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*J Immunol* 2000; 164:5564-5574; doi: 10.4049/jimmunol.164.11.5564

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Inhibition of Lipopolysaccharide-Induced Signal Transduction in Endotoxin-Tolerized Mouse Macrophages: Dysregulation of Cytokine, Chemokine, and Toll-Like Receptor 2 and 4 Gene Expression

Andrei E. Medvedev, Karen M. Kopydlowski, and Stefanie N. Vogel

In this study, the effect of in vitro endotoxin tolerance on LPS-induced mitogen-activated protein kinase activation, transcription factor induction, and cytokine, chemokine, and Toll-like receptor (TLR) 2 and 4 gene expression, as well as the involvement of TNF and IL-1 signaling pathways in tolerance, were examined. Pretreatment of mouse macrophages with LPS inhibited phosphorylation of the extracellular signal-regulated kinases, c-Jun NH2-terminal kinases, and p38 kinase; degradation of I-κBα (inhibitory protein that dissociates from NF-κB) and I-κBβ; and activation of the transcription factors NF-κB and AP-1 in response to subsequent LPS stimulation. These changes were accompanied by suppression of LPS-induced expression of mRNA for GM-CSF, IFN-γ-inducible protein-10, KC, JE/monocyte chemotactant protein-1, macrophage-inflammatory protein-1β, and macrophage-inflammatory protein-2, with concurrent inhibition of chemokine secretion. In contrast to control cells, endotoxin-tolerant macrophages exhibited an increased basal level of TLR2 mRNA, and failed to increase levels of TLR2 mRNA or to down-regulate TLR4 gene expression upon restimulation with LPS. As judged by transcription factor activation, LPS and IL-1 were found to induce a state of cross-tolerance against each other, while no such reciprocal effect was seen for LPS and TNF-α. In addition, macrophages from TNFR I/II double knockout mice were LPS tolerizable, and blocking of endogenous TNF-α with TNFR-Fc fusion protein did not affect the capacity of LPS to tolerize macrophages. These data extend our understanding of LPS-signaling mechanisms that are inhibited in endotoxin-tolerized macrophages and suggest that endotoxin tolerance might result from impaired expression and/or functions of common signalling intermediates involved in LPS and IL-1 signaling. The Journal of Immunology, 2000, 164: 5564–5574.

Lipopolysaccharide, a predominant glycolipid in the outer membrane of Gram-negative bacteria, stimulates monocytes, macrophages, and neutrophils to produce cytokines, increase expression of cell adhesion molecules, and secrete low m.w. proinflammatory mediators (1). LPS induces cellular responses by its complexing with circulating LPS-binding protein and subsequent binding to CD14 (2, 3) that, in turn, facilitates the interaction of LPS with signaling molecules belonging to the Toll-like receptor family. Toll is a type I transmembrane protein that controls dorsoventral pattern formation during embryogenesis of Drosophila (4) and antifungal immune response in the adult fly (5).

Cloning of mammalian analogues of Toll have been published (6–9), defining the family of Toll-like receptors (TLR) (4). Transfection of a constitutively active mutant of human TLR4 results in NF-κB activation, leading to expression of a number of proinflammatory genes and the T cell costimulatory protein B7.1 (7). Positional cloning of the Lpsd locus, which is responsible for LPS hyporesponsiveness in C3H/HeJ mice, mapped it to the trld gene that encodes a TLR4 protein with a single point mutation (P712H) (10, 11). This has been proposed to yield a nonfunctional molecule, capable of suppressing LPS signal transduction through wild-type TLR4 (10), a suggestion recently supported by functional studies of \((Lps^{a} \times Lps^{d})_{F_{1}}\) and \((Lps^{a} \times Lps^{d})_{F_{2}}\) mice (12) and by in vitro overexpression experiments (13). A central role for TLR4 in LPS signaling has been further confirmed by the observations that mice with a natural deletion of the trld gene or its targeted disruption are LPS hyporesponsive (10, 11, 14). A novel accessory molecule, MD-2, has been recently found to impart LPS responsiveness on human TLR4 (15). Although these findings strongly suggest TLR4 as the primary LPS signaling receptor, TLR2 has also been implicated in LPS signaling. Indeed, overexpression of TLR2 confers LPS sensitivity upon 293 human embryonic kidney cells, resulting in NF-κB activation and IL-8 mRNA expression (16–18), although re-extraction of commercial LPS preparations

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Received for publication December 22, 1999. Accepted for publication March 10, 2000.

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1 This work was supported by National Institutes of Health Grant AI-18797 (S.N.V.). This work was conducted by K.M.K. in partial fulfillment of the requirements for the Ph.D. degree from the Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences. The opinions or assertions contained within are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense. Research was conducted according to the principles set forth in Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, DHEW Publication (National Institutes of Health) 85–23.

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3 Abbreviations used in this paper: TLR, Toll-like receptor; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; β-gal, β-galactosidase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; I-κB, inhibitory protein that dissociates from NF-κB; IKK, I-κB kinase; IP-10, IFN-γ-inducible protein-10; IRAK, IL-1R-associated kinase; JNK, c-Jun NH2-terminal kinase; Luc, luciferase; MAP, mitogen-activated protein; MCP, monocyte chemotactant protein; MIP, macrophage-inflammatory protein; TRAF, TNFR-associated factor.

