Stony Brook University

The Inhibitory Effect of *Francisella tularensis* on Endothelial Cells

A Dissertation Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Molecular Genetics and Microbiology

Stony Brook University

May 2012

Stony Brook University
The Graduate School

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ABSTRACT OF THE DISSERTATION

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The endothelium can be activated by various bacterial pathogens to secrete proinflammatory cytokines and recruit circulating leukocytes. However, there is a distinct lack of activation of these cells by *Francisella tularensis*, the causative agent of tularemia. Given the importance of endothelial cells in facilitating innate immunity, we investigated the ability of the attenuated live vaccine strain (LVS) and virulent Schu S4 strain of *F. tularensis* to inhibit the proinflammatory response of human umbilical vein endothelial cells (HUVEC). Living *F. tularensis* LVS and Schu S4 did not stimulate secretion of the chemokine CCL2 by HUVEC, whereas material released from heat-killed bacteria did. Furthermore, the living bacteria suppressed secretion in response to heat-killed *F. tularensis*. This phenomenon was dose- and contact-dependent, and it occurred relatively rapidly upon infection. The living bacteria did not inhibit the activation of HUVEC by *E. coli* LPS, highlighting the relative specificity of this suppression. The endothelial protein C receptor (EPCR) confers anti-inflammatory properties

when bound by activated protein C. When the EPCR was blocked, *F. tularensis* lost the ability to suppress activation of HUVEC. To our knowledge, this is the first report that a bacterial pathogen inhibits the host immune response via the EPCR. As the suppressive effect of *F. tularensis* on endothelial cells requires contact with the cell and a functional EPCR, we investigated the fate of the EPCR. When HUVEC were exposed to live bacteria, there was less EPCR expressed on the cell surface but also less EPCR released into the medium. Furthermore, the EPCR was internalized by endothelial cells shortly after contact with the bacteria.

These studies also investigated what bacterial factors are acting on the endothelial cells to bring about suppression. Few virulence factors for *F. tularensis* have been identified to date. Following a screen of a LVS transposon mutant collection, we found that a strain deficient in AcrA, a part of a multi-drug efflux pump, stimulated secretion of CCL2 by HUVEC. This proinflammatory phenotype was confirmed in a mutant of the virulent Schu S4 strain in which *acrA* was deleted. AcrA is a part of a three-gene operon; however, Schu S4 mutants lacking the other two genes did not activate HUVEC. Although many questions remain, endothelial cells are a critical component of the innate immune response to infection, and suppression of their activation by *F. tularensis* is likely a mechanism that aids in bacterial dissemination and evasion of host defenses.

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ABBREVIATIONS

AIM2	absent in melanoma 2
AP-1	activator protein-1
aPC	activated protein C
BSA	bovine serum albumin
CD	cluster of differentiation
CFU	colony forming units
CCL; CXCL	CC chemokine ligand; CXC chemokine ligand
DAPI	4',6-diamidino-2-phenylindole
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FPI	*Francisella* pathogenicity island
GFP	green fluorescent protein
GPCR	G-protein-coupled receptor
HIF1α	hypoxia-inducible factor-1 alpha
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IKKβ	inhibitor or nuclear factor-kappa B kinase beta
IL-1β	interleukin-1 beta
JAK	janus kinase

LPS	lipopolysaccharide
LVS	live vaccine strain
M199	medium 199
mAb	monoclonal antibody
MAPK	mitogen activated protein kinsase
MH	Mueller-Hinton
MHC	major histocompatibility complex
MOI	multiplicity of infection
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular traps
NF-κB	nuclear factor-kappa B
NLRP3	NLR family, pyrin domain containing 3
OD	optical density
OM	outer membrane
PAMP	pathogen-associated molecular pattern
PAR-1	protease activated receptor-1
PBS	phosphate buffered saline
PC	protein C
PHD3	prolyl hydroxylase 3
PRR	pattern recognition receptors
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RGS4	regulator of G-protein signaling 4
RPS18	ribosomal protein 18s
sEPCR	soluble endothelial protein C receptor
siRNA	small interfering RNA

Smad7	mothers against decapentaplegic homolog 7
ssp.	subspecies
STAT	signal transducer and activator of transcription
TAB3	TGFβ-activated kinase1/MAP3K7 binding protein 3
TF	tissue factor
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
UV	ultraviolet
WGA	wheat germ agglutinin
VCAM	vascular cell adhesion molecule

ACKNOWLEDGEMENTS

For supportive critiques, guidance, and a genuine interest in my success and achievement, I would like to thank my dissertation committee: Laurie Krug, Jorge Benach, Barbara Sherry, Richard Kew, and David Thanassi. Their support and suggestions over the years have helped shape the scientist I have become. I would like to acknowledge the members of the Center for Infectious Disease who have provided a nurturing and collaborative environment to work in. For assistance with experiments and trouble-shooting, I am hugely indebted to Indra Jayatilaka as well as Varya Kirillov, Patricio Mena, Joe McPhee, Ann Marie Torres, and Greg Sabino. Their generous technical help and advice have contributed greatly to this dissertation. Past and present members of the Furie lab have provided an enjoyable work atmosphere. In particular, I must thank Shane McAllister for ideas, advice, and an invaluable friendship. I would also like to extend my gratitude to Jim Bliska, Jorge Benach, Erich Mackow, and Patricia Wright for giving me the opportunity to travel to, and study in, Madagascar. In my time at Stony Brook I have gained a new group of friends and colleagues, as well as countless memories from class, lab, conferences, and all other times together. To my friends and family scattered across the country, I am thankful for your love and support during my graduate studies. Importantly, I am enormously grateful for Martha Furie, my adviser and mentor during these studies. Her kind support and guidance have led to my success as a scientist. I am indebted to her for making my graduate school career an enjoyable and formative experience and am without words to accurately describe my gratitude.

INTRODUCTION

I. Innate Immunity

In the context of pathogenic bacteria, the human body has several lines of defense. One of the first is the innate immune response. Unlike the learned specificity and memory of adaptive immunity, innate immunity is germline-encoded and relatively nonspecific. Cells of innate immunity express pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs). Bacterial PAMPs are molecules specific to pathogens, rather than hosts, but are generally universal across different bacterial species (1). Toll-like receptors (TLRs) are one family of well-characterized PRRs. Two key TLRs are TLR2, which typically recognizes bacterial peptidoglycan and lipoproteins (2-5), and TLR4, which responds to bacterial lipopolysaccharide (LPS) (6, 7). The response to these molecules is typically the same upon each encounter. Therefore, there is no long-term protection conferred by the innate immune response, unlike the memory protection provided by adaptive immune cells. However, these two arms of immunity rely on one another for an effective response to infection. When innate immune cells detect a pathogen, a proinflammatory response is triggered, resulting in inflammation.

Acute inflammation leads to a release of various host-cell factors that function to limit the spread of infection. As cells of the innate immune response are activated, they work to clear the infection while also producing molecules that trigger the adaptive immune response. It is the coordination of both types of immune cells that leads to a robust and effective response to pathogens. However, the body must tightly regulate these processes. An imbalance due to

genetic defects or manipulation by a pathogen can greatly alter the outcome of an inflammatory response, generally to the detriment of the host.

A. Cells of Innate Immunity

i. Leukocytes

There are a number of leukocytes involved in the innate immune response. Leukocytes are derived from the bone marrow and circulate in the bloodstream, lymphatic system, and tissues, acting as sentinels. Neutrophils are one type of leukocyte. These polymorphonuclear phagocytes are typically the first responders to a site of infection, and, as such, they have a range of potent antimicrobial capabilities. Upon contact with a bacterium, neutrophils begin the process of phagocytosis. Phagocytosis is a method of internalization by the host cell, ending with the bacterium encased in a phagocytic vacuole. It is in this vacuole that the bacteria are exposed to an arsenal of molecules designed to kill invading pathogens. Granules that contain defensins, proteinases, and other bactericidal components fuse with the phagocytic vacuole (8-10). These host molecules are released into the phagolysosome to kill bacteria, generally by compromising the integrity of the bacterial cell membrane (11-13).

A second method utilized by neutrophils to destroy bacteria is the oxidative burst. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex assembles on the phagosomal membrane, and a highly reactive superoxide anion is formed by transferring electrons to an oxygen molecule. The superoxide anion is converted into hydrogen peroxide and hypocholorous acid, which can then act detrimentally on bacteria (14, 15). Another more recently described system of microbial containment and clearance are neutrophil extracellular

traps (NETs). NETs are a unique phenomenon of neutrophils. They are comprised of decondensed chromatin and contain various proteins and antimicrobial molecules. These NETs serve to contain as well as destroy bacteria and have been detected in a variety of vertebrate species. While full understanding of these structures is still in its infancy, bacterial virulence is increased *in vivo* when NETs are damaged (16, 17). The extreme importance of neutrophils is highlighted by enhanced susceptibility to life-threatening infection in people suffering from neutropenia (18-20). Despite these powerful antimicrobial activities, neutrophils are short-lived cells.

