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# *Treponema pallidum* and *Borrelia burgdorferi* Lipoproteins and Synthetic Lipopeptides Activate Monocytic Cells via a CD14-Dependent Pathway Distinct from That Used by Lipopolysaccharide[1]

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**Lipoproteins of *Treponema pallidum* and *Borrelia burgdorferi* possess potent proinflammatory properties and, thus, have been implicated as major proinflammatory agonists in syphilis and Lyme disease. Here we used purified *B. burgdorferi* outer surface protein A (OspA) and synthetic lipopeptides corresponding to the N-termini of OspA and the 47-kDa major lipoprotein immunogen of *T. pallidum* to clarify the contribution of CD14 to monocytic cell activation by spirochetal lipoproteins and lipopeptides. As with LPS, mouse anti-human CD14 Abs blocked the activation of 1,25-dihydroxyvitamin $D_3$-matured human myelomonocytic THP-1 cells by OspA and the two lipopeptides. The existence of a CD14-dependent pathway was corroborated by using undifferentiated THP-1 cells transfected with CD14 and peritoneal macrophages from CD14-deficient BALB/c mice. Unlike LPS, cell activation by lipoproteins and lipopeptides was serum independent and was not augmented by exogenous LPS-binding protein. Two observations constituted evidence that LPS and lipoprotein/lipopeptide signaling proceed via distinct transducing elements downstream of CD14: 1) CHO cells transfected with CD14 were exquisitely sensitive to LPS but were lipoprotein/lipopeptide nonresponsive; and 2) substoichiometric amounts of deacylated LPS that block LPS signaling at a site distal to CD14 failed to antagonize activation by lipoproteins and lipopeptides. The combined results demonstrate that spirochetal lipoproteins and lipopeptides use a CD14-dependent pathway that differs in at least two fundamental respects from the well-characterized LPS recognition pathway.** *The Journal of Immunology,* **1998, 160: 5455–5464.**

Syphilis and Lyme disease, two chronic inflammatory disorders caused by the spirochetal pathogens *Treponema pallidum* and *Borrelia burgdorferi*, respectively, share a number of clinical features and are characterized by similar histopathologic abnormalities (1–3). The finding that neither *T. pallidum* nor *B. burgdorferi* possesses LPS (4, 5) prompted efforts to identify spirochetal constituents capable of eliciting inflammatory responses. Consistent with prior studies with murein lipoprotein of *Escherichia coli* (6–8), we and others have shown that the abundant lipoprotein immunogens of both *T. pallidum* and *B. burgdorferi* are potent activators of monocytes/macrophages, B cells, and endothelial cells and that acyl modification is essential for these proinflammatory activities (9–19). Collectively, these observations strongly implicate lipoproteins as major proinflammatory agonists in syphilis and Lyme disease.

Investigation of the immunomodulatory properties of spirochetal lipoproteins is complicated by the difficulty in obtaining adequate quantities of these molecules free of endotoxin contamination. As an extension of findings by Bessler and co-workers (6, 8), we have shown that synthetic lipohexapeptides corresponding to the N termini of spirochetal lipoproteins have in vitro proinflammatory properties that mimic those of their full-length, acylated counterparts (11, 14–16, 18). Moreover, following intradermal injection, synthetic lipopeptides elicited histopathologic changes in mice and rabbits that closely resembled those observed during natural or experimental syphilis and Lyme disease, further substantiating their utility as lipoprotein surrogates (20).

Cells of the innate immune system exhibit an intrinsic ability to recognize the cell wall constituents of bacterial and fungal pathogens (21–27). In this regard, the biologic effects of LPS have been intensively investigated because of the central role played by this highly potent glycolipid in the pathophysiology of sepsis and septic shock by Gram-negative bacteria (28). According to the current paradigm, activation of monocytes/macrophages is initiated when

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LPS binds to membrane CD14 (mCD14)[4] (29, 30), a 55-kDa glycosylphosphatidylinositol-anchored protein that lacks both transmembrane and cytoplasmic domains (31). The serum component known as LPS-binding protein (LBP) acts in a catalytic fashion to facilitate the binding of LPS to CD14 (29, 30, 32, 33). A signaling cascade ensues when the CD14-bound LPS presumably interacts with an as yet uncharacterized signal transducing element (27, 34). In addition to its membrane-bound form, which is found only on cells of myeloid lineage, CD14 exists as a soluble serum protein (sCD14) that mediates LPS signaling in cells lacking CD14 (35–37).

While CD14 has been shown to mediate the activation of monocytic cells by bacterial cell wall constituents other than LPS (21–27), its contribution to lipoprotein/lipopeptide signaling is unclear. Supporting its involvement is the observation that maturation of human myelomonocytic THP-1 cells (38) with 1,25-dihydroxyvitamin $D_3$ (vitamin $D_3$) markedly enhanced their responsiveness to lipoproteins and lipopeptides just as it does for LPS (16). It had previously been shown that vitamin $D_3$ maturation enhanced the responsiveness of THP-1 cells to LPS at least in part by inducing the surface expression of CD14 (39, 40). Arguing against a role for CD14 is the observation that transfection with CD14 rendered murine pre-B 70Z/3 cells exquisitely sensitive to LPS (41) but failed to confer responsiveness to spirochetal lipoproteins and lipopeptides (16).

The objective of the present study was to clarify the role of CD14 in the activation of monocytic cells by spirochetal lipoproteins and lipopeptides. Herein, we report that cellular activation by these spirochetal constituents proceeds predominantly via CD14, although a CD14-independent pathway also was discernible. Of particular interest was our finding that fundamental differences exist in the CD14-dependent signaling pathways induced by LPS and spirochetal lipoproteins/lipopeptides. In addition to providing new insights into the pathogenesis of syphilis and Lyme disease, these findings are potentially relevant to mechanisms of immune effector cell activation by other non-LPS bacterial proinflammatory agonists.

## Materials and Methods

### *Reagents*

*Salmonella minnesota* R5 LPS (Sigma, St. Louis, MO), suspended in PBS containing 0.03% BSA (low endotoxin; catalogue no. A4919; Sigma) or *S. minnesota* wild-type LPS (List Biologics, Campbell, CA) were used as positive controls in cell stimulation assays. *E. coli* LCD25 LPS was enzymatically deacylated (dLPS) as previously described (42). 1,25-Dihydroxyvitamin $D_3$ was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). G-418 sulfate was purchased from Mediatech (Herndon, VA). Great care was taken throughout to minimize contamination by environmental LPS during the preparation of all buffers and reagents by using baked glassware, disposable plasticware, and pyrogen-free $H_2O$.

### *Purification of native OspA (nOspA) from* B. burgdorferi *and recombinant, nonacylated OspA*

*B. burgdorferi* strain TI1-EV, generously provided by Jorge L. Benach (State University of New York, Stony Brook, NY), was used as the source for nOspA. Lipoprotein was affinity purified as previously described (16) and was stored in 33 mM Tris (pH 7.4), 1.6 mM NaCl, and 20 mM *n*-octyl-$\beta$-glucoside at −70°C. Preparations of nOspA contained ≤12 pg LPS/$\mu$g of protein as measured by the QCL-1000 quantitative chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD). Nonacylated recombinant OspA was generated by PCR amplification of the *ospA* gene from *B. burgdorferi* TI1-EV and cloned into the expression vector pGEX-2T as previously described (43). The recombinant OspA was recovered following cleavage of the glutathione *S*-transferase fusion protein with thrombin.

