

# Differential Regulation of Lipopolysaccharide (LPS) Activation Pathways in Mouse Macrophages by LPS-Binding Proteins<sup>1</sup>

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LPS binding to its receptor(s) on macrophages induces the synthesis of inflammatory mediators involved in septic shock. While the signaling mechanism(s) remains to be fully defined, the human LPS-binding protein (LBP) is known to regulate responses to LPS by facilitating its binding to CD14 on human monocytes. The structurally related bactericidal permeability increasing protein (BPI) differs from LBP by inhibiting LPS-induced human monocyte activation. We have demonstrated that, unlike the human monocyte response to LPS, both LBP and BPI inhibited LPS-stimulated TNF- $\alpha$  production in mouse peritoneal macrophages. In contrast, LPS-dependent nitric oxide release was not affected by LBP. LPS induces the phosphorylation of a number of proteins in a dose and time-dependent manner, however, the pattern of LPS-induced phosphorylation was not reduced by either LBP or BPI under conditions that result in selective TNF- $\alpha$  inhibition. Further, activation of the transcription factor NF- $\kappa$ B in response to LPS was also not modified by either LBP or BPI. Finally, no differences were detected in TNF- $\alpha$  or inducible nitric oxide synthase mRNA accumulations induced by LPS in the presence or absence of either protein, whereas a slight decreased mRNA stability was observed in the group with LPS treatment. These results would suggest that many of the early signaling events contribute to LPS-induced macrophage signaling at a point preceding the divergence of pathways that differentially regulate TNF- $\alpha$  and NO production. *The Journal of Immunology*, 1998, 161: 2552–2560.

Lipopolysaccharide, a major component of the outer membrane of Gram-negative bacteria, is known to induce in the host a variety of pathophysiologic responses, including fever, coagulant activity, septic shock, and death. Many approaches designed to study these processes in vitro have utilized human monocytes or mouse macrophages as prototypic inflammatory cells involved in sepsis, since these cells are potently activated by LPS, leading to the production of many inflammatory mediators, including oxygen and nitrogen intermediates, PGs, and cytokines (1). Of potential importance to the pathogenesis of sepsis, many of these mediators have been reported at elevated levels in the circulatory systems of either patients or experimental animals with sepsis (2, 3). These immunomodulatory substances cause pleiotropic biologic effects and play a crucial role in both inflammation and normal immune responses. They are also thought to mediate the systemic inflammatory response characteristic of endotoxin shock, and in fact, therapeutic targeting of these mediators has been pursued as a means to reduce mortality in sepsis (4).

During the past decade, several laboratories have investigated the cellular events leading to the production of the mediators involved in endotoxic shock, including the identification of LPS receptors on target cells and the signal transduction pathways that result in gene activation and cytokine production. It has been reported that LPS, by exerting most of its biologic effect through the lipid A moiety, activates several signal transduction pathways in macrophages (5, 6), but the mechanisms of the signaling cascades of stimulation are still not totally understood. Strong evidence supports the concept that LPS activation of human monocytes involves both the LPS-binding protein (LBP)<sup>6</sup> and CD14. The 60-kDa serum acute phase glycoprotein, LBP, binds to the lipid A with high affinity and appears to catalytically deliver LPS to the 55-kDa membrane glycoprotein CD14 (mCD14) (7, 8). Since this glycosylphosphatidylinositol-anchored molecule is not a transmembrane protein, additional, as yet unidentified transducer proteins have been proposed to initiate the intracellular signals. Further, whereas LPS activation in monocytes/macrophages has been shown to be a CD14-dependent event, some data have shown that it can also occur independently of CD14 (9–11). In fact, the response to LPS by various cell types lacking mCD14, such as B lymphocytes (12),  $\gamma\delta$  T lymphocytes (13), and fibroblasts (14), could be explained by either utilization of serum-soluble CD14 or CD14-independent pathways (15). Studies in our laboratory have identified an as yet to be characterized 73-kDa protein that may serve as an alternative LPS receptor on mouse macrophages (16).

There is solid evidence showing a key role for protein phosphorylation in the early intracellular signaling pathways initiated by LPS. It has been suggested that protein tyrosine kinases (PTK), including nonreceptor p53/56<sup>lyn</sup>, p56/59<sup>hck</sup>, p58<sup>c-frag</sup> (17), p38 (18), and the mitogen-activated protein kinase (MAPK) family (19), are

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<sup>6</sup> Abbreviations used in this paper: LBP, LPS-binding protein; PTK, protein tyrosine kinases; NO, nitric oxide; iNOS, inducible nitric oxide synthase; BPI, bactericidal/permeability increasing protein; EMSA, electrophoretic mobility shift assay; mCD14, membrane CD14.

involved at different levels. In addition, inhibitors of tyrosine kinases have been shown to attenuate LPS-induced cytokine production by mouse macrophages (20), and the targeting of PTK has recently been pursued as a potential therapeutic approach against septic shock (21). At a different level in the LPS-initiated activation cascade, cytokine expression has been reported to depend on the activation/translocation of the transcription factor NF- $\kappa$ B. The members of the Rel family, p50 and p65 of NF- $\kappa$ B, form heterodimer complexes capable of binding to  $\kappa$ B consensus motifs in the promoter/enhancer regions of codifying genes (6). Recent reports have indicated that  $\kappa$ B motifs flanking the TNF- $\alpha$  gene are essential for transactivation in LPS-stimulated macrophages (22), and expression of the inducible nitrite oxide synthase (*iNOS*) gene might also be regulated by NF- $\kappa$ B/Rel (23). The precise contribution of these mechanisms in the ultimate activation cascade induced by LPS, however, is still not clear and may depend on the experimental model.

We have recently studied potential pathways for LPS activation by evaluating the effect of purified LBP on LPS activation events (24). Our data demonstrate that human LBP enhanced LPS-induced human monocyte TNF- $\alpha$  and IL-6 production, but suppressed the equivalent cytokine response in mouse macrophages in a dose-dependent way. Interestingly, LBP showed no inhibitory effect on LPS-induced nitric oxide (NO) secretion by the same macrophages. The bactericidal/permeability-increasing protein (BPI) secreted by polymorphonuclear leukocytes, which presents high homology with LBP (25, 26) but antagonistic effects in human monocytes (27), inhibited both LPS-induced TNF- $\alpha$  and NO secretion by mouse macrophages. Therefore, LBP manifested opposite effects on LPS-induced activation of human monocytes and mouse macrophages, whereas BPI showed inhibitory effects in both cell types. The ability of LBP to modify LPS effects *in vitro* would thus depend upon both the cell type under study and, within a single-cell population, upon the particular response being measured. The fact that LPS can be selectively influenced by both LBP and BPI suggests the existence of at least two different activation pathways in macrophages.

The purpose of our studies has been, therefore, to investigate the relative contribution of several biochemical signaling events implicated in the LPS-initiated cascade of activation that might explain the dichotomy observed between mouse and human cells. In mouse peritoneal macrophages, our data did not support an action of LBP in modifying LPS-induced PTK activation, nuclear transcription factors translocation to the nucleus, or synthesis of new mRNA for TNF- $\alpha$  or *iNOS*, under conditions that nevertheless resulted in significant reductions in TNF- $\alpha$  and IL-6 production. The results suggest that, upon stimulation with LPS, mouse macrophages initiate a cascade of events that lead to the synthesis of mRNA for proinflammatory mediators, independently of the presence of LBP. The fact that the secretion of the final products TNF- $\alpha$  or NO are differentially regulated by LBP, therefore suggests that a downstream control point(s) subsequent to mRNA synthesis provides an important negative feedback mechanism that would then contribute to the dichotomy in response to LBP-LPS in mouse peritoneal macrophages.