As neutrophils die and are removed from an area of inflammation, they are replaced by macrophages, another type of phagocytic cell. At any given time, there is a population of monocytes moving in and out of host tissues, acting as sentinels and monitoring the body for injury and infection. Once activated, a monocyte can mature into a macrophage, a professional phagocyte. Macrophages respond to the same inflammatory signals and PAMPs as neutrophils, and, like neutrophils, they take up bacteria into a phagosomal vacuole. Bacteria-containing phagosomes can then fuse with lysosomal granules. This fusion event leads to a change in the pH of the phagolysosome, creating a more acidic environment. The pH change alone can be destructive to pathogens, but the granules contain a host of enzymes, like those found in neutrophils, that function to degrade bacteria and work optimally at a more acidic pH (21, 22). However, just as these powerful enzymes and reactants are vital to a robust immune response, and are effective against bacteria, they must be tightly regulated. Unrestrained inflammation is equally destructive to host tissues. Phagocytic regurgitation or secretion of granule components prior to the phagosome closing can cause damage to the surrounding host cells (10, 14).

Macrophages tend to produce nitric oxide in response to bacterial factors such as LPS, instead of the reactive oxygen species made by neutrophils (21). Nitric oxide is another potent bactericidal agent, though its precise mode of action is still poorly understood. It may directly interfere with bacteria and/or disrupt DNA machinery, or its effects may be indirect. For one, nitric oxide is known to activate nuclear factor-kappa B (NF-κB), a transcription factor that regulates a number of proinflammatory genes (23).

Macrophages play a more significant role than neutrophils in recruiting further immune cells to control the infection while also initiating the adaptive immune response. These events occur by the production of cytokines and chemokines, which activate and recruit immune cells and will be discussed in further detail later. Stimulation of adaptive immunity is aided by macrophages expressing bacterial peptides in the context of the major histocompatibility complex (MHC) II and co-stimulatory molecules. Bacterial proteins that have been degraded by macrophages are processed into short peptides that are loaded onto MHC II and trafficked for display on the cell surface (24-26). These antigen-presenting macrophages move to lymph nodes, where B and T cells can be primed with the MHC-presented antigens activated by cytokines to begin generation of an acquired immune response.

Dendritic cells are another type of innate immune cell that is vital to activation of the adaptive immune response. Found throughout the body, one type of dendritic cell is myeloid-derived and may arise from monocytes (27-29). These dendritic cells can demonstrate different traits depending on the tissue type and environment (28, 30). In general, dendritic cells act similarly to macrophages. Once bacteria are engulfed by a dendritic cell, they are encased in the phagosome, followed by lysosomal fusion and degradation of the bacteria. Where dendritic cells differ from both neutrophils and macrophages is with respect to their role in activating the

adaptive immune response. Uptake of the bacteria leads to increased expression of co-stimulatory molecules on the surface of the dendritic cell (31, 32). As with macrophages, dendritic cells process bacterial peptides to present them via MHC II. However, these cells also upregulate a number of other co-stimulatory molecules such as cluster of differentiation (CD) 86 and CD40; collectively, these molecules are crucial to activate B and T cells (30, 33, 34). Having engulfed the bacteria, these now-mature dendritic cells migrate to lymphoid tissue, where they can prime CD4+ T cells with antigens presented via MHC II and the other co-stimulatory molecules. Macrophages can serve this same role, however, not as effectively as dendritic cells (35). The role of the dendritic cell in infection and inflammation is the least understood of the innate immune cells, though its importance in controlling inflammation and bridging the innate and adaptive responses is clear.

ii. Endothelial Cells

In addition to the circulating leukocytes, endothelial cells are an integral part of innate immune defense. Endothelial cells are thin cells and relatively small in diameter at ~10 μm, as compared to macrophages which are, on average, twice that size. Endothelial cells line the vasculature, creating a dynamic interface between the tissue and bloodstream. These cells also express PRRs, including TLR2 and TLR4. In the context of infection and innate immunity, endothelial cells have two major roles. The first is to function as a barrier between the blood and the underlying tissue. The second is as a potent activator of the innate immune response. In this way, they regulate the flow of small molecules and cells into and out of the blood, as well as prevent bacteria from infiltrating deeper tissue. Under normal conditions, some types of circulating leukocytes will move across the endothelium as a part of general immune surveillance (36). However, when endothelial cells are activated by LPS, other bacterial

components, or host cytokines, vascular permeability and leukocyte extravasation are increased to help control the infection and localize the response (36-38).

The enhanced extravasation of white blood cells happens in part due to the upregulation of adhesion molecules on the surface of endothelial cells. In general, there are three major types of adhesion molecules expressed on both endothelial cells and leukocytes: selectins, integrins, and members of the immunoglobulin (Ig) superfamily. All three types of molecules are involved in the diapedesis of leukocytes through the endothelium. Selectins are surface glycoproteins that bind carbohydrate ligands (39). L-selectin is expressed on leukocytes, and P- and E-selectin are found on endothelial cells (40-42). Expression of both P- and E-selectin is increased upon stimulation of endothelial cells by bacterial or host factors (39, 43, 43).

Integrins are the second group of molecules and are comprised of an α- and β-chain. Key to the extravasation process of leukocytes are $\alpha_4\beta_1$ (VLA-4), $\alpha_L\beta_2$ (LFA-1), and $\alpha_M\beta_2$ (Mac-1), which are expressed in various combinations on the surface of white blood cells (43). Once leukocytes adhere via selectins, they slow and can respond to chemoattractants being secreted by endothelial cells, other host cells, or pathogens. The chemoattractants induce conformational changes in β_2 integrins, allowing them to interact with members of the Ig superfamily of adhesion molecules, importantly, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Binding of integrins to ICAM-1 and VCAM-1 is a stronger bond than that of selectins and their ligands (39), and this tight adhesion facilitates subsequent movement of leukocytes through the endothelial cell barrier (43, 44). This interaction between endothelial cells and leukocytes is summarized in Figure 1.

B. Proinflammatory Cytokines

i. Cytokines

As mentioned prior, innate immune cells secrete and respond to cytokines and chemokines. Cytokines are a family of small but powerful molecules that modulate immune responses. These small polypeptides are produced by a myriad of cells, including the aforementioned leukocytes and endothelial cells. Cytokines can act in both an autocrine and paracrine fashion and are vital in the proinflammatory response (45). Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) are two of the best characterized cytokines and are potent proinflammatory activators. IL-1β is the mature form of pro-IL-1β and is produced shortly after cells such as macrophages, neutrophils, and endothelial cells are activated by infection or injury (46). TNFα, which is largely produced by macrophages, functionally overlaps with IL-1β, while also triggering its production (47). Nitric oxide is also produced by endothelial cells in response to TNFα, leading to increased vasodilation (23). IL-1β and TNFα both activate white blood cells and endothelial cells, leading to production of further cytokines, upregulation of adhesion molecules, and enhanced permeability of the endothelium (48-50). However, the powerful, multiple functions of these cytokines mean that the effects can be damaging to host cells if they are produced unchecked. Overproduction of proinflammatory agents can lead to inflammatory diseases such as rheumatoid arthritis, as well as organ dysfunction due to vasodilation and vascular leakage, over-recruitment of immune cells, and cellular apoptosis (in particular, endothelial cell apoptosis) (51, 52).

Another cytokine of note is IL-2. IL-2 is produced by T cells and dendritic cells but not macrophages (53, 54). IL-2 triggers activation and proliferation of T and B cells. Activated dendritic cells produce and secrete IL-2, which can then bind the IL-2 receptor on the surface of

T and B cells in the lymphatic tissue. This binding event triggers janus kinase (JAK)/signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase (MAPK) signaling cascades, ultimately leading to cell proliferation and production of proinflammatory molecules (30). This function further highlights the necessity for dendritic cells in bridging the innate and adaptive immune responses to produce a robust reaction to infection.

ii. Chemokines

Chemokines are a subset of cytokines that act as chemoattractants, directing the trafficking of leukocytes responding to injury and infection (45, 55, 56). Chemokines are small polypeptides and are broken into four families based on the arrangement of two cysteines near the N-terminus (57). The two largest families are CC chemokines, where the two cysteines are adjacent, and CXC chemokines, where there is one amino acid between the cysteines. The CX3C family consists of one chemokine, fractalkine, which has three residues between the cysteines. The last family, XC, is also small, comprising just two chemokines that lack one of the two characteristic cysteines (57).

