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**Research Article** **Immunology**

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# Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection

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**Recognition of LPS by TLR4 on immune sentinel cells such as macrophages is thought to be key to the recruitment of neutrophils to sites of infection with Gram-negative bacteria. To explore whether endothelial TLR4 plays a role in this process, we engineered and imaged mice that expressed TLR4 exclusively on endothelium (known herein as Endothelium$^{TLR4}$ mice). Local administration of LPS into tissue induced comparable neutrophil recruitment in Endothelium$^{TLR4}$ and wild-type mice. Following systemic LPS or intraperitoneal *E. coli* administration, most neutrophils were sequestered in the lungs of wild-type mice and did not accumulate at primary sites of infection. In contrast, Endothelium$^{TLR4}$ mice showed reduced pulmonary capillary neutrophil sequestration over the first 24 hours; as a result, they mobilized neutrophils to primary sites of infection, cleared bacteria, and resisted a dose of *E. coli* that killed 50% of wild-type mice in the first 48 hours. In fact, the only defect we detected in Endothelium$^{TLR4}$ mice was a failure to accumulate neutrophils in the lungs following intratracheal administration of LPS; this response required TLR4 on bone marrow–derived immune cells. Therefore, endothelial TLR4 functions as the primary intravascular sentinel system for detection of bacteria, whereas bone marrow–derived immune cells are critical for pathogen detection at barrier sites. Nonendothelial TLR4 contributes to failure to accumulate neutrophils at primary infection sites in a disseminated systemic infection.**

## Introduction

Localized infection with Gram-negative bacteria activates the local microenvironment and causes the influx of neutrophils into the afflicted tissue. The neutrophils eradicate the pathogen without eliciting any notable systemic innate immune responses. By contrast, when Gram-negative bacteria gain access to blood and disseminate, a systemic, often overexuberant innate immune response ensues (sepsis and septic shock), which continues to elude effective therapy with 30%–40% 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. The prevailing view is that the shedding of LPS from Gram-negative bacteria into the circulation incites the systemic release of cytokines and chemokines, leading to the so-called "cytokine storm" (4). Whether the sequestration of neutrophils in the lungs and the cytokine storm are a protective program of the innate immune system or a clever survival mechanism of bacteria to hijack the innate immune system and redirect neutrophils to the lung and away from the bacterial source remains unknown.

The discovery of TLRs as pattern recognition receptors for bacterial, fungal, and viral ligands has revolutionized research in the area of innate immunity (5, 6). In the case of the LPS receptor, TLR4, macrophages and various cell lines have served as useful tools to study the signaling pathways downstream of this receptor. Robust responses (cytokine production) are generated in these model systems in vitro, and similar exuberant responses are also elicited in vivo. However, macrophage-deficient mice have robust neutrophil responses to both TLR4 and TLR2 ligands (7, 8), suggesting that other cell types can contribute in a meaningful way to the inflammatory cascade. In this regard, most leukocytes, platelets, and non–bone marrow–derived parenchymal cells (epithelium, endothelium, etc.) have also been reported to express functionally significant amounts of TLR4 (9–12). In fact, chimeric mice lacking TLR4 on bone marrow rapidly recruited neutrophils to lungs (in the first 4 hours following bacterial wall products exposure [LPS]) as effectively as wild-type mice, suggesting important roles for parenchymal cells in this process (13).

When bacteria and bacterial products such as LPS penetrate physical barriers and enter local tissues, there is an extremely well-coordinated series of events that recruit effector neutrophils. The prevailing view is that the LPS binds TLR4 on sentinel cells in the tissue, including macrophages and mast cells, that then become activated and release both pre- and de novo–synthesized factors, including cytokines, chemokines, and lipid mediators. The endothelium, which separates the circulating effector cells from the

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**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Nonstandard abbreviations used:** BAL, bronchoalveolar lavage; Endothelium$^{TLR4}$ mice, mice that had TLR4 exclusively on endothelium; IRES, internal ribosome entry site.

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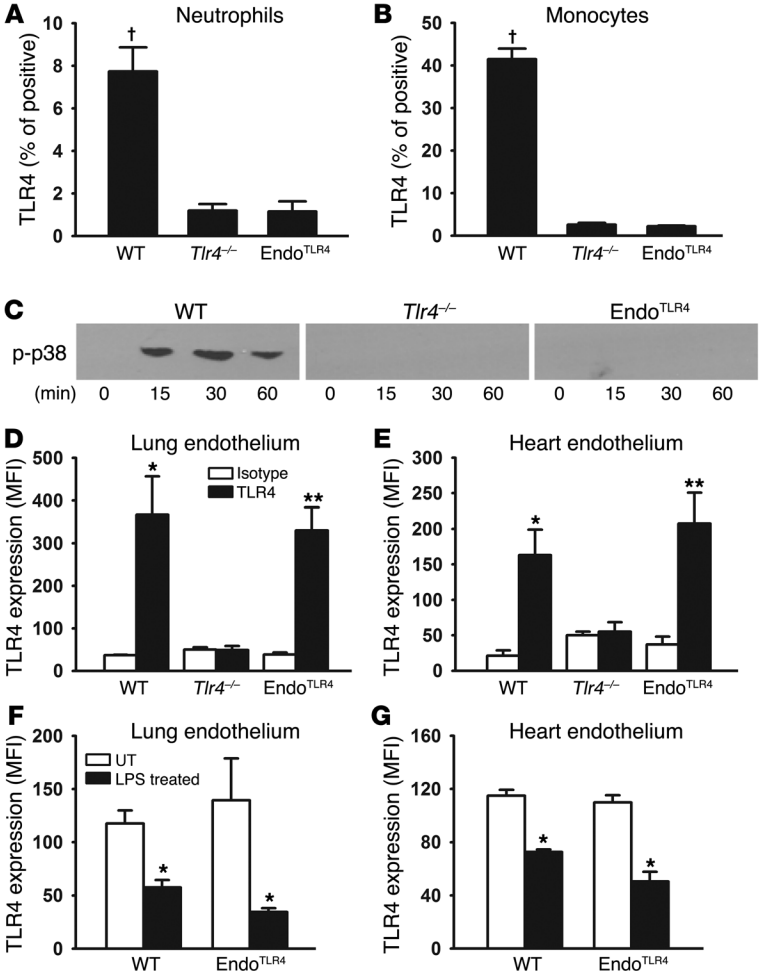


**Figure 1**
TLR4 is only expressed on the endothelium in the Endothelium$^{TLR4}$ mice. TLR4 expression on (**A**) neutrophils and (**B**) monocytes from wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ (Endo$^{TLR4}$) mice was assessed by flow cytometry. (**C**) Phospho-p38 (p-p38) activation of peritoneal macrophages from wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice treated for 0, 15, 30, and 60 minutes with LPS was assessed by Western blot. TLR4 expression on the primary culture endothelial cells of (**D**) lung and (**E**) heart was assessed by flow cytometry. (**F**) Lung or (**G**) heart endothelium was harvested prior to or 4 hours following LPS administration without culturing, and TLR4 expression was immediately measured by flow cytometry. Data are presented as the arithmetic mean ± SEM ($n$ = 3 to 5 in each group). †$P$ < 0.01 versus neutrophils or monocytes from *Tlr4*$^{-/-}$ and Endothelium$^{TLR4}$ mice; *$P$ < 0.05, **$P$ < 0.01 versus isotype or *Tlr4*$^{-/-}$ endothelium. UT, untreated.

tissue resident sentinel cells, is often overlooked. However, endothelium must be activated either directly by LPS or indirectly by mediators released by macrophages to express adhesion molecules necessary for neutrophil recruitment. Since endothelium expresses TLR4 as well as receptors for TNF-α and IL-1β, either scenario is plausible (14). Moreover, the relative importance of endothelial TLR4 versus other cellular sources of TLR4 to the overall local and systemic innate immune response in vivo is completely unknown.

