by H$_2$O$_2$, okadaic acid, PMA, or HIV-tat. These results suggest that IFN-α may act at a step where TNF, ceramide, and LPS converge in the signal transduction pathway leading to NF-κB activation.

**IFN-α inhibits TNF-induced AP-1 activation**

TNF is also one of the most potent activators of AP-1 (15). To determine the effect of IFN-α on TNF-induced AP-1 activation, Jurkat cells were treated with 1, 10, and 100 U/ml of IFN-α for 24, 48, and 72 h and then activated for AP-1 by 0.1 nM TNF for 30 min and examined for AP-1 activation by EMSA. As shown in Fig. 7, A–C, TNF activated AP-1 by 4-fold and IFN-α pretreatment abolished TNF-induced AP-1 activation in a time- and dose-dependent manner. Maximum suppression was observed when cells were treated with 100 U/ml IFN-α for 48 h. IFN-α alone did not activate AP-1 (Fig. 7C). We also noted suppression of TNF-induced AP-1 activation by IFN-α (data not shown). As indicated by the supershift analysis, TNF-induced AP-1 consisted of Fos and Jun subunits (Fig. 7D). Nonspecific Abs had no effect on the supershift. Formation of the AP-1 band could be prevented by unlabeled oligonucleotide (Fig. 7D).

Besides TNF, AP-1 is also activated by a wide variety of other agents including phorbol ester, LPS, okadaic acid, ceramide, and HIV-tat. Therefore, we examined the effect of IFN-α on the activation of AP-1 by these various agents. The results shown in Fig. 5E indicate that IFN-α suppressed the activation of AP-1 induced by LPS and H$_2$O$_2$ but had minimal effect on AP-1 activation induced by ceramide, okadaic acid, PMA, or HIV-tat. These results suggest that IFN-α may act at a step where TNF, H$_2$O$_2$, and LPS converge in the signal transduction pathway leading to AP-1 activation. Although H$_2$O$_2$-induced NF-κB was unaffected by IFN-α, H$_2$O$_2$-induced AP-1 was completely suppressed, suggesting a difference in the mechanism of activation of these two transcription factors. This is in agreement with the results of Meyer et al. (28).
Among the cytokines, TNF is one of the most potent inducers of apoptosis. Modulation of TNF-induced apoptosis was also investigated in Jurkat cells. For this, cells were treated with various concentrations of IFN-\(\alpha\) for 72 h, then treated with 10 pM TNF for an additional 72 h, and then examined for cytotoxicity by the MTT method (Fig. 8A). Both IFN-\(\alpha\) and TNF by themselves had minimal effects on the viability of Jurkat cells. However, preexposure to IFN-\(\alpha\) sensitized the cells to TNF-induced cytotoxicity in a dose-dependent manner. Because the cytotoxic effects of TNF are mediated through the activation of caspases, we also examined the effect of IFN-\(\alpha\) on TNF-induced caspase activation. Activated caspase-2, -3, and -7 are known to cleave PARP protein. As shown in Fig. 8B, in untreated cells 0.1 nM TNF induced a partial cleavage of PARP, but in IFN-\(\alpha\)-pretreated cells TNF induced complete cleavage of PARP substrate. These results thus suggest that IFN-\(\alpha\)-pretreatment also sensitizes the cells to TNF-induced caspase activation.

To determine whether IFN-\(\alpha\) also affected TNF receptors in Jurkat cells, we examined the effect of IFN-\(\alpha\) on TNF receptors by receptor-binding assays. TNF bound to Jurkat cells, and this binding was completely unaffected by pretreatment of cells with IFN-\(\alpha\) for 72 h (data not shown). These results suggest that the suppression of NF-\(\kappa\)B and AP-1 by IFN-\(\alpha\) were not due to down-regulation of TNF receptors.

Our results clearly demonstrate that IFN-\(\alpha\) can down-regulate TNF-induced NF-\(\kappa\)B and AP-1, which most likely contributes to the synergistic apoptotic effects of the two cytokines. The suppression of NF-\(\kappa\)B by IFN-\(\alpha\) is mediated through suppression of IkB \(\alpha\) phosphorylation and degradation. Also we show that IFN-\(\alpha\) acts at a step downstream from NIK. Because only IKK-\(\beta\) mediates TNF-induced phosphorylation of IkB \(\alpha\) at positions 32 and 36, we suggest that IFN-\(\alpha\) may suppress NF-\(\kappa\)B activation through suppression of IKK-\(\beta\).

