Kupffer Cell Activation by Lipopolysaccharide in Rats: Role for Lipopolysaccharide Binding Protein and Toll-like Receptor 4

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Lipopolysaccharide (LPS) binding protein (LBP) is a key serum factor that mediates LPS activation of mononuclear cells. In the presence of LBP, 1/1,000 the concentration of LPS is sufficient to activate peripheral blood monocytes. Previous studies with Kupffer cells have shown a variable effect of serum on LPS activation of these cells and led to the conclusion that, unlike extrahepatic mononuclear cells, Kupffer cells do not respond to LPS in an LBP-dependent fashion. Because there are multiple components in serum other than LBP that might affect LPS activation, these reports with serum are difficult to interpret. To investigate the specific role of LBP in LPS activation of Kupffer cells, we produced a functional recombinant rat LBP using a baculovirus expression system, which we used to selectively examine the role of LBP's on Kupffer-cell function. Isolated Kupffer cells exposed to increasing concentrations of LPS (0, 1, 10 ng/mL) showed a dose-dependent increase in TNF-α production, which was augmented and accelerated by the presence of LBP. The effects of LBP on Kupffer cell activation by LPS are dependent on a functional Toll-like receptor 4 (Tlr 4) because Kupffer cells from C3H/HeJ mice failed to respond to LPS in the presence of LBP. LBP plays an important role in mediating Kupffer cell activation by LPS, and these effects are dependent on the presence of functioning Tlr 4. (HEPATOLOGY 2000;31:932-936.)

Endogenous lipopolysaccharides (LPS) have been implicated as a cofactor in promoting liver injury in many models of liver injury, including alcoholic hepatitis.[1,2] In the Tsukomoto and French model of rat alcoholic hepatitis,[3] the degree of liver injury is diminished by treatment with either antibiotics, lactobacillus, or polymixin, all of which decrease endogenous LPS.[4,5] In this model, Kupffer cells, when activated by LPS, play a prominent role in promoting liver injury.[6] Despite its potentially critical importance, the molecular mechanism by which Kupffer cells are activated by LPS remains largely unknown.

In peripheral blood monocytes, the pathway of LPS activation has been recently delineated. In serum, LPS binds to LPS-binding protein (LBP), which is a 60-kd acute-phase protein produced by hepatocytes.[7,8] This LPS-LBP complex then binds to membrane CD14 resulting in cell activation, nuclear translocation of NF-κβ, and production of cytokines such as TNF-α.[9,10] The critical importance of LBP during *in vivo* responses to LPS and gram-negative bacteria is clearly shown by the inability of LBP knock-out mice to fight intraperitoneal infections[11] as well as the ability of anti-LBP monoclonal antibodies to prevent lethality in the LPS/galactosamine model of endotoxemia.[12] Multiple lines of evidence suggest that the mechanisms by which LPS activates Kupffer cells may differ from those found in blood monocytes. Some authors have suggested that LPS activation in Kupffer cells is not mediated via the LBP/CD14 pathway hypothesized for blood monocytes.[13,14] Support for this idea stems from reports showing relatively low levels of CD14 expression in resting Kupffer cells compared with RAW 264.7 cells (murine macrophage cell line) and peritoneal macrophages.[15] Furthermore, in some studies, LPS activation of Kupffer cells, unlike that in peripheral blood monocytes, is not augmented by the addition of serum, suggesting that the LBP found in serum may act differently in Kupffer cells.[13,14] Because serum contains multiple factors that may alter LPS activation, we sought to focus on LBP's role in Kupffer cell activation by performing experiments using recombinant rat LBP. In addition, because Kupffer cells express relatively low levels of CD14 in the resting state, we sought to examine the role of another candidate LPS receptor, the Toll-like receptor 4 (Tlr 4), in mediating the effects of LBP on LPS activation of Kupffer cells.

MATERIALS AND METHODS

Reagents. LPS from *Escherichia coli* (055:B5) was purchased from Sigma (St Louis, MO), and pronase was obtained from Boehringer Mannheim (Indianapolis, IN).

