Kupffer cells and activation of endothelial TLR4 coordinate neutrophil adhesion within liver sinusoids during endotoxemia

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Submitted 22 February 2013; accepted in final form 5 October 2013

McDonald B, Jenne CN, Zhuo L, Kimata K, Kubes P. Kupffer cells and activation of endothelial TLR4 coordinate neutrophil adhesion within liver sinusoids during endotoxemia. Am J Physiol Gastrointest Liver Physiol 305: G797–G806, 2013. First published October 10, 2013; doi:10.1152/ajpgi.00058.2013.—A key pathological feature of the systemic inflammatory response of sepsis/endotoxemia is the accumulation of neutrophils within the microvasculature of organs such as the liver, where they cause tissue damage and vascular dysfunction. There is emerging evidence that the vascular endothelium is critical to the orchestration of inflammatory responses to blood-borne microbes and microbial products in sepsis/endotoxemia. In this study, we aimed to understand the role of endothelium, and specifically endothelial TLR4 activation, in the regulation of neutrophil recruitment to the liver during endotoxemia. Intravital microscopy of bone marrow chimeric mice revealed that TLR4 expression by non-bone marrow-derived cells was required for neutrophil recruitment to the liver during endotoxemia. Furthermore, LPS-induced neutrophil adhesion in liver sinusoids was equivalent between wild-type mice and transgenic mice that express TLR4 only on endothelium (tlr4−/−; Tie2+), revealing that activation of endothelial TLR4 alone was sufficient to initiate neutrophil adhesion. Neutrophil arrest within sinusoids of endotoxemic mice requires adhesive interactions between neutrophil CD44 and endothelial hyaluronan. Intravital immunofluorescence imaging demonstrated that stimulation of endothelial TLR4 alone was sufficient to induce the deposition of serum-derived hyaluronan-associated protein (SHAP) within sinusoids, which was required for CD44/hyaluronan-dependent neutrophil adhesion. In addition to endothelial TLR4 activation, Kupffer cells contribute to neutrophil recruitment via a distinct CD44/HA/SHAP-independent mechanism. This study sheds new light on the control of innate immune activation within the liver vasculature during endotoxemia, revealing a key role for endothelial cells as sentinel cells in the detection of intravascular infections and coordination of neutrophil recruitment to the liver.

SEVERE SEPSIS IS A SYNDROME of systemic inflammation and organ dysfunction caused by infection (7). Despite intensive research, there are no specific therapies to combat the inflammatory pathology of severe sepsis, and as a result mortality from this disease remains extremely high at ~20–50% (12, 20, 28). In gram-negative bacterial sepsis, shedding of bacterial lipopolysaccharide (LPS) into the blood can stimulate an exuberant systemic innate immune response that results in accumulation of neutrophils in the expansive capillary beds of organs such as the liver and lungs, causing organ damage and dysfunction (1, 10, 36). Septic inflammation in the liver is of particular interest as this organ is both a target of immune-mediated pathology as well as a key site for the clearance of bacteria and bacterial toxins from the blood (17, 38). In particular, neutrophils recruited to sinusoids generate an intravascular defense system against the spread of septic infections (31). However, this antimicrobial response is mounted at the expense of significant neutrophil-mediated injury and vascular dysfunction in the liver (8, 9, 17, 31). Therefore, understanding the mechanisms that initiate and regulate neutrophil recruitment to the liver during sepsis/endotoxemia may reveal ways to therapeutically manipulate the innate immune response to avoid organ damage while optimizing antibacterial defenses.

During endotoxemia, LPS is detected by Toll-like receptor 4 (TLR4) on sentinel cells that initiate the subsequent inflammatory response, including neutrophil recruitment to the liver. It is widely believed that stimulation of TLR4 on neutrophils results in cellular hyperactivation, causing neutrophils to accumulate in the narrow capillaries of the liver (and lungs) (10, 14). However, TLR4 is expressed on many other cells, both leukocytes and nonleukocytes, that may contribute to the coordination of neutrophil recruitment in liver sinusoids. Tissue-resident leukocytes such as macrophages and mast cells are hallmark sentinel cells and have well-described roles in the initiation of inflammatory responses in various disease models (37). In addition, there is increasing evidence that nonleukocytes also play key roles in detecting microbial products and coordinating leukocyte recruitment to inflamed tissues. Notably, several recent studies have uncovered critical roles for the vascular endothelium in initiating and regulating the systemic inflammatory response to endotoxemia and bacterial sepsis (3, 4, 43, 44). Such evidence has led us to hypothesize that vascular endothelial cells are a primary TLR4-expressing sentinel population for the detection of circulating LPS and, as such, can coordinate the recruitment of neutrophils to the liver during endotoxemia.

