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J Immunol (2004) 173 (4): 2683–2691.

<https://doi.org/10.4049/jimmunol.173.4.2683>

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Lipopolysaccharide Binding Protein Binds to Triacylated and Diacylated Lipopeptides and Mediates Innate Immune Responses¹

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LPS binding protein (LBP) is an acute-phase protein synthesized predominantly in the liver of the mammalian host. It was first described to bind LPS of Gram-negative bacteria and transfer it via a CD14-enhanced mechanism to a receptor complex including TLR-4 and MD-2, initiating a signal transduction cascade leading to the release of proinflammatory cytokines. In recent studies, we found that LBP also mediates cytokine induction caused by compounds derived from Gram-positive bacteria, including lipoteichoic acid and peptidoglycan fragments. Lipoproteins and lipopeptides have repeatedly been shown to act as potent cytokine inducers, interacting with TLR-2, in synergy with TLR-1 or -6. In this study, we show that these compounds also interact with LBP and CD14. We used triacylated lipopeptides, corresponding to lipoproteins of *Borrelia burgdorferi*, mycobacteria, and *Escherichia coli*, as well as diacylated lipopeptides, corresponding to, e.g., 2-kDa macrophage activating lipopeptide of *Mycoplasma* spp. Activation of Chinese hamster ovary cells transfected with TLR-2 by both lipopeptides was enhanced by cotransfection of CD14. Responsiveness of human mononuclear cells to these compounds was greatly enhanced in the presence of human LBP. Binding of lipopeptides to LBP as well as competitive inhibition of this interaction by LPS was demonstrated in a microplate assay. Furthermore, we were able to show that LBP transfers lipopeptides to CD14 on human monocytes using FACS analysis. These results support that LBP is a pattern recognition receptor transferring a variety of bacterial ligands including the two major types of lipopeptides to CD14 present in different receptor complexes. *The Journal of Immunology*, 2004, 173: 2683–2691.

The innate immune system is represented by both humoral and cellular elements (1, 2). Soluble CD14 (sCD14)⁴ and LPS binding protein (LBP) are major serum factors with the ability to bind pathogens and initiate innate immune responses (3, 4). The cellular, so-called pattern recognition receptors include the recently identified family of TLRs (2). TLRs are germline-encoded receptors exhibiting homologies to *Toll*, which is involved in embryogenesis and host defense against Gram-positive bacteria and fungi in *Drosophila* (5, 6). TLR-2 was originally described to recognize LPS, a major constituent of the outer membrane of Gram-negative bacteria (7, 8), whereas later studies identified TLR-4 as the central transmembrane component of the LPS receptor (9, 10). The reason for this discrepancy was that LPS preparations used contained contaminating bacterial lipoproteins,

which later were identified to be highly active TLR-2 agonists (11, 12). TLR-2 also has been shown meanwhile to interact with numerous ligands of mainly bacterial origin, including peptidoglycan and lipoteichoic acid (LTA) of Gram-positive bacteria (13, 14) and spirochetal glycolipids (14, 15).

Lipoproteins and lipopeptides have been identified in a large number of microorganisms, the most prominent ones being mycobacteria, mycoplasmas, and spirochetes. They have been found to exhibit both a strong innate inflammatory response in the host and an enduring adaptive immune response in mammalian hosts (16). The strong proinflammatory capacities of lipoproteins were first described for outer surface proteins A and B of *Borrelia burgdorferi*, which are also highly immunogenic (17) and have lately been the basis for a Lyme disease vaccine development (18). These compounds exhibit an triacylated lipid anchor structure comprising an *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R**S*)-propyl]-(*R*)-cysteinylyl (Pam₃Cys) moiety at the N terminus (19), a feature that was previously described for the Braun lipoprotein from *Escherichia coli* (20). Because the N-terminal Pam₃Cys modification is essential for immunoactivation caused by lipoproteins of *B. burgdorferi* as well as of another spirochete, *Treponema pallidum* (21), subsequent studies investigating immune responses to spirochetes used synthetic lipopeptides (22). The Pam₃Cys moiety was also reported to be present in cytokine-inducing lipoproteins of *Mycobacterium* and *Mycoplasma* spp. (23, 24); thus, it can be regarded as a highly conserved molecular motif among different classes of bacteria. In *Mycoplasma fermentans*, the presence of a macrophage stimulating lipopeptide, termed 2-kDa macrophage-activating lipopeptide (MALP-2), was observed, being stimulatory active at picomolar concentrations (25). This compound, in contrast to the predominant lipopeptide structures present in lipoproteins of *E. coli*, *B. burgdorferi*, and mycobacteria, lacks the *N*-palmitoyl

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Received for publication December 16, 2003. Accepted for publication June 14, 2004.

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¹ This work was supported in part by grants given by the Bundesministerium für Bildung und Forschung to R.R.S. (CAPNetz), by Clinique La Prairie Research to C.A., and the Deutsche Forschungsgemeinschaft to R.R.S. and N.W.J.S. (Schr 726/1-1) as well as H.H. (He 2758/3-1).

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⁴ Abbreviations used in this paper: sCD14, soluble CD14; LBP, LPS binding protein; LTA, lipoteichoic acid; MALP-2, 2-kDa macrophage-activating lipopeptide; h, human; LP, lipopeptide.

group, thus containing a diacylated (Pam₂Cys) lipid anchor structure at the N terminus. Following studies revealed the presence of closely related compounds in other *Mycoplasma* spp. (26).

Cytokine induction caused by lipoproteins and lipopeptides is mediated by TLR-2 (11, 12). To interact with a rather wide spectrum of ligands, TLR-2 forms heterodimers with TLR-1 and TLR-6 (27). Studies using knockout mice identified TLR-6 as the coreceptor required for the recognition of diacylated lipopeptides corresponding to MALP-2 (28), whereas TLR-1 apparently forms heterodimers with TLR-2 to recognize triacylated lipopeptide structures corresponding to, e.g., *B. burgdorferi* lipoproteins (29). In contrast to this, a recent study using dominant-negative mutants provided evidence that TLR-6 may also be involved in recognition of these compounds (30).

Activation of immune cells by microbial compounds involves the soluble serum protein LBP (3, 15, 31). LBP is an acute-phase protein synthesized predominantly in the liver exhibiting homologies to other phospholipid binding proteins (3, 32–35). However, there is growing evidence that it is also released by cells of other compartments, because alveolar and intestinal epithelia have been shown to express this protein (36–38). LBP was first described to bind LPS from Gram-negative bacteria catalyzing its transfer to CD14, present as a phosphatidylinositol-anchored receptor on monocytes, macrophages, and neutrophils, and secreted in soluble isoforms (sCD14) by a wide variety of cells (4, 39, 40). LBP knockout mice have been shown to be highly susceptible to *Salmonella* infection (41), apparently due to the impaired recognition of these bacteria. Elevated LBP levels as found during sepsis have been shown to display beneficial effects by inhibiting cytokine response, and protecting the host from septic shock (42, 43). It is well established that CD14 is a pattern recognition receptor recognizing a wide variety of bacterial ligands (44), including LTA and peptidoglycan of Gram-positive bacteria (45, 46) as well as bacterial triacylated lipoproteins (47). In contrast, LBP for some time has been regarded to specifically recognize LPS of Gram-negative bacteria. In previous studies, we were able to show that LBP also recognizes LTA of Gram-positive bacteria as well as structurally similar spirochetal glycolipids (15, 48). Furthermore, LBP was shown to recognize pneumococcal peptidoglycan in a murine meningitis model (31).

In this study, we focus on the interaction of LBP and CD14 with synthetic triacylated and diacylated lipopeptides corresponding to triacylated lipoproteins and MALP-2, respectively. We provide evidence that cellular activation by these compounds is mediated by LBP and CD14 by the use of rLBP and blocking CD14 Abs as well as transfection experiments using Chinese hamster ovary (CHO) cells. In addition, these results were supported by binding studies on microtiter plates, native gel shifts, and FACS analysis. Thus, we provide evidence that LBP enables the innate immune system to recognize not only LPS and glycolipid-like structures such as LTA, but also the N-terminal lipid anchor structures of lipoproteins and lipopeptides, an abundant molecular motif shared by many different bacteria. These data thus provide insight in both function of LBP and pathogenesis of bacterial infections caused by bacteria.

