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*J Immunol* 2011; 186:2592-2601; Prepublished online 7 January 2011;
doi: 10.4049/jimmunol.1002248
http://www.jimmunol.org/content/186/4/2592

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/01/07/jimmunol.1002248.DC1

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The Role of CD14 in Neutrophil Recruitment within the Liver Microcirculation during Endotoxemia

Erin F. McAvoy,*† Braedon McDonald,*† Sean A. Parsons,* Connie H. Wong,* Regine Landmann,† and Paul Kubes*†

During Gram-negative sepsis and endotoxemia, CD14 is essential for the recognition of LPS by the TLR4 complex and subsequent generation of systemic inflammation. However, CD14-independent responses to LPS have been reported in vitro and in vivo in selected tissues including the skin. As the liver is a key target organ for neutrophil sequestration and inflammatory pathology during sepsis and endotoxemia, we investigated the role of CD14 in the recruitment of neutrophils into the liver in a mouse model of endotoxemia. Using dynamic in vivo imaging of the liver, we observed that neutrophil recruitment within the sinusoids and post-sinusoidal venules occurred equivalently between LPS-treated wild-type and CD14-knockout mice. Neutrophil recruitment within the liver was completely independent of CD14 regardless of whether it was expressed on cells of hematopoietic or nonhematopoietic origin or in serum as soluble CD14. Whereas CD14 expression was essential for activation of circulating neutrophils and for the development of LPS-induced systemic inflammation (pulmonary neutrophil sequestration, leukopenia, and increased serum proinflammatory cytokine levels), deficiency of CD14 did not limit the adhesion strength of neutrophils in vitro. Furthermore, wild-type and CD14-knockout mice displayed identical deposition of serum-derived hyaluronan-associated protein within liver sinusoids in response to LPS, indicating that the sinusoid-specific CD44/hyaluronan/serum-derived hyaluronan-associated protein-dependent pathway of neutrophil adhesion is activated independently of CD14. Therefore, the liver microcirculation possesses a unique CD14-independent mechanism of LPS detection and activation of neutrophil recruitment. The Journal of Immunology, 2011, 186: 2592–2601.

E arly recognition of microbial products by the innate immune system is a strict requirement for the production of an effective inflammatory response resulting in leukocyte recruitment to the site of infection and bacterial clearance. Pattern recognition receptors including the TLRs play an essential role in this process. Of the TLRs, TLR4 is the most extensively studied and plays an essential role in innate immune responses to bacterial LPS and the clearance of Gram-negative bacterial infections. However, during disease states such as Gram-negative sepsis, systemic activation of the innate immune system via TLR4 on multiple cell types is known to mediate the development of inflammatory pathology and mortality (1, 2).

Sepsis syndrome results from pathological systemic inflammation in response to infection, culminating in immune-mediated damage and dysfunction of multiple organs (3). Key to the immunopathogenesis of severe sepsis is the activation and recruitment of innate immune effector cells, namely neutrophils, within the microvasculatures of vital organs such as the lungs and liver (3). During Gram-negative sepsis and simplified models such as endotoxemia, recruitment of neutrophils into the liver results in vascular dysfunction, hepatocellular injury, and organ failure (4). Therefore, a further understanding of the molecular mechanisms of LPS recognition and TLR4-dependent signaling may yield new insight into the pathogenesis of sepsis-induced liver inflammation and damage and may also reveal new therapeutic avenues to reduce hepatic inflammation.

Our current understanding of the early events in LPS recognition by the innate immune system involves LPS binding to LPS binding protein and subsequent transfer to CD14, which exists in both membrane bound and soluble forms (5, 6). CD14 is a 55-kDa serum/cell-surface glycoprotein that acts as a pattern recognition receptor. It is expressed on the surface of most cells of hematopoietic origin as well as endothelial cells (7). Because CD14 lacks both transmembrane and cytoplasmic domains, it has no intrinsic signaling capabilities. CD14 is thought to play a crucial role in the detection of LPS by binding LPS and transferring it to the signaling complex containing both TLR4 and the accessory protein myeloid differentiation protein-2 (8). Activation of TLR4 leads to the induction of signaling cascades that result in cellular activation, production of inflammatory molecules, and leukocyte recruitment. The importance of CD14 in LPS-induced inflammation has been documented extensively, best exemplified by the demonstration that CD14-deficient (CD14−/−) mice are resistant to LPS-induced lethality (9). However, recent studies have suggested that CD14-independent responses to LPS can occur in the presence of extremely high doses of LPS or in a tissue-specific manner (9, 10). A systematic assessment of the role of CD14 in the liver during endotoxemia has not been performed.
In the current study, we examined the role of CD14 in leukocyte recruitment within the liver using a mouse model of endotoxia. Because soluble CD14 (sCD14) is an acute-phase protein released by the liver, we sought to further understand the role of CD14 in LPS-induced leukocyte recruitment within the hepatic microvasculature. Surprisingly, our data suggest that despite the central role of the liver in the biosynthesis of CD14, this molecule is dispensable for the generation of local hepatic innate immune responses during endotoxia.

Materials and Methods

Animals

CD14−/− (The Jackson Laboratory, Bar Harbor, ME) and Cx3cr1gfp/+ (a gift from D.R. Littman) mouse colonies were maintained in a pathogen-free facility at the University of Calgary. For wild-type (WT) controls, C57BL/6 mice were purchased from The Jackson Laboratory. At the time of use, all animals were 6–10 wk of age weighing between 20 and 35 g. All experimental animal protocols carried out in this study were approved by the University of Calgary Animal Care Committee and conform to the guidelines established by the Canadian Council for Animal Care.

