Activation of human and mouse Kupffer cells by lipopolysaccharide is mediated by CD14

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KUPFFER CELLS, THE RESIDENT macrophages of the liver, represent the largest population of fixed macrophages within the body. Located in the sinusoidal space, Kupffer cells are in contact with portal blood draining the intestinal tract and are thus exposed to bacteria and bacterial products, such as lipopolysaccharide (LPS), which traverse the intestinal barrier. Kupffer cells play a key role in clearing LPS, as demonstrated by experiments that show that the majority of injected 125I-labeled LPS can be localized to the Kupffer cells within 30 min of injection (18). Kupffer cell interactions with LPS, however, are not limited to clearance. Kupffer cells, when exposed to LPS, are activated and can produce a spectrum of cytokines and reactive oxygen intermediates, including the proinflammatory cytokine tumor necrosis factor-α (TNF-α) (2–4). LPS is a potent stimulator of Kupffer cell TNF-α production, and this pathway has been implicated in the pathogenesis of many types of liver injury, including alcohol-induced liver injury (32). In human alcoholic hepatitis, elevations of TNF-α are associated with a worse prognosis (7). Treatment of severe alcoholic hepatitis with corticosteroids has been shown to be beneficial, suggesting that immune over-activation may be contributing to the pathogenesis of this disease (5, 20). In rodent models of alcoholic hepatitis, the pathogenesis of immune activation has been well studied. Experimental evidence supporting an important role for TNF-α in mediating liver injury has been provided by in vivo studies using anti-TNF-α antibodies and TNF receptor 1 knockout mice (13, 35). A role for Kupffer cell activation by endogenous LPS is further supported by studies showing significantly less liver injury in the presence of antibiotics, lactobacillus, or gadolinium chloride (1, 2, 22).

Despite its importance, the mechanism by which LPS activates Kupffer cells is not clearly understood. In peripheral blood monocytes, LPS activation is mediated through LPS binding protein (LBP) and CD14. In serum, LPS binds to LBP, a 60-kDa glycoprotein produced predominantly by the liver and secreted in serum (25, 33). LBP catalyzes the transfer of LPS to cell surface receptors such as membrane CD14 (9). In the presence of LBP, markedly less LPS is needed to activate peripheral blood monocytes. The LBP and CD14 pathway is critical for interactions at the low concentrations of LPS found under physiological con-
ditions (30). Multiple lines of evidence suggest that Kupffer cells differ significantly from peripheral blood monocytes in their interactions with LPS (15). Unlike peripheral blood monocytes, Kupffer cells have relatively low baseline expression of CD14 (3, 29, 36). Furthermore, Kupffer cells and other macrophages can interact with LPS in a serum-independent fashion (4, 16). This has led many other studies (4, 16) to suggest that Kupffer cell activation by LPS is mediated not through the LBP/CD14 pathway but through some other less-characterized pathway. To directly address this question, we studied the LPS response of human Kupffer cells to LPS in the presence of neutralizing monoclonal anti-CD14 antibodies. In addition, we compared the response of murine Kupffer cells isolated from mice that lack CD14 to the response of their wild-type controls that possess an intact CD14 receptor.

MATERIALS AND METHODS

Reagents. LPS from Escherichia coli (055:B5) and phosphatidylinositol-specific phospholipase C (PIPLC) were purchased from Sigma (St. Louis, MO), and pronase was obtained from Boehringer-Mannheim (Indianapolis, IN). Anti-human CD14 antibody M4 was obtained from Coulter Immunology (Hialeah, FL), and LeuM3 was obtained from Becton, Dickinson Immunocytometry Systems (San Jose, CA).

Animals. Mice studied included 6- to 12-wk-old female CD14 knockout, back-crossed 10 times onto the BALB/c background (10, 11) and age- and/or sex-matched control BALB/c mice (Jackson Laboratory, Bar Harbor, ME). All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

Recombinant LBP. Recombinant human LBP was obtained from LBP-transfected Chinese hamster ovary (CHO) cells as previously described (21). Recombinant rat LBP was produced using a baculovirus expression system as previously described (27). Bioactivity of the rat recombinant LBP in mouse cells was demonstrated using RAW 267.4 cells (27). Because of difficulty in maintaining the bioactivity of the purified protein due to rapid degradation, we used in these experiments supernatants (at a concentration of 3% of total volume) from either human LBP-transfected CHO cells or recombinant baculovirus-infected SF9 cells. The concentration of the human LBP in 3% total volume was ~3 μg/ml as measured by an LBP ELISA previously described by Myc et al. (21).