During the systemic inflammatory response of sepsis or endotoxemia, neutrophils are sequestered within liver sinusoids by an adhesive interaction between neutrophil CD44 and endothelial hyaluronan (HA) (30). Adhesion between CD44 and HA has been shown to support leukocyte-endothelial interactions in a number of inflammatory models. The constitutive expression of CD44 on numerous leukocyte subsets as well as HA on vascular endothelium suggests that their binding is functionally regulated in response to inflammatory stimuli. CD44 on lymphocytes and monocytes can be induced into an HA-avid state by stimulation with cytokines (TNF-α, IL-1α,
IL-1β, IL-2), chemokines (IL-8, MIP-1β, RANTES), as well as bacterial products such as LPS (reviewed in Ref. 35). CD44 binding activity can be modulated by numerous molecular modifications including glycosylation, phosphorylation, sulfation, and expression of splice variant forms of CD44 (21, 27, 34). It was originally hypothesized that sequestration of neutrophils within the liver microvasculature in response to LPS was the result of neutrophil activation causing enhanced CD44 adhesiveness for endothelial HA. Surprisingly, stimulation of neutrophils with many different mediators (including LPS, cytokines such as TNF-α, or chemokines such as MIP-2) was insufficient to induce neutrophil CD44 to bind to HA (30). Such evidence suggests that neutrophil recruitment within liver sinusoids may not be regulated at the level of the neutrophil but may instead be regulated by functional activation of vascular endothelium and its surface HA. Recent studies have demonstrated that HA can be functionally activated into a CD44-avid state through the binding of a serum-derived hyaluronan-associated protein (SHAP, composed of the heavy chains H1 and H2 of inter-α-trypsin inhibitor) (45). Deposition of SHAP on the luminal surface of liver sinusoids during endotoxia has been reported, but its functional role in neutrophil adhesion within these vessels has not been assessed (29, 30).

In this study, we aimed to understand the role of endothelium, and specifically endothelial TLR4 activation, in the regulation of neutrophil recruitment to the liver microvasculature during endotoxia. First, using bone marrow chimeras that express TLR4 only on selected cell populations, we systematically identified vascular endothelial cells as a key TLR4-expressing sentinel population regulating neutrophil recruitment within the liver microvasculature in response to endotoxia. Activation of endothelial TLR4 alone was found to be sufficient to initiate CD44-HA-dependent neutrophil adhesion in liver sinusoids. Furthermore, we examined the contribution of SHAP to the regulation of CD44-HA interactions, revealing that activation of endothelial TLR4 leads to neutrophil adhesion through the induction of SHAP-dependent adhesion of HA to neutrophil CD44. Finally, we investigated the role of another key sentinel population, Kupffer cells, and demonstrated that endothelium and Kupffer cells provide unique and additive contributions to neutrophil recruitment within liver sinusoids. Overall, this study provides important new insight into the molecular mechanisms that govern neutrophil recruitment to the liver during endotoxia and sepsis.

**MATERIALS AND METHODS**

**Animals.** C57BL6J mice (referred to as WT), TLR4 knockout mice (referred to as tlr4−/−), and B6.SJL-Pippca Pep3b/BoyJ mice were purchased from Jackson Laboratories. Bikunin knockout mice (referred to as Bik−/−) were generated in the laboratory of Dr. Kimata as described previously (46). The tlr4−/−/Tie2Δ24 transgenic mice were generated in our laboratory as previously described (4). It is important to note that this line of mice was lost as a result of a breeding error and has not been regenerated to date. All animals were on a C57BL6/J background. At the time of use, mice weighed between 20 and 35 g and were 6 to 10 wk old. All animals were anesthetized with a mixture of 200 mg/kg ketamine (Rogar/STB) and 10 mg/kg xylazine (MTC Pharmaceuticals) administered via an intraperitoneal injection. Experimental animal protocols carried out in this study were approved by the University of Calgary Animal Care Committee and met the guidelines of the Canadian Council for Animal Care.

**Generation of chimeric mice.** Bone marrow chimeras between C57BL/6 and tlr4−/− mice, and C57BL/6 and B6.SJL-Pippca Pep3b/BoyJ mice were generated by following a standard protocol (3). Bone marrow was isolated from donor mice euthanized by cervical spine dislocation. Recipients were irradiated with two doses of 5 Gy (Gammacell 40.137Cs γ-ray source), with a 3-h interval between doses; 8 × 10⁶ isolated donor bone marrow cells were injected via the tail vein into irradiated recipient mice. Transplanted mice received water with 0.2% neomycin and remained in germ-free microisolator cages for 8 wk to allow full hematological reconstitution.

**Intravital microscopy in the mouse liver.** The procedure for transillumination intravital microscopy of the murine liver was performed as outlined previously (41). Following general anesthesia, the right jugular vein was cannulated for administration of additional anesthetic and for injection of antibodies in some experiments. Throughout all procedures the body temperature was maintained at 37°C with infrared heat lamps.