Materials and Methods

Reagents and plasmids

Triacylated lipopeptides (Pam₃CSK₄; lipopeptide (LP)3) and diacylated lipopeptides (Pam₂CSK₄; LP2), first described by Mührlradt et al. (26), as well as synthetic MALP-2 were purchased from EMC Microcollections (Tübingen, Germany). LPS from *Salmonella minnesota* R595 was purchased from Sigma-Aldrich (St. Louis, MO) and found to be devoid of any lipoprotein contaminations in previous studies. Plasmids encoding human

(h)CD14, hTLR-2, hTLR-4, and hTLR-1 as well as β -galactosidase and the ELAM NF- κ B reporter plasmid were kindly provided by C. Kirschning (University of Munich, Munich, Germany). Plasmids encoding for hMD-2 and hTLR-6 were kindly provided by K. Miyake (University of Tokyo, Tokyo, Japan) and O. Takeuchi (University of Osaka, Osaka, Japan), respectively. Recombinant human LBP as well as rabbit polyclonal Ab against human LBP were kindly provided by Xoma (Berkeley, CA). The blocking CD14 Ab My4 was purchased from Beckman Coulter (Krefeld, Germany). IgG2 isotype control Ab was from BD Pharmingen (Hamburg, Germany). The blocking CD14 Ab MEM-18 (49) was a kind gift from V. Horejssi (Institute of Molecular Genetics, Prague, Czech Republic). IgG1 isotype control was from DakoCytomation (Hamburg, Germany).

Transfection of HEK293 and CHO cells

HEK293 cells stably transfected with human CD14 were cultivated at a density of 50,000/well in DMEM (Invitrogen Life Technologies, Eggenstein, Germany) supplemented with 10% FCS (PAA, Linz, Austria), glutamine, and antibiotics (Invitrogen Life Technologies) in 12-well tissue culture plates overnight. Cells were transfected with 0.1 μ g/well β -galactosidase and 0.25 μ g of ELAM and, as indicated, 0.05 μ g of hTLR-2, 0.01 μ g of hTLR-4, 0.05 μ g of hMD-2, and 0.1 μ g of hTLR-1 and -6 using Lipofectamine (Invitrogen Life Technologies) according to the manufacturer's protocol. After 24 h, cells were stimulated with different compounds as indicated for 16 h under serum-free conditions. Luciferase and β -galactosidase activities were estimated using an assay based on chemiluminescence (Boehringer, Mannheim, Germany). CHO cells were cultivated in Ham's Nutrient F12 medium (PAA) supplemented with FCS, glutamine, and antibiotics at a density of 50,000/well in 12-well tissue culture plates overnight, followed by transfection with the plasmids mentioned above and hCD14 (0.1 μ g/well) at the indicated concentrations using Fugene (Roche, Heidelberg, Germany). Stimulation and estimation of chemiluminescence were performed as indicated above.

Isolation and stimulation of human peripheral blood monocytes

Blood from healthy volunteers was drawn with heparin (5 U/ml) and diluted 1/2 in RPMI 1640 (PAA) supplemented with glutamine and antibiotics. A volume of 30 ml was layered on 15 ml of Pancoll (Pan Biotech, Aidenbach, Germany) and centrifuged at 400 \times g and 21°C for 45 min. The resulting interphase containing leukocytes was isolated and washed twice with RPMI 1640 at 400 \times g and 21°C for 10 min. Remaining platelets were removed by centrifugation at 100 \times g for 15 min. Resulting leukocytes were cultivated in 96-well plates in RPMI 1640 containing 2% human albumin (Sigma-Aldrich) for 2 h, followed by two washing steps to remove nonadherent cells. Cells were stimulated with the indicated compounds in the presence or absence of rhLBP. As indicated, the anti-CD14 Ab My4 or its isotype control were added to the cells at a concentration of 5 μ g/ml for 30 min before stimulation. After 4 h, supernatants were collected, and TNF- α levels were estimated. To this end, 96-well Immuno-maxi plates (TPP, Buchs, Switzerland) were coated with 5 μ g/ml rabbit anti-human TNF- α Ab (BD Pharmingen) diluted in 100 mM NaHCO₃. Plates were blocked with PBS containing 10% FCS for 2 h at room temperature, followed by incubation with the samples as well as TNF- α standard at 4°C overnight. After washing, plates were incubated with biotinylated TNF- α Ab (BD Pharmingen) for 1 h, followed by incubation with peroxidase-conjugated streptavidin (1 μ g/ml; Sigma-Aldrich) for 30 min. Detection of TNF- α was conducted using ortho-phenylen-diphosphate (Sigma-Aldrich).

Phast Gel native and SDS gel electrophoresis

An automated form of nondenaturing gel electrophoresis was performed using the Phastsystem apparatus (Amersham Biosciences, Piscataway, NJ). Briefly, 5 μ M recombinant sCD14 (Biometec, Greifswald, Germany) was incubated with or without LP2 and LP3 at concentrations of 50 and 250 μ M in Dulbecco's PBS (Invitrogen Life Technologies) at 37°C for 10 min. No other compounds were present in the mixtures. The samples were chilled on ice, and 4 \times native sample buffer (pH 7.6) was added. Native electrophoresis was performed at 4°C by automated application of 1 μ l of each sample to PhastGel Homogenous-20 gels equipped with native buffer strips, and electrophoresis was performed at 400 V. Integrity of sCD14 was verified by adjusting the remaining part of the sample to denaturing conditions by adding SDS to a final concentration of 2%, heating at 95°C for 5 min, and PhastGel SDS electrophoresis on the same gels as described above. Gels were stained with silver staining according to the manufacturer's protocol.

LBP microtiter binding assay

LBP-binding assays were performed using a published protocol (50) with some modifications. Rabbit anti-human LBP Ab was biotinylated using sulfo-succinimidyl biotin (Sigma-Aldrich). The Ab was dialyzed against PBS, and 100 μg were mixed with 30 μg of biotin dissolved in DMSO (Roth, Braunschweig, Germany) in a total volume of 80 μl and incubated at 4°C overnight. Immunomaxi plates were coated with LPS, LP2, LP3, or MALP-2 at 50 $\mu\text{g}/\text{ml}$ in 20 mM EDTA and 100 mM Na_2CO_3 (pH 9.2) for 3 h at room temperature. Blocking was performed with 150 mM NaCl and 50 mM HEPES (pH 7.4), supplemented with 10 mg/ml BSA (Roth) for 30 min at 37°C. Plates were then incubated with rhLBP (100 ng/ml) with or without LPS (50 $\mu\text{g}/\text{ml}$) in the same buffer supplemented with 1 mg/ml BSA at 37°C for 2 h. Detection of bound LBP was performed using the biotinylated anti-LBP Ab (1 $\mu\text{g}/\text{ml}$) and peroxidase-coupled streptavidin as described above. For certain experiments, LBP was added to microplates coated with LPS together with increasing concentrations of LPS, LP2, LP3, or MALP-2.

FACS analysis

Pam₃CSK₄-FLAG and Pam₂CSK₄-FLAG (LP3-FLAG and LP2-FLAG) were obtained from EMC Microcollections and dissolved in distilled water. Human PBMC were isolated from heparinized blood of healthy adult donors by Ficol-Isopaque density gradient centrifugation and incubated with LP3-FLAG or LP2-FLAG (10 nM) for 15 min at 37°C in serum-free RPMI 1640 with or without recombinant human LBP (0.5 $\mu\text{g}/\text{ml}$). After two washing steps with PBS, cells were incubated with a murine anti-FLAG M2 Ab (Sigma-Aldrich) at 5 $\mu\text{g}/\text{ml}$ for 1 h. Cells were again washed twice, followed by incubation with Cy5-conjugated secondary goat anti-murine IgG Ab (Sigma-Aldrich) for 1 h. Cells were washed twice and fixed in 3% paraformaldehyde, and gated monocytes were analyzed by FACS (FACSCalibur; BD Biosciences, San Jose, CA). For experiments investigating CD14, mononuclear cells were incubated with the CD14 blocking Ab MEM-18 or IgG1 control at 10 $\mu\text{g}/\text{ml}$ for 30 min at 37°C before incubation with the lipopeptides and LBP. After two washing steps with PBS, lipopeptides bound to the cells were detected using biotinylated anti-FLAG-M2 Ab (Sigma-Aldrich) at 5 $\mu\text{g}/\text{ml}$, for 1 h at 37°C, followed by two washing steps and detection with streptavidin-Cy5 (Dianova, Hamburg, Germany). Cells were washed, fixed, and analyzed as described above.

