

Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs

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The rapid and selective accumulation of neutrophils into the lungs is thought to underlie the pulmonary failure that leads to sepsis-related death. In this study we investigated whether neutrophil TLR4 is important in LPS-induced pulmonary neutrophil recruitment by creating chimeric mice (transferring bone marrow between *TLR4*^{+/+} and *TLR4*^{-/-} mice). In *TLR4*^{+/+} mice receiving *TLR4*^{-/-} bone marrow, 6 weeks after transplant TLR4 was absent in all circulating leukocytes as well as in resident macrophages (these mice were termed *LeukocyteTLR4*^{-/-}), and these cells were completely nonresponsive to LPS. In *TLR4*^{-/-} mice receiving *TLR4*^{+/+} bone marrow, endothelial cells but not leukocytes were deficient in TLR4 (*EndotheliumTLR4*^{-/-}). Surprisingly, systemic LPS (0.5 mg/kg) induced a dramatic increase in neutrophil sequestration into the lungs of *LeukocyteTLR4*^{-/-} mice over the first 4 hours. Concomitantly, numbers of circulating leukocytes decreased by 90%. By contrast, *EndotheliumTLR4*^{-/-} mice showed very little increase in neutrophil sequestration in the lungs, suggesting that endothelium rather than leukocyte TLR4 was important. Intravital microscopy of peripheral microcirculation in the cremaster muscle revealed about 30-fold more leukocyte-endothelial cell interactions in LPS-treated *EndotheliumTLR4*^{-/-} mice than in LPS-treated *LeukocyteTLR4*^{-/-} mice. This is consistent with less sequestration of leukocytes into the lungs of *EndotheliumTLR4*^{-/-} mice. In conclusion, our data challenge the view that LPS directly activates neutrophils to trap in lungs and suggest a far more important role than previously appreciated for the endothelial cells.

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Introduction

Gram-negative septicemia continues to elude effective therapy with 50% mortality, translating into the death of approximately 400,000 North Americans per year (1, 2). A consistent finding in rodent models of sepsis and septic patients is that, regardless of the organ in which the sepsis originates, the lungs are generally the first to fail (3). Consequently, pulmonary failure remains the most common cause of sepsis-related death. A key event that, in part, is thought to explain this pathology is the rapid accumulation of neutrophils in the narrow lumen of lung capillaries. Indeed, depletion of neutrophils in animal models preserves the lung during endotoxemia (4).

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Nonstandard abbreviations used: Toll-like receptor-4 (TLR4); myeloperoxidase (MPO); intraperitoneal (i.p.); bronchoalveolar lavage (BAL); mean channel fluorescence (MCF).

However, the mechanism by which these neutrophils sequester in the lungs remains poorly understood.

A major factor contributing to the inappropriate neutrophil infiltration into the lungs is the shedding of LPS from Gram-negative bacteria into the circulation. This proinflammatory molecule may activate macrophages as well as circulating neutrophils and the endothelium of various vascular beds. LPS-induced activation of mammalian cells occurs through Toll-like receptor-4 (TLR4), the dominant LPS receptor. Macrophages from C3H/HeJ and C57BL/10ScCR mice, which exhibit missense and deletion mutations, respectively, in the *tlr4* gene, and macrophages from TLR4-deficient mice are completely nonresponsive to LPS (5, 6). Although the majority of work to date has focused on macrophages, neutrophils and endothelium also express TLR4. Recently we reported that within 4 hours of systemic LPS administration, there is a profound neutropenia and most of the neutrophils preferentially sequester into lungs (7). This occurred despite profound global activation of all vascular beds, not just the lung. Mice lacking the LPS signaling machinery had absolutely no recruitment of neutrophils into lungs, no neutropenia, and no increase in endothelial adhesion molecule expression (7). Clearly, TLR4 on macrophages, neutrophils, and/or endothelium must be the motor for the

inappropriate recruitment of neutrophils into lungs and away from the periphery; however, the exact cell that regulates this important TLR4-dependent event remains unknown.

In this study, we examined the importance of LPS-induced activation of leukocytes versus LPS-induced activation of noncirculating cells (e.g., endothelium). We made chimeric mice and compared the role of functional TLR4 on bone marrow-derived cells (circulating leukocytes and macrophages) and cells not derived from bone marrow (the endothelium) in LPS-induced neutrophil recruitment into lungs. Next, we used a quantitative *in vivo* adhesion molecule expression system to examine whether TLR4 expression by either circulating leukocytes or noncirculating cells influenced endothelial responsiveness to LPS in different organs. Unexpectedly, our data challenge the view that LPS directly activates circulating neutrophils, causing their recruitment into the pulmonary microvasculature, and suggest a more important role than previously appreciated for endothelial cells.

Methods

Mice. C3H/HeJ and TLR4 KO mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA), and C3H/HeN and C57BL/6 mice were purchased from Charles River Laboratories (Montreal, Quebec, Canada). All mice were maintained in a pathogen-free facility until they weighed 20–35 g and were 6–10 weeks old, at which time they were used. To ensure that our model of pulmonary neutrophil recruitment was not simply a selectin- and integrin-dependent (adhesion-independent) model, we obtained E/P-selectin KO and CD18 KO mice, a generous gift from R.G. Collins and A.L. Beaudet (Baylor College of Medicine, Houston, Texas, USA), and subjected them to the same model of endotoxemia as we did the TLR4 chimeric mice.

Bone marrow transplantation. Briefly, bone marrow chimeras were generated following a standard protocol previously described by researchers from our laboratory (8, 9). Two sets of chimeras were generated for this study. The first set used C3H/HeN and C3H/HeJ mice. The second set used C57BL/6 and TLR4 KO mice to ensure that the spontaneously occurring mutants and generated knockouts had similar phenotypes. Bone marrow was isolated from mice euthanized by spinal cord displacement. Recipient mice were irradiated with 2 doses of 5 Gy (Gammacell 40 ¹³⁷Cs γ -irradiation source; Nordion International, Kanata, Ontario, Canada). An interval of 3 hours was allowed to pass between the first and second irradiations. Next, 8×10^6 donor bone marrow cells were injected into the tail vein of recipient irradiated mice. The mice were kept in microisolator cages for 8 weeks to allow full humoral reconstitution. This protocol previously confirmed that about 99% of leukocytes in Thy1.1 and Thy1.2 congenic recipient mice were from donor bone marrow (8).