Leukocytes that remained stationary on the venular or sinusoidal endothelium for ≥30 s were considered adherent cells. Adherent cells were counted per 100 μm in postsinusoidal venules, or per field of view in sinusoids. The percentage of sinusoids perfused was determined by dividing the number of perfused sinusoids by the total number of sinusoids in a field of view.

**Spinning disk confocal microscopy.** Intravital microscopy was performed as previously described, and the anterior surface of the liver was viewed with a spinning disk multichannel confocal microscope. Images were acquired with an Olympus BX51 (Olympus) inverted microscope using a ×10 or ×200/0.75 NA objective. The microscope was equipped with a confocal light path (WaveFx, Quorum) based on a modified Yokogawa CSU-10 head (Yokogawa Electric). Livers were imaged with 488-, 561-, and 680-nm laser excitation (Cobalt), and visualized with the appropriate long-pass filters (Semrock). Typical exposure times for 488-, 561-, and 680-nm laser excitation were 120, 240, and 500 ms, respectively. A 512 × 512 pixel back-thinned EMCCD camera (C9100-13, Hamamatsu, Bridgewater, NJ) was used for fluorescence detection. Velocity Acquisition software (Improvement, Lexington, MA) was used to drive the confocal microscope. Images were acquired as 20 μm z-stacks (1-μm slices) through sinusoids and are presented in extended focus format. Intravital immunofluorescence labeling was performed as previously reported (19, 30, 31). HA expression in sinusoids was visualized with use of 25 μg Alexa Fluor 488-labeled (Molecular Probes) HABP (Calbiochem). SHAP was detected by use of Alexa Fluor 555-labeled antibodies against SHAP [anti-inter-α-trypsin inhibitor (Izol) heavy chains 1 and 2 mAbs, 1.6 μg each; Santa Cruz Biochemicals]. Neutrophils were visualized with 10 μg Alexa Fluor 647-labeled anti-Gr-1 mAb (clone RB6-8C5, BD Pharmingen). Kupffer cell chimerism after bone marrow transplant was evaluated by visualization of Kupffer cells (Alexa Fluor 660-labeled F4/80 mAb) and leukocyte markers to differentiate donor from recipient (PE-labeled anti-CD45.1 and FITC-labeled anti-CD45.2, respectively). All antibodies and labeled proteins were injected intravenously through a jugular vein cannula 30 min prior to imaging.

**Experimental protocols.** For each experiment, mice received 1 mg/kg ultrapure LPS from E. coli O111:B4 (List Biological Laboratory) administered intravenously (iv) 4 h prior to intravital microscopy. This concentration is sufficiently low that all animals survived general anesthesia, and perfusion in the liver vasculature remained such that leukocyte-endothelial interactions occurred under flow conditions. In some experiments, mice were injected with 20 μg of purified anti-mouse CD44 mAb (clone KSM81, Cedarlane) administered iv 30 min before LPS injection. Intravascular HA was depleted by intraperitoneal administration of 20 U/g hyaluronidase type-IV (Sigma-Aldrich), a procedure known to deplete HA from the vascular endothelium (6, 23). The liver was then prepared for intravital microscopy and leukocyte kinetics were investigated as described above.

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**Cytokine measurement.** Mice were anesthetized and blood was collected by cardiac puncture. Blood was allowed to clot at room temperature for 30 min after which samples were centrifuged at 5,000 rpm for 10 min for the retrieval of serum. The serum samples were analyzed for inflammation-relevant cytokines and chemokines by using a Luminex 200 apparatus (Applied Cytometry Systems) and 25-plex MILLIPEX MAP mouse cytokine/chemokine magnetic panel kit from Millipore according to manufacturer’s instructions. The data was analyzed with the StarStation V.2.3 software from Applied Cytometry Systems.

**Statistical analysis.** All data are presented as mean values ± SE. A Student’s $t$-test was used to determine the significance between population means when two groups were compared. When more than two groups were compared, a one-way analysis of variance with post hoc Bonferroni test was used for multiple comparisons. Statistical significance was set at $P < 0.05$.

**RESULTS**

**LPS stimulation of TLR4 on non-bone marrow-derived cells initiates neutrophil adhesion in the liver microvasculature.** We first aimed to define the role of TLR4 on bone marrow-derived vs. non-bone marrow-derived cell populations in the induction of neutrophil recruitment to the liver in response to iv LPS. Bone marrow transplantation between *Tlr4*−/− and wild-type (WT, *Tlr4*+/+) mice generated chimeric animals that expressed TLR4 only on bone marrow-derived cells (WT→*Tlr4*−/−) or only on non-bone marrow-derived cells (*Tlr4*−/−→WT). Syngeneic transplants between WT→WT mice and *Tlr4*−/−→*Tlr4*−/− mice served as controls. In untreated WT→WT control mice, 3 ± 0.3 neutrophils adhered per field of view in liver sinusoids, and 2 ± 0.2 adherent neutrophils were observed per 100 μm in postsinusoidal venules (Fig. 1, A and B). Four hours after iv administration of LPS, neutrophil adhesion increased significantly to 12 ± 0.5 cells/field in sinusoids and 10 ± 0.5 cells/100 μm in venules (Fig. 1, A and B). As anticipated, *Tlr4*−/−→*Tlr4*−/− control mice showed no response to LPS (Fig. 1, A and B).

