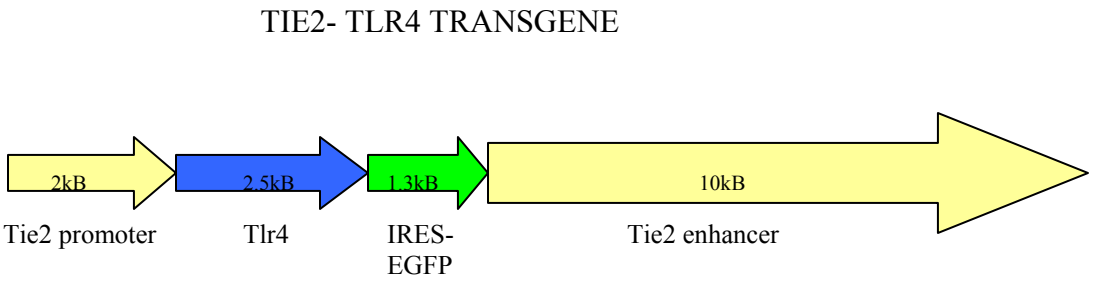
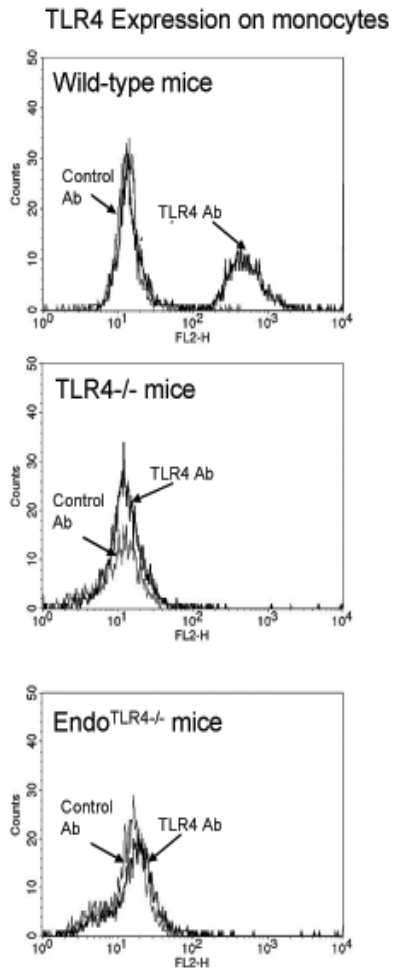
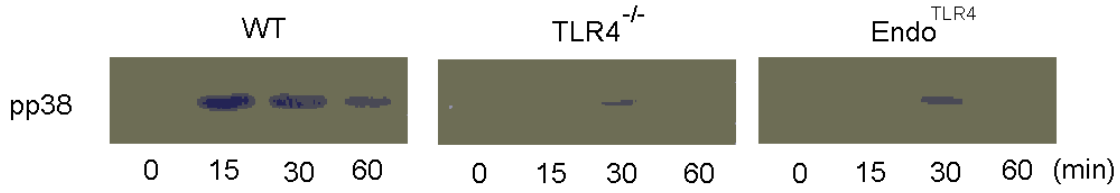
Supplemental Data



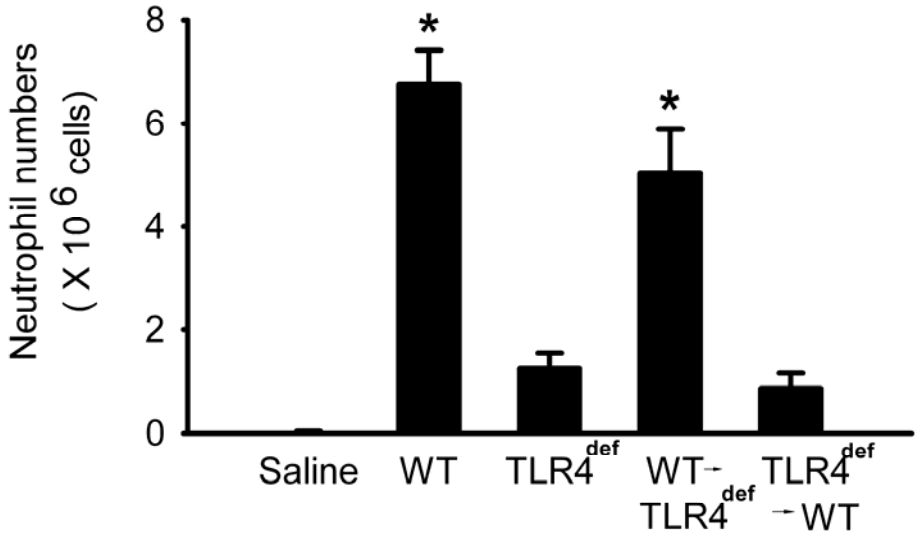
Supplemental Figure 1.- Scheme of the transgene microinjected to obtain TLR4 expression into the endothelium.



