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Bacterial Lipoprotein TLR2 Agonists Broadly Modulate Endothelial Function and Coagulation Pathways In Vitro and In Vivo

Hae-Sook Shin,*† Fengyun Xu,‡ Aranya Bagchi,*† Elizabeth Herrup,§ Arun Prakash,‡ Catherine Valentine,*,† Irshikesh Kulkarni,‡ Kevin Wilhelmsen,‡ Shaw Warren,*†,#,** and Judith Hellman‡

TLR2 activation induces cellular and organ inflammation and affects lung function. Because deranged endothelial function and coagulation pathways contribute to sepsis-induced organ failure, we studied the effects of bacterial lipoprotein TLR2 agonists, including peptidoglycan-associated lipoprotein, Pam3Cys, and murein lipoprotein, on endothelial function and coagulation pathways in vitro and in vivo. TLR2 agonist treatment induced diverse human endothelial cells to produce IL-6 and IL-8 and to express E-selectin on their surface, including HUVEC, human lung microvascular endothelial cells, and human coronary artery endothelial cells. Treatment of HUVEC with TLR2 agonists caused increased monolayer permeability and had multiple coagulation effects, including increased production of plasminogen activator inhibitor-1 (PAI-1) and tissue factor, as well as decreased production of tissue plasminogen activator and tissue factor pathway inhibitor. TLR2 agonist treatment also increased HUVEC expression of tissue plasminogen activator and tissue factor pathway inhibitor. TLR2 agonist treatment also increased lung fibrin levels. These studies show that TLR2 activation by bacterial lipoproteins broadly affects endothelial function and coagulation pathways, suggesting that TLR2 activation contributes in multiple ways to endothelial activation, coagulopathy, and vascular leakage in sepsis. The Journal of Immunology, 2011, 186: 1119–1130.

Although the pathways that are activated during sepsis have been extensively characterized, much remains to be learned about the mechanisms underlying sepsis-induced organ failure. The endothelium, through its effects on inflammation, the coagulation pathways, blood flow, and vascular barrier function, is thought to contribute to the pathogenesis of organ dysfunction in sepsis (1–4). The endothelium produces proinflammatory cytokines such as IL-6 and IL-8, and it participates in leukocyte recruitment to organs (4, 5). It is also centrally involved in modulating the coagulation pathways (2). Sepsis causes alterations in multiple limbs of the coagulation system leading to intravascular coagulopathy. Finally, endothelial activation is a critical contributor to vascular leak during sepsis (5, 6). Endothelial activation, coagulopathy, and vascular leak occur at locations that are remote from sites of infection, causing inflammation, edema, and ultimately “bystander organ injury” syndromes.

TLRs are a family of innate immune receptors that recognize conserved molecular motifs from microorganisms and play a central role in the initiation of inflammatory responses in sepsis (7–10). Although the various TLRs share intracellular pathways, there are differences between the TLRs. For instance, TLR4 recognizes LPS (11, 12), whereas TLR2 mediates the effects of bacterial lipoproteins and components of Gram-positive bacteria and fungi (13–16). Also, different TLRs can elicit different inflammatory responses, which is based in part on their differential utilization of the MyD88-dependent versus the TRIF-dependent signaling pathways (8, 17–20). TLRs have been most extensively studied in macrophages, but they are also expressed by many other cell types, including endothelial cells (21–24).

TLR2 has been viewed as being primarily involved in responses to Gram-positive bacterial infections. Additionally, however, lipoprotein TLR2 agonists such as peptidoglycan-associated lipoprotein (PAL), murein lipoprotein (MLP), and outer membrane protein A are ubiquitously expressed by Gram-negative bacteria, and there is increasing evidence that TLR2 is important in Gram-negative bacterial infections (25, 26). We previously observed that PAL, MLP, and outer membrane protein A are shed into human serum as well
as into the blood of animals with Gram-negative sepsis (27–29). We reported that TLR2 activation modulates inflammatory effects of other TLR agonists, induces systemic, lung, and myocardial inflammation in mice, and reduces contractility of cardiac myocytes in vitro (29–32). We have also found that TLR2 activation in vivo causes impaired vasocnstrictive responses to alveolar hypoxia and reduced partial pressure of arterial oxygen in mice (33).

Because systemic endothelia interact continuously with the circulation, components of microorganisms constantly come into contact with endothelial TLRs during sepsis. We therefore performed studies to test the hypothesis that activation of TLR2 contributes to endothelial dysfunction and coagulopathy in sepsis. We found that treatment with TLR2 agonists has broad effects on the endothelium in vitro and in vivo, including upregulation of endothelial inflammatory responses, increased neutrophil trafficking to the endothelium, altered expression of coagulation pathway factors, increased endothelial permeability, and increased lung levels of fibrin. We also found that TLR2 activation causes endothelial cell apoptosis and potentially upregulates septic responses by increasing expression of TLR2 itself.

### Materials and Methods

#### Reagents

PAL and MLP were prepared from *Escherichia coli* bacteria as described and confirmed to contain a single protein band by gold staining (29, 34). Pam3CysSKKK was purchased (EMC Microcollections, Tubingen, Germany). Preparations of PAL and Pam3Cys contained <5 pg LPS per microgram of protein, and MLP contained <0.25 pg LPS per microgram of protein based on the *Limulus* amoebocyte lysate assay. E. coli O111:B4 LPS was purchased (List Biological Laboratories).

#### Animals

The Institutional Animal Care and Use Committee approved all animal studies. C57BL/6J (wild-type) and TLR2-knockout (TLR2−/−) mice were purchased (The Jackson Laboratory).