Our results indicate that IFN-\(\alpha\) also blocked TNF-induced AP-1 activation. TRAF2, which is known to bind to TNF receptor through TRADD, is also required for AP-1 (16, 17). The suppression of TRAF2 activity may explain how IFN-\(\alpha\) inhibits AP-1 activation. Are NF-\(\kappa\)B and AP-1 suppressed by IFN-\(\alpha\) by a similar mechanism? Recent studies from our laboratory showed that inducing overexpression of cells with either superoxide dismutase (23) or \(\gamma\)-glutamylcysteine synthetase, a rate-limiting enzyme in the glutathione biosynthesis pathway (30), blocked both NF-\(\kappa\)B and AP-1 activation induced by TNF, indicating a similar mechanism of activation of both transcription factors. Though these results also imply that IFN-\(\alpha\) may suppress these factors by regulating the redox status of the cells, it is unlikely because TNF-induced cytotoxicity, which also requires reactive oxygen intermediates (31), was potentiated by IFN-\(\alpha\) through suppression of reactive oxygen intermediates.

We found that IFN-\(\alpha\) blocked NF-\(\kappa\)B-dependent reporter gene expression. Several genes that are involved in various diseases are regulated by NF-\(\kappa\)B. These include inflammatory cytokines, cyclooxygenase-2, metalloproteinases, urinary plasminogen activator, NO synthase, and cell surface adhesion molecules (32–37). Thus it is possible that IFN-\(\alpha\) mediates its immunosuppressive effects through suppression of NF-\(\kappa\)B-regulated genes. Indeed the suppression by IFN-\(\alpha\) of cyclooxygenase-2, NO synthase, and inflammatory cytokities has been reported (38–42). Because NF-\(\kappa\)B-regulated gene products have also been implicated in tumorigenesis, IFN-\(\alpha\) may prove useful in suppressing tumorigenesis in vitro (43) and in vivo (44). Our results also provide the basis for the synergistic apoptotic effects of TNF and IFN-\(\alpha\).

 Constitutively active NF-\(\kappa\)B has been identified in a variety of tumors, including Hodgkin disease; T cell lymphoma; breast, ovarian, and prostate carcinomas; and a variety of others tumors (45–48). The active form of NF-\(\kappa\)B is required for proliferation of these tumors (46, 47), and its suppression inhibits the growth. Thus it is possible that suppression of NF-\(\kappa\)B and also AP-1 in these tumors by IFN-\(\alpha\) may prove useful in inhibition of their proliferation.

In the murine system, IFN-\(\alpha\) is known to induce a protein p202 which blocks phorbol ester-induced activation of NF-\(\kappa\)B and AP-1 reporter gene expression (49). Because p202 did not decrease the DNA binding of NF-\(\kappa\)B as detected by EMSA (49), it is unlikely that in our system human IFN-\(\alpha\) is suppressing TNF-induced NF-\(\kappa\)B activation through p202 protein. In addition phorbol ester-induced NF-\(\kappa\)B activation in our system was unaffected by pretreatment of cells with IFN-\(\alpha\). Besides, the role of p202-like protein in human system has not been reported.

NF-\(\kappa\)B has been proposed as a reasonable target for new anti-cancer drugs (50). Although adenosinov 1k\(\alpha\)B, an NF-\(\kappa\)B inhibitor, has been used for the treatment of arthritis and tumorigenesis (13, 51), and our data suggest its potential. We believe that the use of IFN-\(\alpha\) to suppress NF-\(\kappa\)B activation is preferable. IFN-\(\alpha\) has...
proven to be safe for human administration (which would be required if Ibev was used), there are no real delivery problems associated with IFN-α.

Overall our results indicate that suppressive effects of IFN-α on NF-κB and AP-1 activation may have therapeutic effects in diseases in which TNF plays a major role. We have shown that this potential lies in IFN-α ability to modulate TNF activation of various transcription factors.

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