Reagents

Highly purified LPS (Escherichia coli O111:B4) was purchased from Calbiochem (EMD Sciences, San Diego, CA). This LPS had no effect on leukocyte recruitment in TLR4−/− mice, suggesting no significant contamination by bacterial products other than LPS.

Generation of chimeric mice

Bone marrow (BM) chimeric mice were generated following a standard protocol described in previous studies by our laboratory (11–13). WT C57BL/6 mice and CD14−/− mice were used as recipients and/or donors for sex-mismatched BM transplantation. Male donors were first euthanized by cervical spine dislocation prior to BM isolation. Two doses of 5 Gy (Gammacell 40 Cs gamma-radiation source), with a 3-h interval between doses, were used to irradiate female recipients. Isolated BM cells (8 × 10^6) were injected i.v. via the tail vein into female irradiated recipient mice. BM transplanted mice were kept in clean, pathogen-free microisolator cages for 8 wk to allow for full hematological reconstitution. Preliminary work by researchers in our laboratory confirmed that ∼99% of circulating leukocytes in chimeric mice were derived from donor BM, as determined using Thy-1.1 and Thy-1.2 congenic C57BL/6 mice. C57BL/6 BM was transplanted into C57BL/6 mice and CD14−/− BM was also transplanted into CD14−/− mice as controls to ensure that no untoward effects of the transplant occurred.

Intravital microscopy

Animals were anesthetized by an i.p. injection of a mixture of 10 mg/kg xylazine (Bayer, Toronto, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Bimeda-MTC, Cambridge, Ontario, Canada) prior to surgery. The right jugular vein was cannulated to allow for i.v. administration of Abs and additional anesthetic as required.

Liver preparation

Animals were then prepared for intravital microscopy of the liver as previously reported by our group (14). Single post-sinusoidal venules ranging in size from ∼25–45 μm in diameter and associated sinusoids both above and below the vessel were selected at random for each study. Offline playback analysis was used to quantify leukocyte rolling velocity, the number of rolling cells, as well as the number of adherent cells per 100 μm of post-sinusoidal venule as well as per field of view (FOV) of sinusoids.

Cremaster muscle preparation

The cremaster muscle preparation was performed as previously described by our group (10). Depending on the number of clearly visible vessels, one to five single unbranched vessels of ∼20–35 μm in diameter were selected per cremaster muscle. Each vessel was recorded for ∼2 min for playback analysis at a later time. Leukocyte rolling velocities and the number of rolling, adherent, and emigrated leukocytes were determined during offline playback analysis. Rolling leukocytes were defined as those moving at a velocity less than erythrocytes and demonstrated a clear rolling motion. Leukocyte rolling flux was determined by counting the number of cells that passed through a given point in the vessel per minute. Leukocyte rolling flux fraction was calculated as previously described (15) by dividing leukocyte rolling flux by total leukocyte flux through the venule using the formula: rolling flux fraction = [WBC] × VS × ΔX × 2/π r^2, in which [WBC] is the total leukocyte concentration in blood, VS is the blood flow velocity in the venule, and r is the radius of the venule. Leukocytes were considered adherent if they remained stationary within the vessel for >30 s, and the total adhesion was defined as the number of adherent cells per 100 μm length of venule. Lastly, emigration was quantified by determining the number of extravascular leukocytes per microscopic FOV (×25 objective lens) on either side of the vessel in question.

Wall shear rate (γ) in cremaster venules was calculated as previously described (15) using Poiseille’s Law for Newtonian Fluids: γ = (8 × Vmean × D_venule), in which Vmean is estimated from the midline velocities of RBCs (Vmean = V_RBC/1.6), and D_venule is the diameter of the venule. V_RBC was measured in real time for each venule using an optical Doppler velocimeter (Texas A&M, College Station, TX).

Experimental protocol

Mice (WT and CD14−/−) were treated with an i.p. injection of 0.5 mg/kg (≈12.5 μg/mouse) purified LPS (E. coli O111:B4) dissolved in sterile saline. At 4 h post LPS treatment, mice were anesthetized, and intravital microscopy was performed. The doses of LPS used in this study were sufficiently low that all animals were able to survive anesthesia, and hepatic perfusion was not severely diminished so that leukocyte–endothelial cell interactions could still be observed under flow conditions. In some experiments, mice were treated with mAbs against CD11b (clone M1/70, rat IgG2b; eBioscience, San Diego, CA) 30 min prior to LPS administration. At the end of each experiment, whole blood was harvested via cardiac puncture to determine circulating leukocyte numbers and for serum collection. Liver tissue was harvested for preparation of histological sections.