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to remove bioactive contaminants eliminated signaling in TLR2, but not TLR4, transfecants. TLR2 associates with CD14 on the cell surface, and LPS enhances oligomerization of TLR2 with subsequent recruitment of the IL-1R-associated kinase (IRAK) (18). Common signaling intermediates, including the adaptor protein MyD88, IRAK, TNFR-associated factor-6, and NF-κB-inducing kinase, are shared by the IL-1, TLR2, and TLR4 signaling pathways (16, 18, 19). Yet, recent studies have demonstrated that TLR2 is largely required for signaling by lipoproteins (20–22), numerous Gram-positive ligands (23–26), mycobacteria (25, 27), spirochetes (21, 22, 25), and mycoplasma (25). In contrast, TLR4 fails to confer responsiveness to Gram-positive bacteria and their components (26) and has been postulated as the main LPS signaling receptor based on the data obtained in the mouse. Indeed, TLR2 knockout mice are hypersensitive to several Gram-positive bacterial cell walls and Staphylococcus aureus peptidoglycan, whereas their LPS response is normal (28). However, a profound inhibitory effect of anti-TLR2 Ab on LPS-mediated IL-12 production from human monocytes has been reported (20), suggesting a functional role for TLR2 in LPS signaling in human monocytes. Although TLR4 is clearly critical to LPS signaling in the mouse, additional studies will be required to delineate further the relative contribution of TLR2 vs TLR4 in LPS signal transduction in human cells.

Prior exposure to LPS induces a transient state of cellular hypersensitivity to subsequent stimulation with LPS known as endotoxin tolerance. This mechanism may protect the host from developing a shock syndrome caused by hyperactivation of monocytes and macrophages with persisting bacteria and LPS. On the other hand, it has been recently postulated that suppressed IL-12 production by monocytes and dendritic cells associated with endotoxin tolerance may result in an inability to respond appropriately to secondary infections in survivors of sepsis (29). Although LPS tolerance is manifested by suppressed production of many cytokines, e.g., TNF-α, IL-1β, IL-6, and IL-12, expression of other mediators, e.g., IL-10, IL-1R antagonist, TNFRII, and NO, is not inhibited (reviewed in Ref. 30). Therefore, endotoxin tolerance does not totally inhibit cellular functions, but rather represents a reprogramming of cells, possibly, as a means of adaptation to bacterial infection (31). Endotoxin tolerance is not accompanied by decreased expression of CD14 (32), and inhibition of LPS signaling in endotoxin-tolerized cells occurs very early in the signaling cascade, involving decreased membrane GTP-binding capacity and G protein content (33), and altered expression of phospholipase C-γ1 and phosphatidylinositol-3′ kinase (34). Other changes include suppressed activation of the mitogen-activated protein (MAP) kinases (35–37) and I-κB kinases (IKK) (38), and decreased degradation of I-κBα (38). Contradictory results have been reported on NF-κB translocation (38–45) and the subunit composition of NF-κB in endotoxin-tolerant cells (39, 40, 45–48). LPS-tolerant macrophages have been found to express decreased levels of the protooncogene Jun-B (49), a member of the AP-1 family. Yet, little is known about the effect of endotoxin tolerance on degradation of I-κBβ, as well as on AP-1 DNA binding and transcription. Controversy exists regarding the involvement of the TNF and IL-1 signaling pathways in the induction of tolerance (50–55). Finally, it is not well defined how endotoxin tolerance affects the expression of TLR2 and 4.

In this study, we report inhibited MAP kinase phosphorylation, NF-κB and AP-1 activation, cytokine and chemokine gene expression, and dysregulated transcription of TLR2 and 4 genes in endotoxin-tolerized murine macrophages stimulated with LPS. Our findings demonstrate that LPS and IL-1β induce a state of cross-tolerance, indicating that endotoxin tolerance involves the IL-1 signaling pathway. Conversely, application of several approaches to block TNF-α production or responsiveness, as well as experiments on induction of cross-tolerance, allowed us to rule out a role for TNF-α in the induction of endotoxin tolerance in vitro.

Materials and Methods

Reagents

Protein-free, phenol/water-extracted Escherichia coli LPS K235 was prepared as described previously (56). Human rIL-1β (sp. act., 1.9 × 10^7 U/mg) was kindly provided by Dr. D. Kahn (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD) and had endotoxin content of 2.5 U/mg protein, as determined by a Limulus amebocyte lysate assay. Murine rTNF-α (sp. act., 2.5 × 10^7 U/mg, endotoxin content <1 ng/mg) was purchased from R&D Systems (Minneapolis, MN). Rabbit pAb against active (phosphorylated) extracellular signal-regulated kinases (ERK) 1 and 2, e-Jun NH2-terminal kinases (JNK) 1 and 2, and p38, as well as HRP-conjugated donkey anti-rabbit IgG were obtained from Promega (Madison, WI). Rabbit polyclonal IgG against 1-κBα/ MAD-3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antisera directed to mouse p50 and p65 NF-κB subunits, and to I-κBα, were a kind gift of Dr. N. Rice (Frederick Cancer Research and Development Center, National Cancer Institute). Recombinant soluble TNFR type II-Fc IgG conjugate (TNFR-Fc) was provided by Immunex (Seattle, WA). ELISA kits for detection of TNF-α, JE/monocyte chemotactic protein (MCP)-1, macrophage-inflammatory protein (MIP)-1β, KC, and MIP-2 were purchased from Genzyme (Cambridge, MA), Pharmingen (San Diego, CA), and R&D Systems, respectively.

Mice

C3H/OuJ (Lps−/) and B6129SP/J/V mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TNFR I/II double knockout mice were kindly provided by Dr. George Yap (Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD). Mice were housed in cages with filter tops in a laminar flow hood and fed food and acid water ad libitum.