Given the fact that endothelium lines the entire vascular tree of all organs and would be the first cells to come in contact with bacteria and bacterial products like LPS that have disseminated into the blood stream, it would make sense that the endothelium would actually be the sentinel system for bacteremia and endotoxemia and, thereby, potentially be important in both the detection of invading pathogens and localization of the innate immune response. In this study, to systematically test the importance of endothelial TLR4, we generated mice that had TLR4 exclusively on endothelium (referred to herein as Endothelium$^{TLR4}$ mice). Our results revealed that endothelial TLR4 was entirely sufficient to recruit neutrophils to peripheral tissues when LPS was locally administered. During systemic endotoxemia, neutrophils were hijacked by LPS and sequestered for prolonged periods in lungs of wild-type mice but only transiently in Endothelium$^{TLR4}$ mice, thereby freeing effector neutrophils to traffic to target sites in the latter mice. Most importantly, Endothelium$^{TLR4}$ mice were far more efficient than wild-type mice at clearing i.p. Gram-negative bacteria, due to increased neutrophil delivery in the first 24 hours to the infectious nidus, while not succumbing at all to a dose of *E. coli* that caused the immune response to kill 50% of wild-type mice. TLR4 on neutrophils and macrophages is not essential for systemic bacterial recognition and clearance and, in some instances, may even be detrimental.

## Results

*Characterization of Endothelium$^{TLR4}$ mice*. Tie-2 promoter and full enhancer, along with the TLR4–internal ribosome entry site–EGFP (TLR4-IRES-EGFP), were microinjected into C57BL/6 mice, which were mated with *Tlr4*$^{-/-}$ mice, as described in Methods and Supplemental Figure 1 (supplemental material available online with this article; doi:10.1172/JCI36411DS1), to obtain the Endothelium$^{TLR4}$ mice. Our Endothelium$^{TLR4}$ mice, much like the *Tlr4*$^{-/-}$ mice, were healthy, fertile, displayed no overt phenotype, and had normal hematocrits, white blood cell counts, and differential counts in our specific pathogen–free mouse facility. Tie-2 has been reported on a small number of hematopoietic stem cells in addition to endothelium, but Tie-2 is turned off at the start of differentiation, making it unlikely that nonendothelial cells would express TLR4 (15). Nevertheless, a number of the experiments below were reproduced in Endothelium$^{TLR4}$ mice transplanted with bone marrow from *Tlr4*$^{-/-}$ mice to ensure no contri-

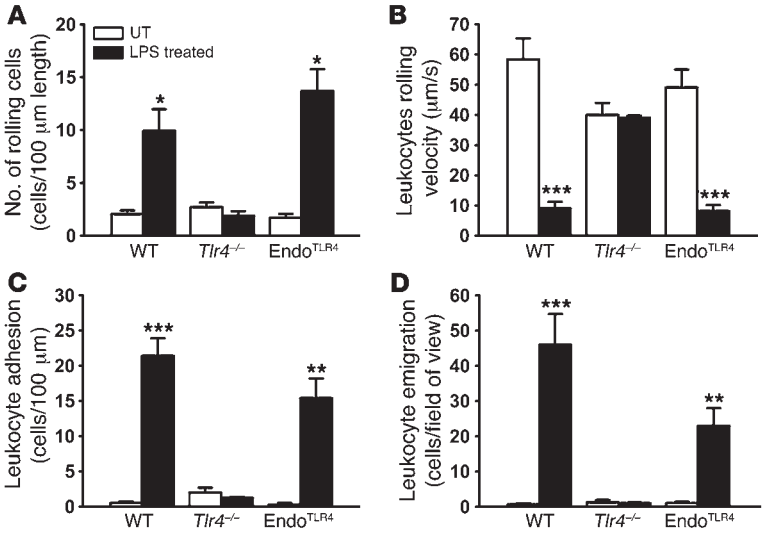


**Figure 2**
Effect of local LPS in the cremaster microcirculation of wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice. Mice were treated with LPS (0.5 μg/kg) locally for 3.5 hours. At this time, for 1 hour, the cremasteric venules were visualized by intravital microscopy. Results represent the leukocyte kinetics observed at 4 hours and show (**A**) number of rolling cells, (**B**) leukocyte rolling velocity, (**C**) leukocyte adhesion, and (**D**) leukocyte emigration in postcapillary venules of wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice. Data are represented as the arithmetic mean ± SEM ($n$ = 4 to 6 mice in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus untreated mice.

bution from any minor bone marrow component. In no case were differences noted (data not shown).

Flow cytometry revealed that neutrophils and monocytes from wild-type mice but not Endothelium$^{TLR4}$ or *Tlr4*$^{-/-}$ mice expressed TLR4 (Figure 1, A and B, and Supplemental Figure 2). Moreover, no other bone marrow–derived humoral cells expressed TLR4 (data not shown). Intracellular signaling in peritoneal macrophages, measured as phosphorylation of p38 MAPK, revealed robust and rapid responses to LPS in wild-type mice but not *Tlr4*$^{-/-}$ and Endothelium$^{TLR4}$ mice at 15 minutes (Figure 1C). On occasion, a small level of p38 MAPK phosphorylation was noted in both *Tlr4*$^{-/-}$ and Endothelium$^{TLR4}$ mice (Supplemental Figure 3), but this never translated to a detectable LPS response. Primary culture of lung and heart endothelial cells from wild-type and Endothelium$^{TLR4}$ mice but not *Tlr4*$^{-/-}$ mice expressed TLR4 to a similar extent (Figure 1, D and E). Finally, in some experiments, endothelium was harvested from lungs (Figure 1F) and hearts (Figure 1G) of wild-type or Endothelium$^{TLR4}$ mice prior to or 4 hours after LPS administration, and TLR4 levels were immediately measured (no culturing) using flow cytometry. Very similar levels of TLR4 were noted in wild-type and Endothelium$^{TLR4}$ mice under basal conditions. TLR4 expression levels decreased following 4 hours of LPS in both strains of mice. These data demonstrated that endothelial cells but not leukocytes in Endothelium$^{TLR4}$ mice do express TLR4 in comparable amounts to wild-type mice and respond similarly to LPS.

