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*J Immunol* 1999; 163:6139-6147; [http://www.jimmunol.org/content/163/11/6139](http://www.jimmunol.org/content/163/11/6139)

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Lipoteichoic Acid inhibits Lipopolysaccharide-Induced Adhesion Molecule Expression and IL-8 Release in Human Lung Microvascular Endothelial Cells¹

Kate Blease,²* Yan Chen, † Paul G. Hellewell, ‡ and Anne Burke-Gaffney³* †

Cell adhesion molecule expression (CAM) and IL-8 release in lung microvascular endothelium facilitate neutrophil accumulation in the lung. This study investigated the effects of lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria, alone and with LPS or TNF-α, on CAM expression and IL-8 release in human lung microvascular endothelial cells (HLMVEC). The concentration-dependent effects of Staphylococcus aureus (S. aureus) LTA (0.3–30 μg/ml) on ICAM-1 and E-selectin expression and IL-8 release were bell shaped. Streptococcus pyogenes (S. pyogenes) LTA had no effect on CAM expression, but caused a concentration-dependent increase in IL-8 release. S. aureus and S. pyogenes LTA (30 μg/ml) abolished LPS-induced CAM expression, and S. aureus LTA reduced LPS-induced IL-8 release. In contrast, the effects of S. aureus LTA with TNF-α on CAM expression and IL-8 release were additive. Inhibitory effects of LTA were not due to decreased HLMVEC viability, as assessed by ethidium homodimer-1 uptake. Changes in neutrophil adhesion to HLMVEC paralleled changes in CAM expression. Using RT-PCR to assess mRNA levels, S. aureus LTA (3 μg/ml) caused a protein synthesis-dependent reduction (75%) in LPS-induced IL-8 mRNA and decreased the IL-8 mRNA half-life from >6 h with LPS to ~2 h. These results suggest that mechanisms exist to prevent excessive endothelial cell activation in the presence of high concentrations of bacterial products. However, inhibition of HLMVEC CAM expression and IL-8 release ultimately may contribute to decreased neutrophil accumulation, persistence of bacteria in the lung, and increased severity of infection. The Journal of Immunology, 1999, 163: 6139–6147.

Evere bacterial infection is the major cause of sepsis, a syndrome characterized by a widespread inflammatory response that triggers organ damage and ultimately may result in multiple organ failure (1, 2). The involvement of Gram-negative bacteria in sepsis is well established (3), but there is an increasing prevalence of Gram-positive bacteria, alone or in mixed infections, as a cause of sepsis; however, less is known about the mechanisms involved (4–6). Gram-positive bacteria are now thought to be responsible for one-third to one-half of all cases of sepsis, and with the resurgence of hospital-acquired Gram-positive infections, it is likely that these will be predominant in the future (5, 6). In recent years, there has also been a change in the main origin of sepsis from the abdomen to the lung (6, 7). Regardless of the organ in which sepsis originates, however, the lung is usually the first to fail (8). This may in part result from a rapid accumulation of neutrophils in the narrow lumen of lung capillaries (9). Toxic products released from sequestered neutrophils damage the endothelial cells lining the capillary walls and lead to an increase in permeability, edema formation, and ultimately pulmonary failure (10).

Mechanisms that facilitate neutrophil recruitment to the lung in response to an inflammatory stimulus may contribute to endothelial cell damage and pulmonary failure. Two mechanisms that play a key role in the recruitment of neutrophils to sites of inflammation are the induction of cell adhesion molecules (CAM)⁴ and the release of chemokines in the vascular endothelium (11–14). Neutrophils bind E-selectin or ICAM-1 expressed on endothelial cells via sialyl-Lewis X and related carbohydrate structures or the β₂ integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), respectively, on neutrophils (11–14). The CXC chemokine, IL-8, acts in concert with CAM to provide a directional cue for neutrophil recruitment and to activate neutrophil function (12). CAM expression and IL-8 release are increased in the lung microvascular endothelium of patients with sepsis, and raised levels of IL-8 and soluble CAM are also detected in the plasma and bronchoaveolar lavage fluid of these patients (15–19).

LPS, a major cell wall component of Gram-negative organisms (20), triggers the inflammatory responses associated with sepsis, including increased CAM expression and release of IL-8 in endothelial cells (21, 22). Little is known, however, about the endothelial-activating properties of cell wall components of Gram-positive bacteria or how these effects contribute to the pathogenesis of sepsis. The cell walls of Gram-positive bacteria are made up of a layer of peptidoglycan (PepG) embedded with teichoic acids, including lipoteichoic acid (LTA) (4). The effects of LTA and PepG on cells other than endothelial cells are quite well characterized and include induction of IL-8 release and CAM expression in monocytes.

