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Profound Differences in Leukocyte-Endothelial Cell Responses to Lipopolysaccharide Versus Lipoteichoic Acid

Bryan G. Yipp,* Graciela Andonegui, † Christopher J. Howlett, ‡ Stephen M. Robbins, § Thomas Hartung, § May Ho,* and Paul Kubes†‡

We have investigated the effects of LPS from Escherichia coli, lipoteichoic acid (LTA), and peptidoglycan (PepG) from Staphylococcus aureus, and live S. aureus on leukocyte-endothelial interactions in vivo using intravital microscopy to visualize muscle microvasculature. Systemic vs local administration of LPS induced very different responses. Local administration of LPS into muscle induced significant leukocyte rolling, adhesion, and emigration in postcapillary venules at the site of injection. LPS given systemically dramatically dropped circulating leukocyte counts and increased neutrophils in the lung. However, the drop in circulating leukocytes was not associated with leukocyte sequestration to the site of injection (peritoneum) nor to peripheral microvessels in muscles. Unlike LPS, various preparations of LTA had no systemic and very minor local effect on leukocyte-endothelial interactions, even at high doses and for prolonged duration. LPS, but not LTA, potently activated human endothelium to recruit leukocytes under flow conditions in vitro. Endothelial adhesion molecule expression was also increased extensively with LPS, but not LTA. Interestingly, systemic administration of live S. aureus induced leukocyte-endothelial cell responses similar to LPS. PepG was able to induce leukocyte-endothelial interactions in muscle and peritoneum, but had no effect systemically (no increase in neutrophils in lungs and no decrease in circulating neutrophil counts). These results demonstrate that: 1) LPS has potent, but divergent local and systemic effects on leukocyte-endothelial interactions; 2) S. aureus can induce a systemic response similar to LPS, but this response is unlikely to be due to LTA, but more likely to be mediated in part by PepG. The Journal of Immunology, 2002, 168: 4650–4658.

A key feature of inflammation is the sequestration of leukocytes from the circulation to the endothelium, resulting in leukocyte emigration into the surrounding tissue. Leukocyte recruitment occurs in a sequential multistep fashion (1, 2). Endothelium activated, for example, with LPS or TNF-α expresses the adhesion molecules that mediate tethering, rolling, and adhesion. Therefore, a localized response to LPS or infection will induce brisk recruitment of leukocytes without undue damage to the surrounding tissue or organs. In contrast, an inappropriate inflammatory response such as systemic sepsis will cause leukocytes to localize to the lung, where they become physically trapped and are not able to recirculate (3–6). Indeed, a key feature in human septic shock from Gram-negative or Gram-positive bacteria is inappropriate leukocyte recruitment predominantly in lung with a tendency for impaired lung function (7).

Gram-negative septic shock is believed to result from the effects of LPS (8, 9). Recent evidence has established a model for LPS recognition that involves both CD14, a glycosylphosphatidylinosi-tol-linked protein, and an associate signal transducer Toll-like receptor 4 (TLR4) (10, 11). Therefore, TLR4 is a critical receptor involved in the LPS detection system (12) that confers responsiveness to circulating and tissue leukocytes as well as endothelium (13). By contrast, lipoteichoic acid (LTA), the Gram-positive cell membrane equivalent to LPS, does not appear to activate TLR4, but rather functions through a second TLR, namely TLR2, to activate immune responses. Nevertheless, in macrophages or cell line equivalents, the in vitro work has suggested very similar mechanisms of action for the two lipoproteins. Both LPS and LTA act by binding a TLR (14–18), activating various tyrosine kinases, and translocating the transcription factor NF-κB (19), resulting in the production and release of numerous proinflammatory mediators, including TNF-α, IL-6, IL-1β, IL-12, and NO (20–24). However, two very recent in vitro studies reported some significant differences in mediator release in response to TLR2 vs TLR4 activation (25, 26). Although in vivo studies have suggested that both LPS and LTA can induce the same end result, namely shock (8, 27, 28), at least for leukocyte-endothelial cell interactions LPS and LTA may elicit very different responses. For example, TLR4, the receptor for LPS, was abundant on endothelium, whereas TLR2, the receptor for LTA, could not be detected (13). Since the endothelium affects leukocyte trafficking by expressing adhesion molecules in lungs as well as peripheral microvasculature, LPS vs LTA responses may be quite different.

