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Francisella tularensis Selectively Induces Proinflammatory Changes in Endothelial Cells

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Naturally acquired infections with Francisella tularensis, the bacterial agent of tularemia, occur infrequently in humans. However, the high infectivity and lethality of the organism in human raise concerns that it might be exploited as a weapon of bioterrorism. Despite this potential for illicit use, the pathogenesis of tularemia is not well understood. To examine how F. tularensis interacts with cells of its mammalian hosts, we tested the ability of a live vaccine strain (LVS) to induce proinflammatory changes in cultured HUVEC. Living F. tularensis LVS induced HUVEC to express the adhesion molecules VCAM-1 and ICAM-1, but not E-selectin, and to secrete the chemokine CXCL8, but not CCL2. Stimulation of HUVEC by the living bacteria was partially suppressed by polymyxin B, an inhibitor of LPS, but did not require serum, suggesting that F. tularensis LVS does not stimulate endothelium through the serum-dependent pathway that is typically used by LPS from enteric bacteria. In contrast to the living organisms, suspensions of killed F. tularensis LVS acquired the ability to increase endothelial expression of both E-selectin and CCL2. Up-regulation of E-selectin and CCL2 by the killed bacteria was not inhibited by polymyxin B. Exposure of HUVEC to either live or killed F. tularensis LVS for 24 h promoted the transendothelial migration of subsequently added neutrophils. These data indicate that multiple components of F. tularensis LVS induce proinflammatory changes in endothelial cells in an atypical manner that may contribute to the exceptional infectivity and virulence of this pathogen. The Journal of Immunology, 2003, 171: 2563–2570.

Tularemia is a febrile disease that was recognized in humans as long ago as 1837. The causative bacterium, Francisella tularensis, was first isolated in 1911 from ground squirrels with a plague-like illness in Tulare County, California (1). F. tularensis is a small, aerobic, nonmotile, Gram-negative, pleomorphic coccobacillus (2). It is a facultative intracellular organism that has been reported to infect hepatocytes (3) and macrophages (4). Two major subspecies that are pathogenic for humans have been identified. The most virulent in humans is F. tularensis subsp. tularensis (also known as type A), and it is also the predominant cause of tularemia in North America. F. tularensis subsp. holarctica (type B) predominates in Eurasia and causes less severe human disease than does type A (5). An attenuated live vaccine strain (LVS), derived from F. tularensis subsp. holarctica, does not cause illness in humans, but is highly infectious for and virulent in mice (6). The molecular basis for attenuation of F. tularensis LVS has yet to be determined.

F. tularensis is perpetuated in nature in an enzootic cycle involving wild mammals (particularly rodents and lagomorphs) and blood-sucking arthropods (including ticks, tabanid flies, and mosquitoes) (7). It is also a relatively hardy organism that can survive free in the environment (2, 5). Transmission to humans can occur through insect bites, handling of infected animals, or contact with contaminated mud or water (2), but spread between humans has not been reported (7, 8). Human tularemia manifests itself in a variety of syndromes. The typhoidal and pneumonic forms are the most deadly, with a mortality rate in untreated patients that can exceed 30% (7).

F. tularensis is highly infectious; fewer than 25 organisms are capable of causing disease when administered to humans s.c. or by aerosol (9). Despite this high infectivity, human cases of tularemia are rather rare. From 1990 to 2000, the number of cases reported annually in the U.S. averaged only 124 (10). Although significant underreporting most likely occurs (7), F. tularensis clearly does not naturally infect humans with high frequency. However, its extreme infectivity, relative hardiness, and ability to cause lethal disease when disseminated by aerosol raise serious concerns that it might be exploited as a biological weapon. Consequently, F. tularensis is one of six pathogens classified by the Centers for Disease Control and Prevention as category A, with the greatest potential for an adverse impact on public health if used in an act of terrorism (8).

Despite these concerns, relatively little is known about the ways in which F. tularensis interacts with mammalian hosts. For example, it has been well established that infection of monkeys (11, 12), rabbits (13), and rodents (3, 14, 15) with F. tularensis results in a pronounced inflammatory response. Indeed, it has been suggested that the response itself may be responsible for much of the tissue damage that occurs (4). However, the components of the bacterium that induce inflammation remain largely a mystery. For most Gram-negative organisms, LPS serves as a potent proinflammatory mediator. The LPS of F. tularensis LVS, though, is atypical and shows low proinflammatory activity in a variety of assays (16–19).