#### Human endothelial cells

Human endothelial cells were incubated at 37°C under humidified 5% CO₂. HUVEC (passage 2–6), human lung microvascular endothelial cells (HMVEC-L, passage 4–5), and human coronary artery endothelial cells (HCAEC, passage 4–5) were purchased (Cambrex Bio Science). HUVEC were grown in EGM-2MV (Clonetics, Walkersville, MD). HMVEC-L and HCAEC were grown in EGM-2-MV (Clonetics, Walkersville, MD). Endothelial growth medium was supplemented with 2% FCS.

#### Stimulation of human endothelial cells

Cells were seeded into wells (4.5–7.5 × 10⁴ cells/cm²), grown to 70–80% confluence, and then incubated with TLR2 agonists. ELISAs were used to quantify cytokines (R&D Systems), tissue factor pathway inhibitor (TFPI; American Diagnostica), tissue plasminogen Ag activator (tPA; Innovative Research, Novi, MI), and plasminogen activator inhibitor-1 (PAL-1; Innovative Research) in supernatants. A cell-based ELISA assessed surface expression of E-selectin and TLR2. Immunobots were used to detect tissue factor (TF) and TLR2 in cell lysates. Viability and apoptosis were assessed using MTT and TUNEL assays, respectively.

#### Cell-based ELISA and for surface expression of E-selectin and TLR2 by human endothelial cells

Surface expression of E-selectin by HUVEC, HMVEC-L, and HCAEC was assessed using a cell-based ELISA at intervals up to 18 h of stimulation with PAL. After removing the supernatants and gently washing the cells with RPMI 1640/FCS, cells were incubated with mouse anti-human E-selectin (R&D Systems) for 45 min and then washed again with RPMI 1640/FCS.

#### Immunobots of HUVEC lysates

After incubation with TLR2 agonists and controls, HUVEC were lysed and protein concentrations of the lysates were estimated using the RCDC protein assay kit (Bio-Rad, Hercules, CA). The cell lysates contained solubilized proteins from all components of the cells, including organelles and membranes, but did not contain nuclear proteins. Total proteins were separated by SDS-PAGE (10% gels for TLR2, 12% gels for TF) and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in 3% BSA for 45 min at room temperature and then were incubated overnight with primary Abs at 4°C. Primary Abs used were anti–human tissue factor mouse monoclonal IgG (1:1000; no. 05-881; Upstate Biotechnology, Waltham, MA), anti-human TLR2 goat polyclonal IgG (0.5 μg/ml; AF2616; R&D Systems), or anti-actin goat polyclonal Ab (Sigma-Aldrich). After developing the immunobots, the same membrane was reprobed with anti-actin to confirm equal amounts of protein in the different samples. Binding of the primary Abs was determined by adding suitable peroxidase-conjugated secondary Abs (Jackson ImmunoResearch Laboratories). Immunobots were developed using a chemiluminescent substrate and then by exposing the blots to imaging films.

#### Neutrophil-endothelial cell adhesion

Studies using human polymorphonuclear neutrophils (neutrophils) were approved by the Institutional Human Studies Committee. Neutrophils were prepared from blood drawn from healthy human volunteers as described (35). The final neutrophil pellet was suspended in HEPES buffer containing Ca²⁺ (1 mM) and the fluorescent label calcein (5 μm; neutrophils, 10⁷/m; membranes, 10⁶/m) before detaching them at 37°C with calcium- and magnesium-free EGTA containing 1 mM calcium, PAL, or Pam3Cys. The cells were then washed three times with Dulbecco’s PBS with magnesium and calcium and incubated for 1 h at 37°C with goat anti-human TLR2 (R&D Systems) and normal goat IgG control (EMD, Gibbstown, NJ). Cells were washed with Dulbecco’s PBS and then incubated with a 1:2000 dilution of bovine-anti-goat-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 37°C. The HUVEC were then washed and developed by addition of TMB solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD). ODs were recorded at a wavelength of 450 nm. Absorbance values were normalized based on the crystal violet staining of adhered cells in each well.

#### Endothelial permeability assays

Permeability was determined by measuring FITC-albumin (Sigma-Aldrich) flux across the HUVEC monolayer using a Transwell system as described (37). HUVEC were seeded into the upper (luminal) chamber of 6.5 mm
Transwells that contained 0.4 μM pore polyester membrane inserts (Corning Life Sciences; 1 × 10⁶ cells/cm²). Cells were allowed to grow for 2–3 d until the monolayers were uniformly confluent, which was assessed by visualization using inverted microscopy and confirmed by measuring transendothelial resistance (38). Transendothelial resistance was measured using a tissue resistance measurement chamber (Endohm-6, World Precision Instruments, Sarasota, FL). Dilutions of Pam3Cys (0–15 μg/ml) were added to the luminal chamber in a volume of 100 μl medium. Cytomix, a mixture of equal amounts of recombinant human IL-1β, INF-γ, and TNF-α (10 ng/ml each; R&D Systems), was the positive control. For all conditions, the lower (abluminal) chamber contained 600 μl EGM-2 medium. After 24 h, FITC-albumin (200 μg/ml) was added to the luminal chamber in 100 μl medium. Two hours later samples were removed from both the luminal and abluminal chambers and their fluorescence was quantified using a standard optical FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC). The excitation and emission wavelengths were 485 and 520 nm, respectively. Using a standard curve of FITC-albumin, the readings were converted to albumin concentrations that were used in the following equation to determine the permeability coefficient of albumin (Pa): Pa = ((A/V × l/a)/(v[L]), where [A] and [L] are the abluminal and luminal FITC-albumin concentrations, t is time (seconds), a is area of membrane (cm²), v is volume of abluminal chamber (ml). Data are expressed as percentages of control (medium alone).

