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*J Clin Invest.* 1998;**102**(9):1645-1652. <https://doi.org/10.1172/JCI3877>.

### Research Article

Recognition of bacterial endotoxin (LPS) elicits multiple host responses, including activation of cells of the innate immune system. LPS exposure occurs repeatedly during septicemia, making strict regulation of gene expression necessary. Such regulation might prevent, for example, the continuous production of proinflammatory cytokines such as tumor necrosis factor (TNF), which could lead to severe vascular collapse. Tolerance to LPS is characterized by a diminished production of TNF during prolonged exposure to LPS, and is therefore likely to represent an essential control mechanism during sepsis. In the present study, which uses mice with genetic deletions of the proteins of NF-kappaB complex, we provide data demonstrating that increased expression of the p50 subunit of NF-kappaB directly results in the downregulation of LPS-induced TNF production. This contention is supported by the following observations: (1) tolerance to LPS is not induced in macrophages from p50<sup>-/-</sup> mice; (2) long-term pretreatment with LPS does not block synthesis of TNF mRNA in p50<sup>-/-</sup> macrophages (in contrast to wild-type macrophages); (3) ectopic overexpression of p50 reduces transcriptional activation of the murine TNF promoter; and (4) analysis of the four kappaB sites from the murine TNF promoter demonstrates that binding of p50 homodimers to the positively acting kappaB3 element is associated with development of the LPS-tolerant phenotype. Thus, p50 expression plays a key role in the development of LPS [...]

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## Regulation of an Essential Innate Immune Response by the p50 Subunit of NF- $\kappa$ B

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### Abstract

Recognition of bacterial endotoxin (LPS) elicits multiple host responses, including activation of cells of the innate immune system. LPS exposure occurs repeatedly during septicemia, making strict regulation of gene expression necessary. Such regulation might prevent, for example, the continuous production of proinflammatory cytokines such as tumor necrosis factor (TNF), which could lead to severe vascular collapse. Tolerance to LPS is characterized by a diminished production of TNF during prolonged exposure to LPS, and is therefore likely to represent an essential control mechanism during sepsis. In the present study, which uses mice with genetic deletions of the proteins of NF- $\kappa$ B complex, we provide data demonstrating that increased expression of the p50 subunit of NF- $\kappa$ B directly results in the downregulation of LPS-induced TNF production. This contention is supported by the following observations: (1) tolerance to LPS is not induced in macrophages from p50<sup>-/-</sup> mice; (2) long-term pretreatment with LPS does not block synthesis of TNF mRNA in p50<sup>-/-</sup> macrophages (in contrast to wild-type macrophages); (3) ectopic overexpression of p50 reduces transcriptional activation of the murine TNF promoter; and (4) analysis of the four  $\kappa$ B sites from the murine TNF promoter demonstrates that binding of p50 homodimers to the positively acting  $\kappa$ B3 element is associated with development of the LPS-tolerant phenotype. Thus, p50 expression plays a key role in the development of LPS tolerance. (*J. Clin. Invest.* 1998. 102:1645–1652.) Key words: lipopolysaccharide • tumor necrosis factor • macrophages • sepsis • regulation of transcription

### Introduction

Nosocomial infections cause substantial morbidity and mortality especially when they progress to shock and multiorgan fail-

ure (1). Infection with Gram-negative bacteria is a leading cause of such problems. This group of pathogens contains a membrane glycolipid known as endotoxin (LPS). LPS activates cells of the innate immune system so that host defense responses to the pathogen are mobilized to eliminate the infection. This includes induction of a multitude of new genes encoding proinflammatory cytokines such as TNF and IL-1 as well as other proteins with pro- or anti-inflammatory properties (2). Septicemia is most likely accompanied by multiple exposures to bacterial products such as LPS (1). In this setting, unregulated production of proinflammatory cytokines could result in systemic changes which progress from protective host defense responses to the injurious events that contribute to the septic shock syndrome. Thus, to fully understand the pathogenesis of septic shock, one must determine how responses to multiple exposures of LPS are regulated. Approximately 50 yr ago Beeson (3) characterized a phenomenon termed endotoxin tolerance that manifests itself with a variety of diminished in vivo responses to sequential injections of LPS. Subsequent studies have revealed that one important feature of LPS tolerance is altered responsiveness of monocytes/macrophages to LPS rechallenge after an initial exposure. Changes in LPS responsiveness are characterized by hypo-, normo-, or hyper-responsiveness to LPS depending on which cellular response is measured (4–9). Now, the phenomenon of endotoxin tolerance is better thought of as adaptation, or reprogramming of cellular responses (10). One notable change is a marked decrease in the synthesis of proinflammatory cytokines such as TNF when an initial LPS challenge is followed by a second exposure some hours later. Importantly, in vitro studies have shown that other stimuli such as heat-killed *Staphylococcus aureus* (HKSA)<sup>1</sup> are fully capable of stimulating LPS-tolerant cells to produce inflammatory mediators such as TNF indicating that all cellular pathways capable of producing TNF are not disabled but rather are selectively repressed (4, 5). Several recent reports have provided data indicating that tolerance to LPS requires synthesis of one or more proteins that interfere with LPS-induced signaling to the nucleus with a consequence of decreased transcription of genes such as TNF (8, 11, 12). Here, we provide data identifying overexpression of the p50 subunit of NF- $\kappa$ B and the binding of p50 homodimers to the  $\kappa$ B3 element of mouse TNF promoter as essential events in the downregulation of TNF expression during the LPS tolerant state.

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Received for publication 29 April 1998 and accepted in revised form 14 September 1998.

