The Journal of Immunology
RESEARCH ARTICLE | APRIL 15 2000

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J Immunol (2000) 164 (8): 4277–4285.
https://doi.org/10.4049/jimmunol.164.8.4277

TNF-α Gene Expression in Macrophages: Regulation by NF-κB Is Independent of c-Jun or C/EBPβ[1]

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The interaction of transcription factors is critical in the regulation of gene expression. This study characterized the mechanism by which NF-κB family members interact to regulate the human TNF-α gene. A 120-bp TNF-α promoter-reporter, possessing binding sites for NF-κB (κB3), C/EBPβ (CCAAT/enhancer binding protein β), and c-Jun, was activated by cotransfection of plasmids expressing the wild-type version of each of these transcription factors. Employing adenoviral vectors, dominant-negative versions of NF-κB p65, and c-Jun, but not C/EBPβ, suppressed ($p < 0.05–0.001$) LPS-induced TNF-α secretion in primary human macrophages. Following LPS stimulation, NF-κB p50/p65 heterodimers bound to the κB3 site and c-Jun to the −103 AP-1 site of the TNF-α promoter. By transient transfection, NF-κB p65 and p50 synergistically activated the TNF-α promoter. In contrast, no synergy was observed between NF-κB p65, with or without NF-κB p50, and c-Jun or C/EBPβ, even in the presence of the coactivator p300. The contribution of the upstream κB binding sites was also examined. Following LPS stimulation, the κB1 site bound both NF-κB p50/p65 heterodimers and p50 homodimers. The binding by NF-κB p50 homodimers to the κB1, but not to the κB3, site contributed to the inability of macrophages to respond to a second LPS challenge. In summary, adjacent κB3 and AP-1 sites in the human TNF-α promoter contribute independently to LPS-induced activation. Although both the κB1 and κB3 sites bound transcriptionally active NF-κB p50/p65 heterodimers, only the κB1 site contributed to down-regulation by NF-κB p50 homodimers. ***The Journal of Immunology*****, 2000, 164: 4277–4285.**

Tumor necrosis factor-α is an important mediator of inflammation and it contributes to the pathogenesis of a variety of conditions, including rheumatoid arthritis, Crohn's disease, and many types of infections (1, 2). Although various cell types are capable of producing TNF-α, monocytes and macrophages are its principal source (1, 3). The regulation of TNF-α gene expression in myelomonocytic cells is complex and stimulus-dependent. Binding sites for multiple transcription factors, including NF-κB, CCAAT/enhancer binding protein β (C/EBPβ),[3] and c-Jun, have been identified in the proximal promoter of the TNF-α gene (4–7). By deletion and mutational analysis, each of these sites has been shown to be capable of contributing to the activation of the TNF-α promoter in macrophages (4–8). Each of these transcription factors may be activated via the extracellular signal-related kinase-1 and -2 and c-Jun N-terminal kinase pathways following LPS stimulation of macrophages (9–13). However, ectopically expressed wild-type or dominant-negative (DN) versions of these transcription factors have not been employed to document their importance in the regulation of the TNF-α gene in primary human macrophages.

Transcriptional activation occurs through the interaction of transcription factors binding to the promoters of specific genes. Members of the NF-κB family interact with one another to activate many genes, particularly those involved with the inflammatory response (reviewed in Ref. 14). The best-characterized interaction is that of the transcriptionally active NF-κB p65 (RelA) with NF-κB p50 (NF-κB1), which does not possess a transactivation domain (14). NF-κB p65 and NF-κB p50 homodimers have also been characterized (15, 16). Other members of the NF-κB family, including c-Rel, NF-κB2 (p52/p100), and RelB, are also capable of interacting through their Rel homology domains (14). LPS stimulation induces activation of multiple forms of NF-κB, including the classical p50/p65 heterodimers, p50 homodimers, and c-Rel/p65 heterodimers (15, 16). How NF-κB family members interact to regulate human TNF-α gene expression is not fully characterized.

In addition, the potential interactions of other transcription factors with NF-κB in the activation of the human TNF-α promoter have not been fully explored. NF-κB and C/EBPβ interact through their Rel and b-Zip domains to synergistically activate other cytokine genes including IL-6, IL-8, and IL-12 p40 (17–21). NF-κB has also been shown to interact with c-Jun and to synergistically activate the HIV 5′ long terminal repeat (17, 18). However, little is known about the interaction of these transcription factors in the activation of the TNF-α gene in macrophages. Our prior studies documented that C/EBPβ and c-Jun interacted following PMA/LPS treatment of U937 myelomonocytic cells to activate TNF-α gene expression (22). ATF-2/Jun and NF-ATp were shown to interact in the regulation of the TNF-α gene in activated T cells (23).

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Received for publication September 15, 1999. Accepted for publication February 2, 2000.

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[1] This work supported in part by Grants AR43642, AR62229, and AR30692 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

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[3] Abbreviations used in this paper: C/EBPβ, CCAAT/enhancer binding protein β; DN, dominant negative; Ad, adenovirus; AdGFP, adenovirus green fluorescence protein; RAW, RAW 264.7 cells; moi, multiplicity of infection.

0022-1767/00/$02.00

This study was performed to characterize the interactions between NF-κB p65 and p50, and C/EBPβ and c-Jun in the LPS-induced activation of the human TNF-α gene in macrophages. Activation was mediated by binding of NF-κB p65/p50 heterodimers to the κB1 and κB3 sites. c-Jun also contributed to LPS-induced TNF-α activation by binding to AP-1 site centered −103 bp 5′ of the transcription start site. NF-κB and c-Jun acted independently, and no synergistic interactions were detected. C/EBPβ does not contribute to LPS-induced TNF-α activation in macrophages. Finally, following LPS-induced activation, NF-κB p50 homodimers were strongly expressed and contributed to LPS-induced tolerance by binding to the κB1, but not to the κB3, binding site in the human TNF-α promoter.

Materials and Methods

Materials

DMEM, RPMI 1640, FCS, penicillin, streptomycin, L-glutamine, and Lipofectamine reagent were obtained from Life Technologies (Gaithersburg, MD). LPS (from *Escherichia coli* serotype 0127:B8), PMA, ATP, Luciferin, and poly(dI-dC) were purchased from Sigma (St. Louis, MO). Rabbit polyclonal Abs specific for c-Jun, C/EBPβ, and the p65 and p50 subunits of NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Reagents for the quantitation of protein were obtained from Bio-Rad (Hercules, CA). [γ-^{32}P]dCTP (3000 Ci/mmol) (Easytides) was from NEN Life Science (Boston, MA).

Cells and cell culture

RAW 264.7 (RAW) cells, a murine macrophage cell line, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. THP-1 human monocytic cells were obtained from ATCC and maintained in RPMI 1640 medium with 10% FCS. Human blood mononuclear cells were isolated by Histopaque-1077 (Sigma) from commercially obtained buffy coats, from which monocytes were isolated by countercurrent elutriation (Beckman Avanti TM J-251 centrifuge, Palo Alto, CA).

Plasmid vector constructs

The TNF-α promoter-reporter constructs containing 120 or 615 bp 5′ of the transcription start site, linked to a luciferase gene, and the expression vectors encoding wild-type and DN C/EBPβ (CMV-C/EBPβ and CMV-DNC/EBPβ, respectively) have been described (5, 22, 24). The vectors expressing wild-type NF-κB p65 and p50 were regulated by the CMV promoter (17, 25). The DN version of NF-κB p65 (NF-κB DN p65) contains a C-terminal truncation possessing the Rel homology domain with the transactivation domain deleted (17, 25). The pCMVc-Jun and pCMVTAM67 plasmids express the wild-type and transactivation domain deletion mutant of c-Jun (22).

