Differential Effects of Lipopolysaccharide and Tumor Necrosis Factor on Monocytic IκB Kinase Signalsome Activation and IκB Proteolysis*

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The inflammatory mediators lipopolysaccharide (LPS) and tumor necrosis factor (TNF) are potent activators of NF-κB. This study compared the effect of these stimuli on endogenous IκB kinase (IKK) signalse activation and IκB phosphorylation/proteolysis in human monocytic cells and investigated the role of the signalsome components IKK-α, IKK-β, NF-κB-inducing kinase (NIK), IKK-γ (NF-κB essential modulator), and IKK complex-associated protein. Kinase assays showed that TNF elicited a rapid but short-lived induction of IKK activity with a 3-fold greater effect on IKK-α than on IKK-β, peaking at 5 min. In contrast, LPS predominantly stimulated IKK-β activity, which slowly increased, peaking at 30 min. A second peak was observed at a later time point following LPS stimulation, which consisted of both IKK-α and -β activity. The endogenous levels of the signalsome components were unaffected by stimulation. Furthermore, our studies showed association of the IKK-α/β heterodimer with NIK, IκB-α and -ε in unstimulated cells. Exposure to LPS or TNF led to differential patterns of IκB-α and IκB-ε disappearance from and re-assembly with the signalsome, whereas IKK-α, IKK-β, and NIK remained complex-associated. NIK cannot phosphorylate IκB-α directly, but it appears to be a functionally important subunit, because mutated NIK inhibited stimulus-induced κB-dependent transcription more effectively than mutated IKK-α or -β. Overexpression of IKK complex-associated protein inhibited stimulus-mediated transcription, whereas NF-κB essential modulator enhanced it. The understanding of LPS- and TNF-induced signaling may allow the development of specific strategies to treat sepsis-associated disease.

Bacterial lipopolysaccharide (LPS), a glycolipid membrane component, accounts for many of the cellular responses to Gram-negative infections, including sepsis and the development of septic shock (1). Once patients exhibit the septic shock syndrome and associated organ failure, a high mortality rate of the affected individuals is observed (1). The interaction of LPS with cells of, for example, the monocytic lineage appears to be especially important because subsequent cellular activation results in the release of highly active proinflammatory molecules, such as tumor necrosis factor (TNF) and interleukin (IL)-1β, which in turn mediate systemic effects (2, 3). One of the key regulators of LPS-induced, as well as TNF- and IL-1β-induced, gene expression at the interface between signal transduction and transcription is the transcription factor NF-κB (4–6).

There is an ever-increasing body of evidence that suggests that NF-κB/Rel plays an important role in gene regulation during inflammatory and immune reactions in a variety of disease settings (7–11). NF-κB regulatory sequences have been found in promoters or enhancers of numerous genes, e.g. coding for the proinflammatory cytokines TNF and IL-1β, the chemokines IL-8 and monocyte chemotactic protein-1; several adhesion molecules, including intercellular adhesion molecule-1 and vascular cell adhesion molecule-1; and the procoagulatory protein tissue factor (5, 12–15). The prototypic NF-κB dimer consists of the subunits p65 (RelA) and p50, although other subunits, such as c-Rel, RelB, and p52 have been identified (5). These dimeric NF-κB complexes are present in the cytosol in an inactive state bound to inhibitory proteins, collectively termed IκB (5, 14, 15). Several IκB proteins have been identified, including IκB-α (16, 17), IκB-β (18), and the more recently cloned IκB-e (19). A variety of agents such as microbial pathogens (for example, LPS), as well as inflammatory or lymphoproliferative cytokines, including TNF and IL-1β, induce the activation of NF-κB (5, 6, 14). This is mediated by a network of kinases leading to the phosphorylation of IκB, which is subsequently degraded in an ubiquitin-dependent step by the proteasome, a multicatalytic high molecular weight protease system (16, 20–22). The removal of the inhibitor IκB allows the translocation of the thus activated NF-κB dimer into the nucleus.