Recombinant Rat LBP. Recombinant rat LBP was produced using the baculovirus expression system. Briefly, the full-length rat LBP complementary DNA (cDNA)[16] was cloned in frame into pBluebacHis2c (Invitrogen, Carlsbad, CA) and used in conjunction with the linearized defective baculovirus DNA, Bac-N-Blue (Invitrogen, Carlsbad, CA) to cotransfect Sf9 insect cells. Recombinant viral clones were analyzed and selected using polymerase chain reaction (PCR) and plaque assay. Optimization of protein production after viral infection of Sf9 cells was carried out with Western blot analysis

Abbreviations: LPS, lipopolysaccharides; LBP, LPS binding protein; Tlr 4, toll-like receptor 4; cDNA, complementary DNA; HDL, high density lipoprotein; sCD14, soluble CD14; TNF-α, tumor necrosis factor α; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; GBSS, Gey's Balanced Salt Solution; FCS, fetal calf serum; mCD14, membrane CD14.

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of cell pellets and supernatants using the anti-Xpress antibody (Invitrogen, Carlsbad, CA), which recognizes an 8 amino acid epitope in the amino terminus of the recombinant fusion protein. A single 60-kd protein consistent with the predicted size of rat LBP is seen both in the cell lysate and supernatant by Western blot (Fig. 1A). The recombinant fusion protein also has a 6-histidine tag in the amino terminus that allows purification of the protein with metal affinity chromatography. Although a single protein is purified using a Nickel column (Fig. 1B and 1C), the binding of the protein on the column requires denaturing conditions that may affect the activity of the protein. Therefore, for the experiments in this article, the supernatants of baculovirus cultures containing the recombinant LBP are used. As control, an irrelevant protein CAT (chloramphenicol acetyltransferase) cloned in the same vector, pBluebacHis2, was also used to cotransfect Sf9 cells in an identical manner as LBP to produce control viral clones. Identical quantities of supernatants from these clones were used as controls for all experiments. For all the experiments, the amount of LBP and control CAT supernatant was 3% of the total volume in the experimental condition. Previous experiments with isolated Kupffer cells have shown that the peak TNF-α levels in response to LPS (1 ng/mL and 10 ng/mL) were achieved with the addition of 3% to 10% of LBP. High volume production of both recombinant proteins were performed in serum-free conditions by infecting High Five insect cells (Invitrogen, Carlsbad,CA) in Excell 400 media (JRH Biosciences, Lenexa, KS). The intact biological activity of the recombinant LBP was shown by its ability to augment IL-6 production in RAW 264.7 cells in response to LPS (Fig. 2).

Isolation and Culture of Kupffer Cells. Kupffer cells were isolated from male Sprague-Dawley rats (Harlan Sprague-Dawley) weighing 200 to 300 grams using the standard techniques of pronase perfusion[17] followed by differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden).[18] All animals received humane care in compliance with the regulations of the University of Michigan and in accordance with the guidelines set forth by the National Institute of Health Guide for the Care and Use of Laboratory Animals. Briefly, livers were perfused through the portal vein with Gey's Balanced Salt Solution (GBSS, Gibco BRL, Gaithersburg, MD) followed by GBSS with 0.2% pronase E. The liver was then excised and minced before incubation with GBSS/pronase solution with continuous stirring at 37°C for 60 minutes. DNase (0.8 μg/mL) was added to prevent cell clumping. The liver slurry was filtered through gauze mesh and washed with culture media and centrifuged at 600*g* for 5 minutes 2 times. Cells were resuspended in PBS with DNase (0.8 μg/mL). Cells were further purified using a discontinuous Percoll gradient of 25% and 50% Percoll as described in detail by Pertoft and Smedsrod.[19] Purified nonparenchymal cells were washed and cultured in media containing Williams E medium supplemented with 100,000 U/L penicillin, 100 mg/L streptomycin, 15 mmol/L HEPES, and 10^{-6} mol/L insulin. Kupffer cells were enriched by differential adherence to tissue culture plates. Cells (either 1.6×10^6 cells/well in a 24-well plate or 4.0×10^5 cells/well in a 96-well plate) were plated in tissue culture plates at 37°C for 1/2 hour before washing and incubating in tissue culture media containing 5% FCS overnight. These cells were approximately 80% pure for Kupffer cells as estimated by their ability to ingest latex beads. Cell viability was always greater than 90% as assessed by trypan blue. All experiments were subsequently performed after washing the cells 3 times with serum free media.