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⁴Abbreviations used in this paper: CAM, cell adhesion molecule; LTA, lipoteichoic acid; PepG, peptidoglycan; HUVEC, human umbilical vein endothelial cells; HLMVEC, human lung microvascular endothelial cells; EihH-1, ethidium homodimer-1; EGM-MV, microvascular endothelial growth medium; KRPD, Krebs-Ringer phosphate buffer.

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(23, 24). LTA also induces the release of hepatocyte growth factor from human gingival fibroblasts (25), and PepG, alone and in combination with LTA, triggers cytokine production in monocytes and up-regulates inducible nitric oxide synthase expression in macrophages (26–28).

The only endothelial-activating property of LTA described to date is CAM induction in human umbilical vein endothelial cells (HUVEC) by *Staphylococcus aureus* LTA (29), although infection of HUVEC with live *S. aureus* induces CAM expression and IL-8 release (30, 31). Also, the effects of LTA in combination with LPS or cytokines on endothelial cell function in vitro have not been investigated. In vivo, LPS-treated rats failed to recruit neutrophils to the lung following aerosolized challenge with LTA or Gram-positive bacteria, resulting in the proliferation of bacteria in the lung, which suggests an inhibitory interaction between LPS and Gram-positive bacteria/bacterial components (32, 33). The aims of the present study were to investigate the effects of LTA from *S. aureus* alone and in combination with LPS or TNF-α on ICAM-1 and E-selectin expression, neutrophil adhesion, and IL-8 release in human lung microvascular endothelial cells (HLMVEC). In selected studies, the effects of LTA from *Streptococcus pyogenes* (*S. pyogenes*) were compared with those of *S. aureus* LTA. These Gram-positive bacteria were chosen because they are often associated with lung infections and may also trigger sepsis (34, 35).

**Materials and Methods**

**Cell culture reagents**

HLMVEC, prepared by Clonetics (San Diego, CA), were obtained from three donors, as cryopreserved third-passage cultures from TCS Biologics (Buckingham, U.K.) and used at passages 5–10. Microvascular endothelial growth medium (EGM-MV) was also obtained from TCS Biologicals.

**Cytokines and other reagents**

Human recombinant (hr) TNF-α was obtained from Boehringer Mannheim U.K. (Lewes, U.K.; sp. act., >1 × 10^6). hr IL-8 (72 aa) was a gift from Dr. J. White, SmithKline Beecham (King of Prussia, PA). LPS from *Escherichia coli* (055:B5) and LTA from *S. aureus* (DSM 20233) and *S. pyogenes* (IID 698) were purchased from Sigma (Poole, U.K.). The preparations of LTA contained <1 ng of LPS/mg LTA (0.03 ng/mL LPS per 30 μg/mL LTA, maximum concentration used in this study) according to the manufacturer’s control. Peptidoglycan from *S. aureus* was a gift from Dr. C. Thiemeermann (William Harvey Research Institute, London, U.K.). Percoll was obtained from Pharmacia Biotech (St. Albans, U.K.). sterone, 5% heat-inactivated FCS, 50 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 5% heat-inactivated FCS, 50 ng/ml amphotericin B, bovine brain extract containing 12 μg/ml protein, and 10 μg/ml heparin. The magnitude of basal and stimulated CAM expression or IL-8 release was not significantly altered as HLMVEC were passaged or by hydrocortisone in the culture medium. Confluent cells were subcultured and seeded onto 96-well plates at 3200 cells/well, as described previously, unless otherwise stated (22). Confluent monolayers (days 4 in culture) were incubated with stimuli diluted in serum-supplemented complete culture medium for the times indicated in **Results**.

**ELISA for ICAM-1 and E-selectin expression**

The ICAM-1 and E-selectin were detected by a specific ELISA method using mouse anti-human ICAM-1 (RR1/1) or E-selectin (ID2) primary mAbs, a peroxidase-linked goat anti-mouse secondary Ab, and the chroomophore 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid as described previously (22, 37). Optical density was determined at 405 nm, and adhesion molecule expression was given as OD₄₀₅. Also, the OD₄₀₅ for the theoretical additive value of CAM expression was calculated by addition of LTA-stimulated CAM expression to that induced by LPS or TNF-α and is the value predicted whether the effects of these agents acting in concert to additive. LPS and TNF-α have been shown previously to have an additive effect on HLMVEC function (39, 40).