The signaling mechanisms that lead to the phosphorylation of IκB, and thereby NF-κB activation, are only partly understood and characterized for TNF, IL-1β, and LPS (23–27). A high molecular weight IκB kinase (IKK) complex, also named the signalsome, has recently been identified, and it is postulated to represent a bona fide IκB kinase (28–32). Several kinase-active components of this complex have been cloned, namely IKK-α, IKK-β, and NF-κB-inducing kinase (NIK) (28–33). Furthermore, two adaptor or scaffold proteins have been found, IKK-γ/NF-κB essential modulator (NEMO)/IKK-associated protein 1 (34–36) and IKK complex-associated protein (IKAP) (37), which have been suggested to stabilize the high molecular weight complex and/or regulate the kinase activity. Additional kinases, such as mitogen-activated protein kinase/
extracellular-regulated kinase kinase kinase-1 (38) or mitogen-activated ribosomal S6 protein kinase (p70S6k) (39), may assemble with the signalosome following activation of cells by certain stimuli. For example, activation of NF-κB by TNF appears to involve several upstream signaling proteins, including TRAF2 and RIP, which in turn activate NIK, thereby initiating a signaling pathway that results in IκB phosphorylation (23–25).

It should be mentioned that the functionality of these IKK signalosome components has been mostly established under relatively unphysiological overexpression conditions in easy to handle cell lines, such as HeLa, 293, or Chinese hamster ovary B (28–32). TNF and IL-1β were almost exclusively used as activators of these systems (28–32), although LPS has recently been reported to activate IKK (27). The regulation of expression and activation of endogenous signalosome components in cell types and conditions relevant for inflammation has not been investigated in great detail. The present study was therefore designed to compare the effect of TNF and LPS on endogenous IKK signalosome activation and IκB phosphorylation/proteolysis in mononuclear cells. In addition, we investigated the involvement of IKK-α, IKK-β and NIK, as well as the adaptor proteins IKK-γ (NEMO) and IKAP, in these signaling cascades.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Reagents—THP-1 human mononuclear cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were maintained in suspension in RPMI 1640 (Glutamax-1, low endotoxin, Seromed, Berlin, Germany) containing 7% fetal calf serum (Myoclon super plus, low endotoxin, BioWhittaker, Walkersville, MD), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc., Eggenstein, Germany) as described (40). For the experiments, the cells were plated at a density of 3 × 10^6 cells/well in six-well culture dishes. Peripheral blood mononuclear cells were isolated from blood samples of normal donors by the Ficoll-Hypaque method as described (41). Monocytes were isolated from mononuclear cells by adherence to achieve a purity of approximately 90% as determined by flow cytometry. The adherent monocytes were cultured overnight in the same medium as THP-1 cells with 10% fetal calf serum before the experiment was started. LPS (Escherichia coli 0111:B4) and TNF-α from Sigma (St. Louis, MO) were also purchased from Sigma. Endotoxin contamination was screened by the limulus amoebocyte lysate assay (BioWhittaker), and only reagents with an endotoxin content of <10 pg/ml were used in the experiments. A potential toxicity of the cell culture conditions applied was monitored by cell morphology and count, trypsin blue dye exclusion, and the WST-1 cell toxicity test (Roche Molecular Biochemicals, Mannheim, Germany).

Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Cytosolic extracts were isolated as described earlier (42). Electrophoresis was performed with 12.5% polyacrylamide gels (0.1% SDS) as described previously (40). The proteins were transferred to a nitrocellulose membrane using the wet blotting technique. After transfer, the membranes were incubated with polyclonal antibodies against the carboxyl-terminal domains of the inhibitors IκBα, -β, and -γ (Santa Cruz Biotechnology, Heidelberg, Germany), or e (a kind gift from Prof. N. Rice, NCI-Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, MD), against the kinases IKK-α, -β, or NIK (Santa Cruz Biotechnology), as well as the scaffold protein IKK-γ (using an antibody raised against the mouse homologue of human IKK-γ, namely NEMO, a gift from Prof. Alain Israel and co-workers, Institut Pasteur, Paris, France) or with a monoclonal antibody against α-actin (Sigma). In some experiments, an antibody recognizing only the form of IκB-α phosphorylated at Ser-32 was used (Calbiochem, Bad Soden, Germany). This incubation was followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany). The protein was visualized on x-ray film using the Western blot chemiluminescent reagent plus (NEN Life Science Products). The protein size was confirmed by molecular weight standards (Amersham Pharmacia Biotech, Braunschweig, and Bio-Rad).

