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LPS appears to activate human endometrial endothelial cells through TLR-4-dependent and TLR-4-independent mechanisms

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Abstract

Problem—Uterine innate immunity remains poorly characterized, and while endometrial endothelial cells are known to express Toll-like receptors, (TLRs), little is known about their function in these cells. The present study evaluated the effect of Gram-negative bacterial lipopolysaccharide (LPS) on human endometrial endothelial cell (HEECs) cytokine secretion and tissue factor expression, and the role of TLR-4 in these responses.

Methods—HEECs were treated with or without LPS +/- LPS-RS a TLR-4 antagonist, via the binding of MD-2. After 24 hours cell-free supernatants were evaluated for cytokines by multiplex analysis and cell lysates were analyzed for tissue factor expression by Western blot.

Results—Treatment of HEECs with LPS significantly upregulated the secretion of IL-6, IL-8, and G-CSF, and this was prevented by LPS-RS. LPS also induced tissue factor expression by the HEECs, however, this was unaffected by LPS-RS.

Conclusion—These findings suggest that TLR-4 is functional in HEECs and its activation by bacterial LPS induces a specific cytokine/chemokine response. However, bacterial LPS also induced tissue factor expression in what seemed to be a TLR-4 independent fashion, suggesting that this bacterial component can act on the HEECs through TLR-4-dependent and -independent pathways. These findings indicate that endometrial endothelial cells may play an active role in uterine innate immunity.

Introduction

Endothelial cells line the lumen of arteries, veins, and capillaries, thus separating blood and extra-vascular tissues¹; and are active responders to stimuli. Under normal physiological conditions, the endothelium is maintained in an anti-coagulant, relatively quiescent state^{2,3}. However in the presence of Gram-negative bacteria or bacterial components, such as lipopolysaccharide (LPS), the endothelium from various organs are induced to aberrantly express tissue factor, the primary initiator of coagulation, and produce inflammatory growth factors, cytokines and chemokines^{2,3}. This suggests that endothelial cells may have the capacity to sense infection and mount an innate immune and pro-thrombotic response. Indeed, several endothelial cell types from different organs have been demonstrated to express Toll-like receptors (TLRs)^{4–8}, innate immune receptors that mediate the pattern recognition of, and response towards, pathogens and host-derived danger signals^{9,10}.

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Previously, studies from our group have demonstrated that the microvascular endothelium within decidual tissue express TLR-4⁵. Since the uterus is an immunologically unique site that must promote tolerance to the semi-allogeneic fetus, it is not surprising that the stromal and endothelial cells of the decidua express TLRs⁵. Similarly, cells of the non-pregnant female reproductive tract play a role in immune protection against infection^{11,12}. Indeed, human endometrial endothelial cells (HEECs) isolated from the non-pregnant uterus also express TLRs, such as TLR-4⁵, however, little is known about how TLR-4 functions in this endothelial subtype. Therefore, in this current study we sought to determine the role of TLR-4 in HEEC cellular responses towards bacterial LPS. Herein, we report that LPS triggers HEECs to generate an inflammatory cytokine/chemokine response in a TLR-4 dependent manner, and to express tissue factor through what appears to be a TLR-4-independent mechanism.

Materials and Methods

Reagents

LPS (*Escherichia coli* 055:B5, purified by phenol extraction) was purchased from Sigma-Aldrich (St. Louis, MO) and the TLR-4 antagonist, *Rhodobacter sphaeroides* LPS (LPS-RS) was purchased from Invivogen (San Diego, CA).

Human endometrial endothelial cells

The human endometrial endothelial cells (HEECs) used in this study have been isolated and characterized as previously described^{5,13,14}. Cells were cultured in flasks coated with 2% gelatin in EGM-2 MV Singlequot Medium with 5% stripped fetal calf serum (Cambrex Bio Science, Inc., Walkersville, MD)¹⁴.

Cytokine studies

HEECs were seeded into 6-well tissue culture plates overnight until ~70% confluent. Cells were then treated in serum-free OptiMEM (Gibco, Grand Island, NY) with or without LPS-RS (5µg/mL) for 4 hours and subsequently treated with or without LPS (1µg/mL) for 24 hours. After this incubation time, cell-free supernatants and cell lysates were collected as previously described¹⁵. The supernatants were evaluated for levels of interleukin (IL)-2, IL-6, IL-8, IL-17, granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), interferon- γ (IF γ), and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) by multiplex analysis using the Bioplex assay (BioRad, Hercules, CA).

Western blot analysis

Cell lysates were analyzed for tissue factor expression by Western blot as previously described⁵ using the LI-COR Odyssey® detection System (Lincoln, Nebraska). Blots were probed using the anti-human tissue factor antibody from R&D systems (Minneapolis, MN). The antibody directed against the loading control, heat shock protein (hsp)-90, and all secondary antibodies were obtained from LI-COR Odyssey® Systems.

