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Lipopolysaccharide-Induced Leukocyte-Endothelial Cell Interactions: A Role for CD14 Versus Toll-Like Receptor 4 Within Microvessels

Graciela Andonegui,* Sanna M. Goyert, † and Paul Kubes2*

The objective of this study was to systematically assess leukocyte-endothelial cell interactions in vivo in response to LPS in CD14-deficient (CD14−/−) and Toll-like receptor 4-deficient (TLR4−/−; C3H/HeJ) mice. Local injection of LPS (0.05 μg/kg) into muscle at a concentration that did not cause systemic effects produced a significant reduction in the speed with which leukocytes roll and a substantial increase in leukocyte adhesion and emigration 4 h postinjection. There was no response to LPS in the muscle microvasculature of CD14−/− mice or TLR4−/− animals. Systemic LPS induced leukopenia and significant sequestration of neutrophils in lungs in wild-type mice but not in CD14−/− or TLR4−/− mice. P-selectin expression was examined in numerous mouse organs using a dual radiolabeling mAb technique. The results revealed a 20- to 50-fold increase in P-selectin expression in response to LPS in all wild-type tissues examined but no response in any TLR4 organs. This work was supported by Canadian Institutes of Health Research and in part by Alberta Heritage Foundation for Medical Research fellow. Within Microvessels

A localized Gram-negative bacterial infection in otherwise healthy individuals is rapidly resolved due to effective recruitment of leukocytes and in the early phase primarily neutrophils. By contrast, systemic, Gram-negative sepsis continues to elude effective therapy with 50% mortality, i.e., the death of ∼200,000 people a year in North America (1, 2). In these cases, it is the inappropriate activation of inflammatory processes including inappropriate leukocyte infiltration into tissues that causes the progression to uncontrolled body inflammation (3). It is thought that a major contributor to 1) localized infections as well as 2) the morbidity associated with systemic infections is the shedding of LPS from Gram-negative bacteria into the circulation (4). Leukocyte recruitment has generally been described as a multistep cascade involving endothelial selectins (E- and P-selectin) and leukocyte selectins (L-selectin) which permit the initial phase of leukocyte recruitment, namely transient attachment to the endothelial surface followed by leukocyte rolling along the vessel wall (5, 6). The second phase of leukocyte recruitment involves the activation of integrins that mediate firm adhesion (5, 7). In many local inflammatory conditions, including, e.g., ischemia/reperfusion of the mesentery or muscle, immunonneutralization of one or more selectins completely inhibited leukocyte rolling and therefore prevented leukocyte recruitment (8–10). Interestingly, using identical antiselctin approaches to try to inhibit LPS-induced leukocyte recruitment has proved to be far more challenging. Although we were able to delay leukocyte rolling and subsequent adhesion in mesentery with antiselctins, ultimately, inhibition of one of the integrins (αi integrin) was also necessary to prevent all leukocyte adhesion in response to LPS (11). Further complexity was identified in tissues like liver and lung where adhesion molecule inhibitor regimen did not reduce leukocyte adhesion in response to LPS (12–14). Therefore, from a therapeutic viewpoint, due to the multitude of known as well as to date unidentified adhesion mechanisms that are evoked with LPS, antiadhesion therapy may not be a viable approach to limiting leukocyte sequestration in endotoxemia. However, interruption of the initiating signal, perhaps at the level of the LPS receptor could conceivably inhibit the multitude of adhesion molecules that are activated and limit the untoward sequestration of leukocytes in the lungs as well as other vascular beds. CD14 is considered the main LPS receptor, present both as a soluble form in blood or as a membrane-bound form in myeloid lineage cells. Indeed, CD14−/− mice are at least 100 times more resistant to LPS-induced mortality, and their macrophages also have greatly reduced responsiveness as assessed by TNF-α and IL-1 production (15). Because CD14 is a GPI-anchored protein devoid of a transmembrane domain, by itself, it presumably cannot transmit the activating signal into the cell. Indeed, in vitro work has demonstrated that Toll-like receptor 4 (TLR4)3 activates the immune system by functioning as a transmembrane coreceptor to
CD14 (16, 17). Both TLR4-deficient (TLR4"/"") mice and mice with a single point mutation at 712 (proline to histidine) in the TLR4 gene (C3H/HeJ) are resistant to the immunostimulatory and pathophysiological effects of LPS (18, 19). Clearly, it seems reasonable to hypothesize that altered leukocyte-endothelial cell interactions observed in lungs and other tissues in response to LPS would be entirely dependent on CD14 and associated TLR4.