Within the sinusoids of *Tlr4*−/− mice that received WT bone marrow (WT→*Tlr4*−/−), LPS administration failed to induce neutrophil adhesion to sinusoidal endothelium, yielding an equivalent response to that observed in the negative control *Tlr4*−/−→*Tlr4*−/− group (Fig. 1A). Conversely, WT mice that received *Tlr4*−/− marrow (*Tlr4*−/−→WT) responded to LPS identically to the WT→WT positive controls (Fig. 1, A and B). These results demonstrate that LPS-induced neutrophil recruitment to the liver is dependent on TLR4 signaling in non-bone marrow-derived cells, with minimal input from TLR4 activation on leukocytes.

Notably, there was a small increase in neutrophil adhesion within the postsinusoidal venules of *Tlr4*−/− mice that received WT marrow (WT→*Tlr4*−/−), although this small response was significantly less than that observed in LPS-treated WT→WT control group (Fig. 1B). This small increase is likely accounted for by the integrin-dependent mechanism of adhesion that neutrophils utilize to bind to endothelium in venules (15, 30). As such, LPS stimulation of TLR4-expressing neutrophils likely generated sufficient upregulation of integrin activity to allow for some increased adhesiveness toward ligands on the venular endothelium.

Although irradiation and bone marrow transplantation result in >95% reconstitution of circulating leukocytes by donor cells, liver-resident Kupffer cells have previously been found to be somewhat radioresistant, resulting in substantial chimerism following bone marrow transplant (24). The origin of Kupffer cells (donor vs. recipient) was investigated by transplantation of C57Bl/6 bone marrow (expressing the leukocyte antigen CD45.2) into irradiated congenic mice expressing CD45.1 (B6.SJL-*Ptprca Pep3b*/BoyJ mice), enabling quantification of donor (CD45.2*F4/80*/+) vs. recipient (CD45.1*F4/80*/+) Kupffer cells within the liver. Indeed, we found that, 8 wk after irradiation and transplantation, 69.2% (±7.1, SE) of Kupffer cells were of donor origin, whereas 30.8% (±7.1, SE) remained of recipient origin. Kupffer cells are key producers of...
TNF-α in response to LPS, and TNF-α can serve as a strong stimulus for neutrophil recruitment in liver sinusoids (15). Therefore, given the substantial chimerism in the Kupffer cell population, we investigated serum levels of TNF-α following LPS administration in the above WT→Tlr4+/− bone marrow-transplanted mice. No significant differences in levels of serum TNF-α were found between WT→Tlr4+/− and Tlr4+/−→WT groups (Fig. 1C), confirming that the differences in neutrophil recruitment observed between these groups of mice are not the result of differing levels of TNF-α production in the setting of incomplete repopulation of Kupffer cells.

Stimulation of TLR4 on endothelium alone is sufficient to initiate neutrophil adhesion in liver sinusoids. Given that neutrophil recruitment to the liver during endotoxemia was dependent on TLR4 from non-bone marrow-derived cells, we hypothesized that the primary LPS-responsive sentinel within this population was the vascular endothelium. To test this hypothesis, our laboratory generated a transgenic mouse that expresses TLR4 exclusively on endothelial cells (4). Briefly, a linearized transgene containing the Tlr4 coding sequence under the control of the Tie2 promoter and enhancer was incorporated into the genome of Tlr4−/− mice, yielding Tlr4−/−Tie2TIE4 animals. In adult Tlr4−/−Tie2TIE4 mice, the only cells that express TLR4 are those that also express Tie2 [exclusively endothelial cells; see previous report (4) for full characterization of TLR4 expression]. Therefore, using these animals that express TLR4 exclusively on endothelial cells, we tested the hypothesis that TLR4 signaling within endothelium alone is sufficient to initiate neutrophil recruitment to the liver during endotoxemia.

In the absence of LPS, Tlr4−/−Tie2TIE4 mice and WT mice had equivalent hematocrits, total white blood cell and differential counts (data not shown), and very few neutrophils within the liver sinusoids (Fig. 2A). Four hours after LPS administration, neutrophil adhesion in liver sinusoids and postsinusoidal venules of Tlr4−/−Tie2TIE4 mice increased significantly to levels that were not different from WT Tlr4+/+ mice (Fig. 2, A and B). Importantly, Tlr4−/−Tie2TIE4 mice fail to produce significant amounts of serum TNF-α (or other proinflammatory cytokines such as IL-1β) in response to endotoxemia (4, 44), indicating that neutrophil recruitment in the liver of these mice is not the result of cytokine production, but rather a direct response to LPS/TLR4 signaling in endothelial cells. These findings reveal that neutrophil adhesion in the liver microvasculature can be initiated entirely by TLR4 signaling within vascular endothelial cells.