*Effect of local LPS in the cremaster microcirculation*. Neutrophil recruitment is a hallmark feature of the innate immune response. We imaged the response using intravital microscopy. In untreated vessels of all groups of mice imaged, approximately 1–4 leukocytes rolled through postcapillary venules (Figure 2A) at relatively high velocities (40–60 μm/s) (Figure 2B) at any given time. Fewer than 2 cells adhered (Figure 2C) and emigrated (Figure 2D) in the postcapillary venules of untreated mice. Local administration of LPS (for 4 hours at 0.5 μg/kg, intramuscular injection) significantly increased the number of rolling cells in wild-type and Endothelium$^{TLR4}$ mice to 10–15 leukocytes/100 μm length venule (Figure 2A). In addition, local administration of LPS decreased leukocyte rolling velocity by 90% in the muscle postcapillary venules of wild-type and Endothelium$^{TLR4}$ mice (Figure 2B). Clearly, LPS activation of endothelial TLR4 was sufficient to elicit these rolling

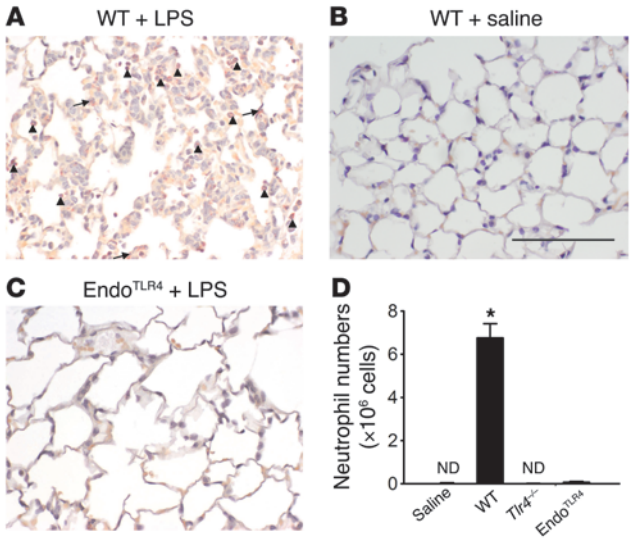


**Figure 3**
Effect of intratracheal LPS on neutrophil sequestration into the lungs. Mice were treated with LPS (intratracheal aerosol) for 24 hours, and the lungs were prepared for histology (esterase staining). Intratracheal aerosol of LPS was administered to (**A**) wild-type and (**C**) Endothelium$^{TLR4}$ mice or saline was administered to (**B**) wild-type mice. (**B**) Representative sections show alveolar spaces and capillaries mainly devoid of neutrophils in the saline-treated lungs, (**A**) while many alveolar spaces contain neutrophils (arrows), with some neutrophils still within capillaries (arrowheads), in the LPS-treated wild-type mice. (**C**) In contrast, LPS treatment in Endothelium$^{TLR4}$ mice resulted in very few neutrophils collecting in the pulmonary capillaries or alveolar spaces. (**D**) BAL was performed, and number of neutrophils was quantified. Data are expressed as the mean ± SEM ($n$ = 3 to 8 mice in each group). ND, not detectable. Original magnification, ×400 (**A**–**C**). In **B**, the black line represents 100 μm. $^{*}P < 0.001$ versus saline-treated mice.

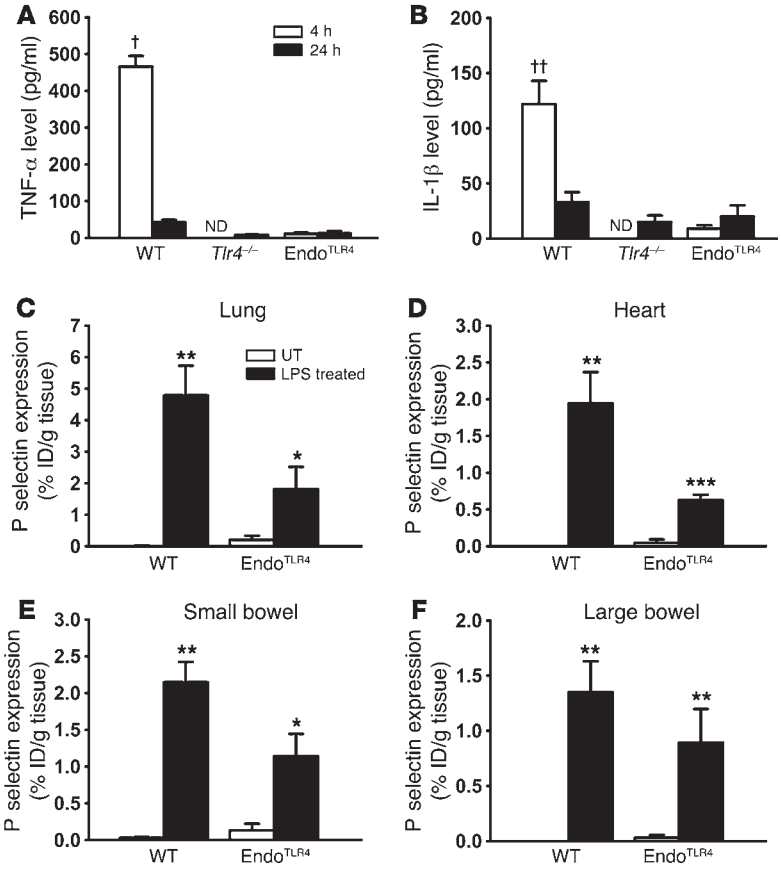


**Figure 4**
Effect of systemic LPS on TNF-α and IL-1β production and P selectin expression on wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice. Mice were treated with LPS (1 mg/kg) systemically for 4 hours and 24 hours. Plasma levels of (**A**) TNF-α and (**B**) IL-1β in wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice were measured. P selectin expression was also quantified after 4 hours of systemic LPS treatment in (**C**) lung, (**D**) heart, (**E**) small bowel, and (**F**) large bowel of wild-type and Endothelium$^{TLR4}$ mice. Data are represented as the arithmetic mean ± SEM ($n$ = 4–5 mice in each group in **A** and **B** and $n$ = 5–6 mice in each group in **C**–**F**). †$P$ < 0.01, ††$P$ < 0.001 versus *Tlr4*$^{-/-}$ and Endothelium$^{TLR4}$ mice treated with LPS for 24 hours; *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001 versus untreated mice. % ID, percentage of the injected dose.

responses, consistent with endothelial selectins regulating these effects (16). The activation of the endothelium by LPS in Endothelium$^{TLR4}$ mice was sufficient to recruit significant numbers of adhering (Figure 2C) and emigrating (Figure 2D) neutrophils in a timely and robust manner, and surprisingly, the magnitude of tissue recruited neutrophils was decreased only 40%–50% in Endothelium$^{TLR4}$ mice relative to wild-type mice. In concordance with previous data shown in TLR4-deficient mice (17), local LPS administration did not induce any changes in leukocyte recruitment in *Tlr4*$^{-/-}$ mice. There was no increase in the number of rolling cells (Figure 2A), no decrease in rolling velocity (Figure 2B), and no increase in adhesion (Figure 2C) or emigration (Figure 2D) of the *Tlr4*$^{-/-}$ cells in response to LPS. No systemic effects were noted in any of the 3 mouse strains; circulating and lung leukocyte counts did not change (data not shown).