### *Synthetic hexapeptides and lipohexapeptides corresponding to the N termini of the spirochetal lipoproteins*

Hexapeptides corresponding to the N termini of the *B. burgdorferi* strain B31 OspA lipoprotein (Cys-Lys-Gln-Asn-Val-Ser) (44) and the *T. pallidum* subspecies *pallidum* 47-kDa lipoprotein (Cys-Gly-Ser-Ser-His-His) (45) were synthesized on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer using standard 9-fluorenylmethoxycarbonyl chemistry as recommended by the manufacturer. For use in cell stimulation assays, quantities of lyophilized hexapeptides were solubilized by vortexing in sterile, pyrogen-free $H_2O$. Lipohexapeptides corresponding to the acylated N termini of the lipoproteins were synthesized as tripalmitoyl-*S*-glyceryl-cysteine derivatives using a solid phase synthesis procedure (11). Hexapeptides and lipopeptides contained undetectable levels of endotoxin (≤1 pg LPS/$\mu$g protein) as measured by the QCL-1000 quantitative chromogenic *Limulus* amebocyte lysate assay.

### *Cell lines*

The human promyelomonocytic cell line THP-1 (38) was maintained in RPMI 1640 medium (Mediatech) containing 2 mM L-glutamine and supplemented with 10% heat-inactivated FBS (HIFBS; heated for 30 min at 56°C; Mediatech), 100 U of penicillin/ml, and 100 $\mu$g of streptomycin/ml. In some experiments, THP-1 cells were preincubated with 50 nM vitamin $D_3$ for 72 to 96 h before stimulation by LPS, nOspA, OspA-L, and 47-L. In others, THP-1 cells stably transfected with either the cloning vector pRc/RSV or pRc/RSV containing a cDNA encoding human CD14 were used.

Chinese hamster ovary (CHO) cells transfected with either the cloning vector pKoNeo or pKoNeo containing a cDNA encoding human CD14 (46) were provided by Douglas T. Golenbock (Boston University School of Medicine, Boston, MA). These cells were maintained in Ham's F-12 medium (Mediatech) containing 2 mM L-glutamine and supplemented with 10% HIFBS, 100 U of penicillin/ml, and 100 $\mu$g of streptomycin/ml.

For experiments, cells were seeded in 6- or 24-well flat-bottom tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at a density of $1 \times 10^6$ or $5 \times 10^5$ cells/ml/well, respectively, and were grown to confluence at 37°C in a humidified atmosphere of 5% $CO_2$ and air. All transfected cells were cultured in the continuous presence of 0.5 mg/ml (active drug) of the aminoglycoside G-418 sulfate to ensure the maintenance of stably transfected DNA conferring neomycin resistance. G-418 sulfate was removed 24 h before experimentation, cells were washed twice with appropriate medium, and LPS or spirochetal lipoproteins/lipopeptides were added in 10-$\mu$l volumes.

### *FACS analysis*

Cell surface expression of CD14 was determined by staining $5 \times 10^5$ cells suspended in PBS containing 3% normal mouse serum (NMS) with a saturating concentration of FITC-conjugated mouse anti-human CD14 mAb (UCHM1, IgG2a; Sigma) or FITC-conjugated isotype-matched control mAb (UPC10; Sigma) for 30 min on ice. Cells were washed twice with PBS containing 3% NMS, and bound mAb was detected with a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

### *IL-8 ELISA*

Levels of IL-8 in culture supernatants were measured in Immulon II 96-well U-bottom plates (Dynatech, Chantilly, VA) using the Duoset ELISA Development System for human IL-8 (Genzyme Diagnostics, Cambridge, MA). The lower limit of detection of IL-8 was 7.8 pg/ml.

### *Inhibition of macrophage activation by anti-CD14 polyclonal serum*

Vitamin $D_3$-matured THP-1 cells ($1 \times 10^6$ cells in 0.1 ml) were chilled for 5 min on ice and then incubated for an additional 20 min with 1/5, 1/25, or 1/50 dilutions of mouse polyclonal Abs directed against a human CD14-IgG1 fusion protein (47). Cells were then stimulated for 3 h with LPS (10 ng/ml), nOspA (75 ng/ml), OspA-L (1 $\mu$g/ml), and 47-L (1 $\mu$g/ml), and the culture supernatants were assayed for IL-8 as described above. Cells incubated with 1/5 dilutions of NMS or mouse anti-human IgG1 ascites (Zymed Laboratories, South San Francisco, CA) were used as negative controls.

[4] Abbreviations used in this paper: mCD14, membrane CD14; LBP, lipopolysaccharide-binding protein; sCD14, soluble CD14; dLPS, deacylated lipopolysaccharide; nOspA, native outer surface protein A; HIFBS, heat-inactivated fetal bovine serum; OspA-L, lipopeptide corresponding to the N terminus of the outer surface protein A lipoprotein of *Borrelia burgdorferi*; 47-L, lipopeptide corresponding to the N terminus of *Treponema pallidum*; RSV, Rous sarcoma virus; NMS, normal mouse serum; EMSA, electrophoretic mobility shift assay.

*Electrophoretic mobility shift assay (EMSA)*

Following stimulation of $1 \times 10^6$ THP-1 or CHO cells for 1 h, nuclear extracts from cell lysates were prepared as previously described (39). The active form of NF-κB, translocated into the nuclei of stimulated cells, was detected by incubating 5 μg of nuclear extract protein with a radiolabeled, double-stranded NF-κB oligonucleotide prepared using the sequences 5′-GTTCGACAGA**GGGGACTTTCC**GAGAGG-3′ and 3′-TGTCT**CCCC TGAAAGG**CTCTCCGTTG-5′ (bolded text indicates the consensus NF-KB binding sequence).

Protein-DNA complexes were resolved in 4% native polyacrylamide gels that were dried onto paper and visualized by exposure to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 6 h. The intensity of protein-DNA complexes was quantified with a PhosphorImager SF (Molecular Dynamics) using the ImageQuant version 3.3 software package. Results are presented in arbitrary phosphorimage units.

*Stimulation and analysis of responses by peritoneal macrophages from CD14-deficient and control mice*

Female CD14-deficient mice (from the fifth backcross with BALB/c) (48) and control mice (BALB/c; Harlan Sprague-Dawley) (8 wk old) were injected i.p. with 3 ml of 3% (w/v) Brewer thioglycolate broth (Difco, Detroit, MI). Four days later, cells were harvested by peritoneal lavage with 5 ml of RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 2 mM L-glutamine and supplemented with 100 U of penicillin/ml and 100 μg of streptomycin/ml. The cells were washed twice, resuspended in the above medium supplemented with 1% autologous serum, and then added to the wells ($5 \times 10^5$ cells/well) of a 24-well tissue culture plate (Nunc, Naperville, IL). The cells were incubated for 3 h and then were washed twice with 1 ml of medium before treatment with the various stimuli. *S. minnesota* wild-type LPS, nOspA, OspA-L, and 47-L were diluted in medium to the indicated concentrations and added to the adherent macrophages (0.5 ml/well). Following a 4-h incubation, cell-free supernatants were collected and assayed for TNF-α by ELISA according to the manufacturer's instructions (Genzyme Diagnostics). The lower limit of detection of TNF-α was 10 pg/ml.