*J. Clin. Invest.*

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0021-9738/98/11/1645/08 \$2.00

Volume 102, Number 9, November 1998, 1645–1652

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1. Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; BrdUrd, 5'-bromo-2'-deoxyuridine; HKSA, heat-killed *Staphylococcus aureus*; PEM, peritoneal exudate macrophages.

## Methods

**Primary macrophage isolation and cell culture.** The generation of the p50<sup>-/-</sup> and cRel<sup>-/-</sup> mice have been described elsewhere (13, 14). Peritoneal exudate macrophages (PEMs) were obtained by lavage 3 d after intraperitoneal injection of thioglycollate. After plating, the nonadherent cells were removed by washing 4 h later. The cells were incubated in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 5% heat-inactivated FCS for 18–24 h before activation. RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin, 1 mM sodium pyruvate, nonessential amino acids, and 10% heat-inactivated FCS at 37°C in 5% CO<sub>2</sub>.

**Cell activators.** LPS was prepared from *Salmonella minnesota* Re595 bacteria as described (15). Before the use, the stock solutions (10 µg/ml) were sonicated. A clinical isolate of *Staphylococcus aureus* was kindly provided by Dr. T. Kirkland (V. A. Hospital, San Diego, CA). After propagation, the bacteria were washed three times in saline and boiled for 2 h. The final stock suspension (2 × 10<sup>11</sup> cells/ml) contained an undetectable amount (< 5 pg/ml) of LPS by *Limulus amoebocyte* lysate assay (corresponding to < 0.025 pg/ml at the concentration used for stimulation by HKSA). A dilution resulting in a ratio of 100 heat-killed bacteria per cell was used for stimulation.

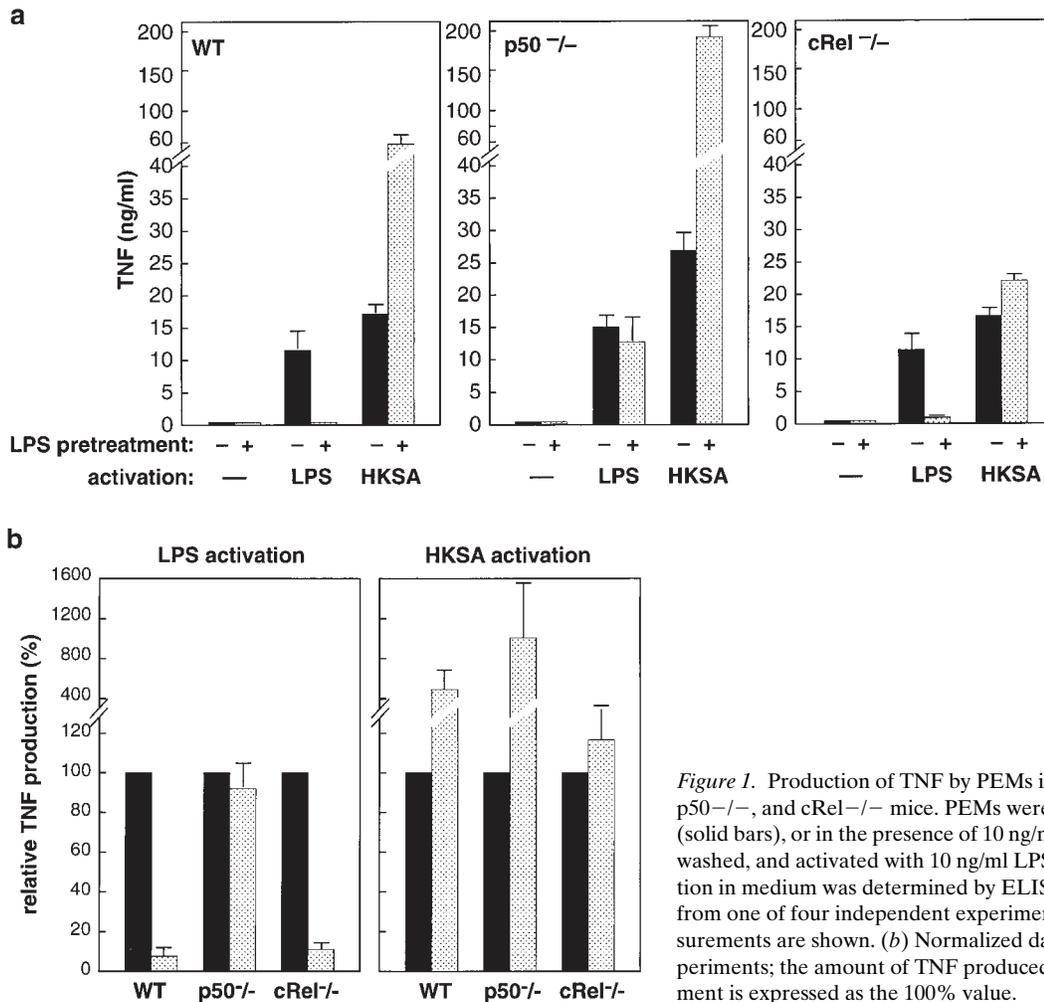
**Quantification of TNF.** PEMs were plated at 2 × 10<sup>6</sup> cells per well in 12-well plates. To induce tolerance to LPS, cells were incu-

bated in the presence of 10 ng/ml LPS for 18 h. After washing, the cells were activated for 6 h, and the supernatants were collected. The concentration of TNF in supernatants was determined by an ELISA, as recommended by manufacturer (PharMingen, San Diego, CA).