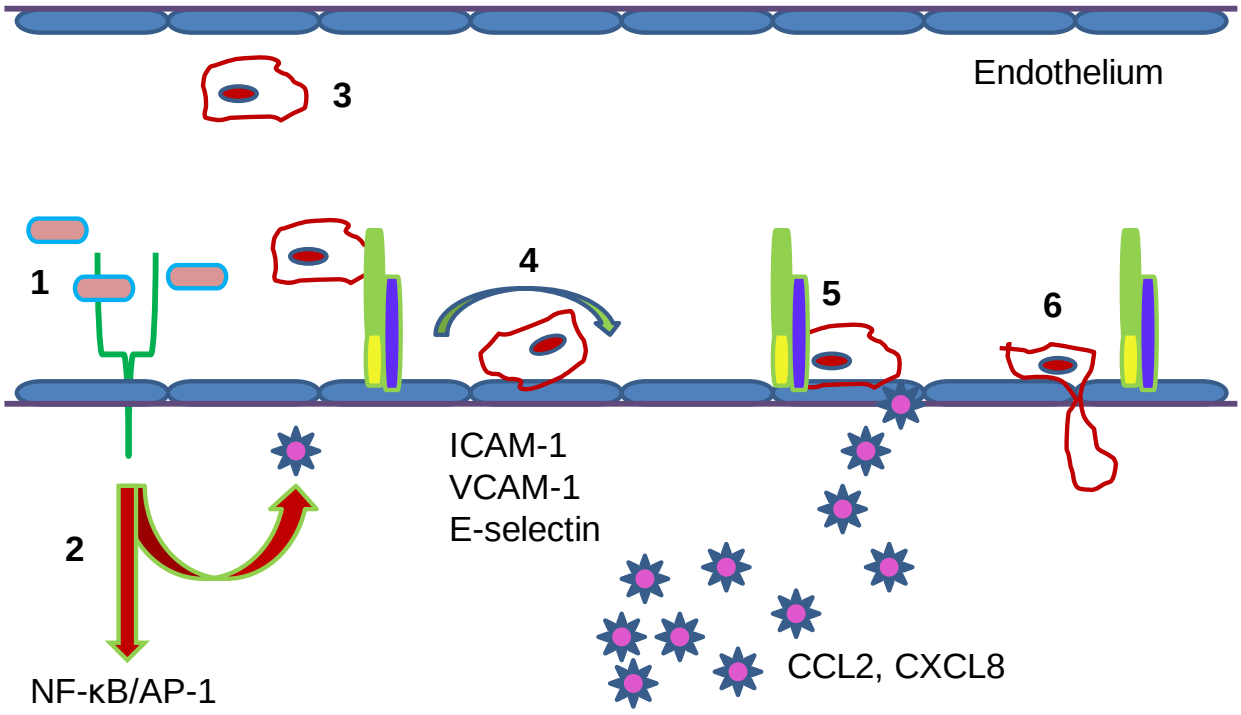
These chemotactic agents are produced by a range of cell types, including endothelial cells. Once the endothelium has been activated by a PAMP, it is induced to secrete chemokines. The molecules then bind their respective receptors on leukocytes, aiding transendothelial migration while also directing the movement of the extravasated immune cells via a chemotactic gradient, helping to localize them to the site of inflammation (56, 58-61). In the context of infection, the CXC and CC families of chemokines are most important. Pertinent to the studies herein are CXCL8 (aka IL-8) and CCL2 (aka MCP-1). CXCL8 was the first chemokine discovered (62-64), and it binds receptors CXCR1 and CXCR2 (38). This chemokine was identified as a powerful chemoattractant for neutrophils. Although this is the primary cell

recruited by CXCL8, this chemokine also attracts mast cells (38). CCL2, on the other hand, primarily recruits monocytes to the site of infection, but also dendritic, natural killer, T, and B cells (38, 65-69). There are many other chemokines that monocytes respond to, but CCL2 is perhaps the most important as mice lacking it or its receptor, CCR2, have a profound defect in recruiting monocytes to most tissues (70). In addition, CCL2 is needed for a proper adaptive immune response, since mice that are unable to produce the chemokine are defective for the production of antibodies by activated lymphocytes (71).

Both leukocytes and endothelial cells can produce CXCL8 and CCL2 in response to PAMPs and cytokines such as IL-1β and TNFα (38, 72), thus creating a cycle of activation, response, and production that enhances the proinflammatory response by attracting immune cells to clear an infection. Because of the potency of both chemokines and cytokines, their production and localization are tightly regulated both by the amount produced and the expression of their receptors (38). Without these measures, there is a potential for an over-accumulation of immune cells, thus exacerbating rather than alleviating the inflammation. In fact, an imbalance in chemokines and their receptors is thought to play a role in a number of disease states, including psoriasis, systemic lupus erythematosus, asthma, and sepsis (57, 73).

FIGURE 1. Interaction between activated endothelial cells and circulating leukocytes.

1. Endothelial cells can recognize and be activated by pathogens. 2. Activation by pathogens leads to signaling cascades that trigger transcription factors NF-κB and/or AP-1 which transcribe genes encoding proinflammatory cytokines, chemokines, and adhesion molecules. 3-4. Circulating leukocytes will begin to bind to selectins and roll along the surface of the endothelium. 5. Chemokines induce conformational changes in integrins, facilitating firm adhesion by the leukocytes. 6. Leukocytes can then respond to, and follow, secreted chemokines. The immune cells will move through the endothelial cell layer and attempt to clear the infection.


Endothelium
3
1
4
5
6
2
ICAM-1
VCAM-1
E-selectin
NF-κB/AP-1
CCL2, CXCL8

II. Tularemia

A. *Francisella tularensis*

F. tularensis is the causative agent of tularemia. This Gram-negative bacterium was first isolated in 1911 from an infected squirrel in Tulare County, California (74). Since that time, there have been limited advances in our understanding of this pathogen, leaving much to be learned. *F. tularensis* is a small, aerobic coccobacillus (75). Currently, there are four primary ssp.: *F. tularensis* ssp. *tularensis*, ssp. *holarctica*, ssp. *novicida*, and ssp. *mediasiatica* (75, 76). Of these ssp., *tularensis* and *holarctica* are the two that generally cause disease in humans. Subspecies *tularensis* (Type A) is the more virulent of the two. The Type A strain is found predominantly in North America (74). In the laboratory, the Schu S4 isolate is commonly used as a representative Type A strain and must be handled under strict biosafety level three conditions. The subspecies *holarctica* is found primarily in Europe and Asia, and though it also causes infection in humans, it is typically less virulent (74). An attenuated version of this subspecies called the live vaccine strain (LVS) was originally developed as a vaccine to be used on the general public. However, its mechanism of attenuation is not understood. This lack of understanding and the general low risk of exposure has led to use of the LVS only in special cases, such as high-risk military and laboratory personnel (77, 78). This strain is useful for research as it is attenuated for causing disease in humans but maintains virulence in mice (79, 80), making it a valuable model of tularemia that does not require biosafety level three protection.

F. tularensis is found naturally in the environment and has adapted to survive in a number of hosts, vectors, and reservoirs. Among these are fresh water amoebas, arthropods,

rodents, and freely in the environment (75). As such, the bacterium can be transmitted to humans in a variety of ways. Natural infection generally occurs from insect bites, handling of infected animals, or ingestion of contaminated food or water (75, 81, 82). Ingestion or introduction through the skin or mucous membranes can result in flu-like symptoms (83), skin ulcers (75), or conjunctivitis (84). Infection by these routes is normally self-limiting. The greater concern is the ability of the bacteria to be transmitted via aerosol, resulting in the more deadly pneumonic tularemia (85). As few as 10 bacteria can cause infection by this route, and pneumonic tularemia in humans carries a 30% mortality rate if left untreated (76). At this time, there have been no reported cases of human-to-human transmission (85). Because of its high infectivity and virulence, *F. tularensis* has been classified as a Category A agent of bioterrorism by the Centers for Disease Control and Prevention (86).

B. Evasion of Host Innate Immunity

i. Intracellular Evasion

F. tularensis has adapted to disseminate efficiently throughout various host tissues but targets primarily the liver, spleen, and lung (87-90). As such, it has developed a number of ways to evade the host's innate immune response. For one, the bacterium replicates intracellularly. Replication occurs primarily within macrophages and dendritic cells (85, 91, 92). Once inside these host cells, the bacteria actively dampen the proinflammatory response. *F. tularensis* prevents fusion of the lysosome to the phagosome and thus prevents acidification of the phagosome. From there, the bacteria move to the cytosol where they replicate (92, 93). *F. tularensis* can also infiltrate and survive within neutrophils; however, it does not replicate therein

(94). The ability to survive within cells that normally kill invading pathogens gives *F. tularensis* a protected niche in which to move throughout the host to distal organs. In addition to infecting leukocytes, *F. tularensis* can also infect non-phagocytic cells including erythrocytes, epithelial cells, and hepatocytes (88, 95, 96). It is not clear yet whether the bacteria can invade endothelial cells; however, they do not replicate within cultured endothelial cells (97), and there is no evidence to date indicating that they replicate in the endothelial cells of infected mice.