*Serum and LBP dependence experiments*

To assess whether the stimulatory activity of spirochetal lipoproteins/lipopeptides was dependent upon serum, THP-1 cells were washed four times with PBS and cultured in Cellgro Complete serum-free medium (Mediatech) for 24 h before experimentation. Following incubation of $1 \times 10^6$ cells for 1 h with various concentrations of LPS, nOspA, OspA-L, and 47-L, the translocation of NF-κB was assessed by EMSA as described above. To determine the effects of exogenous LBP, cells were adapted to growth under serum-free culture conditions for 6 wk in Cellgro Complete serum-free medium. One-tenth milliliter of culture supernatant (CHO-S-SFM II, Life Technologies) from CHO cells transfected with either pRc/RSV or pRc/RSV containing a cDNA encoding human LBP (provided by Peter Tobias, Scripps Research Institute, La Jolla, CA) was added to wells containing $1 \times 10^6$ cells. The cells were incubated for 1 h with LPS (10 ng/ml), nOspA (75 ng/ml), OspA-L (1 μg/ml), or 47-L (1 μg/ml) and then harvested for assessment of NF-κB translocation by EMSA as described above.

*Deacylated LPS antagonism experiments*

Vitamin $D_3$-matured THP-1 cells were treated with 10 nM dLPS (36 ng/ml) for 15 min before and during stimulation by 10 nM LPS (40 ng/ml) or various concentrations of nOspA, OspA-L, and 47-L. Following stimulation of $1 \times 10^6$ cells for 1 h, the translocation of NF-κB was assessed by EMSA. Experiments were conducted in the presence of 10% HIFBS to provide a source of LBP.

## Results

*Spirochetal lipoproteins and lipopeptides induce the secretion of IL-8 by vitamin $D_3$-matured THP-1 cells*

We reported previously that spirochetal lipoproteins and synthetic lipopeptides induced murine and human monocytes/macrophages to produce IL-1β, IL-6, IL-12, and TNF-α (9, 14, 16). One proinflammatory cytokine not examined in these prior studies was IL-8, a potent leukocyte chemoattractant (49, 50). Examination of IL-8 secretion by monocytic cells was warranted for two reasons. First, recent evidence supports the contention that local production of IL-8 at sites of spirochetal infection promotes an infiltration of leukocytes that produces the characteristic histopathologic changes of syphilis and Lyme disease (51, 52). Second, because IL-8 is secreted relatively rapidly (within 1–2 h) by activated monocytes/macrophages, its presence in culture supernatants following short incubation periods reflects the direct actions of the agonists under study as opposed to the autocrine effects of subsequently released cytokines. To ensure that the biologic activities under investigation were not limited to a particular lipoprotein or lipopeptide, parallel studies were conducted throughout using lipopeptides representing both *T. pallidum* and *B. burgdorferi* lipoproteins (47-L and OspA-L, respectively) as well as a purified, native lipoprotein, *B. burgdorferi* OspA (nOspA).

As shown in Figure 1, LPS, nOspA, OspA-L, and 47-L induced the secretion of IL-8 by vitamin $D_3$-matured THP-1 cells in a dose-dependent manner. The finding that vitamin $D_3$ maturation markedly enhanced the responsiveness to both LPS and spirochetal components (Fig. 1) was consistent with earlier observations (16, 39, 40). However, interesting differences in the dose-response curves for these compounds also were observed (Fig. 1). First, the concentration ranges that induced IL-8 were considerably narrower for nOspA and lipopeptides than for LPS. Second, as found in prior studies (14, 16), the lipopeptides were considerably less potent on a molar basis than nOspA. LPS was approximately 1 log more potent than nOspA and 4 logs more potent than the lipopeptides with respect to activation of the vitamin $D_3$-matured cells. These results compare quite favorably with previously reported potencies derived using a murine macrophage cell line (RAW 264.7 cells) (14) and with studies using lipopentapeptide analogues of *E. coli* lipoprotein and peritoneal macrophages from C3H/He mice (53). Lastly, although the thresholds for responsiveness to nOspA and the lipopeptides were considerably higher than that to LPS, the peak cellular responses were comparable (Fig. 1). As previously observed (9, 13–15, 17, 20), the stimulatory activities of both nOspA and the lipopeptides were dependent upon lipid modification and were not due to LPS contamination, as determined by the *Limulus* amebocyte lysate assay and by insensitivity to polymyxin B (10 μg/ml; data not shown).

*Mouse anti-human CD14 Abs inhibit lipoprotein/lipopeptide-mediated activation of vitamin $D_3$-matured THP-1 cells*

To determine whether CD14 contributes to the activation of THP-1 cells by spirochetal lipoproteins and lipopeptides, mouse antiserum directed against recombinant human sCD14 was used in functional blocking studies. Polyclonal Abs were used in lieu of mAbs because of the possibility that LPS and lipoproteins/lipopeptides interact with different CD14 epitopes. Incubation of vitamin $D_3$-matured cells with various dilutions of the antiserum before incubation with LPS, nOspA, OspA-L, and 47-L inhibited secretion of IL-8 in a dose-dependent manner (Fig. 2). A 1/5 dilution of the antiserum reduced the cellular response to each compound by an average of $91 \pm 3$, $85 \pm 2$, $96 \pm 2$, and $91 \pm 3\%$, respectively. Substantial inhibition also was observed with 1/50 dilutions of the antiserum. In contrast, NMS (Fig. 2) and anti-human IgG1 ascites (a control for the IgG1 portion of the CD14 fusion protein used to generate the antiserum; data not shown) were incapable of blocking the responses to these immunomodulators.