Determination of lung myeloperoxidase activity. Lung myeloperoxidase (MPO) activity was used as a biochem-

ical index of neutrophil recruitment into lungs. 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 the lung (10). At the end of each experiment, lung samples were weighed, frozen, and processed for determination of MPO activity. The samples were stored at -20°C for no more than 1 week before the MPO assay was performed as previously described (10, 11), with the volumes of each reagent modified for use in 96-well microtiter plates. Change in absorbance at 450 nm over a 60-second period was determined using a kinetic microplate reader (Molecular Devices Corp., Sunnyvale, California, USA).

Lung histology. Untreated mice or mice treated for 30 minutes, 4 hours, or 12 hours with LPS (*Escherichia coli* 0111:B4; Calbiochem-Novabiochem Corp., San Diego, California, USA) were sacrificed, and lungs were fixed with 10% formalin via tracheal injection for 1 hour, harvested, and resuspended in 10% formalin. Formalin-fixed tissues were embedded in paraffin. Four-micrometer-thick sections were stained with H&E and chloroacetate esterase staining (Leder stain) for neutrophils. The sections were analyzed by light microscopy in a blinded fashion by a pathologist (F. Green). Neutrophil numbers were determined by counting the number of positive-stained cells over 20 fields at a magnification of $\times 40$. The mean number of positive cells per high-power field was then calculated.

Lung electron microscopy. Untreated mice or mice treated for 30 minutes, 4 hours, or 12 hours with LPS were sacrificed, and lungs were harvested as above using 2.5% glutaraldehyde and processed for electron microscopy as previously described (12). Briefly, samples were postfixed for 2 hours at 4°C with 1% osmium tetroxide and subsequently dehydrated in a graded series of acetone solutions. Tissues were then embedded in Epon 812, and ultrathin sections were obtained using an ultramicrotome equipped with a diamond knife (Ultracut E; Reichert-Jung, Vienna, Austria). Sections were stained with uranyl acetate and lead citrate and then viewed with a Hitachi H-7000 electron microscope (TEM Hitachi H-7000, Tokyo, Japan).

Quantification of P-selectin expression. Expression of P-selectin was determined as a measure of endothelial activation using a modified dual-radiolabeled Ab technique (13, 14). The Ab's RB40.34 (against P-selectin; Pharmingen, San Diego, California, USA) and A110-1 (a rat IgG₁, λ isotype control; Pharmingen) were labeled with ¹²⁵I or ¹³¹I, respectively, using the Iodogen (Pierce Chemical Co., Rockford, Illinois, USA) method as previously described (13, 14). A110-1 was used to control for nonspecific binding in the murine system.

To determine P-selectin expression, animals were injected *i.v.* with a mixture of 10 μg ¹²⁵I-RB40.34 and a variable dose of ¹³¹I-A110-1 calculated to achieve a total injected ¹³¹I activity of 400,000–600,000 cpm (total volume 200 μl). The Ab's were allowed to circulate for 5 minutes; then the animals were treated with heparin. A blood sample was obtained from the carotid artery

catheter, and the mice were exsanguinated. The lungs, heart, liver, mesentery, small intestine, large intestine, muscle, skin, and stomach were harvested and weighed. ^{131}I and ^{125}I were measured in plasma and tissue samples. P-selectin expression was calculated per gram of tissue, by subtraction of the accumulated activity of the nonspecific Ab (^{131}I -A110-1) from the accumulated activity of the P-selectin Ab (^{125}I -RB40.34). P-selectin data are represented 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 basal levels of P-selectin can be detected in WT mice relative to P-selectin-deficient mice (8, 13, 14).

Labeling of cells for flow cytometric analysis. To quantify the degree of neutrophil activation in the circulation in response to LPS, levels of L-selectin and CD11b expression were measured using flow cytometry. Briefly, whole blood was collected by cardiac puncture from mice treated with LPS for 4 hours using a 1-ml insulin syringe precoated with heparin. The blood (100 μl) was stained with 1 μg of mAb against L-selectin (MEL-14 rat anti-mouse; Pharmingen), CD11b (Mac-1 rat anti-mouse; Pharmingen), or a nonspecific isotype control (R35-95; Pharmingen) for 30 minutes at room temperature. Red blood cells were lysed with OptiLyse B (Immunotech, Marseille, France), and leukocytes were incubated with FITC-conjugated polyclonal goat anti-rat Ig (Pharmingen) for 30 minutes at room temperature. The cells were washed, resuspended in PBS/0.5% BSA/20 mM glucose solution, and read on a BD FACScan flow cytometer (BD Biosciences, Mountain View, California, USA) using CellQuest Pro software (Becton Dickinson Immunocytometry Systems). Data were compared with results from mice that did not receive LPS.

In a separate set of experiments, we examined *in vitro* direct LPS stimulation of neutrophils from chimeric mice. Using L-selectin shedding as an endpoint, this series of experiments also ensured that neutrophils of the chimeric mice were replaced by bone marrow transplantation and responded to LPS appropriately. From the chimeric mice, 100 μl of whole blood was incubated without or with LPS (100 $\mu\text{g}/\text{ml}$) for 30 minutes in a 37°C shaking water bath. OptiLyse B was used to lyse rbc's, and leukocytes were then stained with L-selectin or isotype control Ab's as described above.

Bronchoalveolar lavage and lung macrophage isolation. To determine the phenotype of lung macrophages ($\text{TLR4}^{+/+}$ or $\text{TLR4}^{-/-}$) in the chimeric mice, these cells were isolated and probed for responses to LPS. Mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of 10 mg/kg xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Rogar/STB, London, Ontario, Canada) and secured on a dissecting board, and the tra-

chea was exposed. Bronchoalveolar lavage (BAL) was performed by slow delivery of up to 1 ml warm saline (~37°C) into the mouse trachea. The fluid was slowly withdrawn by gentle suction immediately after delivery, and this procedure was repeated seven times. BAL was stored on ice until further processing. The lungs were filled with 1 ml Dispase (GIBCO BRL; Invitrogen Life Technologies Inc., Burlington, Ontario, Canada) via the tracheal catheter and allowed to collapse naturally, expelling part of the Dispase. Next, 450 μl 1% low-melting-temperature agarose (warmed to 45°C) was slowly infused via the catheter into the lungs that were covered with crushed ice and cooled for 2 minutes. The lungs were suspended in Dispase for 45 minutes at room temperature, then transferred into DMEM containing 0.01% DNaseI before the tissue was carefully teased away from the airways. The resulting cell suspension was filtered through 100- μm and 40- μm nylon mesh before the cells were pelleted and resuspended in 10 ml DMEM. Single-cell BAL and lung digest suspensions were seeded into 24-well plates at a concentration of 2×10^6 cells per ml for 60 minutes at 37°C. Lung macrophages were isolated by washing away the non-adherent cells in the wells with eight 1-ml rinses of DMEM. Adherent lung macrophages were then cultured with or without 10 $\mu\text{g}/\text{ml}$ LPS for 18 hours. Culture supernatants were removed, and cells were lysed in TRIzol reagent (GIBCO BRL; Life Technologies Inc.).