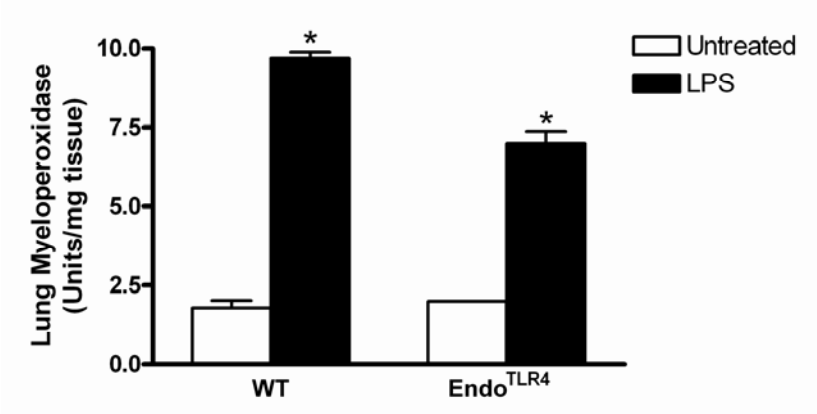
Supplemental Figure 2.- TLR4 is not expressed on monocytes of Endothelium^{TLR4} mice. TLR4 expression on monocytes from wild-type (A), TLR4-/- (B) and Endothelium^{TLR4} (Endo^{TLR4}) mice (C) was assessed by flow cytometry.

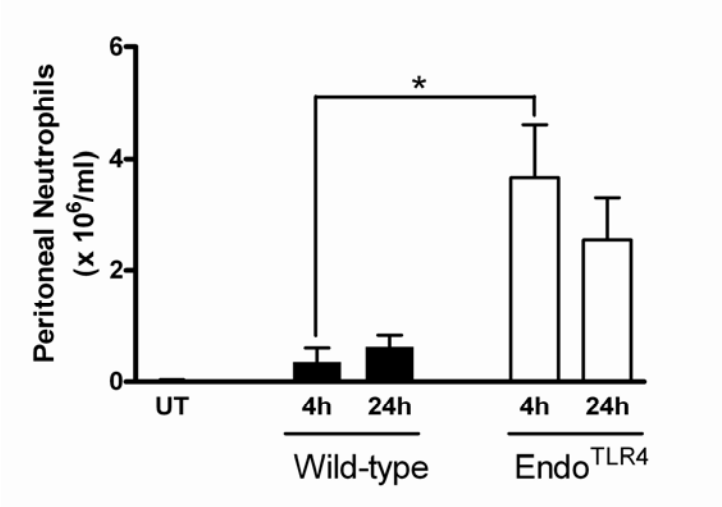


Supplemental Figure 3.- Phospho-p38 activation of peritoneal macrophages from wild-type, TLR4-/- and Endothelium^{TLR4} mice treated for 0, 15, 30 and 60 min with LPS was assessed by Western blot. At no time did LPS translate into any biological phenotype.