As mentioned before, when immune cells contact bacteria, signaling by PRR triggers a proinflammatory response that results in increased secretion of cytokines and expression of various receptors. In the case of tularemia, the bacteria inhibit agonist-induced secretion of proinflammatory cytokines. Primary murine macrophages have reduced secretion of CCL2, IL-6, and TNFα after infection with the LVS isolated from previously infected macrophages (98). Macrophages infected with *F. tularensis* LVS also secrete less TNFα and IL-1β in response to *E. coli* LPS (98, 99). Microarray analysis of human monocytes infected with the virulent Schu S4 strain found general down-regulation of pathways leading to production of inflammatory cytokines (100). In particular, there is reduced activation of NF-κB and activator protein-1 (AP-1) in macrophages infected with *F. tularensis* (101). NF-κB comprises a family of five subunits that form homo- or heterodimers. The p65/p50 heterodimer is the most common form for this transcription factor. NF-κB exists in an inactive form in the cytosol of cells, bound to its inhibitor, I-kappa B alpha. When a cell is activated by proinflammatory stimuli, the inhibitor of nuclear factor-kappa B kinase beta (IKKβ) phosphorylates I-kappa B alpha, leading to release of NF-κB. The NF-κB then translocates to the nucleus and triggers transcription of a number of genes that potentiate a proinflammatory response (102). AP-1 is composed of homo- and heterodimers of the Fos and Jun families of proteins. The Fos family consists of c-Fos, FosB,

Fra-1, and Fra-2, and the Jun family includes c-Jun, JunB, and JunD. AP-1 is similar to NF-κB in that its activation and nuclear translocation are regulated by kinase phosphorylation, and it promotes transcription many of the same genes (103). The proinflammatory factors that are induced by NF-κB and AP-1 include the chemokines CCL2 and CXCL8. In macrophages, escape from the phagosome is required for the inhibitory effect upon AP-1 and NF-κB. *F. tularensis* LVS lacking the gene *iglC* can no longer inhibit the proinflammatory response of macrophages (104). The gene *iglC* is a part of the *Francisella* pathogenicity island (FPI) and is necessary for *F. tularensis* to escape from the phagosome and grow within macrophages (104, 105).

Additionally, the proinflammatory response of dendritic cells of both murine and human origin is blunted when they are infected with *F. tularensis* (91, 106). There is no detectable secretion of TNFα, IL-6, or IL-1β by human dendritic cells infected with the Schu S4 strain. These infected dendritic cells, like macrophages, are also refractory to stimulation with *E. coli* LPS. Importantly, the stunted proinflammatory response is not due to the production of anti-inflammatory cytokines (91). The LVS and Schu S4 strain also evade killing by neutrophils. *F. tularensis* encodes enzymes that neutralize the oxidative burst (107), while also preventing assembly of the NADPH complex in neutrophils (94, 108). The Schu S4 strain may also block the respiratory burst and subsequent activation of proinflammatory genes in macrophages by utilizing an antioxidant scavenging system (109).

The gene transcript levels for MHC molecules are also reduced in leukocytes exposed to *F. tularensis*, which could account for the diminished surface expression of these molecules on infected white blood cells (100, 110). Interestingly, in the case of dendritic cells, this inhibitory effect on surface molecules and secretion of cytokines is seen not only in cells harboring

bacteria, but also uninfected bystander cells (91). When dendritic cells are infected *in vitro*, the suppressive effect appears to be mediated by a soluble factor (91). Furthermore, pulmonary tissue cultured *ex vivo* from mice infected via aerosol with Schu S4 is defective for production of proinflammatory cytokines within the first 48 h (111). Moreover, pulmonary dendritic cells and macrophages of infected animals show no change in expression of MHC II or CD86 vs. cells from control animals. These inhibitory events happen rapidly and allow *F. tularensis* to effectively hide within the host for several days before an overwhelming proinflammatory response is triggered. Often called a "cytokine storm," this late event of large amounts of cytokines and responding leukocytes has been reported during tularemia in mice (112, 113). Such a strong and unregulated immune response is often too late to control the infection and does more damage than good to the host. Evasion of the host immune response, through these and other mechanisms, is likely key to the virulence of *F. tularensis*.

ii. Extracellular Evasion

Despite the importance of phagocytic cells in supporting replication of *F. tularensis*, extracellular bacteria are found in significant numbers in the blood of infected mice, regardless of time point or route of infection (114). During this extracellular phase, *F. tularensis* is exposed to components of the complement system. The complement system is present in plasma and is composed of three pathways. Generally, complement factors target and deposit on bacteria, leading to the formation of the membrane attack complex, which then directly lyses the bacterium. Components of this cascade also aid phagocytosis by opsonizing microorganisms (115). *F. tularensis*, however, avoids complement-mediated lysis. This avoidance is due in part to evasion of C3 deposition on its surface (116). It also seems that *F. tularensis* binds Factor H in plasma. Factor H acts as a cofactor for Factor I, enhancing the affinity between Factor I and C3b.

In turn, the enhanced affinity facilitates cleavage of C3b by Factor I into the inactive form, iC3b (117, 118). This last tactic has a second benefit to the bacteria in that pathogens coated in iC3b are more efficiently targeted and taken up by macrophages, the host cell that *F. tularensis* utilizes for replication (119). Other studies indicate that *F. tularensis* may use host proteases to degrade antibodies that would otherwise aid an immune response against the bacterium (120).

iii. Inhibition of the Function of Endothelial Cells

During the extracellular phase of *F. tularensis*, the blood-borne bacteria will come into contact with endothelial cells (114). As mentioned prior, the endothelium plays a central role during infection and inflammation. In the presence of living LVS organisms, cultured endothelial cells secrete CXCL8, but at a relatively low level compared to what is elicited by even small amounts of the proinflammatory cytokine IL-1β. Moreover, they do not secrete CCL2 above basal levels (97, 121). Although expression of the adhesion molecules ICAM-1 and VCAM-1 is induced, that of E-selectin is not (97). Thus, living LVS organisms elicit a weak and incomplete activation of endothelial cells. In contrast, heat-killed LVS organisms trigger the secretion of significantly more CCL2, as well as higher levels of E-selectin on the endothelial cell surface, than the live bacteria (97). The work herein investigates the interaction between *F. tularensis* and endothelial cells in depth.

C. *F. tularensis* Secretion Systems and Drug Efflux Pumps

F. tularensis has a number of unusual traits beyond its manipulation of the host immune response. For instance, most pathogens that prevent lysosomal fusion do so via secreted effector proteins from the various secretion systems. However, the virulent *F. tularensis* strains have only

a Type I and a putative Type VI secretion system. No strains sequenced to date have a Type III system (105). What this bacterium relies on for intracellular growth and replication is the FPI. The FPI is a group of 17 genes that are present in all strains of *F. tularensis* but are duplicated in ssp. *tularensis* and *holarctica* vs. one copy in the avirulent (for humans) *novicida* subspecies (122). The aforementioned *iglC* is a part of the FPI, in addition to genes that constitute the putative Type VI secretion system (123). This Type VI system is the most likely candidate to secrete effector proteins that would alter the intracellular environment of cells infected with *F. tularensis* (123-129).

F. tularensis also expresses Type IV pili encoded by genes homologous to those that compose Type II secretion systems in other Gram-negative bacteria (130). The pilus system in *F. tularensis* is similar to those found in other Gram-negative bacteria and functions in host-cell adhesion, biofilm formation, and host colonization (130-133). In both the LVS and the Schu S4 strain of *F. tularensis*, these pilus genes are required for full virulence in mice (131, 132, 134). Just as the *novicida* spp. differs from *holarctica* and *tularensis* in the copy number of the FPI, it also differs in the function of the pilus system. The *novicida* spp. seems to secrete effector proteins in a Type II-like fashion, whereas this function appears to have been lost in virulent strains (135-137).

F. tularensis additionally possesses a multi-drug efflux pump that may also act as a Type I secretion system involving the TolC protein (138). Type I secretion systems in other bacteria transport virulence factors across the bacterial cell membrane (139). The machinery involved is made of three parts: an inner membrane pump, a membrane fusion protein, and an outer-membrane channel protein, this last role being filled by TolC. In *F. tularensis* there are two paralogs of TolC: FtlC and SilC (140). Deletion of TolC in the LVS diminishes the bacterium's

resistance to various antibiotics and also causes attenuation *in vivo* (138, 140). Moreover, the Δ*tolC* LVS is hypercytotoxic to macrophages and triggers increased secretion of proinflammatory chemokines from human macrophages and endothelial cells (140). By and large, our understanding of these systems in *F. tularensis* is still in its early stages. A number of genes have been characterized as required for virulence, but many are still classified as hypothetical proteins. To date, there are only two factors reported that may be secreted via the Type VI machinery, and neither is encoded on the FPI (123, 125, 141).