## Materials and Methods

### Animals

Female C3HeB/FeJ, 6- to 10-wk-old mice were obtained from The Jackson Laboratory (Bar Harbor, ME), housed in laminar flow isolation units in the Kansas University Medical Center vivarium under alternate dark-light cycles, and fed *ad libitum* with pellet chow and acidified tap water.

### Reagents

Rough-type LPS from *Salmonella minnesota* or smooth-type LPS from *Escherichia coli* 0111:B4 were purchased from List Biological Laboratories (Campbell, CA). Immediately before use, the LPS stock solution (1 mg/ml in pyrogen-free sterile distilled H<sub>2</sub>O) was sonicated for 3 min (W385; Heat-System Ultrasonic, Farmingdale, NY) and appropriately diluted in culture medium. Human recombinant LBP and BPI (generously provided by Incyte Pharmaceuticals, Palo Alto, CA), were diluted from stock solutions (1 mg/ml at -70°C) immediately before use.

### Macrophage isolation and culture conditions

Exudate macrophages were obtained by peritoneal lavage 5 days after *i.p.* injection of 1.5 ml 4% Brewer thioglycollate (Difco Laboratories, Detroit, MI). Cells in RPMI 1640 medium supplemented with penicillin and streptomycin (JRH Biosciences, Lenexa, KS) were seeded at  $2 \times 10^6$  cells/well into six-well culture plates (Costar, Cambridge, MA) and  $4 \times 10^6$  or  $6 \times 10^6$  cells into 6-mm culture dishes (for nuclear factors or mRNA stability studies, respectively). After incubation at 37°C and 5% CO<sub>2</sub> for 2 to 3 h (or overnight in some experiments to minimize background levels), nonadherent cells were removed by washing twice with the same medium, and the remaining adherent cells stimulated with the indicated concentrations of LPS in the presence or absence of LBP or BPI, then further incubated at 37°C in 5% CO<sub>2</sub> for different amounts of time.

### TNF- $\alpha$ , IL-6, and NO production determination

For production of TNF- $\alpha$ , IL-6, and NO, culture supernatants were collected after 18 h of stimulation (24, 28). The amounts of TNF- $\alpha$  were quantified by assessing the extent of killing of the murine fibroblast cell line L929. IL-6 amounts were determined by ELISA utilizing rat mAb anti-mouse IL-6 (PharMingen, San Diego, CA) following the provider's instructions. The amounts of either TNF- $\alpha$  and IL-6 were calculated by comparison with a recombinant cytokine (Genzyme, Cambridge, MA) standard curve run in each plate. Absorbance were measured using a Dynatech MR5000 microplate reader (Chantilly, VA). NO production in culture supernatants was assessed by measuring the amount the metabolic product nitrite by the Griess reaction.

### Detection of TNF- $\alpha$ and *iNOS* gene transcripts

TNF- $\alpha$  and NO mRNAs were evaluated after 2 and 6 h of stimulation. Cells were lysed with 1 ml/well of Trizol (Life Technologies), and total RNA was extracted according to the manufacturer's instructions. Reverse transcription and 25 cycles of PCR (RT-PCR) were conducted using the GeneAmp RNA PCR kit and the GeneAmp 9600 Thermal Controller apparatus (Perkin-Elmer, Foster City, CA). Mouse TNF- $\alpha$ , IL-6,  $\beta$ -actin (Stratagene, La Jolla, CA), and *iNOS* (Clontech Laboratories, Palo Alto, CA) primers were used as specified by the vendors. The PCR products were electrophoresed on a 1.6% agarose gel and stained with 0.5 mg/ml ethidium bromide. Images were analyzed with a CCD Interactive Tech Videocamera (Philips, The Netherlands) and ITTI 1.31 software (Interactive Technologies International, St. Petersburg, FL) (28).

### Analysis of TNF- $\alpha$ mRNA stability

After 2 h of stimulation, new transcription was inhibited by treatment with 5  $\mu$ g/ml of actinomycin D (Merck, West Point, PA), and mRNA stability was evaluated by Northern blotting performed as described (29). Ten micrograms of total RNA were electrophoresed in a 1% agarose, 2.2% formaldehyde gel and further transferred to a nylon membrane using a Turbo-blotter (Schleicher and Schuell, Keene, NH). The membranes were UV cross-linked and hybridized with a mouse TNF- $\alpha$  cDNA probe using the QuikHyb hybridization solution protocol (Stratagene). The probe (prepared from a cDNA clone kindly given by Dr. Christine Martens, DNAX, Palo Alto, CA) was radiolabeled with [<sup>32</sup>P]dCTP (ICN Pharmaceutical, Costa Mesa, CA) using a Multiprime DNA labeling system (Amersham, Arlington Heights, IL). After blotting overnight exposure of the blot to autoradiography at -70°C, images were processed and analyzed with the ITTI 1.3 software.

### PAGE and phosphotyrosine immunoblotting

PTK activity was evaluated at increasing times of incubation. Cells were lysed by sonication (3 $\times$ , 1 min) at 70 W and boiled for 5 min in SDS reducing sample. Total soluble proteins were electrophoresed in 10% SDS-polyacrylamide gel and further blotted onto nitrocellulose membrane (Schleicher and Schuell) using MiniPROTEAN II electrophoresis and Mini trans-blot electrophoretic transfer cell (Bio-Rad, Richmond, CA). Phosphorylated tyrosines were detected with rat anti-phosphotyrosine mAb

4G10 (Upstate Biotechnology, Lake Placid, NY). The bands were detected using the enhanced chemiluminescence system (ECL; Amersham) protocol. Developed film were subjected to densitometric analysis as described above (30, 31).

#### Electrophoretic mobility shift assay (EMSA) and supershift assay

For transcription factor experiments, cells were stimulated for 1 h. The NF- $\kappa$ B-specific oligonucleotide, synthesized in the Biotechnology Support Facility at the University of Kansas Medical Center, was end-labeled using [ $\gamma$ - $^{32}$ P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase (Life Technologies). For EMSA, nuclear extracts (1  $\mu$ g) were incubated with poly(dI-dC).poly(dI-dC) (Pharmacia LKB Biotechnology, Piscataway, NJ) and the end-labeled DNA, and then the mixture (20  $\mu$ l) was preelectrophoresed and electrophoresed through a native 6% polyacrylamide gel. To identify the components of the NF- $\kappa$ B complex, nuclear proteins were incubated with polyclonal antisera against either p50 or c-Rel subunits or rabbit anti-p65 subunit Ab, as well as with control rabbit antiserum (generously provided by Dr. N. Rice, National Cancer Institute, Bethesda, MD). These mixtures were then subjected to EMSA as described above (32).