*Surface expression of CD14 by monocytic cells enhances responsiveness to spirochetal lipoproteins and lipopeptides*

Ulevitch and co-workers recently noted that undifferentiated THP-1 cells become highly responsive to LPS when stably transfected with CD14 (J. Pugin and R. J. Ulevitch, unpublished observations). The use of this transfected cell line (designated THP-1-CD14) seemed advantageous, as it would enable us to examine

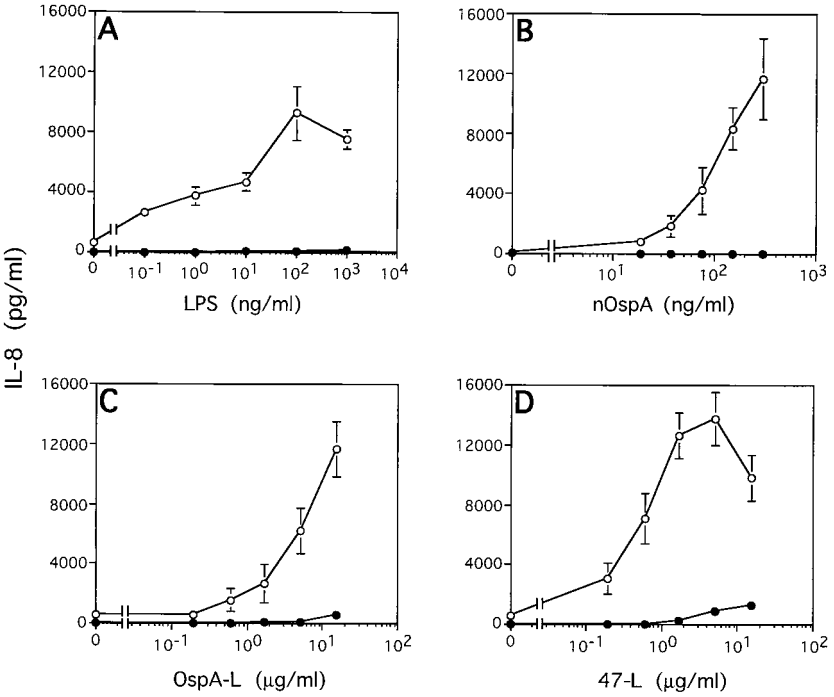


**FIGURE 1.** Spirochetal lipoproteins and lipopeptides induce the secretion of IL-8 by vitamin $D_3$-matured THP-1 cells. Undifferentiated (closed circle) and vitamin $D_3$-matured (open circle) THP-1 cells were incubated in the presence of 10% HIFBS for 3 h with various doses of LPS (*A*), purified *B. burgdorferi* OspA (nOspA; *B*), and OspA-L (*C*), and 47-L (*D*). The concentrations of agonist tested were 0.1 to 1000 ng/ml LPS (0.025–250 nM), 18.8 to 300 ng/ml nOspA (0.63–10 nM), 0.2 to 15 $\mu$g/ml OspA-L (0.15–10 $\mu$M), and 0.2 to 15 $\mu$g/ml 47-L (0.15–10 $\mu$M). Culture supernatants were assayed for IL-8 by ELISA. Unless otherwise indicated, results are presented as picograms per milliliter of IL-8 secreted by $1 \times 10^6$ cells. Shown are the mean ± SE of duplicate determinations for three independent experiments.

CD14 interactions with LPS and spirochetal lipoproteins/lipopeptides in a myeloid background without the pleiotropic effects of vitamin $D_3$ maturation (54). At the outset, flow cytometry was used to compare surface expression of CD14 by transfected cells and that of cells that had undergone vitamin $D_3$ maturation. These studies confirmed that vitamin $D_3$ maturation induces a marked up-regulation of surface CD14 (39, 40, 55). Compared with their vitamin $D_3$-matured counterparts, THP-1-CD14 cells expressed slightly higher levels of CD14 and also expressed the Ag more uniformly (Fig. 3). In contrast, nontransfected immature cells expressed extremely low levels of CD14, which were indistinguishable from those on the cells transfected with the cloning vector alone (designated THP-1-RSV; data not shown).

As shown in Figure 4*A*, THP-1-CD14 cells responded to much lower doses of LPS and produced considerably more IL-8 at each LPS concentration tested than did the THP-1-RSV cells. THP-1-CD14 cells also were more responsive to nOspA and the lipopeptides, showing a shift in sensitivity as well as an increase in the overall amounts of IL-8 secreted (Fig. 4, *B–D*). Interestingly, concentrations of nOspA >150 ng/ml and concentrations of OspA-L and 47-L >0.6 $\mu$g/ml induced secretion of IL-8 by the THP-1-RSV cells (Fig. 4, *B–D*). This potential CD14-independent pathway was most prominent with 47-L (Fig. 4*D*). Finally, in comparing the responsiveness of THP-1 cells to LPS and spirochetal lipoprotein/lipopeptides, it was noted that the CD14-transfected cells consistently showed a higher threshold of sensitivity to both

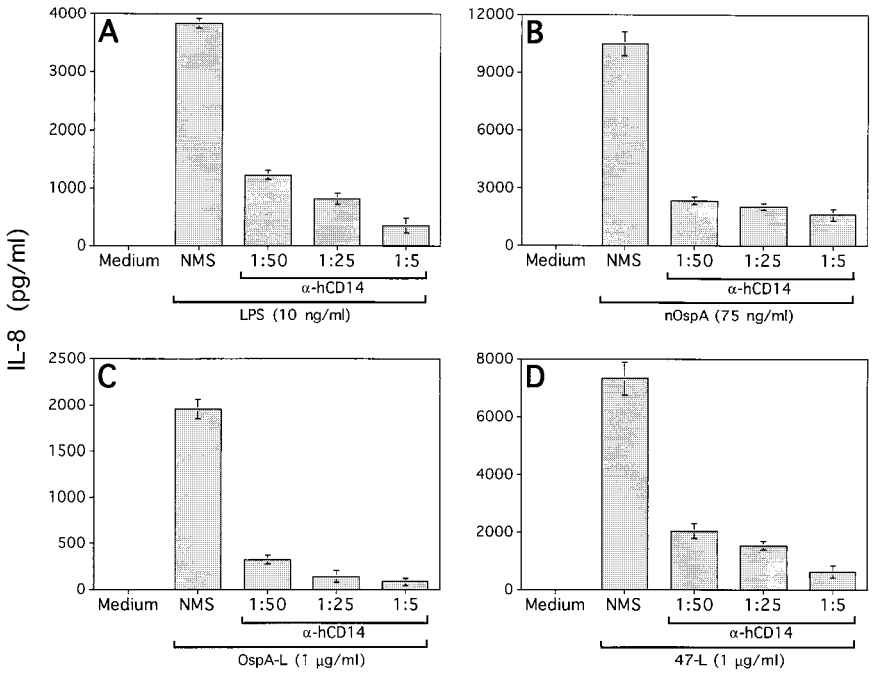


**FIGURE 2.** Mouse anti-hCD14 Abs inhibit both LPS and spirochetal lipoprotein/lipopeptide-mediated activation of macrophages. Vitamin $D_3$-matured THP-1 cells were chilled for 5 min on ice and were then incubated for an additional 20 min with medium alone (medium), NMS diluted 1/5, or various dilutions of mouse anti-hCD14 polyclonal serum ($\alpha$-hCD14). Cells were then stimulated in the presence of 10% HIFBS for 3 h by 10 ng/ml LPS (2.5 nM; *A*), 75 ng/ml nOspA (2.5 nM; *B*), 1 $\mu$g/ml OspA-L (0.67 $\mu$M; *C*), and 1 $\mu$g/ml 47-L (0.67 $\mu$M; *D*). Culture supernatants were assayed for IL-8 by ELISA. Shown are the mean ± SE of duplicate determinations for two independent experiments.