Endothelial viability and apoptosis assays

HUVEC viability was measured using an MTT cell viability assay kit as per the manufacturer’s instructions (Biotium, Hayward, CA). The effects of TLR2 activation on HUVEC apoptosis were assessed using the TUNEL assay as per the manufacturer’s instructions (HT TiterTACS; Trevigen, Gaithersburg, MD). Permeabilized cells treated with endonuclease served as the positive control for the TUNEL assay.

Preparation and stimulation of mouse endothelial cells from wild-type and TLR2 knockout mice

Mouse lung endothelial cells were prepared from lung tissue digests using immunomagnetic selection as described (39). Mice were euthanized by CO₂ asphyxiation and blood was removed by cardiac puncture. Lungs were harvested and then were then rinsed using DMEM/20% FCS, cut into 2-mm² blocks, and digested by incubating in type II collagenase (0.2% w/v) for 1 h at 37°C. The tissue was mechanically disrupted by passing the resultant digest 15–20 times through a blunt-tip needle and 10-ml syringe, and debris were removed by filtering the digested lungs through a 0.7-μm cell strainer. The filtrate was centrifuged (400 × g, 10 min, swinging bucket centrifuge, 4°C). The cell pellet was resuspended and then incubated for 2–3 min in ice cold RBC lysis buffer (Sigma-Aldrich; 3 ml per two mice), and then washed and centrifuged. Cells were then resuspended in Dulbecco’s PBS with calcium and magnesium (Invitrogen, Carlsbad, CA), and then the concentration of cells was adjusted to 10⁶ cells/ml. Immunoselection of endothelial cells was done by sequentially incubating the cells with rat anti-mouse PECAM-1 (BD Pharmingen, San Diego, CA) and rat anti-mouse ICAM-2 monoclonal IgGs (BD Pharmingen) that were covalently attached to magnetic beads (5 μl Ab/50 μl beads) 1–2 d prior to use as described by the manufacturer (Dynabeads; InvivoGen, San Diego, CA). The first magnetic immunoselection was done using anti-PECM-1 IgG. Cells were exposed to magnetic beads conjugated with anti-PECAM-1 IgG (50 μl Ab beads/3 × 10⁶ cells) for 10 min at room temperature, using end-over-end rotation. Approximately 15 ml of basic medium was added to the tube, which was then placed in a magnet, and the liquid was removed from the tube. The beads and the attached cells were then put into gelatin-coated flasks containing basic medium and the following additives: 1% sodium pyruvate, 1% nonessential amino acids, 1 mM l-glutamine, 150 μg/ml endothelial cell growth supplement (Biomedical Technologies, Stoughton, MA), and 12 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated for 3–4 h to allow attachment of the cells to the transwell insert (24-well complete medium). Medium was changed every 2–3 d. When cells reached 70–80% confluence, which took ~10–14 d, they were detached using trypsin/EDTA. Trypsin was neutralized by addition of basic medium (10 ml), and the cells were collected by centrifugation. After this, cells were resuspended in Dulbecco’s PBS, incubated with magnetic beads conjugated with anti-ICAM-2 IgG for the second immunoselection, and processed as described above for the first immunoselection. The high cell-density morphologically unaltered endothelial cells were then centrifuged for 10 min at 200 × g at 4°C and used for all experiments. To verify that the cells used for these studies expressed endothelial cell markers and to assess purity, we analyzed cells that had been frozen down from these experiments by immunofluorescence staining and flow cytometry essentially as described (39). Staining Abs included anti-vascular endothelial cadherin (CD144; Cayman Chemical, Ann Arbor, MI), ICAM-2 (CD102; BD Biosciences; San Jose, CA), and control Abs, including rat and rabbit IgG. Secondary Abs included FITC-labeled goat anti-rabbit IgG and FITC-labeled goat anti-rat IgG (Millipore). Both immunofluorescence staining and flow cytometry confirmed that 84–98% of the cells expressed these endothelial cell markers, which is consistent with the purity obtained by other investigators (39).

The mouse lung endothelial cells were placed in wells of 96-well plates at a density of 4.5 × 10⁵ cells/cm² and were incubated with PAL, LPS (positive control for the TLR2−/− cells), or medium alone. IL-6 levels were quantified in the culture supernatants by ELISA (R&D Systems).

In vivo experiments

Female mice aged 8–10 wk were used for in vivo studies. For studies measuring plasma levels of PAL-1 and E-selectin, as well as lung expression of inflammatory mRNAs, female mice were injected i.v. via the tail vein with carrier (50 mM sodium phosphate [pH 7.4]) or TLR2 agonists. Mice were euthanized by CO₂ inhalation, and blood and lungs were collected. ELISAs were used to quantify plasma levels of soluble E-selectin (R&D Systems) and PAL-1 (Innovative Research) levels. Lungs were analyzed for E-selectin, P-selectin, and MCP-1 mRNA levels using quantitative real-time PCR, and for neutrophil activity by measuring myeloperoxidase (MPO) levels (29).