**Plasmid constructs.** The luciferase reporter construct was provided by Dr. J. Han (The Scripps Research Institute, La Jolla, CA). It contains a 1-kb fragment of the mouse TNF gene promoter (16) cloned into the Bgl II and Hind III sites of pGL2 Basic (Promega, Madison, WI). The reporter plasmid expressing β-galactosidase (pCMVβ) was from Clontech (Palo Alto, CA). The expression plasmids for p50 and p65 (pCMXp50, pCMXp65) were provided by Dr. I.M. Verma (The Salk Institute, San Diego, CA) (17).

**RNA isolation and analysis.** Total RNA was prepared and analyzed by Northern blot hybridization as described (18).

**Transfection and luciferase assay.** The plasmid DNA used for transfection was purified twice by CsCl gradient centrifugation. LPS was removed using endotoxin removal affinity resin (Associates of Cape Cod, Inc., Woods Hole, MA). DNA was introduced into the cells by calcium phosphate mediated transfection followed by the glycerol shock. Each plate received 1–2 µg of luciferase reporter plasmid containing the mouse TNF gene promoter, 0.05 µg of pCMVβ, and variable amounts of expression plasmids pCMXp50 or pCMXp65, as indicated in the text. The total amount of plasmid DNA was kept constant (6 µg) by adding the empty vector pcDNA3. 24 h after transfection, cells were activated for 6 h with 10 ng/ml LPS. Luciferase activity in cell extracts was determined using the Luciferase Assay System (Promega, Madison, WI). β-galactosidase activity was measured using the Galacto-Light β-galactosidase assay



**Figure 1.** Production of TNF by PEMs isolated from wild type (WT), p50<sup>-/-</sup>, and cRel<sup>-/-</sup> mice. PEMs were pretreated in the absence (solid bars), or in the presence of 10 ng/ml LPS (stippled bars) for 18 h, washed, and activated with 10 ng/ml LPS or HKSA. TNF concentration in medium was determined by ELISA. (a) Representative data from one of four independent experiments; data from triplicate measurements are shown. (b) Normalized data from four independent experiments; the amount of TNF produced after the primary LPS treatment is expressed as the 100% value.

(Tropix, Inc., Bedford, MA), and the values representing relative transcription efficiency were used to normalize luciferase activities.

**Electrophoretic mobility shift assay.** The cell extracts for electrophoretic mobility shift assay (EMSA) were prepared by a modification of the method of Dignam et al. (19). In brief, cells in 6-well plates were washed with PBS, harvested, and resuspended in 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, and 10  $\mu$ g/ml aprotinin). After 10 min, 23  $\mu$ l of NP-40 (Boehringer Mannheim GmbH, Mannheim, Germany) was added, and the sample vortexed for 2 s. Nuclei were separated from cytosolic fractions by centrifugation at 13,000 g for 10 s. To isolate nuclear proteins, pelleted nuclei were resuspended in 25  $\mu$ l of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 0.1 mM PMSF) at 4°C. After 30 min, lysates were centrifuged at 13,000 g for 30 s, and supernatants containing nuclear proteins were collected. The oligonucleotides used in EMSA contained sequences (5'→3')  $\kappa$ B1(-850) GGGAGGG-GAATCCTTGAAT;  $\kappa$ B2(-655) GGTCCGTGAATCCCAG-GGC;  $\kappa$ B3(-510) AACAGGGGGCTTCCCTCCT; and  $\kappa$ B4(-210) GATCCGGAGGAGATTCCTTGATG. Antisera used in supershift analysis were sc-114X (p50) and sc109X (p65) (Santa Cruz Biotechnology, Santa Cruz, CA). Antiserum to C terminus of mouse cRel (#1266) was kindly provided by Dr. N. Rice (NCI-FCRDC, Frederick, MD).

**Ultraviolet cross-linking analysis.** A photoreactive  $\kappa$ B probe containing 5'-bromo-2'-deoxyuridine (BrdUrd) was prepared as described (18). DNA-protein complexes bound to the [<sup>32</sup>P]-labeled BrdUrd probe were resolved on native EMSA, UV cross-linked in the gel, excised, eluted from the gel, and analyzed by SDS-PAGE as described (18).

## Results

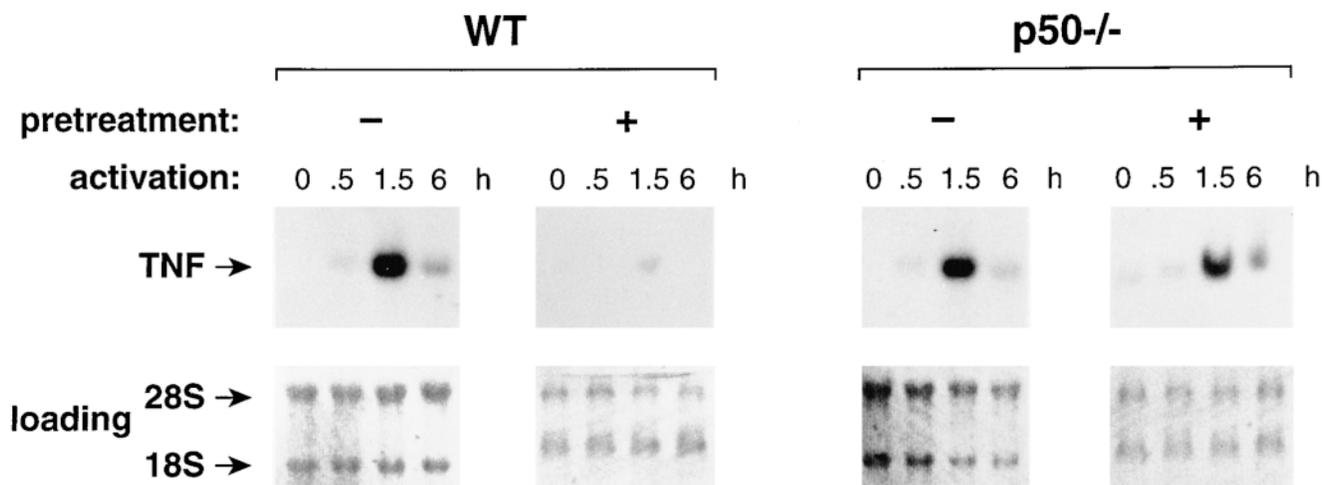
**Macrophages from p50<sup>-/-</sup> mice do not become tolerant to LPS.** NF- $\kappa$ B is known to play an important role in the transcriptional activation of many genes involved in inflammatory responses, including TNF. Here, we used PEMs obtained from wild-type, p50<sup>-/-</sup>, or cRel<sup>-/-</sup> mice to investigate whether these proteins are directly involved in the development of the LPS-tolerant phenotype. In response to an initial dose of LPS, the PEMs from each mouse strain produced comparable amounts of TNF during a 6-h period (Fig. 1 a). This is consis-

