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Transcriptional Inhibition of Interleukin-8 Expression in Tumor Necrosis Factor-tolerant Cells

EVIDENCE FOR INVOLVEMENT OF C/EBPβ*

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There is some evidence that the potent cytokine tumor necrosis factor (TNF) is able to induce tolerance after repeated stimulation of cells. To investigate the molecular mechanisms mediating this phenomenon, the expression of interleukin-8 (IL-8), which is regulated by transcription factors NF-κB and C/EBPβ, was monitored under TNF tolerance conditions. Pretreatment of monocytic cells for 72 h with low TNF doses inhibited TNF-induced (restimulation with a high dose) IL-8 promoter-dependent transcription as well as IL-8 production. Under these conditions neither activation of NF-κB nor IκB proteolysis was affected after TNF re-stimulation, albeit a slightly reduced IκB-α level was found in the TNF pretreated but not re-stimulated sample. Remarkably, in tolerant cells an increased binding of C/EBPβ to its IL-8 promoter-specific DNA motif as well as an elevated association of C/EBPβ protein with p65-containing NF-κB complexes was observed. Finally, overexpression of C/EBPβ, but not p65 or Oct-1, markedly prevented TNF-induced IL-8 promoter-dependent transcription. Taken together, these data indicate that the expression of IL-8 is inhibited at the transcriptional level in TNF-tolerant cells and C/EBPβ is involved under these conditions in mediating the negative-regulatory effects, a mechanism that may play a role in inflammatory processes such as sepsis.

The phenomenon that pre-exposure to a certain substance induces reduced sensitivity to subsequent challenge with the same stimulus is termed tolerance (1). This concept may be considered protective under acute inflammatory conditions like sepsis in prevention of the deleterious effects that would likely result from persistent cytokine signaling (1). There is some evidence from *in vivo* studies that the potent cytokine TNF[1] (2) is able to induce tolerance-like conditions. For example, pretreatment with TNF in several animal species selectively affects certain TNF effects such as fever, anorexia, and lethality (3–5) and partially affects gastrointestinal toxicity (6). Cyclooxygenase inhibitors prevent the induction of tolerance to the toxic effect of TNF (7). In addition, some form of cross-tolerance has been described, *e.g.* pre-exposure to TNF also modulates certain effects of lipopolysaccharide including fever, hypophagia, and lethality (3, 5, 8). The molecular mechanisms underlying TNF tolerance are only poorly understood (4, 9, 10). Interestingly, it has been described that long term (11) as well as short term (12, 13) pretreatment with TNF reduced subsequent TNF-induced activation of transcription factor NF-κB and/or proteolysis of its inhibitor IκB-α.

TNF is a potent activator of NF-κB, which is a dimeric complex most frequently assembled from the subunits RelA (p65) and p50 (14–16). Activation of this transcription factor by TNF is initiated mainly by binding to cell surface TNF receptor 1 (17). Subsequent signaling occurs through the recruitment of cytosolic signaling proteins including TNF receptor-associated death domain protein, receptor-interacting protein, and TNF receptor-associated factor 2, eventually leading to the activation of the IκB kinase complex (17, 18, 19). This high molecular weight assembly kinase phosphorylates the IκB inhibitor proteins (18, 20), which trap the NF-κB dimer in the cytosol in a non-activated state (14, 16). IκB is subsequently degraded in an ubiquitin-dependent step by the proteasome, thereby allowing the liberated NF-κB dimer to translocate to the nuclear compartment (14–16). Within the nucleus, NF-κB is involved in the coordinated expression of numerous target genes, including the potent cytokine and chemokine interleukin-8 (IL-8) (14–16, 21, 22).

IL-8, a member of the CXC family of chemokines, has been implicated in a variety of inflammatory diseases (21, 22). Gene transcription is one major point of control at which expression of IL-8 is regulated (22, 23). Functional studies indicate that IL-8 transcriptional responses to mediators such as TNF are rapid and require only 100 nucleotides of 5′-flanking DNA upstream of the TATA box (22, 23). Within this region DNA binding sites for the inducible transcription factors NF-κB and C/EBPβ (24, 25) were found located next to each other (see Fig. 1; Ref. 23). Transcription factors from these families bind the IL-8 promoter as dimers, and several distinct subunit combinations have been identified (23, 26). C/EBPβ physically interacts with NF-κB, and functional cooperativity among the factors appears to be critical for optimal IL-8 promoter activity in

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[1] The abbreviations used are: TNF, tumor necrosis factor; IL-8, interleukin-8; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; CMV, cytomegalovirus.

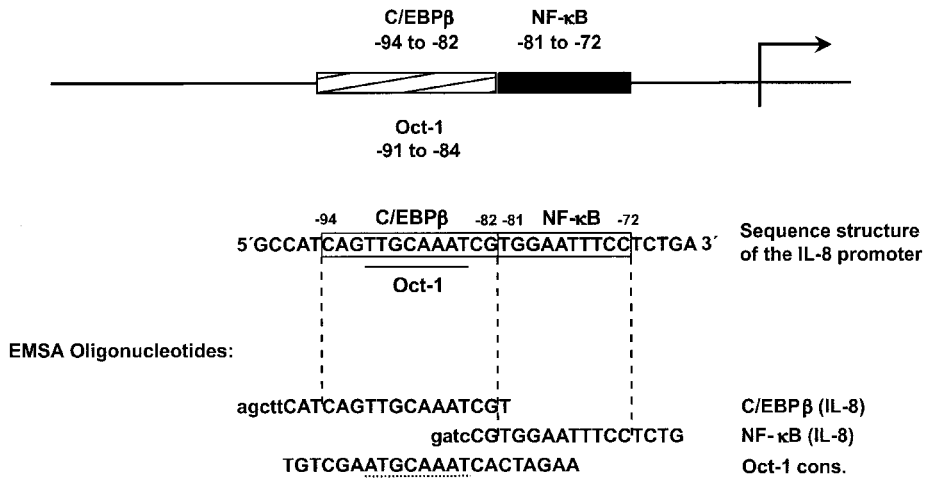

FIG. 1. **Organization of the IL-8 promoter and oligonucleotides used for EMSA.** The depicted IL-8 promoter region contains C/EBPβ (overlapping an Oct-1 motif) and NF-κB binding sites next to each other (marked as *striped* and *dark boxes*). The nucleotide sequence of this region (bp -99 to -67) is shown, and the major elements are identified by *boxes* (C/EBPβ, NF-κB) or *underlined* (Oct-1). The sequences of EMSA oligonucleotides are listed *below*. An additional consensus oligonucleotide was used (*Oct-1 cons.*). Several base extensions, added for Klenow labeling, are shown in *lowercase letters*.