Spinning disc confocal microscopy

In some experiments, untreated mice or mice treated for 4 h with LPS (0.5 mg/kg i.p.) were injected i.v. with 2 μg PE-labeled anti-CD45 Ab (clone 104, mouse IgG2a; eBioscience) to label all leukocytes. In addition, 2 μg FITC-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b; eBioscience) Ab was added to specifically label neutrophils. To ensure that no nonspecific binding occurred, the appropriate FITC- or PE-labeled isotype control Ab was administered. In other experiments, serum-derived hyaluronan-associated protein (SHAP) was detected using Alexa Fluor 555-labeled Abs against SHAP (anti-inter-ac tid Dickinson Systems, Santa Cruz, CA). Intravital microscopy of the murine liver was then performed as described above and imaged using a spinning disc confocal microscope as previously described (16). Images were acquired with an Olympus BX51 (Olympus, Center Valley, PA) upright microscope using a 10× or a 20× 0.75 numerical aperture objective. The microscope was equipped with a confocal light path (WaveFx, Quorum, Guelph, Ontario, Canada) based on a modified Yokogawa CSU-10 head (Yokogawa Electric Corporation, Tokyo, Japan). The liver and labeled cells were imaged using either a 488 or 561 nm laser excitation (Coalt, Stockholm, Sweden) and visualized with the appropriate long-pass filters (Semrock, Rochester, NY). Typical exposure times ranged from 100–500 ms. A 512 × 512 pixel back-thinned EMCCD camera (C9100-13, Hamamatsu, Bridge-water, NJ) was used for fluorescence detection. The confocal microscope was driven by Volocity Acquisition software (Improvision, Lexington, MA).

Cell labeling for flow cytometric analysis

CD11b expression was measured using flow cytometry to quantify the degree of neutrophil activation in the circulation in response to LPS as previously described (11). Cardiac puncture was used to collect whole blood from either untreated mice or mice treated for 4 h with LPS (0.5 mg/kg, i.p.) using a heparinized 1 ml syringe. One microgram FITC-conjugated mAb against CD11b or nonspecific isotype controls (all purchased from BD Biosciences, Mountain View, CA) were incubated with 100 μl blood for 30 min at room temperature. OptiLyse B (Imm unictech, Marseille, France) was then used to lyse RBCs. The cells were then washed and resuspended in PBS/0.5% BSA/0 M glucose solution and read on a BD FACSscan flow cytometer (BD Biosciences) using CellQuest Pro software (Becton Dickinson Immunocytometry Systems).

ELISA for TNF-α and IL-1β

WT or CD14−/− mice were either left untreated or treated with 0.5 mg/kg LPS for 4 h. Blood was then collected via cardiac puncture, and hep-
arinized plasma was isolated. Plasma was assayed for TNF-α and IL-1β concentrations by a commercial ELISA kit (BD Biosciences) according to instructions provided by the manufacturer.

**ELISA for murine sCD14**

As previously reported (17), 96-well plates (Nunc, Rochester, NY) were coated with 2 μg/ml G5A10 anti-CD14 mAb in 0.1 M bicarbonate buffer (pH 9.3) at 4˚C overnight. Plates were then washed three times, and nonspecific binding was blocked with 0.2 M PBS (pH 7.5) containing 2% BSA and 0.01% Tween. Plasma samples or standard (0–500 ng/ml) murine rsCD14-Fc chimera (R&D Systems) were diluted in blocking buffer and incubated in the sCD14-mAb-coated plates for 2 h at 37˚C. Standard curves produced by rsCD14-Fc chimera were used in this study, and the His-tagged rsCD14 used by Landmann and colleagues (17) were identical (data not shown). No differences in standard curves produced by recombinant standards run in CD14−/− murine plasma versus blocking buffer were observed, suggesting that the presence of other plasma proteins had no effect on the ability of the ELISA to detect sCD14 (data not shown). Typically, samples from CD14−/− and untreated C57BL/6 mice were left undiluted, whereas samples from LPS-treated C57BL/6 mice were diluted 1:5 in blocking buffer. Plates were then washed three times, and 100 μl of 500 ng/ml binnonylated polyclonal rabbit anti-CD14 (1617) was added to each well and incubated for 1 h at 37˚C. Plates were then washed extensively followed by a 1 h incubation at 37˚C with streptavidin-HRP (Zymed Laboratories, South San Francisco, CA; 1:50,000 dilution) and a final washing step. Finally, tetramethylbenzidine chromogen (Sigma-Aldrich, St. Louis, MO) was used for detection. The absorbance at 450 nm in the 96-well plates was then determined using a microplate reader (Molecular Devices, Sunnyvale, CA).

**Murine neutrophil isolation for adhesive strength assay**

The protocol used by our laboratory for isolation of murine BM neutrophils has been previously described (18). Whole blood was collected from anesthetized mice via cardiac puncture using a heparinized syringe. Heparinized plasma was then isolated by centrifugation of whole blood at 3000 rpm for 5 min. Mice were then euthanized via cervical dislocation, and the femurs and tibias were dissected free of adherent tissue. The chamber was then assembled and connected to a syringe pump and mouse serum at room temperature for 3 to 4 h to allow for adhesion. Coverslips were coated with 10% heat-inactivated mouse serum diluted in blocking buffer. Plates were then washed three times, and 1 μl of 500 ng/ml binnonylated polyclonal rabbit anti-CD14 (1617) was added to each well and incubated for 1 h at 37˚C. Plates were then washed extensively followed by a 1 h incubation at 37˚C with streptavidin-HRP (Zymed Laboratories, South San Francisco, CA; 1:50,000 dilution) and a final washing step. Finally, tetramethylbenzidine chromogen (Sigma-Aldrich, St. Louis, MO) was used for detection. The absorbance at 450 nm in the 96-well plates was then determined using a microplate reader (Molecular Devices, Sunnyvale, CA).