Transfection and luciferase assay

RAW cells were passed into 6-well plates 1 day before the transfection at the concentration 5×10^5/well. Transfection was performed employing 7 μl of Lipofectamine, according to the protocol provided by the company, keeping the total plasmid concentration constant (3 μg/transfection). After transfection, cells were placed in complete medium for 16–18 h. LPS (10 μg/ml, or as indicated in the individual experiments) was added for 18 h. Cells were harvested, washed, and lysed by freeze-thawing three times, and luciferase activity determined on cell lysates as previously described (5) using a Monolight 2010 luminometer (Analytical Luminesence Laboratory, San Diego, CA). Promoter activities were expressed as relative light units (RLU), normalized for the total protein in each extract. Fold activation was determined by dividing the experimental RLU/μg by that for the control.

Nuclear extracts and EMSA

Nuclear extracts were prepared from 5 to 10×10^6 RAW cells, THP-1 cells, or monocyte differentiated macrophages following LPS or control stimulation, as previously described (5). Oligonucleotide probes spanning the following regions of the TNF-α promoter were employed: −100 to −74 bp (−100/−74), possessing the κB3 (−98 to −89) and the C/EBPβ binding sites; −115 to −98 bp (−115/−98), possessing the AP-1 binding site; −598 to −589 bp (−598/−589) representing the κB1 binding site; and −217 to −200 bp (−217/−200) representing the κB2 binding site (7, 8, 26). In addition, oligonucleotides representing the C/EBPβ binding site of the IL-6 promoter and an AP-1 binding site from the collagenase promoter have been previously described (22). DNA binding reactions were performed by incubation for 20 min at room temperature in a final volume of 20 μl. The reaction mixture contained 100 mmol/L NaCl, 20 mmol/L HEPES, 1 mmol/L EDTA, 4% glycerol, 5%(w/v) Ficoll, 0.25 μg BSA, 1 μg poly(dI-dC), 1 ng of ^{32}P-labeled oligonucleotide, and 5–10 μg of nuclear extract (5, 27). Protein:DNA complexes were separated from free probe by electrophoresis on 5–6% polyacrylamide gels in 0.5× TBE at 160 V for 2–3 h. Gels were dried onto Whatman 3 M paper (Whatman, Maidstone, U.K.) and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). For Ab supershift/inhibition assays, 1–2 μl rabbit monospecific Abs were incubated with the protein extract on ice for 30 min before addition of labeled oligonucleotide to the binding reaction.

TNF-α secretion and quantitation

Human TNF-α was measured by an ELISA using commercially available reagents (R&D Systems, Minneapolis, MN) as described previously (22). Murine TNF-α-mediated cytotoxicity was determined using TNF-sensitive L929 fibroblasts, as previously described (28).

Infection of human macrophages with adenovirus

The freshly isolated human peripheral monocytes were differentiated with 10% FCS alone or with recombinant human M-CSF (100 ng/ml; Genetics Institute, Cambridge, MA) for 7 or 14 days. Adenoviral (Ad) vectors expressing the DN versions of c-Jun, NF-κB p65, and C/EBPβ, AdTAM-67, AdNF-κBDNp65 (29), and AdDNC/EBPβ, respectively, and the control vector expressing green fluorescence protein (AdGFP), were employed (30, 31). Each DN lacked the transactivation domain but retained the ability to dimerize and bind DNA (data not shown). Viruses were propagated in the 293 cell line and purified by ultracentrifugation through cesium chloride gradients (30, 31). Titers of viral stocks were determined by plaque assay in 293 cells. Macrophages were exposed to virus at 100 and 200 multiplicity of infection (moi) for 2 h in serum-free RPMI 1640 medium, followed by addition of serum to the concentration of 5% overnight. The infected cells were washed with PBS, and medium containing 10% FCS and antibiotics was added for 48–72 h. LPS (10 μg/ml) was added for an additional 18 h. Supernatants were harvested for TNF-α ELISA and the cell number was estimated by the MTT method (32), which were used to normalize the TNF-α concentration in each experiment.

Results

Activation of the proximal TNF-α promoter in macrophages

Binding sites for AP-1, NF-κB, and C/EBPβ are present in the proximal promoter of the human TNF-α gene located −115 to −74 bp 5′ of the transcription start site. We employed a −120-bp TNF-α luciferase promoter-reporter, which was highly responsive to LPS in myelomonocytic cells, to examine the relative contribution of each of these transcription factors to TNF-α promoter activation in the RAW macrophage cell line. Expression of NF-κB p65, C/EBPβ, and c-Jun each resulted in significant ($p < 0.01$, 0.05, and 0.05, respectively) activation of the TNF-α promoter (Fig. 1). Cotransfection of the vector expressing NF-κB p65 with the TNF-α promoter-reporter consistently resulted in 2- to 3-fold greater activation compared with vectors expressing C/EBPβ or c-Jun (Fig. 1). As expected, coexpression of either NF-κB p50 or the coactivator CBP alone did not activate the TNF-α promoter. These observations indicate that each of the transcription factors tested was capable of activating the human TNF-α promoter in this macrophage line.

Inhibition of NF-κB p65 and c-Jun suppresses LPS-induced TNF-α secretion in primary macrophages

To determine the contribution of these transcription factors to the LPS-induced expression of the cellular TNF-α gene in primary human macrophages, adenoviral vectors expressing DN versions of NF-κB p65, C/EBPβ, and c-Jun were employed at 100 and 200 moi. Western blots demonstrated that the expression of each of the DNs in macrophages was comparable (data not shown). At an moi

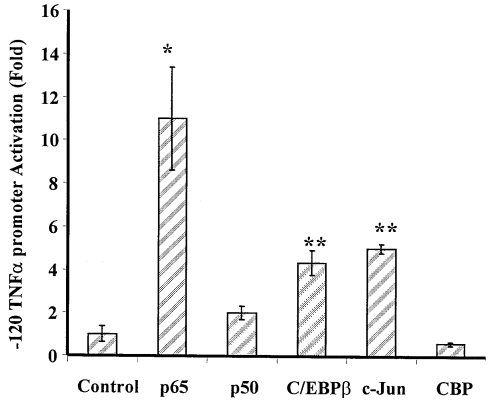

FIGURE 1. Activation of the proximal human TNF-α promoter by NF-κB p65, C/EBPβ, and c-Jun in a macrophage cell line**.** RAW cells were transfected with -120 TNF-α luciferase promoter-reporter (1 μg/transfection) and plasmids expressing NF-κB p65 (p65), NF-κB p50 (p50), C/EBPβ, c-Jun, and CBP (each 1 μg/transfection). Cells were harvested, and cell lysates were obtained by freeze-thaw. Luciferase activity was measured as relative light units (RLU), which was corrected for the total protein in each lysate. The results are presented as fold activation compared with control vector alone. The results presented are the mean $\pm$ 1 SE of an experiment performed in duplicate, which is representative of four experiments. *, $p < 0.01$; **, $p < 0.05$, compared with control vector by *t* test for matched pairs.

of 200, infection of primary human macrophages with AdNF-κBDNp65 resulted in 66% ($p < 0.001$) suppression of LPS-induced TNF-α secretion in primary human macrophages compared the control vector, AdGFP (Fig. 2). In addition, a 43% reduction ($p < 0.05$) of LPS-induced TNF-α secretion was observed in cells transduced with the AdTAM-67 (DN c-Jun) at an moi of 200 (Fig. 2). No reduction of LPS-induced TNF-α secretion was observed in the presence of the DN C/EBPβ in primary human macrophages. These observations indicate that in differentiated macrophages, NF-κB activation was the major contributor to LPS-induced activation. Inhibition of c-Jun resulted in a modest reduction of TNF-α secretion, whereas inhibition of C/EBPβ activity was without effect.