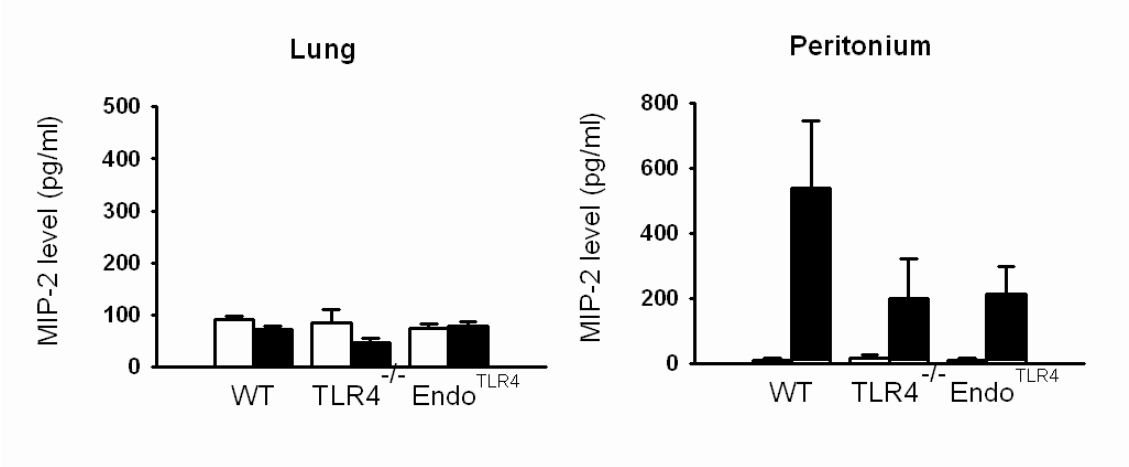


Supplemental Figure 4.- Pulmonary neutrophil sequestration induced by intratracheal LPS. Wild-type mice, TLR4^{def} mice, TLR4^{def} mice that received bone marrow from wild-type mice (WT→TLR4^{def}) and wild-type mice that received bone marrow from TLR4^{def} mice (TLR4^{def}→WT) were treated with intratracheal LPS for 24hrs. At this time the bronchoalveolar lavage was performed and slides were prepared and stained with H&E to analyze the number of neutrophils. Data are expressed as the mean ± SEM of 3 to 8 mice in each group. $P < 0.001$ vs. saline mice.