Tissue culture

Peritoneal exudate macrophages were harvested by peritoneal lavage with ice-cold sterile physiological saline 4 days after i.p. injection of mice with 3 ml of sterile 3% thioglycollate broth. Cells were washed, resuspended in RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 0.3% sodium bicarbonate, and 2% FBS, and plated in six-well plates (4 × 10^5 cells/well). Following overnight incubation, nonadhering cells were removed by washing three times with PBS (BioWhittaker, Walkersville, MD), and macrophages were resuspended in fresh culture medium. The mouse macrophage cell line, RAW 264.7, was obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM medium supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Cells were pretreated for 20 h with medium, LPS, or cytokines, washed three times with PBS (BioWhittaker), and resuspended in culture medium. After incubation for 2 h, macrophages were restimulated as described in the figure legends.

Plasmids, transient transfection, and reporter assays

Construction of the control luciferase reporter plasmid Luciferase (Luc) and reporter plasmids p(NF-κB)Luc and p(AP-1)Luc was previously described (57). Transient transfections were performed essentially as described (57), with small modifications. Briefly, RAW 264.7 cells were plated into 12-well plates (Costar, Cambridge, MA) at 1 × 10^5 cells/well in DMEM/10% FCS, grown overnight, and transfected for 3 h with the reporter plasmids (0.5 μg/well of p(NF-κB)Luc or 0.1 μg/well of p(AP-1)Luc) along with 0.5 μg/well of pHC110 eucaryotic β-galactosidase (β-gal) assay vector (Amer sham Pharmacia Biotech, Piscataway, NJ) by using 7.5 μl/well of SuperFect transfection reagent (Qiagen, Chatsworth, CA). The total amount of plasmid DNA was adjusted to 1.5 μg/well by addition of corresponding amounts of phBluescript II SK+ plasmid (Stratagene, La Jolla, CA). After transfections, cells were washed with PBS, stimulated for 20 h, washed three times with ice-cold PBS, and lysed in a lysis buffer (Analytical Luminescence Laboratory, Sparks, MD) for 30 minutes.
FIGURE 1. Comparison of LPS-mediated activation of the ERK1/2, JNK1/2, and p38 MAP kinases in mouse C3H/OuJ macrophages pretreated with medium or endotoxin. Cells were pretreated for 20 h with either medium or 10 ng/ml LPS, followed by washing with PBS, addition of fresh medium, and incubation for 2 h. Subsequently, macrophages were restimulated for the indicated time points with 10 ng/ml LPS (A, time course) or for 30 min with serial dilution of LPS (B, dose response). Cellular extracts were prepared and MAP kinase phosphorylation was examined by Western blotting with pAb specific for the respective forms of the MAP kinases. The results of a representative experiment (n = 6) are presented.

Preparation of nuclear extracts and EMSA
Nuclear extracts were prepared according to Dignam et al. (59) with small modifications (57). Protein concentration was determined using the Bio-Rad assay kit (Hercules, CA). NF-κB-specific oligonucleotide 5′-AGTT-GAGGGCCCTCCCCAGGC-3′ from the murine Ig κ light chain gene enhancer and AP-1-specific oligonucleotide 5′-CGCTTGTAGGATGGCAGCCGGA-3′ probes were synthesized by the BIC Synthesis and Sequencing Facility (Uniformed Services University of the Health Sciences, Bethesda, MD). DNA probes were 32P end labeled with T4 polynucleotide kinase (Promega), as recommended by the manufacturer. Nuclear extracts (4 μg) were incubated with 0.2 ng radiolabeled DNA probe in a binding buffer (final volume 20 μl) containing 2 μg poly(dI-dC) (Amersham Pharmacia Biotech), 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM EDTA, and 0.25 mg/ml BSA for 30 min at room temperature. For supershift analyses, nuclear extracts were first preincubated with 1 μl of the respective antisera against members of the NF-κB family for 5 min at room temperature in the binding buffer, followed by addition of radiolabeled probe. After incubation, a portion of each reaction (18 μl) was loaded onto a 5% nondenaturing polyacrylamide gel, and the DNA-protein complexes were resolved from free oligonucleotide by electrophoresis (0.25% Triton X-100, 2 h). The gels were dried (80°C, 2 h) and exposed to x-ray films (X-OMAT AR; Eastman Kodak, Rochester, NY).

Western blot analysis
Cellular extracts were prepared as described (60). Twenty micrograms of total protein were added in Laemmli buffer, boiled for 5 min, resolved by SDS-12% PAGE in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS), and blotted onto Immunobilon P transfer membranes (100 V, 1.5 h, 4°C). After blocking for 2 h in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20), containing 1% gelatin and 5% nonfat milk, membranes were washed three times in TBS-T and probed for 1.5 h with anti-phospho-MAP kinase Ab (Promega; dilution 1/3,000), anti-IκBα pAb (Santa Cruz Biotechnology; dilution 1/1,000), or anti-IκBβ antisemur (dilution 1/1,000) in TBS-T/0.5% nonfat milk. Following washing three times in TBS-T, membranes were incubated with secondary HRP-conjugated donkey anti-rabbit IgG (Promega; 1/10,000 dilution) and washed five times in TBS-T, and bands were detected using enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech), according to manufacturer’s description.