We have investigated leukocyte recruitment in response to local and systemic LPS or LTA by directly visualizing leukocyte behavior in vivo, further characterizing the direct effects of each

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3 Abbreviations used in this paper: TLR, Toll-like receptor; HDMEC, human dermal microvascular endothelial cell; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; PepG, peptidoglycan.

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lipoprotein on various microvascular beds. Our findings demonstrate that LTA and LPS induce profoundly different responses in terms of leukocyte recruitment. Whereas LPS elicited profound leukocyte recruitment, LTA had no noticeable effects in vivo or in vitro at the same concentration. Our results provide direct evidence that the in vitro macropathage activity of LTA is not predictive of its physiological effects upon leukocyte-endothelial cell interactions.

Materials and Methods

Reagents

All reagents for tissue culture, unless specified, were purchased from Life Technologies (Gaithersburg, MD). LTA from *Staphylococcus aureus* (Sigma-Aldrich, Oakville, Ontario, Canada) was reconstituted in sterile PBS, filtered, and stored at −20°C. Highly purified LTA, which was >99% pure and contained <6 pg LPS/μg LTA, as assessed by Limulus amebocyte assay, was isolated using a novel isolation procedure (24). Peptidoglycan (PepG) from *S. aureus* (Fluka, Oakville, Ontario, Canada) was reconstituted in sterile PBS and sonicated for 1 h before use. Smooth LPS from *E. coli* was provided by S. Goyert (North Shore University Hospital/New York University School of Medicine, Manhasset, NY.). rTNF-α was purchased from R&D Systems (Minneapolis, MN).

In vivo experiments

Male BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) weighing between 20 and 25 g were used at 6–10 wk of age and were housed in a double barrier pathogen-free facility. One postcapillary venule was visualized under stereomicroscopy, and “n” refers to the total number of mice. For consistency, we chose unbranched vessels that were 25–35 μm in diameter, consistent with postcapillary venules.

Intravital microscopy

The mouse cremaster preparation, which was approved by the animal care committee, University of Calgary, was used to study the behavior of leukocytes in the microcirculation (29). 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; RogarSTB, London, Ontario, Canada). The jugular vein was cannulated and used to administer additional anesthetic. 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.

An intravital microscope (Axioskop; Carl Zeiss Canada, Don Mills, Ontario, Canada) with ×25 objective lens (Wetzlar L25/0.35; E. Leitz, Munich, Germany) and a ×10 eyepiece was used to examine the cremasteric microcirculation. A video camera (Panasonic 5100 HS, Osaka, Japan) was used to project the images onto a monitor, and the images were recorded for playback analysis using a videocassette recorder. Single unbranched cremasteric venules (25–40 μm in diameter) were selected, and to minimize variability, the same section of cremasteric venule was observed throughout the experiment. The number of rolling and adherent leukocytes was determined off-line during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocyte rolling velocity was determined by excluding those cells moving at a velocity less than that of erythrocytes. The number of rolling and adherent leukocytes was determined off-line during video playback analysis. Rolling leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 s or longer. Leukocyte emigration was defined as the number of extravascular leukocytes per microscopic field of view (×25 objective lens), and was determined by averaging the data derived from four to five fields adjacent to postcapillary venules. Venular diameter (*D*) was measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline RBC velocity (*V<sub>mean</sub>*), was measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute), and mean RBC velocity (*V<sub>mean</sub>*), was determined as *V<sub>mean</sub>/1.6. Venular wall shear rate (γ) was calculated based on the Newtonian definition: γ = 8 (V<sub>mean</sub>/Dv)μ.

**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. MPO is an enzyme found in cells of myeloid origin, and has been used extensively as a biochemical marker of granulocyte (mainly neutrophil) infiltration into the lung (3, 30). The lungs were sampled at −20°C for no more than 1 wk before the MPO assay was performed. MPO activity was determined using an assay described previously (31), but with the volumes of each reagent modified for use in 96-well microtiter plates. Change in absorbance at 450 nm over a 90-s period was determined using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

**Circulating and peritoneal neutrophil counts**

At the end of each experiment, whole blood was collected via cardiac puncture. Total leukocyte counts were performed, using a Bright-line hemocytometer (Hauser Scientific, Horsham, PA). Mice were sacrificed, and the peritoneal cavity was washed with 10 ml HBSS. The cells were sedimented by centrifugation at 260 × g for 5 min. The pelleted was resuspended in DMEM containing 5% FCS. The total number of cells in the lavage fluid was counted using a hemocytometer.