Only a few studies have focused on the role CD14 and TLR4 in leukocyte sequestration. Based on initial work by Haziot et al. (15), CD14-deficient mice had reduced dissemination of Gram-negative bacteria. These investigators went on to discover that TLR4-deficient mice as well as TLR4 mutant mice had an early and intense sequestration of neutrophils into the peritoneum, which was absent in wild-type mice. This was unexpected in light of the lack of responsiveness of these mutant mice to LPS. Therefore, we decided to systematically compare and contrast the role of CD14 and TLR4 in local and systemic LPS-induced leukocyte-endothelial cell interactions in vivo in numerous organs using two specialized techniques. First we used an in vivo assay system to visualize and study leukocyte behavior within a number of microvasculatures before and after LPS. Second, we used a sensitive, quantitative in vivo adhesion molecule expression system to examine endothelial responsiveness to LPS in all organs of CD14"/" and TLR4"/" mice. We show that TLR4"/" mice are completely resistant to LPS administration as it pertains to leukocyte-endothelial responses. By contrast, we identified that in some but not all microvascular beds partial endothelial responsiveness (protein expression and leukocyte function) to LPS was noted in CD14"/" mice.

Materials and Methods

Mice

Mice deficient in CD14 were generated by gene targeting in embryonic stem cells as previously described (15) and were backcrossed on a BALB/c background to the 10th generation. Wild-type BALB/c mice (CD14"+/") (The Jackson Laboratory, Bar Harbor, ME) were used as controls for the CD14"/" mice. TLR4"/" mice (C3H/HeJ mice) were obtained from The Jackson Laboratory. Wild-type C3H/HeN mice (TLR4"+/") (Charles River Laboratories, Montreal, Quebec, Canada) were used as controls for TLR4"/" mice. All mice weighed between 20 and 35 g and were between 6 and 10 wk of age at the time of use.

Intravital microscopy

Mice were anesthetized by i.p. injection of a mixture of xylanine hydrochloride (10 mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and xylazine hydrochloride (200 mg/kg; B.Braun, STZB, London, Ontario, Canada). The jugular vein was cannulated and used to administer additional anesthetic, fluorescent dyes, and various drugs. Animals were then prepared for viewing of either the skeletal muscle microcirculation (20) or the dermal microcirculation (21) as previously reported by our laboratory.

Cremaster muscle preparation. The cremaster muscle was dissected free of tissues and exteriorized onto an optically clear viewing pedestal. The muscle was cut longitudinally with a cautery and held flat against the pedestal by attaching silk sutures to the corners of the tissue. The muscle was then superfused with bicarbonate-buffered saline (pH 7.4).

Skin flap preparation. A midline dorsal incision was made. The skin was carefully separated from the underlying tissue, remaining attached laterally. Blood supply to the skin flap remained intact. The skin flap was then extended over a viewing pedestal, secured along the edges using 4-0 suture, exposing the dermal microvasculature. The exposed skin was continuously superfused with bicarbonate-buffered saline to avoid tissue dehydration. Due to the thickness of the skin flap, leukocyte-endothelial cell interactions were not visible by transillumination. Therefore, for this protocol, animals were injected with the fluorescent dye, rhodamine 6G (0.3 mg/kg i.v.; Sigma-Aldrich, St. Louis, MO), immediately before microscopic visualization. Rhodamine 6G at the dose used labels leukocytes and platelets, allowing detection of the same number of rolling leukocytes as transmitted light, but no effect on leukocyte kinetics (22). Rhodamine 6G-associated fluorescence was visualized by epi-illumination at 510–560 nm using a 590-nm pore size emission filter (22).