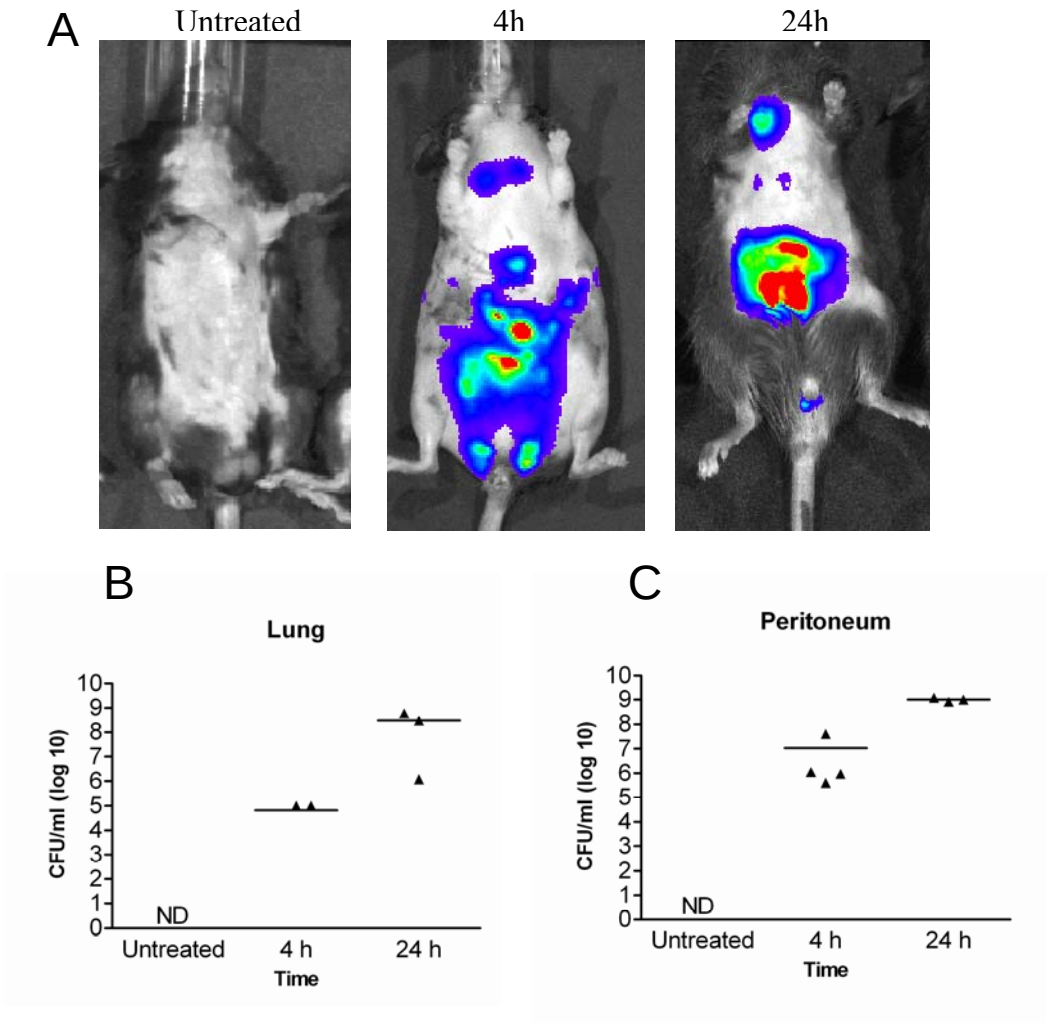




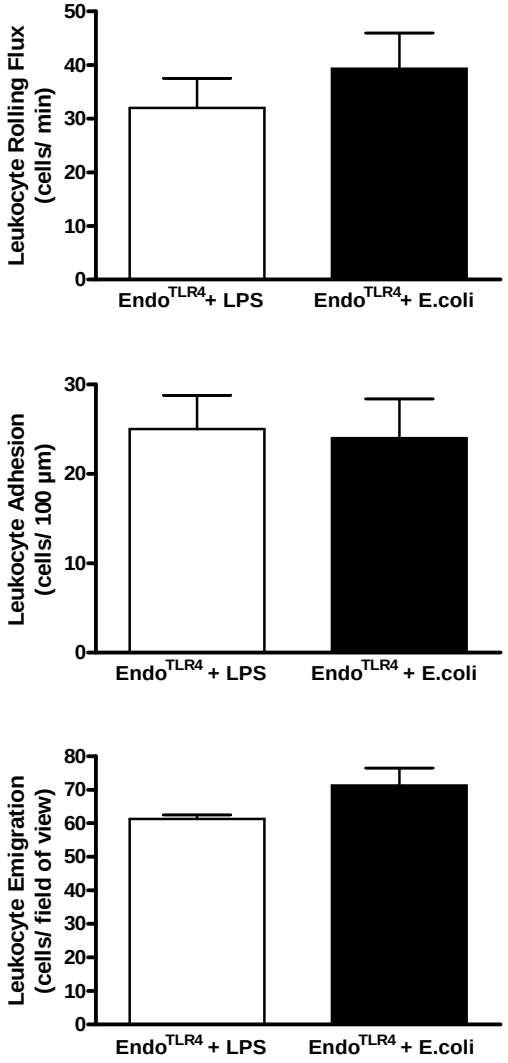
Supplemental Figure 5.- Pulmonary and peritoneal neutrophil sequestration induced by intraperitoneal LPS. Wild-type and Endothelium^{TLR4} mice were untreated or treated with LPS (intraperitoneal injection) for 4 or 24 hrs. At 4 hrs blood was drawn to determine the number of circulating leukocytes (1.7 ± 0.25 vs. 2.4 ± 0.54, wild-type vs. Endothelium^{TLR4} mice), the lungs were harvested to measure myeloperoxidase (A) and at 4 and 24 hrs the peritoneal lavage was collected to determine the number of neutrophil infiltrated (B). Data are expressed as the arithmetic mean ± SEM of n = 3. * $P < 0.05$ vs. untreated mice.



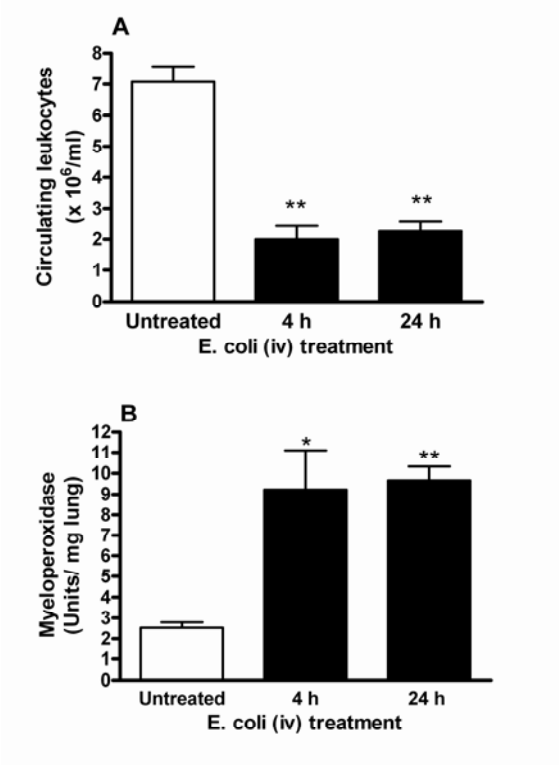
Supplemental Figure 6.- MIP-2 expression in lungs and peritoneal lavage of wild-type, TLR4-/- and Endothelium^{TLR4} mice is similar. Mice were untreated or treated with E. coli for 24h and at this time lungs were harvested and the peritoneal lavage was collected to determine MIP-2 expression by ELISA. Data are expressed as the arithmetic mean ± SEM of n= 3 in each group.