different cell types (27, 28). IL-8 transcription appears to be activated by a promoter recruitment mechanism where inducible transcription factors are required for binding of TATA box proteins and formation of a stable preinitiation complex (22). In addition, the POU-homeodomain transcription factor Oct-1, which binds to an overlapping sequence within the C/EBPβ site, appears to be involved in regulation of basal transcriptional activity of the IL-8 promoter (29).

The aim of this study was to investigate the molecular mechanisms underlying TNF tolerance. To initiate tolerance-like conditions, monocytic cells were pretreated with low TNF concentrations for 72 h and then re-stimulated with a high TNF dose. Under these conditions the expression of the IL-8 gene was monitored as a read-out, and the roles of NF-κB, C/EBPβ, and Oct-1 were examined.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Reagents—THP-1 human monocytic cells (DSMZ, Braunschweig, Germany) were maintained in suspension in RPMI 1640 (Glutamax-1, low endotoxin) containing 7% fetal calf serum (low endotoxin), 100 units/ml penicillin, and 100 μg/ml streptomycin (Biochrom, Berlin, Germany) (30). For the experiments, the cells were plated at a density of 2×10^6 per well in 6-well culture dishes. Human recombinant TNF was obtained from Sigma (Deisenhofen, Germany). HeLa cells (DSMZ) were cultured in Dulbecco's minimum Eagle's medium (Biochrom) (10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin). Endotoxin contamination was screened by the limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD). A potential toxicity of the cell culture conditions applied was monitored by cell morphology/count, trypan blue dye exclusion, and the WST-1 test (Roche Diagnostics).

Transfection of THP-1 Cells—In transfection studies pGL2-IL-8 (420 bp of the IL-8 promoter region), a firefly luciferase reporter plasmid, was utilized (30, 31). This plasmid (1 μg) was transiently co-transfected with 0.2 μg of a constitutively active *Renilla* luciferase control plasmid, pRLtk (Promega, Mannheim, Germany), into THP-1 cells using a DEAE-dextran-based protocol (30, 31). After transfection, cells were plated at a density of 2×10^6/3 ml of RPMI with 7% fetal calf serum in a 6-well plate and incubated for 72 h (without or with TNF pretreatment). After this time, the cells were left untreated or stimulated for 5 h with TNF at 0.1–100 ng/ml. Subsequent to stimulation the cells were lysed, and luciferase activity was determined using the Dual Luciferase Reporter assay system (Promega). Results are expressed as relative luciferase activity, which means that firefly relative light units were divided by *Renilla* relative light units.

Determination of IL-8—The supernatant concentration of IL-8 protein was measured by sandwich type immunoassay (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared and analyzed as described (31, 32). The sense strand sequences of the IL-8 oligonucleotides C/EBP-β (IL-8) as well as NF-κB (IL-8) are listed in Fig. 1 (28, 29). These oligonucleotides were applied as a probe and labeled with the Klenow fragment of DNA polymerase I (Roche Diagnostics) using [α-^{32}P]dCTP (PerkinElmer Life Sciences, Brussels, Belgium). In some experiments the 35-mer (IL-8) oligonucleotide was used, which contains the sequence of both C/EBPβ (IL-8) and NF-κB (IL-8). Oct-1 and Sp-1 binding was analyzed using consensus oligonucleotides (Promega) labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (Promega). Nuclear proteins were incubated with the radiolabeled probes for 30 min at room temperature in 20 μl of binding buffer (12 mM HEPES, pH 7.9, 4 mM Tris, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA, 12% glycerol, 5 mM dithiothreitol, 50 ng/μl poly(dI-dC)) as described (28). Samples were run in 0.25× TBE (10× TBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.0) on non-denaturing 6% polyacrylamide gels. Gels were dried and analyzed by autoradiography.

Supershift and Competition Studies—The nuclear extracts were incubated with 2 μl of appropriate TransCruz gel supershift antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) per 20 μl of reaction volume in binding buffer at 4 °C for 1 h before EMSA. The following antibodies were used: anti-p65, anti-C/EBPβ, and anti-Oct-1. In competition studies, samples were incubated with a 100× excess of unlabeled oligonucleotide: NF-κB (IL-8), C/EBPβ (IL-8), and Oct-1 (Fig. 1) as well as Sp-1 (Promega).

PAGE and Western Blot Analysis—Cytosolic and nuclear extracts were isolated as described (30), and electrophoresis was performed with 12.5% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes using the wet blot technique. After transfer, the membranes were incubated with antibodies against TNF receptor-associated factors 1 and 2, receptor-interacting protein, IκB kinase-α, IκB-α, p65, p50, cyclin B1, Sp-1 (Santa Cruz Biotechnology), or actin (Sigma). This was followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany). The proteins were visualized on x-ray film using the Chemiluminescent Reagent Plus (PerkinElmer Life Sciences).