*Effect of intratracheal LPS on neutrophil recruitment.* Perhaps sentinel macrophages would be necessary to detect LPS and pathogens in tissues in which a barrier existed between the site of the infection and the microvasculature. LPS administered intratracheally would undoubtedly bind epithelium and alveolar macrophages first. Intratracheal LPS administration in wild-type mice induced a statistically significant increase ($P$ < 0.001) in the number of neutrophils infiltrating the alveolar air space of the lungs relative to mice receiving saline (Figure 3, A, B, and D). Interestingly, Endothelium$^{TLR4}$ mice had essentially no neutrophils in the alveoli following challenge with intratracheal LPS (Figure 3, C and D). These mice were oblivious to the LPS, showing no increase in neutrophils, suggesting that activation of the endothelium by LPS is not sufficient to recruit leukocytes from the circulation into the pulmonary alveoli. The bronchoalveolar lavage (BAL) fluid was harvested and the cell types were quantified. Only wild-type mice showed an increase in neutrophils in air space (Figure 3D).

The aforementioned results, therefore, raised the possibility that either other parenchymal cells (epithelium) or bone marrow–derived cells were responsible for neutrophil recruitment in response to intratracheal LPS. We previously used the spontaneous TLR4-deficient mice, C57BL/10sCnJ (TLR4$^{def}$) to determine that bone marrow–derived cells were not necessary for the recruitment of neutrophils into lung capillaries in response to systemic LPS (13). Chimeric mice were made, in which wild-type mice received bone marrow from TLR4$^{def}$ mice. In these mice, TLR4 was expressed only on parenchymal cells (epithelium, endothelium). In striking contrast to systemic LPS treatment, intratracheal LPS treatment did not induce neutrophil infiltration into the lungs of these mice (Supplemental Figure 4), consistent with the complete lack of response observed in Endothelium$^{TLR4}$ mice. Clearly, neither endothelial nor epithelial TLR4, for that matter, was required/necessary for neutrophil recruitment into lungs in response to intratracheal LPS. By contrast, in chimeric mice, in which TLR4$^{def}$ mice received bone marrow from wild-type mice, intratracheal LPS induced a statistically significant increase in neutrophils infiltrating the alveolar air space of the lungs (Supplemental Figure 4), suggesting that the activation of hematopoietic immune cells by LPS plays a critical role in this response. As expected, TLR4$^{def}$ mice had no response to intratracheal LPS (Supplemental Figure 4).

*Systemic effects of LPS in cytokine levels and endothelial activation in vivo.* It is clear that endothelial TLR4 was sufficient for normal neutrophil recruitment, following local administration of LPS in muscle but not in lungs. We next examined the responses to systemic LPS. Animals received 25 μg per mouse of LPS (i.v. for 4 hours or 24 hours). This was the optimal dose to examine innate immune responses without causing any mortality. Plasma levels of both TNF-α and IL-1β increased in a very significant manner in wild-type mice but not Endothelium$^{TLR4}$ or *Tlr4*$^{-/-}$ mice in response to LPS at 4 hours (Figure 4, A and B). Due to the relatively low level of LPS used, the LPS was cleared rapidly and cytokine levels decreased toward control levels by 24 hours (Figure 4, A and B).

Endothelium can be activated directly by LPS or indirectly by IL-1β, TNF-α, and other cytokines released following endotoxemia.

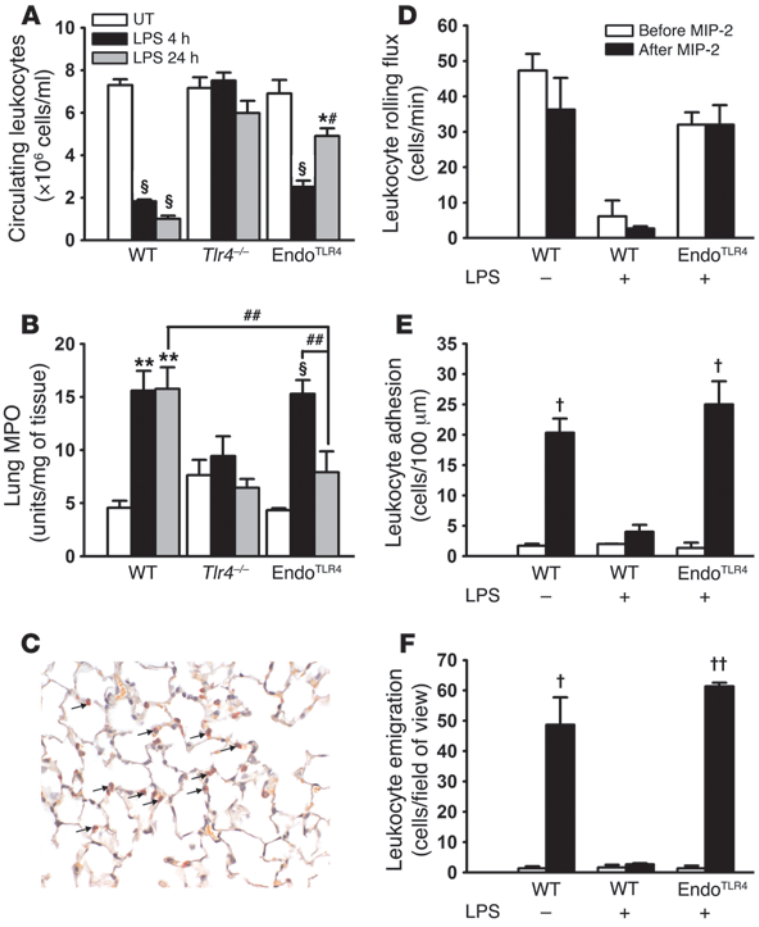


**Figure 5**
Effect of systemic LPS on circulating leukocytes and pulmonary neutrophil sequestration in wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice. Mice were treated with LPS (25 μg/mouse) systemically for 4 hours and 24 hours. The number of (**A**) circulating leukocytes and (**B**) the amount of lung myeloperoxidase (MPO) were measured. (**C**) A representative section of lung shows numerous neutrophils sequestered within the capillaries (arrows) but no neutrophils within the alveolar spaces. (**D**) Leukocyte rolling flux, (**E**) leukocyte adhesion, and (**F**) leukocyte emigration were visualized by intravital microscopy. In some wild-type mice, just MIP-2 was administered (first 2 bars of each graph), while some received LPS 24 hours prior to MIP-2 administration. Data are expressed as the mean ± SEM ($n$ = 4–5 mice in each group in **A** and **B** and $n$ = 3 mice in each group in **D**–**F**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\S}P < 0.001$ versus untreated mice; $^{\#}P < 0.01$ versus Endothelium$^{TLR4}$ mice treated with LPS for 4 hours; $^{\#\#}P < 0.05$ versus Endothelium$^{TLR4}$ mice treated with LPS for 24 hours; $^{\dagger}P < 0.01$, $^{\dagger\dagger}P < 0.001$ versus wild-type mice treated with LPS for 24 hours. Original magnification, ×400 (**C**).