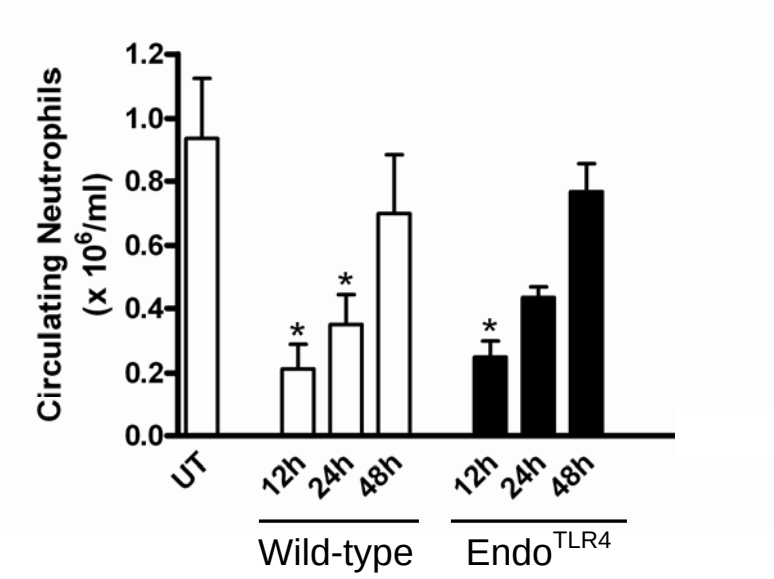
Supplemental Figure 7.- Wild-type mice treated with E.coli showed increased bacterial load in lung and peritoneum. Wild-type mice were untreated or treated with 1 x 10^7 CFU luminescent E. coli for 4 and 24 hrs. At these times, (A) the mice were imaged with the xenogen system and then (B) the lungs were harvested and (C) the peritoneal lavage was collected to determine the bacterial load.



Supplemental Figure 8.- Circulating leukocytes from E.coli treated Endothelium^{TLR4} mice respond to MIP-2 in the same fashion that LPS treated Endothelium^{TLR4} mice. Mice were treated for 24 hrs with LPS (white bars) or E.coli (1 x 10^7/ml) (black bars) and then MIP-2 was superfused onto the cremaster muscle and MIP-2 induced leukocyte rolling flux, leukocyte adhesion and leukocyte emigration was visualized by intravital microscopy. The LPS data are reproduced from Figure 5D, E, F to enable direct comparison. Data are expressed as the mean ± SEM of 3 mice in each group.



Supplemental Figure 9.- Wild-type mice treated with E.coli intravenously behaved in the same fashion that wild-type mice treated intraperitoneally. Wild-type mice were untreated or treated with E.coli (1 x 10^7) intravenously for 4 h and 24 h. A) Blood was drawn to calculate the number of circulating leukocytes and B) the lungs were harvested to determine the myeloperoxidase content. Data are expressed as the arithmetic mean ± SEM of n= 3-5 mice in each group. * $P < 0.05$ vs untreated, * $P < 0.01$ vs. untreated.



Supplemental Figure 10.- Wild-type and Endothelium^{TLR4} mice treated with E. coli showed neutropenia. Wild-type, Endothelium^{TLR4} were untreated or treated with 1 x 10^7 CFU luminescent E. coli for 12, 24 and 48 hrs. At these times, blood was drawn by cardiac puncture to count the number of circulating leukocytes. In addition, a blood smear was done to determine the percentage of the different white blood cell populations to calculate the absolute neutrophil counts. Data are expressed as the arithmetic mean ± SEM , n = 3-6 in each group.

Supplemental Methods

Generation of transgenic mice expressing endothelial TLR4

TLR4 was amplified by RT-PCR from total RNA obtained from mouse blood cells using two sets of primers [TLR4-F1 (224-246): AAA TGC CAG GAT GAT GCC TCC CT; TLR4-R1 (1561-1536): GAT AAG AAC GCT GAG AAT TCT GTG AC and TLR4- F2 (1536-1561): GTC ACA GAA TTC TCA GCG TTC TTA TC; TLR4-R2 (2780-2761): AGG ATC CGC AAA CAG ACT GGG TTT AGG]. TLR4 was cloned into PCR2.1TOPO, digested with BamHI/ScaI and subcloned in pDrive-IRES-EGFP yielding plasmid pDrive-TLR4-IRES-EGFP. Tie2promoter and Tie2 full enhancer, both indispensable to have specific endothelial expression of TLR4, were taken from pT2HLacZpA1I.7 vector (generously donated by Dr Thomas N. Sato, University of Texas, Southwestern Medical Center, Dallas). First, the 2.1 Kb HindIII fragment containing the Tie2promoter was released from pT2HLacZpA1I.7, blunt ended with Klenow fragment and cloned into the blunted-SnaBI site of pDrive-TLR4-IRES-EGFP yielding pDrive-Tie2promoter-TLR4-IRES-EGFP. Second, NotI/XbaI fragment containing Tie2 full enhancer (10 Kb) was released from pT2HLacZpA1I.7 and subcloned into NotI/NheI sites of Tie2promoter-TLR4-IRES-EGFP yielding pDrive-Tie2promoter-TLR4-IRES-EGFP-Tie2enhancer. Its fidelity was confirmed by both restriction endonuclease digestion and DNA sequence analysis.