Immunoprecipitation—Cytosolic extracts were subjected to immunoprecipitation (IP) (35, 43) in either TNT buffer (200 mM NaCl; 20 mM Tris-HCl, pH 7.5; 1% Triton X-100; 1 mM dithiothreitol; 0.5 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride; and 0.75 μg/ml each leupeptin, antipain, aprotinin, pepstatin A, and chymostatin; Sigma) or TN buffer (as TNT but without Triton X-100). Unspecific binding was blocked by incubation with 1 μg of normal rabbit IgG (Santa Cruz Biotechnology) and 25 μl of 6% protein A-agarose (Roche Molecular Biochemicals) for 30 min at 4 °C followed by immunoprecipitation for 2 h at 4 °C with 1 μg of anti-kinase antibody (Santa Cruz Biotechnology) and 25 μl of 6% protein A-agarose. After washing three times with TNT/TN buffer as appropriate and three times with kinase buffer (20 mM HEPS, pH 8.0; 10 mM MgCl2; 100 μM Na3VO4; 20 mM β-glycerophosphate; 50 mM NaCl; 2 mM dithiothreitol; 0.5 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride; and 0.75 μg/ml each leupeptin, antipain, aprotinin, pepstatin A, and chymostatin), the precipitated proteins were either analyzed by Western blot or kinase assay.

Kinase Assay—IP was carried out as described above and followed by the kinase assay as described (35, 43). The kinase reaction was performed in kinase buffer for 30 min at 30 °C in the presence of 5 μCi of [γ-32P]ATP (NEN Life Science Products) and 500 ng of the substrate GST-IκB-α (Santa Cruz Biotechnology). Proteins were analyzed on 12.5% polyacrylamide gels (0.1% SDS), dried, and visualized by autoradiography.

Transfection of THP-1 Cells—The following reporter plasmids were utilized in transfection studies: 3xβ.B luciferase reporter plasmid containing three copies of a prototypic (5’-GGAGGATTTCC-3’) κB site (42); TNFkop.luc, comprising 1108 base pairs of the TNF promoter region (42); and pGL2-IL-8, containing 420 base pairs of the IL-8 promoter region (44). Overexpression plasmids used in our studies were: pcDNA3.1(+) vector plasmid (all gifts from TulipIns, South San Francisco, CA), NEMO, IκB-α (wild type and mutated), IκB-β (gifts from Prof. Alain Israel and co-workers, Institut Pasteur), and antisense IKK-α (a gift from Prof. Michael Karin, University of California-San Diego, La Jolla, CA). RCMV (Invitrogen, Groningen, Netherlands) containing no insert was used as a negative control in the overexpression experiments. These plasmids, either alone or in combination, were transiently co-transfected with a constitutively active Renilla luciferase control plasmid, pRLtk (Promega, Mannheim, Germany), into THP-1 cells using a DEAE-dextran-based protocol (13, 44). After transfection, cells were plated out at a density of 2 × 10^6 cells/ml of RPMI medium with 7% fetal calf serum in a six-well plate and incubated for 2 days. After this time, the cells were stimulated for 5 h with either TNF or LPS. Subsequent to stimulation, the cells were lysed, and the luciferase activity was determined using the dual luciferase reporter assay system (Promega). The results are expressed as firefly luciferase relative light units divided by the values in relative light units obtained for the Renilla luciferase.

Pulse-Chase—Pulse-chase experiments were carried out as described (40). Briefly, THP-1 cells were washed in complete medium (RPMI 1640 medium) without Met/Cys (BioWhittaker) and resuspended in this medium at a density of 3 × 10^6 cells/ml. After incubation in a 12.5-cm² culture flask at 37 °C and 5% CO2 for 30 min, 100 μCi of [35S]Met/Cys (NEN Life Science Products) was added, followed by a labeling time of 3 h. The cells were then washed in the above-mentioned medium plus 500 μM Met/Cys (Boehringer Ingelheim, Ingelheim, Germany), resuspended in this medium, and plated out at a density of 3 × 10^6 cells/well in a 12-well plate. The plate was returned to the incubator, and extracts were made at various times later. Cytosolic and nuclear extracts were prepared as described (42), except that the nuclei were incubated in Buffer B (0.02 μM HEPS, pH 7.0, 0.1 mM KCl, 0.1 mM NaCl, 5 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.75 μg/ml each leupeptin, antipain, aprotinin, pepstatin A, chymostatin, Sigma) plus 0.5% Nonidet P-40 for 30 min on ice prior to disruption by drawing through a narrow pipette tip. Trichloroacetic acid-precipitated proteins were assayed for cpm. IP of equal volumes of protein was carried out using an anti-IKK-α or -β antibody, and the resulting protein precipitate was separated by SDS-polyacrylamide gel electrophoresis. Autoradiographs of the vacuum-dried gels were analyzed densitometrically, the values were corrected for the cpm loaded, and the half-life was calculated for each protein.