**Murine neutrophil isolation for adhesive strength assay**

Neutrophils were either left untreated, treated with 100 ng/ml LPS for 1 h, or treated with 10 μM MMP-2 for 5 min before the start of each experiment to assay adhesive strength of LPS-treated WT and CD14−/− neutrophils in vivo, vascular mimetic microfluidic chambers (a generous gift from Scott Simon, Department of Biomedical Engineering, University of California, Davis, Davis, CA) were used. As previously described (19), the chamber consists of a single channel 600 μm in width and 70 μm in height and uses a network of vacuum channels to reversibly bind to a layer of endothelial cells. Coverslips were coated with 10% heat-inactivated mouse serum at room temperature for 3 to 4 h to allow for adhesion. The chamber was then assembled and connected to a syringe pump and then placed on a DeltaVision microscope (Applied Precision) equipped with a heated stage and imaged using a 10X objective lens. Cells were allowed to flow into the chamber and allowed to adhere to the coverslip for 5 min prior to start of flow. HBSS maintained at 37˚C was then perfused through the chamber at 1 dyne/cm² for 1 min to remove any nonadhering cells. After 1 min, HBSS was perfused at 5 dynes/cm², and the images were recorded for 5 additional min using Velocity Acquisition software (Improvision).

### In vivo assay for bacterial clearance in the liver

Bacterial clearance in the liver of mice was determined as previously described (20). E. coli (Caliper Life Sciences) were grown to mid log phase, then fluorescently labeled with Syto-60 dye (Invitrogen) for 10 min, washed, and resuspended in saline. Four hours after i.p. administration of LPS, mice were prepared for liver intravital microscopy and then received an i.v. infusion of 1 × 10^9 CFU fluorescently labeled E. coli. Bacterial trapping was then quantified within the liver using spinning disk confocal intravital microscopy to determine the number of trapped bacteria per FOV at various time points postinfection.

### Statistical analysis

Data are expressed as the arithmetic mean ± SEM. A Student t test was used in which two groups were compared, and one-way ANOVA with post hoc Bonferroni’s test was used for multiple comparisons. A p value ≤0.05 was considered statistically significant.

### Results

**Systemic LPS administration induces neutrophil infiltration into the liver**

Multichannel fluorescence spinning disk microscopy was used to visualize recruitment kinetics of leukocyte subsets within the liver microvasculature of mice treated with 0.5 mg/kg (≈12.5 μg/ml) LPS from E. coli 4 h prior to imaging. This dose was previously established to induce significant activation of the vascular endothelium and circulating neutrophils in WT animals (10, 11). In addition, preliminary experiments demonstrated that this rather low dose of LPS induced significant recruitment within the hepatic microvasculature without causing mortality or a catastrophic decrease in sinusoidal perfusion. In untreated mice, the addition of low concentrations of FITC-conjugated anti–Gr-1 Ab (selectively labels neutrophils; Supplemental Fig. 1) and PE-conjugated anti-CD45 Ab (labels all leukocytes) demonstrated that very few CD45+Gr-1− leukocytes are found in the liver under baseline conditions (Fig. 1A, 1C). In sharp contrast, numerous CD45+Gr-1− leukocytes (all leukocytes excluding neutrophils) were observed, consistent with the view that the healthy liver houses a large population of resident immune cells including Kupffer cells, NK cells, and lymphocytes, but not neutrophils (Fig. 1A, 1C) (21). Administration of LPS induced a large neutrophil influx into the liver of WT mice, as demonstrated by the dramatic increase in the number of CD45+Gr-1− (arrowhead) neutrophils found in both the post-sinusoidal venules (thick dashed line) and sinusoids (thin dashed line) (Fig. 1B, 1C). The number of CD45+Gr-1− leukocytes did not increase after LPS treatment, indicating that the majority of leukocytes recruited to the liver during acute endotoxemia are, in fact, neutrophils (Fig. 1C). No cells were labeled after the addition of appropriate FITC- and PE-conjugated isotype control Abs (data not shown). In addition, Leder-stained liver sections counterstained with hematoxylin were used to quantify the number of neutrophils and mononuclear cells in the livers of both untreated and LPS-treated mice. Histological examination revealed 6.4 ± 3.2 neutrophils and 89.2 ± 14 mononuclear cells per FOV in untreated WT mice versus 43.8 ± 3.8 neutrophils and 86.2 ± 4 mononuclear cells per FOV in mice treated with 0.5 mg/kg LPS for 4 h (Fig. 1D).

Clearly, predominantly neutrophils, and not mononuclear cells, are recruited to the inflamed liver in this model.

**LPS-induced neutrophil recruitment within the liver microvasculature is independent of CD14**

Administration of LPS to WT C57BL/6 mice resulted in profound recruitment of neutrophils into the hepatic microvasculature (Fig. 2). Within post-sinusoidal venules of endotoxemic WT mice, the number of neutrophils observed rolling along the endothelium was...
increased, and the velocity with which they rolled was decreased compared with untreated controls (Fig. 2A, 2B). Furthermore, the number of neutrophils that firmly adhered to the venular endothelium was significantly greater in LPS-treated mice compared with control animals (Fig. 2C). Within the liver sinusoids, LPS administration in WT mice resulted in a >5-fold increase in the number of neutrophils that adhered to the endothelium (Fig. 2D), as well as a significant reduction in the percentage of perfused sinusoids (Fig. 2E). Therefore, LPS injection in WT mice induced significant hepatic inflammation as demonstrated by the recruitment of neutrophils within both the venular and sinusoidal microvascular compartments of the liver.