Immunoblotting was used to assess the effects of Pam3Cys challenge on lung fibronectin deposition (40). C57BL/6J mice were anesthetized by s.c. administration of the combination of ketamine, xylazine, and acepromazine and then were challenged by intratracheal administration of Pam3Cys (200 μg/mouse) or carrier, each in a volume of 40 μl, followed by 1 ml air. After 20 h, the mice were treated with systemic heparin (20 U i.v.) and then euthanized by CO₂ inhalation. The lungs were perfused with saline, isolated, and homogenized in ice-cold homogenization buffer (20 mM Tris, 150 mM NaCl, and 2 U/ml heparin). Lung tissue digests were then incubated with 0.1 U plasmin at 37°C for 4 h with shaking to release fibrin monomers and then were centrifuged and the supernatants were collected and stored in −80°C. Fibrin levels were measured using immunobolts. The membranes were stained with an Ab against mouse fibrin (American Diagnostics, Stamford, CT). Immunoreactive proteins were detected using SuperSignal West Dura (Thermo Scientific, Rockford, IL) and visualized using FluorChem 5500 imaging system (Alpha Innotech, San Leandro, CA), and band intensities were quantified via spot densitometry. After developing the immunoblots, the same membrane was stripped and reprobed with anti-actin to confirm equal amounts of protein in the different samples.

Quantitative real-time PCR

Quantitative real-time PCR was performed on lungs of wild-type and TLR2−/− mice after treatment with Pam3Cys, PAL, or saline. Quantitative real-time PCR was done using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) and TaqMan premade primers for E-selectin, P-selectin, and MIP-1 (Applied Biosystems). Presence of a single amplification product was verified using dissociation curves, rRNA (18S) was detected using 18S primers (forward, 5′-CGCGTACACCATCAGGGA-3′, reverse, 5′-GCTGAAATTGGGCTCC-3′) (DNA Oligonucleotide Synthesis Core, Massachusetts General Hospital, Boston, MA). Changes in gene expression were normalized to 18S rRNA levels using the relative cycle threshold method.

Statistics

Data are expressed as means ± SD. The data were analyzed using t tests when comparing two conditions, one-way ANOVA with Bonferroni’s post hoc analysis when comparing more than two conditions, and Mann–Whitney tests for data that were not normally distributed. GraphPad Prism was used for statistical analyses. The p values of <0.05 were considered significant. Except where indicated, the following denote p values in the figures: *p < 0.05; **p < 0.01; ***p < 0.001.

Results

TLR2 agonists activate human endothelial cells

In initial experiments, HUVEC were incubated with culture medium alone or with serial dilutions of PAL, a naturally occurring TLR2 agonist that is expressed by enteric Gram-negative bacteria. PAL caused a concentration-dependent increase in IL-6 and IL-8 levels in culture supernatants (Fig. 1A, 1B). Time course experi-
ments with PAL (1 μg/ml) indicated that both IL-6 and IL-8 levels were similar to baseline at 2 h, but both were significantly increased by 6 h, continued to rise through 18 h, and then remained stable through the 24 h time point (Fig. 1C).

Additional experiments were done to assess the effects of two other bacterial lipoproteins: MLP, a naturally occurring TLR2 agonist in the cell wall of Enterobacteriaceae, and Pam3Cys, a synthetic TLR2 agonist. All three TLR2 agonists induced IL-6 production by HUVEC (Fig. 1D). Based on the differences in molecular mass of PAL (18–21 kDa), MLP (5–9 kDa), and Pam3Cys (1.5 kDa), on an equimolar basis, PAL was found to be significantly more potent at inducing IL-6 than either MLP or Pam3Cys.

**PAL activates human lung microvascular and coronary artery endothelial cells**

To assess the effects of TLR2 activation on endothelial cells in other vascular beds, we studied effects of PAL on inflammatory responses of HMVEC-L and HCAEC. Treatment with PAL increased production of IL-6 and IL-8 by both HMVEC-L (Fig. 1E) and HCAEC (Fig. 1F).

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**FIGURE 1.** TLR2 agonists activate human endothelial cells to secrete IL-6 and IL-8. A and B, HUVEC monolayers were incubated with dilutions of PAL (n = 4). Levels of IL-6 (A) and IL-8 (B) were quantified in the culture supernatants at 18 h. *p < 0.05; **p < 0.01 PAL versus medium. C, HUVEC monolayers were incubated with PAL (1 μg/ml) for intervals through 24 h (n = 3). A time course of IL-6 and IL-8 levels induced by PAL was plotted. *p < 0.05; **p < 0.01; ***p < 0.001 IL-6, PAL versus medium; #p < 0.01; ##p < 0.001 IL-8, PAL versus medium. D, HUVEC monolayers were incubated for 18 h with equal concentrations (1.5 μg/ml) of PAL or MLP, both of which are naturally occurring TLR2 agonists, or Pam3Cys, which is a synthetic TLR2 agonist (n = 3). IL-6 levels in supernatants: *p < 0.01; ***p < 0.001 for TLR2 agonist versus medium. E and F, Monolayers of human lung microvascular endothelial cells (E) and human coronary artery endothelial cells (F) were treated with medium or PAL (1 μg/ml) for 18 h (n = 4). Levels of IL-6 and IL-8 were quantified in culture supernatants. *p < 0.001; ***p < 0.001 PAL versus medium. G, Lung microvascular endothelial cells from wild-type and TLR2−/− mice were incubated overnight with PAL (1 μg/ml) or medium (n = 6). Levels of IL-6 were quantified in culture supernatants. LPS (1 μg/ml), a TLR4 agonist, was used as the positive control to verify intact intracellular TLR signaling. ***p < 0.001 PAL versus medium in wild-type endothelial cells; ****p < 0.001 PAL-treated endothelial cells from wild-type versus TLR2−/− mice.
TLR2 mediates activation of mouse lung endothelial cells by PAL

To confirm that the effects of PAL on endothelial inflammatory responses are mediated through TLR2, we incubated lung endothelial cells from wild-type and TLR2−/− mice with PAL or with medium alone. There was no detectable production of IL-6 by endothelial cells that were treated with medium alone, suggesting that the process of preparing and growing the mouse lung endothelial cells did not in itself lead to sustained activation of the endothelial cells. Treatment with PAL increased production of IL-6 by lung endothelial cells from wild-type but not from TLR2−/− mice (Fig. 1G), indicating that the expression of IL-6 induced by PAL is mediated through TLR2. IL-6 levels were equally increased in the supernatants of endothelial cells from wild-type and TLR2−/− mice that were treated with LPS, indicating intact signaling through intracellular TLR signaling pathways.