LPS activation of endothelial TLR4 initiates CD44-dependent adhesion in liver sinusoids. We have previously reported that neutrophil adhesion in murine liver sinusoids in response to LPS is largely dependent on adhesive interactions between neutrophil CD44 and endothelial HA, whereas recruitment within postsinusoidal venules requires classical integrin-mediated adhesion (30). To determine whether stimulation of endothelial TLR4 initiated neutrophil adhesion in liver sinusoids via the CD44/HA axis, WT and Tlr4−/−Tie2TIE4 mice were treated with a blocking antibody against CD44 prior to LPS administration. Indeed, neutralization of CD44 reduced adhesion within sinusoids of Tlr4−/−Tie2TIE4 mice to the same extent as WT animals but had no effect on adhesion within postsinusoidal venules (Fig. 2, A and B).

Neutrophil adhesion in liver sinusoids after endothelial TLR4 stimulation is dependent on SHAP binding to endothelial HA. In 2006, Kimata and colleagues (45) reported that SHAP, composed of the heavy chains of inter-α-inhibitor, binds covalently to HA and greatly enhances its avidity for leukocyte CD44. We therefore tested the hypothesis that endothelial HA may be modified during endotoxemia by the formation of SHAP-HA complexes and that these SHAP-HA complexes were necessary to allow for adhesive interactions between CD44 and HA in the liver sinusoids. Spinning disk confocal intravital microscopy (SD-IVM) was used to visualize the localization of HA [using Alexa Fluor 488-labeled hyaluronan binding protein (HABP)], SHAP (using Alexa 555-labeled anti-IoI H1 and H2 Abs), and neutrophils (using Alexa Fluor 647-labeled anti-GR-1 mAb) within the liver sinusoids in vivo. In the absence of LPS, HA was observed lining the sinusoidal endothelium, but very little SHAP and very few neutrophils were observed within these vessels (Fig. 3A). In stark contrast, 4 h after LPS injection both adherent neutrophils and SHAP were observed in abundance within liver sinusoids (46). Following LPS administration (Fig. 3C). To investigate whether SHAP was in fact binding to endothelial HA, a group of animals was depleted of intravascular HA by treatment with HNase, and 4 h after LPS injection the absence of endothelial HA correlated with an absence of SHAP (and fewer adherent TIE2 and HA staining) within the liver sinusoids.
endothelial TLR4 and neutrophil recruitment to liver

neutrophils) within the liver sinusoids of these animals (Fig. 3D). This evidence suggests that LPS stimulation of endothelial TLR4 results in profound SHAP deposition on endothelial HA within the liver sinusoids.

Finally, the role of SHAP deposition on endothelial HA was probed for its contribution to neutrophil adhesion in the liver sinusoids. Inter-α-inhibitor, the circulating SHAP precursor, is composed of a central protein called bikunin attached to heavy chain proteins (H1 and H2), which are covalently transferred to HA as “SHAP” at sites of inflammation (45). Therefore, Bik<sup>−/−</sup> mice are deficient of IαI, and are thereby deficient of SHAP generation in response to LPS (Fig. 4A) and other inflammatory stimuli (46). Quantitative assessment of neutrophil adhesion in liver sinusoids using intravital microscopy revealed that in SHAP-deficient Bik<sup>−/−</sup> mice, neutrophil adhesion was significantly reduced by over 50% (Fig. 4B). The reduction in sinusoidal neutrophil adhesion in Bik<sup>−/−</sup> mice was equivalent to that seen in anti-CD44 aAb treated WT animals (Fig. 2A above). Furthermore, antibody blockade of CD44 or depletion of HA in Bik<sup>−/−</sup> mice yielded no further decrease in the levels of neutrophil adhesion in liver sinusoids, indicating that SHAP, HA, and CD44 function as a single interdependent