Isolation of RNA and RT-PCR
Total RNA was isolated by using RNA Stat60 isolation reagent (Tel-Test “B,” Friendswood, TX), as specified by the manufacturer and quantified by spectrophotometric analysis. Relative quantities of mRNA for hypoxanthine-guanine phosphoribosyltransferase (HPRT), TNF-α, IL-1β, GM-CSF, KC, IFN-γ-inducible protein (IP)-10, MIP-1β, and MIP-2 were determined by a coupled RT-PCR using the primers and probes, as detailed previously (60, 61). The primers (sense (S) and antisense (AS)) and probes (P) for the genes analyzed in this study were as follows: TLR2, (S) 5′-ACGGAGGCAGGCTGGAGGAC-3′, (AS) 5′-TTGCTGAAGAGGACTGTTATGG-3′, (P) 5′-CTTCACCTCTCCTGTATTTGAC-3′; TLR4, (S) 5′-AGCAGAGGAGAAAAGCTCAGCTATGATGC-3′, (AS) 5′-GGTTTAGGC CCCAGAGTTTTGT TCTCC-3′, (P) 5′-GGTTCACCTCGGCTTAC-3′; MyD88, (S) 5′-CACCCTGTGCTGTTGCATT-3′, (AS) 5′-CG CAGGATATCGGGAAAAATG-3′, (P) 5′-CAAGCTGTCTCCAGGTAGA-3′. The optimal cycle number for each gene was determined empirically and was defined as the number of cycles that resulted in detectable PCR-amplified products under nonsaturating conditions, as specified previously (60). Each cycle consisted of 1 min at 95°C, 1 min at a gene-specific annealing temperature, and 2-min primer extension at 72°C. The annealing temperatures and optimal cycle numbers (shown in parentheses) for each primer set were as follows: 60°C for TNF-α (26 cycles); 54°C for IL-1β (29 cycles), GM-CSF (35 cycles), and HPRT (25 cycles); 60°C for JE/MCP-1 (25 cycles); 55°C for IP-10 (24 cycles); 65°C for MIP-1β (28 cycles), MIP-2 (28 cycles), and KC (28 cycles); 56°C for TLR2 (27 cycles); 58°C for TLR4 (28 cycles); and 55°C for MyD88 (28 cycles). PCR products were electrophoresed and transferred to Hybond N+ membranes (Amersham, Arlington Heights, IL) in 10× SSC by standard Southern blotting techniques. DNA was cross-linked with UV light, baked at 80°C for 18 h, and hybridized with oligonucleotide probes labeled with FITC by using ECL oligolabeling system (Amersham). Following incubation with anti-FITC Ab conjugated with HRP, bands were visualized using ECL reagents (Amersham), according to manufacturer’s description. To determine the magnitude of change in gene expression, cDNA from a sample known to be positive for the expression of the given gene (positive control) was used to generate standard curves by serial 2-fold dilution of the positive control and simultaneous amplification. The signal of each band in the positive control and simultaneous amplification. The signal of each band in the positive control was defined as the number of cycles that resulted in detectable PCR-amplified products under nonsaturating conditions. The equation from this line was used to calculate fold induction in test samples. Data were normalized for the relative quantity of mRNA by comparison with HPRT. Changes in mRNA expression were presented as mean fold induction relative to untreated controls, which were arbitrarily assigned a value of 1.

Cytokine/chemokine ELISA
JE/MCP-1 and TNF-α content in macrophage culture supernatants was measured by ELISA, as specified by the manufacturer (PharMingen, R&D Systems, and Genzyme, respectively). KC and MIP-2 were measured using Ab pairs (rat anti-mouse KC IgG2a, 1 μg/ml; biotinylated goat anti-mouse KC IgG, 0.5 μg/ml; rat anti-mouse MIP-2 IgG2b, 1 μg/ml; and biotinylated goat anti-mouse MIP-2 IgG, 0.5 μg/ml; R&D Systems), according to the manufacturer’s instructions. The lower limit of detection for JE/MCP-1, KC, MIP-2, and TNF-α was 15.6, 15.6, 7.8, and 35 pg/ml, respectively.
Macrophages rendered endotoxin tolerant show inhibited LPS-induced MAP kinase activation

Activation of the ERK, JNK, and p38 MAP kinases is important in mediating many macrophage functions, including activation of various transcription factors and production of pro- and anti-inflammatory cytokines (62). Therefore, we sought to examine whether endotoxin tolerance affects this important upstream stage of LPS signaling. Because phosphorylation of the MAP kinases closely correlates with their activation (63), this parameter was measured in macrophage cellular extracts subjected to Western blotting and staining with Ab specific for phosphorylated forms of the respective MAP kinases.

Results

**Macrophages rendered endotoxin tolerant show inhibited LPS-induced MAP kinase activation**

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**Endotoxin tolerance inhibits activation of NF-κB and AP-1 in response to LPS**

The MAP kinase pathways play an important role in mediating transcription factor activation (62). Hence, it was of interest to examine whether suppression of LPS-induced MAP kinase activation detected in endotoxin-tolerant macrophages is accompanied by decreased induction of the transcription factors NF-κB and AP-1. Before translocation of NF-κB into the nucleus, IκB proteins undergo phosphorylation, ubiquitination, and proteosomal degradation, releasing NF-κB dimers and unmasking their nuclear localization and DNA binding domains (64). Therefore, we first...
IκBα cleaved by 60–120 min. In contrast, LPS-pretreated C3H/OuJ presented.