RT-PCR of iNOS gene expression was used as an index of responsiveness in isolated lung macrophages. Total RNA was isolated from the macrophages using the RNeasy Mini Kit (QIAGEN Inc., Valencia, California, USA). RT-PCR was performed using the OneStep RT-PCR kit (QIAGEN Inc.). The primer sequences used for amplifying iNOS cDNA were sense, 5'-TCAC-TGGGACAGCACAGAAT-3', and antisense, 5'-TGAAGC-CATGACCTTTCGCATTAGCATG-3', with a PCR product size of 1,423 bp. GAPDH cDNA was coamplified as an internal control using the following primer sequences: sense, 5'-CGGAGTCAACGGATTGGTCGTAT-3', and antisense, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3', with a final PCR product size of 302 bp. The RT-PCR conditions were optimized so that both iNOS and GAPDH mRNAs were expanded linearly for 35 cycles, as follows: 100 ng total RNA, 0.5 μM of each iNOS primer, 0.2 μM of each GAPDH primer. PCR products were electrophoresed through 2% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Bands were visualized and analyzed using a Fluor-S MAX MultiImager (Bio-Rad Laboratories Inc., Hercules, California, USA).

Murine pulmonary endothelial cell isolation. Lungs were harvested from control or TLR4 chimeric mice and finely minced with scalpels in HBSS. The tissue was suspended in 0.25% collagenase type II (250 U/mg; Worthington Biochemical Corp., Lakewood, New Jersey, USA) in HBSS with 2% FBS at 37°C for 45 minutes, with vortexing at 10-minute intervals. The resulting cell suspension was immediately filtered through a 100- μm mesh and washed in PBS. The dissociated cells

were resuspended in 100 μ l OptiLyse B solution as a fixative agent. Following a 10-minute incubation, 1 ml ddH₂O was added to lyse the contaminating rbc's. Cells were washed and stained using phycoerythrin:TLR4 (PE:TLR4) mAb (MTS510; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) and FITC:vascular endothelial-cadherin (VE-cadherin) polyclonal Ab (Bender MedSystems, Vienna, Austria) as a marker of endothelial cells, or appropriate isotype controls. Analysis was performed using a BD FACScan and CellQuest Pro software (BD Biosciences).

Intravital microscopy. Mice were anesthetized by i.p. injection of a mixture of 10 mg/kg xylazine and 200 mg/kg ketamine hydrochloride. The left jugular vein was cannulated to administer anesthetic. To view the cremaster muscle microcirculation, an incision was made to separate the scrotal skin from the associated fascia, and a second incision was made on the ventral surface of the cremaster muscle. The testicle and epididymis were separated from the underlying muscle and reintroduced into the abdominal cavity. The muscle was then spread out over an optically clear viewing pedestal and secured along the edges with 4-0 suture. The exposed tissue was superfused with warm bicarbonate-buffered saline (pH 7.4). The microcirculation was observed through an intravital microscope with a $\times 10$ eyepiece and a $\times 25$ objective lens (Axioskop; Carl Zeiss Canada Ltd., Don Mills, Ontario, Canada). The microcirculation was recorded using a video camera (Panasonic 5100 HS; Panasonic, Osaka, Japan). Images of the microcirculation were recorded for 1 hour in untreated mice and 4 hours after LPS stimulation. All experimental parameters were quantified at these time points.

Single unbranched venules (25–40 μ m in diameter) were selected, and to minimize variability, the same section of the venule was observed throughout the experiment. Venular diameter was measured using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas, USA). The number of rolling and adherent leukocytes was determined offline during video playback analysis. Rolling leukocytes were defined as those leukocytes that rolled at a velocity slower than that of erythrocytes within a given vessel. Leukocyte rolling velocity was measured for 20 leukocytes and was determined as the time required for a leukocyte to traverse a 100- μ m length of venule.

Experimental protocol. In all experiments, mice received 0.5 mg/kg (approximately 12.5 μ g/mouse) of purified LPS i.p. This dose was chosen to avoid any mortality during the study period, even following anesthesia. Initially, we used highly purified LPS (*E. coli* 0111:B4) with less than 0.0008% contaminating bacterial proteins, provided by S.M. Goyert (Division of Molecular Medicine, North Shore University/New York University School of Medicine, Manhasset, New York, USA) (15). As our data were identical with this LPS and with purified LPS from Calbiochem EMD Biosciences. (*E. coli* 0111:B4), we used the latter in most studies. At 4 hours, in separate groups of mice, tissue expression of

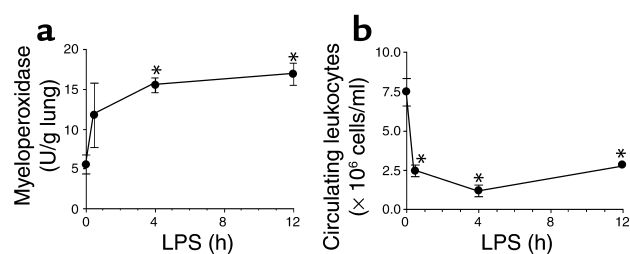


Figure 1

Systemic LPS induces neutrophil sequestration into the lungs and reduces the number of circulating leukocytes in *TLR4*^{+/+} mice. Mice were untreated or treated with LPS for 30 minutes, 4 hours, or 12 hours. At these times the lungs were harvested to determine MPO levels (a), and samples of blood were drawn by cardiac puncture to assess the number of circulating leukocytes (b). Data are expressed as the arithmetic mean \pm SEM of four to five mice per group. **P* < 0.05 vs. untreated mice (0 hours).