Immunoprecipitation (IP)—Nuclear extracts were subjected to IP (30) in TN buffer (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.5 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin, antipain, aprotinin, pepstatin A, chymostatin 0.75 μg/ml each; Sigma). IP was carried out at 4 °C overnight with 2 μg of anti-C/EBPβ, anti-p65, anti-cyclin B1 or anti-Sp-1 (Santa Cruz Biotechnology), and 70 μl of 6% protein A agarose (Roche Diagnostics). After washing five times with TN buffer the precipitated proteins were analyzed by PAGE and Western blot analysis.

Overexpression Experiments—The plasmids used included C/EBPβ, Oct-1 (wild type, mutated), and p65 (14, 33, 34). RcCMV (Invitrogen, Groningen, Netherlands) containing no insert was used as a negative control. These plasmids (10 μg) were transiently co-transfected with 0.2 μg of pRLtk and 1 μg of pGL2-IL-8 into HeLa cells using Superfect (Qiagen, Hilden, Germany). After overnight culture, cells were left untreated or stimulated for 5 h with TNF at 20 ng/ml followed by lysis, and relative luciferase activity was determined.

RESULTS

Long Term Pretreatment with Low TNF Doses Inhibits TNF-induced IL-8 Promoter-dependent Transcription as Well as IL-8 Production—For the present study we decided to pretreat with a low dose of TNF for 72 h to induce a tolerance-like condition as described (11) and then re-stimulate with a significantly

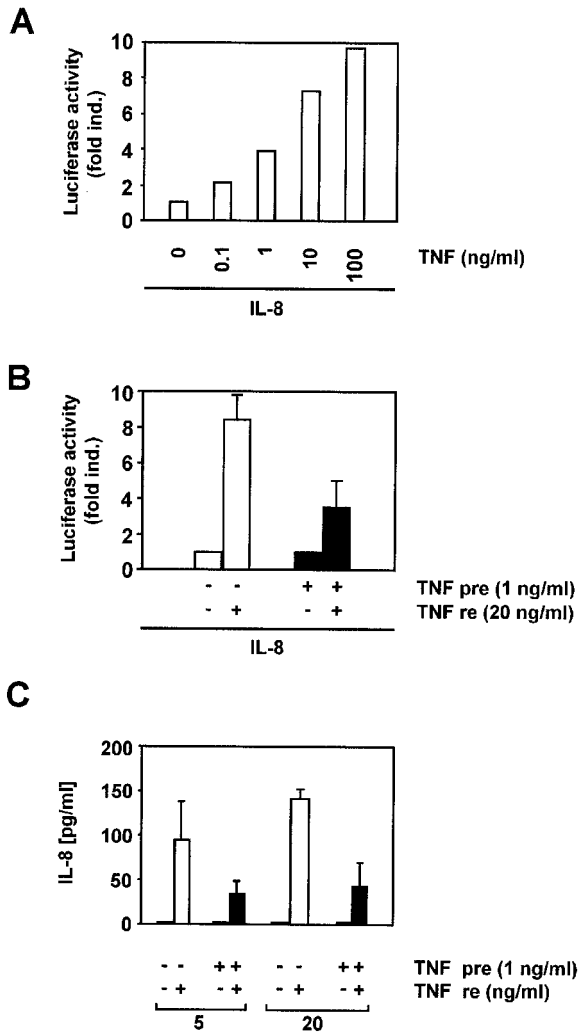

FIG. 2. **IL-8 promoter-dependent transcription as well as IL-8 production is inhibited in TNF-tolerant cells.** *A*, THP-1 monocytic cells were co-transfected with pGL2-IL-8 and the pRLtk *Renilla* plasmid, incubated for 72 h, and then stimulated with increasing TNF doses for 5 h. Cells were lysed, and firefly as well as *Renilla* relative light units were measured. Results are depicted as fold induction (*ind.*) above the value obtained for unstimulated cells. Representative data of three independent experiments are shown. *B*, transfections of cells were performed as described in *A* and followed by culture in medium alone (*white bars*) or medium containing 1 ng/ml TNF (*black bars*) for 72 h. After this incubation period, the cells were left untreated or stimulated with 20 ng/ml TNF for 5 h. Fold induction was calculated as described above. The *bars* show the mean ± S.D. from three independent experiments. *C*, THP-1 cells were preincubated with medium alone (*white bars*) or medium supplemented with 1 ng/ml TNF (*black bars*) for 72 h and subsequently stimulated for 5 h with TNF (5 or 20 ng/ml). The supernatants were analyzed for IL-8 by immunoassay. Data of three independent experiments are shown (mean ± S.D.).

higher dose and monitor for IL-8 promoter-dependent transcription as well as IL-8 expression. Please note that in the following the low dose TNF-pretreated cells are designated as "tolerant cells." Initially, dose response experiments were performed with THP-1 monocytic cells to establish a window in which TNF exerts its effect on IL-8 promoter-dependent transcription measured by luciferase reporter assays (Fig. 2*A*). In these studies we observed a dose-dependent increase in transcriptional activity in a range from 0.1 to 100 ng/ml TNF. The following protocol was selected for most of the experiments: preincubation with 1 ng/ml TNF or medium for 72 h (indicated as *TNF pre* in the Figs. 2, 3, 4, and 6) followed by re-stimulation with a 20-fold higher concentration (20 ng/ml) for 5 h (depicted as *TNF re* in Figs. 2, 3, 4, and 6). Pretreatment with TNF led to a significant reduction in IL-8 promoter-dependent transcriptional activity when cells were re-stimulated with the higher TNF dose compared with cells that were preincubated with medium alone (Fig. 2*B*). Consistent with the results above we found a 70% inhibition of the production of the IL-8 protein measured by immunoassay in tolerant cells (Fig. 2*C*). Similar effects were observed when HeLa cells were treated under the same conditions (data not shown).