The cremaster and dermal microcirculations were observed through an intravital microscope with a ×10 eyepiece (Axioskop; Carl Zeiss Canada, Don Mills, Ontario, Canada), a ×25 objective lens for cremaster, and a ×40 objective lens for skin. A video camera (5100 HS; Panasonic, Osaka, Japan) for the cremaster preparation and a fluorescent camera (model C-2400-08; Hamamatsu Photonics, Hamamatsu City, Japan) for the skin preparation were used to project the images onto a monitor, and the images were recorded for playback analysis using a video cassette recorder. Single unbranched venules (25–40 μm in diameter) were selected, and to minimize variability the same section of the venule was observed throughout the experiment. The number of rolling and adherent leukocytes was determined off-line during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocyte rolling velocity was determined by measuring the time required for a leukocyte to roll along a 100-μm length of venule. Rolling velocity was determined for 20 leukocytes at each time interval. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 s or longer. Leukocyte emigration was defined as the number of extravascular leukocytes per microscopic field of view and was determined by averaging the data derived from four to five fields adjacent to postcapillary venules. Venular diameter (Dv) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline RBC velocity (Vrbc) was also measured on-line using a commercial Doppler velocimeter (Microcirculation Research Institute), and mean RBC velocity (Vmean) was determined as Vmean = Dv γ. Venular wall shear rate (γ) was calculated based on the Newtonian definition: γ = 8 (Vmean/Dv) (23).

Quantitation of endothelial activation

To determine the degree of endothelial activation two endothelial adhesion molecules (P-selectin and VCAM-1) known to contribute to leukocyte recruitment in endotoxemia were measured. Expression of the adhesion molecules P-selectin and VCAM-1 were quantified using a modified dual-radiolabeled Ab technique (21, 24). The Abs RB40.34 (against P-selectin) and 429 (MVCAM.A) (against VCAM-1) were labeled with 125I. The Abs A110-1 (a rat IgG1, isotype standard) and R35-95 (a rat IgG2a, isotype control Ig) were labeled with 131I. In both cases, the Abs were labeled using the IodoGen method, as previously described (21, 24). A110-1 and R35-95 were used to detect nonspecific binding in the murine system.

To study P-selectin, animals were injected i.v. with a mixture of 10 μg of 125I-labeled P-selectin (RB40.34), and a variable dose of 131I-labeled nonbinding Ab (A110-1). To measure VCAM-1, mice were injected with 10 μg of 125I-labeled anti-VCAM-1 (429 (MVCAM.A)), 25 μg unlabeled anti-VCAM-1 (429 (MVCAM.A)), and a variable dose of 131I-labeled nonbinding Ab (R35-95) calculated to achieve a total injected 131I activity of 400,000–600,000 cpm (total volume, 200 μl). This Ab combination was chosen after pilot experiments, conducted over a range of doses of unlabelled 429 (MVCAM.A), showed that this protocol ensured receptor saturation under stimulated conditions. In both cases, the Abs were allowed to circulate for 5 min; then the animals were heparinized. A blood sample was obtained from a carotid artery catheter; then the mice were exsanguinated by blood withdrawal through the carotid artery catheter and simultaneous i.v. infusion with bicarbonate-buffered saline. The lung, muscle, heart, brain, small bowel, large bowel, skin, pancreas, and liver were harvested and weighed. Both 131I and 125I activities were measured in plasma and tissue samples.