P-selectin was quantified, lung tissue was harvested for MPO analysis, and pulmonary macrophages were assessed for TLR4 responsiveness. Since platelets can also be a source of P-selectin, in some P-selectin expression experiments *EndotheliumTLR4*^{-/-} mice received 50 μ l/mouse of rabbit anti-mouse thrombocyte serum (Accurate Chemical and Scientific Corp., Westbury, New York, USA) i.p. 1 hour before LPS treatment. The anti-mouse thrombocyte serum depleted circulating platelets by 96%. Finally, in some experiments, 4 hours after LPS administration, intravital microscopy was used to examine the peripheral microvasculature of the cremaster muscle.

Statistical analysis. All results are expressed as mean \pm SEM. A Student's *t* test with Bonferroni correction was used for multiple comparisons. Statistical significance was set at *P* < 0.05.

Results

LPS induces neutrophil accumulation in the lungs of *TLR4*^{+/+} mice. The effect of systemic LPS on neutrophil sequestration into the lungs was assessed by the MPO assay. Treatment of *TLR4*^{+/+} mice (C57BL/6) with 0.5 mg/kg LPS i.p. for 30 minutes, 4 hours, and 12 hours resulted in a significant increase in MPO levels over time (Figure 1a). This increase in MPO values was accompanied by a significant decrease in circulating-leukocyte counts, which was observed as early as 30 minutes, and for as long as 12 hours, after LPS treatment (Figure 1b). Lung sections stained for neutrophil esterase showed an increase in the number of neutrophils sequestered into the lungs (Figure 2a, right panel). Blinded quantification of the lung sections revealed a significant increase in the number of neutrophils sequestered in capillaries per high-power field (Figure 2b). There was also a very small increase in neutrophil numbers in the alveolar space as well as the venule and arteriole compartments following LPS treatment (Figure 2b). The neutrophils were not found in airways, arteries, or veins of untreated or LPS-treated *TLR4*^{+/+} mice (Figure 2b).

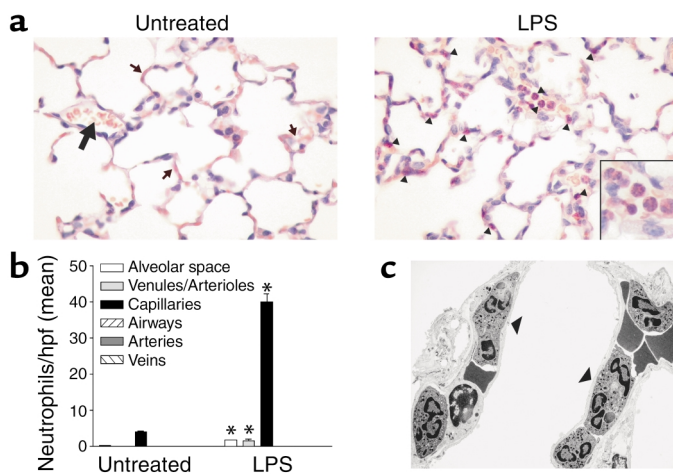


Figure 2

LPS-induced neutrophil sequestration into the lung capillaries of $TLR4^{+/+}$ mice. Mice were treated with LPS for 4 hours, and the lungs were prepared for histology or electron microscopy. (a) Leder (esterase) stain of lung sections. Untreated mice (left panel) show normal alveolar septa with capillaries (small arrows) and small venules (large arrow). No neutrophils are seen in this section. LPS-treated mice (right panel) show infiltrating neutrophils (arrowheads) within capillaries and venules. The inset shows several stained neutrophils in a venule. (b) Quantitative analysis of neutrophil sequestration into the different lung compartments. hpf, high-power field. (c) The electromicrograph shows several neutrophils (arrowheads) within capillaries. * $P < 0.05$ vs. untreated mice.

Electron microscopy confirmed that the sequestered neutrophils were predominantly within capillaries (Figure 2c). From this point on, all remaining experiments were performed after 4 hours of LPS treatment.

LPS activation of circulating neutrophils in vivo. To investigate and quantify the degree of neutrophil activation in vivo in response to LPS, L-selectin shedding (16, 17) and CD11b upregulation (17) were measured using flow cytometry. LPS (0.5 mg/kg) was administered i.p., and, after 4 hours, whole blood was drawn by cardiac puncture from untreated and LPS-treated $TLR4^{+/+}$ mice. As shown in Figure 3, untreated $TLR4^{+/+}$ mice expressed L-selectin with a mean channel fluorescence (MCF) of 35 ± 2 , and in LPS-treated mice, the MCF was significantly reduced to 19 ± 5 ($P < 0.01$). Further evidence that neutrophils were activated in the circulation in response to LPS included a two- to threefold shift in CD11b 62 ± 5 vs. 159 ± 18 , untreated vs. LPS-treated mice; Figure 3).

Sequestration of neutrophils into the lungs of LPS-treated E/P-selectin^{-/-} and CD18^{-/-} mice. To investigate whether the enhanced accumulation of neutrophils into the lungs was adhesion molecule-dependent in our model, we examined the sequestration of cells in E/P-selectin and CD18 KO mice. Both mutant mice have a profound impairment in leukocyte recruitment in peripheral tissues in response to LPS and bacteria (18, 19). Blood and lungs obtained from untreated mice and mice treated i.p. for 4 hours with LPS (0.5 mg/kg) were examined for numbers of circulating leukocytes and for MPO production. As shown in Figure 4a, both E/P-selectin^{-/-} and CD18^{-/-} mice treated with LPS showed an approximately 90% reduction in circulating-leukocyte counts. These mice also showed a profound increase in lung MPO when treated with LPS (Figure 4b). It should be noted that both circulatory counts and lung MPO values were greatly elevated relative to those in WT mice. Nevertheless, these data suggest that neither the endothelial selectins, E-selectin and P-selectin, nor the neutrophil integrin, CD18, are involved in LPS-induced sequestration of neutrophils into the lungs.