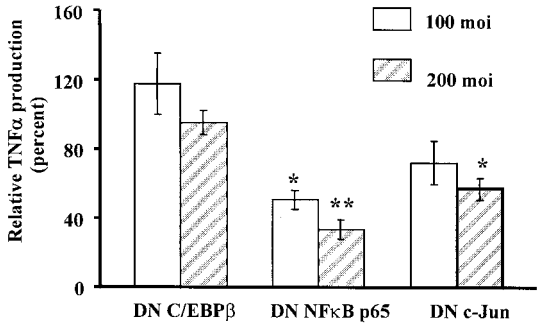

FIGURE 2. Suppression of LPS-induced TNF-α secretion by human macrophages transduced with adenovirus vectors expressing DN versions of NF-κB p65 and c-Jun, but not C/EBPβ. Human peripheral monocytes were differentiated into macrophages in 10% FCS plus 100 ng/ml recombinant human M-CSF for 7 days. The cells were infected with AdNF-κBDNp65, AdTAM-67, AdDNC/EBPβ, or the control AdGFP at 100 and 200 moi. LPS (10 μg/ml) was added 48–72 h after the infection, and the supernatants were harvested 18 h later. TNF-α secretion was determined by ELISA and corrected for the number of cells in each well at the end of the culture as determined by MTT. The results presented are relative TNF-α production compared with control adenovirus (AdGFP), which was employed in each experiment. The results are presented as the mean $\pm$ 1 SE of four experiments each performed in triplicate. ** and *, Significant decreases ($p < 0.001$ and $p < 0.05$, respectively) compared with the control AdGFP (*t* test for matched pairs).

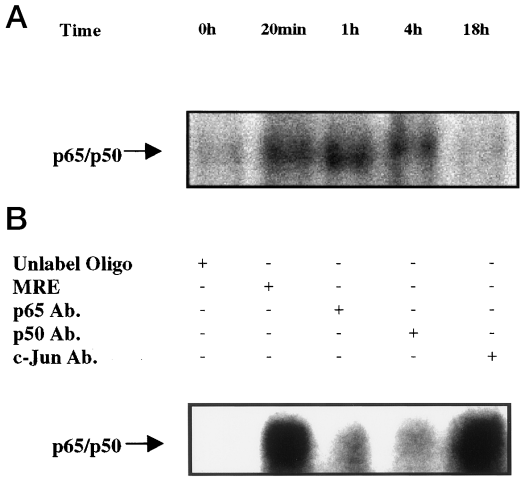

FIGURE 3. LPS-induced binding of NF-κB to the κB3 binding site in the human TNF-α promoter. *A*, Time course after LPS stimulation. EMSAs were performed with nuclear extracts harvested from RAW cells that were unstimulated or treated with LPS (10 ng/ml) for 20 min or 1, 4, or 18 h. EMSAs were performed with a ^{32}P-labeled oligonucleotide representing -100 to -74 bp of the TNF-α promoter containing the κB3 and the C/EBPβ binding sites. The NF-κB complex is identified on the *left* of the figure. *B*, NF-κB p50/p65 heterodimers bind to the TNF-α κB3 site. The nuclear extracts from RAW cells stimulated by LPS for 1 h were employed. Binding of the NF-κB complex to the radiolabeled oligonucleotide was inhibited by excess (100$\times$) unlabeled $-100/-74$ (Unlabel Oligo), but not by an irrelevant oligonucleotide (MRE). The NF-κB complex was diminished by monospecific Abs to NF-κB p65 (p65 Ab.) and NF-κB p5 (p50 Ab.), but not by Abs c-Jun (c-Jun Ab.). The anti-c-Jun served as the control because no AP-1 binding site is present in the $-100/-74$-bp oligonucleotide.

LPS-induced binding of NF-κB and c-Jun to the TNF-α promoter

To define the potential mechanisms for the contribution of these transcription factors to the LPS-induced activation of the TNF-α promoter, EMSAs were performed with nuclear extracts from RAW cells that were unstimulated or treated with LPS. We previously demonstrated that the $-100/-74$ oligonucleotide binds ectopically expressed C/EBPβ (5, 22) and it possesses the κB3 binding site (8). Constitutively, there was minimal binding of NF-κB to this oligonucleotide (Fig. 3*A*). Following LPS treatment, binding increased at 20 min, peaked at 1 h, diminished at 4 h, and returned to basal levels by 18 h (Fig. 3*A*). As determined by inhibition/supershift with monospecific Abs, this complex consisted of NF-κB p50 and p65 heterodimers (Fig. 3*B*). Other NF-κB binding factors cannot be excluded because Abs to p65 and p50 did not result in the disappearance of the entire complex. At no time point was binding of C/EBPβ to the $-100/-74$ oligonucleotide observed employing the nuclear extracts from LPS-treated cells (data not shown).

The binding of c-Jun to the proximal TNF-α promoter was examined with the $-115/-98$ oligonucleotide. Enhanced binding in the nuclear extracts from LPS-stimulated cells was noted at 20 min through 4 h (Fig. 4*A*). This band was identified as c-Jun by inhibition of binding with monospecific Ab (Fig. 4*B*). The anti-C/EBPβ was employed as a control and was without effect on the observed complex. These observations indicate that NF-κB p50/

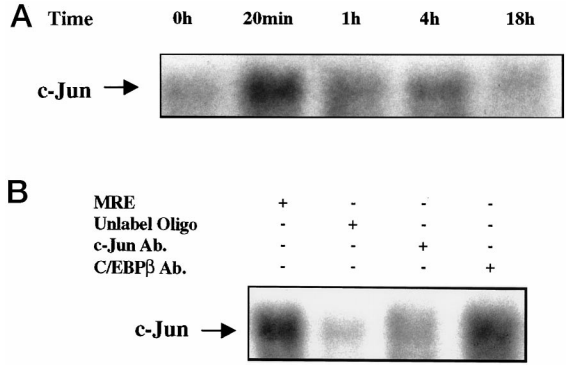

FIGURE 4. LPS-induced binding of c-Jun to the TNF-α promoter. *A*, Time course after LPS stimulation. EMSAs were performed with nuclear extracts harvested from RAW cells that were unstimulated or treated with LPS (10 ng/ml) for 20 min or 1, 4, or 18 h. EMSAs were performed with a ^{32}P-labeled oligonucleotide possessing the AP-1 binding site (−115/−98) of the TNF-α promoter. The c-Jun complex and the unbound oligonucleotides are identified on the *left* of the figure. *B*, Identification of c-Jun employing a monospecific Ab. This experiment was performed employing nuclear extracts from RAW cells stimulated by LPS for 1 h, as described in Fig. 3*B*. The ^{32}P-labeled oligonucleotide −115/−98 was used. The binding of c-Jun to the radiolabeled oligonucleotide was inhibited by excess (100×) unlabeled −115/−98 and by monospecific anti-c-Jun (c-Jun Ab.) but not by an irrelevant oligonucleotide (MRE) or the control anti-C/EBPβ Ab (C/EBPβ Ab.).

p65 heterodimers and c-Jun bind to the proximal promoter of the TNF-α gene following stimulation of macrophages with LPS.

Lack of interaction between transcription factors in the proximal TNF-α promoter

Because NF-κB and c-Jun bind to the proximal TNF-α promoter following LPS stimulation and because inhibition of NF-κB p65 and c-Jun each partially inhibited LPS-induced TNF-α secretion in primary macrophages, studies were performed to determine whether NF-κB functionally interacted with either c-Jun or C/EBPβ to activate the TNF-α gene. Coexpression of either NF-κB p65 alone or NF-κB p65 plus p50 together with either c-Jun or C/EBPβ did not result in synergistic activation of the −120 TNF-α promoter-reporter (Fig. 5, *A* and *B*). There was a consistent trend with the coexpression of C/EBPβ for less activation compared with that observed with NF-κB alone. This observation suggested that C/EBPβ may interfere with activation by NF-κB. To examine this further, the transcriptionally inactive DN C/EBPβ was cotransfected with NF-κB. Expression of the DN C/EBPβ resulted in suppression of TNF-α promoter activation induced by coexpression of NF-κB (Fig. 5*C*).