In screening for factors of *F. tularensis* involved in dampening the proinflammatory response of endothelial cells, the gene encoding the protein AcrA came to light (this report). AcrA is a membrane-fusion protein and a part of a multi-drug efflux pump (142). The gene encoding AcrA is a part of a three-gene operon that includes *acrB* and *dsbB* and is conserved across all strains of *Francisella* sequenced to date (143, 144). Characterized in other bacteria, AcrB is a pump protein, and AcrA is a membrane fusion protein that can associate with various pore proteins, including TolC (143). DsbB functions to facilitate cysteine bond formation in newly-made proteins and may not play a functional role in the efflux pump, but it is regulated with these genes at the transcriptional level. When *acrB* is deleted from the LVS, the bacteria are also more susceptible to antibiotics and attenuated *in vivo* (144), much like the TolC mutants (138, 140). However, Schu S4 strains lacking *acrB* or *acrA* are similar to wild-type in both intracellular replication and *in vivo* virulence. Only when *dsbB* is deleted is the Schu S4 strain attenuated (143).

III. The Endothelial Protein C Receptor

A. Structure

Endothelial cells express a range of receptors, including the aforementioned adhesion molecules, chemokine receptors, and TLRs. Another important, yet less understood, receptor is the endothelial protein C receptor (EPCR). The EPCR is a surface-exposed receptor and classified as a type I transmembrane protein. It has a CD1/MHC-like structure with a phospholipid bound in what would be the antigen-presenting groove on MHC molecules (145, 146). Some receptors in the CD1 family have bound glycolipids, which can be exchanged for pathogen-derived lipids when they are internalized by the presenting cell (147). Unlike some of the other receptors mentioned, the EPCR is not known to signal on its own. The EPCR has only a three-amino acid cytoplasmic domain and thus requires co-receptors or some other method to induce subsequent cell signaling (145, 148, 149). The EPCR was first identified on endothelial cells as the receptor for activated protein C (aPC), and it is expressed in relatively large numbers on these cells. Subsequently, the EPCR has been found at low levels on leukocytes and lymphocytes (150-152). There is also a soluble form of the EPCR (sEPCR). The soluble form of the receptor is capable of binding aPC just as is the membrane-bound form, but it may act as a negative regulator by preventing the anticoagulant and anti-inflammatory effects of aPC (153).

B. Protein C

Protein C (PC) is a zymogen that normally circulates in the plasma in an inactive form. Upon inflammation due to injury or infection, several events occur that lead to activation of PC.

Proinflammatory cytokines such as TNFα and IL-1β and/or bacterial components can trigger the expression of tissue factor (TF). TF forms complexes with Factor VII, subsequently activating several other coagulation factors and ultimately resulting in the generation of thrombin (154). Thrombin is a procoagulant/proinflammatory protease. When activated, thrombin can bind thrombomodulin on the surface of endothelial cells, leading to the secretion of chemokines, upregulation of adhesion molecules, and, subsequently, enhanced recruitment of white blood cells (155). At the same time, PC in the plasma binds to the EPCR on the surface of the endothelium. Binding to the EPCR facilitates cleavage of PC by thrombin, forming aPC, a serine protease. In its active form, PC has anticoagulant and anti-inflammatory properties. The aPC inactivates coagulation Factors Va and VIIIa, thus creating a negative feedback loop for the generation of thrombin (154). Additionally, when bound to the EPCR, aPC activates anti-inflammatory and anti-apoptotic signaling pathways in endothelial cells (156-158). Though the mechanisms remain unclear, aPC inhibits activation of NF-κB, a transcription factor that leads to generation of many proinflammatory molecules, including CCL2 and CXCL8 (Fig. 2) (159, 160).

Expression of adhesion molecules in response to high mobility group box 1 or TNFα is also reduced on aPC-exposed endothelial cells (159, 161). The reduction in proinflammatory chemokines and adhesion molecules is an EPCR-dependent event, despite the fact that the EPCR is not known to signal on its own (148, 162). aPC also binds the EPCR on monocytes and neutrophils. When bound to neutrophils, aPC prevents migration but has no effect on phagocytosis of bacteria or the respiratory burst (163, 164). Human monocytes treated with aPC are longer-lived in the presence of an inducer of apoptosis and secrete fewer cytokines in response to LPS (165).

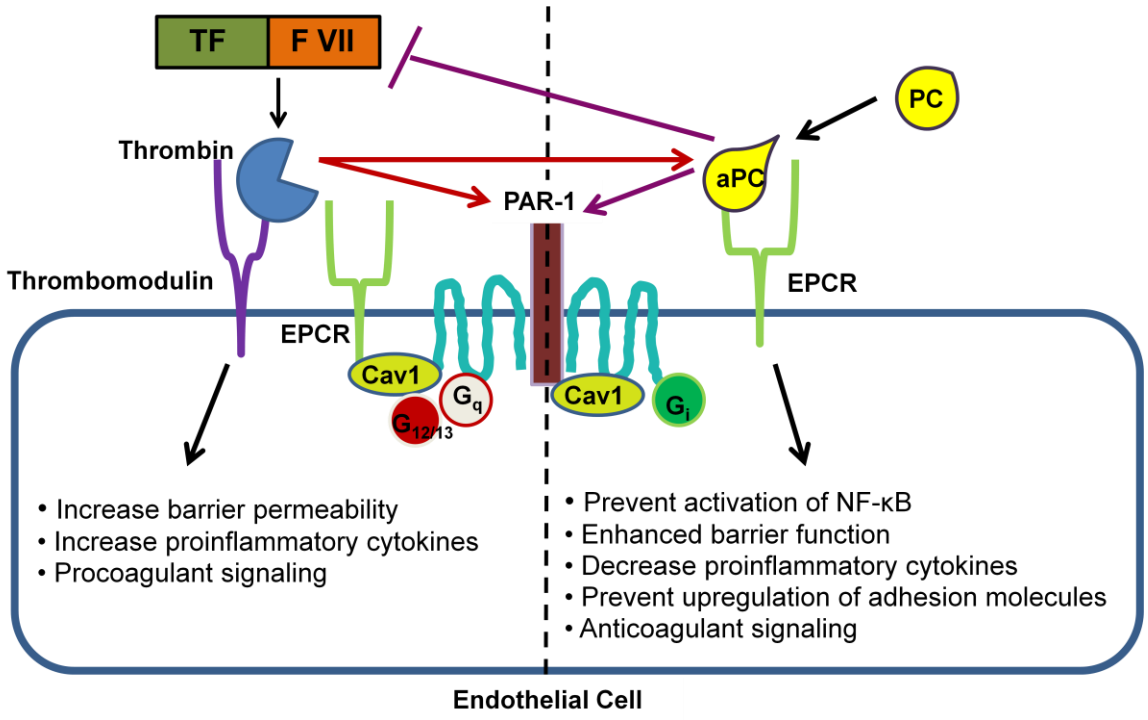
C. Co-receptors

Host-cell signaling events due to the aPC/EPCR complex are conducted via co-receptors. The EPCR is localized to lipid rafts with caveolin-1 and protease-activated receptor-1 (PAR-1). PAR-1 is a G-protein-coupled receptor (GPCR) that is activated by enzymatic cleavage. PAR-1 can be cleaved by both thrombin and EPCR-bound aPC; however, the outcomes change based on the occupancy of the EPCR (149, 162). Cleavage of PAR-1 by thrombin is EPCR-independent and generates a proinflammatory signaling cascade in endothelial cells (158). Interestingly, EPCR occupancy by PC or aPC alters the response of endothelium to PAR-1 cleavage by thrombin from proinflammatory to anti-inflammatory. Activation of NF-κB is reduced, whereas barrier function and resistance to apoptosis are enhanced, in endothelial cells where thrombin has activated PAR-1 in the presence of EPCR bound by PC or aPC (149, 162, 166). A catalytically inactive form of aPC also skews the endothelial cell response to thrombin, indicating that cleavage of PAR-1 by aPC is not required for this phenomenon (156, 162). One thought is that when the EPCR is complexed with aPC, caveolin-1 dissociates, allowing PAR-1 to couple with the G_i-protein rather than G_q or $G_{12/13}$, the latter two of which would lead to downstream activation of NF-κB (Fig. 2) (166).

Ligation of the EPCR by PC or aPC also inhibits migration of human lymphocytes toward chemokines by a mechanism that involves the epidermal growth factor receptor (EGFR) but apparently not PAR-1 (150). How exactly EGFR signaling functions in the aPC/EPCR cytoprotective pathway remains to be fully understood. It is important to highlight that although the EPCR does not signal independently, nearly all cytoprotective effects resulting from aPC require its binding to the EPCR, whereas cleavage of PAR-1 is required for only some of the events (158).