The role of CD14 in the generation of LPS-induced hepatic inflammation was investigated in CD14−/− mice. Remarkably, LPS administration to CD14−/− mice resulted in levels of neutrophil–endothelial interactions within both the post-sinusoidal venules and sinusoids that were the same or even increased compared with LPS-treated WT mice (Fig. 2). Interestingly, the number of rolling cells in post-sinusoidal venules was significantly higher in CD14−/− mice, but the velocity of rolling cells was not different between CD14−/− and WT mice (Fig. 2A, 2B). The reduced rolling velocity has previously been shown to be a direct reflection of endothelial activation (22). The greater number of rolling cells in CD14−/− mice was likely a reflection of the higher number of neutrophils within the circulation of endotoxemic CD14−/− mice, owing to the lack of LPS-induced neutropenia in these mice compared with their WT counterparts (shown later). Fig. 2C and 2D demonstrate a large increase in the number of adherent cells within both the post-sinusoidal venules and sinusoids in endotoxemic CD14−/− mice that did not differ significantly from inflamed WT controls. Furthermore, LPS-induced reductions in sinusoidal perfusion rates were no different between WT and CD14−/− mice (Fig. 2E). Overall, these results lend to the conclusion that inflammation and neutrophil recruitment in the liver in response to LPS does not require CD14.

Importantly, in the microvasculatures of other organs, the same LPS preparation failed to induce neutrophil recruitment in animals that lacked CD14, confirming that the LPS response observed in the liver was not due to a CD14-independent contaminant. Specifically, local administration of LPS into the cremaster muscle for 4 h revealed that within this vascular bed, neutrophil recruitment in response to LPS is entirely dependent on CD14 (Fig. 3). The number of rolling cells (Fig. 3A) as well as the rolling flux fraction were significantly increased in response to LPS in WT mice (untreated: 8.1% versus LPS: 49.3%), whereas CD14−/− mice showed no increase in the number of rolling cells or the rolling flux fraction in response to LPS (untreated: 12.8% versus LPS: 14.6%). Similarly, leukocyte adhesion and emigration out of the vasculature were abrogated in CD14−/− mice compared with WT mice (Fig. 3C, 3D). Importantly, there were no significant differences in venule wall shear rates among any of the above experimental groups (data not shown). Furthermore, after systemic administration of LPS, neutrophil sequestration within the pulmonary vasculature was found to be entirely dependent on CD14 (shown later). Therefore, the neutrophil recruitment observed within the liver in response to LPS represents a unique CD14-independent LPS-sensing mechanism that is not shared by other organs such as the cremaster muscle or lungs.

Neutrophil recruitment in the liver microvasculature is independent of CD14 even at low concentrations of LPS

Previous studies have suggested that the requirement for CD14 in the generation of LPS-induced inflammation can be overcome by extremely high doses of LPS (9). To test the hypothesis that LPS-
induced hepatic inflammation is independent of CD14 regardless of LPS concentration, we compared the quantity of neutrophil adhesion in the livers of CD14<sup>−/−</sup> mice and WT mice in response to varying doses of LPS (0.01–1 mg/kg). Interestingly, equivalent levels of neutrophil adhesion were observed in both the post-sinusoidal venules as well as sinusoids of CD14<sup>−/−</sup> and WT mice,
regardless of the dose of LPS administered (Fig. 4A, 4B). Therefore, even in response to low concentrations of LPS, neutrophil recruitment is activated independently of CD14.

**LPS-induced systemic inflammatory response syndrome is dependent on CD14**

It has previously been reported that CD14<sup>−/−</sup> mice are resistant to LPS-induced shock, pulmonary neutrophil sequestration, and neutropenia (9, 10). To confirm that our model of endotoxemia behaved similarly, we assessed various markers of systemic inflammation in response to LPS in WT versus CD14<sup>−/−</sup> mice. First, we observed severely impaired production of proinflammatory cytokines (TNF-α, IL-1β, as well as IL-6, keratinocyte chemoattractant, and MIP-1α) within the circulation of CD14<sup>−/−</sup> compared with WT control mice (Fig. 5A, 5B, and data not shown). Furthermore, in accordance with previous studies, deficiency of CD14 resulted in abrogation of pulmonary neutrophil sequestration and no reduction in circulating leukocyte counts in response to LPS (Fig. 5C, 5D). Therefore, the profound hepatic neutrophil recruitment that was observed in CD14<sup>−/−</sup> mice occurred despite a lack of significant systemic inflammatory response.

**LPS-induced liver inflammation is not modulated by the presence of CD14 on hematopoietic cells, nonhematopoietic cells, or the presence of sCD14**

Next, we aimed to understand whether the presence of CD14 on leukocytes, on nonhematopoietic cells such as endothelium, or released as sCD14 could modulate the inflammatory response locally within the liver or systemically during endotoxemia. We addressed this question by conducting BM transplantations between CD14<sup>−/−</sup> and WT mice to generate groups of chimeric animals that expressed CD14 only by cells of hematopoietic origin (WT→CD14<sup>−/−</sup>/CD14<sup>−/−</sup>→WT), or nonhematopoietic origin (CD14<sup>−/−</sup>→WT). Control groups were generated by syngeneic transplantation (CD14<sup>−/−</sup>→CD14<sup>−/−</sup>→WT, and WT→WT). Because CD14<sup>−/−</sup> and WT mice were observed to generate identical hepatic inflammation in response to LPS, we hypothesized that neither chimeric mouse would have a reduced phenotype. Indeed, in response to systemically administered LPS, both the CD14<sup>−/−</sup>→WT and WT→CD14<sup>−/−</sup> chimeras demonstrated significantly increased levels of neutrophil adhesion within the post-sinusoidal venules of the liver compared with untreated controls (Fig. 6A). Within the sinusoids, neutrophil adhesion was significantly increased when CD14 was expressed on nonhematopoietic cells (CD14<sup>−/−</sup>→WT) and also appreciably increased in animals that expressed leukocyte CD14 (WT→CD14<sup>−/−</sup>/CD14<sup>−/−</sup>→WT), albeit not as robustly as the reciprocal chimera (Fig. 6B). No differences in neutrophil adhesion were observed between either of the chimeric groups compared with the WT or CD14<sup>−/−</sup> control transplanted groups (WT→WT and CD14<sup>−/−</sup>→CD14<sup>−/−</sup>/CD14<sup>−/−</sup>→WT, Fig. 6A, 6B).