One target through which most forms of LPS stimulate inflammation is the vascular endothelium. LPS, both directly and through
induction of host cytokines, causes endothelial cells to up-regulate expression of adhesion molecules for leukocytes and to secrete chemotaxtractants, including the chemokines CXCL8 (formerly called IL-8) and CCL2 (previously known as monocyte chemotaxtractant protein 1) (20–24). As a consequence, the activated endothelium serves to recruit circulating leukocytes to areas of extravascular infection. In this study, we report that living F. tularensis LVS induces a limited subset of proinflammatory changes in cultured human endothelial cells. When killed, however, the organisms acquire the ability to promote a wider array of alterations. The components of F. tularensis LVS that stimulate endothelium do not appear to use a typical LPS signaling pathway. These observations suggest that F. tularensis incites inflammation in an unusual manner that perhaps contributes to its exceptional virulence.

Materials and Methods

Reagents

mAbs BB11 against E-selectin (25) and 4B9 against VCAM-1 (22) were gifts of R. Lobb (Biogen, Cambridge, MA), mAb R6.5 (26), directed against ICAM-1, was provided by C. Smith (Baylor College of Medicine, Houston, TX). Human rIL-1β (2.2 × 10^7 U/mg) was obtained from BD Biosciences (Lincoln Park, NJ). LPS (Escherichia coli serotype 0111:B4), polymyxin B sulfate (7940 U/mg), and MTT were purchased from Sigma-Aldrich (St. Louis, MO). Gentamicin was obtained from Life Technologies (St. Louis, MO). Polymyxin B sulfate (7940 U/mg), and MTT were purchased from Sigma-Aldrich (St. Louis, MO). Gentamicin was obtained from Life Technologies (Rockville, MD).

Culture of endothelial cells

Endothelial cells were isolated from human umbilical veins via collagenase perfusion and grown to confluence in 60-mm dishes in a 37°C, 5% CO2/95% air environment in medium 199 (M199; Life Technologies) supplemented with 20% FBS (HyClone Laboratories, Logan, UT), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 μg/ml of amphotericin B (27). Cells were cultured for 3–5 days, trypsinized, and passaged onto 24-, 48-, or 96-well plates (all from BD Biosciences) or acellular amniotic tissue. For use as a culture substrate, amniotic tissue was collected several hours postdelivery, fastened to Teflon holders that yielded substrates with a surface area of 2.0 cm², and processed, as previously described (28).

Culture of bacteria

F. tularensis LVS (American Type Culture Collection 29684, Manassas, VA) was a gift of K. Elkins (Center for Biologics, Evaluation and Research, Food and Drug Administration, Rockville, MD). Frozen stocks were prepared from bacteria grown to mid-log phase in Mueller-Hinton broth (BD Biosciences) supplemented with 2% IsoVitalex Enrichment (BD Biosciences), 0.1% glucose, 63 mM CaCl₂, 53 mM MgCl₂, and 34 mM ferric pyrophosphate. For each experiment, a frozen stock was thawed and streaked on solid medium composed of Mueller-Hinton II B (BD Biosciences) supplemented with 1% bovine hemoglobin (both from BD Biosciences) and 1% IsoVitalex Enrichment. The bacteria were grown for 2–3 days at 37°C in a 5% CO₂/95% air environment to allow for colony formation. A single colony then was inoculated into the supplemented Mueller-Hinton broth and grown to late log phase for 16–18 h at 37°C with shaking at 100 rpm and in a 5% CO₂ atmosphere. Aliquots of bacterial culture then were centrifuged, and the pellets were resuspended in M199 supplemented with 25 mM HEPES, pH 7.4, and 20% heat-inactivated (HI) (30 min at 56°C) FBS. Bacteria were routinely resuspended in a volume of medium equal to the volume of broth from which they were derived. The number of viable bacteria in the suspensions was determined by streaking appropriate dilutions on Mueller-Hinton II agar plates and counting colonies 3 days later. Several experiments used sham preparations of bacteria, which consisted of uninoculated Mueller-Hinton broth that was processed simultaneously with and in the same manner as the inoculated broth.