P-selectin and VCAM-1 expression was calculated per gram of tissue, by subtracting the accumulated activity of the nonbinding Ab (131I-labeled Ab) from the accumulated activity of the binding Ab (125I-labeled Ab). Data for P-selectin and VCAM-1 were presented as the percentage of the injected dose of Ab per gram of tissue. It has been previously demonstrated that this approach provides reliable quantitative values of adhesion molecule expression and that radiolabeled binding Ab can be displaced specifically with sufficient amounts of unlabeled Ab. The technique is sufficiently sensitive that very small, basal levels of P-selectin can be detected in wild-type mice relative to P-selectin-deficient mice where values are zero (24, 25).

Determination of tissue myeloperoxidase (MPO) activity

At the end of each experiment, samples of the lung were weighed, frozen on dry ice, and processed for determination of MPO activity. MPO is an enzyme found in cells of myeloid origin and has been used extensively as a biochemical marker of granulocyte (mainly neutrophil) infiltration into tissues (26, 27). The samples were stored at −80°C for no more than 1 wk before the MPO assay was performed. MPO activity was determined using an assay described previously, with the volumes of each reagent modified for use in 96-well microtitre plates (28). Change in OD450 during
a 90-s period was determined using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

Circulating leukocyte counts
At the end of each experiment, whole blood was drawn via cardiac puncture. Total leukocyte counts were performed, using a Bright-line hemocytometer (Hauser Scientific, Horsham, PA).

Experimental protocol
First, CD14<sup>d</sup>−, TLR4<sup>d</sup>, and their respective wild-type mice (CD14<sup>+/+</sup> and TLR4<sup>+/+</sup>) were prepared for intravital microscopy, and the microcirculations were examined for leukocyte-endothelial cell interactions under basal conditions. Circulating leukocyte counts as well as pulmonary MPO levels were also measured at the end of each experiment.

Local LPS administration. In all experiments, LPS from Escherichia coli 0111:B4 dissolved in nonpyrogenic water was added to 0.2 ml saline. This LPS is highly purified with <0.0008% of contaminating bacterial proteins (15, 29). In these studies, LPS was administered locally by s.c. injection beneath the scrotal skin using a 30-gauge needle. Animals were returned to their cages for 3.5 h. The right cremaster muscle was then prepared for intravital microscopy and leukocyte-endothelial cell interactions, and hemodynamic parameters in single postcapillary venules were examined. Preliminary experiments indicated that local administration of 0.05 μg/kg LPS was optimal for examination of leukocyte-endothelial interaction. To examine an LPS-independent inflammatory response, the effect of local administration of TNF-α (0.5 μg) was examined in all mouse strains.

Systemic (i.p.) LPS administration. Mice received 0.5 mg/kg (∼12.5 μg/mouse) of the highly purified LPS i.p. This dose was chosen to avoid any mortality over the study period even after anesthesia. Moreover, preliminary dose-response studies revealed that higher doses of LPS administered i.p. resulted in stagnant peripheral microvascularities. In one set of experiments, we evaluated the expression of P-selectin adhesion molecule in different mouse organs induced by LPS after 4 h treatment by using a dual-radiolabeled Ab technique. To examine the peripheral microvascularity directly, the dorsal skin or the cremaster muscle was prepared for visualization by intravital microscopy and observed for 60 min between 3.5 and 4.5 h post-LPS administration. In all cases, LPS-induced leukocyte sequestration was examined in CD14<sup>d</sup>−, TLR4<sup>d</sup>, and their respective wild-type mice (CD14<sup>+/+</sup> and TLR4<sup>+/+</sup>).

Statistical analysis
All data are displayed as mean ± SEM. All data were analyzed using Student’s t test, and a Bonferroni correction was applied where multiple comparisons were necessary. A value of p < 0.05 was deemed significant.