Bone marrow transplantation studies. To examine the relative importance of TLR4 on leukocytes and on parenchymal cells (endothelium) in the process of neutrophil recruitment into lungs, we made use of bone marrow transplantation (BMT). Two sets of chimeras were created, one using C57BL/6 and $TLR4^{-/-}$ and the other using C3H/HeN and C3H/HeJ mice. Because the spontaneously occurring TLR4 mutant mice and the generated $TLR4^{-/-}$ mice had identical responses, the data were pooled. Nevertheless, in every series of experiments in this study, TLR4 mutant mice were used, and at times, data were confirmed using C3H/HeJ mice. In the remainder of this manuscript, (a) C3H/HeJ and TLR4 KO mice are referred to as $TLR4^{-/-}$ mice, (b) WT mice (C3H/HeN and C57BL/6) are referred to as $TLR4^{+/+}$ mice, (c) irradiated $TLR4^{-/-}$ mice reconstituted with bone marrow from $TLR4^{+/+}$ donors so that they have TLR4-positive leukocytes but TLR4-negative endothelium are referred to as *Endothelium* $TLR4^{-/-}$ mice, and (d) irradiated $TLR4^{+/+}$ mice reconstituted with bone marrow from $TLR4^{-/-}$ donors so that they

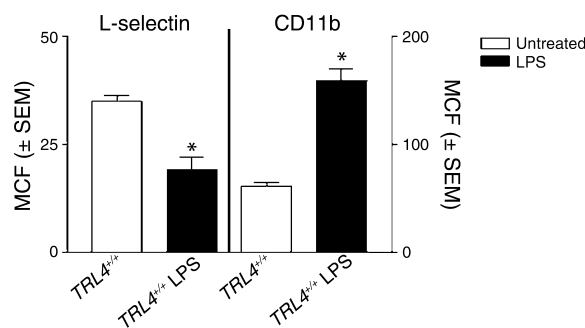


Figure 3

Circulating neutrophils exposed to LPS in vivo exhibit a state of activation. $TLR4^{+/+}$ mice were treated with or without LPS for 4 hours, and then whole blood was drawn by cardiac puncture. Neutrophil L-selectin and CD11b expression were assessed by flow cytometric analysis. Results are shown as the MCF \pm SEM for untreated (white bars) and LPS-treated (black bars) mice ($n = 3$ per group). * $P < 0.05$ vs. untreated mice.

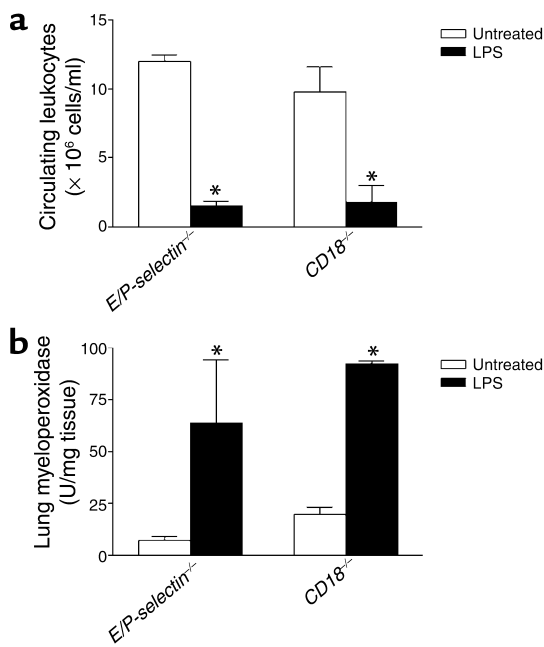


Figure 4
LPS stimulates neutrophil sequestration into the lungs in *E/P-selectin*^{-/-} and *CD18*^{-/-} mice. Mice were treated without LPS (white bars) or with LPS (black bars) for 4 hours, and then whole blood was taken by cardiac puncture and lungs harvested. (a) Circulating-leukocyte counts in harvested lungs. (b) MPO levels in harvested lungs. Data are expressed as the arithmetic mean \pm SEM of four mice per group. * $P < 0.05$ vs. untreated mice.

have TLR4-positive endothelium but TLR4-negative leukocytes are referred to as *LeukocyteTLR4*^{-/-} mice. It is clear that fibroblasts and other cells may also be *TLR4*^{+/+} in the latter chimeric mice, but the leukocyte-endothelial interactions involve primarily leukocytes and endothelium. These bone marrow transfer experiments allowed us to compare the function of TLR4 on bone marrow-derived cells (circulating leukocytes and resident macrophages) with that of TLR4 on cells not derived from bone marrow (vascular endothelium).

LPS induces neutrophil accumulation in the lungs of LeukocyteTLR4^{-/-} but not *EndotheliumTLR4*^{-/-} mice. In each group of mice (WT, KO, and chimeric mice) the effects of systemic LPS on neutrophil sequestration into the lungs was assessed by the MPO assay. Treatment of *TLR4*^{+/+} mice with 0.5 mg/kg LPS i.p. for 4 hours resulted in an approximately threefold increase in MPO levels (Figure 5). Like the *TLR4*^{+/+} mice, *LeukocyteTLR4*^{-/-} chimeras showed a significant increase in MPO levels following LPS treatment (Figure 5), suggesting that TLR4 was not required on neutrophils for their sequestration into lungs. In striking contrast, neither *TLR4*^{-/-} mice nor *EndotheliumTLR4*^{-/-} mice showed any increase in lung MPO levels (Figure 5), suggesting that neutrophils were sequestered into lungs in response to LPS without a need for neutrophil TLR4. Most of the sequestered neutrophils in *LeukocyteTLR4*^{-/-} mice were localized in the capillaries, as in *TLR4*^{+/+} mice (data not shown).

Neutrophils from EndotheliumTLR4^{-/-} mice are responsive to LPS in vitro and in vivo. To confirm that leukocytes from *LeukocyteTLR4*^{-/-} mice were nonresponsive to LPS, whole blood was drawn by cardiac puncture from the *EndotheliumTLR4*^{-/-} and *LeukocyteTLR4*^{-/-} chimeras and incubated with 100 μ g/ml LPS for 30 minutes. L-selectin expression, as assessed by flow cytometry, revealed that neutrophils from LPS-treated *EndotheliumTLR4*^{-/-} mice significantly shed L-selectin compared with those from untreated mice. The MCF of L-selectin expression was 190 ± 36 , and when these cells were treated with LPS, L-selectin expression dropped significantly to an MCF of 83 ± 20 (Figure 6a). By contrast, L-selectin was not shed significantly by direct exposure to LPS of neutrophils from *LeukocyteTLR4*^{-/-} mice (MCF 177 ± 41 vs. 134 ± 29 , untreated vs. LPS-treated mice; Figure 6b).