Statistics

As indicated, experimental data were analyzed for statistical significance using Student's *t* test for unpaired samples. Significant effects are indicated by asterisks.

Results

Interaction of synthetic lipopeptides with different TLRs

The first set of experiments was aimed at confirming that the synthetic compounds used in this study were similar to their naturally occurring counterparts regarding interaction with TLRs. HEK293 cells stably transfected with human CD14 were transiently transfected with β -galactosidase, the ELAM NF- κ B reporter plasmid and TLR-4/MD-2 or TLR-2 alone, followed by stimulation with LPS, LP3, and LP2. Cells were unresponsive to any ligands tested when no TLR was transfected (Fig. 1A), whereas transfection with TLR-4/MD-2 led to activation by LPS only (data not shown). TLR-2 was transfected without additional transfection of MD-2, because MD-2 failed to exhibit any effect toward recognition of TLR-2 ligands in our previous studies (31, 48). Only upon transfection with TLR-2, HEK293 cells could be activated by both synthetic lipopeptides (Fig. 1B), identifying them as TLR-2 agonists. Because no activation of TLR-4/MD-2-transfected HEK293 cells by LP2 or LP3 was detectable, the presence of significant amounts of contaminating LPS was ruled out.

TLR-2 is known to form heterodimers with either TLR-1 or -6, and MALP-2 has been identified to interact with TLR-2/TLR-6 heterodimers (28). In contrast, triacylated lipopeptides have been shown to be recognized via TLR-2/TLR-1 (29). Therefore, we tested whether the synthetic lipopeptides studied here could also be discriminated by these receptors. Because HEK293 cells are

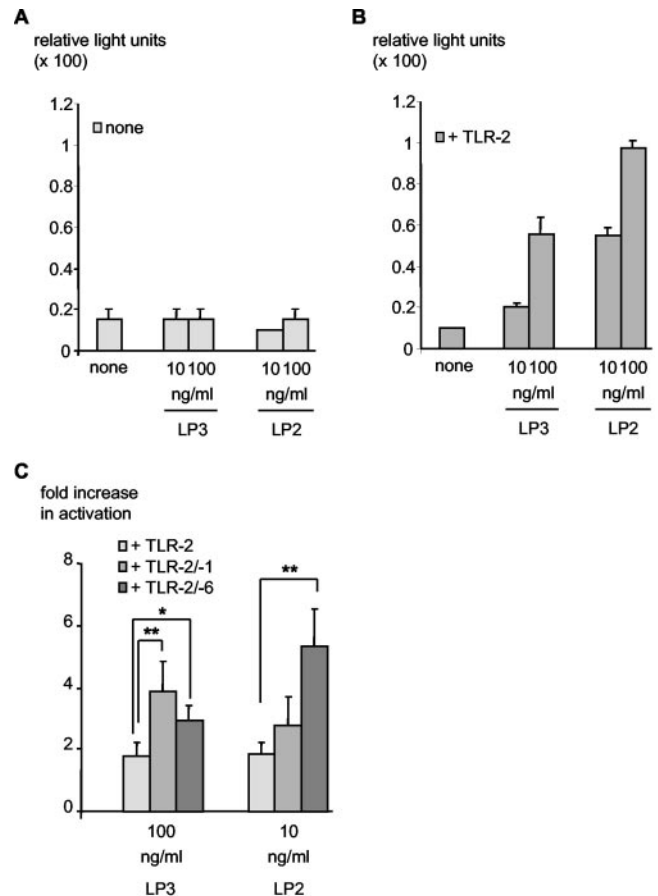


FIGURE 1. Involvement of TLR-2, -1, and -6 in activation of HEK293 cells by synthetic lipopeptides. HEK293 cells stably transfected with CD14 were cultured at a density of 50,000/well in 12-well tissue culture plates in DMEM plus 10% FCS overnight, followed by additional transfection with a plasmid encoding for β -galactosidase and an ELAM NF- κ B reporter plasmid using Lipofectamine (A). Additionally, cells were transfected with human TLR-2 (0.05 $\mu\text{g}/\text{well}$; B) and TLR-2 with human TLR-1 or -6 as indicated (0.1 $\mu\text{g}/\text{well}$ each; C). Twenty-four hours after transfection, cells were stimulated with LP2 or LP3 for 16 h without serum followed by estimation of cellular activation using chemiluminescence. Experiments were performed in triplicate; shown are representatives of three separate experiments (A and B) and combined results of four separate experiments (C). Statistics was performed using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

known to constitutively express both TLR-1 and TLR-6 (51), we overexpressed these receptors and determined whether this had any effect on activation by the lipopeptides. Overexpression of TLR-6 significantly increased the responsiveness to LP2, whereas TLR-1 had the same effect on LP3 (Fig. 1C). However, we also observed a trend toward an increased stimulation of HEK293 by LP3 upon overexpression of TLR-6 (Fig. 1C) confirming a previous report (30).

Interaction of synthetic lipopeptides with CD14

Although triacylated lipopeptides have been shown to interact with CD14 to cause cytokine induction (47), there are no reports on the potential involvement of CD14 in immune responses caused by diacylated lipopeptides. Studies with other *Mycoplasma* lipoproteins reported a stimulatory pathway independent of CD14 (52, 53). Therefore, we tested whether cytokine induction in human monocytes can be blocked by the CD14-blocking Ab My4. Human monocytes isolated from peripheral blood were stimulated, and

TNF- α -production was assessed after 4 h. Both synthetic lipopeptides led to a comparable increase in cytokine production by these cells in the presence of serum; however, LP2 was ~ 10 times more active compared with LP3 (Fig. 2, A and B). When human monocytes were preincubated with My4, no cytokine induction could be observed, indicating that cellular activation caused by diacylated lipopeptides is also CD14 dependent (Fig. 2, A and B).

In a different approach, we aimed at verifying this finding by studying the potential direct interaction of LP2 with CD14 using PhastGel native gel analysis. Soluble CD14, when preincubated with LP2 at a molar excess, displayed a shift toward lower electrophoretic mobility, indicating the formation of complexes of CD14 with LP2 (Fig. 2, C and D).

To further verify these results, we used CHO cells expressing neither CD14 nor functional TLR-2, the latter due to a frameshift mutation that causes a C-terminally truncated protein (54). These cells were transfected with human TLR-2, β -galactosidase, the ELAM NF- κ B reporter plasmid, and either human CD14 or its corresponding empty vector. Baseline NF- κ B activity in CD14-transfected cells was $\sim 30\%$ higher compared with controls (not shown); that is why data were analyzed as fold increase in luciferase activity. Stimulation with both LP3 and LP2 induced a marked increase in NF- κ B-mediated cellular activation, which was significantly greater when CD14 was present (Fig. 3), indicating that cellular recognition of both compounds is facilitated by this coreceptor. However, in these cells, in contrast to the human monocytes, LP3 turned out to be more active compared with LP2, possibly due to different expression ratios of TLR-1 and -6 compared with monocytes.