100 ng/ml PMA, followed by measurement of Luc and phages with mouse rTNF-

Consistent with our MAP kinase data, prior incubation of macrophages previously exposed to LPS, as opposed to the strong LPS-induced response seen in medium-pretreated cells (Fig. 2B). To confirm these results, we next evaluated cytokine secretion by LPS-stimulated macrophages subjected to prior treatment with medium or LPS. Table I shows that LPS stimulation of medium-pretreated macrophages induced the production of high levels of all the cytokines tested. The highest levels of cytokine production were detected for TNF-α, IL-1β, and IL-6 for JE/MCP-1, IP-10, and MIP-2 (61). Prior exposure to LPS led to a profound inhibition of the expression of TNF-α and KC, JE/MCP-1, and MIP-2 mRNA following stimulation with LPS (Fig. 4). To extend these observations further, we examined the effect of in vitro endotoxin tolerance on LPS-induced steady-state levels of mRNA for CXC chemokines IP-10, KC, and MIP-2, as well as CC chemokines JE/MCP-1 and MIP-1β. Detection of mRNA for each chemokine was conducted at their peak levels of LPS induction (i.e., 2 h for KC and MIP-1β, and 6 h for JE/MCP-1, IP-10, and MIP-2) (61).

Previous reports have documented that endotoxin tolerance suppresses TNF-α gene expression and production both in vivo and in vitro (30, 35–37, 42, 48, 55), and inhibits expression of a panel of chemokine genes in response to cecal ligation and puncture in vivo (65). However, controversial results have been published with respect to LPS-induced IL-1β gene expression in endotoxin-tolerant cells (35, 42, 55, 66), and little is known about the effect of in vitro endotoxin tolerance on GM-CSF and chemokine gene expression in primary mouse macrophages. To this end, we performed RTPCR analyses of LPS-induced expression of a number of cytokine and chemokine genes in murine C3H/OuJ macrophages following pretreatment with medium or LPS. LPS pretreatment resulted in suppression of LPS-mediated induction of TNF-α, IL-1β, and GM-CSF mRNA (Fig. 4A), whereas the expression of a housekeeping gene, HPRT, was comparable. To extend these observations further, we examined the effect of in vitro endotoxin tolerance on LPS-induced steady-state levels of mRNA for CXC chemokines IP-10, KC, and MIP-2, as well as CC chemokines JE/MCP-1 and MIP-1β. Detection of mRNA for each chemokine was conducted at their peak levels of LPS induction (i.e., 2 h for KC and MIP-1β, and 6 h for JE/MCP-1, IP-10, and MIP-2) (61).

Prior exposure to LPS led to a profound inhibition of the expression of IP-10, KC, MIP-2, JE/MCP-1, and MIP-1β mRNA following stimulation with LPS (Fig. 4). To confirm these results, we next evaluated cytokine secretion by LPS-stimulated macrophages subjected to prior treatment with medium or LPS. Table I shows that LPS stimulation of medium-pretreated macrophages induced the production of high levels of all the cytokines tested. The highest levels of cytokine production were detected for TNF-α and KC, JE/MCP-1 manifested intermediate LPS inducibility, whereas the lowest induction was detected for MIP-2 (Table I). In accordance with the mRNA data, LPS pretreatment significantly inhibited the production of TNF-α, JE/MCP-1, KC, and MIP-2 induced by LPS after 6 h of stimulation (Table I). Thus, LPS-tolerant macrophages are profoundly refractory in their capacity to induce gene expression and secretion of a broad array of cytokines and chemokines.

**FIGURE 3.** Endotoxin-tolerant macrophages exhibit suppressed activation of the nuclear factor AP-1 in response to LPS, but not to PMA. A, C3H/OuJ mouse macrophages were pretreated as specified in Fig. 2A, washed with PBS, and incubated in a fresh medium for 2 h. Thereafter, cells were stimulated with 10 ng/ml LPS for the indicated time periods, followed by preparation of nuclear extracts and assaying AP-1 DNA-binding activity by EMSA. The figure represents one of four separate experiments conducted. B, RAW 264.7 cells were pretreated for 20 h with medium or 1 ng/ml LPS, washed with PBS, resuspended in a fresh medium, and incubated for 2 h. Following cotransfection with p(AP-1)3 L d Luc and pCH10, cells were stimulated for 24 h with medium, 10 ng/ml LPS, or 100 ng/ml PMA, followed by measurement of Luc and β-gal activity in cellular lysates. The data of a representative experiment (n = 4) are presented.
Dysregulation of LPS-induced TLR2 and TLR4 mRNA expression in endotoxin-tolerant macrophages

Because endotoxin-tolerant phenotype is manifested at the upstream stages of LPS signaling (33–37), it is plausible that LPS tolerance affects expression and/or functions of TLR2 and TLR4. To test this hypothesis, LPS-mediated regulation of the expression of TLR2 and TLR4 mRNA was analyzed by RT-PCR in medium- or LPS-pretreated mouse C3H/OuJ macrophages. Analysis of mRNA induction was the only possible approach due to the lack of available Ab against murine TLR2 and TLR4 required to complement mRNA results with protein expression data. Fig. 5 demonstrates that in medium-pretreated cells, LPS led to a strong increase in the steady-state levels of TLR2 mRNA detectable as early as 1 h; maximal response was reached at 3 h after stimulation, and was sustained throughout 12 h of LPS treatment. In contrast to the pattern of TLR2 gene expression, TLR4 mRNA was constitutively expressed and modestly down-regulated by LPS following activation for 3–6 h (Fig. 5). After 20 h of LPS treatment, the level of TLR4 mRNA was similar to that measured in untreated and unstimulated macrophages, while TLR2 mRNA was present at levels comparable with those detected after 1 h of LPS stimulation of medium-pretreated cells (Fig. 5). As shown in Fig. 5, LPS-pretreated macrophages failed to respond to subsequent LPS exposure by modulating either TLR2 or TLR4 mRNA expression. Of note, the steady-state levels of mRNA for the adaptor