**Quantitation of expression of P-selectin**

Expression of the adhesion molecules P-selectin was quantified using a modified dual-radioabeled Ab technique (32, 33). The Abs RB40.34 (against P-selectin) and A110-1 (a rat IgG1, λ isotype standard) were labeled with either 125I (RB40.34) or 131I (A110-1) using the iodogen method, as previously described (32, 33). A110-1 was used to detect non-specific binding in the murine system.

To study P-selectin, animals were injected i.v. with a mixture of 10 μg 125I-labeled P-selectin (RB40.34), and a variable dose of 131I-labeled 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 the Abs 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 intestine, colon, skin, and muscle were harvested and weighed. Both 125I and 131I 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 (131I-labeled A110-1) from the accumulated activity of the binding Ab (125I-labeled RB40.34). Data for P-selectin were represented as the percentage of the injected dose of Ab per gram of tissue. It has been demonstrated previously 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 zero (32).

**Experimental protocol**

To determine the in vivo effect of LTA, PepG, and LPS, we examined the local and systemic responses in BALB/c mice. Our initial experiments were performed using LTA, PepG, or LPS concentrations that were similar to the concentrations of LPS used in previous studies.

**Local LTA or LPS administration**

The local response to LTA was examined by injecting varying concentrations of LTA in 200 μl sterile saline s.c. beneath the scrotal skin using a 30-gauge needle. The animals were prepared for intravital microscopy at 3.5 or 23.5 h after LTA administration, and the microvasculature was observed for 60 min. At 4.5 or 24.5 h, the animals were sacrificed, the lungs were harvested for MPO assay, and peripheral blood was drawn for circulating leukocyte counts. Preliminary experiments with LPS demonstrated a dramatic effect of 0.05 μg/kg at 4 h on the local microvasculature; therefore, our initial experiments with LTA were performed under the same conditions. Increased concentrations and prolonged times were subsequently examined.

**Systemic LTA, PepG, LPS, or live *S. aureus* administration**

The systemic response was examined by injecting 500 μl of varying concentrations of LTA, PepG, or LPS i.p. for 4 and/or 24 h. The microvasculature was directly observed at 3.5 or 23.5 h for 60 min. At the end of the intravital observations, the lungs from each animal were immediately harvested and frozen at −70°C, peripheral blood was drawn, and a peritoneal lavage was performed. The initial concentration of 500 μg/kg for 4 h was chosen to make a direct comparison between LTA and LPS. Subsequent experiments with LTA determined the effects of increasing concentrations and longer response times. *S. aureus* ATCC strain 25923 was grown on blood agar plates (Difco, Detroit, MI) for 18 h, harvested, and washed twice with saline. Mice were injected i.p. with 104 CFU/g body weight.
**In vitro experiments**

As in vivo studies revealed very minor leukocyte-endothelium response to LTA, we performed in vitro studies: 1) in macrophage cell lines to determine whether our LTA had biological activity, and 2) in a human endothelial-leukocyte system to ensure that the lack of biological activity of LTA was not restricted to mice.

**Activation of macrophage cell lines**

THP-1 (American Type Culture Collection, Manassas, VA) and RAW 264.7 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate at 37°C in a 5% CO₂ atmosphere. To induce the differentiation of the THP-1 cells to the more mature macrophage-like phenotype, cells were suspended to a cell density of 5 × 10⁶ cells/ml in fresh medium containing 5 × 10⁻⁵ M 1,25-dihydrovitamin D3 and incubated for 72 h (34). Both cell lines were stimulated with either LPS (100 ng/ml) or LTA (100 ng/ml) for the indicated times. Western blots were performed on cell-free lysates (35) with the phospho-specific mitogen-activated protein kinase (MAPK) Ab (Promega, Madison, WI), phospho-specific p38 Ab (New England Biolabs, Beverly, MA), or the total p38 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control, according to manufacturer’s specification.