#### Statistical analysis

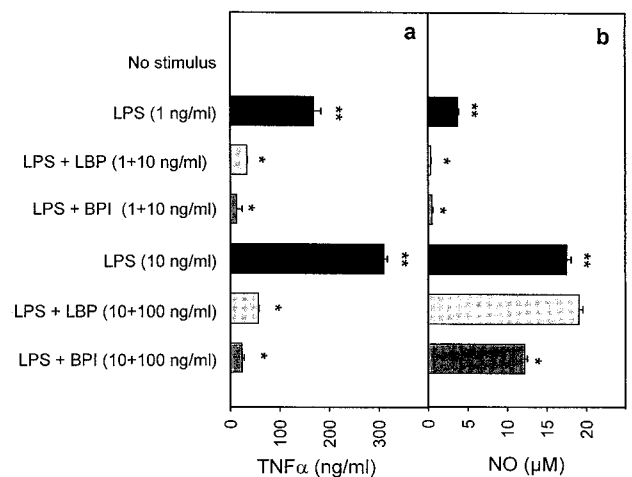
Data were expressed as means and were compared statistically by Student's *t* test. Levels of significance were determined using the Epistat statistical package (T. Gustafson, Round Rock, TX) or Sigmaplot software (Jandel, San Rafael, CA) in a personal computer. A *p* value <0.05 was considered statistically significant.

## Results

#### Effect of LPS-binding proteins (LBP and BPI) on the response of macrophages to LPS

The human serum protein LBP has been extensively reported to mediate LPS activity by facilitating its binding to human monocytes (8). Opposite effects have been demonstrated, however, when mouse peritoneal exudate macrophages were evaluated for response to LPS under otherwise identical conditions (24). Specifically, this protein has been shown to inhibit the ability of LPS to stimulate TNF- $\alpha$  production in a dose-dependent manner, while having no inhibitory detectable effects on the LPS-mediated production of NO. In the present report, therefore, we have extended these investigations on the participation of LBP and other serum proteins in the cascade of activation by bacterial LPS in mouse macrophages.

We used several doses of rough-type LPS, which possesses higher activating capacity than smooth LPS, in these studies; the amounts of TNF- $\alpha$  and nitrite were assessed in culture supernatants of exudate peritoneal macrophages stimulated for 18 h with increasing concentrations of rough type LPS. As indicated by the data depicted in Figure 1, doses of 1 to 10 ng/ml of LPS triggered TNF- $\alpha$  and NO production by elicited mouse peritoneal macrophages. We therefore evaluated the effect of the LBP and BPI on the macrophage activation induced by these doses of LPS. Similar to what had been observed previously in this system, and in contrast to what was reported for human cells, the addition of up to 100 ng/ml of human rLBP markedly inhibited TNF- $\alpha$  production by mouse macrophages induced by up to 10 ng/ml of rough-type LPS (Fig. 1a). Also confirming our previous results, the production of NO induced by LPS was not significantly modified by the presence of this concentration of LBP (Fig. 1b). We have previously reported that BPI, despite its high homology with LBP, presented contrary effects to LBP, resulting in a dose-dependent inhibition of LPS-dependent activation of both human PBMC and mouse macrophages. Confirming those results, the presence of BPI in the two concentrations used inhibited both LPS-induced TNF- $\alpha$  and NO production by mouse peritoneal macrophages. In accordance with our previous report, these data suggest the possibility of



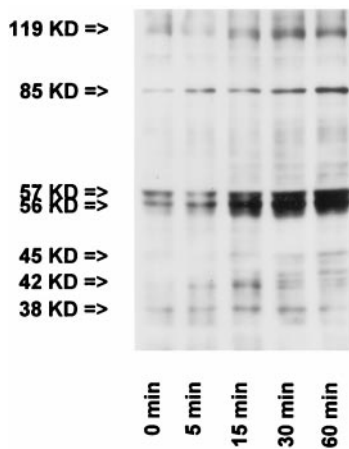
**FIGURE 1.** Effect of LBP and BPI on LPS-induced TNF- $\alpha$  and NO production by mouse macrophages. Supernatants from exudate peritoneal macrophages were evaluated after 18 h of incubation with 0 to 10 ng/ml of rough-type LPS alone or in combination with recombinant human LBP or BPI. TNF- $\alpha$  was quantitated as the extent of cytotoxicity on the fibroblast cell line L929 (a); and the amount of nitrite was assessed by the Griess reaction (b), as described in *Materials and Methods*. The results represent the average of triplicate determinations  $\pm$  SEM from one representative experiment repeated four times under equivalent conditions. \*, Statistical difference with respect to the group treated with LPS alone, *p* < 0.05 (Student's *t* test); \*\*, statistical difference with respect to the control untreated group.

differential activating pathways utilized by mouse macrophages in response to LPS, or even the potential participation of independent receptors in that response.

#### Effect of LBP and BPI on LPS-induced PTK activation

PTK activity appears to correspond to one of the earliest biochemical steps to be triggered by LPS. Experiments were designed, therefore, to evaluate the potential participation of PTK activation in the differential pathways leading to TNF- $\alpha$  vs NO production by mouse macrophages in response to LPS. The key role of the PTK in the cascade of activation induced by LPS was first confirmed by utilizing PTK inhibitors. Pretreatment of mouse macrophages with either 30  $\mu$ g/ml of genestein or 7.5  $\mu$ g/ml of herbimycin A (IC<sub>50</sub>) abolished the secretion of IL-6, TNF- $\alpha$ , and NO induced by LPS treatment (data not shown), thus confirming similar results obtained with human monocytes (33).

The PTK activity of mouse macrophages was assessed by examining the patterns of proteins phosphorylated in response to LPS using Western blot analysis. Upon stimulation with LPS, a number of proteins become phosphorylated, presenting different patterns of phosphorylation over time (Fig. 2). Since the phosphorylation of several proteins (38, 42, 45, 46, 56/57, 85, and 119 kDa) was maximal at 30 min following stimulation with LPS, this time was chosen as the peak time of activation in kinase activity for subsequent analysis, the time at which the effect of both LPS-binding proteins, LBP and BPI, on the LPS-dependent response was evaluated. For these studies, mouse macrophages were incubated with LPS in the presence or absence of LBP or BPI using previously established conditions to result in differential inhibition of TNF- $\alpha$  but not NO secretion (Fig. 1). The results, depicted in Figure 3, indicate that neither the addition of LBP nor BPI detectably diminished, but rather enhanced, the pattern of phosphorylation of

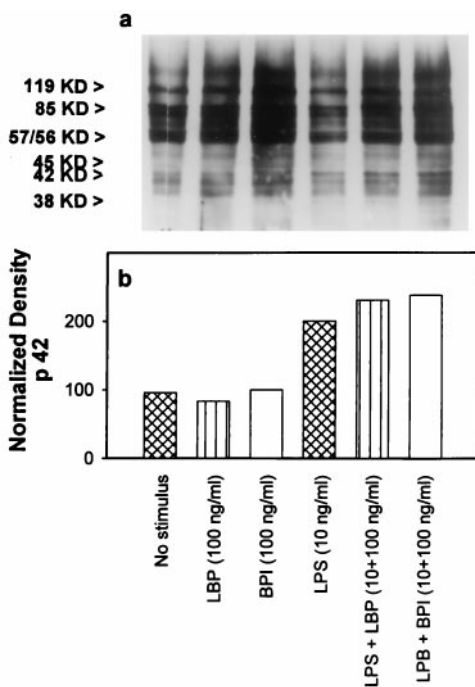


**FIGURE 2.** Time course of PTK activity induced by LPS in mouse macrophages. Cells were stimulated with 10 ng/ml of LPS, and at increasing times of stimulation whole soluble proteins were extracted and subjected to PAGE and immunoblotting using antiphosphotyrosine mAbs as described in *Materials and Methods*. In the representative autoradiograph shown, arrows indicate m.w. of 119, 85, 56/57, 45, 42, and 38 kDa, from top to bottom.