P selectin expression was used as a marker of endothelial activation in all vasculatures of wild-type and Endothelium$^{TLR4}$ mice, following LPS administration (18, 19). In several tissues examined, including lung, heart, and small and large bowel, there was a significant increase in P selectin following systemic LPS treatment (for 4 hours) in Endothelium$^{TLR4}$ mice but not to the same level as in wild-type mice (Figure 4, C–F). Interestingly, LPS-induced pulmonary endothelial P selectin levels in Endothelium$^{TLR4}$ mice were about 30%–40% of the value in wild-type mice and only 20%–25% in heart, 50% in small bowel, and 70%–80% in large bowel (Figure 4, C–F). Clearly, LPS activation of pulmonary endothelium was sufficient to directly express adhesion molecules but to a lesser degree than when all other cell types also expressed TLR4 and cytokines became elevated systemically.

*Systemic effects of LPS in neutrophil trafficking in vivo*. LPS i.v. resulted in a profound drop in the number of circulating leukocytes (Figure 5A) and neutrophils (data not shown) in both wild-type and Endothelium$^{TLR4}$ mice. This was accompanied by a robust increase in the number of neutrophils that sequestered into the lungs, assessed as myeloperoxidase activity (Figure 5B). The neutrophils sequestered in the capillaries, but, unlike with intratracheal administration of LPS, did not emigrate significantly into the lung parenchyma or the alveoli (Figure 5C). Wild-type mice retained neutrophils in the pulmonary vasculature at 24 hours (Figure 5B), excluding them from the peripheral circulation (Figure 5A). By contrast significant numbers of neutrophils were released from the lung capillaries in Endothelium$^{TLR4}$ mice (Figure 5B) into the peripheral circulation by 24 hours (Figure 5A). Circulating and pulmonary neutrophil numbers were identical under baseline conditions in wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice and remained unchanged following LPS treatment in *Tlr4*$^{-/-}$ mice over the entire experimental period (Figure 5, A and B). Instead of i.v., i.p. administration of LPS also induced a systemic response, including a profound drop in circulating leukocytes after 4 hours LPS treatment (i.p. injection = 1.7 ± 0.25 ×10$^6$ cells/ml versus 2.4 ± 0.54 ×10$^6$ cells/ml, wild-type versus Endothelium$^{TLR4}$ mice) and a large infiltration of neutrophils into the lungs (Supplemental Figure 5). The slightly higher numbers of neutrophils in the circulation of Endothelium$^{TLR4}$ mice than wild-type mice following i.p. LPS administration may actually be important, as the former mice were able to recruit some neutrophils into the peritoneum, whereas wild-type mice did not (Supplemental Figure 5).

These data led us to hypothesize that LPS and/or bacteria that sequester neutrophils into the pulmonary vasculature prevent their access to peripheral tissues in which the focus of the infection may lie (e.g., abscess in muscle). To test this possibility, we delivered local chemokine (MIP-2) injection that causes profound neutrophil recruitment into muscle in otherwise healthy mice i.e., no LPS (Figure 5, D–F, first and second bars of each graph). Many cells rolled both before and after MIP-2 administration. There was a very large increase in adhesion (Figure 5E) and emigration (Figure 5F) following MIP-2 administration. By contrast, endotoxemic

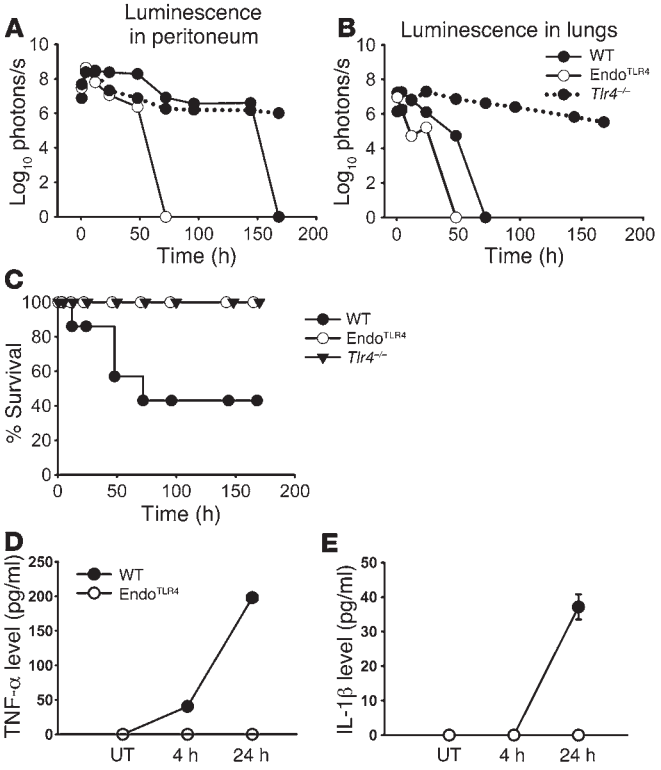


**Figure 6**
Endothelium$^{TLR4}$ mice showed much more efficient bacterial clearance than wild-type mice. Mice were injected with $1 \times 10^7$ CFUs luminescent *E. coli* (i.p.) and imaged at 30 minutes, 4 hours, 12 hours, 24 hours, and every 24 hours after infection using the Xenogen system. Luminescence is expressed as photons per second; this measurement takes into account the intensity and area of luminescent signal, which indicates amount and spread of luminescent bacteria. Luminescence was analyzed in 2 areas, (**A**) peritoneum and (**B**) lungs, and verified in Supplemental Figure 7. (**C**) Percentage of mouse survival after the injection of *E. coli* was also documented. The levels of plasma (**D**) TNF-α and (**E**) IL-1β were analyzed in untreated and *E. coli*–infected wild-type and Endothelium$^{TLR4}$ mice at 4 and 24 hours.

mice had very low numbers of rolling cells that remained low after MIP-2 administration (Figure 5D, third and fourth bars of each graph). No adhesion (Figure 5E) or emigration (Figure 5F) could be seen in the endotoxemic wild-type mice. By contrast, large numbers of rolling cells were seen in endotoxemic Endothelium$^{TLR4}$ mice. The Endothelium$^{TLR4}$ mice did respond to MIP-2, despite the presence of endotoxemia (Figure 5, D–F, fifth and sixth bars of each graph). Clearly, retention of neutrophils in lungs and away from the peripheral vasculature prevents neutrophil recruitment to peripheral sites.