**Measurement of neutrophil adhesion to HLMVEC**

HLMVEC monolayers were washed three times with PBS (containing Ca^{2+}/Mg^{2+}) to remove stimuli before carrying out the adhesion assay. One hundred microliters of KRPD and resuspended at 1.25×10⁶ cells/ml in KRPD containing 2.5% fetal bovine serum supplemented with 5% heat-inactivated FCS, 50 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 5% heat-inactivated FCS, 50 ng/ml amphotericin B, bovine brain extract containing 12 μg/ml protein, and 10 μg/ml heparin. A measure of 100% cell damage was obtained from monolayers treated with saponin detergent (0.1%, 10 min, room temperature). Fluorescence

**Measurement of endothelial cell viability**

Endothelial cell damage was assessed using Eth-1, a high affinity red fluorescent DNA dye that enter cells through damaged cell membranes, as we have described previously (44). Following stimulation, conditioned medium was removed, and monolayers were incubated with 2.5 μM Eth-1 (dissolved in PBS containing 0.025% DMSO) for 1 h at room temperature. A measure of 100% cell damage was obtained from monolayers treated with saponin detergent (0.1%, 10 min, room temperature). Fluorescence
was measured with a Biolite F1 multwell fluorescent plate reader (excitation at 530 ± 25 nm and emission at 645 ± 40 nm). Background fluorescence was subtracted, and results were expressed as arbitrary units of fluorescence.

**RIA detection of IL-8**

IL-8 levels in conditioned medium collected from HLMVEC monolayers (pooled triplicate wells) were determined by a RIA method described previously by Au and colleagues (45). Human IL-8 was iodinated with Na125I using lodogen radioiodination reagent as described previously (46), and the sp. act. of [125I]IL-8 was 65 ± 4 Ci/μg protein (n = 4). Radiolabeled IL-8 was diluted in 10 mM PBS containing 0.1% NaN3, 0.2% gelatin, and 0.5% EDTA (10 mM), and NaN3 (0.1%) and spun at 5420 rpm for 1 h, 4°C) with polyethylene glycol (11%), protamine sulfate as the mean (C) was determined by RIA. Results are shown as the mean ± SEM of five experiments. To ensure clarity, statistics and SEM are given in Results.

**Messenger RNA stability analysis**

The rate of IL-8 mRNA decay in HLMVEC was determined by mRNA decay analysis. HLMVEC monolayers were activated with LPS and/or LTA for 2 h. RNA synthesis was blocked by addition of actinomycin D (5 μg/ml), and total RNA was isolated at 0, 2, 4, and 6 h after addition. Semi-quantitative RT-PCR was then performed for IL-8 and β-actin mRNA, as described in the previous section, and the decay of mRNA was determined from band density ratios.

**Statistics**

Results are expressed as the mean ± SEM of n experiments. Statistical analysis was conducted unless otherwise stated using one-way ANOVA followed by Dunnett’s multiple comparison test, which compares all values with a control. Instat GraphPad software was used to perform statistical analysis. Results were deemed significant if p < 0.05.

**Results**

**Cell adhesion molecule expression and IL-8 release in HLMVEC treated with LTA or PepG**

Constitutive ICAM-1, but not E-selectin, expression was detected on resting HLMVEC monolayers (Fig. 1, A and B). We have reported previously that maximum E-selectin and ICAM-1 expressions in HLMVEC are detected at 6 and 24 h, respectively, following LPS or TNF-α stimulation, and these times were therefore used in the present study. The concentration-dependent effect of *S. aureus* LTA (0.3–30 μg/ml) on ICAM-1 (24 h) or E-selectin expression (6 h) was bell shaped (Fig. 1, A and B). Significant increases in ICAM-1 were detected with *S. aureus* LTA at 1 (p < 0.05), 3 or 10 μg/ml (p < 0.01), but not 0.3 or 30 μg/ml (Fig 1A), and significant induction (p < 0.01) of E-selectin was also detected at these concentrations (Fig. 1B). In contrast, *S. pyogenes* LTA or *S. aureus* PepG did not induce significantly ICAM-1 or E-selectin expression (0.3–30 μg/ml; Fig. 1, A and B). Furthermore, PepG (0.3–30 μg/ml) did not alter *S. aureus* LTA-induced ICAM-1 expression (data not shown).