Activation of NF-κB as Well as IκB Proteolysis Are Not Affected—Because the IL-8 gene is transcriptionally regulated by NF-κB (22, 23), it was next tested to determine whether low dose TNF pretreatment can affect the activation of NF-κB. After preincubation, monocytic cells were re-stimulated with TNF for 15 min, and EMSAs were performed using an oligonucleotide comprising the κB motif of the human IL-8 promoter (*NF-κB (IL-8)*, see Fig. 1). Re-stimulation with TNF induced a significant NF-κB binding activity in non-pretreated cells or a slightly further increased NF-κB binding activity in low dose TNF pretreated cells (Fig. 3*A*). NF-κB binding was confirmed using supershift analysis as well as oligonucleotide competition (Fig. 3*A*). Under the same conditions, TNF-induced IκB-α proteolysis was not affected after re-stimulation, determined by Western blot analysis, albeit a slightly reduced IκB-α level was found in the TNF-pretreated but not re-stimulated sample (Fig. 3*B*). As expected, stimulation with TNF was accompanied by an increase in the nuclear levels of p65 and p50 (Fig. 3*C*). The amount of several upstream signaling molecules involved in TNF-induced NF-κB activation such as TNF receptor-associated factor 1 and 2, receptor-interacting protein, and IκB kinase-α as well as actin were not changed (Fig. 3*B*). The expression of TNF receptor 1 (p55) as well as TNF receptor-associated factor 2 (p75) were not affected under these conditions as shown by flow cytometry (data not shown).

C/EBPβ DNA Binding Activity as Well as Association of C/EBPβ Protein with p65 Are Increased under TNF Tolerance Conditions—Several other transcription factors besides NF-κB are involved in the regulation of the IL-8 gene, including C/EBPβ (28). To evaluate if this transcription factor is involved in TNF tolerance, EMSAs were performed using an oligonucleotide solely encompassing the C/EBPβ binding site of the IL-8 promoter (*C/EBPβ (IL-8)*, see Fig. 1) in cells that were pretreated with TNF (1 ng/ml) (TNF preincubation) or medium for 72 h and then re-stimulated with a 20-fold higher dose (TNF re-stimulation) for 15 min. Under these conditions an elevated binding of nuclear proteins to the C/EBPβ site was observed in TNF-tolerant cells (Fig. 4*A*). Supershift analysis as well as oligonucleotide competition demonstrated the presence of C/EBPβ but also a small amount of Oct-1 in these complexes. In the same nuclear extracts we also examined the binding of nuclear proteins to oligonucleotides comprising the Sp-1 consensus sequence (*Sp-1*) to monitor quality and equal loading, which was not changed under these conditions (Fig. 4*A*). Previous studies demonstrate a functional and physical association between NF-κB and C/EBP family proteins (27, 28, 35). Therefore, in the following we attempted to determine by co-immunoprecipitation studies whether C/EBPβ associates with NF-κB complexes in cells that were first pretreated with TNF and then re-stimulated with this cytokine as described above. C/EBPβ was immunoprecipitated in nuclear extracts, and p65 was detected in the precipitate by Western blot analysis or vice versa. Remarkably, these experiments demonstrated an increased association of C/EBPβ protein with p65-containing NF-κB complexes in TNF-tolerant cells (*TNF pre*), which was most intensive after TNF re-stimulation (*TNF re*) (Fig. 4*B*). To show the specificity of the bands the IP was carried out without