CD14 can also be expressed in a soluble form in the serum (sCD14), thereby complicating the strict tissue distribution of CD14 expression assumed in the aforementioned chimeric mice. Levels of serum sCD14 increased dramatically after LPS administration in WT mice, indicating that sCD14 behaves as an acute-phase reactant (Supplemental Fig. 2). Quantification of serum sCD14 in each of the groups of BM chimeric animals revealed that levels of sCD14 were only upregulated in animals that expressed CD14 on nonhematopoietic cells (WT→WT and CD14<sup>−/−</sup>→WT) (Fig. 6C). Although controversy has existed over the primary source of sCD14 expression in vivo, the present data support the view that CD14 is an acute-phase reactant most prominently induced by parenchymal cells including liver hepatocytes in response to inflammatory mediators (23, 24).

Finally, as a marker of systemic inflammatory response to LPS, a drop in circulating leukocyte counts was observed in all groups except animals completely lacking CD14 (CD14<sup>−/−</sup>→CD14<sup>−/−</sup>→WT control group) (Fig. 6D). Therefore, the presence of CD14 expression in serum (sCD14), on parenchymal cells, or on leukocytes alone was sufficient to induce a drop in circulating leukocyte counts in response to LPS, whereas neutrophil recruitment within the liver during endotoxemia proceeds unaffected by the presence or absence of CD14 on any cell type or in any form (membrane bound or soluble).

**Endotoxemia-induced neutrophil activation is impaired without limiting adhesion in CD14<sup>−/−</sup> mice**

We have recently reported that adhesion of neutrophils within inflamed sinusoids of the liver is dependent on an adhesive interaction between neutrophil CD44 and endothelial hyaluronan (HA) (16). In response to LPS in WT mice, CD44-HA–dependent adhesion is activated when HA is covalently modified by the binding of SHAP, heralded by the deposition of large amounts of SHAP within the liver sinusoids (16, 25) (Fig. 7A, 7B). Identically to WT mice, large amounts of SHAP were observed within the liver sinusoids of CD14<sup>−/−</sup>→WT mice in response to LPS (Fig. 7C, 7D). Therefore, this data suggests that neutrophil adhesion within sinusoids is activated independently of CD14 and that the major adhesive mechanism for neutrophil adhesion within sinusoids, namely SHAP, was increased identically between WT and CD14<sup>−/−</sup> mice.

Within the venules of the liver, this CD44/HA/SHAP pathway of adhesion plays no role. Instead, neutrophils adhere to venular endothelium using integrins (including β2 integrins CD11a/CD18 [LFA-1] and CD11b/CD18 [Mac-1] as well as α<sub>4</sub> integrins) (16). Expression levels of the β2 integrin CD11b/CD18 (Mac-1) on

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**FIGURE 4.** CD14 deficiency has no effect on neutrophil recruitment within the endotoxemic liver regardless of LPS concentration. WT or CD14<sup>−/−</sup> mice were either left untreated or were treated with 0.01, 0.1, 0.5, or 1 mg/kg LPS for 4 h (A, B). Neutrophil adhesion was quantified in both the post-sinusoidal venules (A) and sinusoids (B) using intravital microscopy. Data are presented as the arithmetic mean ± SEM of at least four animals per group.
neutrophils harvested from LPS-treated CD14−/− or WT mice were determined by flow cytometry. Neutrophils from WT mice treated with LPS significantly upregulated CD11b expression compared with neutrophils from untreated mice (Fig. 8A). In contrast, neutrophils from CD14−/− mice failed to upregulate CD11b compared with untreated neutrophils, indicating that these

FIGURE 5. CD14 deficiency impairs the systemic inflammatory response to LPS. Whole blood was isolated from WT or CD14−/− mice that were either left untreated or were treated with an i.p. injection of 0.5 mg/kg LPS for 4 h. The concentrations of TNF-α (A) and IL-1β (B) in plasma were determined by ELISA. C. Myeloperoxidase levels were quantified in pulmonary tissues as an index of neutrophil sequestration within this organ. D. Circulating leukocyte numbers were quantified in whole blood obtained by cardiac puncture. Data are presented as the arithmetic mean ± SEM of five mice per group. *p < 0.05, **p < 0.01 versus untreated controls unless otherwise indicated.