IL-8 release from resting HLMVEC monolayers was 39 ± 8 pM. Increasing concentrations of *S. aureus* LTA (0.3–30 μg/ml) had a bell-shaped effect on IL-8 release at 24 h (Fig. 1C). Significant increases were measured with LTA at 1 (p < 0.05), 3, or 10 μg/ml (p < 0.01), but not 0.3 or 30 μg/ml (Fig 1C). *S. aureus* PepG (0.3–30 μg/ml) did not alter basal (Fig 1C) or *S. aureus* LTA-induced IL-8 release (data not shown). In contrast to the bell-shaped effect seen with *S. aureus* LTA, the effect of increasing concentrations of *S. pyogenes* LTA on IL-8 release was sigmoid shaped (Fig. 1C). Significant increases (p < 0.01) were detected with 10 and 30 μg/ml *S. pyogenes* LTA (Fig 1C).
LTA inhibits LPS-induced ICAM and E-selectin expression

Incubation of HLMVEC monolayers with LPS (0.01 or 0.1 μg/ml) significantly induced ICAM-1 and E-selectin expression (Figs. 2 and 3), and these responses were deemed submaximal and maximal, respectively, because 1 μg/ml LPS did not further increase CAM expression (data not shown). LPS-induced ICAM-1 or E-selectin expression was not increased further in the presence of S. aureus LTA, despite significant induction with 1, 3, and 10 μg/ml LTA alone (Figs. 1, A and B; 2, and 3). Expression of ICAM-1 and E-selectin in HLMVEC treated with LPS in combination with LTA was also less than the predicted additive effect of these stimuli (Figs. 2 and 3). We have described these predicted effects as theoretical additive values, and they are within the range of maximal CAM induction detected previously in HLMVEC (22). LTA at 30 μg/ml abolished LPS-induced ICAM-1 and E-selectin expression (Figs. 2 and 3). A lower concentration of LTA (10 μg/ml) also significantly inhibited (p < 0.01) E-selectin induced by 0.1 μg/ml LPS, but had no effect on expression induced by 0.01 μg/ml LPS (Fig. 3). Finally, although S. pyogenes LTA (0.3–30 μg/ml) had no effect on either ICAM-1 or E-selectin expression, 10 μg/ml significantly inhibited (p < 0.01) E-selectin induced by 0.1 μg/ml LPS, and 30 μg/ml abolished LPS-induced expression of these CAM (Fig. 4).

Additive effect of LTA on TNF-α-induced ICAM and E-selectin expression

Tumor necrosis factor-α (0.1 ng/ml) significantly induced ICAM-1 (24 h) and E-selectin expression (6 h) in HLMVEC monolayers (Fig. 5). The ICAM-1 and E-selectin expression in HLMVEC treated with TNF-α and S. aureus LTA in combination were similar to the theoretical additive values for these stimuli (Fig. 5). In particular, E-selectin induced by TNF-α in combination with 1, 3, or 10 μg/ml LTA was significantly greater (p < 0.05) than that induced by TNF-α alone (Fig. 5B). In contrast to the inhibitory effect on LPS-induced ICAM-1 or E-selectin expression of LTA at 30 μg/ml, TNF-α-induced expression was not significantly altered by S. aureus LTA at this concentration (Fig. 5).

Neutrophil adhesion to HLMVEC pretreated with LPS, TNF-α, and LTA

In these experiments we investigated whether the effects of S. aureus LTA on LPS- and TNF-α-induced CAM expression in HLMVEC were paralleled by similar effects on neutrophil adhesion. Stimulation of HLMVEC monolayers (6 h) with LPS (0.1 μg/ml) and with S. aureus LTA at concentrations of 1, 3, and 10 μg/ml, but not 0.3 or 30 μg/ml, caused a significant increase (p < 0.01, 0.05, 0.01, and 0.05, respectively) in neutrophil adhesion (Fig. 6A). However, pretreatment of HLMVEC with LPS and LTA together gave a percent adhesion of neutrophils that was less than that predicted for an additive effect. Also, pretreatment of HLMVEC with LPS in combination with 30 μg/ml LTA reduced neutrophil adhesion to basal levels (Fig. 6A). Pretreatment of HLMVEC with TNF-α (0.1 ng/ml, 6 h) also significantly increased (p < 0.05) neutrophil adhesion from basal levels (Fig. 6B). Neutrophil adhesion to HLMVEC pretreated with TNF-α combined with 1, 3, or...
LTA inhibits LPS-induced ICAM-1 and E-selectin expression in HLMVEC monolayers. HLMVEC were treated for 24 h (A) or 6 h (B) with culture medium (○), LPS (■, 0.1 µg/ml), and S. aureus LTA (0.3–30 µg/ml) alone (△) or with LPS (●). ICAM-1 (A) and E-selectin (B) expressions were determined by ELISA. Results show the mean ± SEM of three experiments. *, p < 0.01 (significant induction of ICAM-1 or E-selectin expression compared with treatment with culture medium). #, p < 0.01 (significant inhibition of LPS-induced ICAM-1 or E-selectin expression).