**TLR2 activation increases endothelial E-selectin expression**

HUVEC, HMVEC-L, and HCAEC were incubated with PAL or Pam3Cys (1–2 μg/ml) or with medium for intervals up to 18 h, after which the surface expression of E-selectin was assessed using a cell-surface ELISA and flow cytometry. For all three types of endothelial cells, PAL upregulated E-selectin at the cell surface (Fig. 2A, shown for 2 h). In contrast to IL-6 and IL-8 levels, which gradually rose to a maximum at 18 h, surface E-selectin expression was significantly increased at 2 h, was maximal at 4 h, and remained elevated at the 18 h time point (Fig. 2B). Flow cytometry using HUVEC that were treated for 18 h with PAL (1 μg/ml) or Pam3Cys (1 μg/ml) confirmed that TLR2 agonist treatment upregulates surface expression of E-selectin (Fig. 2C).

**Lipoprotein TLR2 agonists enhance neutrophil adherence to HUVEC monolayers**

Neutrophil–endothelial cell interactions are thought to be important in the migration of neutrophils to areas of inflammation. We therefore assessed binding of calcein-labeled human neutrophils to HUVEC that had been preincubated for 2 h with PAL, MLP, and Pam3Cys (1.5–12 μg/ml). The 2 h time point was chosen based on our data, indicating that surface expression of E-selectin on HUVEC was increased by 2 h of exposure to PAL. All three TLR2 agonists caused a dose-dependent increase in binding of neutrophils to pretreated HUVEC (Fig. 2D, shown for TLR2 agonist concentration of 1.5 μg/ml). A representative fluorescent micrograph that shows the increased binding of calcein-labeled neutrophils to endothelial cell monolayers is included in Fig. 2D.
Systemic activation of TLR2 increases plasma E-selectin levels in mice

Wild-type and TLR2<sup>−/−</sup> mice were injected i.v. with Pam3Cys (25 µg/mouse) or PAL (25 µg/mouse), and plasmas were collected after 90 min. Treatment with PAL or Pam3Cys increased soluble E-selectin levels in the plasma of wild-type mice but not in TLR2<sup>−/−</sup> mice (Fig. 3A, shown for PAL only). Soluble E-selectin levels were increased equally in the plasma of both wild-type and TLR2<sup>−/−</sup> mice after treatment with LPS, indicating intact signaling through intracellular TLR signaling pathways (data not shown).

Systemic activation of TLR2 upregulates expression of mRNAs for E-selectin, P-selectin, and MCP-1

Quantitative real-time PCR was used to assess mRNA expression in the lungs of mice. Pam3Cys and PAL both increased expression of mRNAs encoding the adhesion molecules E-selectin and P-selectin and the chemotactic cytokine MCP-1 (Fig. 3B, 3C). For all three markers, lung mRNA expression peaked 2 h after challenge with Pam3Cys or PAL. However, only MCP-1 mRNA remained elevated at 22 h (Fig. 3B). The time course experiments were performed using high dosages of Pam3Cys (400 µg/mouse). This dose was chosen based on the dosages of LPS that are often used to study lung physiology and inflammatory responses in mice. However, because we also found that lower doses induced lung inflammatory mRNAs, we used lower dosages (25–50 µg/mouse) for subsequent in vivo experiments that compared responses of wild-type and TLR2<sup>−/−</sup> mice. Looking at the previously defined 2 h point of rising E-selectin expression in vitro, we found increased expression of E-selectin, P-selectin, and MCP-1 mRNAs in the lungs of wild-type but not TLR2 knockout mice (Fig. 3C). Again, treatment with LPS equally increased mRNA levels in lungs of wild-type and TLR2<sup>−/−</sup> mice.
We next tested the expression of PAI-1 in wild-type and TLR2 knockout mice, indicating that TLR signaling pathways were intact after TLR2 knockout (data not shown).

**Systemic activation of TLR2 causes neutrophil infiltration of the lung**

We previously reported that peripheral blood neutrophil counts are decreased in mice treated with Pam3Cys (33). Because we found that TLR2 activation also increases expression of adhesion molecules and chemokines, we hypothesized that activation of endothelial TLR2 would stimulate neutrophil trafficking to the lungs, which can be indirectly assessed by measuring MPO levels. Challenge with Pam3Cys (25 μg/mouse) or PAL (25 μg/mouse) increased MPO levels in the lungs of wild-type mice but not in the lungs of TLR2−/− mice (Fig. 3D, shown for Pam3Cys only). In contrast, lung MPO levels were equally increased in the lungs of LPS-challenged wild-type and TLR2−/− mice, indicating that downstream TLR signaling is intact.

**TLR2 agonists increase PAI-1 production by HUVEC**

Sepsis causes multiple coagulation abnormalities, including derangements in the fibrinolytic portion of the coagulation system. PAI-1 is an inhibitor of fibrinolysis. We incubated HUVEC monolayers with Pam3Cys and quantified PAI-1 in culture supernatants. Treatment with Pam3Cys increased PAI-1 levels in HUVEC culture supernatants in a concentration-dependent manner (Fig. 4A).

