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 mononuclear 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 IkB proteolysis was affected after TNF re-stimulation, although a slightly reduced IkBα 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 (2) is able to induce tolerance-like conditions. For example, pre-treatment 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
different cell types (27, 28). IL-8 transcription appears to be activated by a promotor recruitment mechanism where inducible transcription factors are required for 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 (DSMZ, Braunschweig, Germany) were maintained in suspension in RPMI 1640 (Glutamax-1, low endotoxin) containing 7% fetal calf serum in 200 μl of binding buffer (20 m M HEPES-I, pH 7.9, 4 mM Tris, pH 7.9, 60 mM KCl, 5 mM MgCl2, 0.6 mM EDTA, 12% glycerol, 5 mM dithiothreitol, 50 ng/ml 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 unlabelled 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 factor 1 and 2, receptor-interacting protein, IκBα, 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-cyclopalin B1 or anti-Sp-1 (Santa Cruz Biotechnology), and 70 μl of 8% 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). RecMV (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 pRIK and 1 μg of pGDL-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

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/EBPβ (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-32P]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-32P]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 m M HEPES-I, pH 7.9, 4 mM Tris, pH 7.9, 60 mM KCl, 5 mM MgCl2, 0.6 mM EDTA, 12% glycerol, 5 mM dithiothreitol, 50 ng/ml 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.

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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...
higher dose and monitor for IL-8 promoter-dependent transcrip-
tion 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 per-
formed with THP-1 monocytic cells to establish a window in
which TNF exerts its effect on IL-8 promoter-dependent tran-
scription measured by luciferase reporter assays (Fig. 2A). In
these studies we observed a dose-dependent increase in tran-
scriptional 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. 2B). 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. 2C). 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 Af-
fected**—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 oligo-
nucleotide 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. 3A). NF-κB binding was confirmed
using supershift analysis as well as oligonucleotide competition
(Fig. 3A). Under the same conditions, TNF-induced IκB-α pro-
teolysis 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.
3B). As expected, stimulation with TNF was accompanied by
an increase in the nuclear levels of p65 and p50 (Fig. 3C). The
amount of several upstream signaling molecules involved
in TNF-induced NF-κB activation such as TNF receptor-associ-
ted factor 1 and 2, receptor-interacting protein, and IκB ki-
nase-α as well as actin were not changed (Fig. 3D). The expres-
sion 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 oligo-
nucleotide 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. 4A). 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 con-
sensus sequence (Sp-1) to monitor quality and equal loading,
which was not changed under these conditions (Fig. 4A). Pre-
vious studies demonstrate a functional and physical associa-
tion 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 in-
creased 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.
4B). To show the specificity of the bands the IP was carried out without
any extract (marked by an asterisk). In addition, control IP reactions were performed using antibodies against cyclin B1 (Fig. 4B) 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. 5A). When we performed control experiments with cells expressing C/EBPβ, we observed an elevated DNA binding of this transcription factor (Fig. 5B) 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. 5B, 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. 5C). 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. 6A). Specificity of the bands was confirmed by oligonucleotide competition experiments and supershift analysis (Fig. 6A, 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. 6B).

**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.
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, the slightly reduced IκB-α level was found that may be due to a low

![Fig. 4. DNA binding activity of C/EBPβ as well as association of C/EBPβ protein with p65 are increased in TNF-tolerant cells.](image)

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.
level of ongoing signaling associated with IkB-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 IkB 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.
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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