FIGURE 6. The effect of hematopoietic versus nonhematopoietic cell CD14 expression on neutrophil recruitment during endotoxemia. BM chimeric mice were generated using either WT BM transplanted into WT recipients (WT→WT), CD14−/− BM into WT recipients (CD14−/−→WT), WT BM into CD14−/− recipients (WT→CD14−/−), or CD14−/− BM into CD14−/− recipients (CD14−/−→CD14−/−). Neutrophil recruitment was assessed via intravital microscopy of the liver, and the number of adherent neutrophils within the post-sinusoidal venules (A) and in the sinusoids (B) were quantified. C. Levels of sCD14 in the serum of mice were determined by ELISA and expressed as nanograms per milliliter of serum. D. Circulating leukocyte numbers were quantified in whole blood obtained by cardiac puncture. Data are presented as the arithmetic mean ± SEM of at least three mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 versus untreated controls.
cells were not activated as profoundly in the circulation as neutrophils in endotoxemic WT mice (Fig. 8A).

We next questioned whether the impairment in LPS-induced neutrophil activation observed in CD14$^{-/-}$ mice (Fig. 8A) was substantial enough to reduce the neutrophils ability to adhere. The effect of CD14 deficiency on neutrophil adhesive strength was assessed in vitro using a flow chamber assay. BM-derived neutrophils from WT and CD14$^{-/-}$ mice were either left untreated, treated with MIP-2 as a positive control, or were treated with 100 ng/ml LPS ex vivo for 1 h and allowed to adhere on a coverslip. A high shear force was then applied to the cells (5 dynes/cm$^2$), and the percentage of cells remaining adhered was determined over a 5-min period. Untreated CD14$^{-/-}$ neutrophils adhered similarly to WT neutrophils, as only 20–30% of neutrophils adhered after 5 min exposure to the high shear force (Fig. 8B). Moreover, neutrophils from WT and CD14$^{-/-}$ mice that were stimulated with 10 nM MIP-2 for 5 min and then allowed to adhere to the coverslip showed very similar adhesion in the two strains of mice (Fig. 8C). Despite the differences observed in LPS-induced neutrophil activation (CD11b upregulation) and observed between WT and CD14$^{-/-}$ mice (Fig. 8A), CD14 deficiency did not affect the ability of neutrophils to functionally adhere when stimulated with LPS (Fig. 8D). Of note, selective inhibition of CD11b alone did not significantly reduce neutrophil adhesion within sinusoids or post-sinusoidal venules in WT or CD14$^{-/-}$ mice (Supplemental Fig. 3), which is consistent with reports that demonstrated neutrophil adhesion in post-sinusoidal venules requires multiple integrins (both $\beta_2$ and $\alpha_4$ integrins) (16). Therefore, despite an impairment in neutrophil activation and CD11b upregulation, CD14$^{-/-}$ neutrophils demonstrated effective integrin-dependent adhesion in an in vitro flow chamber and in vivo in the post-sinusoidal venules of the liver.

Efficient clearance of circulating bacteria within the liver of CD14$^{-/-}$ mice

Previous studies of the role of CD14 in Gram-negative bacterial sepsis have demonstrated that CD14$^{-/-}$ mice are able to clear bacteremia as well or better than WT mice (9, 26). The liver is a primary site of bacterial clearance during sepsis, and neutrophils from WT and CD14$^{-/-}$ mice were either left untreated, treated with MIP-2 as a positive control, or were treated with 100 ng/ml LPS ex vivo for 1 h and allowed to adhere on a coverslip. A high shear force was then applied to the cells (5 dynes/cm$^2$), and the percentage of cells remaining adhered was determined over a 5-min period. Untreated CD14$^{-/-}$ neutrophils adhered similarly to WT neutrophils, as only 20–30% of neutrophils adhered after 5 min exposure to the high shear force (Fig. 8B). Moreover, neutrophils from WT and CD14$^{-/-}$ mice that were stimulated with 10 nM MIP-2 for 5 min and then allowed to adhere to the coverslip showed very similar adhesion in the two strains of mice (Fig. 8C). Despite the differences observed in LPS-induced neutrophil activation (CD11b upregulation) and observed between WT and CD14$^{-/-}$ mice (Fig. 8A), CD14 deficiency did not affect the ability of neutrophils to functionally adhere when stimulated with LPS (Fig. 8D). Of note, selective inhibition of CD11b alone did not significantly reduce neutrophil adhesion within sinusoids or post-sinusoidal venules in WT or CD14$^{-/-}$ mice (Supplemental Fig. 3), which is consistent with reports that demonstrated neutrophil adhesion in post-sinusoidal venules requires multiple integrins (both $\beta_2$ and $\alpha_4$ integrins) (16). Therefore, despite an impairment in neutrophil activation and CD11b upregulation, CD14$^{-/-}$ neutrophils demonstrated effective integrin-dependent adhesion in an in vitro flow chamber and in vivo in the post-sinusoidal venules of the liver.
infiltration within the liver contributes to bacterial capture within the sinusoids (20, 27). Given our observation that neutrophil recruitment within the liver is CD14-independent during endotoxemia, we hypothesized that CD14−/− mice would effectively clear bacteria within the liver during a Gram-negative sepsis. To test this hypothesis, we injected fluorescently labeled E. coli into the circulation of endotoxemic mice and used intravital microscopy to visualize bacterial clearance within the liver over time, as previously described (20). No difference in the number of captured bacteria was observed at any time point between WT and CD14−/− mice (Fig. 9). Therefore, as hypothesized, CD14−/− mice displayed effective bacterial clearance within the liver microvasculature.