10 µg/ml of LTA was similar to the theoretical additive values and was not inhibited in the presence of 30 µg/ml LTA (Fig. 6B).

Treatment of HLMVEC monolayers with LTA, LPS, or TNF-α did not alter cell viability

Uptake of EtH-1 was used to determine whether decreases in CAM expression and neutrophil adhesion were due to a reduction in HLMVEC viability. Fluorescence was increased 3.6-fold after treatment of HLMVEC with saponin (0.1%, 10 min; Table I) compared with that in control cells. Treatment of HLMVEC monolayers for 24 h with S. aureus LTA alone or in combination with LPS or TNF-α did not significantly increase fluorescence (Table I). Similar data were obtained with S. pyogenes LTA (data not shown). These results suggest that the inhibitory effects of LTA are unlikely to be due to decreased HLMVEC viability.

Effect of LTA in combination with LPS or TNF-α on IL-8 release in HLMVEC

In the experiments described in this section, the effects of activating HLMVEC for 24 h with S. aureus LTA in combination with LPS (0.1 µg/ml) or TNF-α (0.1 ng/ml) and also with S. pyogenes LTA in combination with LPS on IL-8 release were assessed. Induction was normalized so that LPS- or TNF-α-induced IL-8 was 100%, and the effects of LTA alone and in combination were expressed as a percentage of the effect of LPS or TNF-α alone, where appropriate. The theoretical additive values of IL-8 release in these experiments are within the range of maximal release from HLMVEC (data not shown).

LPS-induced IL-8 release was not increased by S. aureus LTA (Fig. 7A), was less than the theoretical additive values, and was significantly inhibited (p < 0.01) with 30 µg/ml LTA (Fig. 7A).

Similar data were obtained with S. pyogenes LTA, although an inhibitory effect with 30 µg/ml LTA was not detected (Fig. 7B). Finally, IL-8 release in HLMVEC treated with TNF-α and S. aureus LTA in combination was similar to the theoretical additive value for these stimuli and was not inhibited in the presence of 30 µg/ml LTA (Fig. 7C).

LTA inhibits LPS-induced, but not TNF-α-induced, IL-8 mRNA expression

In these experiments we investigated whether the effects of S. aureus LTA with LPS or TNF-α on IL-8 release in HLMVEC were paralleled by similar effects on IL-8 mRNA induction. Total RNA was isolated from HLMVEC following 2-h stimulation, and semi-quantitative RT-PCR analysis was used to assess the level of IL-8 and β-actin mRNA. Induction was expressed as a ratio of IL-8/β-actin band densities. Figs. 8 and 9 show blots for β-actin (A) or IL-8 (B) RT-PCR products from representative experiments and bar graphs (C) of mean IL-8/β-actin ratios for three experiments. The ratio for HLMVEC monolayers treated with LPS (0.1 µg/ml) or LTA (3 µg/ml) was significantly increased (p < 0.001 or p < 0.01) compared with that in untreated monolayers (Fig. 8C). However, LTA in combination with LPS significantly decreased (p < 0.05) the IL-8/β-actin ratio compared with that in LPS-treated monolayers (Fig. 8C). TNF-α also increased IL-8 mRNA, but this was not significantly altered by LTA (Fig. 9C).

Inhibition of protein synthesis increases LPS/LTA-induced IL-8 mRNA expression

To assess whether levels of IL-8 mRNA expression were regulated by de novo protein synthesis, HLMVEC monolayers were treated...
for 2 h with cycloheximide (5 μg/ml), alone or in combination with LPS, LTA, TNF-α, LPS/LTA, and TNF-α/LTA. Cycloheximide is a protein synthesis inhibitor that can cause the superinduction of many genes by preventing the degradation of otherwise labile mRNA. Cycloheximide significantly increased (p < 0.01) the IL-8/β-actin ratio in control cells, but not in cells treated with LPS, TNF-α, or LTA alone (Figs. 8 and 9). The IL-8/β-actin ratio for HLMVEC treated with LPS/LTA was, however, significantly increased (p < 0.01) in the presence of cycloheximide by ~6-fold (Fig. 8), whereas the ratio for TNF-α/LTA-treated cells was not increased significantly (Fig. 9).