tent with previous observations and suggests that other members of the NF- $\kappa$ B family may compensate for the absence of the deleted transcription factors in the knockout mice (13, 20, 21).

As we (4, 5) and others (7, 8, 11) have previously reported, pretreatment with LPS for 18 h rendered wild-type PEMs tolerant to LPS upon rechallenge (Fig. 1 a, left). We also observed the LPS-tolerant phenotype when PEMs from cRel<sup>-/-</sup> mice were treated in the same manner as wild-type PEMs (Fig. 1 a, right). In sharp contrast, production of TNF by PEMs lacking p50 was not reduced by the 18-h pretreatment with LPS (Fig. 1 a, middle), and even when the primary exposure of LPS was increased to 100 ng/ml, we failed to induce tolerance (data not shown). This did not result from a generalized block in the TNF pathway because PEMs from all three strains were still responsive to treatment with suspension of HKSA (Fig. 1 a, all panels). Moreover, we noted the development of hyperresponsiveness (up to a 10-fold increase in TNF production) of wild-type and p50<sup>-/-</sup> but not cRel<sup>-/-</sup> PEMs to HKSA after a primary exposure to LPS treatment used to induce tolerance. Normalized data from four independent experiments comparing responses to LPS and HKSA by PEMs from all three mouse strains are provided in Fig. 1 b.

**Failure to induce LPS tolerance in p50<sup>-/-</sup> macrophages results from impaired downregulation of TNF mRNA synthesis.** Northern blot analysis revealed that stimulation of wild-type PEMs with a primary dose of LPS resulted in strong induction of TNF mRNA synthesis (Fig. 2). In contrast, no TNF mRNA was induced in wild-type PEMs that were pretreated with LPS for 18 h and subsequently restimulated with LPS. This is in agreement with our earlier reports showing downregulation of TNF mRNA synthesis in LPS-tolerant rabbit macrophages (4) and murine macrophage cell line RAW 264.7 (5). In marked contrast, pretreatment of p50<sup>-/-</sup> macrophages with LPS did not impair the ability of these cells to produce TNF mRNA upon rechallenge with LPS (Fig. 2).

**Expression of p50 modulates the activity of TNF promoter.** The previous data prompted us to investigate how p50 expression modulates transcription of the murine TNF gene and to determine if such modulation is stimulus dependent or independent. Cotransfection of RAW 264.7 cells, a cell line previ-



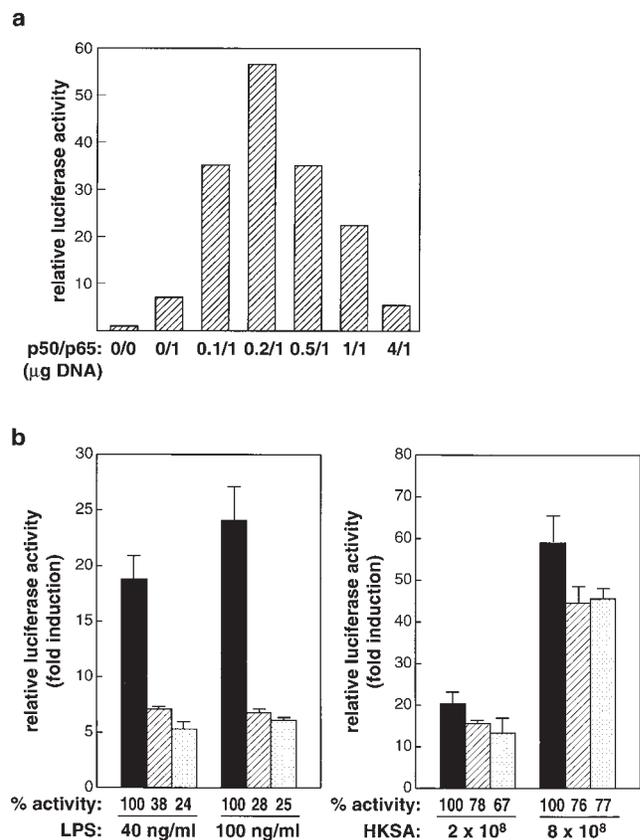
**Figure 2.** Analysis of TNF mRNA production by PEMs isolated from wild type (WT) and p50<sup>-/-</sup> mice. PEMs were pretreated in the absence, or in the presence of 10 ng/ml LPS for 18 h, washed, and activated with 10 ng/ml LPS for various times, as indicated. Expression of TNF mRNA was determined by Northern blot analysis. An ethidium bromide-stained gel is shown to ensure approximately equal sample loading.

ously used to study LPS tolerance (4), with increasing amounts of a p50 expression vector together with constant amounts of a p65 expression vector dramatically increased transactivation of a luciferase reporter gene construct containing a 1-kb fragment of the murine TNF promoter. However, as the amount of expressed p50 was further increased, we observed a dose-dependent inhibition of transcription (Fig. 3 *a*). This dual effect most likely resulted from the formation of either transcriptionally active p50–p65 complexes or inactive p50 homodimers depending on the relative amounts of the expressed proteins.