All the experiments presented in this article were performed using Kupffer cells isolated by the method described above with the exception of the ones isolated with counterflow elutriation. For these cells, portal vein perfusion with pronase and digestion were carried out as described earlier. However, after the liver slurry was filtered through gauze and centrifuged, cells were resuspended in GBSS/0.8 μg/mL DNAse and elutriated before plating on tissue culture plates. A Beckman J2/21ME centrifugal elutriator (Beckman Instruments, Palo Alto, CA) with a JE-6B rotor at 4°C was used. The flow rate was calibrated at the start of each experiment. Kupffer cell viability was assessed with trypan blue and for purity by their ability to ingest latex beads. Kupffer cells were plated overnight at a concentration of 5×10^5 cells/well in a 24-well plate. Cells were washed 3 times with serum free media before all experiments.

Identical procedures were used to isolate mice Kupffer cells from 6- to 8-week-old C3H/OuJ and C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME) with the exception that the inferior vena cava was cannulated and retrograde perfusion was performed.

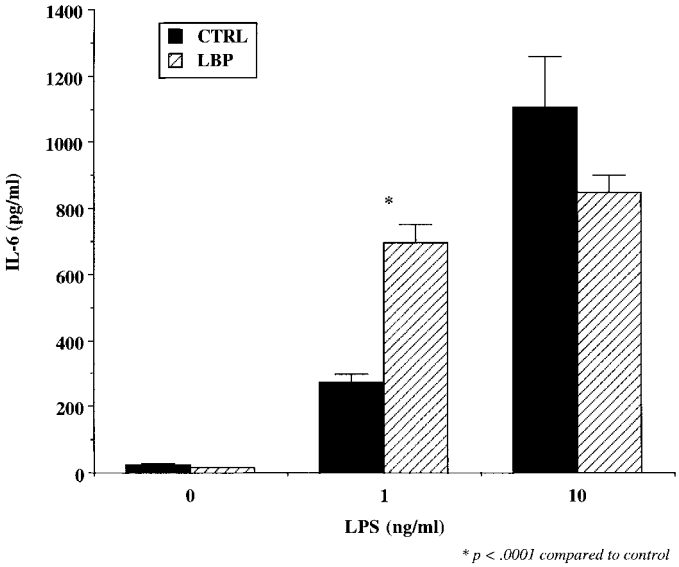

FIG. 2. RAW 267.4 cells were plated in a 24-well plate at a concentration of 5×10^5 cells/mL. Cells were incubated with increasing concentrations of LPS (0, 1, 10 ng/mL) in the presence of either the irrelevant control protein (CTRL) or recombinant LBP (LBP) for 6 hours. Supernatants were assayed for mouse IL-6 by ELISA. All the experimental conditions were performed in triplicate. This is a representative graph from the 3 experiments performed.

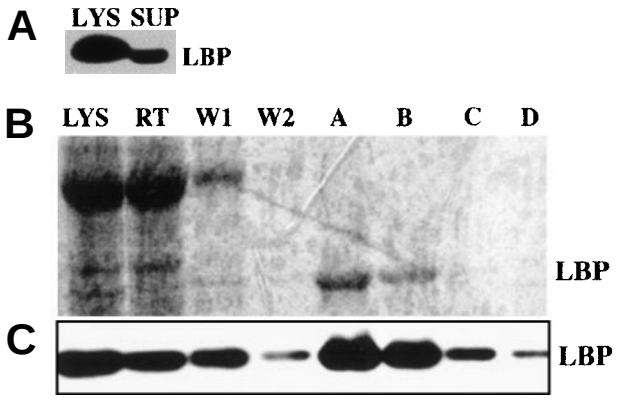

FIG. 1. (A) Western blot analysis of lysates (LYS) of cell pellets and supernatant (SUP) from Sf9 insect cells infected with recombinant LBP baculovirus at a Multiplicity of Infection (MOI) of 14 shows a 60-kd protein in both the cell pellet and supernatant. (B and C) Coomassie stain of SDS PAGE and Western blot showing the purification of recombinant LBP over a Nickel Column. All the fractions, lysate (LYS), run through (RT), washes 1 and 2 (W1 and W2), and elutions (A,B,C,D) were collected and run simultaneously on 2 gels. One gel was stained with Coomassie stain and the other immunoblotted with the Anti-Xpress antibody (Invitrogen, Carlsbad, CA). Cell pellets were lysed using a freeze-thaw lysis in the presence of protease inhibitors and bound to a Nickel Column (Qiagen,Valencia, CA) under denaturing conditions containing 8 mol/L Urea and 5 mmol/L imidazole, pH 8.0. The column was washed with 10 mmol/L imidazole and eluted with 250 mmol/L imidazole (Sigma, St. Louis, MO).