In some studies, F. tularensis LVS was killed by incubation with 100 μg/ml of gentamicin. The effects of such treatment were examined by adsorbing bacterial suspensions onto polyvinyl formal/carbon-coated grids (Ernest F. Fullam, Latham, NY), fixing with 1% glutaraldehyde, washing sequentially with PBS and water, staining with 0.5% phosphotungstic acid, and viewing specimens in a JEOL 1200EXII transmission electron microscope (JEOL USA, Peabody, MA). E. coli (K12 strain MC4100) was provided by D. Thanassi (Stony Brook University, Stony Brook, NY) and cultured in Luria-Bertani broth at 37°C with aeration.

Viability of HUVEC exposed to F. tularensis LVS

To determine whether F. tularensis LVS killed endothelial cells, second-passaged HUVEC were plated on collagen-coated dishes (2.5 × 10^5 cells/well in 100 μl of culture medium) and grown to confluence. Aliquots (100 μl) of F. tularensis LVS then were added, and dishes were incubated at 37°C in 5% CO₂ for various times. Cultures were washed, and the relative numbers of live cells in cultures that were or were not exposed to F. tularensis LVS were measured using MTT, as previously described (24). The ability of F. tularensis LVS to infect endothelium was tested by plating HUVEC in 24-well dishes at a density of 2.2 × 10⁵ cells/well and culturing to confluence. F. tularensis LVS then was added at a ratio of 66 or 136 bacteria per endothelial cell in two separate experiments. After 24 h, cultures were washed, and incubation was continued for an additional 1 or 24 h in medium supplemented with 5 μg/ml of gentamicin to kill remaining extracellular bacteria. This concentration of antibiotic was sufficient to kill F. tularensis LVS in control samples incubated without HUVEC, but, in separate studies, permitted its replication in murine bone marrow-derived macrophages (data not shown). Cultures then were washed with medium lacking antibiotic, and HUVEC were lysed by addition of 0.5 ml of 0.1% deoxycholate in PBS. Lysates were diluted in M199 supplemented with 20% serum and plated to determine the numbers of intracellular bacteria.

Quantitation of E-selectin, ICAM-1, and VCAM-1

Induction of expression of adhesion molecules for leukocytes on the surface of endothelium in response to F. tularensis LVS was evaluated by whole-cell ELISA. HUVEC at second passage were seeded in 96-well Primaria tissue culture plates at 2.0 × 10⁵ to 2.5 × 10⁶ cells/well and cultured to confluence. HUVEC then were incubated with 100 μg/ml of control medium and M199 with 20% FBS and 25 ml of LVS, F. tularensis LVS, a sham preparation of bacteria, or IL-1β at 37°C for indicated times. ELISAs to detect adhesion molecules on the surfaces of living, intact monolayers of HUVEC then were performed, according to Sellati et al. (29).

Quantitation of CXCL8 and CCL2

To assess the ability of F. tularensis LVS to stimulate production of chemokines, HUVEC at second passage were plated in 24- or 48-well plates at densities of 1.0 × 10³ to 1.7 × 10³ cells/cm² and cultured to confluence. F. tularensis LVS, a sham preparation of bacteria, or IL-1β, or control medium was added to the 24- or 48-well plates in volumes of 1.0 and 0.5 ml, respectively. Conditioned medium was collected and clarified by centrifugation. Amounts of CXCL8 and CCL2 in the supernatants were quantitated using commercially available ELISA kits (Antigenix America, Franklin Square, NY). Some experiments used cell culture inserts with a pore size of 0.1 μm (Corning, Corning, NY) to assess whether production of chemokines by HUVEC in response to F. tularensis LVS requires direct contact between the bacteria and endothelium.