RESULTS

Differential Activation of IKK-α and -β—Initial experiments were performed to assess potential selective effects of LPS and TNF on endogenous kinase active signalosome subunits in human mononuclear cells. Kinase assays were carried out to monitor the activity of IKK-α and -β following incubation with these stimuli. Cytosolic extracts from THP-1 cells were subjected to IP with an anti-kinase antibody and the ability of the precipitated proteins to phosphorylate GST-IκB-α in vitro was analyzed. It was shown that 1% Triton X-100, present in
The peak IKK activities induced by TNF (at 5 min) or LPS (at 30 and 75 min) were analyzed by densitometry and compared. In general, the activation of IKK by TNF was much more pronounced than that mediated by LPS at the time points of peak activation (Fig. 1C). The effect of TNF on IKK-α was 3-fold greater than that on IKK-β at 5 min of incubation, whereas LPS almost exclusively activated IKK-β after a 30-min stimulation period (Fig. 1, C and D). However, at 75 min of incubation, LPS appeared to induce both IKK-α and -β activity, to approximately the same extent (Fig. 1D).

**Stimulus-induced IKK-α and -β Activity ex Vivo in Human Monocytes**—Kinase assays were also performed with cytosolic extracts from human adherent monocytes following either TNF (1.6 ng/ml) or LPS (100 ng/ml) stimulation. The experiments showed that TNF rapidly induced IKK activity (IKK-α > IKK-β) with an early peak at 5 min (Fig. 2A). Similar to the effect seen in THP-1 cells, LPS exposure led to a slow increase in predominantly IKK-β activity with a peak at 30 min (Fig. 2B).

**The Role of NIK in IκB Phosphorylation**—Next, we tested whether monocytic endogenous NIK, another kinase-active signalosome component, can itself directly phosphorylate IκB-α substrate. For this purpose, IP with an antibody raised against NIK was performed under different conditions. As already mentioned, the presence or absence of Triton X-100 in the IP buffer is important with respect to the integrity of the precipitated complex. TN buffer (without detergent) or TNT buffer (1% Triton X-100) was used in the IP step following LPS or TNF stimulation of THP-1 cells. The subsequent kinase assay showed that in the absence of detergent, a strong kinase activity following LPS stimulation was detected (Fig. 3, TN). However, when detergent was present and the precipitated protein was almost exclusively NIK alone (data not shown), no phosphorylation of the substrate was observed (Fig. 3, TNT). A similar result was seen when TNF was used as the stimulus (Fig. 3). It should be noted that in some experiments, the peak activity following exposure to TNF was observed at an earlier time point than 5 min. The results suggest that in monocytic cells, NIK itself cannot phosphorylate the substrate IκB-α. The activity seen in the absence of Triton X-100 must be due to kinases that have co-precipitated with NIK in the signalosome complex.

**Fig. 1. Differential activation of endogenous IKK-α and -β by TNF and LPS in monocytic cells.** A, cytosolic extracts from THP-1 cells incubated with TNF (1.6 ng/ml) were subjected to IP using TNT buffer with either IKK-α or -β antibodies as indicated. A kinase assay was performed using the substrate GST-IκB-α, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. 32P-Phosphorylated (circled P) GST-IκB-α is shown by the arrow. B, THP-1 cells were incubated with LPS (1 μg/ml) for the times indicated, and IP followed by kinase assay for IKK-α and -β activity was performed as in A. C, comparison between LPS- and TNF-induced IKK activity. Experiments were performed as described in A and B, and the peak activities (TNF, 5 min; LPS, 30 min) were analyzed. Representative films were scanned and quantified densitometrically. Induction above base line was calculated, and the results are depicted. D, kinase assays were performed as described in B, and the two peak activities of IKK after LPS stimulus (30 and 75 min) were analyzed as in C.