ELISA. Mouse IL-6 and TNF-α were measured by ELISA using the following antibodies: antimouse IL-6 antibody, MP5-20F3, biotinylated antimouse IL-6, MP5-32C11, antimouse TNF-α antibody, G281-2626, and biotinylated antimouse TNF-α, MP6-XT3 from Pharmingen (San Diego, CA) per the manufacturer's instructions. Recombinant mouse IL-6 and TNF-α from Pharmingen (San Diego, CA) were used for the standard. Rat TNF-α levels were measured with a rat TNF ELISA kit (Biosource, Camarillo, CA) per manufacturer's instructions.

Western Blots. Total protein was measured with the BCA protein assay method (Pierce Chemical, Rockford, IL). Cell extracts and supernatants were separated by SDS-PAGE using a 10% to 12.5% gel under reducing condition using the methods of Laemmli.[20] Transfer was carried out electrophoretically by the methods of Towbin et al.,[21] to nitrocellulose (Schleicher and Schuell). The membrane was probed with a primary antibody followed by a horseradish peroxidase-linked secondary antibody. Detection was carried out with the ECL western blotting kit (Amersham, UK).

Polymerase Chain Reaction. Total RNA was isolated by standard methods with Trizol (Gibco BRL, Rockville, MD).[22] Reverse transcription PCR was carried out as previously described.[16] The conditions for PCR amplification were as follows: 94°C for 1 minute, 57°C for 2 minutes, and 72°C for 3 minutes per cycle for 30 cycles. For each reaction, 500 ng of total RNA was used for reverse transcription and used as the template. The primers for mouse Tlr 4 were designed from the reported sequence (GenBank Accession # AF092076) and are as follows:

5′TCTGCCTTCACTACAGAGACT

3′AGTCTTCTCCAGAAGATGTGC

The primer location span from 2,315 base pairs (bp) to 2,633 bp to create a PCR product of 318 base pairs.

Statistical Analysis. Data were analyzed using analysis of variance and 2-tail Student's *t* test when the data had a normal distribution (Statview program, Abacus Concepts/SAS Institute, Cary, NC). Statistical significance was assigned at a *P* value of $< .05$. All the figures are graphed with the mean ± standard error.

RESULTS

LBP Augments Kupffer Cell Interactions with LPS. Kupffer cells were isolated by differential adherence and incubated with varying doses of LPS (0, 1, 10 ng/mL) in the presence of either recombinant LBP or irrelevant control protein (CTRL) for 6 hours in serum-free media. Supernatants were then harvested and assayed for TNF-α by ELISA. Kupffer cells responded to increasing concentrations of LPS in the absence of LBP but the addition of LBP resulted in significantly more total TNF-α production ($P < .01$) in response to LPS at both 1 ng/mL and 10 ng/mL (Fig. 3). Under the identical conditions, normal rat serum (3%) and media alone with no additives were also used (data not shown). Similar levels of TNF-α were produced with either media alone or the irrelevant control protein. Normal rat serum had a variable effect on TNF-α production by Kupffer cells in response to LPS with some experiments showing more TNF-α production than media alone and some showing less TNF-α production than media alone at the LPS doses of 1 ng/mL and 10 ng/mL. In some of the experiments, 100 ng/mL of LPS was also tested, and even at these higher concentrations of LPS, there was a significant increase in TNF-α production with the addition of LBP.