Fig. 3. Endothelial TLR4 stimulation initiates serum-derived hyaluronan-associated protein (SHAP) deposition on endothelial hyaluronan. Spinning disk confocal intravital microscopy was used to visualize hyaluronan expression (green, AF488-HABP) and detect the presence of SHAP [red, AF555 anti-inter-α-trypsin inhibitor (IαI) H1 and H2 Abs] within liver sinusoids of untreated WT mice (A), LPS-treated WT mice (B), LPS-treated Tlr4<sup>−/−</sup>Tie2<sup>−/−</sup> mice (C), and mice pretreated with HNase [to depleted intravascular hyaluronan (HA)] prior to LPS (D). Neutrophils are displayed in blue (AF647 anti-Gr1 Ab). Representative of 3 experiments. Scale bars denote 50 μm.
Resident macrophages in the liver augment neutrophil adhesion in a SHAP/HA/CD44-independent manner. The results presented above demonstrate that stimulation of endothelial TLR4 is sufficient to initiate neutrophil adhesion within liver sinusoids during endotoxemia. However, the liver contains a large population of intravascular macrophages (Kupffer cells) that are intimately involved in many inflammatory and immunological responses in the liver (11) and may also affect neutrophil recruitment during endotoxemia. In fact, an important role for Kupffer cells in the development of sepsis-induced liver inflammation and pathology has previously been reported (13, 40). To determine whether Kupffer cells contribute to LPS-induced neutrophil recruitment in liver sinusoids, a group of mice was depleted of Kupffer cells by iv administration of clodronate liposomes (CLL) 48 h prior to LPS injection. This treatment has been shown by us and others to deplete >99% of Kupffer cells from the liver (16, 26). SD-IVM of the liver with staining for Kupffer cells with the pan-macrophage marker F4/80 confirmed the depletion of Kupffer cells by CLL in our experiments (Fig. 5A). Importantly, injection of CLL alone did not induce any neutrophil recruitment in sinusoids or venules (not shown). Interestingly, neutrophil adhesion in sinusoids was reduced by ~40% in endotoxemic CLL-treated mice compared with positive control animals that received LPS alone (Fig. 5, A and B).

Surprisingly, SHAP deposition in liver sinusoids was unchanged in the absence of Kupffer cells, yet neutrophil adhesion was decreased (Fig. 5A). This suggested the possibility that Kupffer cells may contribute to neutrophil adhesion in sinusoids independently of the CD44-HA/SHAP axis. To investigate this possibility, mice lacking Kupffer cells (CLL treated) were treated with anti-CD44 mAb prior to LPS administration, which resulted in further reduction of neutrophil adhesion in sinusoids beyond CLL-treatment alone (Fig. 5B). The additive effects of Kupffer cell depletion and CD44 blockade suggest that these pathways are independent contributors to neutrophil adhesion. Given that Kupffer cells are an important source of serum cytokines during endotoxemia, we investigated the early cytokine response in Kupffer cell-depleted mice vs. controls following LPS administration. Interestingly, few differences were seen for notable cytokines and chemokines that have been implicated in promoting neutrophil recruitment within the liver (TNF-α, KC, MIP-2), whereas reduced levels were seen for cytokines such as IL-1 and IL-12 (Table 1).

DISCUSSION

In this study, we have identified resident non-bone marrow-derived cells as the sentinels responsible for LPS detection (and the induction of neutrophil recruitment) in the liver during endotoxemia. Using transgenic animals that expressed TLR4 only on endothelium, we further specified that endothelial cells are the primary sentinels responsible for LPS detection and initiation of neutrophil adhesion in sinusoids. Stimulation of endothelial TLR4 was found to initiate neutrophil adhesion through a mechanism involving functional activation of HA on

Fig. 4. Impaired neutrophil adhesion in the liver sinusoids of SHAP-deficient mice. A: representative spinning-disk intravital microscopy images of liver sinusoids in LPS-treated WT (left) and SHAP-deficient bikunin knockout mice (Bik−/−, right). SHAP is shown in red (AF555 anti-I antibodies), and neutrophils are shown in green (AF488 anti-Gr-1 Ab). Scale bar denotes 50 μm. B: intravital microscopy was used to quantify the number of adherent neutrophils in liver sinusoids of WT and Bik−/− mice left untreated (open bar) or treated with LPS (solid bars). In some experiments, bikunin knockout mice were pretreated with anti-CD44 mAb, or HNase prior to LPS administration. Data are presented as the arithmetic means ± SE of at least 5 animals per group. *P < 0.05 relative to untreated controls (open bars), #P < 0.05 relative to LPS-treated WT (positive control) animals.
the luminal surface of the sinusoidal endothelium through binding of SHAP, enabling firm adhesion to neutrophil CD44.

Sequestration of neutrophils within the microcirculation of highly vascular organs such as the liver and lungs is a hallmark of the systemic inflammatory response of endotoxemia and sepsis (8). For many years, the prevailing dogma has been that blood-borne bacterial products (LPS) cause hyperactivation of circulating neutrophils, resulting in their accumulation in narrow capillaries of the liver and lungs (5, 14, 18, 22, 32, 42). Our data contradict this view, instead revealing that TLR4 signaling in neutrophils (and other marrow-derived cells) is not required for their recruitment to the liver. Interestingly, similar observations have been reported for neutrophil sequestration in the pulmonary microvasculature during endotoxemia/sepsis, wherein TLR4 on bone marrow-derived cells (leukocytes) was shown to be dispensable for recruitment to the lungs (3). Therefore, contrary to the notion that neutrophil sequestration in the liver (and lungs) is the result of cellular hyperactivation by LPS, our data suggest that recruitment is coordinated by non-leukocyte-tissue-resident sentinels.