Transendothelial migration assay

The ability of F. tularensis LVS to promote transendothelial migration of neutrophils was examined using a procedure similar to that of Randolph and Fairbairn (30). Primary cultures of HUVEC were trypsinized, seeded onto amniotic tissue at a density of 1.5 × 10⁶ cells/cm², and cultured for 7–10 days, at which time transendothelial electrical resistances are maximal (27). HUVEC cultured on amnion then were incubated for 24 h at 37°C with 0.5 ml of control medium, F. tularensis LVS, a sham bacterial preparation, or IL-1β. The effects of F. tularensis LVS on the integrity of the endothelial monolayers were monitored in certain experiments by staining some of the cultures with silver nitrate to mark intercellular junctions, as previously described (28). Neutrophils were obtained from the venous blood of healthy adult volunteer donors, using 0.12% disodium EDTA as an anticoagulant. Erythrocytes first were sedimented with 0.6% dextran (Pharmacia, Piscataway, NJ). Neutrophils then were isolated from the plasma by gradient centrifugation using Accu-Prep Lymphocytes (Accurate Chemical, Westbury, NY), as described previously (31). Following treatment, HUVEC-amnion cultures were washed, and neutrophils were applied to the apical surfaces for 30 min. The cultures then were fixed in 10% buffered Formalin. Neutrophils associated with the cultures were visualized using Wright stain (Sigma-Aldrich) and bright-field microscopy. Neutrophils were counted in nine randomly selected ×400 fields for each sample, and the average number of neutrophils per field was determined. To distinguish neutrophils that had migrated across the endothelium from those that were merely attached to the apical surface, a portion of each culture was embedded in glycol methacrylate (Polysciences, Washington, PA). Sections, 2.2 μm thick, were cut perpendicularly to the plane of the endothelial monolayer and stained with toluidine blue. The positions with
respect to the endothelium of at least 100 neutrophils per sample were determined to calculate the percentage of migrated cells. Corrections were made for loss of adherent cells during the embedding procedure, as previously described (30).

**Statistics**

GraphPad Instat version 3.01 (GraphPad Software, San Diego, CA) was used for analysis of all data. All experimental groups were subjected to an unpaired ANOVA using the Tukey-Kramer multiple comparison test to determine statistical significance.

**Results**

The capacity of *F. tularensis* LVS to induce proinflammatory changes in endothelium was assessed in vitro using cultured HUVEC. Three different indicators of endothelial activation were examined: increased secretion of chemokines, up-regulation of endothelial adhesion molecules for leukocytes, and promotion of the transendothelial migration of neutrophils. Although it has been reported that *F. tularensis* LVS proliferates poorly in DMEM supplemented with 10% FBS (32, 33), the organism grew well in endothelial cell culture medium (M199 with 20% HIFBS). Inocula ranging from $10^6$ to $5 \times 10^7$ bacteria/ml all reached final concentrations of $\sim 10^8$ to $2 \times 10^8$ bacteria/ml when grown overnight at 37°C in a 5% CO$_2$/95% air environment. HUVEC cultured with *F. tularensis* LVS at an initial multiplicity of infection (MOI) as high as 180 showed no decrease in viability over 24 h, although some death was seen if coinoculation was extended to 48 h (data not shown). Infectivity assays revealed that, after 24 h of coinoculation with HUVEC, some *F. tularensis* LVS were protected from killing by gentamicin, indicating that they were located within the endothelial cells. However, in two separate experiments, the numbers of such bacteria were quite low, averaging fewer than 1 for every 50 HUVEC. Even smaller amounts of intracellular bacteria were recovered from cocultures after a further 24 h of incubation in the presence of gentamicin, demonstrating that *F. tularensis* LVS neither replicates nor survives well within the endothelial cells.

**Live and killed *F. tularensis* LVS stimulate endothelial cells to secrete different arrays of chemokines**

The ability of *F. tularensis* LVS to stimulate endothelial secretion of chemokines was tested by incubating HUVEC with the bacteria for 24 h at 37°C. Concentrations of CXCL8 and CCL2 in the cell-free conditioned medium then were measured by ELISA. *F. tularensis* LVS induced the endothelial cells to secrete significantly more CXCL8 than did untreated HUVEC (Fig. 1). In 15 independent experiments, initial inocula of *F. tularensis* LVS ranging from $\sim 10^9$ to $1500$ bacteria per endothelial cell increased production of CXCL8 on average by 5.6 ± 4.0-fold. *F. tularensis* LVS was a relatively weak stimulus, comparable in its effect to rather low amounts (0.05–0.1 U/ml) of the proinflammatory cytokine IL-1β. CXCL8 was not induced in response to a sham preparation of bacteria, which consisted of un inoculated bacterial growth medium subjected to the same experimental manipulations as the bacteria themselves (Fig. 1). Thus, secretion in response to *F. tularensis* LVS was not due to components of its growth medium nor to inadvertent introduction of environmental contaminants.