The coactivator p300 has been shown to cooperate with NF-κB, and c-Jun in the activation of a variety of genes (33, 34). Therefore, p300 was coexpressed with NF-κB p65 and with c-Jun to determine the effect on activation of the −120 TNF-α promoter. A greater than additive activation of the TNF-α promoter was observed when p300 was coexpressed with c-Jun (Fig. 6). In contrast, no enhancement of activation by NF-κB p65 expressed alone or together with c-Jun was observed in the presence of p300 (Fig. 6). Cotransfection of the plasmid expressing wild-type c-Jun with the −95 TNF-α promoter reporter, which possesses the −63-bp AP-1 site, but not the −103-bp AP-1 or the κB3 binding sites, failed to activate the TNF-α promoter (data not shown). These observations suggest that the affect of c-Jun was mediated by the −103-bp AP-1 binding site.

Regulation of the TNF-α promoter by NF-κB p50 and p65

Studies were performed to determine how NF-κB p65 and p50 might interact with the TNF-α promoter to regulate gene expression. NF-κB p65/p50 heterodimers, but not p65 or p50 homodimers, bound the −100/−74 oligonucleotide following LPS stimulation (Fig. 3). This may be due to the inability of the κB3 site to bind other NF-κB species or to the absence NF-κB species other than p65/p50 heterodimers. Functional studies were performed by increasing the concentration of the NF-κB p50 expression plasmid, keeping the p65 constant, employing TNF-α promoter constructs possessing either the κB3 binding site alone (−120 TNF-α promoter-reporter), or one containing the κB1, κB2, and κB3 binding sites (−615 TNF-α promoter-reporter). With the

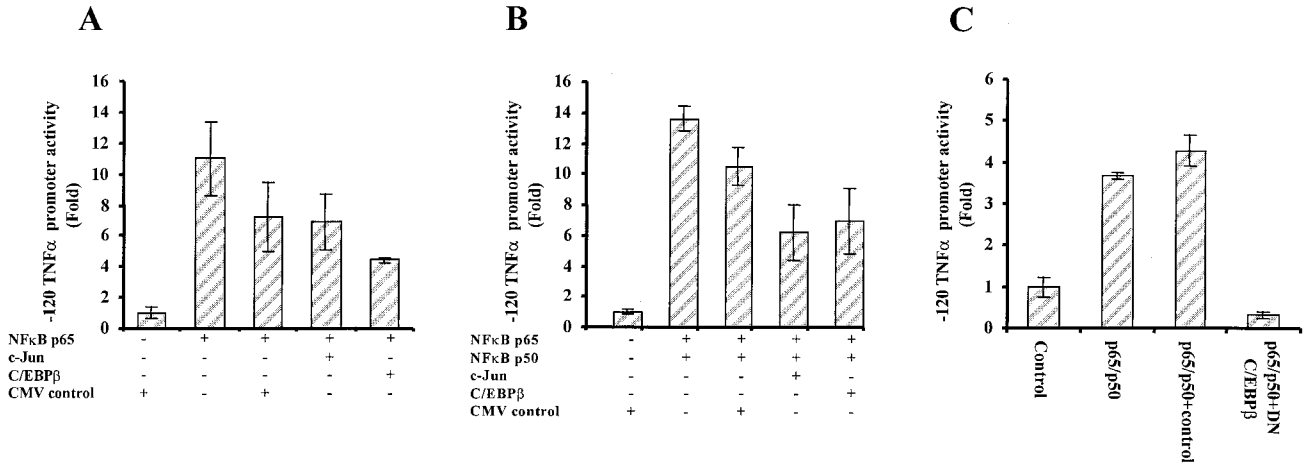

FIGURE 5. Lack of synergy between NF-κB and c-Jun or C/EBPβ in the activation of the human TNF-α promoter. Plasmids expressing NF-κB p65 (1.0 μg/transfection)(*A*) or NF-κB p65 plus p50 (0.5 μg of each/transfection) (*B*) were cotransfected alone or with the plasmids expressing c-Jun (1.0 μg), C/EBPβ (1 μg), or the control plasmid, together with the −120 TNF-α promoter-report (1 μg). The total plasmid concentration was kept constant (3 μg/transfection). The results of each panel presented are the mean ± SE of an experiment performed in duplicate, which was representative of four experiments. *C*, The DN C/EBPβ inhibits NF-κB-induced TNF-α promoter activation. The −120 TNF-α promoter-reporter (1 μg) was cotransfected with NF-κB p65 (0.5 μg) plus p50 (0.5 μg) plus a CMV control plasmid (1 μg), or one expressing the DN C/EBPβ (1 μg). The results are presented as the mean ± 1 SE of one experiment performed in duplicate, which is representative of two experiments.

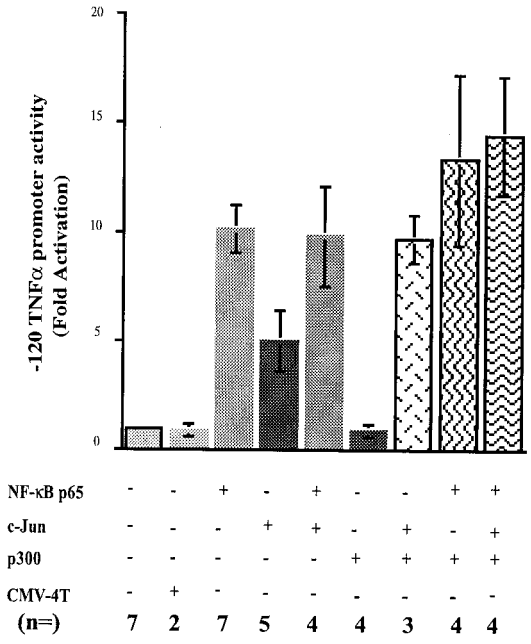

FIGURE 6. Lack of synergy between NF-κB p65 and c-Jun plus p300 in the activation of the human TNF-α promoter. RAW cells were transfected with −120 TNF-α-luciferase promoter-reporter (1 μg/transfection) and plasmids expressing NF-κB p65, c-Jun, p300, or control vector (CMV-control). The total concentration of plasmid was kept constant in each experiment (3 μg/transfection). The results are reported as fold activation compared with the control transfection. The number of experiments performed with each combination is indicated at the bottom of the figure (n =). The results presented are the mean ± 1 SE for all of the experiments performed.

−120 TNF-α promoter-reporter (Fig. 7*A*), increasing concentrations of NF-κB p50 resulted in increased TNF-α activation ($p <$ 0.05 at 1:3 and 1:4). Western blot analysis of lysates from 293 transfected cells demonstrated that NF-κB p65 and p50 were comparably expressed (data not shown). In contrast, with the −615 TNF-α promoter-reporter, although the coexpression of NF-κB p50 resulted in increased activation at ratios of 1:1 to 1:3, at the 1:4 ratio promoter activation was significantly ($p < 0.05$) reduced (Fig. 7*B*). These observations suggest that the κB1 or κB2 binding sites might bind to p50 homodimers, which may be formed at the higher ratios, thus resulting in suppression.

Therefore we examined the ability of NF-κB to bind to the κB1 and κB2 binding sites employing nuclear extracts from LPS-stimulated RAW cells. No LPS-induced binding to the κB2 site was observed (data not shown), consistent with previous observations (7). In contrast, NF-κB in the nuclear extracts bound strongly to the κB1 site. The constitutive binding of p50 homodimers was greater than the p65/p50 heterodimers (Fig. 8*A*). Following LPS stimulation there was a marked increase of the heterodimers at 20 min and 1 h. By densitometry, NF-κB p65/p50 heterodimer binding to κB1 was 2- to 3-fold greater than the p50 homodimers. By 4 h, binding of the NF-κB p50 homodimers was greatly increased, and by 18 h it was the dominant species present in the nuclear extracts (Fig. 8*A*). At 18 h NF-κB p50 homodimer binding to κB1 was >20-fold greater than the binding of the p50/p65 heterodimers. These binding activities were very similar to those observed with the Ig/HIV consensus oligonucleotide (Fig. 8*B*). The specific bands possessing the NF-κB p50 homodimers and the p65/p50 heterodimers present in the nuclear extracts at 0, 1, and 4 h were identified by monospecific Abs (Fig. 8*C*).