Discussion

Severe sepsis and septic shock continue to elude effective therapies in hospital intensive care units, resulting in the deaths of hundreds of thousands of North Americans each year (28). In Gram-negative sepsis, LPS shed from the surface of bacteria is thought to be an important contributor to the pathogenesis of this disease (29, 30). Regardless of the initial source of infection, systemic activation of circulating leukocytes by mediators such as LPS results in inappropriate sequestration of neutrophils in the pulmonary and hepatic microvasculatures, resulting in organ damage and dysfunction (3, 31, 32). Our group has previously demonstrated that LPS-induced neutrophil recruitment in both the lung and liver during endotoxemia is solely dependent on TLR4 and that CD14 is required at least for LPS-induced sequestration of neutrophils in the pulmonary microvasculature (10, 11). However, a role for CD14 in LPS-induced recruitment within the hepatic microvasculature has yet to be defined. To our knowledge, this study is the first to report that neutrophil recruitment in the hepatic microvasculature during acute endotoxemia occurs independently of CD14. Within both the post-sinusoidal venules and the sinusoids of the liver, neutrophil recruitment was observed to occur equivalently between CD14−/− and CD14+/+ (WT) mice, regardless of the dose of LPS or the presence of CD14 on any cell type. This was in contrast to other organs such as the cremaster muscle or lungs, in which LPS-induced neutrophil infiltration was entirely dependent on CD14. Therefore, the liver possesses a unique mechanism for LPS detection and initiation of inflammatory signaling that does not rely on CD14 during endotoxemia.

The liver, much like the lungs, is a key target organ for neutrophil-mediated inflammatory pathology during sepsis and endotoxemia (10, 11). It is largely unknown why these organs are involved, but it has been hypothesized that they are incidentally targeted by systemically activated circulating neutrophils that become inadvertently sequestered in the dense microvascular beds in these organs. However, in this study, it was observed that neutrophils lacking CD14 expression failed to become activated in the circulation in response to LPS, yet still retained their ability to strongly adhere to a coverslip in vitro and to endothelium in the hepatic microcirculation in vivo. This observation is in line with previous studies demonstrating that LPS activation of endothelium and possibly other nonhematopoietic cells, rather than neutrophils themselves, is the primary determinant of neutrophil recruitment into tissues (11, 33, 34). Indeed, we have previously reported using a systematic screen of endothelial activation levels in all major organs that endothelial cells within the liver of CD14−/− mice are activated in response to LPS (demonstrated by upregulation of VCAM-1 expression) (10). Taken together, these results suggest that CD14-independent activation of the liver vascular endothelium by LPS may be necessary and sufficient for the recruitment of neutrophils into the liver during endotoxemia. In line with this conclusion, we have previously demonstrated that adhesion of neutrophils within liver sinusoids occurs through an interaction between neutrophil CD44 and endothelial HA, and this interaction was not dependent on activation of neutrophil CD44 but was instead controlled at the level of endothelium through modification of HA by SHAP (16). In the current study, we demonstrate that equivalent levels of SHAP are deposited in liver sinusoids of WT and CD14−/− mice in response to LPS, thereby explaining the observation that equivalent amounts of neutrophils adhered in the sinusoids of both strains of mice.

It remains unclear why neutrophils become sequestered within the liver microvasculature during sepsis/endotoxemia, but it has been hypothesized that hepatic neutrophil sequestration represents a coordinated effort by the host to enhance the clearance of circulating bacteria (20, 27). Previous studies have demonstrated enhanced bacterial clearance in CD14−/− mice during Gram-negative sepsis (9, 26). In our present study, we found that CD14−/− mice were able to trap circulating bacteria in the liver as effectively as WT mice. Clearance of blood-borne bacteria within the liver during Gram-negative sepsis is known to be highly dependent on the presence of recruited neutrophils in the sinusoids and the release of neutrophil extracellular traps that ensnare circulating bacteria (20). Together, this suggests that CD14-independent neutrophil recruitment into the liver may be a coordinated effort by the host to enhance protection against bacterial dissemination during Gram-negative sepsis.

The evidence presented in this study supports the view that the liver is an exception to the general rule that CD14 is required to sense LPS and initiate an inflammatory response. Our finding that endotoxemic CD14−/− mice generated pronounced hepatic inflammation, yet failed to generate evidence of a systemic inflammatory response (pulmonary neutrophil sequestration, increased serum proinflammatory cytokines, drop in circulating neutrophil counts), suggests the existence of a unique LPS sensing system that is specifically positioned within the liver to respond to intravascular danger such as LPS. We believe this represents a unique role for the liver in intravascular surveillance for circulating bacteria or bacterial products, which is consistent with the importance of the liver for clearance of circulating bacteria and bacterial products (27). CD14-independent responses to LPS in the liver microcirculation make this dense vascular bed uniquely sensitive to the presence of LPS and allow the generation of local inflammatory responses independently of the development of pathological systemic inflammation and neutrophil activation.

**FIGURE 9.** CD14−/− mice effectively clear bacteria in the liver. WT or CD14−/− mice were treated with an i.p. injection of 0.5 mg/kg LPS for 4 h and were then infused intravenously with 1 × 10⁷ CFU fluorescently labeled E. coli. The livers were then imaged using spinning disk confocal intravital microscopy, and the numbers of trapped bacteria were determined per FOV at the indicated time points after administration of bacteria. Data are presented as arithmetic mean ± SEM of at least three experiments.
Acknowledgments

We thank Carla Badick, Dean Brown, Lori Zbytnuik, and Varinder Gill for excellent technical assistance and Dr. Pina Colarusso and the Live Cell Imaging Facility at the University of Calgary.

Disclosures

The authors have no financial conflicts of interest.

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