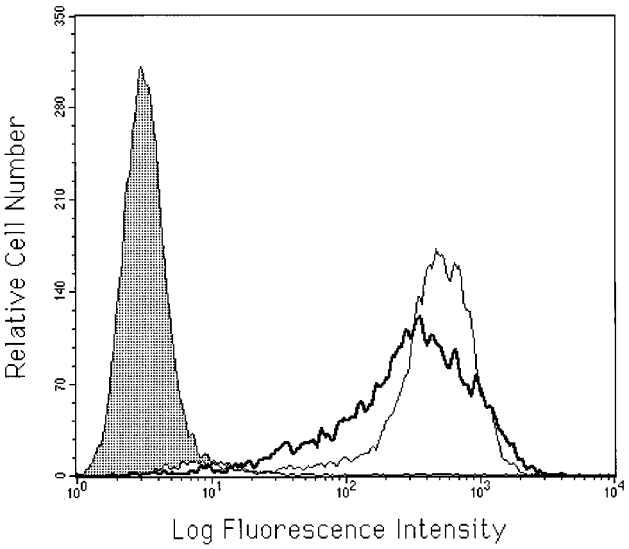


**FIGURE 3.** Vitamin $D_3$-matured and CD14-transfected THP-1 cells express comparable levels of CD14. Undifferentiated THP-1 cells (shaded) and cells treated with 50 nM vitamin $D_3$ for 96 h (thick line) or transfected with CD14 (thin line) were resuspended in cold PBS containing 3% NMS and stained with $\alpha$-hCD14 mAb (UCHM1) or an isotype-matched control mAb. The undifferentiated THP-1 cells stained with mAb UCHM1 are presented as a negative control. Shown are results from one of two independent experiments.

LPS and lipoprotein/lipopeptides than did their vitamin $D_3$-matured counterparts and, for each concentration of immunomodulator tested, produced considerably less IL-8 (compare Figs. 1 and 4, which show experiments conducted in parallel). Given the comparable levels of CD14 expressed on both cell populations (Fig. 3), the difference in responsiveness may relate to the differentiation state of the cells.

As a final confirmation that CD14 can potentiate the responses of monocytic cells to spirochetal lipoproteins and lipopeptides, we measured TNF-$\alpha$ secretion by thioglycolate-elicited peritoneal macrophages from control ($CD14^+$) and CD14-deficient BALB/c mice. In these experiments, the macrophages were incubated in medium supplemented with 1% autologous serum to eliminate potential activation of the CD14-deficient macrophages by sCD14-LPS complexes (48, 56). As found previously (48, 56, 57), cells from the CD14-deficient animals produced considerably less TNF-$\alpha$ in response to LPS, although cytokine secretion was noted at higher concentrations (Fig. 5*A*). Macrophages from CD14-deficient animals also were less responsive to nOspA and the two lipopeptides than were cells from $CD14^+$ mice (Fig. 5, *B–D*). Only a minimal response by the CD14-deficient macrophages was detected at the highest concentration of nOspA tested (Fig. 5*B*), whereas a CD14-independent component was more evident with the two lipopeptides and with 47-L in particular (Fig. 5, *C* and *D*). It is worth noting that, despite the different cytokines studied, the responses of the $CD14^+$ and the CD14-deficient macrophages roughly paralleled those of the THP-1-CD14 and THP-1-RSV cells (compare Figs. 4 and 5). Furthermore, the use of autologous serum in these experiments suggests that sCD14 from FBS played a nominal role in lipopeptide-mediated activation of undifferentiated and RSV-transfected THP-1 cells (Figs. 1 and 4, respectively).

### *Activation of THP-1 cells by spirochetal lipoproteins and lipopeptides is serum and LBP independent*

The mCD14-mediated recognition of low concentrations of LPS (typically <40 ng/ml) is enhanced by the serum component LBP (29, 30, 32, 33). Having shown that mCD14 can potentiate the response of monocytes/macrophages to spirochetal lipoproteins and lipopeptides, experiments next were conducted to examine the potential involvement of LBP or other serum components in this process. Here we assessed cell activation by nuclear translocation of NF-$\kappa$B, a transcriptional activator implicated in cytokine induction in LPS- and lipoprotein/lipopeptide-stimulated immune cells (16, 17, 58, 59). The rapid kinetics of NF-$\kappa$B translocation (within 15 min of stimulation) should preclude autocrine effects due to subsequently secreted cytokines (39).

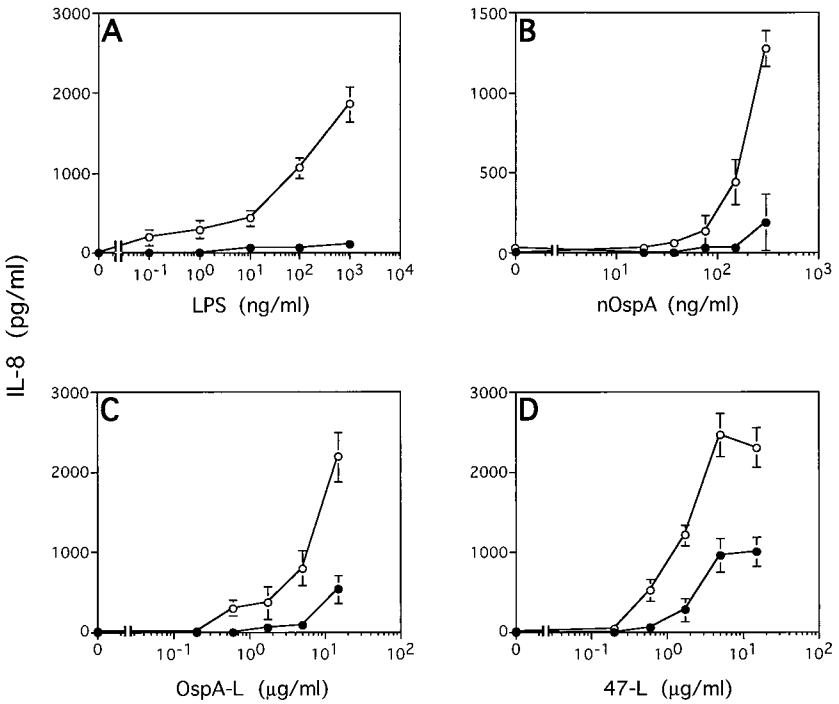


**FIGURE 4.** Transfection of THP-1 cells with CD14 enhances responsiveness to spirochetal lipoproteins and lipopeptides. THP-1 cells transfected with cloning vector alone (closed circles) or CD14 (open circles) were incubated in the presence of 10% HIFBS for 3 h with various doses of LPS (*A*), nOspA (*B*), OspA-L (*C*), and 47-L (*D*). Culture supernatants were assayed for IL-8 by ELISA, and the results are presented as picograms per milliliter of IL-8 secreted by $5 \times 10^5$ cells. Shown are the mean ± SE of duplicate determinations for three independent experiments.