**Treatment with Pam3Cys reduces HUVEC secretion of tPA**

tPA is produced by the endothelium and plays an important role in fibrinolysis. We measured tPA total Ag in HUVEC culture supernatants after 24 h of treatment with dilutions of Pam3Cys. Treatment with Pam3Cys reduced HUVEC secretion of tPA concentration-dependently (Fig. 4B).

**TFPI levels are reduced in supernatants of HUVEC treated with TLR2 agonists**

TFPI regulates the TF coagulation pathway. To assess the effects of TLR2 on the modulation of this pathway, TFPI levels were quantified in HUVEC culture supernatants after 24 h of treatment with PAL (20 μg/ml). TFPI levels were reduced in the supernatants of the PAL-treated versus medium-treated HUVEC (Fig. 4C).

**TF levels are increased in lysates of HUVEC treated with TLR2 agonists**

TF initiates coagulation by activating the extrinsic pathway of plasma coagulation and is the primary initiator of cell-based coagulation. We used immunoblots to assess TF expression in HUVEC lysates following treatment with PAL (20 μg/ml) or medium. A representative immunoblot is shown in Fig. 4D. TF expression was increased in the lysates of HUVEC at 1, 6, and 24 h of treatment with PAL (Fig. 4D).

**Systemic TLR2 activation increases plasma PAI-1 in mice**

We next tested the expression of PAI-1 in wild-type and TLR2−/− mice after i.v. challenge with TLR2 agonists. We found that PAI-1 levels were increased in the plasma of wild-type mice injected with either PAL (25 μg/mouse) or Pam3Cys (50 μg/mouse) when compared with the plasmas of mice injected with carrier (Fig. 5A, 5B). PAI-1 levels were not increased in the plasma of TLR2−/− mice treated with Pam3Cys, indicating that challenge with Pam3Cys induces PAI-1 specifically through TLR2 (Fig. 5B).

**TLR2 agonist treatment increases lung fibrin in mice**

To further explore the effects of TLR2 on lung coagulation, immunoblots were performed to assess fibrin deposition in the lungs of C57BL/6 mice 20 h after intratracheal challenge with Pam3Cys (200 μg/mouse). Fig. 5C shows a representative immunoblot, and the graph shows the fibrin band intensity relative to the actin band intensity of carrier-treated versus Pam3Cys-treated mice as assessed by spot densitometry (n = 4). Increased fibrin was detected in the lungs of mice that were treated with Pam3Cys as compared with carrier (Fig. 5C).

**Treatment with Pam3Cys increases endothelial permeability**

Increased vascular permeability to albumin and other macromolecules is a hallmark of septic organ dysfunction. We used a Transwell system to assess the effects of Pam3Cys on permeability. HUVEC monolayers were incubated in the upper (luminal) chamber with Pam3Cys (5–15 μg/ml) for 24 h, after which FITC-labeled albumin was added to the luminal chamber, and flux was quantified based on fluorescence intensity measurements of samples taken from both the luminal and abluminal chamber.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** TLR2 agonists alter coagulation pathway factor expression in vitro. A, HUVEC monolayers were treated with medium or increasing concentrations of Pam3Cys as indicated in the figure (n = 4), and PAI-1 levels were quantified at 24 h. *p < 0.05; **p < 0.01 Pam3Cys versus medium. B, HUVEC monolayers were treated with medium or increasing concentrations of Pam3Cys as indicated in the figure (n = 4), and tPA levels were quantified at 24 h. *p < 0.05; **p < 0.01 Pam3Cys versus medium. C, HUVEC monolayers were treated with PAL (20 μg/ml) or medium (n = 3) and TFPI levels were quantified in the cultures supernatants after 24 h. ***p < 0.001 Pam3Cys versus medium. D, A representative immunoblot showing expression of TF (upper panel) in HUVEC lysates after 1, 6, and 24 h of exposure to PAL (20 μg/ml). The actin blot (lower panel) indicates equal loading of different lanes. Protein (250 μg) was loaded per lane.
Pam3Cys concentrations of $\geq 10 \mu g/ml$ increased the permeability of HUVEC monolayers to FITC-albumin (Fig. 6).

**Pam3Cys decreases HUVEC viability and increases HUVEC apoptosis**

To understand mechanisms by which TLR2 agonists change endothelial permeability, we considered the hypothesis that they might induce apoptosis or otherwise cause cell death. The effects of the TLR2 on HUVEC viability were therefore studied using the MTT assay, and the effects on apoptosis were studied using a TUNEL assay (Pam3Cys, 1.67–40 $\mu g/ml$). Low concentrations of Pam3Cys minimally affected viability, whereas viability was significantly reduced at Pam3Cys concentrations $\geq 15 \mu g/ml$ (Fig. 7A). Pam3Cys also induced apoptosis in the TUNEL assay at concentrations $\geq 5 \mu g/ml$ (Fig. 7B).