### Discussion

The complex interaction between an infectious agent and the host may result in induction or suppression of the inflammatory response, and the balance is critical in determining the outcome of infection. This study shows that *S. aureus* LTA had a bell-shaped effect on CAM expression and release of IL-8 in HLMVEC, inhibited LPS-induced expression of these properties, but had an additive effect with TNF-α. In contrast, the IL-8/β-actin band density ratios in LTA/LPS-treated HLMVEC were significantly (p < 0.01) reduced, compared with LPS, at 4 and 6 h to 40% of the ratio at time zero (Fig. 10). The half-life of IL-8 mRNA in LTA/LPS-treated cells was therefore ~2 h. Destabilization of IL-8 mRNA in LTA/LPS-treated cells was reversed by cycloheximide (5 μg/ml; data not shown).

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**Table I. Uptake of ethidium homodimer-1 by HLMVEC monolayers after 24-h treatment**

<table>
<thead>
<tr>
<th>LTA (μg/ml)</th>
<th>+ Medium (0.1 μg/ml)</th>
<th>+ LPS (0.1 μg/ml)</th>
<th>+ TNF-α (0.1 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>583 ± 23</td>
<td>597 ± 32</td>
<td>556 ± 10</td>
</tr>
<tr>
<td>0.3</td>
<td>608 ± 22</td>
<td>566 ± 26</td>
<td>641 ± 27</td>
</tr>
<tr>
<td>1</td>
<td>546 ± 14</td>
<td>584 ± 15</td>
<td>553 ± 16</td>
</tr>
<tr>
<td>3</td>
<td>573 ± 13</td>
<td>548 ± 14</td>
<td>609 ± 28</td>
</tr>
<tr>
<td>10</td>
<td>609 ± 27</td>
<td>612 ± 34</td>
<td>579 ± 28</td>
</tr>
<tr>
<td>30</td>
<td>576 ± 28</td>
<td>618 ± 4</td>
<td>551 ± 28</td>
</tr>
<tr>
<td>Saponin b</td>
<td>2071 ± 49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b Uptake of EtH-1 (2.5 μM, 1 h) was assessed by fluorescence expressed as arbitrary units (AU).

<sup>a</sup> HLMVEC exposed to saponin (0.1%) for 10 min before EtH-1.

<sup>*</sup> p < 0.01.
manifestation locally. Thus, local down-regulation in response to bacteria may limit leukocyte-mediated tissue/organ damage without negating the systemic inflammatory response. This protection may be of particular importance in the lung, because the capillary microvascular endothelium is more likely than other vascular beds, with the exception of the gut, to be exposed to high concentrations of bacterial products.

In contrast to the effects seen with *S. aureus* LTA on CAM expression, *S. pyogenes* LTA did not induce ICAM-1 or E-selectin in HLMVEC under the conditions used in this study. It is of note that clinically a striking feature in some patients with *S. pyogenes* infection is an absence of inflammatory cells at infection sites and also a relatively high mortality (50). In contrast, *S. aureus* infection is associated with brisk neutrophil infiltrates at the site of infection resulting from the orchestrated expression of CAM on endothelial cells (35). The observation that CAM expression in endothelial cells stimulated with LTA parallels neutrophil accumulation in patients infected with *S. aureus* or *S. pyogenes* establishes an important link between in vitro and in vivo results.

The stimulatory effects of *S. aureus* and *S. pyogenes* LTA on IL-8 release in HLMVEC were similar, apart from a lack of effect with *S. aureus* LTA at 30 μg/ml. In contrast to the largely stimulatory effects of LTA, PepG had no effect on IL-8 release or CAM expression in HLMVEC or on similar properties in HUVEC (27).

FIGURE 7. Effects of *S. aureus* LTA in the presence of LPS or TNF-α and of *S. pyogenes* LTA with LPS on IL-8 release in HLMVEC monolayers. HLMVEC were treated for 24 h with LPS (0.1 μg/ml) and *S. aureus* LTA (0.3–30 μg/ml; A), LPS and *S. pyogenes* LTA (0.3–30 μg/ml; B), and *S. aureus* LTA and TNF-α (0.1 ng/ml). IL-8 release was normalized so that LPS-induced (A and B) or TNF-α-induced (C) IL-8 was 100%, and release in the presence of other stimuli was expressed as a percentage of these. Results show the mean ± SEM of four experiments. #, p < 0.01 (significant difference compared with LPS- or TNF-α-induced IL-8 release from HLMVEC monolayers; analysis was performed on nontransformed data). Basal release of IL-8 (95 ± 18 pM (A and C) or 35 ± 5 pM (B)) was increased to 708 ± 56 pM (p < 0.01; A) or 1831 ± 303 pM (p < 0.001; B) with LPS and with TNF-α to 425 ± 75 pM (p < 0.05; C). To ensure clarity, statistical significance for the effects of LTA alone is given in Results.