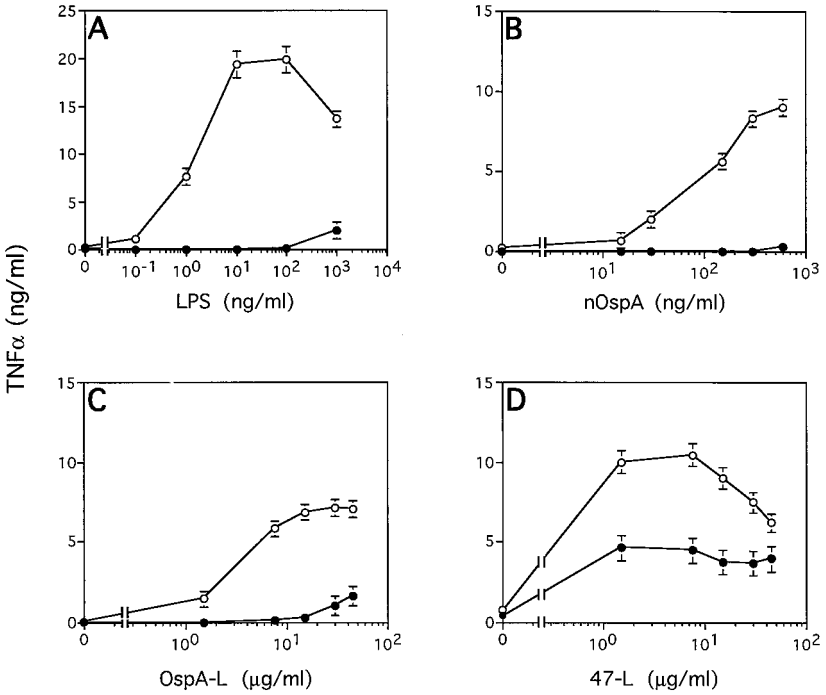


**FIGURE 5.** CD14-deficient peritoneal macrophages are hyporesponsive to spirochetal lipoproteins/lipopeptides as well as to LPS. CD14-deficient (closed circle) and $CD14^+$ (open circle) peritoneal macrophages were incubated for 4 h with various doses of LPS (*A*), nOspA (*B*), OspA-L (*C*), and 47-L (*D*). Culture supernatants were assayed for TNF-$\alpha$ by ELISA. Shown are the mean ± SE of duplicate determinations for two independent experiments.

In one series of experiments, vitamin $D_3$-matured THP-1 cells were washed extensively and maintained in serum-free medium for 24 h. They then were incubated in the absence or the presence of 10% HIFBS with various concentrations of LPS, nOspA, or lipopeptides. As predicted (29, 30, 32, 33), the cellular response to LPS differed markedly depending upon the absence or the presence of serum; the serum dependence of the LPS response was particularly striking at LPS concentrations ≤10 ng/ml (Fig. 6*A*). In contrast, nearly identical dose-response curves for nOspA and the two lipopeptides were obtained in the absence or the presence of serum (Fig. 6, *B–D*). Similar results were obtained when measuring secretion of IL-8 or when parallel experiments were conducted with THP-1-CD14 cells (data not shown).

Despite the above results, it was still possible that exogenous LBP could augment responses to spirochetal lipoproteins and lipopeptides. To examine this issue, THP-1 cells cultured in serum-free medium for >6 wk were treated with vitamin $D_3$ and then stimulated by LPS, nOspA, OspA-L, and 47-L in the absence or the presence of recombinant LBP (added as serum-free culture supernatant from CHO-LBP transfectants). In preliminary experiments, it was determined that 0.01 ml of culture supernatant was sufficient to dramatically enhance the LPS response; nevertheless,

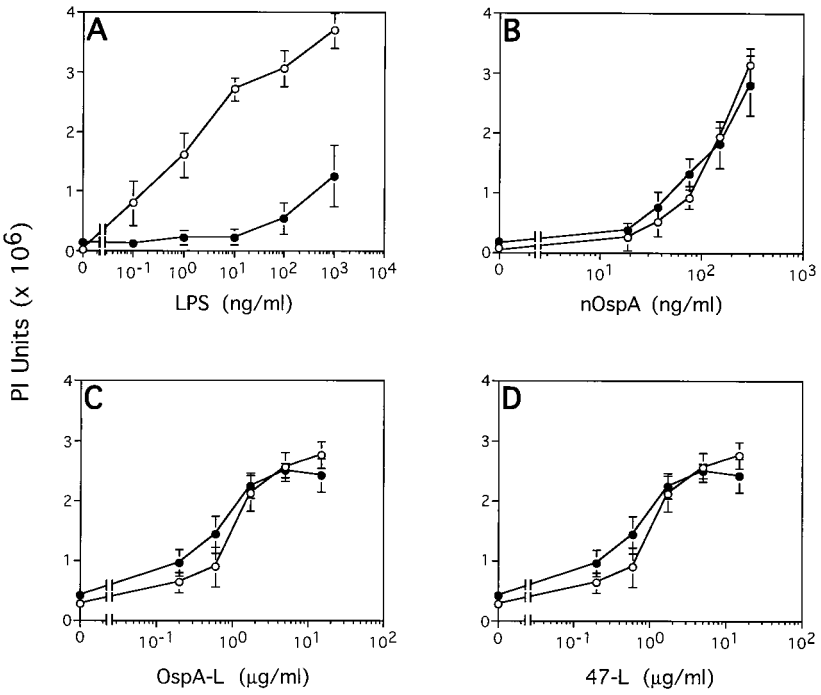


**FIGURE 6.** Activation of THP-1 cells by spirochetal lipoproteins and lipopeptides is serum independent. Vitamin $D_3$-matured THP-1 cells were incubated for 1 h with various doses of LPS (*A*), nOspA (*B*), OspA-L (*C*), and 47-L (*D*) in the absence (closed circles) or the presence (open circles) of 10% HIFBS. Nuclear extracts containing 5 $\mu$g of protein were analyzed by EMSA for binding to a $^{32}$P-labeled, double-stranded NF-$\kappa$B consensus oligonucleotide. The radioactivity of protein-DNA complexes was quantified by PhosphorImager analysis and reported in phosphorimage (PI) units. Shown are the mean ± SD for three independent experiments.

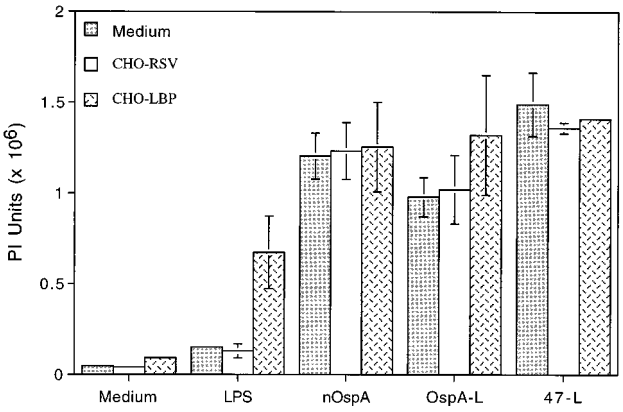


**FIGURE 7.** Exogenous LBP fails to augment the responsiveness of THP-1 cells to spirochetal lipoproteins and lipopeptides. THP-1 cells grown for 6 wk under serum-free conditions were treated with vitamin $D_3$ and plated at a density of $1 \times 10^6$ cells/ml. These 1-ml cultures were either unsupplemented (Medium, shaded columns) or supplemented with 0.1 ml of culture supernatant from CHO cells transfected with pRc/RSV (CHO-RSV, open columns) or pRc/RSV containing a cDNA encoding human LBP (CHO-LBP, hatched columns). Cells were then incubated for 1 h with medium alone (medium), 10 ng/ml LPS (2.5 nM), 75 ng/ml nOspA (2.5 nM), 1 $\mu$g/ml OspA-L (0.67 $\mu$M), and 1 $\mu$g/ml 47-L (0.67 $\mu$M). Nuclear extracts were analyzed by EMSA for translocation of NF-κB; results were quantified by PhosphorImager analysis. Shown are the mean ± SD for three independent experiments.

a 10-fold excess (0.1 ml) of culture supernatant was used to ensure adequate amounts of LBP in subsequent experiments. As shown in Figure 7, while LBP dramatically enhanced responsiveness to LPS, it had no effect on the responses of the cells to nOspA or lipopeptides. It should be noted that immunomodulator concentrations were employed that generate submaximal responses to ensure that the ability of LBP to augment cellular responses would not be masked.