**TLR2 agonists upregulate HUVEC TLR2 expression in a concentration- and time-dependent manner**

To determine whether TLR2 agonist treatment regulates TLR2 expression, immunoblots were done to detect TLR2 in lysates of HUVEC that were treated with Pam3Cys at intervals from 1 to 24 h. TLR2 expression was minimal at baseline, and Pam3Cys upregulated TLR2 expression in a concentration- and time-dependent manner (Fig. 8A). Whereas TLR2 was strongly upregulated at the higher concentrations of Pam3Cys, TLR2 expression was visibly increased at the lowest concentration that we tested (0.31 $\mu g/ml$). TLR2 expression was strongly increased at 8 h, continued to rise until 20 h, and remained elevated through 24 h. Cell-based ELISA and flow cytometry studies were done to determine whether TLR2 agonist treatment upregulates surface expression of TLR2. Binding of anti-TLR2 IgG, but not isotype control IgG, was slightly increased in cells that were treated with Pam3Cys as compared with cells treated with medium alone (Fig. 8B). However, much higher concentrations of Pam3Cys were required to upregulate surface expression of TLR2 (10 $\mu g/ml$) than were required to upregulate TLR2 in HUVEC lysates. In fact, TLR2 was increased in HUVEC lysates at the lowest concentration of Pam3Cys tested (0.31 $\mu g/ml$). Flow cytometry studies also showed slight upregulation of TLR2 expression at the HUVEC surface by treatment with the higher concentration (10 $\mu g/ml$) of Pam3Cys and PAL (Fig. 8C). The differences in concentrations required to induce TLR2 in cell lysates versus at the cell surface suggest that a significant proportion of the TLR2 that we detected in the lysates is intracellular. This finding is consistent with another published study showing predominant expression of TLR2 intracellularly in HUVEC, with slight increased expression at the cell surface after stimulation with inflammatory mediators (41).

**Discussion**

During sepsis there is extensive interplay between disparate systems, including those involved in systemic inflammation, coagulation, and vascular barrier function. The endothelium plays a critical role in regulating these systems, and derangements as a result of endothelial activation or dysfunction can contribute to organ failure during sepsis (2–4). Our studies demonstrate that activation of TLR2 has broad effects on endothelial phenotype and function. We found that TLR2 activation increases endothelial cell expression of cytokines and of other inflammatory mediators in vitro and in the lungs of mice. Consistent with the upregulated expression of chemokines and adhesion molecules, more neutrophils adhere to endothelial monolayers after activation of TLR2, and MPO levels are increased in the lungs of mice challenged with TLR2 agonists. Our studies also indicate that TLR2 activation modulates endothelial cell expression of factors involved in coagulation and in fibrinolysis. A functional effect of TLR2 activation on coagulation is suggested by the increased fibrin levels that we found in the lungs of Pam3Cys-challenged mice. We also found that activation of TLR2 increases endothelial permeability, a novel finding suggesting that TLR2 activation may contribute to the vascular leak seen clinically in sepsis. Finally, on a cellular level, we observed that HUVEC treatment with TLR2 agonists causes upregulation of TLR2 expression, decreased cell viability, and increased apoptosis.

Our data show that TLR2 agonists activate endothelial cells from different vascular beds, including from the lung (HMVEC-L) and the heart (HCAEC). The fact that TLR2 agonists induce inflammatory responses in multiple endothelial cell types suggests that TLR2 activation has widespread effects on the endothelium in different tissues during sepsis. We have previously shown that TLR2 agonists are shed into the blood by bacteria in animal models of sepsis (28, 29). Thus, TLR2 agonists have access to a vast endothelial cell surface throughout the body during sepsis, and...
circulating TLR2 agonists could contribute to the induction of diffuse endothelial activation and dysfunction as well as coagulopathy, and through these effects to the development of multiple organ failure. In addition to playing a role in sepsis, studies have implicated TLR2 in the pathogenesis of atherosclerotic vascular disease (42). Our data support the notion that microbial components acting through endothelial TLR2 might be involved in the progression of coronary artery disease or even potentially in acute ischemic events.

Whereas some investigators have reported that TLR2 agonists induce endothelial inflammatory responses (43–47), others have reported that TLR2 agonists do not induce endothelial inflammatory responses (41, 48, 49). There are several plausible explanations for the discrepancies in different studies. The plating density and presence of serum during the period of stimulation may affect results. Additionally, differences in batches of endothelial cells could potentially contribute to variability in responses due, for instance, to TLR polymorphisms (50). Our data that baseline TLR2 expression by HUVEC is low corroborate several reports that unstimulated human endothelial cells have only low levels of baseline TLR2 expression (41, 43, 48, 51). Human TLR2 has been shown to be upregulated by cytokines, histamine, and LPS, but it has not been heretofore shown to be upregulated by TLR2 agonists themselves (41, 43, 49, 51). We found that TLR2 expression was upregulated over time by treatment with Pam3Cys. We observed that the degree of upregulation of TLR2 on the cell surface by cell-based ELISA and flow cytometry did not seem to be as marked as that observed in immunoblots of cell lysates. Additionally, higher concentrations of Pam3Cys were required to upregulate TLR2 at the cell surface than in the cell lysates, which suggests that much of the increased TLR2 was intracellular. This is in line with another study showing predominantly intracellular localization of TLR2 in HUVEC at baseline and an increase in TLR2 at the surface of the HUVEC following treatment with IFN-γ or IL-1β (41). In contrast to our results, other investigators have found that lipoteichoic acid, a nonlipoprotein TLR2 agonist, does not upregulate endothelial TLR2 expression (49). Although the cause for the differences in induction of TLR2 between these two TLR2 agonists is not clear, the considerable difference in structure between the two agonists could be responsible. The possibility that different TLR2 agonists have different potencies is supported by the differences that we observed in induction of IL-6 production and neutrophil-endothelial adhesion by PAL, Pam3Cys, and MLP.