Table I. Production of JE/MCP-1, KC, MIP-2, and TNF-α in culture supernatants of LPS-tolerized peritoneal macrophages

<table>
<thead>
<tr>
<th>Pretreatment/Challenge</th>
<th>JE/MCP-1b (pg/ml)</th>
<th>KCb (pg/ml)</th>
<th>MIP-2b (pg/ml)</th>
<th>TNF-αb (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium/medium</td>
<td>157 ± 18</td>
<td>7 ± 4</td>
<td>±15</td>
<td>±70</td>
</tr>
<tr>
<td>LPS/medium</td>
<td>4,928 ± 160</td>
<td>1,022 ± 60</td>
<td>1,075 ± 117</td>
<td>1,092 ± 51</td>
</tr>
<tr>
<td>Medium/LPS</td>
<td>14,397 ± 1,091</td>
<td>19,109 ± 2,085</td>
<td>10,499 ± 1,537</td>
<td>22,208 ± 4,241</td>
</tr>
<tr>
<td>LPS/LPS</td>
<td>5,675 ± 169c</td>
<td>3,728 ± 499c</td>
<td>590 ± 166c</td>
<td>3,041 ± 152c</td>
</tr>
</tbody>
</table>

a Macrophage cultures were pretreated with medium or LPS (100 ng/ml) 24 h prior to challenge with medium or LPS (10 ng/ml). Culture supernatants were collected 2 h (MIP-2) or 6 h (JE/MCP-1, KC, and TNF-α) after challenge.

b Data are represented as the mean ± SEM from four separate experiments.

c Data from LPS/LPS were significantly lower (p ≤ 0.01) than medium/LPS.
protein, MyD88, were not influenced by LPS stimulation and were expressed at the same levels in both control and LPS-tolerant cells (Fig. 5).

Involvement of the IL-1, but not TNF, signaling pathway in the induction of LPS tolerance

Next, the involvement of TNF and IL-1 signaling pathways in the induction of endotoxin tolerance was examined. Fig. 2B shows that similar kinetics of LPS-induced NF-κB translocation was detected in mouse C3H/OuJ macrophages pretreated with either medium or mouse rTNF-α, whereas endotoxin pretreatment markedly inhibited the NF-κB response to LPS. Similarly, pretreatment of RAW 264.7 cells with mouse rTNF-α did not affect LPS-induced NF-κB transactivation, although it completely suppressed the TNF-α response (data not shown). To verify these results, two additional approaches were employed. First, LPS-induced NF-κB activation was examined in LPS-pretreated macrophages isolated from either TNFR I/II-deficient macrophages and cells from TNFR I/II knockout mice revealed strong TNF-α-induced NF-κB, no NF-κB translocation was seen in TNFR I/II double knockout mice or wild-type controls. Whereas TNFR I/II translocation was detected in TNFR I/II knockout macrophages from both LPS and IL-1β stimulation (Fig. 7, A and B). Consistent with these data, prior exposure of macrophages to IL-1β also exerted a marked suppression of NF-κB and AP-1-dependent transcription in response to both LPS and IL-1β stimulation (Fig. 7, A and B). Furthermore, a marked suppression of LPS-induced expression of TNF-α mRNA was seen when macrophages were pretreated with either LPS or IL-1β, but not with TNF-α, whereas their response to TNF-α was unaffected (data not shown). Collectively, these results indicate the involvement of the IL-1, but not TNF, signaling pathway in the induction of macrophage tolerance in vitro.

Discussion

Studies on the mechanisms of in vivo endotoxin tolerance have provided compelling evidence for a key role for macrophages in
this process (30). Pretreatment of macrophages in vitro with LPS also induces a refractory state to subsequent stimulation with LPS (30, 35–37, 44), although the precise relationship between this in vitro phenomenon and in vivo endotoxin tolerance has not been delineated fully. Prior exposure to LPS inhibits expression of many LPS-inducible cytokine genes, whereas others are unaffected or even up-regulated (30). Hence, a concept of reprogramming has been proposed that explains such changes in terms of macrophage adaptation to coexisting bacteria (31). Recent studies have identified intracellular mechanisms affected by in vitro endotoxin tolerance. These include, but are not limited to, impaired G protein expression and function (33), suppressed MAP kinase activation (35–37), diminished NF-κB translocation (38, 42) and transactivation (40, 46), changes in the ratio between p50 and p65 subunits of NF-κB (40, 45–47), and induction of immunosuppressive proteins (30). However, as discussed below, controversial results have been reported even when similar models have been used. The purpose of this work was to analyze in a greater detail the effect of in vitro endotoxin tolerance on select signaling pathways known to be activated in macrophages by LPS in an attempt to clarify contradictory results and to extend our understanding of mechanisms of endotoxin tolerance.

Activation of the MAP kinases ERK, JNK, and p38 is important in mediating a broad array of cellular responses, including cell proliferation and differentiation, transcription factor activation, and cytokine gene expression and production (62). Several recent reports have shown a down-regulation of activation of ERK, JNK, and p38 MAP kinases in endotoxin-tolerant mouse macrophages (35–37). However, the interpretation of some of these findings is seriously complicated by the fact that the authors used commercial LPS that was likely to be contaminated with LPS-associated proteins, as evidenced by its activity in C3H/HeJ macrophages (35). Using highly purified protein-free LPS, we have demonstrated very weak LPS-mediated phosphorylation of the ERK1/2, JNK1/2, and p38 MAP kinases, and, conversely, pretreatment with TNF-α did not suppress LPS responses, indicating specific inhibition of LPS signaling pathways.