Results
TLR4<sup>d</sup> and CD14-deficient mice are completely resistant to the effects of local LPS in the cremaster microcirculation
First, we chose to examine a normal inflammatory response to local LPS administration (0.05 μg/kg). This concentration of LPS had absolutely no effect on hemodynamic parameters (data not shown) but induced profound changes in leukocyte-endothelial cell interactions in wild-type mice (Fig. 1). In untreated vessels of all four groups of mice (BALB/c (CD14<sup>+/+</sup>), CD14<sup>−/−</sup>, C3H/HeN (TLR4<sup>+/+</sup>), C3H/HeJ (TLR4<sup>−/−</sup>)), ∼50–100 leukocytes can be seen rolling through postcapillary venules every min (Fig. 1A). These cells roll at relatively high velocities (40–80 μm/s) (Fig. 1B). Fewer than three cells are seen adhering (Fig. 1C) in the postcapillary venules. Local administration of LPS into muscle significantly decreased leukocyte rolling velocity in the muscle postcapillary venules of wild-type mice (CD14<sup>+/+</sup> and TLR4<sup>+/+</sup>), suggesting activation of the local vasculature in these mice (Fig. 1B). Interestingly, absolutely no change in the rolling velocity of leukocytes was noted in postcapillary venules of CD14<sup>−/−</sup> and TLR4<sup>−/−</sup> mice (Fig. 1B). The flux of rolling leukocytes was not altered by the dose of LPS used in this study (Fig. 1A). Unlike local LPS, one feature of systemic LPS is a profound drop in both the circulating leukocytes and the flux of rolling leukocytes (later section) in wild-type mice.

Hemodynamic parameters in response to systemic LPS
In preliminary data, we observed profound reductions in blood flow using concentrations of LPS higher than 0.5 mg/kg i.p. Therefore, we chose 0.5 mg/kg (∼12.5 μg/mouse) LPS, which induces leukocyte sequestration without affecting hemodynamic parameters including venular diameter, RBC velocity, and calculated wall shear rates.
Table II. Hemodynamic parameters in CD14<sup>+/−</sup> and TLR4<sup>d</sup> mice and their control CD14<sup>+/+</sup> and TLR4<sup>+/+</sup> mice, respectively, with or without LPS treatment<sup>a</sup>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD14&lt;sup&gt;+/+&lt;/sup&gt; Mice</th>
<th>CD14&lt;sup&gt;+/−&lt;/sup&gt; Mice Treated with LPS</th>
<th>CD14&lt;sup&gt;+/−&lt;/sup&gt; Mice</th>
<th>TLR4&lt;sup&gt;d&lt;/sup&gt; Mice Treated with LPS</th>
<th>TLR4&lt;sup&gt;d&lt;/sup&gt; Mice</th>
<th>CD14&lt;sup&gt;+/+&lt;/sup&gt; Mice Treated with LPS</th>
</tr>
</thead>
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<tr>
<td>Venular diameter (μm)</td>
<td>30 ± 2</td>
<td>33 ± 2</td>
<td>29 ± 2</td>
<td>29 ± 1</td>
<td>33 ± 2</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>RBC velocity (mm/s)</td>
<td>2.3 ± 0.6</td>
<td>2.3 ± 0.4</td>
<td>2.7 ± 0.6</td>
<td>2.8 ± 0.8</td>
<td>2.1 ± 0.4</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Wall shear rate (s&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>372 ± 88</td>
<td>344 ± 72</td>
<td>470 ± 106</td>
<td>359 ± 89</td>
<td>318 ± 51</td>
<td>349 ± 75</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice were treated with 0.5 mg/kg LPS i.p. for 3.5 h. At this time for 1 h, the cremasteric postcapillary venules were visualized by intravital microscopy. Data are expressed as the arithmetic mean ± SEM from three mice in each group.