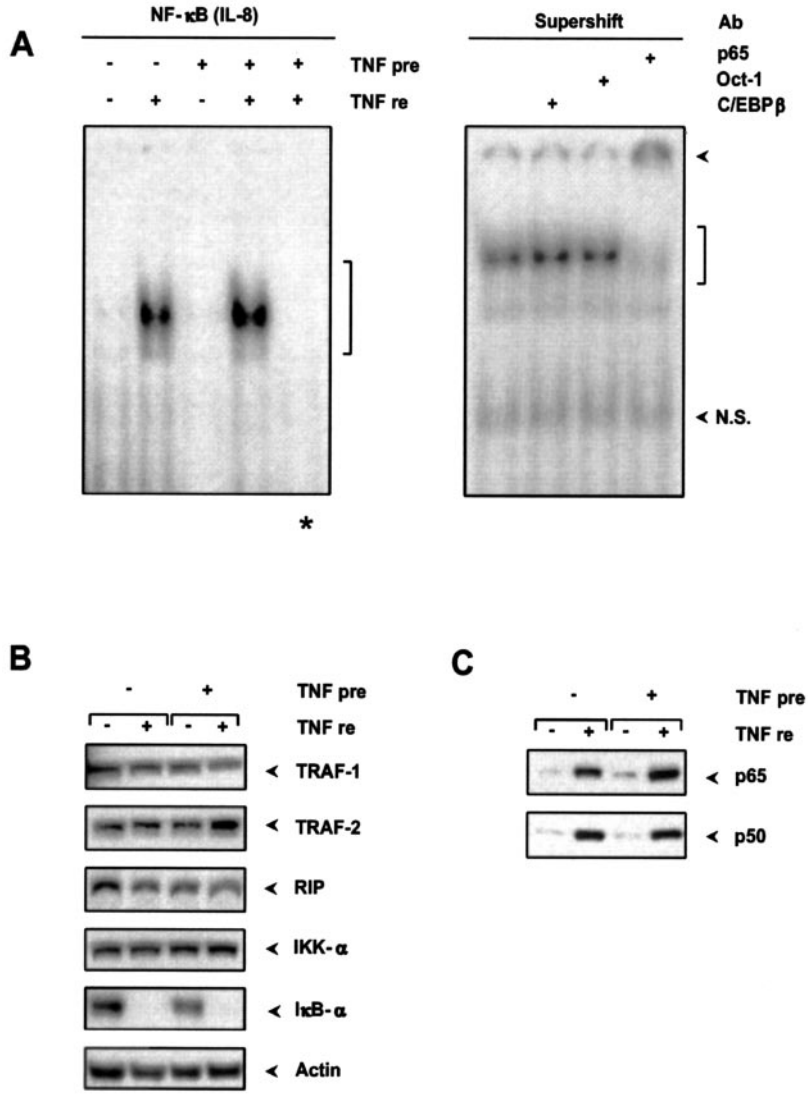

FIG. 3. **NF-κB activation as well as IκB proteolysis are not affected.** Monocytic cells were incubated with medium alone or medium containing 1 ng/ml TNF (*TNF pre*) for 72 h followed by stimulation with TNF (20 ng/ml, *TNF re*) for 15 min. *A*, nuclear extracts were subjected to EMSA using an oligonucleotide containing the NF-κB site of the IL-8 promoter. *Brackets* indicate NF-κB binding. The *asterisk* marks a control reaction in which a 100-fold concentration of unlabeled oligonucleotide was added to the EMSA. *Supershift* experiments were performed with the extract of the pretreated, re-stimulated cells (*TNF pre*, *TNF re*) using antibodies (*Ab*) against p65 and Oct-1 as well as C/EBPβ, and the *arrow* marks the complexes remaining in the loading slot by preincubation with an antibody against p65. *N.S.*, nonspecific band. Cytosolic (*B*) as well as nuclear (*C*) extracts were examined by Western blot for the presence of the indicated proteins (*arrows*). Representative experiments are shown for each condition, which were repeated at least five times. *RIP*, receptor-interacting protein; *IKK*, IκB kinase.

any extract (marked by an *asterisk*). In addition, control IP reactions were performed using antibodies against cyclin B1 (Fig. 4*B*) or Sp-1 (data not shown), demonstrating comparable levels of these precipitated proteins in the nuclear extracts regardless of TNF treatment. Furthermore, no specific signal was detected when IP was performed with an unspecific IgG antibody (data not shown).

Overexpression of C/EBPβ Prevents TNF-induced IL-8 Promoter-dependent Transcription—To further determine if C/EBPβ is involved in the negative regulation of TNF-induced IL-8 promoter-dependent transcription we performed transfection studies. HeLa cells were transiently transfected with the CMV control vector or expression plasmids coding for C/EBPβ wild type or a mutated form, respectively. Most importantly, these experiments showed that the presence of C/EBPβ significantly inhibited TNF-induced IL-8 promoter-dependent transcription, whereas no effect was found when the mutated protein was expressed (Fig. 5*A*). When we performed control experiments with cells expressing C/EBPβ, we observed an elevated DNA binding of this transcription factor (Fig. 5*B*) using the C/EBPβ (IL-8) as well as the 35-mer (IL-8) oligonucleotide (see "Experimental Procedures"). After TNF stimulation of these transfected cells, we detected a broadened band using the 35-mer (IL-8) oligonucleotide, which enables binding of both C/EBPβ and NF-κB (Fig. 5*B*, *right*; data not shown). Furthermore, p65 overexpression experiments demonstrated a direct effect of p65 on IL-8 promoter-dependent transcription, but in contrast to the studies with C/EBPβ, no inhibitory effect on TNF-induced transcriptional activity was found (Fig. 5*C*). The presence of the expressed proteins was also monitored by Western blot analysis (data not shown).

Effect of Oct-1 on TNF-induced IL-8 Promoter-dependent Transcription—The transcription factor Oct-1 has been suggested to be negatively involved in the regulation of the IL-8 gene (29). Therefore, DNA binding activity was examined using an oligonucleotide solely encompassing an Oct-1 consensus sequence. In these experiments we also observed an increased binding of nuclear proteins to the Oct-1 oligonucleotide in TNF-tolerant cells (Fig. 6*A*). Specificity of the bands was confirmed by oligonucleotide competition experiments and supershift analysis (Fig. 6*A*, data not shown). However, when Oct-1 was expressed in transfection experiments no inhibitory effect on TNF-induced activation of IL-8 promoter-dependent transcription was observed (Fig. 6*B*).

DISCUSSION

Several studies describe the existence of TNF tolerance *in vivo* and suggest that this phenomenon may play an important role in disease states such as inflammation and sepsis (4, 5, 8, 9). However, the underlying molecular and cellular mechanisms have not been very well established.