**Endothelial cell isolation**

Microvascular endothelium was isolated using a previously established protocol (36). Discarded neonatal human foreskin was cut into 2- to 3-mm² pieces and digested with 0.5 mg/ml collagenase type A1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) in supplemented M199 for 16 h at 4°C. Each segment of skin is placed keratinized side down, and microvessels were obtained by compressing the tissue with a spatula. The collected endothelial cells were cultured in EBM-2 with supplements (Clonetics, San Diego, CA) on 35-mm gelatin-coated tissue culture dishes. Human dermal microvascular endothelial cell (HDMEC) was grown to confluence for each flow chamber experiment, and cells were used before the seventh passage. Adhesion molecule expression was previously shown to be stable up to and including the seventh passage. This protocol was approved by the Ethics Committee of the University of Calgary.

**Leukocyte recruitment under flow conditions**

Leukocyte recruitment was determined using a parallel plate flow chamber (36). All experiments were performed with heparinized whole blood obtained from healthy human donors. The blood was diluted (10%) in sterile HBSS at 37°C. The human blood was drawn through the flow chamber and over the HDMEC at a rate of 2 dynes/cm² using an infusion pump. Experiments were recorded and analyzed off-line. Leukocyte rolling flux was determined by counting the number of leukocytes that roll past a given plane that is perpendicular to the direction of the flow. Leukocytes that stayed stationary for at least 10 s were counted as adherent cells. For each condition, at least six fields were visualized.

**Statistical analysis**

Data are shown as mean ± SEM. The mean for intravital experiments was determined by combining the data from three time points (3.5, 4, and 4.5 h postinjection) for each vessel. All data were analyzed using one-way ANOVA, and Bonferroni correction was applied where multiple comparisons were necessary.

**Results**

**Local LPS, but not LTA, causes profound leukocyte-endothelial cell interactions**

Under normal conditions, baseline leukocyte rolling flux within a postcapillary venule was approximately 75 cells/min (Fig. 1A). None of these cells adhered (Fig. 1B) or emigrated out of the microvessels (Fig. 1C). Four hours after local administration of LPS, the rolling flux ranged between 100 and 200 cells/min (Fig. 1A). Leukocyte adhesion was apparent, with an average of 12.5 cells/100 μm (Fig. 1B). Additionally, an average of 20 cells emigrated into the surrounding tissue (Fig. 1C). In contrast, LTA did not have any effect on leukocyte rolling, adhesion, or emigration (Fig. 1A–C). It was conceivable that the effective dose of LTA may be higher than LPS, so we decided to increase the dose by 10- and 100-fold (0.5 and 5 μg/kg) (Fig. 1). Even under these ultrapathological concentrations of LTA, no increase in leukocyte-endothelial cell interactions was observed. Local LPS administration at these concentrations causes stasis of the local microcirculation (P. Kubes, unpublished observation). No vascular changes, including decreased blood flow or a change in vessel diameter, were noted with LTA (data not shown). As the effect of local LTA administration may not follow the same time course as local LPS administration, we examined the response 24 h after the initial administration of LTA. LTA did not have any effect on leukocyte rolling (Fig. 1A), adhesion (Fig. 1B), or emigration (Fig. 1C) at 24 h.

A recent report (24) demonstrated that a highly active form of LTA could be purified using butanol instead of phenol. The local effect of this highly active form of LTA was investigated. Highly purified LTA did not have any local effect on leukocyte rolling, adhesion, or emigration (Table I).

**Systemic LPS, but not LTA, causes profound changes in the microcirculation**

Although the local administration of LPS had no apparent systemic effects (e.g., no change in circulating leukocyte counts), systemic administration of LPS induced a striking decline in peripheral circulating leukocytes (Fig. 2A). Concurrent with a reduction in circulating leukocytes was a dramatic increase of neutrophil accumulation in the lung (Fig. 2B). Interestingly, addition of LPS i.p.
Table I. Effect of local and systemic administration of highly purified LTA on leukocyte kinetics