Isolation and culture of Kupffer cells. Human Kupffer cells were isolated from normal liver tissue obtained from fresh surgical hepatectomy specimens with the assistance of the University of Michigan Tissue Procurement Core. We used the standard technique of pronase digestion previously described for human Kupffer cells (12) followed by differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden) (26). This research protocol was reviewed and approved by the University of Michigan Medical Institutional Review Board. Briefly, the liver was excised and minced before incubation with Gey's balanced salt solution (GBSS)-pronase solution with continuous stirring at 37°C for 60 min. DNase (0.8 μg/ml) was added to prevent cell clumping. The liver slurry was filtered through gauze mesh, washed with culture media, and centrifuged two times at 600 g for 5 min. 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 Pertoo and Smedsrod (23). Purified nonparenchymal cells were washed and cultured in media containing Williams E medium supplemented with 100 000 U/ml penicillin, 100 mg/l streptomycin, 15 mM HEPES, and 10^-6 M insulin. Kupffer cells were enriched by differential adherence to tissue culture plates. Cells (4.0 × 10^6 cells/well in a 96-well plate) were plated in tissue culture plates at 37°C for one-half hour before washing and incubating in tissue culture media containing 5% FCS overnight. These cells were ~80% pure for Kupffer cells as estimated by their ability to ingest latex beads. The remaining cells are mainly endothelial and stellate cells. Cell viability was always >90% as assessed by trypan blue. All experiments were subsequently performed after washing the cells three times with serum-free media. For each experiment, Kupffer cells were isolated from one individual liver. The amount of liver used was variable and dependent on the amount of excess liver tissue available from each surgical operation.

Identical procedures were used to isolate mouse Kupffer cells with the exception that the livers were perfused retrograde through the inferior vena cava with GBSS (GIBCO, BRL, Gaithersburg, MD) followed by GBSS with 0.2% pronase E before excision and mincing. For each isolation, four mouse livers were used and the Kupffer cells were pooled.

Inner salt assay. Cell viability was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) per the manufacturer's instructions. This assay utilized the novel tetrazolium compound 3(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) and the electron coupling reagent phenazine ethosulfate.

Western blots. Kupffer cells were lysed with a lysis buffer containing 1% IGEPAL, 5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Total protein was measured with the BCA protein assay method (Pierce, Rockford, IL). Total protein (12 μg) was loaded into each lane. Cell extracts were separated by SDS-PAGE using a 10–12.5% gel under denaturing conditions using the methods of Laemmli (14). Transfer was carried out electrophoretically with the methods of Towbin et al. (31) to nitrocellulose (Schleicher & Schuell, Keene, NH). The membrane was probed with a primary antibody followed by a horseradish peroxidase-linked secondary antibody. Detection was carried out with the enhanced chemiluminescence Western blotting kit (Amersham, Little Chalfont, UK).

ELISA. Human TNF-α was measured by sandwich ELISA using antibodies and standards obtained from Pharmingen (San Diego, CA). Mouse TNF-α levels were measured with a rat TNF ELISA kit (Biosource, Camarillo, CA) per manufacturer's instructions.

Statistical analysis. Data were analyzed using ANOVA and two-tailed Student's t-test when the data had a normal distribution (StatView: Abacus Concepts/SAS Institute, Cary, NC). Statistical significance was assigned at a P value of <0.05. All the figures are graphed with the means ± SE.

RESULTS

LBP augment human Kupffer cell TNF-α production in response to LPS. Isolated Kupffer cells were incubated with varying concentrations of LPS (0, 1, or 10 ng/ml) in the presence of either serum-free media or serum-free media with recombinant human LBP. After 6 h at 37°C, the supernatant was collected and human TNF-α levels were measured using ELISA. Without LPS, isolated Kupffer cells produce little detectable

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TNF-α. After incubation with LPS, Kupffer cells produced increasing concentrations of TNF-α in response to increasing concentration of LPS in the presence and absence of LBP. However, at low concentrations of LPS (1 ng/ml), the presence of LBP significantly augmented the production of TNF-α (Fig. 1) \( (P < 0.001 \) for control vs. LBP).

**Inhibition of human Kupffer cell response to LPS by PIPLC.** To indirectly assess the role of CD14 in mediating LPS activation of human Kupffer cells, we utilized the enzyme PIPLC. PIPLC cleaves glycosyl phosphatidyl inositol (GPI)-anchored proteins such as CD14, releasing them from the cell membrane. We analyzed Kupffer cell responses to LPS with and without enzyme treatment. Isolated human Kupffer cells were preincubated with PIPLC (500 mU/ml) or serum-free media for 1 h at 37°C before stimulation with LPS. Subsequently, the Kupffer cells were washed and incubated with increasing concentrations of LPS (0, 1, 10 ng/ml) in serum-free media for an additional 6 h. Supernatants were assayed for TNF-α. Minimal TNF-α was detected in isolated Kupffer cells without LPS in both the PIPLC and control groups. However, preincubation of Kupffer cells with PIPLC significantly decreased the amount of TNF-α produced in response to 1 or 10 ng/ml of LPS \( (P < 0.001; \) Fig. 2), suggesting the importance of CD14 in mediating Kupffer cell response to LPS.