Cytokine induction in human monocytes is increased in the presence of human LBP

In the first experiments on cytokine induction caused by synthetic lipopeptides, we observed an increase in cellular activation in the

presence of serum (data not shown). To test whether this increase was caused by LBP present in serum, we used human monocytes stimulated with lipopeptides in the presence or absence of $1 \mu\text{g/ml}$ recombinant human LBP. Both lipopeptides induced TNF- α release by human monocytes in the absence of serum; however, cytokine levels induced by LP3 were lower as compared with LP2 (Fig. 4, A and B). In the presence of LBP, cytokine levels caused by both compounds were significantly increased. Both lipopeptides led to a marked cytokine production at concentrations of 0.17 ng/ml when LBP was present, whereas in the absence of LBP, higher concentrations of lipopeptides were required for detectable induction of TNF- α in serum-free medium (Fig. 4, A and B). When these higher concentrations of LP3 and LP2 were used, cytokine levels were increased ~ 25 -fold in the presence of LBP (Fig. 4, A and B).

LBP has been shown by us to inhibit cytokine induction caused by LPS both in vivo and in vitro when present at high concentrations similar to serum concentrations found during sepsis (32, 42). In subsequent studies, we were able to demonstrate that high-dose LBP also is able to inhibit the immunostimulatory activity brought about by LTA of Gram-positive bacteria (48). Thus, we wanted to know whether cellular activation caused by lipopeptides is also differentially influenced by varying concentrations of LBP. To this end, we stimulated human monocytes with different concentrations of lipopeptides in the presence of increasing concentrations of LBP (Fig. 4, C and D). Surprisingly, we failed to observe any inhibitory effect of high-dose LBP on cellular activation by lipopeptides even if these compounds were administered at low concentrations. As expected, inhibitory LBP effects were also absent when LP2 and LP3 were added at higher concentrations (data not shown).

LBP binds to diacylated and triacylated lipopeptides

We next aimed at verifying the data obtained for cytokine induction by binding studies with LBP and lipopeptides. Because both

FIGURE 2. Involvement of CD14 in cellular recognition of lipopeptides. Monocytes isolated from human peripheral blood were plated in 96-well culture plates and stimulated with LP3 (A) or LP2 (B) in the presence of 1% human serum with or without preincubation with My4 ($5 \mu\text{g/ml}$) or the isotype control Ab for 30 min at 37°C . After 4 h, supernatants were collected, and TNF- α concentration was estimated by ELISA. Experiments were performed in triplicate; shown is one representative of two separate experiments. *, $p < 0.01$. C and D, Formation of complexes of CD14 and LP2 were studied using PhastGel native gel electrophoresis. Following incubation at 37°C for 10 min, the samples containing recombinant human sCD14 with or without LP2 at the indicated concentrations were analyzed by native gel electrophoresis (C). Integrity of sCD14 was verified by PhastGel SDS gel electrophoresis (D). Both lipopeptides failed to induce a mobility shift of LBP in this assay (not shown).

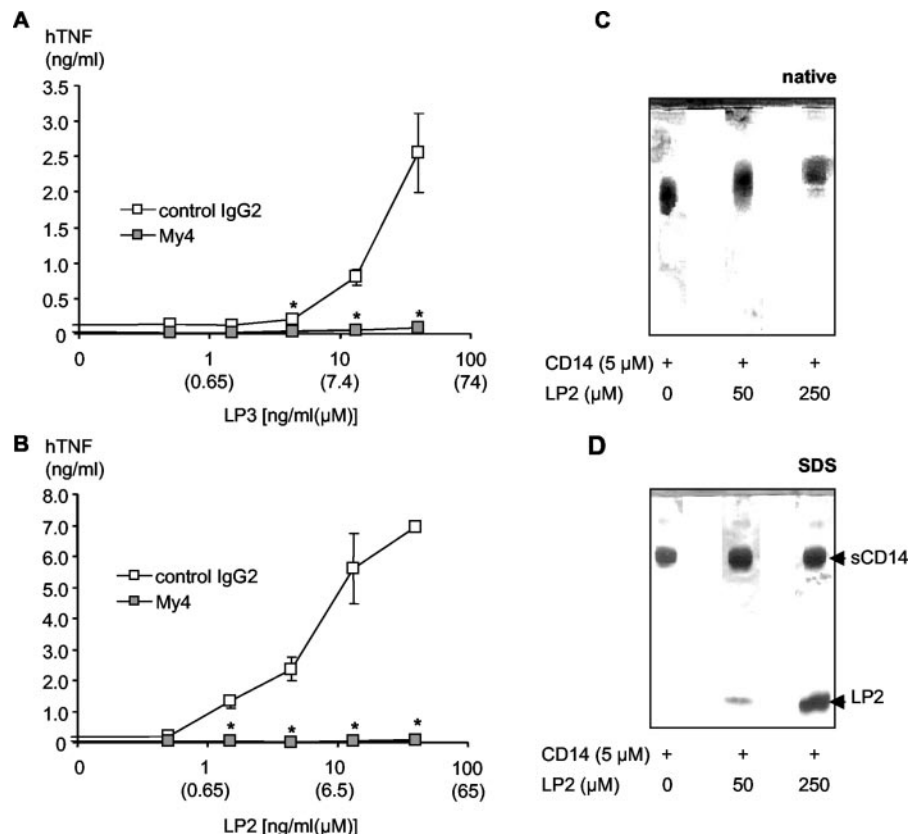
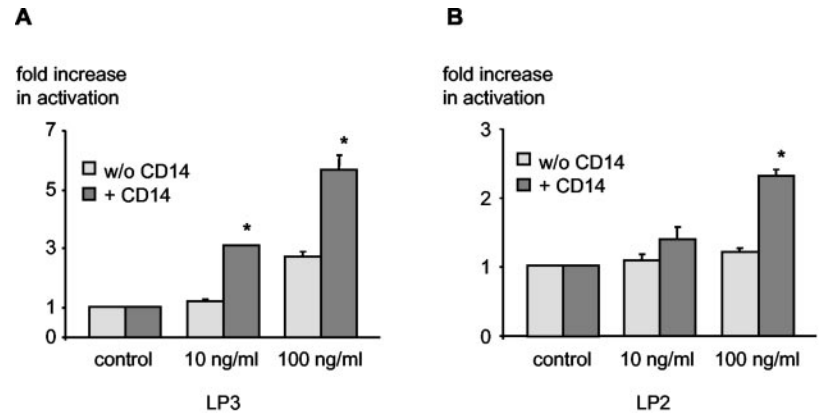


FIGURE 3. Activation of CHO cells by lipopeptides is enhanced by CD14. CHO cells were cultured in Ham's nutrient F12 medium plus 10% FCS at a density of 50,000 cells/well in 12-well tissue culture plates overnight. The following day, cells were transfected with β -galactosidase, ELAM, and human TLR-2 (0.05 μ g/well) with or without CD14 (0.1 μ g/well). After 24 h, cells were stimulated with LP3 (A) and LP2 (B) under serum-free conditions at the given concentrations for 16 h. Baseline activity (set to 1 here) was \sim 30% higher in CD14-transfected cells compared with nontransfected cells. Activation of the cells was estimated using an assay based on chemiluminescence. Data are shown as fold increase in luciferase activity compared with unstimulated controls. Experiments were performed in triplicate; shown is one representative of three separate experiments. *, $p < 0.01$.



compounds did not cause a marked shift in electrophoretic mobility of LBP when investigated by PhastGel analysis (data not shown), we performed an LBP-binding assay with different microbial ligands coated to microtiter plates. Plates were coated with LPS, LP3, and LP2, as well as its naturally occurring counterpart, MALP-2, followed by incubation with recombinant human LBP and subsequent detection using biotinylated anti-LBP Abs and streptavidin peroxidase. We observed a marked increase in absorption when the wells were coated with LP2, LP3, or MALP-2, comparable to the increase seen with LPS, indicating that LBP binds to these lipopeptide ligands (Fig. 5A). This increase was not present when LBP was added to the plates with LPS, suggesting a competition of LPS with lipopeptides for the binding to LBP. In another set of experiments, plates coated with LPS were incubated with LBP together with increasing concentrations of LP2, LP3, and MALP-2 (Fig. 5B). In these assays, the two diacylated lipopeptides, LP2 and MALP-2, inhibited binding of LBP to LPS immobilized on the plates in a comparable fashion to LPS itself (Fig. 5B), indicating a competition for the same binding site. However, triacylated LP3 did not display this inhibition, even at the highest concentration tested.