<table>
<thead>
<tr>
<th></th>
<th>Leukocyte Rolling Flux (cells/min)</th>
<th>Leukocyte Adhesion (cells/100 μm)</th>
<th>Leukocyte Emigration (cells/field of view)</th>
<th>Circulating Leukocytes (&gt;10⁹/ml)</th>
<th>Lung MPO (U/mg tissue)</th>
<th>Peritoneal Leukocytes (&gt;10⁹/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local control</td>
<td>75.0 ± 9.0</td>
<td>0.5 ± 0.5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>7.2 ± 0.6</td>
<td>5.66 ± 0.95</td>
</tr>
<tr>
<td>Local LTA (0.05 μg/kg)</td>
<td>59.1 ± 18.27</td>
<td>0.33 ± 0.33</td>
<td>0.5 ± 0.5</td>
<td>0.375 ± 0.183</td>
<td>7.2 ± 0.6</td>
<td>5.79 ± 0.83</td>
</tr>
<tr>
<td>Systemic control</td>
<td>70.37 ± 7.12</td>
<td>0.125 ± 0.125</td>
<td>0.36 ± 0.36</td>
<td>7.38 ± 14.79</td>
<td>9.0 ± 0.5</td>
<td>1.47 ± 0.16</td>
</tr>
<tr>
<td>Systemic LTA (500 μg/kg)</td>
<td>73.83 ± 14.79</td>
<td>2.3 ± 0.66</td>
<td>1.66 ± 0.33</td>
<td>9.6 ± 0.33</td>
<td>7.96 ± 0.36</td>
<td>1.35 ± 0.22</td>
</tr>
</tbody>
</table>

*LTA was administered i.p. at 0.05 or 500 μg/kg for 4 h. Leukocyte kinetics were determined by observing the microvasculature of the cremaster muscle under an intravital microscope. Harvesting lung tissue and assaying for MPO determined the level of neutrophil sequestration to the lung. Leukocyte counts were determined under light microscopy from blood drawn by cardiac puncture or peritoneal lavage. No significant differences were found between any of the groups. Each parameter represents n = 3.

at concentrations that had systemic effects did not recruit any leukocytes to the site of injection, as there was no change in peritoneal leukocyte numbers (Fig. 2C). In direct contrast, LTA did not cause a drop in the circulating leukocyte counts (Fig. 2A) nor an increase in neutrophil sequestration to the lung within the same 4-h time frame (Fig. 2B). Moreover, administration of LTA for 24 h did not have any effect on the circulating leukocyte counts, lung MPO values, or peritoneal leukocyte counts (Fig. 2, A–C). We examined systemic LTA at 10 times (5,000 μg/kg) and 100 times (50,000 μg/kg) the LPS dose. High doses of LTA did not have any effect on the number of peripheral circulating leukocytes, neutrophil sequestration to the lung, or peritoneal sequestration (Fig. 2, A–C). We administered the highly purified LTA systemically, but did not observe any effect on leukocyte rolling, adhesion, or emigration (Table I).

**Systemic LPS prevents leukocyte-endothelial cell interactions in the periphery**

The decrease in circulating leukocyte counts may have been responsible for the lack of recruitment into the peritoneum. To further explore leukocyte-endothelial cell interactions in the periphery following systemic LPS administration, we exteriorized the cremaster muscle. Remarkably, the number of white blood cells rolling through the vessels dropped from 50–100 cells/min in the control group to less than 5 cells/min in the LPS group (Fig. 3A).

Due to the 95% reduction in rolling cells, very few leukocytes adhered (Fig. 3B) and emigrated (Fig. 3C) out of the vessels compared with local LPS administration and consistent with the observation in the peritoneum. Fig. 3 highlights that neither LTA (500 μg/kg) nor concentrations of LTA 10- or 100-fold higher lowered the number of rolling leukocytes (Fig. 3A), but did have a minor effect on leukocyte adhesion (Fig. 3B), which did not result in emigration (Fig. 3C). Twenty-four-hour administration of LTA did not have any effects on leukocyte-endothelial cell interactions (Fig. 3). Therefore, whereas systemic LPS caused profound changes in leukocyte trafficking, systemic LTA had very minor effect.

Fig. 4 summarizes quantitative P-selectin expression in various murine vascular beds. All tissues had minimal basal P-selectin expression and responded very significantly to LPS. The pulmonary, stomach, and cremaster muscle microvasculature revealed minimal P-selectin expression (Fig. 4, A–C) following LTA administration. Similar lack of effect was noted in heart, other parts of the gastrointestinal tract (small and large intestine), skin, and spleen (data not shown). By contrast, the mesentery (Fig. 4D) and pancreas (data not shown) demonstrated some significant increase in P-selectin in response to LTA, but the values were still much lower than with LPS.