There is emerging evidence that the vascular endothelium is critical to the orchestration of inflammatory responses, especially those involving blood-borne microbes or microbial products. Although tissue-resident leukocytes including macrophages, mast cells, and others have long been considered the primary sentinels for the detection of pathogens and orchestration of acute inflammation, vascular endothelial cells express many pattern recognition receptors (including TLRs, NLRs, RLRs, and CLRs) and are capable of expressing cytokines, chemokines, and adhesion molecules (33). Furthermore,
endothelial cells are the first cell type to contact circulating bacteria or bacterial products, and they substantially outnumber leukocytes in the liver and lungs (2). In sepsis and endotoxemia, the central role of endothelial cells has recently been highlighted in a number of studies. Ye et al. (43) demonstrated using models of endotoxemia and polymicrobial sepsis that endothelium-specific inhibition of NF-κB reduced neutrophil accumulation in multiple organs (including the liver), reduced endothelial permeability, and attenuated hypotension and multiple organ dysfunction. In addition, the generation of Tlr4−/−−Tie2Tlr4−/− mice that express TLR4 exclusively on endothelial cells has allowed our laboratory to perform focused investigations of the role of endothelial TLR4 in a number of organs during endotoxemia and bacterial sepsis. In response to systemic LPS administration, endothelial TLR4 is entirely sufficient to induce neutrophil recruitment to the pulmonary microvasculature (4), central nervous system (44), and liver (present study). More recently, the importance of endothelium as a sentinel system was expanded to antiviral responses, in which it was found that cytokine production and leukocyte recruitment to the lungs following influenza virus infection was coordinated entirely at the level of the vascular endothelium (39).

We have previously reported using the same model of endotoxemia that the majority of neutrophil adhesion in liver sinusoids is mediated by interactions between neutrophil CD44 and endothelial HA (30). Importantly, it was found that binding between neutrophil CD44 and endothelial HA was not induced by neutrophil activation, since stimulation with LPS, various cytokines, or chemokines did not increase neutrophil CD44 binding to HA under either static or flow conditions. Instead, it was hypothesized that functional activation of endothelial HA in response to LPS may regulate neutrophil adhesion in liver sinusoids. A number of previous reports have provided in vitro evidence that covalent binding of SHAP to HA in response to inflammatory stimuli augments the adhesiveness of HA for CD44 (11a, 45). Using an in vitro flow-chamber assay, Zhuo and colleagues (45) demonstrated that CD44-expressing T-cells failed to bind to native HA under flow conditions but would adhere in abundance to HA following SHAP binding. Our study represents the first in vivo evidence that functional modification of HA by SHAP is important for leukocyte adhesion during inflammation. Although the precise mechanism underlying activation of HA by SHAP has not been fully elucidated, there is evidence that covalent binding between HA and SHAP may yield structural changes in HA polymers that generate increased avidity for CD44 on leukocytes (11a).

Evidence presented in this study and others demonstrates that endothelial cells are important sentinels in the detection of circulating microbial products and coordination of neutrophil recruitment, yet other important sentinel cell populations exist that also contribute to the recruitment of neutrophils. Within the liver, Kupffer cells are optimally positioned within the vasculature to encounter circulating bacteria and bacterial products and are critical for their clearance from the bloodstream during steady state and during sepsis (11, 13, 26). Furthermore, Kupffer cells have well-defined roles in the coordination of hepatic inflammation in a number of diseases (11, 26, 40). Importantly, although our bone marrow transplant experiments suggest that TLR4 signaling in Kupffer cells was dispensable, depletion of Kupffer cells with CLL resulted in an ~40% reduction in neutrophil adhesion in sinusoids of endotoxemic mice. These data indicate that Kupffer cells have an important role in neutrophil recruitment, albeit downstream of TLR4 signaling in other sentinels (including endothelium), possibly through the production of cytokines or chemokines that influence neutrophil trafficking. Surprisingly, data presented in Table 1 demonstrate that many cytokines and chemokines known to affect neutrophil recruitment were not different between controls and Kupffer cell-depleted mice during the early response to LPS. However, it is possible that Kupffer cell TLR4 and cytokine/chemokine response may contribute to the later stages of LPS-induced hepatic inflammation.