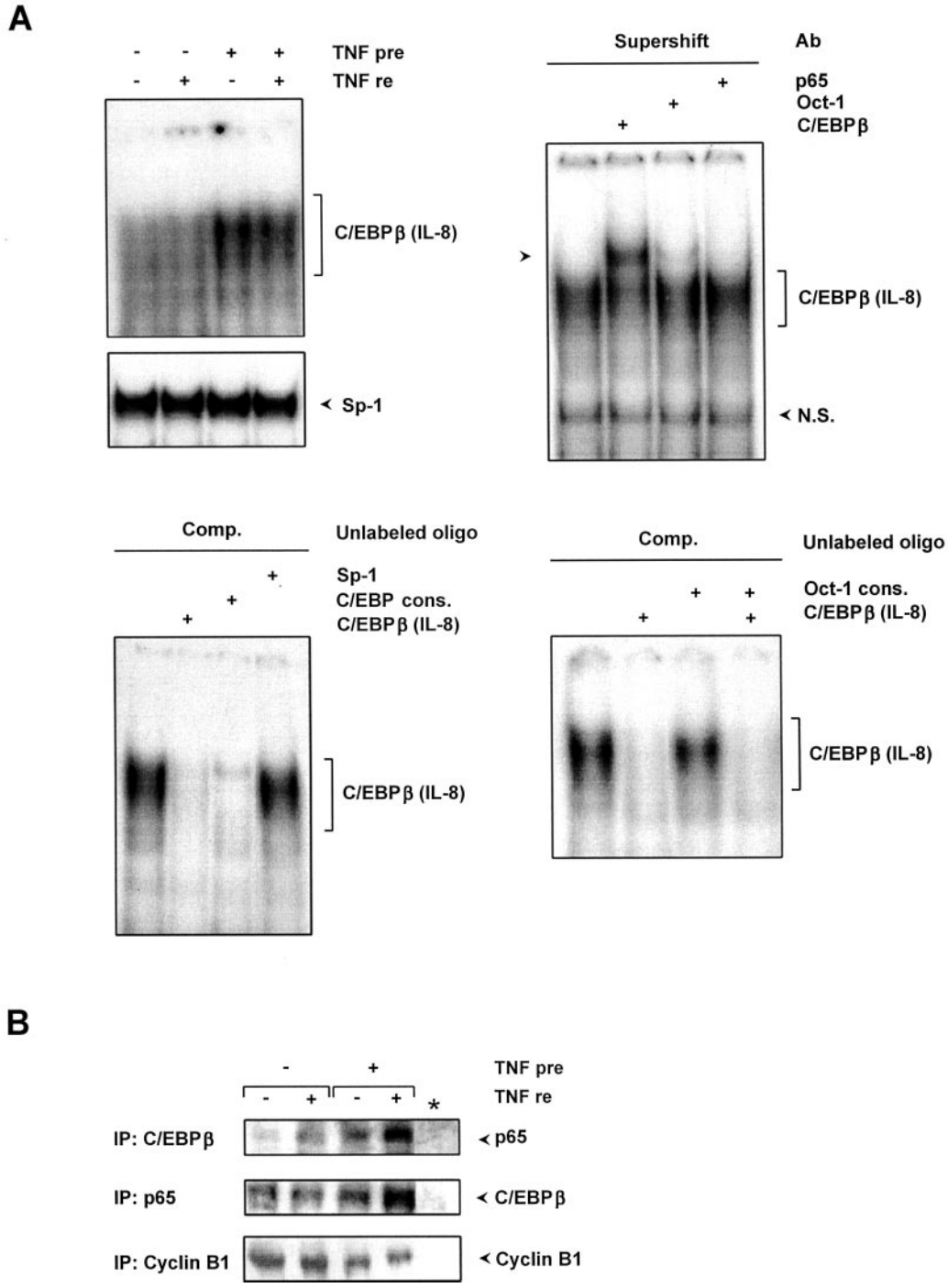

FIG. 4. **DNA binding activity of C/EBPβ as well as association of C/EBPβ protein with p65 are increased in TNF-tolerant cells.** THP-1 monocytic cells were preincubated with medium alone or medium with 1 ng/ml TNF for 72 h (*TNF pre*) and then left untreated or stimulated with TNF (20 ng/ml, *TNF re*) for 15 min. *A*, nuclear extracts were examined by EMSA using a radiolabeled C/EBPβ (IL-8) or Sp-1 consensus oligonucleotide. Specific binding to these oligonucleotides is indicated by *brackets* for C/EBPβ (IL-8) or an *arrow* (*Sp-1*). *Supershift* experiments were performed on nuclear extracts of the pretreated, re-stimulated cells (*TNF pre*, *TNF re*) using antibodies (*Ab*) against C/EBPβ, Oct-1, and p65. The *arrow* shows the position of the bands shifted by preincubation with antibodies against C/EBPβ (intense signal) or Oct-1 (very faint band), respectively. *N.S.*, nonspecific band. The same sample was examined in competition studies (*Comp.*) with a 100-fold excess of oligonucleotide (*Unlabeled oligo: C/EBPβ* (*IL-8*), *C/EBP cons.*, *Sp-1*, or *Oct-1 cons.*). *B*, nuclear extracts were isolated and subjected to IP with anti-C/EBPβ (*upper panel*) or anti-p65 (*middle panel*). The precipitated proteins were then analyzed by Western blot for the presence of p65 (*upper panel*) or C/EBPβ (*middle panel*). The *asterisk* marks an IP reaction without extract. Furthermore, a control IP was performed under the same conditions, and the protein (*cyclin B1*) was detected by Western blot. At least five independent experiments were performed for each condition.

The present study demonstrates that long term preincubation with low TNF doses induced a tolerant state in monocytic cells. Under these conditions, when cells were re-stimulated with a subsequent high dose of TNF, we observed a significant inhibition of IL-8 promoter-dependent transcription as well as protein production of IL-8, which is regulated by NF-κB transcription factors (Refs. 22 and 23; Fig. 1). The observation that in TNF-tolerant cells neither the activation of NF-κB nor IκB proteolysis was affected after TNF restimulation suggests transcriptional regulatory mechanisms. It should also be mentioned that in TNF-pretreated, but not restimulated samples, a slightly reduced IκB-α level was found that may be due to a low