To assess whether an alternate method of Kupffer cell enrichment would alter the effect of LBP on LPS activation of Kupffer cells, Kupffer cells were isolated and enriched using counterflow elutriation. Similar results were found on the

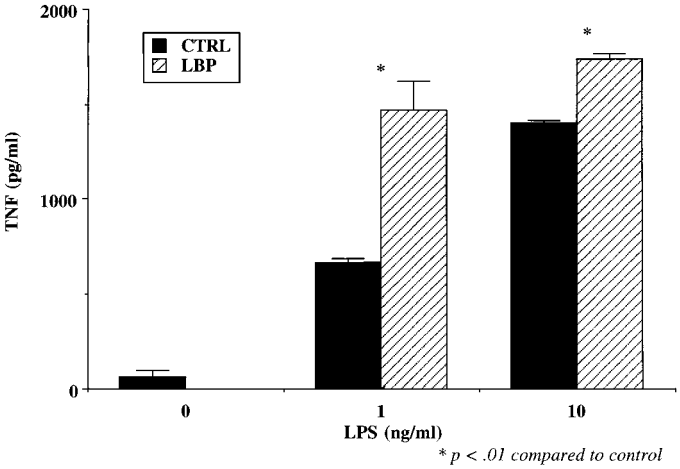

FIG. 3. TNF-α production by Kupffer cells exposed to LPS in the presence of either an irrelevant control protein (CTRL) or recombinant LBP (LBP). All experiments were carried out in triplicate and repeated 3 times. This is a representative graph of the experimental results.

effect of LBP on Kupffer cell response to LPS in Kupffer cells isolated by counterflow elutriation (Fig. 4). The relative increase in TNF-α production at LPS 1 ng/mL and 10 ng/mL seems greater, suggesting that there are less LBP-independent pathways of LPS activation in the elutriated Kupffer cells. This may be a result of less contamination in the elutriated Kupffer cells by other types of nonparenchymal cells.

LBP Decreases the Amount of Time Required for Kupffer Cells to Interact With LPS. One potential mechanism by which LBP can augment Kupffer cell responses to LPS may be via its ability to accelerate the interaction of LPS with its receptor. To examine this possibility, we repeated the experiments but limited the exposure time of LPS with Kupffer cells. Isolated Kupffer cells were exposed to 10 ng/mL of LPS in the presence and absence of LBP for limited periods of time (5, 10, or 60 minutes). After the exposure time to LPS, the supernatant was replaced with media alone containing polymixin (50 U/mL) to block the actions of any remaining LPS. The cells remained in culture for a total of 6 hours from the time LPS was added. Supernatants were then collected for TNF-α quantitation. As noted in Fig. 5, near maximal TNF-α

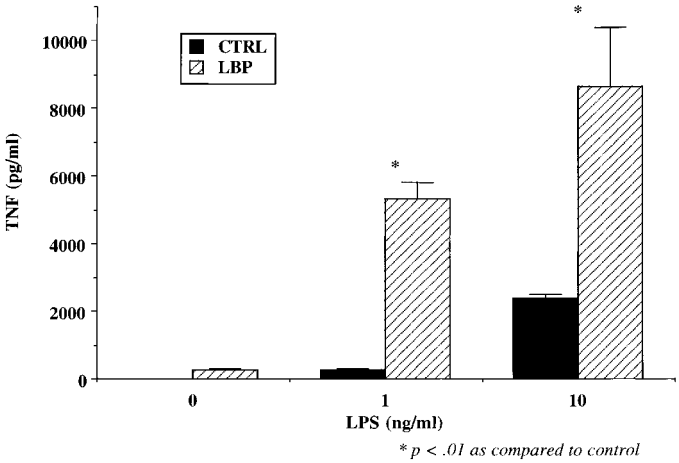

FIG. 4. TNF-α production by Kupffer cells isolated by counterflow elutriation.

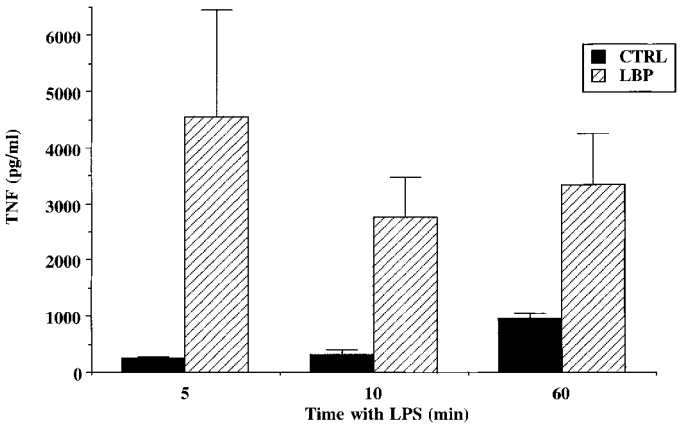

FIG. 5. TNF-α production by Kupffer cells with limited exposure time to LPS in the presence of either the irrelevant control protein (CTRL) or recombinant LBP (LBP). Each condition was performed in triplicate, and the experiment was repeated twice.

production was noted after 5 minutes of exposure in the presence of LBP, which was not achieved in the absence of LBP.