Although conditioned media from HUVEC exposed to *F. tularensis* LVS contained elevated levels of CXCL8, amounts of CCL2 in the same conditioned media were comparable to those secreted by unstimulated endothelium. However, when gentamicin (100 µg/ml) was included during coinoculation of the HUVEC and bacteria, secretion of CCL2 was markedly increased (Fig. 2A). In contrast, levels of CXCL8 were similar whether or not gentamicin was present (Fig. 2B). By the end of the 24-h period of incubation, no viable *F. tularensis* LVS remained in the samples that contained gentamicin. Moreover, these samples contained few intact organisms, as determined by negative-stain electron microscopy, and those bacteria that were observed displayed extensive blebbing of their membranes (data not shown). Taken together, these results indicate that the dying organisms, unlike the live bacteria, produce a stimulus that induces endothelial secretion of CCL2.

To determine whether direct contact between *F. tularensis* LVS and HUVEC is necessary to induce secretion of CXCL8, bacteria were separated from the endothelium during coincubation by placing them in inserts containing filters with 0.1-µm-diameter pores.

**FIGURE 1.** Living *F. tularensis* LVS induces endothelial secretion of CXCL8. HUVEC were incubated for 24 h at 37°C with medium alone (Unstim), a sham preparation of bacteria, *F. tularensis* LVS at a MOI of 280 (Ft), or 0.05 or 0.1 U/ml of IL-1β. Cell-free conditioned medium was collected, and amounts of CXCL8 were measured by ELISA. Bars represent the means ± SD of three replicate samples. *, Significantly different from unstimulated and sham controls, $p < 0.05$.

**FIGURE 2.** Killed, but not live, *F. tularensis* LVS induces endothelial secretion of CCL2. HUVEC were incubated for 24 h at 37°C with medium alone (Unstim), a sham preparation of bacteria, *F. tularensis* LVS at a MOI of 150 (Ft), or 0.1 U/ml of IL-1β, in the presence or absence of 100 µg/ml of gentamicin. Amounts of CCL2 (A) or CXCL8 (B) were measured in cell-free conditioned medium by ELISA. Bars represent the means ± SD of three replicate samples. *, Significantly different from stimulus in the absence of gentamicin, $p < 0.05$. This experiment was repeated twice more, yielding similar results.
No increased secretion of CXCL8 was seen in response to the bacteria that had been placed within inserts, whereas both F. tularensis LVS placed in direct contact with the HUVEC and IL-1β placed within inserts caused a significant up-regulation of the chemokine. Furthermore, bacteria killed with gentamicin retained the ability to induce secretion of CCL2 when separated from the HUVEC by inserts (data not shown).

Endothelial adhesion molecules are up-regulated in different patterns in response to live and killed F. tularensis LVS

The ability of F. tularensis LVS to stimulate expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 was determined using whole-cell ELISA, which allows measurement of the amounts of surface molecules on intact, living monolayers of endothelial cells (29). Although expression of E-selectin by endothelium in response to many proinflammatory mediators peaks at 4–6 h (29), exposure of HUVEC to F. tularensis LVS for 4 h did not increase levels of E-selectin compared with unstimulated cells. In contrast, both E. coli and IL-1β up-regulated expression of E-selectin at this time (Fig. 3A). F. tularensis LVS also failed to stimulate expression of E-selectin after 6, 12, or 24 h of incubation with HUVEC and at initial MOIs ranging from 0.5 to 2900. Addition of bacteria in the presence of gentamicin for 4 or 24 h did not increase levels of E-selectin. If, however, bacterial suspensions were first incubated overnight with gentamicin and then added to HUVEC for 4 h, they acquired the ability to induce expression of E-selectin (Fig. 3B). Similar induction of E-selectin also was observed if bacteria in suspension were killed by heating at 56°C for 15 min, incubated overnight, and then added to HUVEC for 4 h (data not shown).