Interestingly, the reduction of neutrophil adhesion by ~40% following Kupffer cell depletion may in fact be seen as paradoxical, since it could have been predicted to enhanced neutrophil adhesion. Kupffer cells occupy a great deal of endothelial surface area within the sinusoids, and therefore the removal of these cells may have been predicted to expose more binding sites for neutrophils to adhere to endothelium. However, removal of Kupffer cells did not result in enhanced neutrophil-endothelial interactions and instead reduced adhesion significantly through an unknown mechanism. The additive inhibition provided by CD44 blockade in Kupffer cell-depleted mice suggests that CD44-dependent adhesion and Kupffer cell-dependent adhesion are unique and independent pathways. The precise mechanism by which Kupffer cells augment neutrophil adhesion in sinusoids is unknown, but it is possible that some neutrophils adhere directly on Kupffer cells using adhesion molecules other than CD44, or that other signaling pathways in Kupffer cells (including HMGB-1 or PPARγ as has been proposed by others) lead to other unique mechanisms of adhesion. Further studies are needed to fully characterize the molecular mechanism that enables Kupffer cells to contribute to neutrophil recruitment in endotoxemic liver.

Overall, this study sheds new light on the control of innate immune activation within the vasculature of the liver during endotoxemia and sepsis. Neutrophil recruitment to the liver represents an important contributor to the intravascular immune response mounted during sepsis that protects the host against dissemination of blood-borne bacterial infections. Our

### Table 1. Serum cytokine levels following Kupffer cell depletion

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<th>UT Control</th>
<th>LPS Control</th>
<th>CLL + LPS</th>
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<td>TNF-α</td>
<td>0.78 ± 0.34</td>
<td>1.023 ± 48.9</td>
<td>977.8 ± 222.7</td>
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<tr>
<td>IL-6</td>
<td>3.52 ± 1.78</td>
<td>32.418 ± 721.3</td>
<td>30.083 ± 650.5*</td>
</tr>
<tr>
<td>KC</td>
<td>254.2 ± 78.9</td>
<td>33.014 ± 1.603</td>
<td>30.985 ± 1.808</td>
</tr>
<tr>
<td>MIP-2</td>
<td>8.9 ± 8.9</td>
<td>23.245 ± 199</td>
<td>23.140 ± 238</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4.49 ± 1.74</td>
<td>228.7 ± 32.53</td>
<td>76.6 ± 14.64</td>
</tr>
<tr>
<td>IL-12</td>
<td>20.8 ± 5.7</td>
<td>299.3 ± 40.9</td>
<td>171.6 ± 21.7*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>28.0 ± 10.5</td>
<td>25.026 ± 1.449</td>
<td>24.834 ± 247.8</td>
</tr>
<tr>
<td>IL-10</td>
<td>27.6 ± 26.1</td>
<td>2.422 ± 464</td>
<td>821.2 ± 185.2†</td>
</tr>
<tr>
<td>G-CSF</td>
<td>304.2 ± 59.3</td>
<td>61.734 ± 413.9</td>
<td>60.171 ± 881</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>60.0 ± 31.0</td>
<td>125.2 ± 23.9</td>
<td>199.5 ± 50.9</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.38 ± 3.8</td>
<td>20.0 ± 2.5</td>
<td>101.0 ± 27.9*</td>
</tr>
<tr>
<td>IP-10</td>
<td>82.8 ± 13.1</td>
<td>17585 ± 1095</td>
<td>16.781 ± 1082</td>
</tr>
<tr>
<td>RANTES</td>
<td>38.1 ± 9.8</td>
<td>686.4 ± 112.5</td>
<td>686.1 ± 108.2</td>
</tr>
<tr>
<td>IL-1α</td>
<td>19.6 ± 6.4</td>
<td>207.9 ± 12.9</td>
<td>148.3 ± 13.2†</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>58.2 ± 36.1</td>
<td>18.553 ± 3.975</td>
<td>15.664 ± 3.088</td>
</tr>
</tbody>
</table>

Concentrations (pg/ml) of various cytokines and chemokines in serum 90 min following administration of LPS (1 mg/kg) in the indicated groups of mice. Data are presented as means ± SE, UT, untreated; CLL, clodronate liposomes. *P < 0.05 (LPS control vs. CLL+LPS). †P < 0.01 (LPS control vs. CLL+LPS).
ACKNOWLEDGMENTS

The authors thank Lori Zbytnuik, Mandy Tse, and the late Carla Badick for excellent technical assistance.

GRANTS

This work was supported by grants from the Canadian Institutes for Health Research and the Canadian Foundation for Innovation. B. McDonald is supported by an MD/PhD studentship from the Alberta Heritage Foundation for Medical Research (Alberta Innovates Health Solutions). P. Kubes is an Alberta Heritage Foundation for Medical Research (AIHS) Scientist and the Snyder Chair in Critical Care Medicine.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

B.M., L.Z., K.K., and P.K. conception and design of research; B.M. and C.N.J. performed experiments; B.M., C.N.J., and P.K. analyzed data; B.M., C.N.J., and P.K. interpreted results of experiments; B.M. and P.K. prepared figures; B.M. and P.K. drafted manuscript; B.M., C.N.J., and P.K. edited and revised manuscript; B.M., C.N.J., L.Z., K.K., and P.K. approved final version of manuscript.

REFERENCES


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