**LTA does not induce leukocyte-endothelial cell interactions in a human system**

To ensure that these observations were not related to species resistance to LTA, we examined leukocyte-endothelial cell interactions in surrogate human blood vessels (flow chamber seeded with
human endothelium). Perfusion of leukocytes across unstimulated endothelium caused no rolling or adhesion. Addition of LPS for 4 h caused a profound increase in rolling and adhesion that was comparable with values observed with our most potent inducer (TNF-α) of leukocyte-endothelium interactions in human systems (Fig. 5). In direct contrast, LTA at similar concentrations to LPS caused no leukocyte rolling or adhesion (Fig. 5). Twenty-four-hour incubations with LTA had no effect on leukocyte rolling and adhesion (data not shown). The concentration of LTA was examined at 0.1, 1, 10, and 100 μg/ml. None of the LTA concentrations induced leukocyte rolling and adhesion. The highly purified form of LTA failed to induce leukocyte-endothelial cell interactions (data not shown). Each concentration was tested at least four times. These data are consistent with the observations that LPS and TNF-α induced E-selectin and VCAM-1 on human endothelium, but LTA failed to induce any response (data not shown).

FIGURE 3. Effect of systemic LTA or LPS on leukocyte kinetics. Mice were treated with LTA (500, 5,000, or 50,000 μg/kg) or LPS at 500 μg/kg and observed using intravital microscopy at 4 or 24 h postinjection. A, Leukocyte rolling flux; B, leukocyte adhesion; C, leukocyte emigration. Data are expressed as the arithmetic mean ± SEM from at least three mice in each group. *, p < 0.05 compared with control groups; ***, p < 0.001 compared with control groups.

LPS and LTA both elicit biological responses in murine and human macrophages

To ensure that our LTA was active, we used a monocyte cell line THP-1 that is known to respond to LTA. Unstimulated THP-1 cells did not adhere to plastic, whereas when LPS (100 ng/ml) was added to the cell suspension, essentially all THP-1 cells firmly adhered. LTA (100 ng/ml) induced an identical proadhesive phenotype (Fig. 6A), but was quantitatively lower than with LPS (Fig. 6B). In addition, 100 ng/ml of both LPS and LTA induced the phosphorylation of MAPK (p42/p44, also known as extracellular signal-related kinase 1/2) (Fig. 6C). Finally, message for various chemokines was detected with both lipoproteins (data not shown).

To eliminate the possibility that LTA only activates human THP-1 cells, we also tested the murine macrophage cell line RAW 264.7. These cells were stimulated with the same concentrations of LTA or LPS, and again induction of MAPK was determined using Western blot analysis. Both LPS and LTA induced the activation of MAPK (p42/p44) as well as another member of the MAPK family, p38 (Fig. 6D). Interestingly, in both cell lines, there was a noticeable difference between LPS and LTA stimulation in terms of amplitude and timing. LPS induced a more rapid and robust

FIGURE 5. The direct effect of LTA and LPS on HDMEC-mediated leukocyte recruitment. HDMEC was stimulated with 100 μg/ml LTA for 4 h. Diluted fresh whole blood was then perfused over the endothelium, and leukocyte-endothelial cell interactions were observed. TNF-α and LPS were used at 20 ng/ml and 0.1 μg/ml, respectively. Data are expressed as the arithmetic mean ± SEM of six separate experiments for LTA and three separate experiments for LPS and TNF-α.
kinase activation, which was easily detected within 15 min of stimulation compared with the weaker activation by LTA, which was first detected at 30 min. This difference in signaling between LPS and LTA was also consistent when highly purified LTA was used (data not shown).

Gram-positive bacteria induce leukocyte-endothelial cell interactions

To ensure that Gram-positive bacteria could induce leukocyte-endothelial cell interactions, animals received an i.p. injection of live *S. aureus* (10^10 CFU/g). In 4 h, the bacteria caused a 50% decrease in circulating leukocyte counts (Fig. 7A). The MPO assay showed that a significant number of neutrophils had sequestered in the lung (Fig. 7B). Interestingly, the numbers of neutrophils sequestered in the lung were similar between *S. aureus*- and LPS-treated animals. The smaller drop in circulating leukocyte counts allowed for some infiltration into the peritoneum (Fig. 7C). Intravital microscopy revealed that *S. aureus* induced the characteristic drop in the number of rolling leukocytes that was seen with LPS, but not LTA (Fig. 7D). Much like LPS, which essentially eliminated all rolling, only a few leukocytes rolled and adhered with *S. aureus* (Fig. 7, D and E) with some emigration into the surrounding tissue (Fig. 7F).