*Bacterial eradication in wild-type, Endothelium$^{TLR4}$, and Tlr4$^{-/-}$ mice.* To determine whether similar mechanisms are at play in a more physiologic bacterial model of sepsis, a dose of *E. coli* ($1 \times 10^7$ CFUs) in log phase of growth was injected into the peritoneum. There was an approximately 10-fold increase over the first 4 hours in wild-type and Endothelium$^{TLR4}$ mice (Figure 6A). In both strains of mice, bacteria disseminated to the lungs as early as 30 minutes after i.p. injection (Figure 6B, first value). Over the first 48 hours of infection, more than half of the wild-type mice died (Figure 6C). The remaining mice cleared the pulmonary bacteria within the first 70 hours (Figure 6B), but the peritoneal infection was not cleared until 1 week (Figure 6A). In striking contrast, all Endothelium$^{TLR4}$ mice cleared bacteria from the peritoneum within the first 72 hours of infection (Figure 6A). In fact, most of these mice had cleared the infection within the first 48 hours from both peritoneum as well as the lungs (Figure 6B). Most importantly, no Endothelium$^{TLR4}$ mice died during this time frame (Figure 6C). To be thorough, *Tlr4*$^{-/-}$ mice were also injected with *E. coli*, and the bacteria persisted for at least as long in the peritoneum (Figure 6A) and for much longer in lungs (Figure 6B). By 168 hours, about 50% of the *Tlr4*$^{-/-}$ mice cleared bacteria (data not shown). The strain of *E. coli* used was not lethal, so that even very high titers in *Tlr4*$^{-/-}$ mice failed to cause mortality (data not shown). Wild-type mice died despite lower levels of bacteria in lungs than *Tlr4*$^{-/-}$ mice, likely due to an inappropriate or overexuberant TLR4-dependent immune response in wild-type mice. Indeed, Figure 6, D and E, revealed increased levels of plasma TNF-α and IL-1β in response to *E. coli* in wild-type mice between 4 and 24 hours of infection but not in Endothelium$^{TLR4}$ mice.

In addition, we measured chemokine production in the lungs, blood, and peritoneum of wild-type and Endothelium$^{TLR4}$ mice and observed that the neutrophil-selective chemokine, MIP-2, did not increase in lungs of all 3 strains of mice (Supplemental Figure 6). There was an increase in MIP-2 levels in the peritoneum of all 3 strains of mice, and the largest increase was in wild-type mice (Supplemental Figure 6).

The luminescence of these bacteria allowed us to track these infections over time in the same mice. The luminescence correlated with the bacterial load in these organs. In untreated wild-type mice, luminescence was never observed, and the cultures of both the peritoneal lavage and lungs were negative. After *E. coli* treatment for 4 and 24 hours, wild-type mice had luminescence in the peritoneum and the lungs, and the cultures from peritoneal lavage and the lungs verified increased bacterial load in both (Supplemental Figure 7).

To test for an altered immune response, we also tracked neutrophils in the *E. coli* infection model. A very robust increase in neutrophil sequestration into the lungs was observed in wild-type mice that slowly decreased with time (Figure 7A). Simultaneously, a subtle 2- to 3-fold increase in neutrophil number in peritoneum was observed at 4 hours in wild-type mice (Figure 7B). Circulating neutrophil counts were significantly decreased at 12 and 24 hours but returned to normal levels at 48 hours in both groups of mice (Supplemental Figure 10).

Although the Endothelium$^{TLR4}$ mice also had an increase in neutrophil number in the lungs (Figure 7A), it was not as profound, and peritoneal neutrophil counts were dramatically enhanced at both 4 and 24 hours above wild-type values. To verify that neutrophils from Endothelium$^{TLR4}$ mice could respond to chemoattractants despite having bacteremia, MIP-2 was placed into the cremaster muscle and neutrophil recruitment was examined. Much like in the endotoxemia model, neutrophils rolled, adhered, and emigrated in response to MIP-2, despite the presence of a systemic infection (Supplemental Figure 8). The *Tlr4*$^{-/-}$ mice had small numbers of neutrophils in the lungs and peritoneum over the first 72 hours (data not shown). Clearly, complete absence of TLR4 impairs the detection mechanism present in Endothelium$^{TLR4}$ mice.

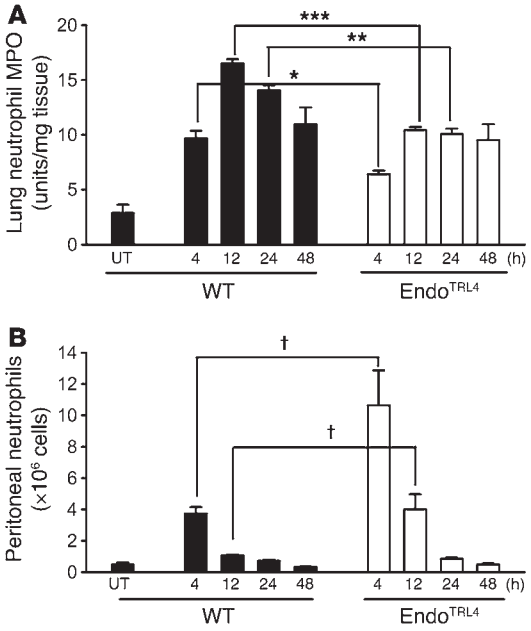


**Figure 7**

Endothelium$^{TLR4}$ mice showed more efficient neutrophil recruitment to the peritoneum than wild-type mice. Mice were injected with $1 \times 10^7$ CFUs luminescent *E. coli* and imaged using the Xenogen system. (**A**) At 4, 12, 24, and 48 hours, some mice were sacrificed, the lungs were harvested to determine myeloperoxidase values, and (**B**) peritoneal lavage was performed to count peritoneal neutrophils. Data are expressed as the arithmetic mean ± SEM ($n$ = 3–6 mice in each group). $^*P < 0.05$ versus wild-type mice treated for 4 hours; $^{**}P < 0.01$ versus wild-type mice treated for 24 hours; $^{***}P < 0.001$ versus wild-type mice treated for 12 hours; $^{\dagger}P < 0.01$ versus wild-type mice treated for 4 and 12 hours.

To be thorough, *E. coli* was also administered i.v., instead of i.p., into wild-type mice, and neutrophil counts dropped precipitously and neutrophils migrated into lungs (Supplemental Figure 9).

## Discussion

When considering sentinel cells, macrophages have been highlighted, due to their prominence in all tissues and their ability to release many of the mediators thought to recruit neutrophils to induce inflammation. Macrophages are also implicated in many other functions, including growth regulation, repair, and scavenging. In fact, recent studies suggest that at least 2 populations of macrophage exist, and the tissue macrophages are noninflammatory cells, unlikely to be responsible for the inflammatory response (20, 21). Indeed, macrophage-deficient mice have impairments in growth but retain inflammatory responses to TLR ligands (7, 8). Mast cells have also received some attention as sentinel cells, as they have pre-stored TNF-α, histamine, and other mediators that can be rapidly mobilized upon activation (22). A cell type that has not been thought of as a sentinel cell to date, despite the fact that it out numbers all other cells in every organ, is the endothelium. Over 60 trillion endothelial cells make up the largest interconnected organ in the human body, weighing 3 kilograms and covering a span of 4,000 square meters (23). Due to this vast coverage, the endothelium could be a very efficient sentinel cell, at least in the event of disseminating bacteria, in which the endothelium would be the first cell to come in contact with the pathogen. However, even in this regard, a recent study has suggested a population of monocytes continuously crawling in blood vessels might serve this function (20). Clearly, the importance of endothelium in innate immunity and, specifically, as it pertains to TLR4 and Gram-negative sepsis remains unappreciated.