Stimulation of RAW 264.7 cells with LPS (40 and 100 ng/ml, respectively) resulted in a strong increase in the transcription of a reporter gene containing the murine TNF promoter (Fig. 3 *b*, *left*). Consistent with the previous experiment, cotransfection of p50 reduced LPS activation of the TNF promoter in a dose-dependent manner with the highest dose almost completely suppressing LPS stimulation (75% of inhibition). In contrast, activation of the TNF promoter by a suboptimal or optimal dose of HKSA was reduced only moderately (23–33% of inhibition) under experimental conditions identical to those used with LPS. Thus, p50 overexpression appears to result in a relatively selective inhibition of LPS induced transcription from the TNF promoter further supporting our hypothesis that p50 plays a key role in regulating of TNF expression in the LPS-tolerant cell.

*The role of the individual  $\kappa$ B sites of the TNF promoter in the establishment of the LPS tolerant state.* Four  $\kappa$ B sites have been identified in the murine TNF promoter (22, 23). A previous study showed that each of these sites appears to contribute to transcriptional activation by LPS (24). Here, we have used a series of oligonucleotides containing each of the four  $\kappa$ B sites in EMSA to evaluate binding of NF- $\kappa$ B during the time period leading to the LPS-tolerant phenotype. Incubation of wild-type PEMs with LPS for 45 min resulted in the induction of NF- $\kappa$ B complexes that bound to each of these  $\kappa$ B sites (Fig. 4, *a–d*), although the composition and affinity of these complexes for each site was unique (see below). The faster migrating complex observed with  $\kappa$ B2 (indicated by asterisk) represents constitutive binding of nonspecific proteins (23). Only the  $\kappa$ B3 site exhibited significant binding activity after extended incubation with LPS as observed in samples prepared at 6 and 18 h (Fig. 4 *c*); importantly, the composition of  $\kappa$ B3 binding complexes changed with time. Faster migrating complexes became dominant after incubation with LPS for 6 h or longer, while the more slowly migrating complex disappeared. In contrast to  $\kappa$ B3, binding to  $\kappa$ B1,  $\kappa$ B2, and  $\kappa$ B4 sites decreased substantially within 6 h of incubation with LPS and minimal binding was detected at later time points (Fig. 4, *a*, *b*, and *d*). The absence of binding to each of these sites in cRel<sup>-/-</sup> PEMs (Fig. 4, *i*, *j*, and *l*) suggests the presence of cRel in complexes bound to  $\kappa$ B1,  $\kappa$ B2, and  $\kappa$ B4. In addition, p65 but not p50 was detected in complexes bound to  $\kappa$ B2 and  $\kappa$ B4, while the  $\kappa$ B1 site bound p65 as well as p50 (data not shown).

Analysis of  $\kappa$ B3 bound nuclear complexes by UV cross-linking confirmed the substantial increase in binding of p50 in macrophages that were activated with LPS for increasing periods of time and a concomitant decrease in p65 (Fig. 5). These results were further confirmed by supershift analysis that identified the  $\kappa$ B3 bound, faster migrating complex, as a p50–p50 homodimer and the slower migrating complex as a p50–p65 heterodimer (see Fig. 8). In agreement with these results, NF- $\kappa$ B complexes from p50<sup>-/-</sup> PEMs exposed to LPS for > 6 h