The transgene, a 15.8 Kb fragment containing Tie2 promoter-TLR4-IRES-EGFP-Tie2 enhancer (Supplemental Fig. 1), was released from the plasmid and microinjected into fertilized eggs derived from the matings of inbred C57BL/6 mice (at the UAB Transgenic Mouse Facility). Positive female founder lines were mated with TLR4-/-

males to obtain Tie2-TLR4 transgenic mice. The mice were screened by PCR to confirm double heterozygosity by using three primers for TLR4-/-; Tlr4 WT: CGT CTA AAC CAG CCA GGT TTT GAA GGC; Tlr4 Common: TGT TGC CCT TCA GTC ACA GAG ACT CTG and NEO: ATC GCC TTC TAT CGC CTT CTT GAC GAG and 4 primers for EGFP; Tie-2-wt-forward: CTA GGC CAC AGA ATT GAA AGA TCT, Tie-2-wt-reverse: GTA GGT GGA AAT TCT AGC ATC ATC C, Tie-2-mutant-forward: ATT CTC GTG GAA CTG GAT GG and Tie-2-mutant-reverse: GGA CAG GTA ATG GTT GTC TGG. Three lines of mice were then set up as breeding pairs. All the offsprings were screened for Tlr4-/-/EGFP/EGFP and line 3 revealed optimal endothelial TLR4 expression.

Neutrophil isolation

Blood was collected from anesthetized mice by cardiac puncture with a heparinized syringe. The heparinized blood was centrifuged at 500g, 4°C, for 15 min. The clear layer of plasma was removed using a transfer pipette, diluted to 10% in HBSS, and stored at 4°C until needed. Mice were then euthanized and the femurs and tibias removed. The ends of the bones were resected and the bone marrow was removed by perfusion of 5 ml ice-cold PBS. The bone marrow was then suspended by drawing it through a 20-gauge needle. Marrow cells were then pelleted in a centrifuge (250g, 4°C, 12 min) and resuspended in 2 ml PBS. The cell solution was placed over a discontinuous Percoll gradient consisting of a stock Percoll solution (90 ml Percoll, 10 ml 10× HBSS) diluted to 72, 64, and 52% in HBSS. The cell solution was spun at 1100g, 4°C, for 30 min. Purified murine neutrophils localized to a band between the 72 and 64% layers. This

band was removed with a transfer pipette, washed in PBS, and suspended in HBSS + 10% murine plasma at 1.0×10^7 cells/ml.

Culture of endothelial cells

Mouse primary lung, heart and vena cava endothelial cells were isolated from 5–7-d-old wild-type, TLR4-/- and Endothelium^{TLR4} mice and were cultured according to the protocols described previously (1). Freshly isolated mouse endothelial cells were cultured in microvascular endothelial cell medium-2 (Clonetics EGM-2MV BulletKit; Cambrex Bio Science) in 35-mm Petri dishes precoated with 20 μg/ml mouse laminin (Upstate Biotechnology). After reaching confluence in 5–6 d, the cells were either used for Western blotting or trypsinized to determine TLR4 expression by flow cytometry.

Isolation of endothelial cells from adult mice

In some experiments endothelial cells from lungs and heart of adult untreated and LPS treated mice were purified to determine TLR4 expression in wild-type and Endothelium^{TLR4} mice. The lung and heart tissues were homogenized and resuspended in sucrose buffer (0.32 M sucrose, 3mM HEPES) to separate microvessels by microcentrifugation. Then microvessels were washed once in PBS and further treated with 1 mg/ml of collagenase (Boehringer Mannheim) for 30 minutes at 37 ºC to isolate the endothelial enriched cells. Then, these cells were washed with PBS and passed through a 40 μm mesh filter to obtain a single cell suspension. TLR4 expression on these cells was determined by flow cytometry.

Expression of TLR4

Expression of TLR4 on isolated neutrophils and isolated blood monocytes from wild-type, TLR4-/- and Endothelium^{TLR4} mice was determined using a BD FACScan flow cytometer (BDBiosciences, Mountain View, California, USA) and CellQuest Pro software (Becton Dickinson Immunocytometry Systems). Briefly, 1 x 10^6 cells were stained with phycoerythrin (PE) anti-mouse TLR4 mAb (UT41; eBioscience, San Diego, CA, USA) or an immunoglobin isotype control (PE Rat IgG1, k from eBioscience, San Diego, CA, USA) and FITC conjugated anti-mouse Ly-6G and Ly-6C mAb (RB6-8C5, BD Biosciences, Mississauga, ON, Canada) or an immunoglobin isotype control (FITC Rat IgG2b, κ chain from BD Biosciences, Mississauga, ON, Canada).