The Effects of LBP on Kupffer Cell Activation by LPS Require a Functional Toll-like Receptor 4. To examine the role of Tlr 4 in mediating the effects of LBP on Kupffer cell activation by LPS, we carried out experiments using Kupffer cells isolated from either C3H/HeOuJ mice, which carry the wildtype Tlr 4 gene, or C3H/HeJ mice, which carry a mutant Tlr 4 gene. Isolated Kupffer cells from C3H/HeOuJ and C3H/HeJ mice have both CD14 and Tlr 4 (Fig. 6A and 6B) as shown by Western blot and PCR, respectively. However, as shown by sequence analysis (Fig. 6C) of the Tlr 4 PCR product, C3H/HeJ mice have a mutation in the Tlr 4 message that leads to a dominant negative receptor product.

Isolated Kupffer cells from C3H/HeOuJ mice respond to LPS in a dose-dependent manner similar to that seen with Kupffer cells isolated from normal rat livers (Fig. 7). Furthermore, the addition of LBP augments this response as was seen with normal rat Kupffer cells. In contrast, Kupffer cells from C3H/HeJ mice did not produce any detectable levels of TNF-α in response to the same doses of LPS, and this lack of activation is not altered by the addition of LBP.

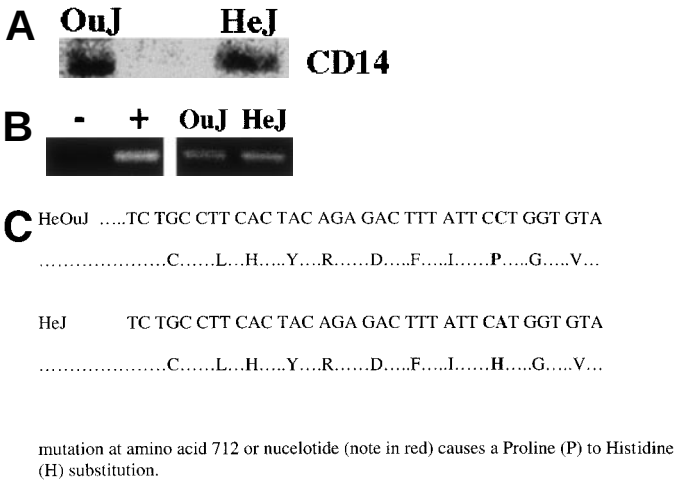

FIG. 6. (A) CD14 expression in isolated Kupffer cells before experimentation in C3H/HeOuJ and C3H/HeJ mice. Western blots performed using monoclonal rat antimouse CD14 antibody, rmC5-3 (Pharmingen, San Diego, CA), shows a 55-kd protein band. (B) Tlr 4 PCR of negative (−, no template) and positive (+, RAW 264.7 cell cDNA) controls, and cDNA from livers of C3H/HeOuJ and C3H/HeJ mice show the 318-bp band. (C) Sequence analysis of the PCR band after cloning into a TA vector (Invitrogen, Carlsbad, CA) shows the mutation at amino acid 712 in the C3H/H3J cDNA.

DISCUSSION

We have shown in the current studies that, although Kupffer cells can be activated by low concentrations of LPS in the absence of LBP, the presence of LBP greatly augments this response. Given identical cell numbers and a set period of time, more TNF-α is produced in response to LPS if LBP is present. This effect is particularly important at low concentrations of LPS such as 1 ng/mL where a greater than two-fold increase in the total amount of TNF-α is measured over 6 hours of incubation. Interestingly, unlike RAW 264.7 cells, a persistent effect of LBP is noted at even higher concentrations of LPS (>1 ng/mL). The explanation for this difference between the 2 cell types is not clear. The effect of LBP on Kupffer cell activation, however, appears to be in part explained by the fact that LBP's presence decreases the amount of time needed for LPS to interact with Kupffer cells. After 5 minutes of exposure to LPS, Kupffer cells produce significantly more TNF-α in the presence of LBP than in its absence. This property of LBP is consistent with previous reports describing LBP as a lipid-transferring protein greatly accelerating the transfer of monomeric LPS to mCD14 and sCD14 as well as high density lipoprotein.[23-25]