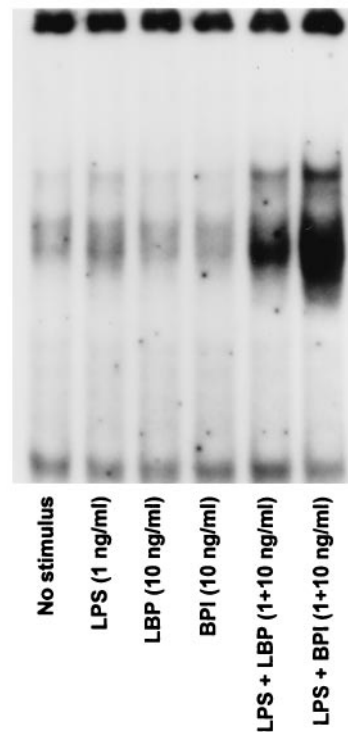
proteins that was induced by LPS treatment of mouse macrophages.

*Effect of LBP or BPI on the translocation of transcription factors*

The activation of transcription factors and their subsequent translocation into the nucleus have been reported as early events in the



**FIGURE 3.** Effect of LBP or BPI on PTK activity induced by LPS treatment in mouse macrophages. After 30 min of stimulation, as previously described, whole soluble proteins were extracted and subjected to SDS-PAGE and immunoblotting as described in *Materials and Methods*. The autoradiograph (a) shown is a representative experiment of two that gave similar results. From top to bottom, arrows indicate m.w. of 119, 85, 56/57, 45, 42, and 38 kDa. The density of the 42-kDa protein (b) was normalized with respect to the background in each lane.

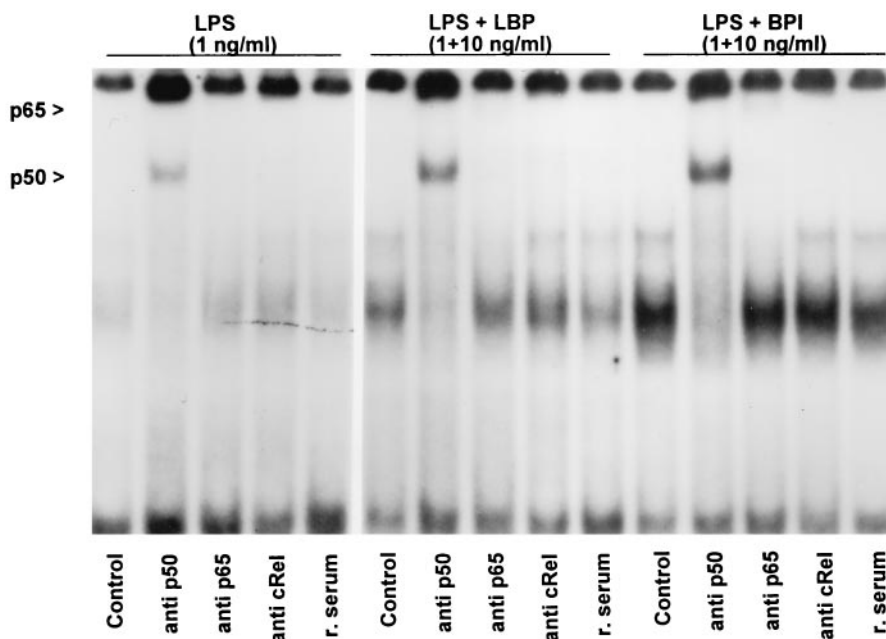


**FIGURE 4.** Effect of LBP or BPI on NF- $\kappa$ B translocation to the nucleus induced by LPS in mouse macrophages. After 1 h of stimulation with LPS alone or in combination with LBP or BPI as described, nuclear proteins were extracted and incubated with a  $^{32}$ P-labeled HIV NF- $\kappa$ B consensus motif-containing oligonucleotide and then subjected to EMSA as described in *Materials and Methods*. The autoradiograph shows a representative of two separate experiments that gave similar results.

activation cascade induced by LPS in macrophages. The next experiments, therefore, were designed to evaluate the potential participation of different transcription factors in the differential response induced by LBP and BPI on peritoneal macrophages activation by LPS. The presence of transcription factors in the nucleus after 30 min of stimulation was assessed by EMSA. In Figure 4 shows that the translocation of NF- $\kappa$ B to the nucleus induced stimulation with as little as 1 ng/ml LPS in mouse macrophages. The addition of TNF- $\alpha$ -inhibitory concentrations of LBP did not induce any detectable decrease in NF- $\kappa$ B; rather, an increase in the translocation of the proteins was observed when peritoneal macrophages were stimulated with LPS-LBP complexes. Similarly, no decrease could be detected in NF- $\kappa$ B translocation to the nucleus upon treatment with BPI plus LPS, in comparison to LPS alone. Equivalent results were observed when 10-fold increased concentrations of each component were used, independently of the nature of LPS (rough or smooth; data not shown).

Recent reports have demonstrated the importance of translocation of specific subunits of NF- $\kappa$ B to present "active" forms. The heterodimeric complex of the p65 and p50 forms of NF- $\kappa$ B leads to the transactivation of genes, while some homodimers (p50) present an impaired binding to DNA (34, 35). Therefore, to further evaluate whether the observed inhibition in TNF- $\alpha$  production was due to an impaired translocation of the appropriate transcription factors, the composition of the NF- $\kappa$ B complex was evaluated by supershift assays using specific Abs against the different components of the NF- $\kappa$ B complex. As shown by the data in Figure 5, treatment with LPS induced the formation of a NF- $\kappa$ B complex, primarily composed of p50, and p65 to a lesser extent. As demonstrated previously, the addition of either LBP or BPI appears to

**FIGURE 5.** LPS-induced translocation of the different components of the NF- $\kappa$ B complex is not affected by LBP or BPI. Nuclear proteins were incubated with Abs against the p50 and p65 subunits of NF- $\kappa$ B complex, c-Rel, or nonimmune rabbit serum, and then subjected to EMSA as described in *Materials and Methods*. The autoradiograph shows a representative of two equivalent experiments that gave similar results.



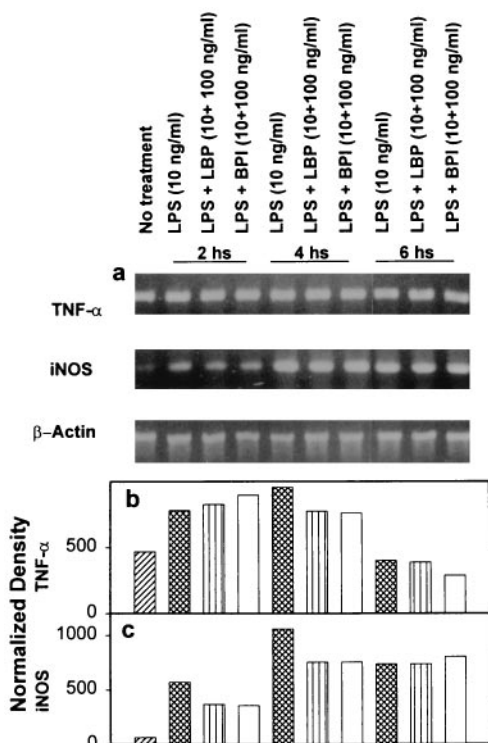
enhance the translocation of the whole NF- $\kappa$ B complex, while neither of these LPS-binding proteins seems to drastically modify the conformation of the complex induced by LPS treatment.