The κB1 site contributes to LPS-induced tolerance

Further studies were performed to determine whether the binding of NF-κB p50 homodimers to the κB1 site might contribute to LPS-induced tolerance by pretreatment with LPS 18 h before LPS stimulation. Pretreatment of macrophages with LPS (10 ng/ml), but not PMA, resulted in a marked shift in the ratio of NF-κB p50 homodimers to heterodimers 1 h following LPS (10 μg/ml) stimulation (Fig. 9*A*). Prior treatment with LPS did not effect heterodimer binding to κB3 (data not shown). Similar observations were made with primary human monocyte differentiated macrophages and with human THP-1 monocytic cells differentiated in the presence of vitamin D_3 (data not shown). To determine the functional significance of the NF-κB p50 homodimers, transient transfection assays were performed employing the −120 and the −615 TNF-α promoter-reporters. Employing the −615 TNF-α promoter, pretreatment with LPS resulted in suppression ($p <$

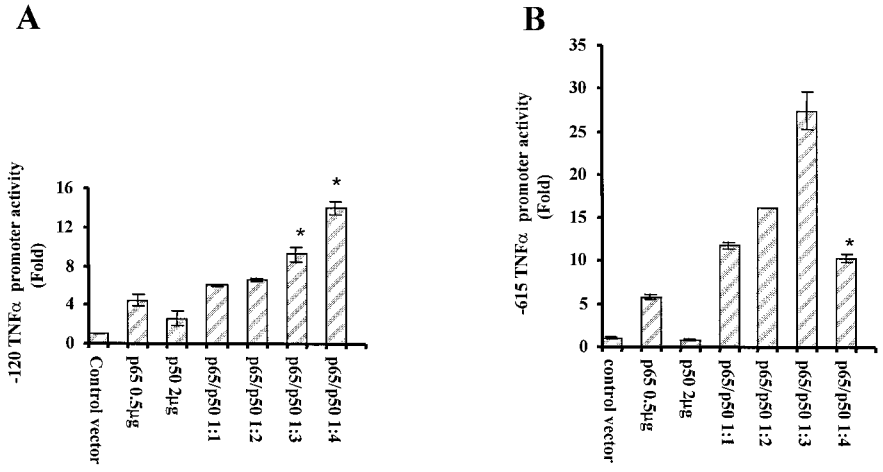

FIGURE 7. Differential regulation of TNF-α promoter-reporter activation by NF-κB p65 and p50. Plasmids expressing NF-κB p65 (p65, 0.5 μg), or NF-κB p50 (2.0 μg) alone, or NF-κB p65 plus increasing concentrations of p50 which resulted in ratios of p65/p50 from 1:1 (0.5:0.5 μg) to 1:4 (0.5:2.0 μg) were cotransfected together with the −120 TNF-α promoter-reporter (1 μg) (*A*) or −615 TNF-α promoter-reporter (1 μg) (*B*) into RAW cells. The total plasmid concentration was kept constant (3 μg). The results of each panel presented are the mean ± SE of an experiment performed in duplicate, which is representative of three and four experiments in *A* and *B*, respectively. The ∗ in *A* indicates a significant difference ($p < 0.05$) compared with p65 alone, and in *B* compared with p65/p50 at 1:3.

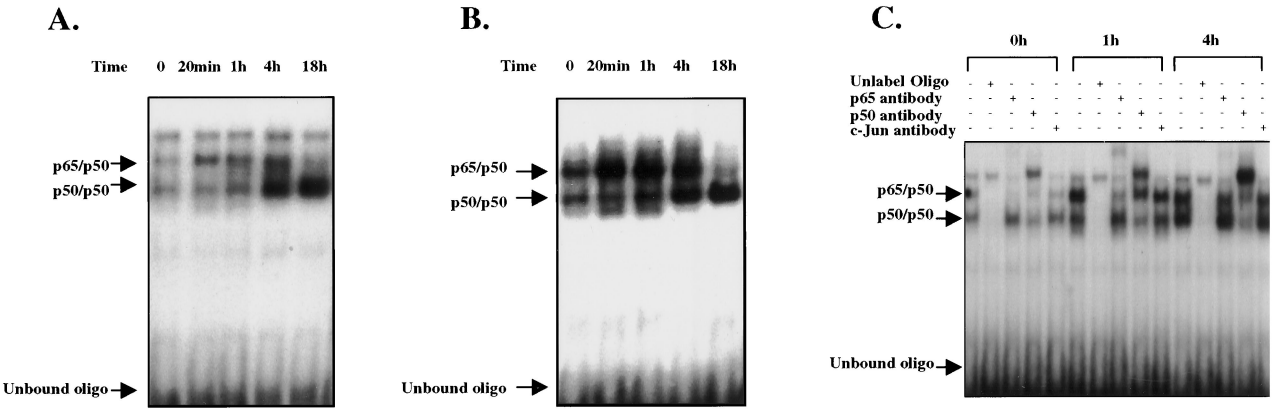

FIGURE 8. Binding of different NF-κB complexes to the κB1 site of TNF-α promoter and Ig/HIV NF-κB oligonucleotide. *A*. The κB1 site of the human TNF-α promoter binds both NF-κB p65/p50 heterodimers and p50 homodimers. EMSA was performed with nuclear extracts from RAW cells that were unstimulated or treated with LPS (10 ng/ml) for the time indicated at the *top* of the figure. The ^{32}P-labeled oligonucleotide represented −598 to −589 bp of the human TNF-α promoter. The location of the NF-κB p65/p50 heterodimers and the p50 homodimers are indicated on the *left* of the figure. *B*, Ig/HIV NF-κB oligonucleotide also binds NF-κB p65/p50 heterodimers and p50 homodimers. EMSA was performed as described in *A* except the ^{32}P-labeled Ig/HIV NF-κB oligonucleotide was employed. *C*, Identification of LPS-induced NF-κB p50 and p65 containing complexes binding to the TNF-α κB1 binding site. The nuclear extracts obtained from RAW cells unstimulated or stimulated by LPS (10 ng/ml) for 1 h and 4 h were employed. The experiments were performed as described in Fig. 3*B*. Rabbit monospecific Abs to NF-κB p65 (p65 Ab) or p50 (p50 Ab) were incubated with the protein extract on ice for 30 min before addition of ^{32}P-labeled κB1 oligonucleotide. The NF-κB complexes were inhibited by excess (100×) unlabeled −598/−589 oligonucleotide (Unlabel Oligo) and Abs to NF-κB p65 and p50, but not c-Jun.

0.02) of subsequent promoter activation (Fig. 9*B*). In contrast, no suppression of subsequent LPS-induced TNF-α promoter activation was observed employing the −120 TNF-α promoter (Fig. 9*C*). When RAW cell supernatants were examined, pretreatment with LPS resulted in suppression of TNF-α activity in the culture supernatants (Fig. 9*D*). These observations indicate that the NF-κB p50 homodimers, present in the nuclear extracts at 18 h, contributed to LPS-induced tolerance by binding to the κB1 binding site in the proximal human TNF-α promoter.

Discussion

This study contributes two novel observations concerning the activation of the human TNF-α gene. First, LPS-induced TNF-α secretion by primary human macrophages was independently regulated by both NF-κB and c-Jun. The contribution of NF-κB was recently documented by inhibition of LPS-induced TNF-α secretion in primary macrophages following infection with an adenoviral vector expressing IκBα (Ref. 35 and data not presented). Inhibition by IκBα suggests that complexes containing NF-κB p65, c-Rel, or RelB may be involved, since each possesses a transactivation domain and binds avidly to IκBα (36). Our study extends this observation by identifying the dominant role of the endogenous, cellular NF-κB p65. Activation of the TNF-α promoter-reporter constructs possessing either the κB3 site alone (−120 bp) or both κB1 and κB3 binding sites (−615 bp), by cotransfection of the plasmid expressing NF-κB p65 alone, suggests that p65 homodimers were capable of binding both sites. Following AdNF-κBDNp65 infection, homodimers of the DN NF-κB p65 accumulate in the nucleus and cytoplasm (data not shown and Refs. 29 and 37). Binding of DN homodimers suppresses promoter activation by inhibiting transactivation due to the wild-type NF-κB (29, 37). Inhibition of LPS-induced TNF-α secretion from primary macrophages by the DN NF-κB p65 documents the contribution of wild-type NF-κB p65.