FIGURE 8. Effects of *S. aureus* LTA on LPS-induced IL-8 mRNA expression in HLMVEC. Total RNA was isolated from HLMVEC after treatment for 2 h with culture medium, LPS (0.1 μg/ml), LTA (3 μg/ml), or LPS/LTA in the absence (□) or the presence (●) of cycloheximide (CHX; 5 μg/ml), and semiquantitative RT-PCR was used to analyze IL-8 or β-actin mRNA. Induction of mRNA is expressed as a ratio of the band densities for IL-8/β-actin RT-PCR products. Blots for β-actin (A) or IL-8 (B) mRNA for a representative experiment and a bar graph (C) of the mean ± SEM of IL-8/β-actin ratios from three separate experiments are shown. *, p < 0.01; **, p < 0.001 (significant increases in IL-8 mRNA expression compared with control cells treated with medium alone and without CHX). #, p < 0.05 (significant inhibition of IL-8 mRNA expression compared with LPS-induced expression without CHX). +, p < 0.01 (significant superinduction of IL-8 mRNA expression by CHX compared with respective controls without CHX). Statistical analysis was conducted using one-way ANOVA on log-transformed data, followed by the Tukey-Kramer multiple-comparison test.

FIGURE 9. Effects of *S. aureus* LTA on TNF-α-induced IL-8 mRNA expression in HLMVEC. Total RNA was isolated from HLMVEC after treatment for 2 h with culture medium, TNF-α (0.1 μg/ml), LTA (3 μg/ml), or TNF-α/LTA in the absence (□) or the presence (●) of cycloheximide (CHX; 5 μg/ml), and semiquantitative RT-PCR was used to analyze IL-8 or β-actin mRNA. Induction of mRNA is expressed as a ratio of the band densities for IL-8/β-actin RT-PCR products. Blots for β-actin (A) or IL-8 (B) mRNA for a representative experiment and a bar graph (C) of the mean ± SEM of IL-8/β-actin ratios from three separate experiments are shown. *, p < 0.01 (significant increases in IL-8 mRNA expression compared with control cells treated with medium alone and without CHX). +, p < 0.01 (significant superinduction of IL-8 mRNA expression by CHX compared with control without CHX). Statistical analysis was conducted using one-way ANOVA on log-transformed data, followed by the Tukey-Kramer multiple comparison test.
HLMVEC monolayers were stimulated with LPS (0.1 µg/ml), LTA (5 µg/ml), or LPS/LTA; after 2 h, actinomycin D (5 µg/ml; an inhibitor of mRNA translation) was added to the monolayers, and total RNA was isolated at 0, 2, 4, or 6 h after actinomycin D addition. This allows assessment of mRNA stability over time. Semi-quantitative RT-PCR was used to measure mRNA for IL-8 or the housekeeping gene β-actin. The mRNA induction is expressed as a ratio of IL-8/β-actin mRNA band density, normalized so that the ratio at 2 h, before actinomycin D addition, for LPS, LTA, and LTA plus LPS was 100. Results show the mean ± SEM of IL-8/β-actin ratios for six experiments. *, p < 0.01 (significant decreases in IL-8/β-actin ratio compared with the ratio for LPS-treated HLMVEC monolayers at the corresponding time).

Despite the fact that PepG makes up 50% of the cell wall of staphylococcal bacteria (35). The lack of effect of PepG may result from the absence of membrane-associated CD14 on endothelial cells, a receptor to which PepG and also LPS and LTA bind, or the inability of PepG to bind soluble CD14 in serum (27).

In the second part of this study we investigated the effects of LTA in combination with LPS or TNF-α on HLMVEC CAM expression and IL-8 release. When several bacteria are present during infection, the interactions between the products of these organisms and/or the cytokines they induce may be instrumental in determining the outcome of infection. An understanding of these interactions may be essential for improving therapeutic intervention for bacterial infection. In this study we showed that while LTA had an additive effect with TNF-α on HLMVEC function, the effects of LTA with LPS were inhibitory. This provides evidence that local down-regulation of an inflammatory response in the lung, as suggested above, may also have the advantage of being stimulus specific.