Transfer of FLAG-tagged lipopeptides to human peripheral blood monocytes by LBP

Next, we investigated whether LBP was able to mediate the transfer of lipopeptides to CD14-bearing cells. For this purpose, we used FLAG-tagged derivatives of LP2 and LP3 sharing the Pam₂Cys and Pam₃Cys motifs with the original nonderivatized lipopeptides while containing an additional FLAG epitope at their C-terminal lysine residues. The FLAG sequence did not alter the biologic activities of these compounds in comparison to LP2 and LP3 (data not shown). Human peripheral blood monocytes were incubated with 10 nM LP3-FLAG or LP2-FLAG (14 and 15 ng/ml, respectively) in the presence or absence of recombinant human LBP. Then, cells were incubated with anti-FLAG Abs and subsequently with Cy5-labeled anti-IgG Abs, and fluorescence of gated monocytes was assessed by FACS analysis. In the presence of both lipopeptides, a small increase in fluorescence was observed (Fig. 6, A and B), which was stronger for LP2-FLAG compared with LP3-FLAG, in line with the data on cytokine induction indicating a lower potency of LP3-FLAG to interact with cells in serum-free medium (Figs. 2, A and B; 4, A and B). However, in the presence of LBP, a profound increase in fluorescence was observed for both lipopeptides in a comparable manner (Fig. 6, A and B), indicating that LBP is able to transfer lipopeptides irrespective of their acylation pattern to monocytes. To verify that the transfer of lipopeptides observed was specific, we used a blocking CD14 Ab. In these experiments, no transfer of either LP3-FLAG and LP2-FLAG oc-

curred in the presence of MEM-18 (Fig. 6C), indicating that LBP transfers both lipopeptides to CD14.

Discussion

Lipoproteins are well-defined molecular patterns of a large variety of pathogenic microorganisms with the capability to stimulate APCs. They have long been known as strong cytokine inducers and have recently become the basis for vaccine development, making them key molecules of innate and adaptive immunity (16, 55). We show in this study that a host acute-phase protein, LBP, which previously has been known to be a key molecule for pathogen recognition via LPS, LTA, and peptidoglycan, is also crucial for lipoprotein recognition. Moreover, we obtained several lines of evidence that CD14 is also involved in cellular responses to triacylated and diacylated lipopeptides. In confirmation of prior studies, lipopeptides displayed a pattern of recognition via TLR-2 in differential combinations with TLR-1 and -6 related to their different degree in acylation. Thus, LBP apparently has the ability to recognize a wide variety of microorganisms and to mediate the initiation of signal transduction not only via TLR-4/MD-2, but also via TLR-2/-1 or TLR-2/-6 as suggested in Fig. 7.

The Pam₃Cys motif of lipoproteins is abundant among different bacteria and has been described for *Enterobacteriaceae* (20), spirochetes (19, 21), mycobacteria (23), and mycoplasmas (24). In contrast, the Pam₂Cys motif exhibiting two fatty acids has only been shown to be present in mycoplasmas (25). Because the Pam₃Cys motif is present in such a large variety of microorganisms and corresponding molecular structures are absent in higher vertebrate species, it may represent a common structure discriminating pathogens from the host. In this study, we investigated the biological characteristics of synthetic triacylated lipopeptides corresponding to triacylated lipoproteins, as well as diacylated lipopeptides corresponding to MALP-2. These derivatives have been previously described to induce cytokines in immune cells in a dose-dependent fashion comparable to their naturally occurring counterparts (26), and therefore appear to be suitable for studying innate immune responses to lipopeptides in general. Although the Pam₃CSK₄ lipopeptide was previously demonstrated to resemble naturally occurring lipoproteins regarding use of TLRs (28, 29), no data have been available for the Pam₂CSK₄ lipopeptide. We show in this study that both lipopeptides interact with TLR-2 in synergy with TLR-1 or -6 as previously reported. We were able to confirm the data obtained with MALP-2 (28) with its synthetic counterpart LP2: We found that TLR-2 was essential for stimulation of HEK293 cells by this compound, and its stimulatory capacity could be significantly enhanced by overexpression of TLR-6. Co-transfection of both TLR-1 and, to a lesser extent, TLR-6 increased

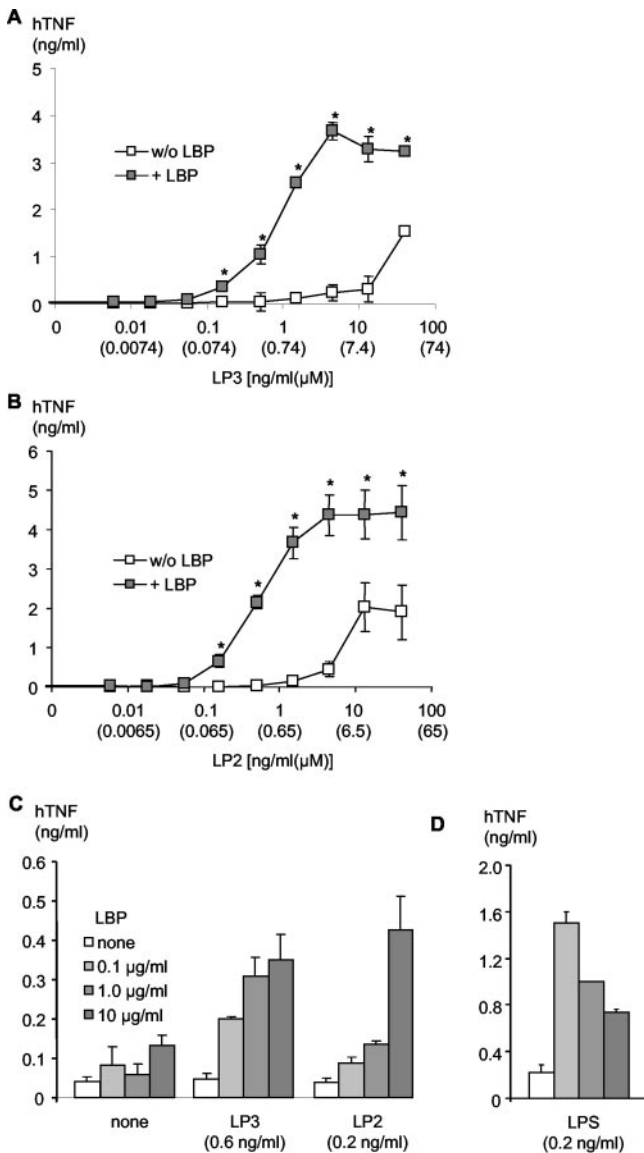


FIGURE 4. Cytokine induction in human monocytes caused by lipopeptides is enhanced in the presence of LBP. Human monocytes were cultured in RPMI 1640 in 96-well tissue culture plates. *A* and *B*, Stimulation was performed with LP3 (*A*) and LP2 (*B*) at the given concentrations in the presence or absence of human recombinant LBP (1 μ g/ml). *C* and *D*, Human monocytes were stimulated with LP3, LP2, or LPS at the concentrations indicated in the presence of varying concentrations of LBP. Due to lesser activity of LP3 compared with LP2 in previous experiments, an increased concentration was used here. After 4 h, supernatants were harvested, and TNF- α levels were assessed using ELISA. Shown is one representative of three separate experiments. Experiments were performed in triplicate. *, $p < 0.01$.

stimulation of HEK293 cells by triacylated lipopeptides. This finding is partially contradictory to previous reports identifying TLR-1 as the sole coreceptor (29), while supporting results of another group using a dominant-negative mutant of TLR-6 (30).