Statistical Analysis

Experiments were performed 4 times and data presented as mean $\pm$ SEM. Statistical analysis was conducted by one way ANOVA with the Holm-Sidak post-hoc test using the Sigma Stat program (Ashburn, VA). $p < 0.05$ was considered significant.

Results

Following 24 hour treatment of HEECs with LPS, multiplex analysis revealed a significant upregulation of IL-6, IL-8, and G-CSF secretion (Figure 1A). In contrast, the basal secretion of IL-2, IL-17, GM-CSF, IFN- γ , and RANTES by the HEECs was not altered following treatment with LPS (Figure 1B). To determine whether the LPS-induced upregulation of HEEC cytokine secretion was due to the activation of TLR-4, cells were treated with LPS in the presence and absence of LPS-RS an inhibitor of TLR4 by binding to MD-2. As shown in Figure 2A, the LPS-induced increase in IL-6, IL-8, and G-CSF secretion was significantly reduced by the presence of LPS-RS. Treatment with LPS-RS alone had no significant effect on the cell's basal cytokine production (Figure 2A). While Western blotting revealed that LPS treatment significantly induced HEEC tissue factor protein expression, the presence of LPS-RS has no significant affect on this response (Figure 2A). Treatment with LPS-RS alone had no significant effect on basal tissue factor expression (data not shown).

Discussion

Bacterial infection of the female genital tract can cause pelvic inflammatory disease, infertility, endomyometritis, septic pelvic thrombophlebitis, and pregnancy complications, such as preterm labor^{16–19}. While non-immune cells of the female reproductive tract and at the maternal-fetal interface have the capacity to sense and respond to infection and infectious components¹², little is known about the precise mechanisms involved, particularly in endothelial cells. Previous studies from our laboratory have demonstrated that TLR-4 is expressed by uterine endothelial cells⁵. Therefore, this study sought to determine the functional role of TLR-4 in human endometrial endothelial cell (HEEC) responses to bacterial LPS.

Our findings demonstrate that stimulation of HEECs with LPS induces a highly specific inflammatory cytokine/chemokine response, characterized by the secretion of IL-6, IL-8 and G-CSF. Moreover, this inflammatory response appears to be mediated by TLR-4 since cytokine/chemokine secretion was reduced to near basal levels in the presence of a specific TLR-4 antagonist. This TLR-4-mediated inflammation in response to LPS has also been observed in other endothelial subtypes^{20,21}. In contrast, although tissue factor protein expression by the HEECs was also induced by LPS, this response was not inhibited by LPS-RS. Several studies have also demonstrated that LPS induces endothelial tissue factor expression^{22–24}, however to our knowledge, no other studies have directly assessed the role of TLR-4. While an animal model of antiphospholipid syndrome-associated thrombosis shows that TLR-4 mediates antiphospholipid antibody (aPL)-induced endothelial tissue factor expression²⁵, other studies have shown that aPL and LPS induce distinct TLR-4 mediated responses²⁶. Therefore, based on this and our own findings, LPS-induced tissue factor expression in endothelial cells may be TLR-4-independent. Nonetheless, the finding that LPS induces tissue factor in HEECs may provide an etiology responsible for certain pathologies associated with endometrial infections^{27,28}.

In summary, this study suggests that TLR-4 is functional in HEECs and that bacterial LPS can act on HEECs through what seems to be a TLR-4-dependent and -independent pathways. These findings indicate that endometrial endothelial cells may play an active role in uterine innate immunity.

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Figure 1. HEEC cytokine secretion in response to LPS

HEECs were treated for 24 hours with media alone (Ctl) or LPS (1 μ g/ml). after which cell-free culture supernatants were evaluated by multiplex analysis. Bar charts show secreted cytokine/chemokine levels after being adjusted to total cell protein content. n=4, * p <0.01 relative to Ctl.

Figure 2. Role of TLR-4 in LPS induced cytokine and tissue factor production

HEECs were pretreated with or without the TLR-4 antagonist, LPS-RS (5 μ g/ml) for 1 hour after which cells were treated with media (Ctl) or LPS (1 μ g/ml) for 24hrs. (A) Supernatants were measured for IL6, IL8, and G-CSF by multiplex analysis. n=4, a: $p < 0.01$ relative to Ctl; and b: $p < 0.01$ relative to LPS. (B) Tissue factor expression in cell lysates was analyzed by Western blot. hsp-90 served as a loading control so that densitometry could be performed. Bar chart shows the ratio of tissue factor band density to hsp-90 band density. n=4; * $p < 0.05$ relative to Ctl.