LPS-mediated activation of the transcription factors NF-κB and AP-1 requires phosphorylation of IKK and JNK kinases, respectively. JNK subsequently phosphorylates AP-1, inducing its transactivation (68), whereas activated IKK phosphorylates the family of IκB proteins, targeting them for degradation via the ubiquitin-proteasome pathway (64). As a result, NF-κB dimers are released from their complex with IκB proteins, followed by their translocation into the nucleus, wherein NF-κB activates transcription of genes encoding cell adhesion and MHC molecules, as well as cytokines and cytokine receptors (64). This study confirms and extends earlier published observations of mitigated LPS-induced degradation of IκBα in LPS-tolerant cells (38, 43). In addition, it shows that degradation of another member of the IκB family, IκBβ, is also markedly repressed in endotoxin-tolerant macrophages stimulated with LPS. Consistent with the ability of IκB proteins to sequester NF-κB in the cytoplasm (64), we observed significantly lower amounts of NF-κB translocated into the nucleus of endotoxin-tolerant macrophages, which confirms some of previously published observations (38, 42). In addition, this study shows that endotoxin-tolerant state of murine macrophages was associated with increased levels of the p50 and decreased amounts of the p65 subunits of NF-κB. Whereas previous publications also demonstrated the predominance of p50 subunits in endotoxin-tolerant cells, no defect in NF-κB translocation was reported (40, 45–47), in contrast to other studies (38, 42) and to our results (Fig. 2). Differences in cell types used, NF-κB oligonucleotide probes utilized, as well as in experimental approaches employed to induce endotoxin tolerance (e.g., long-term vs short-term pretreatment) could potentially account for these inconsistent findings. Our results correspond to the well-established function of IκB proteins in preventing NF-κB translocation (64), showing that decreased LPS-induced degradation of both IκBα and IκBβ caused by prior incubation with endotoxin is accompanied by lower NF-κB translocation into the nucleus in response to subsequent LPS stimulation. Furthermore, our data support the involvement of p50 in transcriptional repression due to its lack of transactivation domains (64). Indeed, consistent with earlier results obtained in other cell
lines (40, 45–47), our transient transfection experiments demonstrated significantly lower LPS-induced NF-κB transactivation. In this respect, it is of great importance that macrophages from p50 knockout mice have been reported to fail to exhibit an endotoxin-tolerant phenotype following pretreatment with LPS (69). We also demonstrate that LPS-tolerant macrophages exhibited markedly repressed activation of the transcription factor AP-1 in response to LPS, evident both at the level of its DNA-binding activity and transactivation, with a concurrent decrease of LPS-induced activation of JNK1/2. These data extend the results of a recent report that showed a down-regulation of the protooncogene Junb, a member of the AP-1 family, in the LPS-tolerant mouse macrophage cell line P388D1 (49). Thus, an endotoxin-tolerance state of murine macrophages induced in vitro is underpinned by inhibited activation of both NF-κB and AP-1.

It was previously shown in a model endotoxin tolerance in vivo that expression of a number of chemokine genes was suppressed in response to polymicrobial sepsis caused by cecal ligation and puncture (65). This study extends these data and demonstrates a profoundly down-regulated ability of LPS to induce steady-state mRNA levels for the CXC chemokines IP-10, KC, and MIP-2, as well as CC chemokines JE/MCP-1 and MIP-1β in macrophages rendered endotoxin tolerant in vitro. Thus, the observation that chemokine gene expression is globally repressed in LPS-tolerized macrophages is also consistent with the reported mitigation of neutrophil infiltration into the lungs of mice tolerized in vivo (65). Likewise, endotoxin-tolerant macrophages showed suppressed LPS-induced expression of mRNA for TNF-α and IL-1β, as well as GM-CSF. Recent studies have demonstrated that GM-CSF knockout mice are less sensitive to LPS, as evidenced by decreased levels of circulating IFN-γ, IL-1α, and IL-6, but not TNF-α (70), suggesting the role for GM-CSF in endotoxicity. Therefore, diminished GM-CSF induction may contribute to the LPS hypersensitivity seen in endotoxin-tolerant mice. Inhibited expression of cytokine and chemokine genes seen in endotoxin-tolerant macrophages stimulated with LPS is likely to result from decreased activation of the transcription factors NF-κB and AP-1 that are pivotal in governing expression of a variety of chemokine, cytokine, and cytokine receptor genes (64, 68). In addition, repressed LPS-mediated activation of the p38 MAP kinase may also contribute to mitigated cytokine gene expression manifested by endotoxin-tolerant macrophages, due to the ability of p38 to bind to and phosphorylate the transcription factor TFIIID, thereby facilitating its interaction with p65 subunit of NF-κB (71).