The LPS receptor on Kupffer cells that mediates the effects of LPS and LBP remains uncertain. Although membrane CD14 is important in mediating the effects of LPS/LBP complexes on peripheral blood monocytes, it is unlikely to be the receptor that can transmit LPS-induced signals across a cell membrane. Because mCD14 is anchored to the cell surface only by a glycosylphosphatidylinositol linkage, it lacks a transmembrane component and thus the ability to transduce cytoplasmic signals.[26,27] It has long been postulated that mCD14 might act to shuttle LPS/LBP to yet another

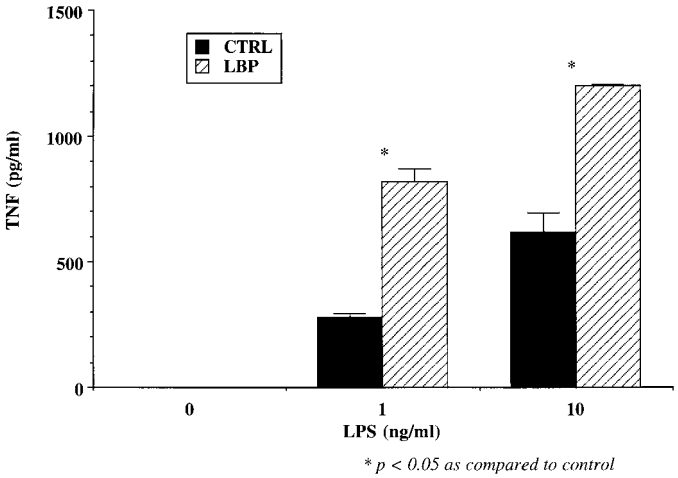

FIG. 7. TNF-α production by isolated Kupffer cells from C3H/HeOuJ mice that were exposed to increasing concentrations of LPS in the presence of either the control protein (CTRL) or recombinant LBP (LBP). All conditions were carried out in triplicate, and the entire experiment was repeated 3 times. Identical experiments carried out in C3H/HeJ Kupffer cells did not yield any detectable TNF-α (not pictured).

transmembrane receptor. Recently, it has been proposed that this candidate LPS receptor may be of the Toll-like receptor family, specifically Tlr 4.[27-29] Toll is a transmembrane protein found in *Drosophila,* which is important in the host defense against fungal infections. Because signaling through the *Drosophila* Toll was found to be via the NF-κβ–like pathway, it was suspected that this receptor system may be important in human innate immunity.[30] Subsequently, a number of human homologues of Toll were identified and named Toll-like receptors.[31,32] Evidence supporting Tlr 4's role as a LPS receptor stems from *in vitro* cell transfection experiments utilizing human Tlr 4 constructs.[27] In these experiments, transfection of Tlr 4 confers LPS responsiveness in HEK 293 cells, which is augmented by the addition of sCD14. Furthermore, in mice, Tlr 4 knockout mice and naturally occurring Tlr 4 mutants (C3H/HeJ and C57BL/10SScCr mice) are not responsive to LPS *in vivo*. The C3H/HeJ mice have a missense mutation in the third exon of the Tlr 4 gene that replaces a proline with histidine at position 712 of the polypeptide chain. This change results in a dominant negative mutant receptor.[28]

Our results suggest that Kupffer cells from C3H/OuJ mice have Tlr 4 mRNA expression and respond normally to LPS. LBP augments the response to LPS in a manner similar to that found in normal rats. Kupffer cells from C3H/HeJ mice, in contrast, express Tlr 4 mRNA that have the missense mutation and do not respond to LPS; this effect cannot be corrected or altered by presence of LBP, suggesting that an intact Tlr 4 receptor is necessary for the activities of LBP on LPS activation. The presence of CD14 on Kupffer cells from both strains of mice does not alter the need for an intact Tlr 4 receptor, supporting the hypothesis that Tlr 4 signaling occurs downstream of LBP and CD14.

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