*CD14-dependent activation by LPS and spirochetal lipoproteins/lipopeptides appears to involve different transmembrane signaling elements*

Previously, we reported that mouse pre-B 70Z/3 cells transfected with CD14 responded to low concentrations of LPS, but not to nOspA or spirochetal lipopeptides even at concentrations as high as 1 and 15 $\mu$g/ml, respectively (16). One potential explanation for this dichotomy between LPS and lipoprotein/lipopeptide responsiveness is that these nonmyeloid cells possess the putative LPS signal transducer but lack a comparable element required for CD14-dependent lipoprotein/lipopeptide signaling. To test this hypothesis, we investigated whether responses to LPS and lipoproteins/lipopeptides could be dissociated in other nonmyeloid cells. Golenbock and co-workers showed that CHO cells, which are normally LPS nonresponsive, become highly responsive to LPS following transfection with human CD14 (46). It was of interest, therefore, to evaluate the responses of these cells to spirochetal lipoproteins and lipopeptides. As previously observed, CHO cells transfected with CD14 were exquisitely sensitive to LPS (Fig. 8*A*). In contrast, the responses of these same cells to nOspA, OspA-L, and 47-L were not significantly different ($P < 0.05$, by ANOVA) from those of cells transfected with the cloning vector alone (Fig. 8, *B–D*, respectively).

Kitchens et al. (39, 60), previously showed that dLPS by leukocyte-derived acyloxyacyl hydrolase can antagonize LPS-mediated stimulation of THP-1 cells at concentrations too low to inhibit LPS binding to LBP or mCD14. This effect was interpreted as reflecting antagonism at a site in the LPS recognition pathway downstream of CD14, presumably at the putative signal transducer (60). Blocking experiments with dLPS were conducted, therefore, to investigate further the possibility that LPS and spirochetal lipoproteins/lipopeptides use disparate downstream signaling elements. As shown in Figure 9, 10 nM dLPS (36 ng/ml), a concentration well below the $K_d$ for binding of dLPS to CD14 (100 ng/ml) (60), completely blocked cell activation by an equimolar concentration of LPS. In contrast, the same concentration of dLPS had no

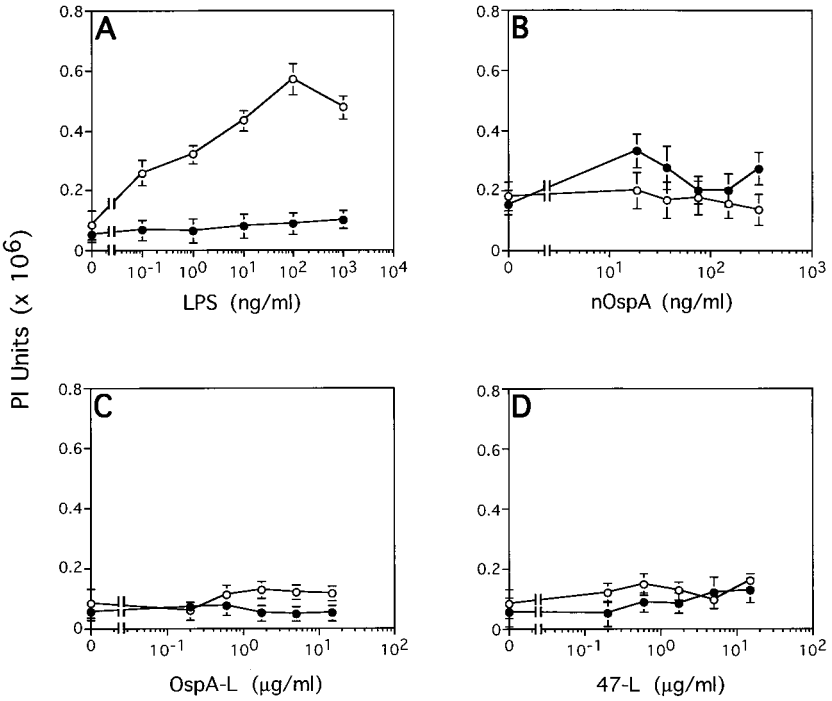


**FIGURE 8.** CHO cells transfected with CD14 are responsive to LPS but not to spirochetal lipoproteins and lipopeptides. CHO cells transfected with pKo-Neo alone (closed circles) or pKoNeo containing a cDNA encoding CD14 (open circles) were incubated in the presence of 10% HIFBS for 1 h with various doses of LPS (*A*), nOspA (*B*), OspA-L (*C*), and 47-L (*D*). Nuclear extracts were analyzed by EMSA for translocation of NF-κB; results were quantified by PhosphorImager analysis. Shown are the mean ± SD for three independent experiments.

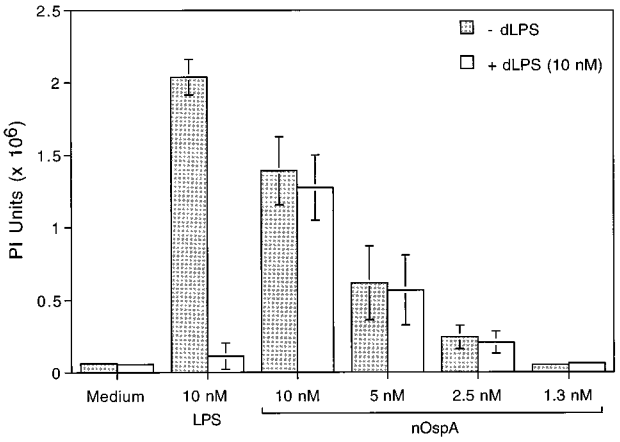


**FIGURE 9.** Deacylated LPS blocks LPS, but not spirochetal, lipoprotein/lipopeptide signaling in THP-1 cells. Vitamin $D_3$-matured THP-1 cells were incubated in the presence of 10% HIFBS for 1 h with medium alone (medium), 10 nM LPS (40 ng/ml), or graded doses of nOspA (1.3–10 nM or 37.5–300 ng/ml) in the absence (shaded columns) or the presence (unshaded columns) of 10 nM dLPS (36 ng/ml). Nuclear extracts were analyzed by EMSA for translocation of NF-κB; results were quantified by PhosphorImager analysis. Shown are the mean ± SD for three independent experiments.

inhibitory effect on nOspA-mediated activation of macrophages, even when used in a fourfold molar excess (10 nM dLPS to 2.5 nM nOspA). Similarly, dLPS was unable to block cell activation by either of the two lipopeptides (data not shown).