Endothelium from CD14<sup>+/−</sup> but not TLR4<sup>d</sup> mice showed P-selectin increase induced by systemic LPS

Leukocyte recruitment into peripheral tissues is dependent on the ability of the endothelium to express selectins to allow for leukocyte rolling followed by subsequent integrin-dependent leukocyte adhesion. P-selectin expression was used as a marker of endothelial activation in all vasculatures of wild-type and mutant mice, because this molecule was previously shown to be an important selectin in endotoxin-induced leukocyte sequestration.
TLR4−/− but not CD14−/− mice are completely resistant to the effects of systemic LPS in the skin microcirculation

To assess whether the organ-specific, differential adhesion molecule responsiveness in different microvasculatures translated to altered leukocyte function, we directly visualized leukocyte trafficking in skin and muscle postcapillary venules using intravital microscopy. LPS induced in both wild-type mice (CD14+/+ and TLR4+/+), a >90% reduction in leukocyte rolling flux within the skin (Fig. 6A), which may reflect our work (Fig. 2) and previous reports of leukocytopenia (3, 13). By contrast, LPS did not alter the flux of rolling cells in either CD14−/− or TLR4−/− mice treated with LPS (Fig. 6A). The velocity of leukocyte rolling is indicative of increased tether upon the leukocyte by activated endothelium (expression of selectins and chemokines). A very significant reduction in leukocyte rolling velocity was noted in CD14+/+ mice (Fig. 6B). If LPS had no impact in skin vasculature of CD14−/− mice, no reduction in rolling velocity would be expected. However, in CD14−/− mice, the velocity of rolling cells was significantly reduced, indicating that the endothelium was activated in CD14−/− skin postcapillary venules (Fig. 6B). No decrease in rolling velocity was seen in TLR4−/− skin postcapillary venules. In the two strains of wild-type mice, leukocyte adhesion increased 5- to 6-fold (Fig. 6C). Most importantly, a significant increase in leukocyte adhesion was noted in CD14−/− mice (Fig. 6C) consistent with the increased adhesion molecule expression in skin microvasculature. The latter responses were completely absent in TLR4−/− mice (Fig. 6C) consistent with the view that TLR4 is absolutely essential in LPS signaling even in tissues where CD14 was not needed.

TLR4−/− and CD14-deficient mice are completely resistant to the effects of systemic LPS in the cremaster microcirculation

Next, we examined leukocyte trafficking in the muscle microvasculature wherein no endothelial activation (no P-selectin) was detected in CD14−/− or TLR4−/− mice stimulated with LPS. Under basal conditions, ~50–100 cells/min rolled through microvessels (Fig. 7A). By contrast after LPS treatment, we noted that both CD14+/+ and TLR4+/+ mice had a profound reduction in leukocyte rolling flux within the cremaster (Fig. 7A), similar to that seen in the skin. The velocity of leukocyte rolling, which is indicative of local endothelial activation, was reduced by 95% in both wild-type mice strains after 4 h LPS administration (Fig. 7B). In contrast, neither the flux of rolling cells nor the velocity of these cells was altered in either CD14−/− or TLR4−/− mice, suggesting that the endothelium was not activated in the postcapillary venules of these

FIGURE 3. P-selectin expression in lung, muscle, heart, brain, small bowel, and large bowel in CD14−/−, TLR4−/−, and their wild-type mice, respectively. Mice were treated with 0.5 mg/kg LPS i.p. for 4 h. After this time, P-selectin expression was quantified using a modified dual-radiolabeled Ab technique (Materials and Methods). Data are expressed as the arithmetic mean ± SEM from five mice in each group. %I.D., percentage of the injected dose. *p < 0.01 vs untreated mice.
mice. It is interesting that in the muscle postcapillary venules, very few cells ultimately adhered in response to systemic LPS (Fig. 7C) in either wild-type or the mutant mouse strains. Only a 2- to 3-fold increase in adhesion was noted perhaps due to less effective synthesis of proinflammatory molecules (chemokines, etc.) in the muscle vasculature. The same scale is used as in Fig. 1C, highlighting the far more effective adhesion of leukocytes in local (Fig. 1) than in systemic (Fig. 7) LPS. Regardless, the data in Fig. 7

FIGURE 4. P-selectin expression in skin and pancreas in CD14−/−, TLR4−/− and their wild-type mice respectively. Mice were treated with 0.5 mg/kg LPS i.p. for 4 h. After this time, P-selectin expression was quantified using a modified dual-radiolabeled Ab technique (Materials and Methods). Data are expressed as the arithmetic mean ± SEM from five mice in each group. %I.D., percentage of the injected dose. *, p < 0.01 vs untreated mice.