Multiple abnormalities of coagulation pathways occur in sepsis, including activation of procoagulant pathways, reduced production of anticoagulants, and decreased fibrinolysis. At the extreme, coagulation disturbances manifest as disseminated intravascular coagulopathy (52). Even in the absence of overt manifestations, disturbances in the balance of coagulation and anticoagulation occur that likely contribute to organ dysfunction during sepsis by impairing microcirculation and affecting the delivery of oxygen and nutrients to organs. PAI-1 inhibits fibrinolysis, thereby favoring perpetuation of already formed clots. Recent studies indicate that higher PAI-1 levels were associated with an increased incidence of organ failure and death in sepsis, pneumonia, and acute lung injury (53–57). Our findings that TLR2 agonists upregulate PAI-1 expression by HUVEC in vitro as well as increase PAI-1 in vivo in the circulation of mice suggest that TLR2 activation contributes to increased PAI-1 expression during sepsis. We also observed that TLR2 activation increased TF expression and decreased TFPI secretion by endothelial cells. We speculate that TLR2 activation may contribute to sepsis-induced coagulopathy through its broad effects on both the coagulation and fibrinolytic sides of coagulation and thrombosis. The global pattern of decreased anticoagulation (TFPI), increased coagulation (TF), decreased production of tPA, which facilitates fibrinolysis, and increased inhibition of fibrinolysis (PAI-1) should favor a hypercoagulable state and predispose to microvascular thrombosis.

Respiratory dysfunction is common in patients with septic shock. TLR2 agonists have been shown by us and other investigators to induce systemic and pulmonary inflammation (29, 33, 58, 59). We also have found that activation of TLR2 causes impaired hypoxic pulmonary vasoconstriction in mice (33). Hypoxic pulmonary vasoconstriction is a physiological response to alveolar hypoxia that helps to maintain arterial blood oxygenation by facilitating ventilation/perfusion matching. Impairment of hypoxic pulmonary vasoconstriction, as occurs in sepsis, results in shunting of blood through unventilated areas of the lung, thereby contributing to hypoxemia. In the same study, we found that mice treated with Pam3Cys had reduced arterial partial pressure of arterial oxygen
Because of these findings, we hypothesize that during sepsis, TLR2 activation may have additional functional effects on the lungs. Both endothelial cell activation and dysfunction and neutrophil sequestration and activation within the lung contribute to lung injury during sepsis (60). In early sepsis, endothelial cells express chemotactic cytokines and adhesion molecules, which promote neutrophil trafficking and transmigration across the endothelium. Our data indicating that TLR2 activation upregulates multiple processes involved in leukocyte trafficking to the lung and increases lung fibrin deposition support the hypothesis that TLR2 may contribute to the pathogenesis of sepsis-induced respiratory failure.

Increased vascular permeability is also prominent in septic shock. It allows fluid and protein to leak out of the intravascular space into the surrounding tissues, causing tissue edema and intravascular hypovolemia (6). These can contribute to shock and organ hypoperfusion. In the lung, sepsis-induced vascular leak leads to noncardiogenic pulmonary edema, which contributes to respiratory failure. The mechanisms underlying vascular leak in sepsis have not been fully defined. We observed that TLR2 agonist treatment increases endothelial permeability, which suggests that TLR2 activation might contribute to vascular leakage during sepsis.

The concentrations of TLR2 agonists required to induce responses varied depending on the endpoint. Whereas 40 ng/ml to 1 μg/ml concentrations were capable of upregulating the expression of cytokines, adhesion molecules, and some of the coagulation pathway factors, higher concentrations (≥10 μg/ml) were required for other effects, such as permeability and apoptosis. This suggests that the strength of TLR2 activation may result in variable intracellular signaling pathways being used for the different responses observed in endothelial cells. However, further studies will be required to define the precise pathways leading from TLR2 activation to alterations in coagulation, apoptosis, and increased permeability. Apoptosis may have contributed to the increased permeability. We speculate that coagulation pathway intermediaries also may be involved in the increased permeability that we observed. Prior studies support a role for coagulation pathway factors in regulating vascular permeability during inflammation (61–63). For instance, TF activity has been reported to contribute to the increased permeability induced by TNF-α and IL-1.

The inflammatory effects of TLR2 activation have been described in a number of studies, but the effects of TLR2 activation on endothelial cell activation and function during sepsis have not been defined. We have demonstrated that bacterial lipoprotein TLR2 agonists broadly affect endothelial functions, including causing increased endothelial permeability, and that they modulate coagulation pathways in vitro and in vivo. The observed alterations in

![FIGURE 8. Pam3Cys upregulates TLR2 expression by HUVEC. A. Immunoblot analyses of lysates of HUVEC monolayers that were incubated with Pam3Cys at different concentrations (upper panel) and for intervals up to 24 h (lower panel, Pam3Cys concentration 20 μg/ml). Fifteen micrograms of protein was loaded per lane. Primary Abs for the immunoblot were goat antihuman TLR2 IgG and goat anti-actin IgG. Molecular mass markers are indicated at the left. B. Cell-based ELISA for surface expression of TLR2 after 20 h of treatment with Pam3Cys (1 and 10 μg/ml). * p < 0.05 TLR2 agonist versus medium. There was no increase in binding of control IgG to the cell surface at either concentration of Pam3Cys. C. Flow cytometry for surface expression of TLR2 after 20 h of treatment with PAL (1 and 10 μg/ml). Similar results were obtained using Pam3Cys (not shown).]
endothelial function and coagulation pathways closely resemble those that occur in patients with sepsis. Our studies suggest that TLR2 activation contributes to sepsis-induced endothelial dysfunction, coagulopathy, and organ failure, and they raise the possibility that interventions for sepsis should target endothelial TLR2 pathways.

Disclosures

The authors have no financial conflicts of interest.

References