Further, this study identified the composition of the complexes possessing NF-κB 65 that were responsible for the LPS-induced activation of the human TNF-α promoter. As early as 20 min following stimulation with LPS, NF-κB p65/p50 heterodimers bound to the κB1 and κB3 binding sites of the proximal TNF-α promoter, consistent with earlier studies (7, 8). The current study extends these observations by directly documenting the functional relevance of these heterodimers, because expression of NF-κB p65 and p50 resulted in greater than additive, or synergistic, activation of the TNF-α promoter. Synergism was observed with the TNF-α promoter-reporter possessing the κB3 site only (−120 bp), as well as the promoter-reporter possessing both sites (−615 bp), which was consistently more active (Fig. 7, discussed below). These observations suggest that both κB1 and κB3 binding sites contribute to the greater than additive activation of the TNF-α promoter by NF-κB p65 plus p50. Supporting the importance of κB1, mutation of this site reduced LPS-induced activation (7, 8). Increased promoter activity was observed following transient transfection with the increasing concentrations of the p50 expression plasmid (ratios of 1:3 for both, and 1:4 for the −120-bp promoter). This may have been due to binding of active p65/p50 heterodimers that may have been displaced by p50 homodimers, from unrelated sites in the nucleus capable of binding both species, or by increased heterodimer formation between ectopically expressed p50 and cellular NF-κB p65.

Inhibition of NF-κB, employing the DN NF-κB p65 or IκBα, did not completely abolish LPS-induced TNF-α secretion (this study and Ref. 35), suggesting the contribution of other transcription factors, either independently or synergistically. The DN c-Jun also effectively suppressed LPS-induced TNF-α secretion, demonstrating the importance of both NF-κB and c-Jun in the LPS-induced TNF-α secretion in primary human macrophages. NF-κB and c-Jun synergistically activate a number of genes, including the 5′ long terminal repeat of HIV-I and the promoters of the tissue factor and GM-CSF genes (25, 38, 39). However, employing either the −615 or the −120 TNF-α promoter-reporters, no synergy between NF-κB p65, alone or with p50 and c-Jun was observed, even in the presence of the coactivator p300, which enhanced the activation due to c-Jun alone. In addition, no cooperative binding was observed when an oligonucleotide containing both the −103 bp AP-1 and the adjacent κB3 sites of the human TNF-α promoter was employed in EMSA (data not shown). These

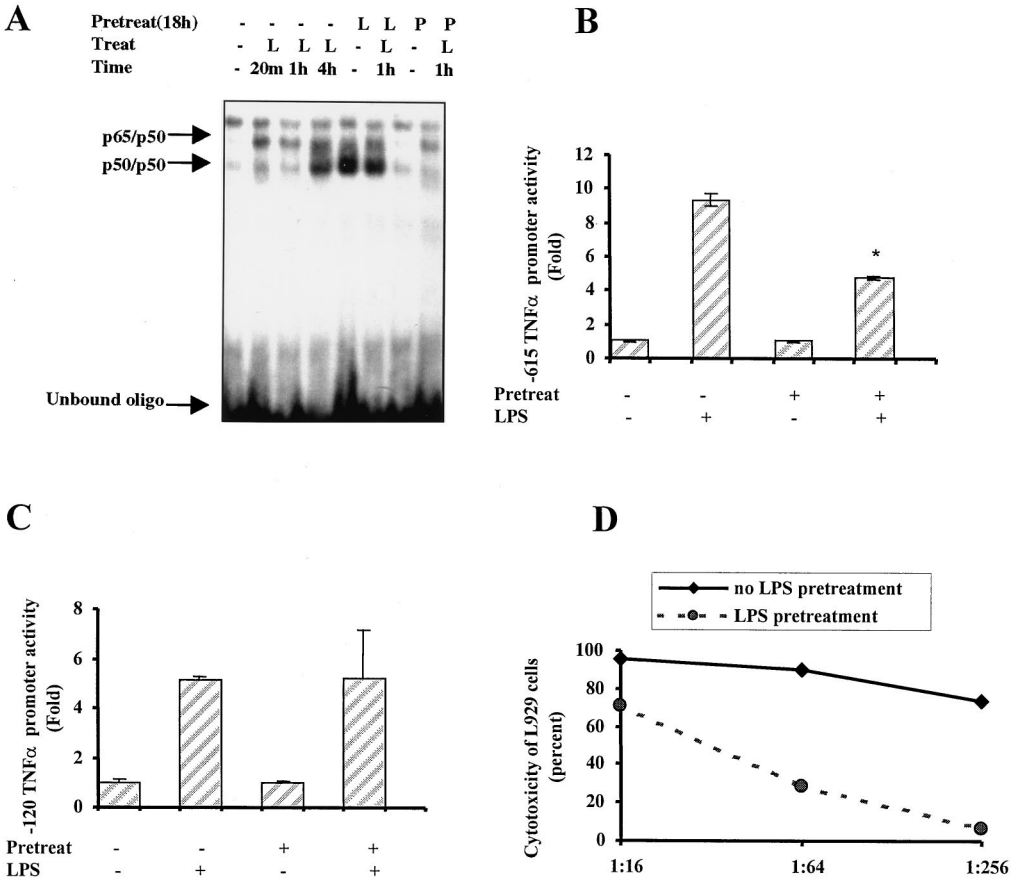

FIGURE 9. Binding of NF-κB p50/p50 homodimers to the human TNF-α κB1 site contributes to LPS-induced tolerance. *A*, Prior LPS treatment alters the ratio of NF-κB p65/p50 heterodimers to p50/p50 homodimers in response to subsequent LPS. RAW cells were treated with LPS (100 ng/ml) for 20 min (m) or for 1or 4 h (h) as indicated at the *top* of the figure. Cells were pretreated (Pretreat 18 h) with LPS (10 ng/ml, L) or PMA (20 ng/ml, P) for 18 h. After pretreatment, the cells were washed twice with PBS before LPS treatment (100 ng/ml). The nuclear extracts were harvested after 1 h of LPS treatment and employed for EMSA with the ^{32}P-labeled κB1 oligonucleotide. The locations of the NF-κB p65/p50 heterodimers and the p50/p50 homodimers are indicated on the *left* of the figure. *B*, Pretreatment with LPS resulted in suppression of subsequent LPS-induced activation of the −615 TNF-α promoter-reporter. RAW cells were pretreated (Pretreat) with LPS (100 ng/ml) or control medium for 18 h. After washing, they were transfected by −615 TNF-α promoter-reporter (1 μg), then were treated with LPS (10 μg/ml) (+) or control medium (−) for another 18 h. The cells were harvested and the cell extracts employed to detect luciferase activity. The fold activation compared with the untreated controls is presented as the mean ± 1 SE of an experiment performed in duplicate, which is representative of three experiments. ∗, Significant difference ($p < 0.02$) compared with no pretreatment. *C*, Pretreatment with LPS does not result in suppression of subsequent LPS-induced promoter activation using −120 TNF-α promoter-reporter. The experiments were performed as described in *B* except that the −120 TNF-α luciferase promoter-reporter was employed. *D*, Pretreatment with LPS resulted in suppression of subsequent LPS-induced L929 cytotoxicity by RAW cell supernatants. The supernatants of RAW cells stimulated for 8 h with LPS (10 μg/ml), without or with pretreatment (100 ng/ml LPS), were collected to determine cytotoxicity employing the TNF-sensitive L929 cell line. Before the addition of second dose of LPS at 18 h, the supernatants were removed and replaced with fresh medium. The results are presented as cytotoxicity at different dilutions of culture supernatants. The results presented are from one experiment and are representative of three experiments.

observations demonstrate that, in contrast to other genes with adjacent NF-κB and AP-1 binding sites, NF-κB and c-Jun independently activate the proximal TNF-α promoter.