#### *Effect of LBP or BPI on cytokine gene transcription*

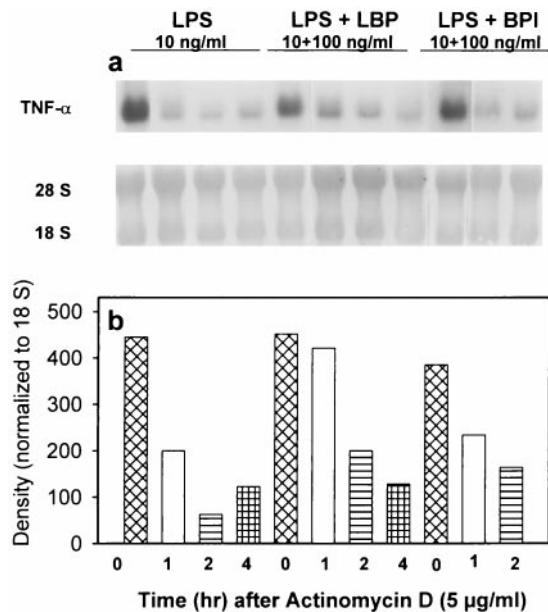
The next series of experiments was performed to determine whether the divergent responses observed in terms of TNF- $\alpha$

and NO production might result from the blockage in the synthesis of mRNA for both TNF- $\alpha$  and *iNOS*. Therefore, the presence of mRNAs for these proteins was semiquantitated by RT-PCR amplification at different times of mouse macrophage stimulation with LPS in the presence or absence of either LBP or BPI. The results indicate significant amounts of TNF- $\alpha$  transcript after 2 h of LPS stimulation (Fig. 6). Relatively high basal mRNA levels, however, were detected in the control, probably due to the degree of activation that peritoneal macrophages manifest upon stimulation for 5 days with thioglycollate and adherence to plastic. Interestingly, when LBP-LPS complexes were used as stimulus, no inhibition could be detected in the TNF- $\alpha$  or *iNOS* transcripts accumulation, compared with treatment with LPS alone. Similar results were obtained with macrophages treated with BPI plus LPS. For the same cells, the LPS-induced *iNOS* mRNA was shown to be increased at later times compared with TNF- $\alpha$ , reaching the maximum at 6 h postactivation. As observed with TNF- $\alpha$ , combined treatment of mouse macrophages with LPS and either LBP or BPI did not seem to detectably modify the extent of gene expression relative to cells treated with LPS alone. Analyses of the expression of other proinflammatory and anti-inflammatory cytokines (IL-6 or IL-10) yielded results similar to those observed with TNF- $\alpha$  and *iNOS* (data not shown).

While an inhibitory effect of LBP or BPI on LPS-dependent expression of TNF- $\alpha$  or *iNOS* mRNA was not detected by RT-PCR, LBP-LPS complexes could differentially regulate the synthesis vs stability of mRNA, which leads to the final TNF- $\alpha$  protein. To test this hypothesis, the decay of the TNF- $\alpha$  mRNA induced by LPS alone or in the presence of LBP or BPI was monitored by Northern blot. According to our previous results with RT-PCR, 2 h of treatment with LPS induced the expression of TNF in mouse macrophages (Fig. 7). After subsequent treatment with 5  $\mu$ g/ml of actinomycin D, a concentration that completely inhibited the incorporation of tritiated uridine in pilot experiments (data not shown), a rapid decay of  $\sim$ 50% could be observed at 1 h in the group treated with LPS alone. While degradation of mRNA was observed upon treatment with LBP-LPS or BPI-LPS complexes, a slower rate of degradation of mRNA was observed with the LBP-LPS complex in comparison with the LPS treatment alone. These data suggest that the inhibition exerted by LBP



**FIGURE 6.** Effect of LBP or BPI on LPS-induced TNF- $\alpha$  and *iNOS* gene expression. Peritoneal macrophages were stimulated as previously indicated for 2 to 6 h. Total RNA was extracted and subjected to RT-PCR as described in *Materials and Methods*. One representative experiment, repeated three times under similar conditions, is shown in the photograph (a). The density of each PCR product for TNF- $\alpha$  (b) and *iNOS* (c) was normalized against  $\beta$ -actin.



**FIGURE 7.** Effect of LBP or BPI on LPS-induced TNF- $\alpha$  mRNA stability. Peritoneal macrophages were stimulated for 2 h, at which time new transcription was stopped by treatment with actinomycin D (5  $\mu$ g/ml). At increasing times total RNA was extracted, and the presence of TNF- $\alpha$  was analyzed by Northern blotting. *a*, One representative experiment, repeated four times under similar conditions, is shown in the autoradiograph. *b*, The density of each TNF- $\alpha$  band was normalized against the 18S band.

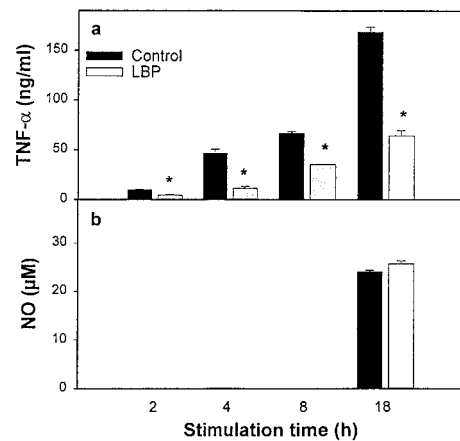
in LPS-stimulated macrophages might be related to an increased mRNA stability, as well as to a later, posttranscriptional step in the activation cascade.

#### Time kinetic of the effect of LBP or BPI on the response to LPS

To further evaluate potential explanations for the differences observed in TNF- $\alpha$  vs NO production induced by LBP-LPS complexes, which could not be attributed to differences in PTK activity, NF- $\kappa$ B translocation to the nucleus, or induction of specific gene expression, we have assessed the kinetics of production of these mediators following stimulation. Peritoneal macrophages were cultured as described above, and supernatants were collected at different times of stimulation. The time course of TNF- $\alpha$  synthesis induced by LPS is presented in Figure 8*a*, where it can be seen that this cytokine was produced at the very early points following stimulation, with levels continuing to increase at later times. The addition of LBP once again induced a decrease in TNF- $\alpha$  production that could be readily detected even at the earliest time points tested, ruling out the possibility of an overexpression induced by LBP and a consequent late down-regulation in TNF- $\alpha$  production. When NO production was evaluated, no nitrite could be detected in supernatants at early stimulation times, and no inhibition in the pattern of LPS-induced NO synthesis was caused by LBP treatment (Fig. 8*b*).

## Discussion

During the last several years, many laboratories have conducted experiments designed to elucidate the mechanisms involved in the activation pathway(s) induced by LPS in host cells. However, several different results were obtained in different laboratories, most probably reflecting differences in the experimental designs utilized. In a previous report, we demonstrated that LPS-induced pro-



**FIGURE 8.** Kinetics of TNF- $\alpha$  (*a*) and NO (*b*) induced by LPS. Exudate peritoneal mouse macrophages were harvested and treated as described in the Figure 1 legend, and supernatants were collected at increasing times poststimulation. TNF- $\alpha$  and NO were quantitated in supernatants as previously described. \*, Statistical difference with respect to the group treated with LPS alone,  $p < 0.05$  (Student's *t* test).

duction of proinflammatory mediators by human monocytes and mouse macrophages could be differentially stimulated or inhibited, respectively, by LBP (24). The related protein BPI, however, inhibited the LPS-induced cytokine response in both cell populations. The production of NO by mouse macrophage, on the other hand, was inhibited only by BPI, but it was not affected by the presence of LBP. To our knowledge, only one laboratory has reported an inhibitory capacity of peptides derived from LBP or BPI on LPS-induced TNF- $\alpha$  synthesis (36).