Compared with E-selectin, ICAM-1 and VCAM-1 are induced by most stimuli more slowly, and their expression is more sustained (29). We therefore tested whether expression of these two adhesion molecules on HUVEC was increased in response to F. tularensis LVS after 24 h of coincubation. Despite its inability to up-regulate E-selectin, living F. tularensis LVS stimulated expression of both ICAM-1 and VCAM-1 as strongly as did 1.0 U/ml of IL-1β (Fig. 4).

Exposure of endothelium to F. tularensis LVS promotes the subsequent transmigration of neutrophils

Because CXCL8 is a potent attractant for neutrophils (34), we examined whether stimulation of its production by F. tularensis LVS was sufficient to cause neutrophils to traverse endothelium. To do so, we used a well-characterized model of the blood vessel wall that consists of HUVEC grown to confluence on acellular human amniotic tissue (27). Living F. tularensis LVS was incubated for 24 h with HUVEC cultured on amnion. The cultures then were washed, and purified human neutrophils were added for 30 min. As shown in Fig. 5A, the number of neutrophils that migrated across endothelium exposed to F. tularensis LVS was significantly greater than the amount that migrated across unstimulated endothelium and approached the number that migrated across HUVEC stimulated with 0.1 U/ml of IL-1β. With either stimulus, nearly all of the neutrophils associated with the cultures were located beneath the endothelial monolayers; neutrophils adherent to the apical surfaces of the endothelial cells were rarely seen. Coincubation of F. tularensis LVS and HUVEC for only 4 h was not sufficient to induce migration of neutrophils, whereas IL-1β stimulated migration significantly at both 4 and 24 h (data not shown).

HUVEC-amnion cultures that had been exposed for 24 h to living F. tularensis LVS were treated with silver nitrate, which stains the intercellular junctions. In some of the cultures, the staining revealed small areas in which cells had either retracted from one another or become detached (data not shown). It is unlikely that such changes, by themselves, could account for the increased transendothelial migration of neutrophils that we observed, because very few neutrophils adhered to amnion on which no HUVEC were grown (Fig. 5B). Nonetheless, we repeated the transmigration study using gentamicin-killed, rather than live, F. tularensis LVS to stimulate expression of ICAM-1 and VCAM-1 (Fig. 4B).


F. tularensis LVS as a stimulus. The killed organisms had no discernible effect on the integrity of the endothelial monolayers (Fig. 5, C and D), but promoted migration of subsequently added neutrophils to the same extent as did the living bacteria (Fig. 5B). This finding is consistent with our observation that live and killed F. tularensis LVS augmented endothelial production of CXCL8 to similar degrees (Fig. 2B).

Activation of endothelium by F. tularensis LVS is inhibited by polymyxin B, but not by absence of serum

Proinflammatory activation of human monocytes by LPS from many types of Gram-negative bacteria can be blunted by polymyxin B (35). The effect of polymyxin B on stimulation of HUVEC by F. tularensis LVS was measured using ELISAs for both CXCL8 and VCAM-1. Addition of polymyxin B significantly decreased the ability of the bacteria to up-regulate CXCL8 (Fig. 6). In four separate experiments, polymyxin B reduced the enhanced secretion of CXCL8 due to F. tularensis LVS by an average of 78 ± 22%. As shown in Fig. 7, increased expression of VCAM-1 was partially inhibited by inclusion of polymyxin B. The inhibition, however, was less profound and more variable than that observed for CXCL8, averaging 43 ± 26% and reaching statistical significance in only two of three experiments performed. Despite its ability to reduce the proinflammatory activities of live F. tularensis LVS, polymyxin B had no effect on induction of CCL2 or E-selectin by gentamicin-killed organisms (data not shown). As expected, polymyxin B prevented purified E. coli LPS from inducing expression of both CXCL8 and VCAM-1, whereas HUVEC stimulated with IL-1β were unaffected by the presence of the LPS inhibitor (Figs. 6 and 7). Polymyxin B also had no effect on proliferation of F. tularensis LVS during the 24-h course of the assays (data not shown).