In separate experiments, we examined in vivo activation of circulating neutrophils in the chimeric mice. We observed that expression of L-selectin on neutrophils from *EndotheliumTLR4*^{-/-} mice treated with LPS for 4 hours in vivo was reduced by more than 50% compared with that of L-selectin on neutrophils from untreated mice (MCF 48 ± 6 vs. 20 ± 9 , $P < 0.05$). This reduction is comparable to that observed for *TLR4*^{+/+} mice as shown in Figure 3. By contrast, L-selectin expression was not decreased significantly on neutrophils from *LeukocyteTLR4*^{-/-} mice treated with LPS for 4 hours in vivo (MCF 74 ± 5 vs. 78 ± 4).

Endothelium is activated in the lungs of LeukocyteTLR4^{-/-} mice and is dependent on TLR4 expression. The aforementioned results suggest that LPS-induced neutrophil accumulation into the lungs of *LeukocyteTLR4*^{-/-} mice involves activation of TLR4 on endothelium rather than neutrophils. We examined the expression of P-selectin in response to LPS as a measure of endothelial activation. Only small levels of P-selectin were noted in lungs of mice not receiving LPS. By contrast, a significant

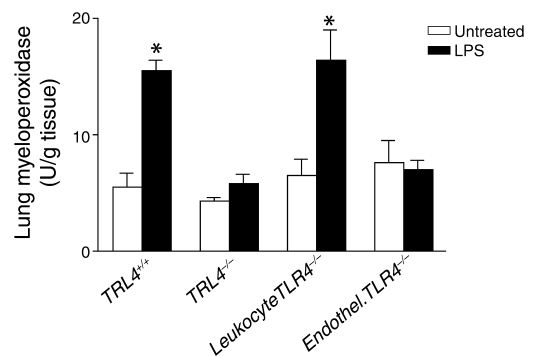


Figure 5
LPS-induced neutrophil sequestration into the lungs of *LeukocyteTLR4*^{-/-} mice. Mice were untreated (white bars) or treated with LPS for 4 hours (black bars), and then lungs were harvested and MPO levels determined in *TLR4*^{+/+}, *TLR4*^{-/-}, *LeukocyteTLR4*^{-/-}, and *EndotheliumTLR4*^{-/-} (*Endothel.TLR4*^{-/-}) chimeric mice. Data are expressed as the arithmetic mean \pm SEM of five mice per group and represent experiments pooled from both sets of chimeric mice. * $P < 0.05$ vs. untreated mice.

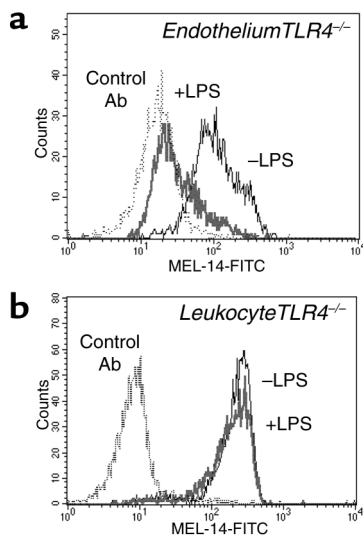


Figure 6
L-selectin shedding from LPS-treated neutrophils differs between TLR4 chimeric mice. Whole blood drawn by cardiac puncture was untreated or treated with LPS for 30 minutes prior to analysis of L-selectin expression by neutrophils from *EndotheliumTLR4^{-/-}* mice (**a**) and *LeukocyteTLR4^{-/-}* mice (**b**). Flow cytometric analysis is shown for a nonspecific isotype control (broken line), L-selectin expression on untreated cells (thin solid line), and L-selectin expression on LPS-treated cells (heavy solid line). Results shown are representative of three experiments per mouse group using C57BL/6 vs. *TLR4^{-/-}* chimeric mice.

increase in endothelial activation was noted in LPS-treated *LeukocyteTLR4^{-/-}* mice (Figure 7). This value was within the same range as LPS responsiveness observed in *TLR4^{+/+}* mice (data not shown). Although a significant increase in endothelial activation was also noted in LPS-treated *EndotheliumTLR4^{-/-}* mice, the response was only 30% of that seen in the *LeukocyteTLR4^{-/-}* mice. The small but significant increase in P-selectin expression on LPS-treated *EndotheliumTLR4^{-/-}* mice could reflect some donor *TLR4^{+/+}* bone marrow replacing the recipient endothelium. This, however, was not the case, as flow cytometric analysis revealed that TLR4 expression on the surface of isolated pulmonary endothelium (identified by VE-cadherin) was comparable between *TLR4^{-/-}* and *EndotheliumTLR4^{-/-}* chimeras. In contrast, more than 85% of the VE-cadherin-positive cells from the WT mice expressed TLR4 (data not shown).

Platelets appeared to contribute to the minor increase in P-selectin expression observed in *EndotheliumTLR4^{-/-}* mice, as, when these mice were made thrombocytopenic, a further reduction in P-selectin expression was noted (% ID/g 0.97 ± 0.66 , $n = 3$). Furthermore, when we compared other organs and tissues, we consistently observed significantly more P-selectin expression in *LeukocyteTLR4^{-/-}* mice than in *EndotheliumTLR4^{-/-}* mice (data not shown).

Resident lung macrophages have the same phenotype as circulating leukocytes. Although the data strongly implicate the endothelium of *LeukocyteTLR4^{-/-}* mice as the cell respon-

sible for neutrophil recruitment, it is possible that, in *LeukocyteTLR4^{-/-}* mice, resident macrophages may have retained their *TLR4^{+/+}* phenotype after BMT and responded to LPS to activate the endothelium. We therefore examined whether the resident lung macrophages within our chimeric mice were replaced by donor macrophages following BMT. Macrophages isolated from lung digests of *EndotheliumTLR4^{-/-}* and *LeukocyteTLR4^{-/-}* chimeric mice were stimulated with LPS for 18 hours, and induction of iNOS mRNA expression was assessed. As shown in Figure 8, macrophages from *EndotheliumTLR4^{-/-}* chimeras responded to LPS with increased iNOS mRNA expression. In contrast, resident lung macrophages from *LeukocyteTLR4^{-/-}* chimeras did not respond at all to LPS for iNOS mRNA induction. Similar results were observed using BAL macrophages from the chimeric mice (data not shown). Clearly, these results suggest that the resident lung macrophages are replaced following BMT, an observation entirely consistent with reports that the mean turnover time of lung macrophages is 6 days (20) (our experiments were done 6–8 weeks after transplantation). More importantly, these results support our hypothesis that, in *LeukocyteTLR4^{-/-}* mice, leukocyte sequestration into the lungs is due to direct activation of endothelial cells by LPS.