We provide evidence that innate immune recognition of lipoproteins is mediated by LBP, a molecule that until recently was thought of as interacting with LPS exclusively. Cytokine induction in human monocytes caused by diacylated and triacylated lipopeptides was greatly enhanced in the presence of LBP, and the interaction of LBP with the lipopeptides could be verified by microplate binding assays. LBP bound to plates coated with diacylated

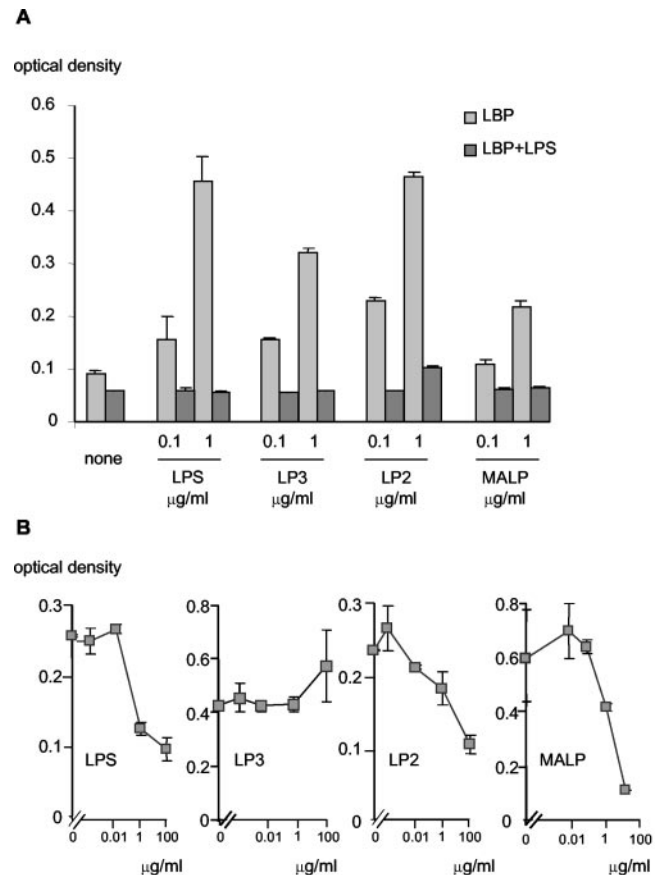


FIGURE 5. Binding of LBP to lipopeptides. *A*, Ninety-six-well microtiter plates were coated with LPS, LP3, LP2, or MALP-2 at the concentrations indicated. After washing, drying, and blocking, as described in *Materials and Methods*, plates were incubated with recombinant human LBP (20 ng/ml) in the presence or absence of LPS (10 μ g/ml). LBP bound to the plates was detected using a biotinylated anti-human LBP Ab, peroxidase-labeled streptavidin, and ortho-phenylen-diphosphate as a substrate. Experiments were performed in triplicate. Shown is one representative of three separate experiments. *B*, Ninety-six-well microtiter plates were coated with LPS. After washing, drying, and blocking, plates were incubated with LBP together with LPS, LP3, LP2, or MALP at the concentrations indicated (highest concentrations, 100 μ g/ml, except for MALP-2, 20 μ g/ml). LBP bound to the plates was detected as described above. Shown are representatives of three (LPS and MALP) as well as five (LP2 and LP3) separate experiments.

and triacylated lipopeptides as well as MALP-2 in a manner comparable to LPS, and the specificity of this binding could be demonstrated by inhibition of LBP binding to the plates when LPS was added. However, when microtiter plates coated with LPS were incubated with lipopeptides, only LP2 and MALP-2 were able to compete with LPS, whereas LP3 did not block binding of LBP to LPS immobilized on the plate. At this point, it cannot be ruled out that this was caused by a lesser affinity of LP3 to LBP compared with LP2 and MALP-2. However, it is tempting to speculate that the lack of competition of LP3 with LPS revealed in these experiments is caused by the use of different binding sites within LBP. In contrast, LP2 and MALP-2 obviously bind to LBP via the same or at least adjacent binding sites within the N-terminal part (50), a feature that we previously demonstrated for two glycolipids exhibiting a lipid anchor with two fatty acids, LTA of Gram-positive bacteria and *Treponema* glycolipids (15, 56). As demonstrated by FACS analysis, LBP transfers both triacylated and diacylated lipopeptides to CD14 because transfer was blocked by an anti-CD14

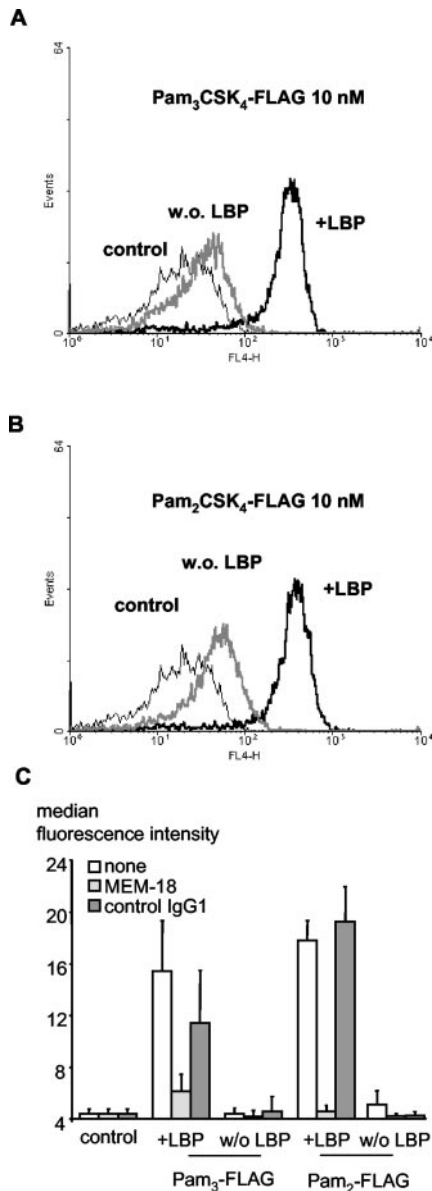


FIGURE 6. LBP transfers lipopeptides to CD14 on peripheral blood monocytes. PBMC were incubated with Pam₃CSK₄-FLAG (A; 10 nM) or Pam₂CSK₄-FLAG (B; 10 nM) for 15 min at 37°C in serum-free RPMI 1640 with or without recombinant human LBP (0.5 μg/ml). Cells were washed and incubated with a murine anti-FLAG Ab followed by incubation with a Cy5-conjugated goat anti-murine IgG Ab. Fluorescence of cells was estimated by FACS. Shown is one representative of three separate experiments. C, Mononuclear cells were incubated with MEM-18 or control IgG1 (10 μg/ml) for 30 min at 37°C in serum-free RPMI 1640 with or without recombinant human LBP (0.5 μg/ml) before the incubation with Pam₃CSK₄-FLAG or Pam₂CSK₄-FLAG (10 nM each). Cell were washed and incubated with murine biotinylated anti-FLAG Ab, followed by streptavidin-Cy5. Fluorescence of gated monocytes was estimated by FACS. Experiments were performed five times with comparable results. Shown are averaged results of two separate experiments.

Ab. Our results imply that the function of LBP is not restricted to structures bearing carbohydrate polymers, such as LPS, LTA (15, 48), mycobacterial lipomannan (57), or muropeptides linked to teichoic acid (31), but also includes interaction with lipoproteins or lipopeptides, both of the triacylated and diacylated subtypes. Additionally, by using inhibiting CD14 Abs, native shift gel electrophoresis, transfection studies, and FACS analysis, we were able to

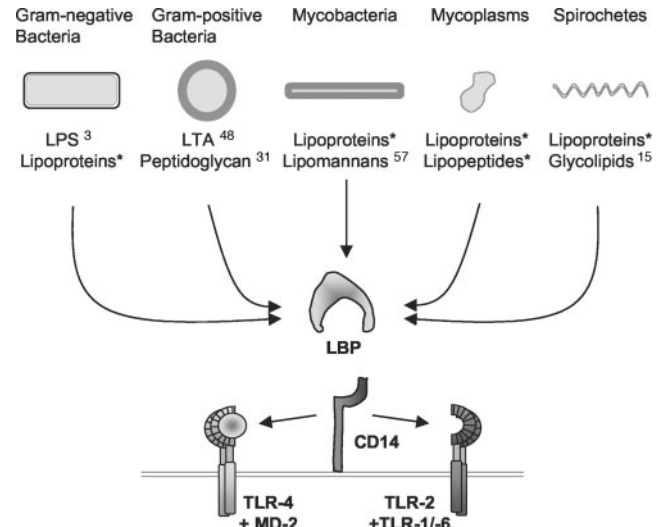


FIGURE 7. Overview of bacterial ligands recognized by LBP. Gram-negative and Gram-positive bacteria as well as mycobacteria, mycoplasmas, and spirochetes exhibit ligands within their cell wall or membrane, respectively, that are bound by LBP and transferred to CD14. CD14 then facilitates the recognition of these ligands by TLR-4 or TLR-2 in synergy with TLR-1 or -6. Corresponding references are indicated by numbers. *, Data presented in this study.

demonstrate for the first time that CD14 is also involved in recognition of diacylated lipopeptides, a feature that was previously demonstrated only for triacylated lipopeptides (47).