FIGURE 2. Summary of the effects of thrombin and aPC on endothelial cells

Proinflammatory cytokines such as TNFα and IL-1β and/or bacterial components can trigger the expression of tissue factor (TF). TF forms complexes with Factor VII (F VII), subsequently activating several other coagulation factors leading to the generation of thrombin. Thrombin can bind thrombomodulin on the surface of endothelial cells leading to proinflammatory and procoagulant signaling. At the same time, PC in the plasma binds to the EPCR on the surface of the endothelium. Binding to the EPCR facilitates cleavage of PC by thrombin, forming aPC, a serine protease. aPC activates anti-inflammatory and anti-coagulant signaling cascades in endothelial cells. Both thrombin and aPC cleave and activate PAR-1 but with different outcomes. One thought is that when the EPCR is complexed with aPC, caveolin-1 (Cav1) dissociates, allowing PAR-1 to couple with the G_i-protein rather than G_q or $G_{12/13}$, the latter two of which would lead to downstream activation of NF-κB.


TF
F VII
Thrombin
PC
aPC
PAR-1
Thrombomodulin
EPCR
EPCR
Cav1
Cav1
G_q
$G_{12/13}$
G_i
• Increase barrier permeability
• Increase proinflammatory cytokines
• Procoagulant signaling
• Prevent activation of NF-κB
• Enhanced barrier function
• Decrease proinflammatory cytokines
• Prevent upregulation of adhesion molecules
• Anticoagulant signaling
Endothelial Cell

D. Role in Sepsis

Most work dealing with the aPC/EPCR complex focuses on the coagulation cascade, but it has also been shown to greatly impact the outcome of infection and sepsis. Sepsis is a complication of infection due to a dysregulated, excessive host response and is a leading cause of death in hospitalized patients. Specifically, sepsis can lead to thrombosis, edema, and organ failure (167). When aPC is bound to the EPCR, it prevents the proinflammatory activation of endothelial cells by counteracting many of the events that lead to a septic state, including clot formation, secretion of cytokines and chemokines, and permeability of the endothelium (158, 167).

The importance of the aPC/EPCR complex in inflammation has been demonstrated *in vivo*. Mice and baboons either lacking the EPCR or treated with a blocking antibody succumb to otherwise sublethal doses of *E. coli* (148, 168). In addition, when such animals are inoculated with a lethal dose of *E. coli* or endotoxin and then treated with recombinant aPC, they survive the challenge (148, 168). Recombinant aPC has been used to treat severe sepsis in humans. However, the increased risk of bleeding and contention over its efficacy has led to its use being discontinued in the United Kingdom (169). Even so, there are significant *in vivo* data to support a role for the aPC/EPCR complex in mediating sepsis and the host response to bacterial infection and sepsis (148, 168, 170). As reported here in, we have found that both the attenuated LVS and the virulent Schu S4 strain of *F. tularensis* require access to the EPCR to prevent the proinflammatory activation of endothelial cells (171). This is the first demonstration that a bacterium uses the EPCR to manipulate the host immune response, and the phenomenon is explored in this dissertation.

AIMS OF THE RESEARCH

Francisella tularensis is the causative agent of tularemia. As a highly virulent bacterial pathogen, *F. tularensis* is able to evade immune detection by replicating intracellularly. However, the bacteria also have a significant extracellular phase where they would come into contact with endothelial cells lining the blood vessels. Endothelial cells act as a critical component of the innate immune response by secreting proinflammatory cytokines and up-regulating the expression of adhesion molecules, all of which serve to recruit and localize circulating leukocytes to the area of infection. The inflammatory responses of phagocytic cells, such as macrophages and dendritic cells, are modulated by *F. tularensis*. The work herein shows that *F. tularensis* also suppresses activation of endothelial cells and explores the mechanisms underlying this event. *F. tularensis* does not stimulate a strong inflammatory response via its LPS like other Gram-negative bacteria, and it lacks typical secretion systems such as the Type III system of *Yersinia pestis* that might be implicated in regulating the host immune response. As such, I hypothesized that *F. tularensis* utilizes unique mechanisms to inhibit the proinflammatory response of endothelial cells, a property that likely aids in its dissemination and virulence.

This research sought to characterize the ability of *F. tularensis* to suppress proinflammatory activation of endothelial cells and identify the responsible bacterial factors. Additionally, this work investigated the host-cell signaling pathways affected by *F. tularensis* and the involvement of endothelial cell receptors.

MATERIALS AND METHODS

I. Isolation and Culture of Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated via collagenase perfusion of umbilical veins as previously described (172). Cells were grown for up to 5 days in 60-mm dishes (Corning) in Medium 199 (M199; Invitrogen) containing 20% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) (HyClone), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 μg/ml of amphotericin B. Upon reaching confluence, HUVEC were detached with trypsin and plated at 2.3 x 10^5 cells/ml in 6-, 12-, 24- or 48-well plates (BD Biosciences) for use in experiments. Experiments were performed using antibiotic-free M199 supplemented with 20% heat-inactivated FBS (referred to as "20% medium" throughout).

II. Culture of Bacteria

A. Live *F. tularensis*: Stocks of *F. tularensis* LVS (ATCC 29684), a gift from Dr. Karen Elkins (Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD), and *F. tularensis* Schu S4 (Biodefense and Emerging Infections Research Resources Repository) were prepared as described (97). For the immunofluorescence experiments, a strain of *F. tularensis* LVS made to express green fluorescent protein (GFP) by transformation with pFNLTP6-gro-gfp, a plasmid provided by Dr. Thomas Zahrt (Medical College of Wisconsin), was used (114). The Δ*acrA*, Δ*acrB*, and Δ*dsbB* Schu S4 strains were kind gifts from Dr. Barbara Mann (University of Virginia), whereas the pilus and Δ*tolC* mutants in the LVS and Δ*tolC*, Δ*ftlC*, and Δ*silC* mutants in the Schu S4 strain were all used with permission from Dr. David

Thanassi (Stony Brook University). For each experiment, a frozen stock was streaked and grown for 2 days on Chocolate II Agar (BD Biosciences) at 37°C in the presence of 5% CO_2. A single colony was used to inoculate Mueller-Hinton (MH) broth supplemented with 2% IsoVitaleX Enrichment (both from BD Biosciences), 0.1% glucose, 625 µM $CaCl_2$, 530 µM $MgCl_2$, and 335 µM ferric pyrophosphate, and the culture was grown to mid-log phase at 37°C with shaking at 100 rpm. All mutants were grown like the wild-type strains except Δ*pilT*, where the growth medium was supplemented with 10 µg/ml of kanamycin. The approximate number of colony forming units (CFU)/ml was estimated for experiments by measuring the optical density (OD) at 600 nm. The actual number of viable bacteria was determined by making serial dilutions, spotting 10 µL of each dilution on Chocolate II Agar plates, and counting colonies two days later.

B. Material Released from Killed *F. tularensis*: Material released from killed *F. tularensis* was prepared by centrifuging 10 ml of bacteria, grown as described above, for 10 min at 2560 x *g*, resuspending in 2 ml of M199, and placing at 56°C for 1 h with mild agitation every 15 min. The heat-killed bacteria were then incubated for 24 h at 37°C to allow for lysis, at which point the preparations were centrifuged for 10 min at 2560 x *g* to remove debris. Supernatants were aliquoted and frozen at -80°C until use. A portion of each supernatant was plated to confirm killing. Prior to killing, dilutions of the bacterial suspension were plated to determine the number of CFU present. For some experiments, a sham preparation was used. The sham consisted of uninoculated MH broth treated in the same manner as the samples containing live bacteria.

C. Inactivated *F. tularensis*: To test the role of viability and production of proteins by *F. tularensis* in suppression, bacteria were treated with formalin, ultraviolet (UV) irradiation, or

chloramphenicol. For formalin-fixed bacteria, the LVS was prepared as described for the live bacteria but resuspended in 1 ml of fixation buffer (Biolegend) for 1 h in the dark. After that time, the bacteria were washed twice by centrifugation at 2560 x *g* in 20% medium before a final resuspension in 1 ml of 20% medium for later dilution to the proper multiplicity of infection (MOI). For UV-inactivation, LVS organisms were diluted to a MOI of ~200, 500, or 700, plated into 6-well culture dishes at 500 µl/well, and exposed to UV light for 2 h. Lastly, *F. tularensis* was treated with 25 µg/ml of chloramphenicol (Sigma) for 15 min prior to use. The chloramphenicol-treated bacteria were centrifuged and resuspended in fresh 20% medium before application to the HUVEC. In all cases, portions of the bacterial preparations were plated to confirm that they were non-viable.