The TNT buffer used for IP, disrupts the integrity of the signalosome complex (data not shown; see also Fig. 3), allowing us to distinguish between IKK-α and -β activity.

TNF stimulation (1.6 ng/ml) elicited a rapid but rather short-lived effect, with a maximum response from both IKK-α and -β at 5 min of incubation (Fig. 1A), although the IKK-α activity was greater than that of IKK-β. TNF-induced IKK-α activity then decreased sharply after 20 min and had reached near base-line levels by 75 min. A similar pattern was observed for IKK-β (Fig. 1A and data not shown).

On the other hand, LPS stimulation (1 μg/ml) had a major effect on IKK-β activity, which slowly increased and peaked at 30 min (Fig. 1B), whereas an almost negligible increase in the activity of IKK-α was observed at this time point. We also detected a second peak of IKK activity, comprising both IKK-α and -β activation, following LPS stimulation at 75 min (Fig. 1B and see below).
Level of the Signalsome Components in Monocytic Cells—Before further analysis of the signalsome complex (see below), we wanted to establish the presence and level of endogenous IkB kinase-active components and associated subunits of the signalsome in monocytic cells. An additional aim was to examine whether the endogenous concentration of these molecules is changed following incubation with potent monocyte activators, such as LPS and TNF. For this purpose, THP-1 monocytic cells were incubated with LPS or TNF, and the level of IKK-α, -β, and -γ (NEMO), as well as of NIK, was determined by Western blot analysis.

In unstimulated monocytic cells, we detected a significant endogenous level of all the above-mentioned signaling proteins (IKK-α, IKK-β, NIK, and IKK-γ), as shown in Fig. 4. Following treatment with LPS or TNF, the levels of IKK-α and IKK-β (Fig. 4A and data not shown), as well as NIK and IKK-γ (Fig. 4B and data not shown), were not significantly altered, suggesting a tight regulation of these components in monocytes. As a control, the concentration of α-actin was also monitored; its level was not influenced by any of the incubation conditions used (Fig. 4A).

Northern blotting for the mRNA coding for IKK-α and -β revealed only a weak signal in both stimulated and unstimulated cells (data not shown). Because this low level of mRNA contrasts with the relatively high constitutive expression of the proteins in this system, we decided to investigate the half-lives of these kinases by pulse-chase analysis. Using this technique the half-life of endogenous IKK-α and -β was found to be 12.8 ± 0.1 h (n = 2) and 12.7 ± 0.1 h (n = 2), respectively, which indicates a relatively high stability of both signaling molecules in monocytic cells.

Analysis of the Signalsome Complex—The association of the endogenous kinase-active signalsome components (IKK-α, IKK-β, and NIK) and the inhibitor proteins IkB-α and -β was investigated in monocytes under various conditions. Initially, IP using a monoclonal antibody raised against IKK-α was performed on cytosolic extracts, followed by Western blot analysis of the co-precipitated proteins. In the absence of detergent in the IP buffer, we saw the complex components IKK-β and NIK, as well as bound IkB-α and -β, co-precipitated by the anti-IKK-α antibody from unstimulated cell extracts (Fig. 5 and data not shown), whereas in the presence of Triton X-100, only the directly precipitated protein was observed (data not shown). This demonstrated the occurrence of IKK-α/β heterodimers in monocytic cells and showed that these subunits are already associated with NIK as well as IkB-α and -β in unstimulated cells. The complex precipitated in the absence of detergent, as far as we were able to ascertain, did not alter in IKK-α, IKK-β, or NIK composition throughout treatment with LPS or TNF (Fig. 5 and data not shown). A different pattern was observed for IkB-α, which, following LPS stimulation, slowly disappeared from the complex over 60 min, being undetectable at this time, and then gradually reassociated (Fig. 5A). This agrees with the phosphorylation and proteolysis pattern that we observed in parallel studies (Fig. 5A). In contrast, TNF stimulation led to a clear phosphorylation of IkB-α after 1 min of incubation (Fig. 5B). This phosphorylated form remained bound to the complex until 5 min, at which time it disappeared. The inhibitor protein then reassembled with the signalsome complex by 45 min, in its unphosphorylated form. The other inhibitory protein, IkB-β, was also initially associated with the signalsome complex and, after TNF stimulation, gradually disappeared. It was not found to dissociate, which is presumably due to the limited time frame studied here (see also Fig. 8).