Previous studies have shown that endotoxin tolerance inhibits LPS signal transduction at upstream levels (33, 34). These changes may result from suppression of the very first LPS signal transduction events that involve the engagement of TLR4 and/or TLR2 (10, 11, 13–19), whose expression levels are of significance for enabling optimal LPS responsiveness (11, 72). Due to a lack of available Ab against mouse TLR that would be required to estimate protein expression, we took advantage of the extreme sensitivity of semiquantitative RT-PCR to address this question. As demonstrated herein, unstimulated C3H/OuJ mouse macrophages constitutively expressed high levels of TLR4 mRNA that were decreased within 3–6 h after LPS stimulation, followed by their return to basal levels at later time periods. TLR2 mRNA was constitutively expressed at very low levels, but in contrast to TLR4 mRNA, was strongly up-regulated by LPS, and high expression levels of TLR2 mRNA were maintained within the entire period of stimulation. In contrast, endotoxin-tolerant macrophages did not exhibit any modulation in the expression levels of either TLR4 or TLR2 mRNA in response to LPS. Poltorak et al. (10) also showed an LPS-mediated decrease of TLR4 mRNA expression in the mouse macrophage cell line RAW 264.7 (10), whereas human cardiomyocytes have been recently published to increase TLR4 mRNA in response to LPS (73). The reason for these differing results is presently unknown, but could reflect species- or cell specificity of LPS-induced responses. Our data argue against a previous suggestion that an LPS-mediated decrease in levels of TLR4 mRNA mediates the induction of an endotoxin-tolerant state (10), because control and endotoxin-tolerant cells expressed equivalent levels of TLR4 mRNA. Instead, we show that LPS-tolerized macrophages express significantly higher amounts of TLR2 mRNA than control cells. Assuming that the increased levels of TLR2 mRNA are paralleled by an increase in TLR2 protein expression, our data suggest that up-regulation of TLR2 cannot compensate for disabled LPS signaling through murine TLR4 in endotoxin-tolerized mouse macrophages. Nonetheless, it is tempting to speculate that a significant change in the ratio of TLR2 to TLR4 molecules could result in altered TLR oligomerization, including possible interactions with each other or with other TLR molecules, that, in turn, leads to faulty recruitment of downstream signaling molecules. However, future experiments will be required to test this hypothesis.

LPS signal transduction via TLR4 and/or TLR2 occurs via engagement of the adaptor protein MyD88, IRAK kinases, and TRAF6 (17–19, 74). The same signaling intermediates are involved in IL-1, but not TNF-α, signal transduction (19, 75, 76), suggesting that endotoxin tolerance could be mediated by defective expression or function of these molecules. Hence, we reasoned that if this suggestion were true, then endotoxin-tolerant macrophages would be unresponsive to both LPS and IL-1, and, conversely, IL-1 would be able to induce tolerance both to itself and LPS. Indeed, this study demonstrates that pretreatment of mouse macrophages with either LPS or IL-1β effectively induced a state of cross-tolerance, as evidenced by significantly lower MAP kinase phosphorylation, activation of the transcription factors NF-κB and AP-1, and TNF-α gene expression in response to these stimuli. Because IL-1 does not signal through TLR4 or TLR2 (75), it could also be indicative of impaired expression and/or functions of molecules downstream of TLRs. MyD88 is prerequisite for both LPS- and IL-1-initiated recruitment of downstream signaling intermediates IRAK and TRAF6 (74). Therefore, we compared the expression of MyD88 mRNA in endotoxin-tolerant and control macrophages by semiquantitative RT-PCR. In contrast to TLR2 and TLR4, steady-state levels of MyD88 mRNA were unaffected by LPS stimulation or tolerance induction. However, this does not exclude a possible effect of endotoxin tolerance on the amount of MyD88 specifically recruited to TLRs, and, hence, on recruitment of IRAK. Experiments are in progress to address functional significance of dysregulated LPS-induced transcription of TLR2 and TLR4 mRNA in LPS-tolerant macrophages.

In contrast to IL-1, TNF-α or PMA-evoked cellular responses were not suppressed in endotoxin-tolerant cells, suggesting specific inhibition of signaling molecules shared by LPS and IL-1. Furthermore, a similar capacity to be tolerated by LPS preexposure was shown by either mouse macrophages rendered deficient in their TNF responses by targeted deletion of the *infr* I and II genes or TNFR II*+/−* cells. In addition, blocking of endogenous TNF-α with the TNFR-Fc fusion protein did not prevent the ability of LPS to induce a tolerant state. Conflicting results have been published with respect to the involvement of IL-1 and TNF-α in endotoxin tolerance (50–55). Using three different approaches, our data rule out a role for TNF-α in the induction of endotoxin tolerance in vitro, as evidenced by MAP kinase phosphorylation, transcription factor activation, and TNF-α gene expression. Our data confirm and extend the reports that show the involvement of IL-1 in this process in the mouse model in vivo (50, 51, 54). In addition, our
results are consistent with the fact that different upstream signaling molecules are utilized by TNF-α and LPS pathways, whereas LPS and IL-1 engage the same signaling intermediates downstream of TLR (19, 74–76).

It is plausible that endotoxin tolerance could interfere with expression and functions of TLR4, TLR2, and downstream signaling intermediates MyD88, TRAF6, TGF-β activating kinase 1 (TAK1), and evolutionarily conserved signaling intermediate in Toll pathways (ECSIT). However, it does not rule out the possibility that immunosuppressive cytokines, e.g., TGF-β and IL-10, produced by endotoxin-in-tolerant macrophages indirectly contribute to an LPS-tolerant state. Further studies are required to underscore the mechanisms of endotoxin tolerance in a greater detail.

Acknowledgments

We thank Dr. George Yap (Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD) for kindly providing us with TNFRII/II double knockout mice, Dr. N. Rice (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD) for her gift of rabbit polyclonal antisera against NF-κB and Dr. N. Rice (Frederick Cancer Research and Development Center, National Institutes of Health, Bethesda, MD) for kindly providing us with TNFRI/II double knockout mice, Dr. George Yap (Immunobiology Section, Laboratory of Parasitology, National Institutes of Health, Bethesda, MD) for critical discussions.

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EFFECT OF LPS TOLERANCE ON SIGNAL TRANSDUCTION IN MOUSE MACROPHAGES