Activation of endothelium by LPS from enteric bacteria at less than 1 μg/ml is dependent on two plasma proteins, soluble CD14 and LPS-binding protein (36). Consistent with this observation, HUVEC exposed to 0.5 ng/ml of LPS from E. coli did not up-regulate expression of VCAM-1 in medium supplemented with 1% BSA instead of 20% HIFBS. In contrast, induction of VCAM-1 by F. tularensis LVS was reduced slightly or not at all by an absence of serum (Fig. 8). In two of three independent experiments, enhanced expression of VCAM-1 due to the bacterium was unaffected by removal of serum; in the third, it was reduced by only 20%. As expected, induction of VCAM-1 by IL-1β was the same
in medium supplemented with either BSA or HIFBS (Fig. 8). However, HUVEC treated with IL-1β produced significantly less CXCL8 in the absence of serum than in its presence (data not shown), even though signaling by IL-1β in medium supplemented with either 20% HIFBS or 1% BSA. Stimuli included the supplemented medium alone (Unstim), a sham preparation of bacteria, F. tularensis LVS at a MOI of 130 (Fl), 0.25 ng/ml of purified E. coli LPS, or 0.1 U/ml of IL-1β. Amounts of VCAM-1 were measured by whole-cell ELISA. Bars represent the means ± SD of four replicate samples. * Significantly different from the same stimulus in the presence of HIFBS, p < 0.001.

Discussion

Our results demonstrate that live F. tularensis LVS up-regulates only selected proinflammatory functions of endothelial cells. IL-1β, TNF-α, and LPS from E. coli induce expression of E-selectin, ICAM-1, and VCAM-1 by HUVEC (20, 22–24). Similarly, these agents induce endothelial secretion of both CXCL8 and CCL2 (21, 24, 37). In contrast, living F. tularensis LVS potently up-regulated ICAM-1 and VCAM-1, but did not induce expression of E-selectin at all. Moreover, it promoted endothelial secretion of CXCL8, which attracts neutrophils, but not CCL2, which attracts monocytes and lymphocytes. How, then, does F. tularensis LVS elicit a pronounced inflammatory response in vivo despite its seeming inability to induce a full panel of responses from endothelium? A possible answer lies in the observation that dead or dying organisms acquire the ability to up-regulate E-selectin and CCL2, presumably because they release one or more factors that the living bacteria do not provide.

For F. tularensis, a vigorous inflammatory response may ultimately be to its advantage. Neutrophils are essential to controlling infections with F. tularensis LVS in mice (38). However, it is well established that F. tularensis is capable of evading the antimicrobial weaponry of macrophages and, indeed, can replicate within these cells (4, 15, 32, 33, 39). It has been suggested that, in vivo, F. tularensis is an obligate intracellular pathogen and that its principal reservoir is the macrophage (4). In addition, nearly all F. tularensis LVS in the blood of bacteremic mice appear to be contained within leukocytes (40), raising the possibility that the organisms exploit macrophages not only as protected sites of replication, but also as vehicles for their dissemination.

Our finding that polymyxin B reduced stimulation of endothelial cells by F. tularensis LVS suggests that LPS is responsible, at least in part, for the proinflammatory activity of the living organism. Because polymyxin B only partially suppressed endothelial activation, LPS is perhaps not the only proinflammatory mediator produced by living F. tularensis LVS. However, it may be that LPS is the sole factor, but is inhibited inefficiently by polymyxin B, which is not equally effective against all types of LPS (35). There is ample precedent, though, to support the conclusion that LPS from F. tularensis LVS lacks typical proinflammatory properties. Like other types of LPS, the LPS of F. tularensis LVS activates complement (41). However, it shows no toxicity in sensitized mice; reacts poorly with lysates of Limulus amebocytes (16); fails to activate murine B lymphocytes in vitro (17, 18); does not induce murine splenocytes to secrete IL-12, IL-6, IL-4, or IFN-γ (18); does not stimulate murine macrophages to produce TNF-α (17); and induces their synthesis of NO very poorly (17).