Overexpression of Wild Type or Mutated Kinase-active Signalsome Components—Next, we examined the effect of the kinase-active proteins IKK-α and -β, as well as NIK, on LPS- and TNF-induced NF-κB-dependent transcription. We transfected THP-1 cells with overexpression vectors for both wild type and mutated kinases together with luciferase reporter plasmids.

Initially, transfection efficiency was assessed by Western blotting of cytosolic extracts from kinase-transfected THP-1 cells, using an antibody raised against the Flag tag present on the overexpressed proteins. This confirmed that the protein was being overexpressed at equal levels in our system and that the control vector without a tagged overexpression protein showed no signal (data not shown).

The effect of both wild type and mutated IKK-α, -β, and NIK was investigated using TNF and LPS as stimuli and 3xκB.luc as a reporter plasmid. The mutated, kinase-inactive proteins were all able to reduce LPS- or TNF-induced NF-κB-dependent transcription below the level seen with the wild type proteins, with an effectiveness of IKK-α < IKK-β < NIK (Fig. 6A). NIK wild type alone, in the absence of a stimulus, was also capable of causing an increase in NF-κB-dependent transcription (4.7-fold over the base-line level of transcription observed in the presence of mutated, kinase-inactive NIK). Neither wild type IKK-α nor -β was able to affect transcription in the absence of a stimulus. In addition, antisense IKK-α, when overexpressed in THP-1 cells, was able to inhibit LPS-induced NF-κB-dependent, as well as TNF promoter-dependent, transcription (data not shown).
and IP in TN buffer with anti-IKK-
lowing activation. THP-1 cells were incubated with LPS (either 3xk
To obtain some insight into possible functional aspects of
plex—
strong as that seen when all three kinases were inactive.
the weakest effect and mutated NIK having an effect almost as
proteins were then analyzed by Western blot for the presence of the
In some cases, an antibody that exclusively recognizes this protein in its
for I
LPS- and TNF-induced
effects. IKAP overexpression led to a partial inhibition of both
IKAP or NEMO in THP-1 cells resulted in two contrasting
experiments were carried out. Interestingly, overexpression of
for I
k
B-dependent transcription, seen with either 3xkB.luci or IL-8 and TNF promoter-dependent con-
structs (Fig. 7A and data not shown). Both LPS- and TNF-
induced 3xkB.luci or IL-8 promoter-dependent transcription were inhibited by approximately 50%. IKAP alone influenced kB-dependent transcription to a certain degree, leading to an increase of 2.2-fold above base-line. NEMO, however, caused a slight rise (1.6-fold) in the level of kB-dependent transcription, over and above the effect seen with LPS and TNF alone (Fig. 7B). In the absence of external stimuli, NEMO-transfected cells also showed a small increase (1.8-fold) in transcriptional activity
over the base-line levels.

IskB Proteolysis and Overexpression of IskB-ε and -β—To in-
vestigate whether the differential activities of IKK after LPS or
TNF stimulation affect the proteolysis and resynthesis of the
recently cloned IskB-ε, as well as IskB-β, we studied the kinetics of IskB degradation and subsequent resynthesis following either LPS or TNF stimulation. Maximal degradation of IskB-ε occurs relatively late following LPS exposure, after a 90-min stimulus (Fig. 8A). This inhibitory protein was rapidly resynthesized, reaching base-line expression levels by 120 min of incubation. A similar degradation pattern was found for IskB-β after LPS challenge, but in contrast to IskB-ε, this was not followed by resynthesis of the IskB-β protein, at least within the time frame studied here (Fig. 8A). IskB-ε was degraded much more rapidly by incubation with TNF, with an almost complete degradation achieved by 10 min (Fig. 8B), followed by a relatively long lag phase and thereafter resynthesis of the protein by 75 min. IskB-β also quickly underwent proteolysis after TNF stimulation, with no significant resynthesis up to 120 min later (Fig. 8B).