FIGURE 5. VCAM-1 expression in pancreas, muscle, lung and small intestine in CD14−/−, TLR4−/− and their wild-type mice respectively. Mice were treated with 0.5 mg/kg LPS i.p. for 4 h. After this time, VCAM-1 expression was quantified using a modified dual-radiolabeled Ab technique (Materials and Methods). Data are expressed as the arithmetic mean ± SEM from five mice in each group. *, p < 0.01 vs untreated mice.

FIGURE 6. Effect of systemic LPS on leukocyte kinetics in dermal skin microcirculation in CD14−/−, TLR4−/−, and their wild-type mice, respectively. Mice were treated with 0.5 mg/kg LPS i.p. for 3.5 h. At this time for 1 h, the dermal postcapillary venules were visualized by intravital microscopy. Results represent the leukocyte kinetics observed at 4.5 h and show leukocyte rolling flux (A), leukocyte rolling velocity (B), and leukocyte adhesion (C) in postcapillary venules of CD14−/− mice, TLR4−/− mice, and their wild-type mice, CD14+/+ and TLR4+/+, respectively. Data are expressed as the arithmetic mean ± SEM from five mice in each group. *, p < 0.01 vs untreated mice. In TLR4+/+, no rolling was seen at 4.5 h, but small amounts of rolling were seen at early time points.
cumulation into this tissue (12, 13). This is thought to be due to physical trapping of activated leukocytes within this microvascular bed due to the more narrow architecture of pulmonary capillaries (26, 27). Wang et al. (30) have reported that the endothelium increases in rigidity and perhaps contributes to the physical trapping of the general circulating pool of leukocytes. There may also be a direct increase in leukocyte rigidity in response to LPS and thereby increased sequestration within the pulmonary microvascular bed (30). Clearly, it is not surprising that some studies have reported only partial or no decrease in LPS-induced leukocyte sequestration with anti-adhesion therapy (13, 31). In this study, we for the first time have identified a genetic intervention (CD14<sup>−/−</sup> or TLR4<sup>−/−</sup>) that completely prevents any systemic LPS-induced leukocyte sequestration into the pulmonary microvasculature. Clearly, LPS stimulates the CD14 and TLR4 complex, which activates both leukocytes and endothelium to induce adhesion and/or physical trapping within the pulmonary microvasculature.

Although at least 10 TLRs have been identified, TLR2 and TLR4 have received the most attention as recognition receptors of distinct bacterial cell wall components and more specifically LPS (19, 32). Much evidence suggests that TLR4 is the dominant LPS TLR. Initial work demonstrated that LPS stimulated NF-κB-mediated gene expression in HEK 293 cells only when those cells were transfected with TLR4 cDNA (33). Mouse strains that lacked functional TLR4 (C3H/HeJ and C57BL/10ScCr) revealed resistance to LPS assessed as a lack of cytokine production and no mortality (18). More recently, TLR4<sup>−/−</sup> mice showed hyporesponsiveness to LPS to an extent similar to that of C3H/HeJ mice (19). Although TLR2 was initially also thought to function as an LPS receptor, this was likely a result of contaminant found in commercial LPS preparations (29, 34). Current evidence argues against a major role for TLR2 in the physiological response to LPS (19, 35, 36). For example, TLR2<sup>−/−</sup> mice have normal cytokine profiles and a similar degree of mortality in response to LPS (19). Our study examined leukocyte-endothelial cell interactions directly with highly purified LPS, and the data support the view that TLR2 is not involved in LPS-induced leukocyte sequestration inasmuch as in the absence of functional TLR4 there was no detectable sequestration of leukocytes or endothelial activation. Clearly, our data are in agreement that TLR4 is the only TLR involved in LPS-mediated leukocyte-endothelial cell interactions.