In contrast to our observations, others have suggested an interaction between c-Jun and NF-κB in the proximal TNF-α promoter (7). This study (7) employed an artificial system of tandem repeats possessing the AP-1 and the NF-κB binding sites from the proximal human TNF-α promoter. Increasing the distance between the two sites suppressed LPS-induced activation of the promoter-reporter (7). However, employing a TNF-α promoter-reporter with a mutated −103 AP-1 site, which would interrupt any potential interactions between c-Jun and NF-κB, LPS-induced TNF-α promoter activation was reduced ~15%, which is not consistent with synergistic interactions between c-Jun and NF-κB (7). Nonetheless, interactions of the −103-bp AP-1 site with adjacent sites have been documented. We observed that c-Jun binding to the −103-bp AP-1 site synergistically interacted with adjacently bound C/EBPβ in PMA/LPS-stimulated U937 cells (22). In addition, in T cells, the −103-bp AP-1 site binding c-Jun/ATF-1 interacted with the κB3 site binding NF-ATp (23). However, in macrophages, both NF-κB and c-Jun independently contributed to the activation of the TNF-α gene.

The second novel observation in this study was the characterization of the functional differences between the human κB1 and κB3 binding sites following activation with LPS. Controversy exists as to the ability of the human κB3 site to activate the human TNF-α promoter (7, 8, 40). Our data clearly documented the functional relevance of this binding because the −120 TNF-α promoter-reporter possessing only the κB3 site was activated not only by LPS, which activates many factors, but also directly by NF-κB p65, alone or with p50. These observations suggest that the differences observed in prior studies were likely due to cell-type differences and not the ability of NF-κB to activate the human TNF-α promoter through the κB3 binding site.

Although both the κB1 and κB3 binding sites bound NF-κB p65/p50 heterodimers by 20 min (Fig. 3), and both sites contributed functionally to TNF-α promoter activation (Fig. 7), only the κB1 site bound NF-κB p50 homodimers, which were suppressive (Figs. 8 and 9). This study for the first time identifies the functional consequences of the differential binding of NF-κB p50 homodimers by the human κB1, but not κB3, binding site. An earlier study noted that NF-κB p50 homodimers bound to the −510-bp murine κB3 site (15). However, this site is not conserved in the human TNF-α promoter (41). The human κB1 site, which like the murine κB3 site, binds NF-κB p50 homodimers, has a different sequence (see below) and relationship to the transcription start site compared with the murine TNF-α promoter (−594 bp for human and −510 for murine) (15, 41). Employing the −615 bp promoter-reporter, which contains both the human κB1 and κB3 binding sites, increasing the ratio of NF-κB p50, relative to p65, resulted in suppression of TNF-α promoter activity and increased detection of NF-κB p50 homodimers on EMSA (data not shown). These observations suggest that LPS-induced tolerance was due to NF-κB p50 homodimers. A promoter-reporter possessing 1 kb of the murine TNF-α promoter, which contained the murine κB3 site, that binds p50 homodimers, also resulted in LPS-induced tolerance (15).

The current study extends the observations (15) obtained with the murine promoter in several ways. First, the functional consequence of the difference in the complexes bound to the human κB1 and κB3 sites was defined. With the human TNF-α promoter possessing both sites (−615 bp), even at the highest concentration of NF-κB p50 examined, TNF-α promoter activity remained, consistent with that observed with the promoter-reporter containing only the κB3 site (Fig. 7). This further suggests that both binding sites may be independently activated. In addition, this study demonstrates that the NF-κB p50 homodimers, present 18 h following pretreatment with LPS, were suppressive, but only when the TNF-α promoter-reporter possessed the κB1 site (−615 bp) and not when the κB3 site alone (−120 TNF-α promoter-reporter) was present (Fig. 9). These observations document the functional significance of the differential binding of NF-κB p50 homodimers to the κB1, compared with the κB3, binding site.

The differential binding by the human κB1 and κB3 sites is likely due to sequence differences of the sites. The human κB1 site (GGGACAGCCC) is consistent with the NF-κB consensus sequence of (GGGRNNYYCC) (in which R is a purine, Y is a pyrimidine, and N is any base pair), except for the G in the seventh base pair position, which is not critical (42). In contrast, the human κB3 site (GGGTTTCTCC) possesses a pyrimidine (T) rather than a purine (A/G) in the fourth base pair position, suggesting that this difference might contribute to the weaker binding noted with this oligonucleotide and the differential effects noted with NF-κB p50 homodimers. However, the NF-κB site that appears responsible for LPS-induced tolerance of the murine TNF-α gene (GGGCTTT CCC), also has a pyrimidine (C) in the fourth base pair position (15), suggesting that this change alone may not be sufficient to explain the differences noted between the κB1 and κB3 sites of the human TNF-α promoter. Sequences flanking the κB binding sites might contribute to the differences observed.

A dramatic and sustained activation of p50 homodimers was observed at 18 h, which was not seen for the p65/p50 heterodimers and did not occur in response to PMA (Figs. 8 and 9; Ref. 15). The mechanism responsible for the differential expression of NF-κB p65/p50 heterodimers compared with p50 homodimers is unclear. A novel TNF-α inhibiting factor was recently identified in the conditioned medium from murine macrophages that resulted in the increased expression of NF-κB p50 homodimers and suppression of LPS-induced TNF-α promoter activation in murine macrophages (43). In summary, our observations demonstrated that both NF-κB and c-Jun contributed to LPS-induced TNF-α expression in primary human macrophages. Although commonly observed with other genes, no evidence for synergistic interactions of NF-κB with c-Jun or with C/EBPβ were observed. The variable binding of different NF-κB complexes to the κB1 and κB3 binding sites within the proximal TNF-α promoter greatly influenced the LPS-induced expression of the TNF-α gene.