We also examined the effect of overexpression of the inhibi-
proteins IskB-ε and -β in THP-1 cells to investigate whether these proteins are involved in shutdown of NF-xB activity. A contrasting result was seen between the two proteins. Trans-
faction of an IskB-ε wild type vector led to a reduction in stim-
ulus intensity of kB-dependent transcription following incuba-
tion with LPS or TNF (Fig. 8C). The mutated form of IskB-ε, which cannot be phosphorylated, also caused inhibition of the above-mentioned stimulus-induced transcription and was in fact even more effective than the wild type molecule (Fig. 8C). Both 3xkB.luci and IL-8 promoter-dependent transcription was similarly affected. In contrast, IskB-β overexpression led to a reinforcement, rather than an inhibition, of the LPS- and TNF-
induced stimulation for all the plasmids examined (Fig. 8D). The signal intensity was more markedly increased (3-fold) in 3xkB.luci-transfected than in IL-8 promoter construct-trans-
fected cells. IskB-β overexpression alone, in the absence of ex-
ternal stimuli, led to a marginal rise in the level of transcription, more clearly visible in IL-8 promoter construct-transfected cells than in those transfected with 3xkB.luci.

DISCUSSION

Both LPS and TNF belong to the most potent activators of
NF-xB in cells of the monocytic lineage (5, 6, 14). However, the signaling cascades leading to NF-xB activation induced by these stimuli appear to differ markedly (24, 26, 43). The present paper elucidates the effects of LPS, as well as TNF, on endogenous monocytic IKK signalosome activation and subse-
quent phosphorylation/proteolysis of the xB inhibitory pro-
teins. Our study reveals that a completely different signalosome activation pattern results from incubation of cells with these two highly effective stimulating molecules.

Incubation of human monocytic cells with LPS predomi-
nantly activated IKK-β in our kinase experiments using IskB-α as a substrate with a slow increase leading to a peak of activa-
tion at 30 min. At this time point, only a negligible activation of IKK-α by LPS was observed. In addition, a second IKK activation peak was seen at a later time point (75 min), which

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**Fig. 5. Composition of the monocytic signalosome complex following activation.** THP-1 cells were incubated with LPS (A) or TNF (B) for the times indicated. IP+WB, Cytosolic extracts were prepared, and IP in TN buffer with anti-IKK-α was carried out. The precipitated proteins were then analyzed by Western blot for the presence of the signalosome components IKK-α and -β, as well as IskB-α and -ε. The double arrowhead for 1IskB-α in B indicates the phosphorylated (upper) and unphosphorylated (lower) form. WB, in parallel experiments, cytosolic extracts were examined by Western blot for the presence of IskB-α. In some cases, an antibody that exclusively recognizes this protein in its stimulus-induced phosphorylated form (circled P) was applied.
consisted of both IKK-β and IKK-α activity. This may be caused by continuous stimulation induced by the presence of LPS in the medium and/or autocrine mechanisms. A recent report describes the activation of IKK by LPS in THP-1 cells (but does not distinguish between IKK-α and -β) with a maximum at 60 min (27). In these experiments, the exclusive dominant-negative expression of IKK-β, but not IKK-α, partially inhibited LPS-induced transcriptional activity (27). This is in contrast to our study, as well as recently published results (26), in which the expression of both dominant-negative IKK-β and IKK-α exerted inhibitory effects. The differences between these studies (peak of activation and effect of dominant-negative IKK-α) could be due to differences in THP-1 sublines/cell types (13). When these data are taken together, our study indicates that IKK-β is the major target of LPS signaling but also suggests a modest activation of IKK-α, especially at a later time point.

In contrast, TNF preferentially activated IKK-α with a dramatic, very early peak of activation at 5 min. Furthermore, a significant effect of TNF on IKK-β activity was observed that was approximately 3-fold lower than that on IKK-α but showed a similar time course of activation. No second peak at a later time point was observed in the presence of TNF. A TNF-induced early peak of IKK activity was also observed in HeLa cells, when the whole signalsome complex was isolated and used for kinase assays, as well as in IKK-α and IKK-β overexpression studies (28, 29, 32). In the latter experiments, equal effects of TNF and IL-1 on IKK-α and IKK-β were observed (28), which contrasts with our TNF studies and may potentially be due to the fact that the functionality of overexpressed proteins does not precisely mirror the endogenous situation.