D. *F. tularensis* Outer-Membrane (OM) Preparation: An OM preparation from *F. tularensis* was made by centrifuging 10 ml of culture and resuspending in 1 ml of 20 mM Tris (pH 8.0) with 1X broad-spectrum protease inhibitor tablet (Pierce). The resuspended pellet was sonicated on ice water for 15 sec on, 15 sec off a total of four times. The lysed cells were then centrifuged at 8600 x *g* for 2 min at 4°C, the supernatant was moved to a clean tube, and 50 µl of 10% sarkosyl in distilled H_2O was applied. The tube was rocked for 5 min at room temperature and then centrifuged for 30 min at 18,500 x *g* at 4°C to pellet the OM. The supernatant was aspirated, and the pellet was resuspended in 1 ml of M199 and washed twice more by centrifugation. After the last wash, the pellet was resuspended in 2 ml of M199 and used at the dilutions described in Results.

III. Quantitation of Chemokines and Soluble EPCR

Conditioned media collected from HUVEC in the various experiments were analyzed using enzyme-linked immunosorbent assay (ELISA) kits for chemokines CXCL8 and CCL2 (both from Antigenix America, Inc.) or soluble EPCR (R&D Systems) according to the manufacturer's protocol.

IV. Activation of HUVEC by Live or Killed *F. tularensis*

To examine whether live *F. tularensis* suppresses the proinflammatory response of endothelial cells, HUVEC were seeded in 0.5 ml of 20% medium in 48-well dishes (BD Biosciences). Upon reaching confluence, some HUVEC were treated with living LVS at an estimated MOI of ~75 for 1 h, after which a 1:10 dilution of material released from killed LVS was added alone or to some of the cells pretreated with live bacteria. After 24 h, the conditioned media were collected, centrifuged to remove debris, and stored at -80°C until they were assayed for content of CCL2 or CXCL8.

Various pre-incubation times were used to examine how long HUVEC must be exposed to live *F. tularensis* LVS before being challenged with killed bacteria to yield reduced activation of HUVEC. To this end, living LVS organisms were added to HUVEC at a MOI of ~75 at the same time as or 30, 60, or 120 min before challenge with a 1:10 dilution of material released from the killed LVS. To determine the number of organisms required for optimal suppression, living LVS was added to HUVEC at concentrations of 1 to 500 bacteria/cell at the same time as a 1:10 dilution of killed LVS. Based on the results of this study, all subsequent experiments were performed using a MOI of ~200 to elicit near-maximal suppression. The time-course of

suppression was evaluated by adding to HUVEC a 1:10 dilution of the material released from heat-killed LVS alone or simultaneously with living LVS. The cultures then were incubated for a total of 4, 8, 12, 16, 18, 20, or 24 h.

The role of the EPCR was investigated using a rat IgG_1 monoclonal antibody (mAb) directed against the human EPCR (Clone RCR-252; Hycult Biotech) or a rat IgG_1 control mAb (eBioscience) at 20 µg/ml. The involvement of PAR-1 was tested using HUVEC that were incubated with the mAb WEDE15 (Beckman Coulter) at 25 or 30 µg/ml, the polyclonal antibody H-111 (Santa Cruz Biotechnology) at 20 µg/ml, or both WEDE15 and H-111 at 25 and 20 µg/ml, respectively, for 1 hour prior to challenge with the LVS at a MOI of ~200 for 24 h. In some experiments, HUVEC were stimulated with 5 or 10 µg/ml of human alpha thrombin (Haematologic Technologies, Inc.) or 1 ng/ml of *E. coli* (serotype 0111:B4) LPS (Sigma-Aldrich) either alone or added simultaneously with living LVS organisms. For all of these studies, conditioned media were collected, centrifuged, and assayed for chemokines.

Experiments with the Schu S4 strain were performed similarly, using optimal parameters as defined for the LVS. Specifically, a MOI of ~200 was employed, and the living organisms were added to the HUVEC at the same time as the material released from the heat-killed bacteria.

V. Analysis of Contact Dependency

To evaluate whether live *F. tularensis* must contact HUVEC to exert its effects, HUVEC were seeded in 1.0 ml of 20% medium in 24-well dishes (BD Biosciences). At confluence, cells were stimulated for 24 h with living LVS (MOI of ~200), a 1:10 dilution of material released

from killed LVS, or a combination of the two. In some samples, *F. tularensis* was separated from the HUVEC using polyester Transwell® inserts with 0.4-μm pores (Corning). A portion of the medium outside of each Transwell® was plated to confirm the absence of bacteria. Conditioned media then were collected, centrifuged to remove particles, and stored at -80°C until assayed for content of CCL2.

To test if a suppressive soluble factor was induced by cellular contact between *F. tularensis* and the endothelial cells, HUVEC were incubated for 24 h at 37°C with either 20% medium (control conditioned medium) or the LVS at a MOI of ~200 (LVS conditioned medium). The conditioned media were collected, and LVS organisms were removed with 0.22-μm Ultra Free® MC centrifugal devices (Millipore). All conditioned media were further clarified by centrifugation. The control conditioned medium and LVS conditioned medium then were added for 24 h to a second set of HUVEC, either alone or combined with material released from killed LVS. Conditioned media from both the first and second set of incubations were assayed by ELISA for secretion of CCL2. To correct for carry-over, the average amount of CCL2 in the control conditioned medium or LVS conditioned medium was subtracted from the amounts measured in the appropriate samples from the second set of incubations.

VI. Flow Cytometry for Surface Expression of the EPCR

HUVEC were seeded in 3.0 ml of 20% medium in 6-well dishes and grown to confluence. The cells were stimulated with living LVS at a MOI of ~200, a 1:10 dilution of material released from killed LVS, or a combination of the two in 6.4 ml total, for 24 h. HUVEC were washed once with phosphate buffered saline (PBS) without Ca^{+2} or Mg^{+2} (Invitrogen). The

cells were then detached with 1 ml of freshly prepared 15 mM lidocaine hydrochloride (Sigma) in PBS for 5 to 10 min at 37ºC. Cells were collected and blocked with M199 containing 5% FBS for 15 min. The cells were then washed by centrifugation at 409 x *g* for 5 min and resuspended in 1 ml of fluorescence-activated cell sorting (FACS) buffer [PBS without Ca^{+2} or Mg^{+2} containing 0.5% very low-endotoxin bovine serum albumin (BSA; Millipore)]. From this point forward, cells were kept on ice and in the dark. The cells were counted, aliquoted at 2.5 x 10^5 cells/tube, washed, resuspended in 200 µl of FACS buffer, and incubated with either 0.5 µg of a polyclonal antibody to EPCR or control goat IgG (both from R&D Systems) for 30 min, except for the unstained and propidium iodide-only control groups. After this time, HUVEC were washed, resuspended in 200 µl of FACS buffer, and incubated with 0.25 µg of phycoerythrin-conjugated donkey anti-goat IgG (R&D Systems) for 30 min. The cells were washed, treated with 0.5 µg of propidium iodide (except the unstained control), washed again, and incubated for 30 min on ice with 500 µl of fixation buffer (Biolegend). The HUVEC then were washed twice, resuspended for a final time in 0.5-1.0 ml of FACS buffer, placed in a fresh 5-ml tube (BD Bioscience), and analyzed using a BD FACSCalibur.

VII. Microarray Analysis of HUVEC Exposed to Live and Heat-Killed *F. tularensis*

HUVEC were seeded in 3.0 ml of 20% medium in 6-well dishes and grown to confluence. The cells were stimulated with living LVS (MOI of ~200), a 1:10 dilution of material released from killed LVS, or a combination of the two in 6.4 ml of 20% medium for 8 h. After this time, a portion of the conditioned medium was collected from each well to be tested by ELISA for CCL2. The HUVEC were washed once with PBS, incubated with 2 ml of RNAlater

(Ambion) per well, and kept at 4ºC prior to isolation of RNA. RNA was extracted using an RNeasy mini-kit (Qiagen), whereas concentration and quality of the RNA were assessed by the NanoDrop spectrophotometer and Bioanalyzer (Agilent Technologies), respectively. The RNA was then processed at the University DNA Microarray Core Facility for analysis using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Genes of interest were confirmed by real-time quantitative RT-PCR (qRT-PCR) (see Section IX). Further analysis to group genes altered two-fold or greater into functional pathways was conducted using WebGestalt created by Vanderbilt University (http://bioinfo.vanderbilt.edu/webgestalt/).

VIII. Treatment of HUVEC with Small Interfering RNA (siRNA)

Three ml of 20% medium were placed in 6-well plates and equilibrated at 37°C and 5% CO_2. Trypsinized HUVEC were counted and aliquoted into microcentrifuge tubes at 5 x 10^5 cells/tube. Aliquots were centrifuged at 200 x *g* for 10 min at room temperature and resuspended in 100 µl of nucleofector solution (Lonza). Ontarget plus Smartpool siRNA for prolyl hydroxylase 3 (PHD3) or a scrambled negative control siRNA (Dharmacon/Thermo) were diluted in 1X siRNA buffer (Dharmacon/Thermo) and added to the HUVEC to final concentrations of 100, 150, or 200 nM. The cells were transferred to a cuvette and electroporated using the Amaxa Nucleofector system and program U-001. Following electroporation, 250 µl of the pre-equilibrated medium was added to the cuvette. The cells were transferred to the culture plates and incubated for 24, 36, or 42 h at 37°C and 5% CO_2. The cell medium was then removed, and the HUVEC were stored at 4°C in 2 ml of RNAlater per well. Total RNA was

collected as described for the microarray and used for real-time qRT-PCR. The optimal condition for knockdown of PHD3 in HUVEC was 200 nM and 42 h.