Although the majority of work regarding TLR4 and CD14 has been performed in isolated macrophages, which are definitely activated during endotoxemia, it is quite likely that the early vascular responses to LPS occur via a direct effect of LPS on endothelium. Indeed, LPS will rapidly activate endothelium to induce synthesis of proadhesive molecules that sequester leukocytes independent of macrophages (37). Although endothelium also expresses TLR4 (38–40), unlike monocytes and neutrophils, endothelium is thought not to constitutively express membrane CD14 (41–44). However, this cell will respond to LPS via a soluble form of CD14 (sCD14) present in blood (45–48). Therefore, in the presence of plasma proteins, endothelium has all of the machinery necessary to rapidly respond to LPS and synthesize P-selectin, E-selectin, VCAM-1, and ICAM-1 as well as chemokines to induce leukocytes to roll, adhere, and ultimately emigrate into tissues. Our data would suggest that much like macrophages, endothelium responds to LPS exclusively via TLR4 to induce leukocyte sequestration.

Although this is to our knowledge the first report of LPS-induced leukocyte sequestration via a CD14-independent, TLR4-dependent pathway, a number of in vitro studies support this view. For example, Tsan et al. (49) reported that LPS was unable to induce TNF and manganese superoxide dismutase in isolated, CD14<sup>−/−</sup> peritoneal macrophages at low concentrations but could...
induce TNF and manganese superoxide dismutase at higher concentrations of LPS. Similar concentration-dependent findings have been reported in peritoneal macrophages for TNF message and for certain LPS-inducible genes including IL12 (p35 and p40) and COX-2 (50). Finally, in the original study by Haziot et al. (15), CD14−/− mice produced no cytokines at 20 mg/kg LPS but could produce IL-6 but not TNF at 200 mg/kg. To avoid concentration-dependent effects, we used 0.5 mg/kg LPS in this study (400-fold less), which did not induce cytokine production in CD14−/− mice (15) yet in the dermal microcirculation induced an increase in leukocyte-endothelium interactions. One could argue that, because we injected LPS i.p., CD14−/− peritoneal macrophages were exposed to relatively high concentrations of LPS, inducing release of cytokines that would have activated distal vasculatures. However, this seems very unlikely because 1) we used 400-fold less LPS than is required to induce cytokine production from CD14−/− macrophages, 2) no systemic signs of inflammation were noted, and 3) the activation was site specific, i.e., occurred in skin but not muscle or lung.

Interestingly, TLR4α mice were resistant to LPS responses that occurred independent of CD14. For example, TLR4α mice were resistant to the increase in P-selectin expression in all the tested organs; in the skin microcirculation, TLR4α mice were completely devoid of leukocyte effects in response to systemic LPS (Fig. 6). Clearly, our data suggest a very sensitive second CD14-independent, TLR4-dependent pathway of leukocyte sequestration. The selectivity of this mechanism in skin rather than lung or muscle may reflect LPS-binding proteins on dermal endothelium or extravascular cells (dermal macrophages, fibroblasts, keratinocytes) that have evolved alternative LPS detection systems.

Potential candidates as substitutes for CD14 include β2 integrins (CD11/CD18), the macrophage scavenger receptor and L-selectin, that have evolved alternative LPS detection systems. In this study, we have for the first time described in vivo an LPS-induced leukocyte sequestration via a CD14-independent, TLR4-dependent pathway in skin. In addition, we have reported that the absence of CD14 or TLR4 prevents any leukocyte sequestration into the lungs and muscle. Thus, taken together, our results suggest that there are CD14-dependent and CD14-independent responses to LPS, but in both cases the responses are dependent on the presence of TLR4, suggesting that other proposed LPS receptors besides CD14 likely require TLR4 in the leukocyte sequestration response.

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References


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