The relative inability of purified LPS from F. tularensis LVS to promote proinflammatory changes strengthens the likelihood that other components of the bacterium are additionally required to induce the inflammation that is seen in infected hosts. For example, the purified LPS incites little secretion of TNF-α by murine macrophages (17), but TNF-α is up-regulated in the livers of mice infected with F. tularensis LVS (42). Our findings further suggest that the proinflammatory activity released by killed F. tularensis LVS is distinct from LPS, because it is unaffected by polymyxin B and induces endothelial changes that the living bacterium does not. Potentially, lipoproteins also may contribute to the proinflammatory properties of F. tularensis. Borrelia burgdorferi, the agent of Lyme disease, lacks LPS and instead induces proinflammatory changes in both endothelial cells (43) and leukocytes (44) via its abundant lipidated outer surface proteins. Eleven strains of F. tularensis tested to date contain a 17-kDa lipoprotein with characteristics similar to those of lipoproteins in B. burgdorferi (45, 46). The 17-kDa lipoprotein is immunogenic in humans (45) and mice (47), but its capacity to elicit inflammatory changes has not been reported. Live F. tularensis LVS did not stimulate HUVEC unless the two cell types were in direct contact. Thus, proinflammatory components, no matter what their identity, do not appear to be shed from or secreted by the living organisms in appreciable quantities.

Our data indicate that the LPS of F. tularensis LVS does not signal endothelial cells via the same pathway as most types of LPS, a pathway that involves interactions among soluble CD14, LPS-binding protein, and Toll-like receptor (TLR) 4 (36). Instead, the lack of a requirement for serum implies that the LPS of F. tularensis LVS must make use of a different pattern recognition receptor, as has been previously suggested by others (17, 18). When injected into mice, LPS purified from F. tularensis LVS protects against a lethal challenge of F. tularensis LVS in both wild-type mice and mice that are deficient in TLR4 (18). Furthermore, the ability of more typical varieties of LPS to bind to murine bone marrow cells or to induce various responses in murine leukocytes is not blocked by LPS from F. tularensis LVS (17). Together with our data, these observations strongly suggest that LPS from F. tularensis LVS signals through a receptor other than TLR4. A possible alternative is TLR2. Interestingly, live B. burgdorferi, which signals several types of cells via TLR2 (48, 49), differs from living F. tularensis LVS in that it induces HUVEC to express both E-selectin (29) and CCL2 (50). Perhaps, then, activation of endothelium by F. tularensis LVS is mediated through a receptor distinct from either TLR2 or TLR4.

From our observations, it is clear that F. tularensis LVS can interact directly with endothelium to induce proinflammatory changes. In vivo, activation of endothelial cells potentially could be further enhanced by release of IL-1β and TNF-α from leukocytes exposed to the bacterium. Although purified LPS of the LVS has little effect on leukocytes (17, 18), it is not known whether the intact organism also fails to stimulate production of proinflammatory cytokines by neutrophils, monocytes/macrophages, or other host cells. The extent to which different strains of F. tularensis might vary with respect to ability to incite inflammation is also
unexplored. *F. tularensis* LVS, which is virulent in mice, but not humans, and Schu4, a fully virulent type A strain, contain LPS of similar antigenicity (51), but whether the molecules are identical remains an open question. Variants of *F. tularensis* LVS with altered antigenicity and activity of their LPS have been described (52). These variants induce higher levels of NO in rat macrophages than does the parental strain and, most likely as a consequence, grow more poorly in the leukocytes. However, the variants and the parental strain do not differ in their ability to proliferate in murine macrophages, and their comparative behavior in human monocytes/macrophages has not been reported. It would be of interest to extend study of the proinflammatory activation of endothelium by *F. tularensis* to fully virulent strains. Differences between fully virulent strains and the LVS may lend insight into why the latter fails to produce disease in humans. Alternatively, strains that are virulent for humans might prove to activate endothelium in the same, atypical manner as the LVS. Such a result would indicate that the virulence of the LVS for different hosts is not linked to the pattern of proinflammatory changes that it induces in endothelium.

Overall, our data suggest that *F. tularensis* LVS stimulates endothelium in a manner that is not typical of many other Gram-negative bacteria. Given that activation of endothelium is a key event in inflammation, a better understanding of the way in which *F. tularensis* interacts with this tissue may ultimately help to explain the extraordinary infectivity and virulence of this pathogen. We propose that an incomplete response elicited by the living bacterium may foster the establishment of infection. Subsequently, a fuller spectrum of inflammatory changes induced by dead *F. tularensis* may enhance recruitment of the macrophages that serve as its hosts.

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**References**