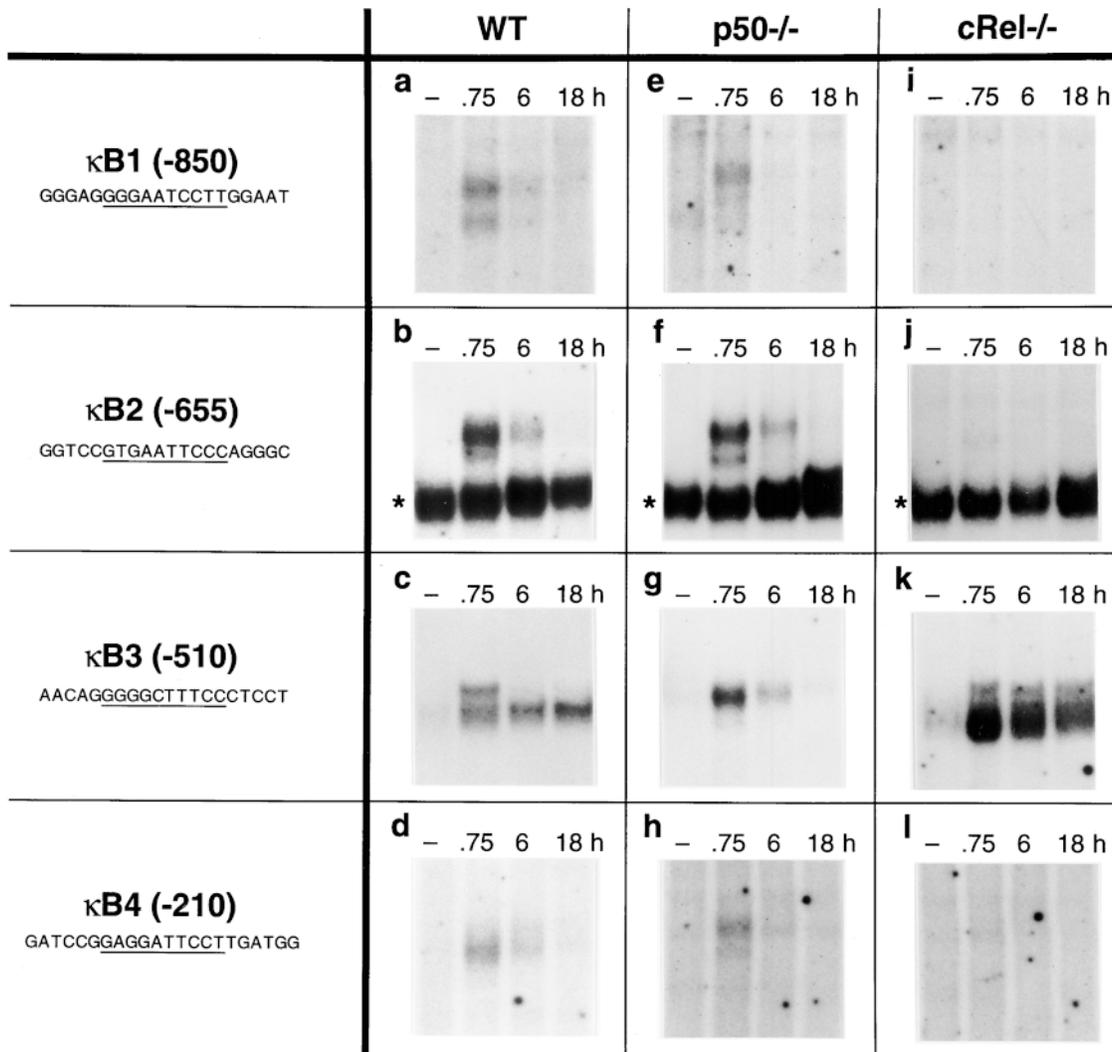


**Figure 3.** Regulation of the TNF promoter inducibility by p50. (*a*) RAW 264.7 cells were cotransfected with 1 µg of luciferase reporter plasmid containing the murine TNF promoter, 0.05 µg of pCMVβ, and the indicated amounts of the p50 and p65 expression plasmids (pCMXp50, pCMXp65). Luciferase activity in cell extracts was determined. The values represent normalized luciferase activities relative to the control transfection using reporter plasmid only. One representative experiment from three independent studies is shown. (*b*) RAW 264.7 cells were cotransfected with 2 µg of luciferase reporter plasmid containing the murine TNF promoter, 0.05 µg pCMVβ, and increasing amounts of the p50 expression plasmid pCMX p50, 0 µg (solid bars), 1 µg (dashed bars) and 4 µg (stippled bars), respectively. Transfected cells were activated in the presence of indicated amount of LPS (*left*) and HKSA (*right*), respectively, and luciferase activity in cell extracts was determined. This is typical of three independent experiments.

failed to bind to the  $\kappa$ B3 site (Fig. 4 *g*). In contrast, nuclear extracts from cRel<sup>-/-</sup> PEMs contained proteins, which bound to  $\kappa$ B3 site during the entire time course (Fig. 4 *k*), with p50 homodimers gradually displacing p50–p65. Thus, increased binding of p50 homodimers to the  $\kappa$ B3 site of the TNF promoter is a distinctive characteristic of PEMs that acquire an LPS-tolerant phenotype.

Incubation of PEMs with primary dose of HKSA for 45 min induced binding of nuclear proteins to the  $\kappa$ B3 site. Binding to the  $\kappa$ B1,  $\kappa$ B2, and  $\kappa$ B4 sites was very weak or undetectable in EMSA (Fig. 6). Supershift analysis revealed that the HKSA-induced  $\kappa$ B3-bound complexes contained p50 and p65, while no cRel was detected (data not shown).

As HKSA represents an agent that efficiently induces TNF expression in the LPS-tolerant state, we decided to study occu-



**Figure 4.** Analysis of the  $\kappa$ B binding activity induced in PEMs from wild type (WT), p50<sup>-/-</sup> and cRel<sup>-/-</sup> mice by LPS. PEMs were incubated with 10 ng/ml of LPS for the indicated periods, and  $\kappa$ B binding activity in nuclear extracts was determined by EMSA. The decameric  $\kappa$ B motifs present in the TNF promoter are indicated (5'→3'). The asterisk indicates the position of the constitutively bound complexes.

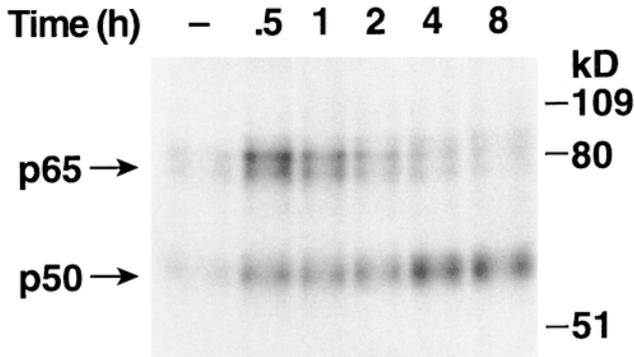
pation of the individual  $\kappa$ B sites of the murine TNF promoter under these conditions. We prepared nuclear extracts from PEMs that were pretreated with LPS for 18 h and rechallenged with HKSA. EMSA revealed binding to the  $\kappa$ B3 site but not to the sites  $\kappa$ B1,  $\kappa$ B2, and  $\kappa$ B4 (Fig. 7). Importantly, rechallenge with HKSA, in contrast to LPS, changed the composition of the complexes bound to the  $\kappa$ B3 site; a slower migrating complex appeared in addition to the p50 homodimers. To analyze changes in the composition of  $\kappa$ B3 binding complexes during different stages of LPS responsiveness, we performed supershift analysis. Complexes induced by incubation of PEMs with LPS for 45 min were supershifted by antibodies to p50 and p65 but not cRel (Fig. 8, *left*). This reflects binding of the p50-p50 (faster complex) and p50-p65 (slower) complexes to the  $\kappa$ B3 site (see also Fig. 4 c). Analysis of the samples prepared after 18-h pretreatment with LPS revealed substantially increased binding of p50 homodimers to the  $\kappa$ B3 site at the LPS-tolerant PEMs (Fig. 8, *middle*; see also Figs. 4 and 5). In addition to these complexes, rechallenge of the LPS-tolerant cells with