Several important conclusions can be drawn initially from our observations reported here. First, the modulatory properties of LBP on LPS responses likely depend upon the cellular target used. Perhaps most striking is the fact that LBP differentially affects the ability of LPS to stimulate mouse peritoneal macrophage responses depending upon the phenotypic response being measured. This observation leads to the concept of two separate pathways in activation of macrophages by LPS; the first would contribute to TNF- $\alpha$ /IL-6 synthesis and could be inhibited by both human LBP and BPI, and the latter could stimulate NO production and be inhibited only by BPI. Finally, such findings might not be inconsistent with the existence of at least two independent membrane-binding sites for LPS on mouse macrophages. Potential candidates for these binding sites would include mCD14, CD11b/CD18 (37), the scavenger receptor (38), and the p73 LPS-binding protein identified earlier by our laboratory (16). Based on those previous results, therefore, the purpose of the work summarized in this report was to investigate the participation of different alternative pathways of signal activation in mouse macrophages, which could be dissected using LBP and BPI.

It has been reported that both LBP and BPI, by competing for binding to lipid A, inhibited the LPS activity in the *Limulus* amoebocyte lysate assay, while presenting opposite effects on the production of IL-6, IL-8, and TNF- $\alpha$  by human monocytes (27, 39). LBP is known to form molecular complexes with LPS, thus facilitating its transfer in monomer form to mCD14 and initiating signal transduction pathway(s) (7, 8). Therefore, LPS should bind with a higher affinity to mCD14 than to LBP to be correctly transferred. In contrast, if mouse mCD14 had a lower binding affinity for LPS than did human mCD14, the transfer of LPS might be not

be facilitated. Thus, the addition of LBP might conceivably suppress mouse macrophage cytokine responses by preventing LPS transfer to mouse mCD14. Such an hypothesis would necessarily require that the immediate events involved in LPS activation signaling pathways were inhibited by LBP, whereas our data reported here would not favor that conclusion.

LPS has been shown to activate several PTK implicated in the activation of gene expression (18, 40). In our experiments, we corroborated the participation of PTK in the cascade of activation leading to the synthesis of TNF- $\alpha$ , IL-6, and NO by mouse macrophages. Surprisingly, while LPS induced a time-dependent phosphorylation in a number of proteins in mouse macrophages, this pattern was not diminished by the addition of LBP or BPI; rather, phosphorylation appeared to be substantially increased in some cases. These observations might suggest several conclusions. First, they suggest that LPS binding to its putative receptor on the surface of mouse macrophages is not impeded by either LBP or BPI. In this respect, it has been recently reported that, independently of signal transduction initiation, LBP-LPS complexes internalize after binding to CD14 (41). Second, they may argue against the role of PTK activation as a necessary and sufficient event for LPS-induced proinflammatory mediators production by mouse macrophages. It has already been suggested that, although MAP kinases participate in LPS signaling, they cannot account for independent events leading to NF- $\kappa$ B activation, TNF- $\alpha$  mRNA accumulation, and protein secretion (11). Moreover, it has recently been reported that events such as NF- $\kappa$ B translocation could be induced by LPS in CD14-transfected cells without PTK involvement (42). In agreement, Mukaida et al. have shown that at least two pathways, one staurosporine-sensitive and the second staurosporine-insensitive and PTK-dependent, would converge to activate NF- $\kappa$ B (43).

At this level, our results demonstrate that NF- $\kappa$ B translocation to the nucleus occurred following either LPS-LBP or LPS-BPI treatment; the process was even enhanced in comparison with treatment with LPS alone. Even though promoter analyses have strongly implicated NF- $\kappa$ B in macrophage response to LPS (8), and its translocation is one of the first detected events, our findings indicate that such biochemical changes might not represent a crucial event(s) leading to proinflammatory mediators synthesis, i.e., NF- $\kappa$ B induction alone may not be sufficient for LPS action. Indeed, activation of NF- $\kappa$ B has been reported to occur upon exposure to LPS in macrophages from both C3H/HeN (LPS-responsive) and C3H/HeJ (LPS-hyporesponsive) mice, although TNF- $\alpha$  and iNOS expression could only be detected in the first (44). On the other hand, when we analyzed the composition of the NF- $\kappa$ B complexes induced by LPS and LBP or BPI, p50 appeared to be translocated mainly upon stimulation, while p65 seemed to be translocated in a lesser extent. If LPS-induced translocation of the NF- $\kappa$ B complex, and predominantly p50, were enhanced by treatment with the LPS-binding proteins plus LPS, these findings might support the concept that the inhibitory effect of the LPS-binding proteins on LPS-induced cytokine production by mouse macrophages involves an altered composition of the NF- $\kappa$ B complex rather than an actual absence of translocation. In this regard, in tolerant-rendered monocytes, which minimally respond to LPS stimulation, it has been recently reported that NF- $\kappa$ B is still efficiently mobilized but is unable to transactivate the TNF- $\alpha$  promoter, probably due to a predominance of p50 homodimers in the complex (35). However, the precise involvement of p50 homodimers as a primary reason for the failure of NF- $\kappa$ B activation in tolerant cells is controversial (45). Further studies may be needed to fully

explain the precise role of these compounds and the contribution to our results.

In our studies, we were not able to detect a fully inhibitory effect of LBP or BPI on LPS-dependent expression of TNF- $\alpha$  or iNOS mRNA. These data suggest that the inhibition exerted by LBP in LPS-stimulated macrophages might be related to a later step in the activation cascade. Expression of TNF- $\alpha$  has been reported to be regulated at several levels: transcriptional, post-transcriptional, translational, and posttranslational (46, 47). Posttranscriptional regulation has been similarly observed for iNOS, the synthesis of which may be linked to negative feedbacks on NF- $\kappa$ B (48). Moreover, the expression of both proinflammatory mediators seems to be reciprocally controlled (49, 50). One logical conclusion could be, therefore, that LBP-LPS complexes differentially regulate the synthesis vs stability of mRNA that leads to the final products. Our analyses of this issue have shown an increased stability of TNF- $\alpha$  mRNA induced by LPS in the presence of LBP at earlier times, which could account for some of the inhibitory effect of LBP on the LPS-induced TNF- $\alpha$  response. Recent reports showing differences in the production of surface vs secreted TNF- $\alpha$  would certainly indicate the complexity in the regulation of synthesis of this protein (51). Another alternative explanation might be that higher initial TNF- $\alpha$  synthesis induced by LBP potentiation on LPS stimulation, and a consequent down-regulation, has contributed to a decreased final product. However, no differences could be verified in the kinetics of TNF- $\alpha$  synthesis, since LBP was inhibitory at all times after stimulation. On the other hand, while we could not determine differences in other cytokines transcription (data not shown), the contribution of anti-inflammatory cytokines, such as IL-10, that could be "deactivating" macrophage response to LPS (52) cannot be ruled out.

Although events such as PTK or NF $\kappa$ B activation were demonstrated to occur in association with CD14 occupancy by LPS in human monocytes, our results would indicate that they might not be unique determinants of activation in mouse macrophages. In fact, their actual participation in the LPS signaling cascade seems controversial, since non-LPS structures such as spirochetal lipoproteins could initiate monocyte activation via different cell surface events, but the signaling pathways ultimately converge to NF- $\kappa$ B activation and to produce qualitatively similar cellular responses (53). In fact, several non-LPS-related products might induce some set of genes similar to LPS, as well as activate PTK (54), and even Gram-positive structures could activate monocytic cells via CD14-dependent mechanisms (55). In this respect, it is possible to hypothesize that the final response observed might depend rather on the strength of activation and that alternative, yet to be identified events might also participate. Additionally, the strength of activation might, in turn, depend on the stochastic occupancy of the number of receptors for LPS in the surface of the cells.