The predominant biologic effect of LBP initially was thought to be an amplification of cytokine response during sepsis, potentially leading to deleterious effects such as septic shock (58, 59). Studies with LBP knockout mice indicated that LBP serves in preventing bacterial infections, because LBP^{-/-} mice are more susceptible to infection by Gram-negative bacteria compared with their wild-type littermates (41). Additionally, our previous results showed that LBP could protect mice from an otherwise lethal septic shock induced by LPS or whole bacteria (42). These findings most likely are based on the fact that LBP is able to bind bacterial ligands and transfer them from the cellular receptor into other compartments of the host for detoxification (60). However, in the experiments described in this paper, lipopeptides differed from LPS and LTA regarding the fact that cytokine induction could not be inhibited by high concentrations of LBP.

LBP is not only synthesized in hepatocytes, but has also been found to be expressed in alveolar epithelial cells (36) and intestinal cells (37). Thus, LBP is apparently present in compartments of the organism exposed to large quantities of microorganisms, and may play a key role in early recognition of pathogens. Lipoproteins and lipopeptides bearing the Pam₂Cys or Pam₃Cys motif are abundant among pathogens of the respiratory tract, such as *Mycoplasma pneumoniae* and *Mycobacterium tuberculosis*. Our own previous studies have revealed that major cell wall components of *Streptococcus pneumoniae* and *Staphylococcus aureus* causing pneumonia, are also recognized by LBP (31, 48). Thus, recognition of molecular patterns by LBP may be an important step in the initiation of a host response during pneumonia. In addition, our data suggest that LBP, by recognizing lipoproteins, may also play a role in chronic or cyclic infections: With the exception of *Mycoplasma*-caused pneumonia, clinically important pathogens carrying lipoproteins represent causes of slow, cyclic or chronic infectious diseases, e.g., Lyme disease or tuberculosis. Even mycoplasma pneumonia, although frequent in children, is often afebrile and

self-limiting in contrast to pneumonia caused by other pathogens (61). Our data suggest that recognition of these slow-acting microorganisms may also be accomplished by LBP. Synthetic lipopeptides have been the basis for broad vaccine development lately for a wide variety of diseases including HIV, malaria, CMV, and HSV (reviewed in Ref. 16). Understanding of the molecular mechanisms of host recognition of lipopeptides and lipoproteins in more detail may further help to further improve these innovative strategies.

Acknowledgments

We acknowledge the excellent technical assistance of Sabine Bobbe and Diana Woellner (Berlin, Germany) as well as Nina Grohmann and Ina Goroncy (Borstel, Germany).

References

- Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91:295.
- Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1:135.
- Schumann, R. R., S. R. Leong, G. W. Flaggs, 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.
- Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249:1431.
- Hashimoto, C., K. L. Hudson, and K. V. Anderson. 1988. The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 52:269.
- Lemaître, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann. 1996. The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* 86:973.
- Kirschning, C. J., H. Wesche, T. Merrill Ayres, and M. Rothe. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* 188:2091.
- Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 395:284.
- Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 282:2085.
- Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162:3749.
- Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem.* 274:33419.
- Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. *J. Immunol.* 163:2382.
- Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem.* 274:17406.
- Opitz, B., N. W. Schröder, I. Spreitzer, K. S. Michelsen, C. J. Kirschning, W. Hallatschek, U. Zähringer, T. Hartung, U. B. Göbel, and R. R. Schumann. 2001. Toll-like receptor-2 mediates *Treponema* glycolipid and lipoteichoic acid-induced NF- κ B translocation. *J. Biol. Chem.* 276:22041.
- Schröder, N. W., B. Opitz, N. Lamping, K. S. Michelsen, U. Zähringer, U. B. Göbel, and R. R. Schumann. 2000. Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by *Treponema* glycolipids. *J. Immunol.* 165:2683.
- BenMohamed, L., S. L. Wechsler, and A. B. Nesburn. 2002. Lipopeptide vaccines—yesterday, today, and tomorrow. *Lancet Infect. Dis.* 2:425.
- Ma, Y., and J. J. Weis. 1993. *Borrelia burgdorferi* outer surface lipoproteins OspA and OspB possess B-cell mitogenic and cytokine-stimulatory properties. *Infect. Immun.* 61:3843.
- Steere, A. C., V. K. Sikand, F. Maurice, D. L. Parenti, E. Fikrig, R. T. Schoen, J. Nowakowski, C. H. Schmid, S. Laukamp, C. Buscarino, and D. S. Krause. 1998. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant: Lyme Disease Vaccine Study Group. *N. Engl. J. Med.* 339:209.
- Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard. 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. *Infect. Immun.* 58:983.
- Braun, V., and K. Rehn. 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall: the specific effect of trypsin on the membrane structure. *Eur. J. Biochem.* 10:426.
- Weis, J. J., Y. Ma, and L. F. Erdile. 1994. Biological activities of native and recombinant *Borrelia burgdorferi* outer surface protein A: dependence on lipid modification. *Infect. Immun.* 62:4632.
- Radolf, J. D., L. L. Arndt, D. R. Akins, L. L. Curetty, M. E. Levi, Y. Shen, L. S. Davis, and M. V. Norgard. 1995. *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytes/macrophages. *J. Immunol.* 154:2866.
- Brightbill, H. D., D. H. Librarty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* 285:732.
- Mühlradt, P. F., H. Meyer, and R. Jansen. 1996. Identification of *S*-(2,3-dihydroxypropyl)cystein in a macrophage-activating lipopeptide from *Mycoplasma fermentans*. *Biochemistry* 35:7781.
- Mühlradt, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung. 1997. Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from *Mycoplasma fermentans* acting at picomolar concentration. *J. Exp. Med.* 185:1951.
- Mühlradt, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung. 1998. Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. *Infect. Immun.* 66:4804.
- Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. USA* 97:13766.
- Takeuchi, O., T. Kawai, P. F. Muhlrardt, M. Morr, J. D. Radolf, A. Zychlinsky, K. Takeda, and S. Akira. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* 13:933.
- Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin, and S. Akira. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* 169:10.
- Bulut, Y., E. Faure, L. Thomas, O. Equils, and M. Arditi. 2001. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and *Borrelia burgdorferi* outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J. Immunol.* 167:987.
- Weber, J. R., D. Freyer, C. Alexander, N. W. Schröder, A. Reiss, C. Kuster, D. Pfeil, E. I. Tuomanen, and R. R. Schumann. 2003. Recognition of pneumococcal peptidoglycan: an expanded, pivotal role for LPS binding protein. *Immunity* 19:269.
- Schumann, R. R., C. J. Kirschning, A. Unbehauen, H. P. Aberle, H. P. Knopf, N. Lamping, R. J. Ulevitch, and F. Herrmann. 1996. The lipopolysaccharide-binding protein is a secretory class I acute-phase protein whose gene is transcriptionally activated by APRF/STAT3 and other cytokine-inducible nuclear proteins. *Mol. Cell. Biol.* 16:3490.
- Elsbach, P., J. Weiss, R. C. Franson, S. Beckerditte-Quagliata, A. Schneider, and L. Harris. 1979. Separation and purification of a potent bactericidal/permeability-increasing protein and a closely associated phospholipase A₂ from rabbit polymorphonuclear leukocytes: observations on their relationship. *J. Biol. Chem.* 254:11000.
- Kirschning, C. J., J. Au-Young, N. Lamping, D. Reuter, D. Pfeil, J. J. Seilhamer, and R. R. Schumann. 1997. Similar organization of the lipopolysaccharide-binding protein (LBP) and phospholipid transfer protein (PLTP) genes suggests a common gene family of lipid-binding proteins. *Genomics* 46:416.
- Schumann, R. R., N. Lamping, and A. Hoess. 1997. Interchangeable endotoxin-binding domains in proteins with opposite lipopolysaccharide-dependent activities. *J. Immunol.* 159:5599.
- Dentener, M. A., A. C. Vreugdenhil, P. H. Hoet, J. H. Vernooij, F. H. Nieman, D. Heumann, Y. M. Janssen, W. A. Buurman, and E. F. Wouters. 2000. Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: implications for local defense to bacterial endotoxins. *Am. J. Respir. Cell Mol. Biol.* 23:146.
- Vreugdenhil, A. C., M. A. Dentener, A. M. Snoek, J. W. Greve, and W. A. Buurman. 1999. Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. *J. Immunol.* 163:2792.
- Vreugdenhil, A. C., A. M. Snoek, J. W. Greve, and W. A. Buurman. 2000. Lipopolysaccharide-binding protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice. *J. Immunol.* 165:4561.
- Goyert, S. M., E. Ferrero, W. J. Rettig, A. K. Yenamandra, F. Obata, and M. M. Le Beau. 1988. The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. *Science* 239:497.
- Tapping, R. I., and P. S. Tobias. 2000. Soluble CD14-mediated cellular responses to lipopolysaccharide. *Chem. Immunol.* 74:108.
- Jack, R. S., X. Fan, M. Bernheiden, G. Rune, M. Ehlers, A. Weber, G. Kirsch, R. Mentel, B. Furl, M. Freudenberg, et al. 1997. Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection. *Nature* 389:742.
- Lamping, N., R. Dettmer, N. W. Schröder, D. Pfeil, W. Hallatschek, R. Burger, and R. R. Schumann. 1998. LPS-binding protein protects mice from septic shock caused by LPS or Gram-negative bacteria. *J. Clin. Invest.* 101:2065.
- Zweigner, J., H. J. Gramm, O. C. Singer, K. Wegscheider, and R. R. Schumann. 2001. High concentrations of lipopolysaccharide-binding protein in serum of patients with severe sepsis or septic shock inhibit the lipopolysaccharide response in human monocytes. *Blood* 98:3800.