Having established that LTA inhibited LPS-induced HLMVEC function, we addressed the mechanism(s) that may be responsible for the inhibitory effects. Our results provide insight into the mechanisms that may be involved and also help to eliminate others. A mechanism that is unlikely to account for the inhibitory effect is competitive antagonism for a receptor such as CD14, as shown for LTA inhibition of LPS-induced responses in monocytes and fibroblasts (51). For competitive antagonism, a greater inhibitory effect would normally be expected in the presence of a lower concentration of LPS, whereas our results show the effect was greater in the presence of a higher concentration. Evidence that CD14-dependent antagonism in particular is unlikely to account for the inhibitory effects is provided by our observation that LPS-induced CAM expression in HLMVEC becomes independent of CD14 with increasing concentrations of LPS, being <20% dependent at 0.1 µg/ml (our unpublished observation). A similar independence of CD14, with increasing concentrations of LPS, has also been reported in macrophages (52).

One explanation for the inhibitory effects of LTA is that LTA induces the synthesis of inhibitory proteins that limit HLMVEC activation by bacteria stimuli. At high concentrations of LTA (30 µg/ml), sufficient inhibitory proteins may be induced to prevent its own action and thus account for the bell-shaped effect. However, LPS may enhance LTA-induced synthesis of such inhibitory proteins, and this may in part account for the greater effect with a higher (0.1 µg/ml), rather than a lower (0.01 µg/ml), concentration of LPS. Further evidence that the synthesis of inhibitory protein(s) plays an important role in the inhibitory effects is provided by the observation that low levels of IL-8 mRNA in LPS/LTA-treated HLMVEC were increased by cycloheximide, a protein synthesis inhibitor. Cycloheximide also significantly increased levels of IL-8 mRNA in resting HLMVEC, which suggests that the inhibitory protein(s) regulating IL-8 production is synthesized constitutively, but that expression is increased with LTA or LPS in combination with LTA. Similar inhibitory proteins are also thought to regulate IL-8 production in monocytes and account for the inability of smooth muscle cells to express E-selectin (23, 53). Constitutive expression of inhibitory proteins that limit basal expression of inflammatory genes and are also inducible in response to appropriate inflammatory stimuli may provide an important control mechanism for inflammation.

We further investigated the inhibitory effect of LTA on LPS-induced IL-8 release and established that LTA destabilized LPS-induced IL-8 mRNA and reduced the half-life from 6 h to ~2 h. The mRNAs of certain cellular proteins, including IL-8, share a common AUUUA pentamer in their 3' untranslated region that confers instability (54). A number of groups have searched for proteins that could potentially modulate the turnover of AUUUA mRNA, and an AU-binding factor, Auf, has been identified that binds to the AUUUA region (54). It is speculated that the binding of this factor leaves the mRNA unprotected and susceptible to degradation (54). At present it is not known, however, whether LTA alone or in combination with LPS induces the expression of Auf or similar AU-binding factors.

Finally, the extent of neutrophil accumulation at sites of infection in patients with mixed Gram-negative and Gram-positive bacterial infection has not, to our knowledge, been specifically addressed. In animal models of mixed infection, aerosolized challenge of LTA or whole Gram-positive bacteria in LPS-treated rats prevented neutrophil accumulation in the lung. However, the contribution to suppressed neutrophil accumulation of CAM expression or chemokine release in lung endothelial cells was not specifically addressed in these studies (32, 33). To establish a direct link, these parameters would need to be determined in tissue taken from an animal model following measurement of appropriate in vivo parameters and is the subject of further studies. Clinical predictions based on findings with in vitro or in vivo models should be made with caution, but, on balance, these results together with ours suggest that inhibition of neutrophil accumulation in the lung may occur in mixed bacterial infection.

In conclusion, our results suggest that LTA from Gram-positive bacteria can limit the extent of HLMVEC activation induced either by itself or by LPS from Gram-negative bacteria, and the effect is dependent on synthesis of inhibitory protein. HLMVEC do not completely lose the ability to express CAM and release IL-8 in response to LTA because their effect with TNF-α remains additive. Whether a selective decrease in endothelial cell function has evolved to provide local protection from bacterial insult in the lung, as we have suggested, or a mechanism exists by which bacteria evade detection can only be speculated. However, these results increase our knowledge of the effects and mechanisms of action of bacteria on endothelial cell inflammatory properties and may have important implications for the treatment of bacterial infections in the future.