Many groups have demonstrated potent endothelial responses to LPS in vitro (24–29), as a result of not just the expression of TLR4 but also membrane CD14 (27), MD2 (30), and Myd88 (31). In fact, primary endothelial monolayers in vitro have all of the necessary machinery to increase expression of adhesion molecules (32) and chemokines (33) to allow neutrophils to roll, adhere, and emigrate across the endothelium. Whether this is important in vivo, with tissue macrophages and mast cells also present, remained unclear. Herein, we demonstrated that the endothelium is sufficient on its own to respond to the bacterial product LPS to initiate the effector phase of innate immunity in peripheral tissues, like muscle. Moreover, TLR4 on neutrophils and macrophages was not essential for their recruitment by endothelium. Clearly, the endothelium must release chemokines abluminally to induce neutrophils to emigrate out of the vasculature, a phenomenon also seen on activated endothelial monolayers in vitro (33). Simply getting neutrophils to the abluminal side of vessels may not translate to functional eradication of bacteria. However, our data would suggest that the neutrophil recruitment was productive, since Endothelium$^{TLR4}$ mice eradicated *E. coli* in a timely manner, while mice lacking TLR4 were unable to eradicate infection. It is certain that once neutrophils cross the endothelium, *E. coli* would inadvertently activate numerous other detection mechanisms, including complement receptors and receptors for exogenous bacterial chemoattractants like fMLP, and help neutrophils to converge on bacteria for final elimination.

In all models of sepsis, including cecal ligation and puncture, endotoxemia, or bacterial injections, neutrophils enter the lungs and sequester in capillaries of this organ (3, 34). The response is robust, occurs rapidly (as early as 30 minutes after exposure), and accounts for the disappearance of the majority of neutrophils from the circulation. Neutropenia, neutrophil sequestration in lungs, and pulmonary failure are also hallmarks of severe sepsis in humans (35). When neutrophils are sequestered in lungs, they are unavailable for entry into other sites of infection. As such, only limited numbers of neutrophils reach the bacterially infected peritoneum in wild-type mice. Although neutrophils were mobilized to the lungs in both wild-type and Endothelium$^{TLR4}$ mice, fewer neutrophils entered the lungs and more entered the periphery in the Endothelium$^{TLR4}$ mice, while the reverse was seen in wild-type mice. In the wild-type mice, not only did the neutrophils not eradicate bacteria as effectively, but in about 50% of the cases, either the infection overwhelmed the mice or the activated immune system killed the mice. Our data would suggest it was the hyperactivated immune system, since in the complete absence of TLR4 in the *Tlr4*$^{-/-}$ mice, the immune response was greatly attenuated, so that few of the mice completely cleared the infection, yet none of the mice died. Clearly, the bacteria alone were not sufficient to kill the host.

It would seem counterproductive to have neutrophils sequester into the lungs just to cause pulmonary dysfunction and injury. Indeed, even in the Endothelium$^{TLR4}$ mice, neutrophils migrated to lungs in response to *E. coli*, suggesting some as yet unknown, necessary immune response is initiated by endothelial TLR4. One possibility may be the recently proposed neutrophil extracellular traps (NETs) that are formed to enhance bacterial trapping (36, 37). Upon adhesion in the lung capillaries, neutrophils release

large net-like structures or NETs composed of DNA loaded with proteases, which greatly increase bacterial trapping and killing capacity but also increase injury to underlying endothelium (38). Why the lung is the target organ is unclear, but perhaps the capillaries have the right geometry to allow neutrophils to sequester and optimally trap bacteria as they flow through these conduits. The initial mobilization of neutrophils into the lungs was dependent upon endothelial TLR4. However, with time the Endothelium$^{TLR4}$ mice mobilized neutrophils back into the circulation, whereas wild-type mice did not. Clearly, at some point, the retention of neutrophils in lungs becomes nonproductive and/or even a subversive tactic on the part of the bacteria. The dramatically increased circulating cytokine levels and associated prolonged retention of neutrophils in the lung capillaries both were dependent upon a nonendothelial source of TLR4. This cytokine storm may in fact sustain neutrophil sequestration in lungs, causing inadvertent damage.

It is tempting to conclude that there is little need for bone marrow–derived TLR4 in host response to infection. However, this is definitely not the case, inasmuch as our data demonstrated a critical role for bone marrow–derived immune cells and not endothelium when LPS and presumably bacteria gained entry into sites such as alveoli, where the host is in direct contact with the external environment. Indeed, without bone marrow–derived TLR4, neutrophils were unable to detect bacterial ligands and enter the alveoli and perhaps other sites such as the intestinal tract, skin, the eyes, mouth, and reproductive organs. Our data also suggested little or no role for TLR4 on pulmonary epithelium in the presence of Gram-negative ligands.

Using live animal imaging, we observed that in *Tlr4*$^{-/-}$, wild-type, and Endothelium$^{TLR4}$ mice, the initial injection of *E. coli* resulted in a similar, rapid growth and dissemination of bacteria to other organs, including the lung parenchyma, suggesting that the initial phase of bacterial kinetics was quite similar among the 3 strains. However, while bacteria in the *Tlr4*$^{-/-}$ mice continued to grow and disseminate with limited neutrophil recruitment, the Endothelium$^{TLR4}$ mice were able to resolve the infection, with a robust neutrophil response in all afflicted organs studied. In fact the Endothelium$^{TLR4}$ mice were much more efficient than their wild-type counterparts, eliminating the bacteria from the original site in less than half the time, with no mortality. This suggests that rather than being beneficial, the net contribution of nonendothelial sources of TLR4 in systemic sepsis were detrimental, delaying bacterial clearance and killing half the mice. Although we only tested *E. coli* in this study, we have evidence that both TLR2 ligands and gram-positive bacteria also induce neutrophil sequestration into lungs, suggesting this mechanism may apply to numerous, if not all forms of sepsis (8).

In a recent report, Ye et al. (39) showed that selective inhibition of NF-κB in endothelium also revealed an important role for this cell, but in this case, the mortality and morbidity were lowered and bacterial clearance was not affected. The key difference is that whereas we examined the role of a single TLR, namely TLR4, and showed it was sufficient to help clear bacteria, Ye et al. (39) inhibited NF-κB downstream of many inflammatory receptors on endothelium, which might have a much greater immunosuppressive effect and as such prevent bacterial clearance. Indeed, our data suggested that fully responsive endothelium is of great benefit in clearing unwanted pathogens without harming the host.

However, it is worth mentioning a few caveats with respect to the Endothelium$^{TLR4}$ mice. Although the endothelium from these mice had similar levels of TLR4 as endothelium from wild-type mice in vitro and in vivo and responded similarly in vivo, as assessed by P selectin expression, we cannot exclude the possibility that the lack of TLR4 on nonendothelial cell types may prevent LPS detoxification and increased LPS levels for endothelium stimulation. Also, although similar downregulation of TLR4 occurred on endothelium in Endothelium$^{TLR4}$ and wild-type mice, we cannot exclude the possibility that one or more signaling pathways downstream of TLR4 might have altered responses. Finally, the complete lack of response to intratracheal LPS in the Endothelium$^{TLR4}$ mice does not necessarily mean there is no role for endothelial TLR4. For example, increased permeability to LPS across epithelium may be required to allow activation of endothelial TLR4 and neutrophil recruitment. This intriguing possibility cannot be discounted. These issues will require further investigation in the future, perhaps in mice lacking TLR4 just on endothelium.