HKSA-induced binding of distinct cRel-containing complexes to the  $\kappa$ B3 site (Fig. 8, *right*). These data are consistent with the failure of HKSA to induce hyperresponsive levels of TNF in LPS-tolerant cRel<sup>-/-</sup> PEMs (Fig. 1) suggesting an important role for cRel in HKSA-mediated induction of TNF on LPS-tolerant PEMs.

## Discussion

Herein, we provide new information about molecular mechanisms that are involved in LPS tolerance. Multiple independent observations support the contention that the p50 subunit of NF- $\kappa$ B plays an indispensable role in the suppression of TNF gene transcription in LPS-tolerant monocytes/macrophages.

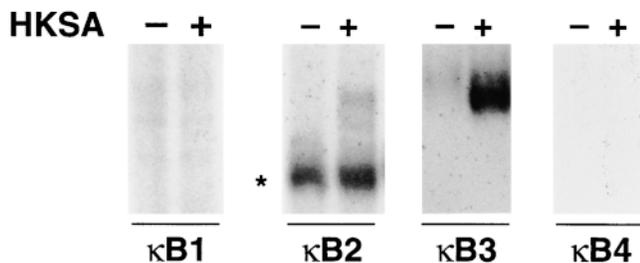
Induction of several genes, including TNF, that were supposed to be activated through p50 containing NF- $\kappa$ B complexes was not diminished by the absence of p50 (13). This suggests that other NF- $\kappa$ B family members may effectively compensate for absence of p50 in transcriptionally active com-



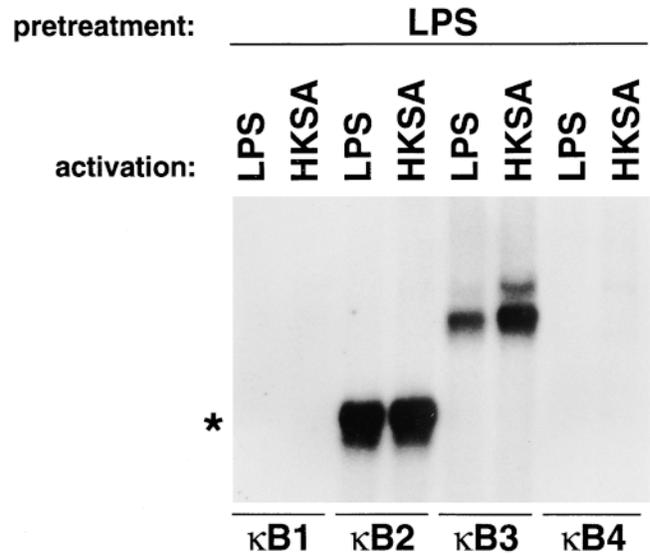
**Figure 5.** Cross-linking analysis of  $\kappa$ B3 binding complexes. PEMs were activated with 10 ng/ml LPS for indicated times. Complexes bound to the  $^{32}$ P-labeled photoreactive  $\kappa$ B3 probe were UV-cross-linked and the resulting adducts were solubilized, and analyzed by SDS-PAGE. Arrows indicate positions of p50- and p65-containing adducts.

plexes. Herein, we demonstrate that p50<sup>-/-</sup> PEMs do not become tolerant to LPS. We have shown that the presence of p50 is essential for the negative regulation of transcription from TNF gene in the setting of LPS tolerance. Overexpression of p50 was shown previously by others to inhibit transcriptional activation from synthetic promoters consisting of multiple  $\kappa$ B motifs (25, 26). We have demonstrated that ectopic overexpression of p50 inhibits LPS-induced transcription from the murine TNF promoter. Importantly, this inhibition was most pronounced when LPS was used as a stimuli. Response to HKSA in cells overexpressing p50 was only slightly reduced (Fig. 2). Thus, overexpression of p50-induced changes that mimicked LPS tolerance insofar as suppression of TNF gene transcription is concerned.

Each of the four  $\kappa$ B sites identified in the murine TNF promoter (22, 23) is thought to contribute to transcriptional activation by LPS (24). However, the involvement of each of these four sites in the LPS-tolerant state has not been addressed. Here, we demonstrate unique binding characteristics of these sites during different stages of LPS activation. When the LPS-tolerant state was established, only the  $\kappa$ B3 site revealed inducible binding activity. This consisted mostly of p50 homodimers; the amount of p50-p65 complex bound to the  $\kappa$ B3 site was strongly decreased when normal and tolerant PEMs were compared. Differences in binding among the individual  $\kappa$ B sites apparently result from specific differences in their se-



**Figure 6.** Induction of  $\kappa$ B binding activity by HKSA. PEMs were activated with HKSA for 45 min.  $\kappa$ B binding activity in nuclear extracts was determined by EMSA. Asterisk indicates position of the constitutively-bound complexes.