Since PepG, an alternative Gram-positive molecule, has previously been shown to have some biologic activity, we examined leukocyte responses to this substance. Surprisingly, our data revealed that some, but not all aspects of Gram-positive or LPS responses were seen with i.p. PepG administration. Although PepG had no effect on circulating leukocyte counts (Fig. 7A) or the number of leukocytes that sequestered into the lung (Fig. 7B), there were some effects on leukocyte behavior in the periphery. A subtle increase in peritoneal leukocyte recruitment was noted with PepG (Fig. 7C). Whereas a very profound drop in rolling cells was seen in the peripheral muscle microcirculation with LPS or Gram-positive bacteria, the PepG did not decrease rolling leukocytes, and in fact an increase was noted (Fig. 7D). Moreover, a very significant rise in adhesion (Fig. 7E) and some increase in emigration were also noted (Fig. 7F). Addition of LTA to PepG in no way enhanced the PepG response (data not shown).

Discussion

Sepsis and septic shock are characterized as widespread inflammatory responses that ultimately lead to failure of multiple organs. Regardless of the organ of septic origin, the lung is generally the first to fail, at least in part because of rapid accumulation of neutrophils in the narrow pulmonary capillaries (7). It is well appreciated that Gram-negative bacteria can cause sepsis and that LPS,
a major cell wall component of these organisms, is involved. Indeed, local exposure of the microcirculation to LPS can result in a decrease in microcirculatory flow and an increase in leukocyte-endothelial cell interactions (37). The impact of systemic LPS administration on leukocyte-endothelial cell interaction in peripheral microcirculatory beds is less well characterized, although it is well appreciated that as an early event many of the leukocytes infiltrate the pulmonary vasculature (7). With the increased prevalence of Gram-positive sepsis, many laboratories have begun to investigate the molecular mechanisms leading to activation of the immune system by components of these organisms. LTA has received a tremendous amount of attention as the key cell wall component of Gram-positive bacteria that functions as an immune activator with characteristics similar to LPS. The impact of local or systemic LTA on leukocyte-endothelial interactions in vivo remains unknown.

In this study, we directly visualized the activation of the innate immune system in the microvasculature in response to local or systemic LTA or LPS administration in an attempt to establish the importance of these two molecules as inducers of leukocyte-endothelial interactions in vivo. Our in vivo data reveal very profound differences between LPS and LTA with respect to leukocyte-endothelial cell interactions. Whereas local LPS induced a cascade of events, including leukocyte rolling, firm adhesion, and migration of leukocytes into the surrounding tissue, LTA had a very limited impact on any of these parameters. Neither extremely high concentrations of LTA, different preparations of LTA, nor longer times of exposure to LTA could induce leukocyte-endothelial cell interactions. These data are not consistent with the many studies that have demonstrated that LTA, devoid of other contaminants, could stimulate various macrophage cell lines to produce and release cytokines and chemokines in vitro (21–24, 38). Clearly, our data would suggest that if indeed these cells were activated in vivo, the amounts of cytokine and chemokine were simply not sufficient to induce leukocyte-endothelial cell interactions.

An alternative explanation is that the Gram-positive and Gram-negative sepsis has very different pathological mechanisms, with only the latter recruiting neutrophils to the sites of infection. However, this does not appear to be the case. The inappropriate inflammatory immune responses observed with Gram-positive and Gram-negative bacteria are strikingly similar and are in fact indistinguishable clinically (39). Comparing experiments with live S. aureus (Gram positive) or E. coli (Gram negative) reveal endpoint similarities, including similar cardiovascular dysfunction (40), rapid neutrophil infiltration into lungs, and increased cytokine and adhesion molecule expression (41). In fact, in our study, administration of S. aureus induced a similar pattern of leukocyte responses, as did LPS, but not LTA, suggesting perhaps that the latter molecule does not account for the leukocyte responses observed in Gram-positive sepsis.