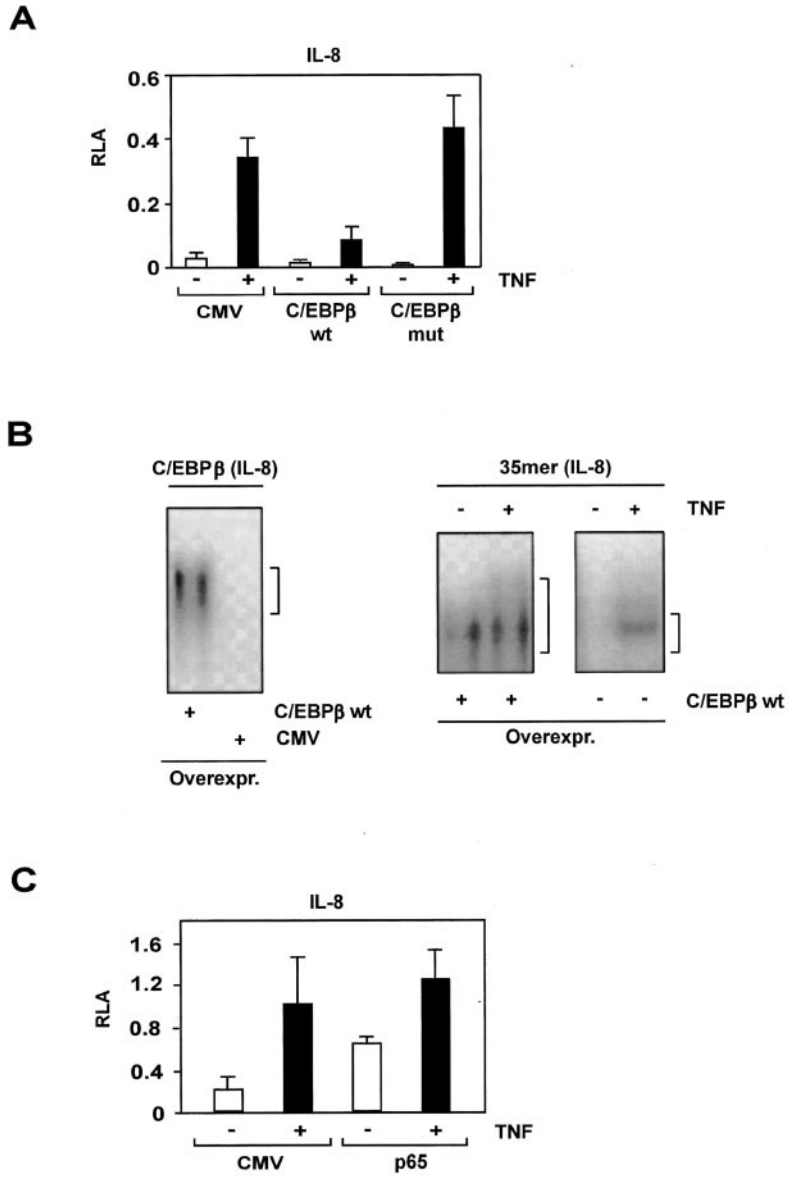

FIG. 5. **TNF-induced IL-8 promoter-dependent transcription is inhibited by overexpression of C/EBPβ.** *A*, HeLa cells were co-transfected with pGL2-IL-8, pRLtk, and C/EBPβ overexpression plasmids (*wt*, wild-type; *mut*, mutated) or the CMV control. After overnight incubation cells were left untreated (*white bars*) or stimulated with TNF (20 ng/ml) for 5 h (*black bars*) followed by lysis, and firefly as well as *Renilla* relative light units were measured. Results are expressed as relative luciferase activity (*RLA*) of three independent experiments (mean ± S.D.). *B*, nuclear extracts from HeLa cells (transfected with C/EBPβ wt or the CMV control) as well as untransfected cells were subjected to EMSA using the C/EBPβ (IL-8) (*left*) or 35-mer (IL-8) (*right*) oligonucleotide. *Brackets* indicate the position of DNA binding complexes containing C/EBPβ and/or NF-κB. *Overexpr.*, overexpression. *C*, HeLa cells were co-transfected with pGL2-IL-8, pRLtk, and the p65 overexpression plasmid or the CMV control. Transfected cells were treated with TNF, and luciferase assays were performed as described in *A* (mean ± S.D., three independent experiments).

level of ongoing signaling associated with IκB-α degradation under the conditions of TNF pretreatment, as similarly described (13). In an earlier study, contrasting results were reported in which long term TNF pretreatment inhibited TNF-induced NF-κB activation determined by EMSAs in a human adenocarcinoma cell line (11), indicating cell type-specific differences. Interestingly, in a recent study it was suggested that TNF and ceramide preconditioning differentially modulates NF-κB-mediated transactivation in astrocyte cultures by a p300-dependent mechanism (36).

It is of note that short term pretreatment of THP-1 cells appears to lead to a different form of TNF tolerance since under these conditions an inhibition of NF-κB activation as well as IκB degradation was found (12) that in our hands goes along with reduced expression of TNF receptors (data not shown). Interestingly, several mechanisms have also been found in lipopolysaccharide-tolerant cells in which transcriptional mechanisms (*e.g.* increase of inhibiting p50/p50 homodimers) (37–39) as well as impaired translocation of NF-κB (11, 40) are responsible for this condition.

Remarkably, under TNF tolerance conditions (long term, low dose preincubation) we observed an increased DNA binding of C/EBPβ to its IL-8 promoter-specific motif using EMSAs. C/EBPβ is a transcriptional protein that is involved in negative and positive regulation of a variety of genes including IL-8 (23–25,28). It was also suggested earlier (35) that simultaneous binding of C/EBP proteins and NF-κB to DNA may strengthen the inhibitory effect of C/EBP possibly by forming a more stable protein-protein-DNA complex. In addition, in coimmunoprecipitation studies we observed an increased association of C/EBPβ protein with p65-containing NF-κB complexes in the nucleus of tolerant cells, which was most intensive after TNF re-stimulation. In this context, it should be noted that functional and physical associations between NF-κB subunits (p65, p50) and C/EBP family members as well as other ATF bZIP proteins have been reported (27, 28, 41, 42) that are mediated by a Rel domain-bZIP interaction. Most importantly, the present study was able to show functional consequences using transfection experiments, demonstrating that overexpression of C/EBPβ, but not p65, inhibited TNF-induced IL-8 promoter-dependent transcription. Similar results were obtained in a previous report, which shows that C/EBPβ overexpression inhibits p65-directed transcriptional activity dependent on an IL-8 promoter fragment (28). Taken together our findings imply C/EBPβ as a key molecule in mediating the inhibitory effects on IL-8 expression in TNF-tolerant cells.