**Anti-CD14 antibodies inhibit LPS-mediated TNF-α production in Kupffer cells.** The effect of the monoclonal anti-human CD14 antibodies (6, 36) MY4 (previously shown to block CD14-mediated function) and LeuM3 (a CD14-specific nonblocking isotype control monoclonal antibody) on Kupffer cell production of TNF-α in response to LPS was analyzed. Kupffer cells were incubated with either MY4 (10 μg/ml), LeuM3 (10 μg/ml), or serum-free media for 1 h at 37°C before wash with serum-free media. Cells were then incubated with increasing concentrations of LPS (0, 1, and 10 ng/ml) in the presence of the same antibodies or serum-free media for 6 h before assay of the supernatant for TNF-α. As presented in Fig. 3, increasing concentrations of TNF-α were noted in the supernatant of cells treated with increasing concentrations of LPS. This increase in TNF-α was blocked by the addition of MY4 but not by the addition of LeuM3. Decrease in TNF-α production in the MY4 groups was due to loss of cell viability; MTS assay of the cells after incubation with MY4, LeuM3, or media showed equal viability in all groups (data not shown). Furthermore, the effect of MY4 is similar in the presence or absence of recombinant human LBP (Fig. 4).

**Kupffer cells isolated from CD14 knockout mice respond to LPS in a LBP/CD14-dependent manner.** Kupffer cells were isolated from CD14 knockout mice or the control mice (BALB/c). As expected, CD14 expression was not found on isolated Kupffer cells in CD14 knockout mice in contrast to control BALB/c mice (Fig. 5). We then examined the response of Kupffer cells to LPS in vitro. Isolated Kupffer cells

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**Fig. 1.** Tumor necrosis factor-α (TNF-α) production by isolated human Kupffer cells in response to lipopolysaccharide (LPS) in the presence and absence of recombinant human LPS binding protein (LBP, 3 μg/ml). All experimental conditions were performed in triplicate. The figure is representative of the results from each of the three experiments. \( *P < 0.001, \) control vs. LBP.

**Fig. 2.** Human Kupffer cells were preincubated with either serum-free media or phosphatidylinositol-specific phospholipase C (PIPLC) before exposure to LPS. Each experimental condition was performed in quadruplicate. \( *P < 0.001, \) control vs. PIPLC.

**Fig. 3.** Human Kupffer cells were stimulated with LPS in the presence of serum-free media (control) and either a neutralizing anti-CD14 antibody (MY4) or a noninhibitory anti-CD14 antibody (LeuM3). Experiments were performed in triplicate and repeated on two separate isolations. \( *P < 0.05, \) control vs. MY4.
from both control and CD14 knockout mice produced no detectable TNF-α in the absence of LPS. Kupffer cells from control mice produced increasing amounts of TNF-α in the presence of increasing LPS (1 and 10 ng/ml) (Fig. 6). CD14 knockout mice produced little or no TNF-α at either LPS concentration (Fig. 6). In the presence of LBP, Kupffer cells from control mice produced significantly more TNF-α in response to LPS (1 ng/ml) than in the absence of LBP (Fig. 7). Under identical conditions, Kupffer cells from the CD14-deficient mice produced little or no TNF-α compared with wild-type animals; however, significantly more TNF-α was produced in the presence of LBP compared with its absence. (Fig. 7).

DISCUSSION

In this article, we demonstrated that the secretion of TNF-α by human Kupffer cells in response to low concentrations of LPS is mediated via membrane CD14. In the presence of neutralizing antibody to human CD14 or after pretreatment of cells with an enzyme (PIPLC) that removes CD14 from the surface by cleavage of its GPI anchor, LPS activation is significantly inhibited. Consistent with these results, Kupffer cells from CD14 knockout mice also show little or no sensitivity to LPS. Previous studies (4, 16) casting doubt on the role of both LBP and CD14 in Kupffer cell activation in rats and mice have been inferred from results showing that serum was not absolutely necessary for activation by LPS compared with elicited peritoneal macrophages or the mouse macrophage cell line RAW 264.7. Because serum contains many LPS binding factors in addition to LBP (such as soluble CD14 and high-density lipoprotein), which could affect cellular responses to LPS, we chose to perform all of our experiments in the presence or absence of recombinant LBP. We have shown that, although Kupffer cells can interact with LPS at concentrations of 10 ng/ml in the

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**Fig. 4.** Human Kupffer cells were incubated with increasing concentrations of LPS in the presence of human LBP (media) with the addition of either CD14 antibody (MY4 or LeuM3). Experiments were performed in triplicate and repeated with two separate isolations. (*P < 0.001, control vs. MY4)

**Fig. 5.** CD14 expression in isolated Kupffer cells before experimentation in CD14 knockout (KO) and BALB/c (wild-type) mice. Western blots performed using monoclonal rat anti-mouse CD14 antibody, rmC5–3 (Pharmingen, San Diego, CA) demonstrates a 55-kDa protein band consistent with mCD14 in Kupffer cells isolated from wild-type mice and not from Kupffer cells isolated from CD14 KO mice.