In conclusion, we have demonstrated that, in contrast with what has been shown to occur in human monocytes, LBP inhibited cytokine production of mouse macrophages stimulated with LPS. Activation of PTK, NF- $\kappa$ B translocation, and cytokine gene transcription were not significantly modified by the treatment with LPS-LBP complexes. Even, to some extent, the stability of the TNF- $\alpha$  mRNA induced by LPS seemed not to be so drastically altered by LBP as to fully explain the inhibitory action of LBP. These early signaling events for LPS-dependent activation of macrophages seem to be involved in signal transduction initiated by LPS at a point preceding the divergence of the pathways that differentially regulate the expression of TNF- $\alpha$  and NO production, which might rather, or additionally,

be controlled by some as yet to be defined posttranscriptional regulatory mechanism. The evaluation of every single step in the activation pathway induced by LPS, wherein the arrest takes place, or the different types of signals that might contribute to the final products is beyond the scope of this manuscript. Further studies are required to achieve the full characterization of all of the intricate mechanisms that could be involved in the cascade of activation induced by LPS.

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## References

- Morrison, D. C., R. L. Danner, C. A. Dinarello, R. S. Munford, C. Natanson, M. Pollack, J. A. Spitzer, R. J. Ulevitch, S. N. Vogel, and E. McSweeney. 1994. Bacterial endotoxins and pathogenesis of Gram-negative infections: current status and future directions. *J. Endotoxin Res.* 1:71.
- Tracey, K. J., and A. Cerami. 1992. Tumor necrosis factor and regulation of metabolism in infection: role of systemic versus tissue levels. *Proc. Soc. Exp. Biol. Med.* 200:233.
- Walley, K. R., N. W. Lukacs, R. M. Standford, R. M. Strieter, and S. L. Kunkel. 1996. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. *Infect. Immun.* 64:4733.
- Abraham, E., R. Wunderink, H. Silverman, T. M. Perl, S. Nasraway, H. Levy, R. Bone, R. P. Wenzel, R. Balk, R. Allred, J. E. Pemmington, and J. C. Wherry. (TNF- $\alpha$  mAb sepsis group). 1997. Efficacy and safety of monoclonal antibodies to tumor necrosis factor alpha in patients with sepsis syndrome: a randomized, controlled, double-blind, multicenter clinical trial. *JAMA* 273:934.
- Raetz, C. R., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan. 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* 5:2652.
- Sweet, M. J., and D. A. Hume. 1996. Endotoxin signal transduction in macrophages. *J. Leukocyte Biol.* 60:8.
- Hailman, E., H. S. Lichenstein, M. M. Wurfel, D. S. Miller, D. A. Johnson, M. Kelley, L. A. Busse, M. M. Zukowski, and S. D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J. Exp. Med.* 179:269.
- Ulevitch, R. J., and P. S. Tobias. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* 13:437.
- Kirikae, F., T. Kirikae, N. Qureshi, K. Takayama, D. C. Morrison, and M. Nakano. 1995. CD14 is not involved in *Rhodobacter sphaeroides* diphosphoryl lipid A inhibition of tumor necrosis factor alpha and nitric oxide induction by taxol in murine macrophages. *Infect. Immun.* 63:486.
- Lynn, W. A., Y. Liu, and D. T. Golenbock. 1993. Neither CD14 nor serum is absolutely necessary for activation of mononuclear phagocytes by bacterial lipopolysaccharide. *Infect. Immun.* 61:4452.
- Hambleton, J., M. McMahon, and A. L. DeFranco. 1995. Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partially mimics lipopolysaccharide-induced signaling events. *J. Exp. Med.* 182:147.
- Cong, Y. Z., E. Rabin, and H. H. Wortis. 1991. Treatment of murine CD5-B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. *Int. Immunol.* 3:467.
- Nitta, T., H. Imai, Y. Ogasawara, and M. Nakano. 1994. Mitogenicity of bacterial lipopolysaccharide on the T lymphocyte population bearing the  $\gamma\delta$  T cell receptor. *J. Endotoxin Res.* 1:101.
- Helfgott, D. C., L. T. May, Z. Stoeber, I. Tamm, and P. B. Sehgal. 1987. Bacterial lipopolysaccharide (endotoxin) enhances expression and secretion of beta 2 interferon by human fibroblasts. *J. Exp. Med.* 166:1300.
- Frey, E. A., D. S. Miller, T. G. Jahr, A. Sundan, V. Bazil, T. Espevik, B. Finlay, and S. D. Wright. 1992. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J. Exp. Med.* 176:1665.
- Morrison, D. C., M. G. Lei, T. Kirikae, and T. Y. Chen. 1993. Endotoxin receptors on mammalian cells. *Immunobiology* 187:212.
- Stefanova, I., M. L. Corcoran, E. M. Horak, L. M. Wahl, J. B. Bolen, and I. D. Horak. 1993. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56<sup>lkn</sup>. *J. Biol. Chem.* 268:20725.
- Lee, J. C., and P. R. Young. 1996. Role of CSBP/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukocyte Biol.* 59:152.
- Weinstein, S. L., C. H. June, and A. L. DeFranco. 1993. Lipopolysaccharide-induced protein tyrosine phosphorylation in human macrophages is mediated by CD14. *J. Immunol.* 151:3829.
- Orlicek, S. L., E. Meals, and B. K. English. 1996. Differential effects of tyrosine kinase inhibitors on tumor necrosis factor and nitric oxide production by murine macrophages. *J. Infect. Dis.* 174:638.
- Novogrodsky, A., A. Vanichkin, M. Patya, A. Gazit, N. Osherov, and A. Levitzki. 1994. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science* 264:1319.
- Kuprash, D. V., I. A. Udalova, R. L. Turetskaya, N. R. Rice, and S. A. Nedospasov. 1995. Conserved kappa B element located downstream of the tumor necrosis factor  $\alpha$  gene: distinct NF- $\kappa$  B binding pattern and enhancer activity in LPS activated murine macrophages. *Oncogene* 11:97.
- Murphy, W. J., M. Muroi, C. X. Zhang, T. Suzuki, and S. W. Russell. 1996. Both basal and enhancer  $\kappa$ B elements are required for full induction of the mouse inducible nitric oxide synthase gene. *J. Endotoxin Res.* 3:381.
- Amura, C. R., L. C. Chen, N. Hirohashi, M. G. Lei, and D. C. Morrison. 1997. Two functionally independent pathways for LPS-dependent activation of mouse peritoneal macrophages. *J. Immunol.* 159:5079.
- Schumann, R. R., S. R. Leong, G. W. Flagg, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. *Science* 249:1429.
- Gray, P. W., G. Flagg, S. R. Leong, R. J. Gumina, J. Weiss, C. E. Ooi, and P. Elsbach. 1989. Cloning of the cDNA of a human neutrophil bactericidal protein: structural and functional correlations. *J. Biol. Chem.* 264:9505.
- Dentener, M. A., E. J. von Asmuth, G. J. Francot, M. N. Marra, and W. A. Buurman. 1993. Antagonistic effects of lipopolysaccharide binding protein and bactericidal/permeability-increasing protein on lipopolysaccharide-induced cytokine release by mononuclear phagocytes: competition for binding to lipopolysaccharide. *J. Immunol.* 151:4258.
- Hirohashi, N., and D. C. Morrison. 1996. Selective effects of serum factors on LPS on bacterial LPS-induced IL-6 and nitric oxide production in murine peritoneal macrophages. *J. Endotoxin Res.* 3:395.
- Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell. 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. *J. Biol. Chem.* 268:1908.
- Caligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober. 1992. Isolation and analysis of proteins. In *Current Protocols in Immunology*. J. E. Caligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds. Greene Publishing Associates and Wiley Interscience, New York, p. 8.1-8.16.
- Amura, C. R., P. A. Fontan, N. Sanjuan, M. M. Nociari, F. R. Buzzola, and D. O. Sordelli. 1995. Tumor necrosis factor  $\alpha$  plus interleukin 1  $\beta$  treatment protects granulocytopenic mice from *Pseudomonas aeruginosa* lung infection: role of an unusual inflammatory response. *APMIS* 103:447.
- Muroi, M., Y. Muroi, K. Yamamoto, and T. Suzuki. 1993. Influence of 3' half-site sequence of NF- $\kappa$  B motifs on the binding of lipopolysaccharide-activatable macrophage NF- $\kappa$  B proteins. *J. Biol. Chem.* 268:19534.
- Beatty, C. D., T. L. Franklin, Y. Uehara, and C. B. Wilson. 1994. Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction. *Eur. J. Immunol.* 24: 1278.
- Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel. 1990. The DNA binding subunit of NF- $\kappa$  B is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* 62:1007.
- Ziegler Heitbrock, H. W., A. Wedel, W. Schraut, M. Strobel, P. Wendelgass, T. Sternsdorf, P. A. Baeuerle, J. G. Haas, and G. Riethmuller. 1994. Tolerance to lipopolysaccharide involves mobilization of nuclear factor kappa B with predominance of p50 homodimers. *J. Biol. Chem.* 269:17001.
- Battafarano, R. J., P. S. Dahlberg, C. A. Ratz, J. W. Johnston, B. H. Gray, J. R. Haseman, K. H. Mayo, and D. L. Dunn. 1995. Peptide derivatives of three distinct lipopolysaccharide binding proteins inhibit lipopolysaccharide-induced tumor necrosis factor- $\alpha$  secretion in vitro. *Surgery* 118:318.
- Ingalls, R. R., and D. T. Golenbock. 1995. CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. *J. Exp. Med.* 181:1473.
- Brown, M. S., and J. L. Goldstein. 1985. Scavenger cell receptor shared. *Nature* 316:680.
- Gazzano Santoro, H., K. Meszaros, C. Birr, S. F. Carroll, G. Theofan, A. H. Horwitz, E. Lim, S. Aberle, H. Kasler, and J. B. Parent. 1994. Competition between rBPI23, a recombinant fragment of bactericidal/permeability-increasing protein, and lipopolysaccharide (LPS)-binding protein for binding to LPS and Gram-negative bacteria. *Infect. Immun.* 62:1185.
- Hambleton, J., S. L. Weinstein, L. Lem, and A. L. DeFranco. 1996. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc. Natl. Acad. Sci. USA* 93:2774.
- Gegner, J. A., R. J. Ulevitch, and P. S. Tobias. 1995. Lipopolysaccharide (LPS) signal transduction and clearance: dual roles for LPS binding protein and membrane CD14. *J. Biol. Chem.* 270:5320.
- Delude, R. L., M. J. Fenton, R. Savedra, Jr., P. Y. Perera, S. N. Vogel, R. Thieringer, and D. T. Golenbock. 1994. CD14-mediated translocation of nuclear factor- $\kappa$  B induced by lipopolysaccharide does not require tyrosine kinase activity. *J. Biol. Chem.* 269:22253.
- Mukaida, N., Y. Ishikawa, N. Ikeda, N. Fujioka, S. Watanabe, K. Kuno, and K. Matsushima. 1996. Novel insight into molecular mechanism of endotoxin shock: biochemical analysis of LPS receptor signaling in a cell-free system targeting NF- $\kappa$ B and regulation of cytokine production/action through  $\beta_2$  integrin in vivo. *J. Leukocyte Biol.* 59:145.