## Discussion

It is now well established that bacterial lipoproteins and synthetic analogues possess proinflammatory properties. In addition to providing valuable insights into syphilis and Lyme disease immunopathogenesis, elucidation of the mechanisms underlying these activities could facilitate the identification of novel therapeutic approaches for these chronic inflammatory disorders. While most syphilis patients respond well to appropriate therapy, inflammatory sequelae can persist, and even progress, after spirochetes have been eradicated (1). In the case of Lyme disease, the need for new therapeutic modalities is highlighted by clinical studies showing that patients often are refractory to antimicrobial therapy (61, 62). Here we used a combination of immunologic and genetic approaches to demonstrate that mCD14 enhances the response of monocytic cells to these spirochetal constituents, albeit in a manner distinct from that of LPS.

The observation that bacterial lipoproteins and lipopeptides with diverse N-terminal sequences can activate monocytes/macrophages (6, 14, 53, 63) has been difficult to reconcile with the presumption that these immunomodulators exert their effects via specific receptor-ligand interactions. Also difficult to explain has been the finding that the biologic activities of these immunomodulators are dependent upon lipid modification (13–15, 17, 64). We now postulate that there must be a region in CD14 that recognizes an amphipathic structural motif at the N terminus of bacterial lipoproteins. Additional support for this conjecture derives from recent native gel electrophoresis experiments in which it was found that acylation was necessary for the binding of recombinant OspA to sCD14 (65). Using both natural and synthetic bacterial lipopeptides, Jung and co-workers have shown that the presence of ester-bound fatty acids is a prerequisite for biologic activity, whereas the amide-bound fatty acid is dispensable (63, 66); thus, it can be inferred that the amide-linked fatty acid is of limited importance for CD14 binding.

The idea that proinflammatory activity can be regarded as a generic property of spirochetal lipoproteins and lipopeptides has important implications for syphilis and Lyme disease pathogenesis. A large proportion of the membrane immunogens of both *T. pallidum* and *B. burgdorferi* are lipid modified (67–69), and it is reasonable to propose that these molecules act in concert to promote the inflammatory processes that culminate in clinical manifestations. Moreover, there is now a substantial body of evidence that *B. burgdorferi* does not express OspA and OspB following tick transmission (70–73), while other antigenically unrelated lipoproteins are selectively expressed in the mammalian host (71–74). Promiscuous binding by CD14 would permit differentially expressed lipoproteins to assume the immunomodulatory roles that have been proposed for OspA and OspB based upon in vitro studies (9, 10, 12–17, 19, 75). Consistent with this idea is our finding that synthetic lipopeptides derived from the N termini of two lipoproteins expressed during infection, OspC and the OspF homologue BbK2.10 (74), have in vitro proinflammatory activities comparable to those of OspA and OspB lipopeptides (T. J. Sellati and J. D. Radolf, unpublished observations).

One of the most important findings reported here is that the CD14-dependent signaling pathways used by LPS and spirochetal lipoproteins/lipopeptides differ in at least two fundamental respects. In contrast to LPS, activation by spirochetal lipoproteins was not facilitated by LBP or other serum components. This finding is not without precedent; Wright and co-workers have shown recently that *Staphylococcus aureus* cell wall extract stimulates human PBMC in a CD14-dependent, LBP-independent fashion (24). LBP is thought to enhance LPS responsiveness by transferring LPS monomers out of LPS aggregates to a binding site(s) on CD14 (32). The lack of involvement of LBP in cell activation by lipoproteins and lipopeptides suggests that these amphiphilic compounds either interact with CD14 as aggregates or that monomers bind to CD14 unassisted by a serum intermediary. The latter scenario, rather than lower binding affinities, could explain the ostensibly lower potencies of lipoproteins and lipopeptides, compared with LPS, inasmuch as the amphiphilic spirochetal constituents (especially the lipopeptides) are extremely insoluble and undoubtedly exist in a highly aggregated state in an aqueous environment.

Particularly intriguing was the finding that the LPS and lipoprotein/lipopeptide signals appear to be transduced via distinct transmembrane elements. Indirect evidence for this was the observation that CHO cells transfected with CD14 were exquisitely sensitive to LPS but were insensitive to lipoproteins and lipopeptides, a result that parallels earlier findings with 70Z/3 cells (16). Additional evidence was provided by the observation that substoichiometric concentrations of an LPS antagonist were unable to block lipoprotein and lipopeptide signaling in vitamin $D_3$-matured THP-1 cells under the same conditions in which LPS signaling was completely ablated. Interestingly, transfection of THP-1 cells with CD14 did enhance responsiveness to lipoproteins and lipopeptides, suggesting that, unlike 70Z/3 and CHO cells, THP-1 cells constitutively express the putative lipoprotein/lipopeptide transducer. Previously, we showed that spirochetal lipoproteins and lipopeptides activate macrophages from LPS-nonresponsive C3H/HeJ mice (9, 14); the existence of distinct LPS and lipoprotein/lipopeptide signal transducers is one plausible explanation for this dichotomy. Nevertheless, because the responses of monocytic cells to lipoproteins/lipopeptides so closely resemble those elicited by LPS (14), we believe that these two CD14-dependent pathways subsequently must overlap and/or converge. Golenbock and co-workers also

have proposed that LPS and lipoarabinomannan from *Mycobacterium tuberculosis* share CD14 as a binding receptor but differ with respect to downstream elements necessary for specific cellular activation (26, 76). However, in contrast to spirochetal lipoproteins and lipopeptides, signaling by lipoarabinomannan can be inhibited by LPS partial structures and is LBP dependent (26).

CD14-independent as well as CD14-dependent pathways have been described for bacterial products other than LPS; these pathways tend to be engaged at higher immunomodulator concentrations than those needed for CD14-dependent stimulation (24, 25, 77). Thus, it was not entirely surprising that CD14-independent responses became apparent at progressively higher concentrations of the spirochetal constituents. Two observations regarding this CD14-independent effect were noteworthy. First, it was observed only in the cellular backgrounds (i.e., THP-1 cells and murine peritoneal macrophages) in which CD14 expression enhanced lipoprotein/lipopeptide responsiveness, suggesting that CD14-dependent and -independent signaling are mechanistically interrelated and potentially nondissociable. Second, the CD14-independent response was more prominent with the synthetic lipopeptides, most notably 47-L. Thus, while lipoproteins and lipopeptides induce qualitatively similar responses in different effector cells of innate immunity and appear to exert these effects through highly similar mechanisms, data presented in this report raise the possibility that differences in biologic activity may exist between lipoproteins and their synthetic analogues. Further studies should clarify this issue while delineating the structural features of lipoproteins and lipopeptides that influence the engagement of CD14-dependent and -independent pathways.

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