**Fig. 6.** Kupffer cells were isolated from control BALB/c mice or CD14 KO mice exposed to increasing concentrations of LPS. Experiments were performed in triplicate and repeated three times.

**Fig. 7.** Kupffer cells from BALB/c (control) and CD14 KO mice were exposed to LPS (1 ng/ml) in the presence of either serum-free media or rat recombinant LBP (rLBP). Experiments were performed in triplicate and repeated three times. *P < 0.005.
absence of recombinant LBP at low concentrations of LPS (1 ng/ml, levels found in the serum of septic patients), the presence of recombinant LBP significantly (>10-fold) augmented the response. Therefore, although LBP is not critical for responses to LPS at high concentrations, it is required for the Kupffer cell response at physiologically relevant low concentrations of LPS. This is consistent with the concept that LBP facilitates the transfer of LPS to its receptors. The fact that LBP is not critical for the response at higher LPS concentrations does not preclude the need for membrane CD14. In fact, our results show that neutralizing antibodies to CD14 and cleavage of CD14 receptors block responses to LPS both in the presence and absence of LBP. Our in vitro studies with isolated human and rodent Kupffer cells are consistent with the concept that Kupffer cells represent a major source of TNF-α during endotoxemia. In vivo studies (24) using a rabbit endotoxemia model and neutralizing anti-rabbit CD14 antibodies show diminished TNF-α production in response to LPS. Similarly, in CD14 knockout mice, a lack of TNF-α production is also seen after LPS injection (10).

Despite the relatively low expression of CD14 on Kupffer cells, this receptor pathway appears to be important in mediating LPS responses. Although we have shown that activation of Kupffer cells by LPS utilizes the LBP/CD14 pathway in a manner similar to peripheral blood monocytes, we have not specifically addressed how Kupffer cells differ from peripheral blood monocytes. Multiple studies including our own (19, 28, 29) have shown that Kupffer cells have low baseline levels of CD14 but can be induced to upregulate CD14 expression with different stimuli, including LPS injection and alcohol-induced liver injury. Takai et al. (29) demonstrated 5- to 40-fold increase in mRNA for CD14 in Kupffer cells isolated after in vivo LPS injection and after in vitro exposure to LPS. Consistent with this finding, CD14 expression in vivo as measured with immunohistochemical staining was minimal in normal livers but increased after LPS injection, peaking at 6 h after intraperitoneal LPS injection (20 μg/mouse) (19). In contrast to Kupffer cells, peritoneal macrophages do not show these marked increases in CD14 mRNA. Rather, peritoneal macrophages have a higher baseline level of CD14 mRNA than Kupffer cells (19, 29). Similarly, peripheral blood monocytes have high baseline levels of CD14 expression that is refractory to further increases (3, 36).

In addition to LPS administration, marked increases in CD14 expression on Kupffer cells are seen after chronic intragastric feeding with ethanol as opposed to isocaloric dextrose (28). The physiological significance of these elevations in Kupffer cell CD14 is not clear, but we suspect that such increases sensitize Kupffer cells to LPS. Transgenic mice that overexpress CD14 are exquisitely sensitive to LPS (8). Thus upregulation of Kupffer cell CD14 in multiple disease models, such as common bile duct ligation and alcoholic hepatitis, may cause the observed liver injury by sensitizing Kupffer cells to the effects of endogenous LPS. Consistent with this hypothesis is a recent report showing decreased liver injury in CD14 knockout mice given ethanol compared with the CD14 wild-type animals (34). CD14 wild-type animals had an increase in liver-to-body weight ratio, serum alanine aminotransferase, and increases in steatosis and necrosis histologically when intragastrically fed ethanol compared with dextrose control diets. These changes due to ethanol were blunted in CD14 knockout animals fed an identical diet. An associated increase in TNF-α mRNA levels was seen in the CD14 wild-type animals fed ethanol diets, which was not seen in ethanol-fed CD14-deficient animals. These studies and our own support the speculation that blocking CD14 may have a benefit in diseases characterized by overexpression of Kupffer cell CD14.

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