Expression of TLR4 on endothelial cells from wild-type, TLR4$^{-/-}$ and Endothelium^{TLR4} mice was determined by flow cytometry. Briefly, 0.5 x 10^6 endothelial cells from the culture were digested with Accutase (Chemicon, CA) under 37 °C for 5-10 minutes, then cells were washed once and incubated with FITC labeled anti-ICAM-2; (3C4, BD Bioscience) and PE labeled anti-mouse TLR4 (UT41, eBioscience) or their appropriate isotype controls for 30 minutes; after two washing with ice-cold FACS washing buffer, cells were collected on a BD FACScan flow cytometer (BD Biosciences) and data was analyzed using CellQuest Pro software (Becton Dickinson Immunocytometry Systems).

Intratracheal aerosolization and bronchoalveolar lavage (BAL)

Mice were anesthetized with isoflurane (MTC Pharmaceuticals, Cambridge, ON, CA) and suspended from their upper front teeth in a vertical, upright position. Oral intratracheal (IT) intubation was achieved under direct visualization using an operating microscope and a small animal laryngoscope (Penn-Century, Philadelphia, PA). Solutions were aerosolized directly into the distal trachea using a MicroSprayer (Model IA-1C, Penn-Century) attached to a stainless steel syringe (FMJ-250, Penn-Century).

Lungs were harvested at 24 h post-aerosolization. The trachea was exposed and cannulated using a blunted 18-gauge needle and BAL was performed. Aliquots of 1000-μl pyrogen-free Dulbecco's PBS were injected and withdrawn until approximately 10 mL of fluid was obtained. BAL fluid cells were counted using a standard hemocytometer followed by centrifugation at 300*g* for 5 min. The cell pellet was resuspended in 100 μl of PBS and cytocentrifuge slides prepared and stained with H&E (Cytospin 3; Shandon Scientific, Sewickley, PA). Differential cell counts were performed on 400 cells by one investigator blind to the experimental conditions.

After BAL, the heart and lungs were removed *en bloc* from the thoracic cavity as previously described (2). The lungs were inflated with 10% neutral buffered formalin, the trachea tied off and the lungs placed in formalin for 24-48 h for subsequent paraffin embedding. Sections were cut at 4 μm and stained with H&E and chloroacetate esterase (Leder stain, Sigma) for histological assessment using light microscopy in a blinded fashion.

Intravital microscopy

Mice were anesthetized by i.p. injection of a mixture of xylazine hydrochloride (10 mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and ketamine hydrochloride (200 mg/kg; Rogar/STB, London, Ontario, Canada). The jugular vein was cannulated and used to administer additional anesthetic, fluorescent dyes, and various drugs. Animals were then prepared to view the skeletal muscle microcirculation as previously reported by our laboratory.

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

The cremaster microcirculation was observed through an intravital microscope with a x10 eyepiece (Axioskop; Carl Zeiss Canada, Don Mills, Ontario, Canada) and a x25 objective lens. A video camera (Panasonic 5100 HS, Osaka, Japan) preparation was used to project the images onto a monitor, and the images were recorded for playback analysis using a videocassette recorder. Single unbranched venules (25-40 μm in diameter) were selected, and to minimize variability, the same section of the venule was observed throughout the experiment. The number of rolling and adherent neutrophils was determined off-line during video playback analysis. Rolling neutrophils were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Neutrophil rolling velocity was determined by measuring the time required for a neutrophil to roll along a 100 μm length of venule. Rolling velocity was determined for 20 neutrophil at each time interval. Leukocytes were considered adherent to the venular endothelium if they remained

stationary for 30s or longer. Neutrophil emigration was defined as the number of extravascular neutrophils per microscopic field of view, and was determined by averaging the data derived from four to five fields adjacent to postcapillary venules. Venular diameter (Dν) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline RBC velocity (V_{RBC}) was also measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute), and mean RBC velocity (V_{mean}) was determined as $V_{RBC}/1.6$. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 (V_{mean}/ D\nu)$.