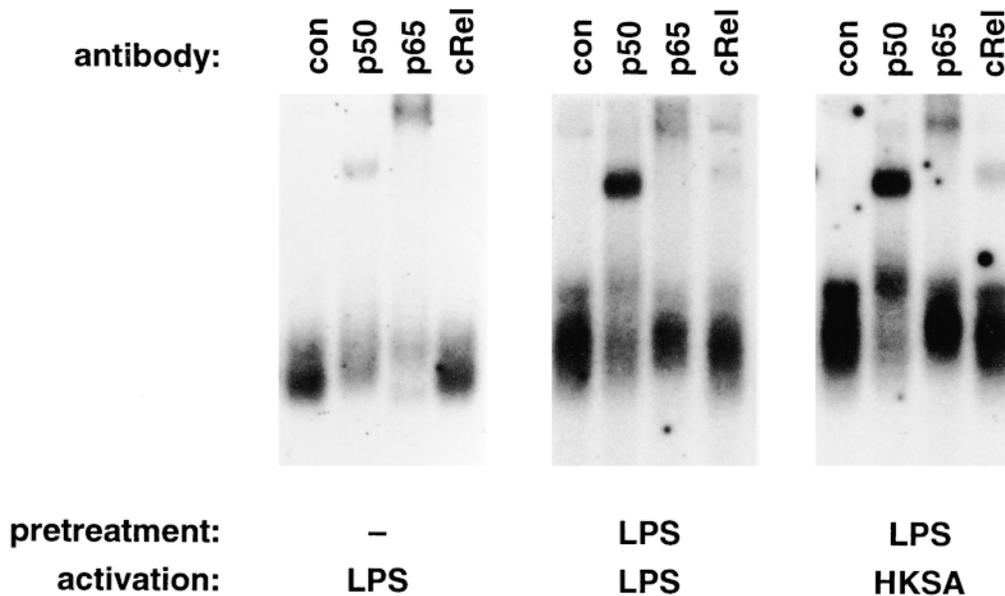


**Figure 7.** Analysis of  $\kappa$ B binding complexes induced in LPS tolerant cells by HKSA. PEMs were pretreated with 10 ng/ml LPS for 18 h, and activated with 10 ng/ml LPS, or HKSA for 45 min.  $\kappa$ B binding activity was determined by EMSA. Asterisk indicates position of the constitutively bound complexes.

quences.  $\kappa$ B2 and  $\kappa$ B4 sites do not meet the requirements for p50 binding due to the lack of a GGG motif at the 5' end (27-29), and the  $\kappa$ B1 site lacks a 3'-end motif CCC (present in  $\kappa$ B3 site), favorable for binding of the p50 homodimers (28, 30, 31) that are induced at later time points.

Extended treatment with LPS results in transcriptional activation of the p50-precursor p105 but not other  $\kappa$ B family members (11, 32). Upregulation of p50 expression apparently favors formation of p50 homodimers in the cytoplasm, which due to their inability to bind to the inhibitor I $\kappa$ B (27), may directly translocate to nucleus. As we demonstrate, binding of p50 homodimers to the positively acting  $\kappa$ B3 element of the murine TNF promoter results in inhibition of LPS-induced  $\kappa$ B-dependent induction of TNF mRNA. Interestingly, inducibility by HKSA is preserved during the LPS-tolerant state. Our data show that activation of LPS-tolerant cells with HKSA results in activation of complexes containing substantial amounts of cRel, which may then compete effectively with p50 homodimers at the  $\kappa$ B3 site. We suggest that such competition for the  $\kappa$ B3 site may overcome the inhibitory effect of p50 homodimers formed in the LPS-tolerant cells. In support of this contention are data showing that p50<sup>-/-</sup> and wild-type PEMs produce substantially more TNF than cRel<sup>-/-</sup> PEMs when LPS-pretreated cells (tolerant phenotype) are challenged with HKSA (Fig. 1). However, cRel is not absolutely required for TNF activation by HKSA since cRel<sup>-/-</sup> PEMs are still HKSA inducible. Furthermore, we have not observed any HKSA-inducible cRel binding on PEMs unless the cells were first treated with LPS.

Tolerance to LPS may have evolved as a physiological mechanism to prevent cellular injury during sepsis by overproduction of endogenous proinflammatory mediators such as TNF. Nevertheless the innate immune system is not globally disabled during the LPS-tolerant state, since the responses to other microbial pathogens are not impaired. Paradoxically, the



**Figure 8.** Analysis of the  $\kappa$ B binding complexes at different stages of LPS responsiveness. PEMs were pretreated in the absence or in the presence of 10 ng/ml LPS for 18 h, and activated with LPS or HKSA for 45 min, as indicated. Composition of nuclear complexes bound to  $\kappa$ B3 site was determined by supershift analysis employing antisera to p50, p65, and cRel.

same mechanism may contribute to a hyperresponsive state when infection with Gram-negative as well as Gram-positive organisms occurs. The specific binding characteristics of the individual  $\kappa$ B sites present in genes of the various proinflammatory mediators may represent a fine tuning mechanism for the innate immune response during infection. Such sites may also be susceptible to modulation by pharmacologic agents and thus provide new means for therapeutic intervention in the setting of septicemia and/or septic shock.

## Acknowledgments

We thank Dr. J. Mathison and P. Oeth for help with isolation of macrophages, Dr. N. Rice for antisera to p50, p65, and cRel, and Dr. D. Baltimore for providing p50<sup>-/-</sup> mice.

This work was supported by grants from the National Institutes of Health AI15136, GM28485 (R.J. Ulevitch), HL56609 (G.C.N. Parry), and HL16411 (N. Mackman).

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