References

1. Vassalli, P. 1992. The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol. 10:411.*
2. Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol. 14:397.*
3. MacNaul, K. L., N. I. Hutchinson, J. N. Parson, E. K. Bayne, and M. J. Tocci. 1990. Analysis of IL-1 and TNF-α gene expression in human rheumatoid synoviocytes and normal monocytes by in situ hybridization. *J. Immunol. 145:4154.*
4. Economou, J. S., K. Rhoades, R. Essner, W. H. McBride, J. C. Gasson, and D. L. Morton. 1989. Genetic analysis of the human tumor necrosis factor α/cachectin promoter region in a macrophage cell line. *J. Exp. Med. 170:321.*
5. Pope, R. M., A. Leutz, and S. A. Ness. 1994. C/EBPβ regulation of the tumor necrosis factor α gene. *J. Clin. Invest. 94:1449.*
6. Wedel, A., G. Sulski, and H. W. L. Ziegler-Heitbrock. 1996. CCAAT/enhancer binding protein is involved in the expression of the tumor necrosis factor gene in human monocytes. *Cytokine 8:335.*
7. Yao, J., N. Mackman, T. S. Edgington, and S.-T. Fan. 1997. Lipopolysaccharide induction of the tumor necrosis factor-α promoter in human monocytic cells. *J. Biol. Chem. 272:17795.*
8. Udalova, I. A., J. C. Knight, V. Vidal, S. A. Nedospasov, and D. Kwiatkowski. 1998. Complex NF-κB interactions at the distal tumor necrosis factor promoter region in human monocytes. *J. Biol. Chem. 273:21178.*
9. Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira. 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. USA 90:2207.*
10. Trautwein, C., C. Caelles, P. van der Geer, T. Hunter, M. Karin, and M. Chojkier. 1993. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature 364:544.*
11. Scherle, P. A., E. A. Jones, M. F. Favata, A. J. Daulerio, M. B. Covington, S. A. Nurnberg, R. L. Magolda, and J. M. Trzaskos. 1998. Inhibition of MAP kinase prevents cytokine and prostaglandin E_2 production in lipopolysaccharide-stimulated monocytes. *J. Immunol. 161:5681.*
12. Nakano, H., M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, and K. Okumura. 1998. Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc. Natl. Acad. Sci. USA 95:3537.*
13. Foey, A. D., S. L. Parry, L. M. Williams, M. Feldmann, B. M. J. Foxwell, and F. M. Brennan. 1998. Regulation of monocyte IL-10 synthesis by endogenous IL-1 and TNF-α: role of the p38 and p42/44 mitogen-activated protein kinases. *J. Immunol. 160:920.*
14. Baldwin, A. S. J. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol. 14:649.*
15. Bohuslav, J., V. V. Kravchenko, G. C. N. Parry, J. H. Erlich, S. Gerondakis, N. Mackman, and R. J. Ulevitch. 1998. Regulation of an essential innate immune response by the p50 subunit of NF-κB. *J. Clin. Invest. 102:1645.*
16. Oeth, P. A., P. G.C.N., C. Kunsch, P. Nantermet, C. A. Rosen, and N. Mackman. 1994. Lipopolysaccharide induction of tissue factor gene expression in monocytic cells is mediated by binding of c-Rel/p65 heterodimers to a κB-like site. *Mol. Cell. Biol. 14:3772.*
17. Stein, B., P. C. Cogswell, and A. S. Baldwin, Jr. 1993. Functional and physical associations between NF-κB and C/EBP family members: Rel domain-b-ZIP interaction. *Mol. Cell. Biol. 13:3964.*
18. LeClair, K. P., M. A. Blanar, and P. A. Sharp. 1992. The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. *Proc. Natl. Acad. Sci. USA 89: 8145.*
19. Plevy, S. E., J. H. M. Gemberling, S. Hsu, A. J. Dorner, and S. T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol. Cell. Biol. 17:4572.*
20. Kunsch, C., R. K. Lang, C. A. Rosen, and M. F. Shannon. 1994. Synergistic transcriptional activation of the IL-8 gene by TNFα-p65 (RelA) and NF-IL-6. *J. Immunol. 153:153.*
21. Matsusaka, T., K. Fujikawa, Y. Nishio, N. Makaida, K. Matsushima, T. Kishimoto, and S. Akira. 1993. Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines interleukin 6 and interleukin 8. *Proc. Natl. Acad. Sci. USA 90:10193.*
22. Zagariya, A., S. Mungre, R. Lovis, M. Birrer, S. Ness, B. Thimmapaya, and R. Pope. 1998. Tumor necrosis α gene regulation: enhancement of C/EBPβ-induced activation by c-Jun. *Mol. Cell. Biol. 18:2815.*
23. Tsai, E. Y., J. Jain, P. A. Pesavento, A. Rao, and A. E. Goldfeld. 1996. Tumor necrosis factor α gene regulation in activated T cells involves ATF-2/Jun and NF-ATp. *Mol. Cell. Biol. 16:459.*

24. Rhoades, K. L., S. H. Golub, and J. S. Economou. 1992. The regulation of the human tumor necrosis factor α promoter region in macrophage, T cell and B cell lines. *J. Biol. Chem. 267:22102.*
25. Stein, B., A. S. Baldwin, Jr., B. W. Ballard, W. C. Greene, P. Angel, and P. Herrlich. 1993. Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potential biological function. *Mol. Cell. Biol. 12:3879.*
26. Goldfeld, A. E., J. L. Strominger, and C. Doyle. 1991. Human tumor necrosis factor α gene regulation in phorbol ester stimulated T and B cell lines. *J. Exp. Med. 174:73.*
27. Baer, M., S. C. Williams, A. Dillner, R. C. Schwartz, and P. F. Johnson. 1998. Autocrine signals control CCAAT/enhancer binding protein β expression, localization, and activity in macrophages. *Blood 92:4353.*
28. LaCour, E. G., M. H. Grayson, C. F. Ware, and R. M. Pope. 1990. Enhanced cytotoxicity in the rheumatoid joint. *Clin. Immunol. Immunopathol. 54:431.*
29. Soares, M. P., A. Muniappan, E. Kaczmarek, K. Koziak, C. J. Wrighton, F. Steinhauslin, C. Ferran, H. Winkler, F. H. Bach, and J. Anrather. 1998. Adenovirus-mediated expression of a dominant negative mutant of p65/RelA inhibits proinflammatory gene expression in endothelial cells without sensitizing to apoptosis. *J. Immunol. 161:4572.*
30. Furtado, M. R., S. Subramanian, R. A. Bhat, D. M. Fowlkes, B. Safer, and B. Thimmappaya. 1989. Functional dissection of adenovirus VAI RNA. *J. Virol. 63:3423.*
31. Ghadge, G. D., S. Swaminathan, M. Katze, and B. Thimmapaya. 1991. Binding of the adenovirus VAI RNA to interferon induced 68 kDa protein kinase correlates with function. *Proc. Natl. Acad. Sci. USA 88:7140.*
32. Kaspers, G. J., A. J. Veerman, R. Pieters, C. H. Van Zantwijk, L. A. Smets, E. R. Van Wering, and A. Van Der Does-Van Den Berg. 1997. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. *Blood 90:2723.*
33. Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. A. Heyman, D. W. Rose, G. a. C.K., and M. G. Rosenfeld. 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell 85:403.*
34. Perkins, N. D., L. K. Felzien, J. C. Betts, K. Leung, D. H. Beach, and G. J. Nabel. 1997. Regulation of NF-κB by cyclin-dependent kinases associated with the p300 coactivator. *Science 275:523.*
35. Foxwell, B., K. Browne, J. Bondeson, C. Clarke, R. DeMartin, F. Brennan, and M. Feldmann. 1998. Efficient adenoviral infection with IκBα reveals that macrophage tumor necrosis factor α production in rheumatoid arthritis is NF-κB dependent. *Proc. Natl. Acad. Sci. USA 95:8211.*
36. Baldwin, A. S., Jr. 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol. 14:649.*
37. Anrather, J., V. Csizmadia, C. Brostjan, M. P. Soares, F. H. Bach, and H. Winkler. 1997. Inhibition of bovine endothelial cell activation in vitro by regulated expression of a transdominant inhibitor of NF-κB. *J. Clin. Invest. 99:763.*
38. Oeth, P., G. C. N. Parry, and N. Mackman. 1997. Regulation of the tissue factor gene in human monocytic cells. *Arterioscler. Thromb. Vasc. Biol. 17:365.*
39. Thomas, R. S., M. J. Tymms, L. H. McKinlay, M. F. Shannon, A. Seth, and I. Kola. 1997. ETS1, NF-κB and AP1 synergistically transactivate the human GM-CSF promoter. *Oncogene 14:2845.*
40. Trede, N. S., E. Castigli, R. S. Geha, and T. Chatila. 1993. Microbial superantigens induce NF-κB in the human monocytic cell line THP-1. *J. Immunol. 150:5604.*
41. Kuprash, D. V., I. A. Udalova, R. L. Turetskay, D. Kwiatkowski, N. R. Rice, and S. A. Nedospasov. 1999. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J. Immunol. 162:4045.*
42. Baeuerle, P. A. 1991. The inducible transcription factor NF-κB: regulation by distinct protein subunits. *Biochim. Biophys. Acta 1072:63.*
43. Baer, M., A. Dilliner, R. C. Schwartz, C. Sedon, S. Nedospasov, and P. F. Johnson. 1998. Tumor necrosis factor α transcripition in macrophages is attenuated by an autocrine factor that preferentially induces NF-κB p50. *Mol. Cell Biol. 18:5678.*