Endothelial sequestration of leukocytes into lungs affects peripheral vasculatures. It has previously been observed that LPS-induced neutrophil recruitment into lungs is associated with a profound neutropenia and a reduction in leukocyte trafficking in peripheral vessels of endotoxemic mice (4, 21–23). Whether the lung neutrophil sequestration and trafficking in peripheral vessels are causally related is unclear. We used intravital microscopy to visualize leukocyte-endothelial cell interactions in peripheral vasculatures. Following LPS treatment, circulating-leukocyte counts dropped by more than 90% in *TLR4^{+/+}* mice (Figure 9a), and this was accompanied by a significant drop in the number

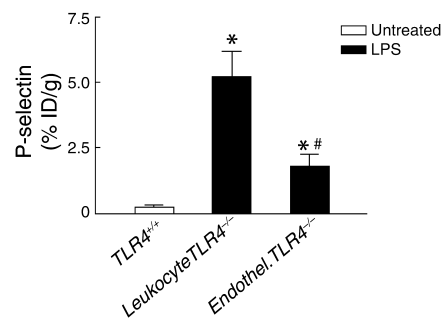


Figure 7
Effect of LPS on P-selectin expression in TLR4 chimeric mice. Expression of P-selectin in lung tissue of untreated *TLR4^{+/+}* mice (white bar) and LPS-treated *LeukocyteTLR4^{-/-}* and *EndotheliumTLR4^{-/-}* (*Endothel.TLR4^{-/-}*) chimeric mice (black bars). Data are presented as the arithmetic mean \pm SEM of four mice per group and represent experiments using chimeric mice generated with C57BL/6 vs. *TLR4^{-/-}* mice. * $P < 0.05$ vs. untreated *TLR4^{+/+}* mice; # $P < 0.05$ vs. *LeukocyteTLR4^{-/-}* mice. ID, injected dose.

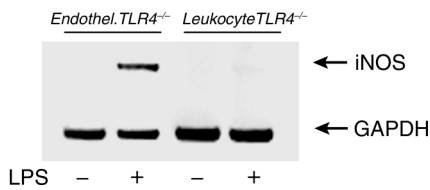


Figure 8
Expression of TLR4 on bone marrow-derived and non-bone-marrow-derived lung macrophages. Macrophages were isolated from *EndotheliumTLR4^{-/-}* (*Endothel.TLR4^{-/-}*) and *LeukocyteTLR4^{-/-}* chimeric mouse lungs, and then treated with LPS for 18 hours or left untreated. Complementary DNA was synthesized, and RT-PCR of iNOS and GAPDH expression was performed. Results shown are representative of three experiments per mouse group using chimeras generated with C57BL/6 and *TLR4^{-/-}* mice.

of rolling leukocytes in the periphery (Figure 9b). Under normal conditions, as many as 60–100 leukocytes per minute roll through peripheral blood vessels of the muscle. However, following LPS treatment, less than 5 rolling leukocytes/min were observed in *TLR4^{+/+}* mice. In contrast, circulating leukocytes remained unchanged in *TLR4^{-/-}* mice (Figure 9a). Furthermore, the average number of rolling cells in *TLR4^{-/-}* mice was approximately 80 per minute, and addition of LPS did not significantly alter the number of rolling cells (Figure 9b). When we examined leukocyte trafficking in LPS-treated chimeric mice, LPS induced a significant reduction in the number of circulating leukocytes in *LeukocyteTLR4^{-/-}* mice (Figure 9a) and a significant decrease in the flux of rolling cells in these mice (Figure 9b). Although some reduction (albeit not significant) in circulating leukocytes was observed in *EndotheliumTLR4^{-/-}* mice (Figure 9a) and a reduction in leukocyte rolling flux in peripheral vessels was noted (Figure 9b), the rolling flux in LPS-treated *EndotheliumTLR4^{-/-}* mice was approximately 30-fold more than in the LPS-treated *LeukocyteTLR4^{-/-}* mice. This is consistent with the idea that neutrophil sequestration into lungs in *LeukocyteTLR4^{-/-}* mice leads to neutropenia and a reduction in neutrophil trafficking in peripheral tissues. It is intriguing that there is some reduction in leukocyte trafficking in LPS-treated *EndotheliumTLR4^{-/-}* mice, suggesting leukocyte sequestration perhaps in other organs such as liver or spleen. Nevertheless, the more profound effect of LPS was on *LeukocyteTLR4^{-/-}* chimeras, suggesting that endothelial TLR4 plays a larger role in regulating systemic leukocyte trafficking.

Discussion

Early and inappropriate neutrophil sequestration into lungs appears to be a key initiator of the pathology associated with sepsis. Our greatly simplified model of sepsis (systemic LPS administration) led to very rapid sequestration of neutrophils into lungs, which appeared to occur even in the absence of the key endothelial selectins (E-selectin and P-selectin) and the primary neutrophil integrin CD18. Associated with the

increase in pulmonary neutrophil sequestration was a rapid decrease in circulating neutrophils and the activation of remaining circulating neutrophils (assessed by L-selectin shedding and increased CD11b expression). However, the very basic mechanisms of how the neutrophils are sequestered within the pulmonary circulation are still unknown. Although, to date, increased activation of circulating neutrophils in response to LPS has been accepted as a likely mechanism of action, this has not been tested systematically (22, 24). The fact that deletion of TLR4 completely inhibits neutrophil sequestration into the lung microvasculature provided us with a tool to begin to explore the mechanisms by which neutrophils are retained in the pulmonary microvasculature. We made chimeric mice lacking TLR4 either on bone marrow-derived cells (including circulating leukocytes and resident macrophages) or on endothelium (and other parenchymal cells). Our data clearly demonstrate that direct activation of circulating neutrophils with LPS is not sufficient to induce their sequestration within the lung. Surprisingly, removal of TLR4 from the circulating neutrophils did not reduce their sequestration into the lungs. In these mice (*LeukocyteTLR4^{-/-}* mice), resident macrophages also lacked functional responses to