44. Ding, A., S. Hwang, H. M. Lander, and Q. W. Xie. 1995. Macrophages derived from C3H/HeJ (*Lpsd*) mice respond to bacterial lipopolysaccharide by activating NF- $\kappa$ B. *J. Leukocyte Biol.* 57:174.
45. Takasuka, N., K. Matsuura, S. Yamamoto, and K. S. Akagawa. 1995. Suppression of TNF- $\alpha$  mRNA expression in LPS-primed macrophages occurs at the level of nuclear factor- $\kappa$ B activation, but not at the level of protein kinase C or CD14 expression. *J. Immunol.* 154:4803.
46. Beutler, B. 1992. Application of transcriptional and posttranslational reporter constructs to the analysis of tumor necrosis factor gene regulation. *Am. J. Med. Sci.* 303:129.
47. Biragyn, A., and S. A. Nedospasov. 1995. Lipopolysaccharide-induced expression of TNF- $\alpha$  gene in the macrophage cell line ANA-1 is regulated at the level of transcription processivity. *J. Immunol.* 155:674.
48. Chen, F., D. C. Kuhn, S. C. Sun, L. J. Gaydos, and L. M. Demers. 1995. Dependence and reversal of nitric oxide production on NF- $\kappa$ B in silica and lipopolysaccharide-induced macrophages. *Biochem. Biophys. Res. Commun.* 214:839.
49. Eigler, A., J. Moeller, and S. Endres. 1995. Exogenous and endogenous nitric oxide attenuates tumor necrosis factor synthesis in the murine macrophage cell line RAW 264.7. *J. Immunol.* 154:4048.
50. Salkowski, C. A., G. Detory, R. McNally, N. van Rooijen, and S. N. Vogel. 1997. Regulation of inducible nitric oxide synthase messenger RNA expression and nitric oxide production by lipopolysaccharide in vivo. *J. Immunol.* 158:905.
51. Chaundri, G. 1997. Differential regulation in biosynthesis of cell surface and secreted TNF- $\alpha$  in LPS-stimulated murine macrophages. *J. Leukocyte Biol.* 62:249.
52. Sivo, J., and S. N. Vogel. 1996. IL-10 differentially regulates mRNA expression induced by lipopolysaccharide and interferons in murine peritoneal macrophages. *J. Endotoxin Res.* 3:407.
53. Norgard, M. V., L. L. Arndt, D. R. Akins, L. L. Curetty, D. A. Harrich, and J. D. Radolf. 1996. Activation of human monocytic cells by *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides proceeds via a pathway distinct from that of lipopolysaccharide but involves the transcriptional activator NF- $\kappa$ B. *Infect. Immun.* 64:3845.
54. Vogel, S. N., C. L. Mantey, P. Y. Perera, Z. Y. Li, and B. E. Henricson. 1995. Dissection of LPS-induced signaling pathways in murine macrophages using LPS analogs, LPS mimetics, and agents unrelated to LPS. *Prog. Clin. Biol. Res.* 392:421.
55. Cleveland, M. G., J. D. Gorham, T. L. Murphy, E. Tuomanen, and K. M. Murphy. 1996. Lipoteichoic acid preparations of Gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Infect. Immun.* 64:1906.