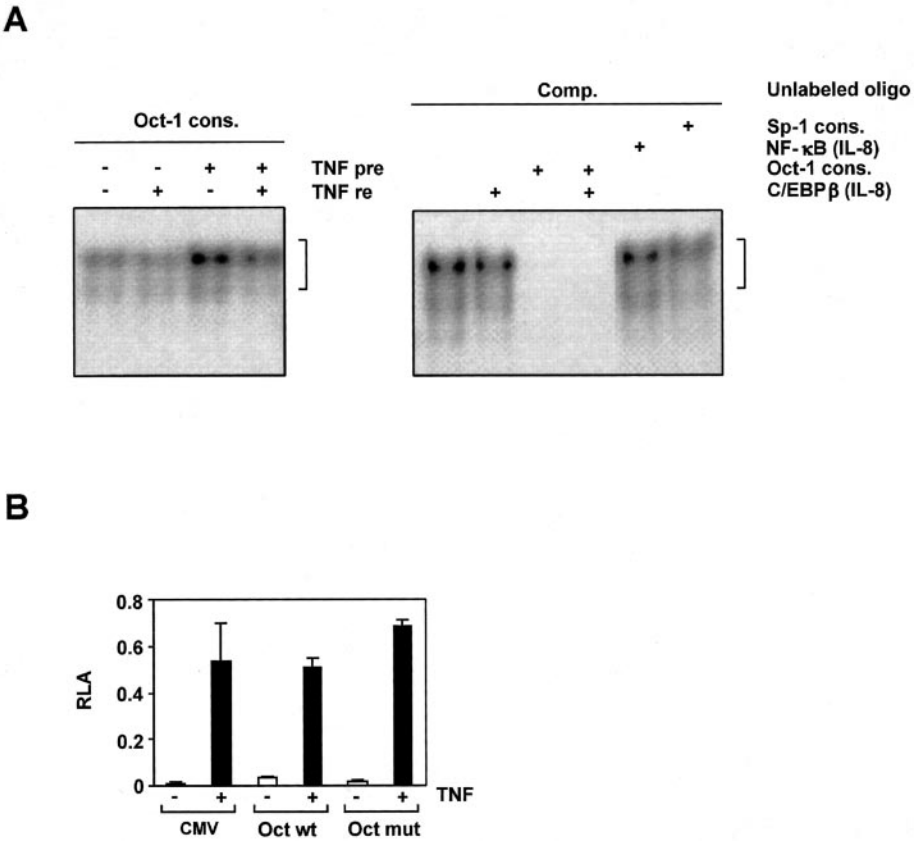

FIG. 6. **Effect of Oct-1 on TNF-induced IL-8 promoter-dependent transcription.** *A*, THP-1 monocytic cells were preincubated with medium alone or medium with 1 ng/ml TNF for 72 h (*TNF pre*) and then left untreated or stimulated with TNF (20 ng/ml, *TNF re*) for 15 min. Nuclear extracts were analyzed by EMSA as well as competition analysis (*Comp.*) using a radiolabeled *Oct-1 cons.* sequence as probe. *Brackets* indicate Oct-1 binding. A 100-fold excess of oligonucleotide (*Unlabeled oligo*) was used for competition studies using nuclear extracts of the pretreated, re-stimulated cells (*TNF pre*, *TNF re*). *B*, HeLa cells were co-transfected with pGL2-IL-8, pRLtk, and an overexpression plasmid for Oct-1 (*wt*, wild type; *mut*, mutated) or CMV. After overnight culture the cells were left untreated (*white bars*) or stimulated with TNF (20 ng/ml) for 5 h (*black bars*). After lysis, firefly as well as *Renilla* relative light units were measured. Data are expressed as relative luciferase activity (*RLA*; mean ± S.D.) of three independent experiments.

We also found an increased binding activity of the transcription factor Oct-1 in TNF-pretreated cells. It has been shown that Oct-1 represses basal transcriptional activity of the IL-8 promoter by binding independently to an element overlapping that of C/EBPβ (29) and acts as a transcriptional repressor for a number of other regulatory regions (43–45). However, overexpression of Oct-1 had no effect on TNF-induced IL-8 promoter-dependent transcription, suggesting that Oct-1 is not involved in negative regulation under TNF tolerance conditions.

The role of C/EBPβ in regulating promoters with NF-κB and C/EBP binding sites appears to be complex. In general, NF-κB and C/EBP synergistically activate promoters with C/EBP binding sites but inhibit promoters with κB binding sites (27). As an example for the latter, C/EBP family proteins have been shown to negatively influence NF-κB-mediated activation of the angiotensinogen gene acute-phase response element (46) and p65-dependent cytomegalovirus IE1/2 enhancer/promoter activity (35) as well as expression of the NF-κB target gene c-*myc* (47). In the case of the IL-8 promoter, in which the C/EBP binding site is adjacent to the NF-κB site (23), similar to the IE1/2 enhancer/promoter (35), a subtle interaction between NF-κB and C/EBP appears to determine whether the IL-8 promoter is activated or inhibited (28). As discussed above, in TNF-tolerant cells we find an increased binding of C/EBPβ to its IL-8 promoter motif as well as association of this protein with p65. The physical interaction found between these transcription proteins may be responsible for the observed inhibitory effects of C/EBPβ on NF-κB. Indeed, it has been suggested earlier (27) that C/EBP blocks the ability of NF-κB to interact with a critical co-activator of the basal transcriptional machinery and/or that C/EBPβ and NF-κB form a higher order transcription factor complex with reduced transcriptional activity at κB enhancers.

In summary, the present study describes a new molecular mechanism modulating IL-8 gene expression in TNF-tolerant cells. Induction of tolerance to control deleterious effects of TNF may play a role in preventing excessive influx of IL-8-responsive granulocytes in infection and inflammation and may potentially improve survival and outcome in processes such as septic shock.

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Transcriptional Inhibition of Interleukin-8 Expression in Tumor Necrosis Factor-tolerant Cells: EVIDENCE FOR INVOLVEMENT OF C/EBP β

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