At odds with our results are reports that LTA has been able to induce E-selectin, VCAM-1, and ICAM-1 expression on human endothelium from both umbilical vein (42) and lung (43), leading to leukocyte adhesion. Although it is tempting to speculate that our data differ due to human vs mouse sensitivity to LTA, when we stimulated human endothelium with LTA, no notable increase in leukocyte rolling and adhesion was observed. The other difference between in vivo and previously published in vitro systems is that the in vitro work was done under static conditions. This is not entirely reflective of the dynamic shear conditions that limit leukocyte-endothelial interactions. Indeed, our in vitro work was also performed under shear through flow chambers, but when we arrested flow and allowed the cells to settle onto the endothelium, leukocyte adhesion was still not observed (data not shown). An alternative explanation is that some LTA preparations have been demonstrated to contain sufficient LPS to account for cellular activation (44). Although the aforementioned studies that observed endothelial responses to LTA also included studies that attempted to discount potential LPS-contaminating effects, LPS as a potential synergistic or priming factor for LTA remains a serious concern. Consistent with this concern is the fact that the receptor essential for LTA effects, namely TLR2, was not detected on endothelium (13). Presently, the work as a whole suggests that LTA does not directly stimulate endothelium sufficiently in vivo or under shear conditions in vitro to induce leukocyte-endothelial cell interactions. Our only caveat at this stage is the small increase in P-selectin in mesentery and pancreas, which raises some minor tissue specificities.

Our data also provide some interesting new observations with respect to leukocyte-endothelial cell responses with systemic LPS. A number of investigators have reported that leukocyte recruitment is reduced to the primary site of infection in systemic septic response (45). Lack of adhesiveness or responsiveness perhaps due to shedding of critical adhesion molecules or internalization of chemokine receptors has been proposed as an explanation for the lack of leukocyte recruitment (46–48). Our data suggest another response to systemic LPS worth noting. Addition of LPS to the peritoneal cavity at concentrations that had systemic effects caused a very profound drop in circulating leukocytes and dramatic sequestration of leukocytes in the lung. Interestingly, this leukocytopenia in wild-type animals translated into fewer leukocyte-endothelial cell interactions in peripheral vasculature and an inability to recruit leukocytes (adhesion and emigration) into peripheral microcirculations, including the peritoneum and skeletal muscle. This delay in leukocyte recruitment into nonpulmonary tissues in septic humans has been appreciated for many years and may account for the multiorgan dysfunction associated with septic shock (49). Although LPS has been shown to increase endothelial adhesion molecule expression in all tissues (32), the preferential recruitment of leukocytes into lungs may not be related to molecular adhesive events, but rather due to physical trapping of activated (rigid) leukocytes within narrow architecture of pulmonary capillaries (3, 6, 30), which precedes the adhesion molecule expression in other tissues. In this regard, our PepG data are consistent with the importance of the lung in systemic endotoxemia. PepG did not increase leukocyte trapping in the lung; hence, no reduction in circulating counts and no decrease in leukocyte rolling in the periphery were observed, allowing for substantial leukocyte adhesion and recruitment.

In some respects, it is not entirely surprising that LTA is not the activator of leukocyte-endothelial cell interactions. First, LTA represents a very small fraction of the outer membrane of Gram-positive bacteria, whereas LPS makes up a substantial amount of Gram-negative outer membrane. Second, Gram-positive bacteria are often coated with capsules so that from a teleological standpoint, evolving recognition receptors against a reasonably rare outer membrane molecule that is often not seen by the innate detection system may not be likely. This of course begs the question as to which Gram-positive molecules do activate leukocyte-endothelial cell interactions? Certainly, there are many potential candidates. PepG have been postulated as important inducers of the immune system. Indeed, our data do suggest that PepG may be responsible for the adhesion of leukocytes in peripheral microvascular with Gram-positive bacteria. However, the trapping of leukocytes in the lung and the drop in leukocyte numbers (leading to fewer rolling leukocytes) clearly require some molecule other than PepG or LTA or PepG together with LTA (P. Kubes, M. Ho, and B. G. Yipp, unpublished data).
Many groups have demonstrated dramatic LTA responses via TLR2 in numerous macrophage cell lines. Although the tendency is to assume that the detection systems on macrophage cell lines and endothelium/neutrophils are the same, our data suggest that only macrophages are activated via LTA and this is not sufficient to induce an inflammatory response similar to that seen with LPS or S. aureus in vivo. Indeed, a recent study has reported ample TLR4, the LPS receptor, but minimal TLR2, the LTA receptor, on endothelium, suggesting that endothelium may use other mechanisms to detect Gram-positive bacteria.

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References


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