Our studies suggest that endogenous NIK, another kinase-active signalsome component (30, 31), is not able to directly phosphorylate IkB-α following LPS or TNF exposure of mono-
Differential Effects of LPS and TNF on the IKK Complex

In unstimulated monocytic cells, the endogenous signalsome as well as TNF-stimulated, transcriptional activity. In 293 cells, an increased level of IKAP has been shown to inhibit TNF- and IL-1-induced NF-κB-dependent transcription, and it was suggested that it may inhibit cytokine signaling by titrating out each individual component of the IKK signalsome (37), e.g. a higher level of IKAP may compete with kinase-active molecules. In contrast, the presence of exogenous NEMO enhanced both LPS- and TNF-induced transcription in our experiments. Recently, it has been shown that IKK associated protein 1 preferentially interacts with IKK-β (36), and it has been suggested that this protein provides a scaffold upon which IKK-β-containing complexes could be localized to the upstream components of the NF-κB activation cascade (34–36). Alternatively, a higher level of IKK-γ in the signalsome may lead to an increased recruitment of the IκB substrate or proteasome particles, which could also be a reason for the observed enhancement.

One technical issue should be discussed, and that is the reliability of transfection/overexpression studies. In our experiments, dominant-negative expression of IKK-α, IKK-β, or NIK in monocytic THP-1 cells led only to a partial inhibition of both LPS- and TNF-activated NF-κB-dependent transcription, with triple transfection most effective, in contrast to earlier studies using HeLa or 293 cells demonstrating a much more profound effect (26, 28, 31, 45). In addition, using the transfection strategies, we were not able to confirm the LPS- or TNF-induced differential pattern of IKK-α and IKK-β activation seen in our kinase assays, which we consider a more clean and direct approach. Furthermore, we were not successful in establishing a direct effect of wild type IKK-α and IKK-β on NF-κB-regulated transcriptional activity observed under the cell culture and overexpression conditions already mentioned (28, 31). One reason for these discrepancies may be a technical problem, which is the low transfection efficiency using monocytic cells. Another reason could be that overexpression of proteins may create unphysiological conditions (46). For example, exogenously added IKK may form nonphysiological dimers/complexes, which may display lower or dysregulated kinase activities (31, 47). Therefore, endogenous IKK-α/β heterodimers could overcome at least partially the effect of the specifically overexpressed proteins in monocytic cells and bias our results such that we did not achieve a clear cut kinase activation pattern.

When these data are taken together, LPS appears to only modestly but preferentially stimulate IKK-β activity over a longer time interval, whereas the effect of TNF on the IKK kinase-active molecules (IKK-α > IKK-β) is marked and rapid but appears to be rather limited. Because the effect of LPS on...
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IKK activity was much weaker than that of TNF, one could argue that the IKK pathway represents just one of several parallel signaling pathways induced by LPS leading to NF-κB activation. In fact, LPS is known to initiate signal transduction cascades resulting in a spray-like activation of a network of kinases, G-proteins, cyclic nucleotide metabolizing enzymes, lipid second messengers, and ion channels, some of which even may be not related to NF-κB (2). Furthermore, additional kinases may be involved in LPS-mediated IκB phosphorylation (39, 48, 49). On the other hand, a modest but continuous activation of IKK by LPS may prove essentially as effective as the rapid but short-lived activation of IKK seen following TNF challenge of cells. In this context, it should be noted that LPS or TNF led to a different proteolysis pattern of IκB-α, -β, and -ε in our work, which incidentally completely agreed with the IKK activation pattern, but finally led under both activation conditions to a marked removal of the inhibitory proteins.

One important issue that has to be addressed in the future is the question of why LPS induces slow motion signaling, whereas TNF activates a rapid motion pathway, and what the question of why LPS induces slow motion signaling, or TNF led to a different proteolysis pattern of IκB-α, -β, and -ε in our work, which incidentally completely agreed with the IKK activation pattern, but finally led under both activation conditions to a marked removal of the inhibitory proteins.

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