## Methods

*Materials*. All reagents were from Sigma-Aldrich unless otherwise stated. *E. coli* (0111:B4) LPS was from Calbiochem EMD Biosciences. LPS was used at a concentration of 1 mg/ml and diluted in pyrogen-free saline. The total dose of LPS administered per aerosolization was 50 μg. For i.p. or i.v. injection, 1 mg/kg of LPS was administered.

*Mice*. C57BL/6 mice were purchased from The Jackson Laboratory. *Tlr4*$^{-/-}$ mice were provided by Douglas T. Golenbock (University of Massachusetts Medical School, Worcester, Massachusetts, USA). Mice (20–35 g, 4–6 weeks old) were maintained in a pathogen-free environment. The mice had access to food and water ad libitum. All procedures performed were approved by the University of Calgary Animal Care Committee and were in accordance with the Canadian Guidelines for Animal Research.

*Methodology*. Standard methodology for Western blots, circulating neutrophil/leukocyte counts, quantitation of cytokines, and determination of tissue myeloperoxidase activity as a measure of neutrophil influx are previously published (8, 13, 17) and details are found in Supplemental Methods.

*Generation of transgenic mice expressing endothelial TLR4*. TLR4 was amplified by RT-PCR from total RNA obtained from mouse blood cells, using 2 sets of primers, as outlined in Supplemental Methods. TLR4 was cloned into PCR2.1TOPO, digested with BamHI/ScaI, and subcloned in pDrive-IRES-EGFP yielding, plasmid pDrive-TLR4-IRES-EGFP. Tie-2 promoter and Tie-2 full enhancer, both indispensable to have specific endothelial expression of TLR4, were taken from pT2HLacZpA1I.7 vector (provided by Thomas N. Sato, University of Texas, Southwestern Medical Center, Dallas, Texas, USA). The transgene, a 15.8-Kb fragment containing Tie-2 promoter–TLR4-IRES-EGFP–Tie-2 enhancer (Supplemental Figure 1), was released from the plasmid and microinjected into fertilized eggs derived from the matings of inbred C57BL/6 mice (at the University of Alabama at Birmingham Transgenic Mouse Facility). Positive female founder lines were mated with *Tlr4*$^{-/-}$ males to obtain Tie-2–TLR4 transgenic mice. The mice were bred and screened by PCR to confirm double heterozygosity. Three lines of mice were then set up as breeding pairs. All the offspring were screened for *Tlr4*$^{-/-}$/EGFP/EGFP, and line 3 revealed optimal endothelial TLR4 expression.

*Bone marrow transplantation*. Briefly, bone marrow chimeras were generated following a standard protocol previously described by our laboratory (40, 41). Bone marrow was isolated from mice euthanized by spinal cord displacement. Recipient mice were irradiated with 2 doses of 5 Gy (Gammacell 1000; Nordion International Inc.). An interval of 3 hours was allowed to pass between the first and second irradiations. Next, $8 \times 10^6$ 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 99% of leukocytes from Thy1.1 into Thy1.2 congenic recipient mice were from donor bone marrow (40).

*Expression of TLR4.* Expression of TLR4 on neutrophils, monocytes, and endothelium from wild-type, *Tlr4*$^{-/-}$, and Endothelium$^{TLR4}$ mice was determined using a BD FACScan Flow Cytometer (BD Biosciences) and CellQuest Pro software (Becton Dickinson — Immunocytometry Systems).

*Intratracheal aerosolization and BAL.* Mice were anesthetized with isoflurane (MTC Pharmaceuticals) and suspended from their upper front teeth in a vertical, upright position. Oral intratracheal intubation was achieved under direct visualization, using an operating microscope and a Small-Animal Laryngoscope (PennCentury). Solutions were aerosolized directly into the distal trachea using a MicroSprayer (model IA-1C; PennCentury) attached to a stainless steel syringe (model FMJ-250; PennCentury). At 24 hours after aerosolization, BAL and histology of the lungs was performed as previously described.

*Intravital microscopy*. Mice were anesthetized by i.p. injection of a mixture of xylazine hydrochloride (10 mg/kg; MTC Pharmaceuticals) and ketamine hydrochloride (200 mg/kg; Rogar/STB). The jugular vein was cannulated and used to administer additional anesthetic, fluorescent dyes, and various drugs. Animals were then prepared for the viewing of the skeletal muscle microcirculation, as previously reported by our laboratory (13). Wild-type and Endothelium$^{TLR4}$ transgenic mice were treated either locally for 4 hours with LPS (0.5 μg/kg) or systemically with LPS (1 mg/kg, i.v.) for 4 or 24 hours. At this time the cremaster was exteriorized and visualized by intravital microscopy. In additional systemic LPS experiments at 24 hours, the cremaster muscle was exteriorized and superfused with MIP-2 (CXCL2; R&D Systems) at 5 nM in superfusion buffer at 37°C, mimicking a secondary abscess site in the periphery that might require neutrophil recruitment. The neutrophil recruitment was monitored at different time points for up to 90 minutes. This dose of MIP-2 induces optimal neutrophil emigration into the tissue of otherwise healthy mice (42).

*Quantitation of endothelial activation*. To determine the degree of endothelial activation in vivo, P selectin, which is known to contribute to leukocyte recruitment in endotoxemia, was measured. We used a modified dual-radiolabeled Ab technique that permits quantification of in vivo measurements of P selectin as previously described (43, 44).

P selectin expression was calculated per gram of tissue, by subtracting the accumulated activity of the nonbinding Ab ($^{131}$I-labeled Ab) from the accumulated activity of the binding Ab ($^{125}$I-labeled Ab). Data for P selectin were 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 very small, basal levels of P selectin can be detected in wild-type mice relative to P selectin–deficient mice, in which values are true 0 (43, 44).

*Induction of bacterial infection and monitoring of clearance by bioimaging.* Briefly, *E. coli* (Caliper Life Sciences) were grown to mid-log, washed, and then suspended in saline. Mice were anesthetized and had their hair removed by chemical depilation, and approximately $1 \times 10^7$ CFUs of the bacterial preparation was injected i.p. in a volume of 1 ml. The rate of spread and clearance of the bacteria was then monitored using an IVIS Lumina (Caliper Life Sciences), with imaging conducted 30 minutes, 4 hours, 12 hours, and 24 hours after infection and every 24 hours thereafter until the infection was cleared. Clearance was defined as 3 consecutive readings with no detectable luminescence.

*Statistics*. Data were analyzed using GraphPad Prism (version 4, GraphPad Software Inc.). Reported values are expressed as mean ± SEM unless otherwise described. We assessed the statistical significance of the difference between 2 sets of data using an unpaired, 2-tailed *t* test. Where the difference between more than 2 sets of data was analyzed, we used a 1-way analysis of variance, followed by Bonferroni multiple-comparisons test. All comparisons were 2-tailed, and *P* values of less than 0.05 were considered to be significant.

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