Western blot for p38

Western blots were performed as previously described (3). Peritoneal macrophages were isolated by peritoneal lavage with saline from wild-type, TLR4-/- and Tie2-TLR4 transgenic mice. Non-adherent cells were removed before treatment with LPS (100 ng/ml) for 15, 30 and 60 minutes. Samples were prepared using 2x Laemmli's buffer and were resolved on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes, which were then blocked by incubation in 5% BSA in TBS plus 0.05% Tween 20 (TBST). The membranes were then incubated at 4°C overnight in the primary Ab (anti phospho- p38, Cell Signaling Technology, Danvers, MA), washed for 40 min in TBST, incubated for 1 h in secondary Ab (rabbit anti-mouse HRP, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and washed for another 40 min in TBST. To determine the total p38 the Abs from membranes were stripped, blocked in 5% BSA in TBS plus 0.05% Tween 20 and incubated with anti total p38 antibody (Cell Signaling

Technology, Danvers, MA) overnight, washed and then incubated with the secondary antibody for 1 h. The membranes were developed using an ECL substrate.

Quantitation of endothelial activation

To determine the degree of endothelial activation P-selectin known to contribute to leukocyte recruitment in endotoxemia was measured. We used a modified dual-radiolabeled Ab technique that permits in vivo measurements of endothelial P-selectin as previously described (4;5). The Ab RB40.34 against P-selectin was labeled with ^{125}I. The Ab A110-1 (a rat IgG_1, λ isotype standard) was labeled with ^{131}I. In both cases the Abs were labeled using the iodogen method, as previously described. A110-1 was used to detect nonspecific binding in the murine system.

To study P-selectin, animals were injected i.v. with a mixture of 10 μg ^{125}I-anti-labeled P-selectin (RB40.34) and a variable dose of ^{131}I labeled nonbinding Ab (A110-1). The Abs were allowed to circulate for 5 min, then the animals were heparinized. A blood sample was obtained from a carotid artery catheter, and then the mice were exsanguinated by blood withdrawal through the carotid artery catheter and simultaneous i.v. infusion with bicarbonate-buffered saline. The lung, heart, pancreas, mesentery, small bowel and muscle were harvested and weighed. Both ^{131}I and ^{125}I activities were measured in plasma and tissue samples.

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, that radiolabeled binding Ab can be displaced specifically with sufficient amounts of unlabeled Ab. The technique is sufficiently sensitive that very small, basal levels of P-selectin can be detected in wild-type mice relative to P-selectin-deficient mice where values are true zero(4;5).

Quantitation of cytokines

Plasma from wild-type, TLR4-/- and Endothelium^{TLR4} mice untreated or treated with LPS (1 mg/kg, intravenously) was collected at various times to measure the levels of TNF-α, IL-1β, and MIP-2 by ELISA (BD OptEIA sets, BD Biosciences, San Diego, CA). The sensitivity of ELISA was 5 pg/ml for both cytokines. All the assays were performed according to the manufacturer's instructions. All samples were assayed in duplicates. The ELISA was read in a spectrophotometer (SpectraMax Plus, Molecular Devices Corporation, Sunnyvale, CA, USA).

Determination of Tissue Myeloperoxidase Activity

At the end of each experiment samples of the lung were weighed, frozen on dry ice and processed for determination of myeloperoxidase (MPO) activity. Myeloperoxidase is an enzyme found in cells of myeloid origin, and has been used extensively as a biochemical marker of granulocyte (mainly neutrophils) infiltration into the lungs (6;7). The samples were stored at -20^{o}C for no more than one week before the MPO assay was performed. MPO activity was determined using an assay described previously, with the volumes of each reagent modified for use in 96-well microtitre plates. Change in absorbance at 450

nm over a 90 second period was determined using a kinetic microplate reader (Molecular Devices, Canada).

Circulating leukocyte counts

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

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, had their hair removed by chemical depilation, and $1x10^7$ CFU of the bacterial preparation was injected intraperitoneally in a volume of 1mL. The rate of spread and clearance of the bacteria was then monitored using an IVIS Lumina (Caliper Life Sciences), with imaging conducted 30 min, 4hrs, 12hrs, 24hrs post-infection (PI), and every 24hrs thereafter until the infection was cleared. Clearance was defined as 3 consecutive readings with no detectable luminescence. Experiments from other groups have demonstrated that the lux cassette is not lost in these *in vivo* models, over this time period, despite the lack of a selective antibiotic during the infection(8). Clearance of the bacteria was measured using three criteria. First, using Living Image software (Caliper Life Sciences) the area and luminescence of the infected area was measured. Mean area of the infected region, and the luminescence relative to the first imaging (30min PI) was then plotted. To normalize mice, the amount of luminescence above the diaphragm but below the head was an

estimate of bacteria escaping the peritoneal cavity and infiltrating the lung. Lastly, the time to clearance was measured.

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