44. Pugin, J., I. D. Heumann, A. Tomasz, V. V. Kravchenko, Y. Akamatsu, M. Nishijima, M. P. Glauser, P. S. Tobias, and R. J. Ulevitch. 1994. CD14 is a pattern recognition receptor. *Immunity* 1:509.
45. Weidemann, B., J. Schletter, R. Dziarski, S. Kusumoto, F. Stelter, E. T. Rietschel, H. D. Flad, and A. J. Ulmer. 1997. Specific binding of soluble peptidoglycan and muramyl dipeptide to CD14 on human monocytes. *Infect. Immun.* 65:858.
46. Dziarski, R., R. I. Tapping, and P. S. Tobias. 1998. Binding of bacterial peptidoglycan to CD14. *J. Biol. Chem.* 273:8680.
47. Sellati, T. J., D. A. Bouis, R. L. Kitchens, R. P. Darveau, J. Pugin, R. J. Ulevitch, S. C. Gangloff, S. M. Goyert, M. V. Norgard, and J. D. Radolf. 1998. *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that used by lipopolysaccharide. *J. Immunol.* 160:5455.
48. Schröder, N. W., S. Morath, C. Alexander, L. Hamann, T. Hartung, U. Zähringer, U. B. Göbel, J. R. Weber, and R. R. Schumann. 2003. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* 278:15587.
49. Bazil, V., V. Horejsi, M. Baudys, H. Kristofova, J. L. Strominger, W. Kostka, and I. Hilgert. 1986. Biochemical characterization of a soluble form of the 53-kDa monocyte surface antigen. *Eur. J. Immunol.* 16:1583.
50. Lamping, N., A. Hoess, B. Yu, T. C. Park, C. J. Kirschning, D. Pfeil, D. Reuter, S. D. Wright, F. Herrmann, and R. R. Schumann. 1996. Effects of site-directed mutagenesis of basic residues (Arg⁹⁴, Lys⁹⁵, Lys⁹⁹) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. *J. Immunol.* 157:4648.
51. Fujita, M., T. Into, M. Yasuda, T. Okusawa, S. Hamahira, Y. Kuroki, A. Eto, T. Nisizawa, M. Morita, and K. Shibata. 2003. Involvement of leucine residues at positions 107, 112, and 115 in a leucine-rich repeat motif of human Toll-like receptor 2 in the recognition of diacylated lipoproteins and lipopeptides and *Staphylococcus aureus* peptidoglycans. *J. Immunol.* 171:3675.
52. Kostyal, D. A., G. H. Butler, and D. H. Beezhold. 1994. A 48-kilodalton *Mycobacterium fermentans* membrane protein induces cytokine secretion by human monocytes. *Infect. Immun.* 62:3793.
53. Rawadi, G., J. Garcia, B. Lemercier, and S. Roman-Roman. 1999. Signal transduction pathways involved in the activation of NF- κ B, AP-1, and *c-fos* by *Mycobacterium fermentans* membrane lipoproteins in macrophages. *J. Immunol.* 162:2193.
54. Heine, H., C. J. Kirschning, E. Lien, B. G. Monks, M. Rothe, and D. T. Golenbock. 1999. Cutting edge: cells that carry a null allele for Toll-like receptor 2 are capable of responding to endotoxin. *J. Immunol.* 162:6971.
55. Bessler, W. G., L. Heinevetter, K. H. Wiesmuller, G. Jung, W. Baier, M. Huber, A. R. Lorenz, U. V. Esche, K. Mittenbuhler, and P. Hoffmann. 1997. Bacterial cell wall components as immunomodulators. I. Lipopeptides as adjuvants for parenteral and oral immunization. *Int. J. Immunopharmacol.* 19:547.
56. Hermann, C., I. Spreitzer, N. W. Schröder, S. Morath, M. D. Lehner, W. Fischer, C. Schutt, R. R. Schumann, and T. Hartung. 2002. Cytokine induction by purified lipoteichoic acids from various bacterial species: role of LBP, sCD14, CD14 and failure to induce IL-12 and subsequent IFN- γ release. *Eur. J. Immunol.* 32:541.
57. Vignal, C., Y. Guerardel, L. Kremer, M. Masson, D. Legrand, J. Mazurier, and E. Elaiss. 2003. Lipomannans, but not lipoarabinomannans, purified from *Mycobacterium chelonae* and *Mycobacterium kansasii* induce TNF- α and IL-8 secretion by a CD14-Toll-like receptor 2-dependent mechanism. *J. Immunol.* 171:2014.
58. Glauser, M. P., G. Zanetti, J. D. Baumgartner, and J. Cohen. 1991. Septic shock: pathogenesis. *Lancet* 338:732.
59. Schumann, R. R., N. Lamping, C. Kirschning, H. P. Knopf, A. Hoess, and F. Herrmann. 1994. Lipopolysaccharide binding protein: its role and therapeutical potential in inflammation and sepsis. *Biochem. Soc. Trans.* 22:80.
60. Thompson, P. A., P. S. Tobias, S. Viriyakosol, T. N. Kirkland, and R. L. Kitchens. 2003. Lipopolysaccharide (LPS)-binding protein inhibits responses to cell-bound LPS. *J. Biol. Chem.* 278:28367.
61. McIntosh, K. 2002. Community-acquired pneumonia in children. *N. Engl. J. Med.* 346:429.