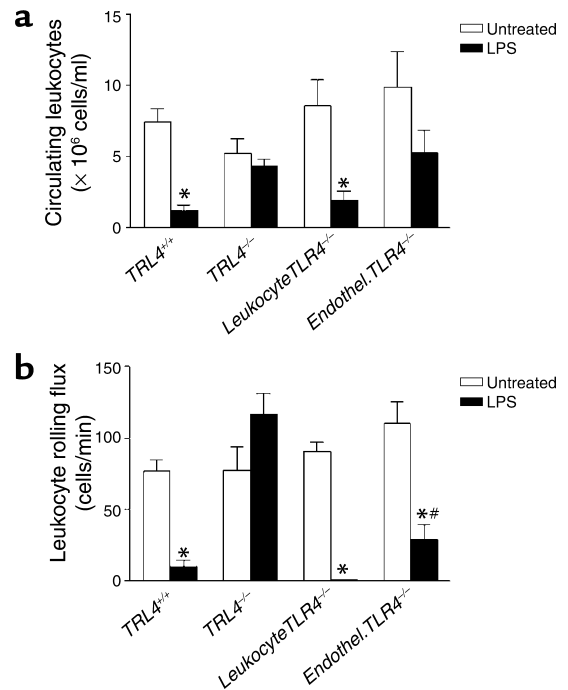


Figure 9
Effect of systemic LPS on circulating leukocytes and leukocyte kinetics in cremaster postcapillary venules in *TLR4^{+/+}*, *TLR4^{-/-}*, and *TLR4* chimeric mice. (a and b) Circulating leukocytes (a) and leukocyte rolling flux (b) in cremaster tissue of untreated (white bars) and LPS-treated (black bars) *TLR4^{+/+}*, *TLR4^{-/-}*, *LeukocyteTLR4^{-/-}*, and *EndotheliumTLR4^{-/-}* (*Endothel.TLR4^{-/-}*) chimeric mice. Data are presented as the arithmetic mean \pm SEM of six to eight mice per group and represent experiments using both sets of chimeras. * $P < 0.05$ vs. untreated mice; ** $P < 0.05$ vs. LPS-treated *LeukocyteTLR4^{-/-}* mice.

LPS, whereas endothelium was very responsive to LPS, as assessed by abundant levels of TLR4 and significant increases in P-selectin expression. This suggests that the endothelium may be the active cell that recruits neutrophils into the endotoxemic lung. Finally, use of intravital microscopy revealed that, in mice in which neutrophils did not sequester into the lungs (*EndotheliumTLR4^{-/-}* mice), circulating-neutrophil counts dropped by only 50% and leukocyte trafficking (rolling) in peripheral organs was 30-fold higher than in mice lacking TLR4 on their neutrophils.

TLR4 is considered to be the key LPS receptor. The evidence that TLR4 is an LPS signaling molecule has been demonstrated by the activation of numerous signaling molecules and production of proinflammatory molecules in the presence but not the absence of TLR4 (25, 26). Furthermore, the molecular defects in the LPS-hyporesponsive C3H/HeJ and C57BL/10ScCr strains of mice were identified as missense and deletion mutations, respectively, of the *tlr4* gene (5). Although TLR2 has also been implicated in LPS-mediated signaling in transfected cell lines (27–29) and in human macrophages (30), our own data demonstrate that neutrophil recruitment into lungs in response to LPS is entirely absent in TLR4-deficient mice. In support of this are publications that show that TLR4-deficient mice, but not TLR2-deficient mice, lack responses to LPS (6, 31). One possible explanation for the discrepancies related to TLR2 as the LPS receptor can be found in the work of Hirschfeld and colleagues (32). In these experiments, commercial LPS preparations, which activated TLR2 and TLR4 transfectants, were repurified to remove highly bioactive, endotoxin-associated proteins. Following repurification, the LPS preparations activated only TLR4 transfectants, suggesting that TLR2 is very sensitive to the contaminants within these commercial LPS preparations.

Clearly, one interpretation of our data is that endothelial cells rather than neutrophils are the LPS-sensitive cells that lead to neutrophil sequestration into lungs. There is ample evidence that endothelium can indeed express TLR4 and respond avidly to LPS (33–35). First, LPS has been shown to induce numerous endothelial cell responses in vitro, in the absence of any other cell. For example, LPS induces the synthesis of a number of adhesion molecules and chemokines in endothelial cells (36, 37). The expression of these molecules is sufficient to induce leukocyte rolling, adhesion, and emigration in vitro. Although macrophages are considered to be very important sources of cytokines (TNF and IL-1) in response to LPS and can induce endothelial adhesion molecule expression, we show here that during the first 4 hours, the macrophages were not essential for neutrophil recruitment into the pulmonary vasculature. This is shown by the fact that in *LeukocyteTLR4^{-/-}* mice, which had no TLR4 on their leukocytes, so that neither neutrophils nor resident macrophages could respond to LPS, there was still very effective recruit-

ment of neutrophils into lungs. This observation is consistent with previous reports that macrophages must synthesize cytokines (which requires 4–6 hours) that then must activate endothelium (another 4–6 hours); these reports make a direct effect of LPS on endothelium during the first 4 hours a far more likely scenario. Nevertheless, by 8–12 hours, it is quite likely that macrophages may become involved.

Our data also highlight that, as neutrophils are trapped in the lungs, they are not available for recruitment in the peripheral circulation. This is exemplified by the profound neutropenia and the 98% reduction in rolling leukocytes in the peripheral vasculature in *TLR4^{+/+}* and *LeukocyteTLR4^{-/-}* mice. By contrast, when the leukocytes had TLR4 and the endothelium did not, the neutropenia was not as profound, and significant numbers of leukocytes could still be seen rolling within the peripheral vasculature. Clearly, the endothelium rather than cells of hematopoietic origin is the dominant regulator of inappropriate leukocyte trafficking during endotoxemia in peripheral vasculatures. Furthermore, our data demonstrate that endothelial cells are a first line of defense against invading microbial agents, actively participating in innate immune responses to LPS via TLR4.

In summary, we have shown, for the first time to our knowledge, that the presence of TLR4 on the endothelium is more important than that of TLR4 on the leukocytes in early LPS responses. We show that, when TLR4 is present on the endothelium, the neutrophils, without a need for neutrophil TLR4, are sequestered into the lungs independently of P-selectin, E-selectin, and CD18 integrin. In addition, when TLR4 was present on the endothelium, and not on the leukocytes, LPS induced endothelial activation, demonstrated by P-selectin expression. Taken together, our results suggest that endothelial cells are more important than previously appreciated in inappropriate neutrophil recruitment in the lungs.

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