IX. Real-time Quantitative RT-PCR

Total RNA was collected as described previously, and reverse transcription was carried out on 500 ng of RNA using a Verso cDNA synthesis kit (Thermo). Real-time qRT-PCR was performed with a Solaris qPCR gene expression Low ROX Master Mix (Dharmacon/Thermo) in a reaction volume of 25 µl using 100 ng of cDNA template. Transcript levels for human PHD3 were measured for each condition in quadruplicate via Solaris probe and primer pairs (Dharmacon/Thermo) in 96-well assay plates using the Applied Biosystems 7500 Real Time PCR System and the following thermocycling program: enzyme activation at 95°C for 15 min, 1 cycle; denaturation at 95°C for 15 sec; and annealing/extension at 60°C for 60 sec, 40 cycles. The primer sequences used in this study were as follows: **PHD3:** forward - GAATTGGGATGCCAAGCTAC; reverse – AATGGGCTCCACATCTGCTATG; **CCL2:** forward – TCGCTCAGCCAGATGCAAT; reverse – ACAGCTTCTTTGGACAC; **CXCL8:** forward – CTTCTAGGACAAGAGCCAGGAA; reverse – TTAGCACTCCTTGGCAAAACTG; **SMAD7:** forward – CTCCAGATACCCGATGGATT; reverse – CCAGGCTCCAGAAGAAGT; **IL-6:** forward – AAGCTCTATCTCCCCTCCAGGA; reverse – GCAACTGGACCGAAGGC.

The transcript levels of ribosomal protein s18 (RPS18) were also measured and used as an endogenous control. **RPS18** primers were as follows: forward – GTGATCCCTGAAAAGTTCCAG; reverse – CACATGAGCATATCTTCGG. Control wells

where no template was present were run concomitantly with these experiments. The relative amount of transcripts in each sample was calculated using the $\Delta\Delta C_q$ method (173).

X. Activation of NF-κB and AP-1

HUVEC were passaged in 3.0 ml of 20% medium into 6-well plates and treated with 20% medium, live LVS (MOI of ~200), heat-killed LVS (1:10 final dilution), or both live and killed LVS. After 4.5 h, the HUVEC were harvested, and their nuclei were extracted using a TransAM^{TM} Nuclear Extraction kit (Active Motif). All procedures were done according to the manufacturer's protocol with the exception of the cells being lifted by trypsinization rather than scraped. Total protein was measured for each sample by a Bradford assay (Pierce). The amount of activated NF-κB or AP-1 subunits in 10 µg of total protein per sample was measured using the TransAM^{TM} NF-κB p65 or AP-1 Family Transcription Factor Assay Kit (Active Motif).

XI. LVS Transposon Collection Screen

A LVS transposon mutant collection was created using the EZ-Tn5^{TM} plasmid system (Epicentre). Some of these mutants were screened for ability to activate endothelial cells. Frozen stocks of mutants and wild-type LVS were spotted into 100 µl of MH broth in 96-well plates and incubated with shaking at 200 rpm at 37°C. After 48 h, the OD_{600} was measured, and only mutants at an OD_{600} between 0.100 and 0.250 were used. HUVEC were inoculated with 4 µl (MOI of ~200) of each mutant, wild-type LVS, or MH broth (used as a negative control). A 1:10 dilution of heat-killed LVS or 20% medium only was also included in some screenings. The endothelial cells were incubated for 24 h, and conditioned media were collected. Contents of the

20% medium, sham, wild-type, and heat-killed wells were combined independently, whereas the conditioned media from the mutants were combined in groups of 4, 5, 6, or 7 mutants per tube. Results indicated that combining samples from 5 or 6 mutants was optimal for screening for activation of HUVEC. Pooled conditioned media were assayed by ELISA for CXCL8. For pools that activated HUVEC, mutants therein were screened individually to identify the specific mutant that was responsible for the stimulation.

XII. Immunofluorescent Detection of Internalized EPCR in HUVEC

HUVEC were cultured as described above, seeded in 3.0 ml of 20% medium in 6-well dishes, and grown to confluence. The cells were incubated with 20% medium, living LVS or the GFP-expressing LVS, both at a MOI of ~200, or a 1:10 dilution of material released from killed LVS for 2 h. HUVEC were then washed once with PBS without Ca^{+2} or Mg^{+2}. The cells were detached with 1 ml of freshly prepared 15 mM lidocaine hydrochloride in PBS for 5 to 10 min at 37ºC. Prior to fixation, some HUVEC were incubated for 10 min at 37°C with 8 µg of wheat germ agglutinin conjugated to Alexa Fluor 488 (Invitrogen) to stain the plasma membrane. The cells were fixed in 500 µl of BD 1X Cytofix (Buffer A) for 20 min at room temperature. Following fixation, the cells were washed in 1 ml of 5% BSA in PBS with 1X human FcR blocking reagent (Wash Buffer; Miltenyi Biotec), and centrifuged at 300 x *g* for 5 min. Some HUVEC were permeabilized using 500 µl of a 1:50 dilution of BD Cytoperm in Buffer A for 30 min at room temperature followed by two washes as described above. The EPCR was probed using 8 µg/ml of the rat-anti human EPCR mAb (RCR-252) for 40 min at room temperature, washed twice, and then stained with a Texas Red® goat-anti rat secondary antibody at a 1:800

dilution for 30 min. The HUVEC were washed twice more and resuspended in 1 ml of the Wash Buffer. Seventy µl of cells from the various conditions were attached to slides using Shandon EZ Single Cytofunnels (Thermo Scientific) centrifuged at 20 x *g* for 3 min. All samples were mounted with VectaShield mounting medium (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a Nikon E600 microscope and processed using AutoQuant 2.0 (MediaCybernetics) where needed.

XIII. Statistics

Data were analyzed with GraphPad Instat version 3.06 (GraphPad Software) or SigmaPlot 11.0 (Systat Software) using an unpaired analysis of variance followed by the Tukey-Kramer multiple comparison test. Because the level of responsiveness of primary HUVEC to inflammatory stimuli is inherently variable, the results of a representative experiment are shown for many of the studies. The number of experiments that were performed for each study, using independently isolated HUVEC, is given in the figure legends.

RESULTS

I. Inhibition of Proinflammatory Activation of Endothelial Cells by *F. tularensis*

A. *F. tularensis* LVS Suppresses the Proinflammatory Activation of Endothelial Cells

The chemokine CCL2 is secreted by endothelial cells in response to infection and serves to recruit mononuclear leukocytes to the site of injury (67, 174). Living *F. tularensis* LVS does not activate HUVEC to secrete CCL2 above basal levels, while heat-killed *F. tularensis* elicits a robust response (97). Since *F. tularensis* actively suppresses the proinflammatory response of monocytes/macrophages (99, 101), we wanted to investigate whether the poor response of HUVEC to the living bacteria was due to lack of stimulation or an active suppression. The ability of *F. tularensis* to down-regulate the proinflammatory response of endothelial cells was tested by incubating HUVEC with living *F. tularensis* LVS and/or material released from heat-killed bacteria. As previously reported (97), living *F. tularensis* LVS did not induce HUVEC to secrete significantly greater amounts of CCL2 than were produced by unstimulated endothelial cells and triggered secretion of CXCL8 at relatively low levels (Fig. 3). In contrast, HUVEC activated with material released from the heat-killed bacteria secreted high levels of CCL2 and CXCL8. HUVEC that were pre-incubated with living *F. tularensis* LVS for 1 h prior to challenge with the heat-killed organisms showed a significant decrease in secretion of CCL2 and CXCL8 compared to cells stimulated with killed LVS alone (Fig. 3). This last observation suggested that living *F. tularensis* indeed suppresses the proinflammatory activation of endothelial cells.

B. Inhibition of Proinflammatory Activation of Endothelial Cells is Conserved in the Virulent Schu S4 Strain

Prior observations in our laboratory indicated that, like the LVS, the living Schu S4 strain does not stimulate endothelial secretion of CCL2 (C. E. Noah and M. B. Furie, unpublished data). We therefore wanted to determine whether the highly virulent Schu S4 strain could also inhibit the response of endothelial cells to heat-killed Schu S4. To test this premise, HUVEC were incubated with living *F. tularensis* Schu S4, material released from heat-killed Schu S4, or the live and killed bacteria together. As was the case with the LVS, endothelial cells exposed to living *F. tularensis* Schu S4 and then challenged with material released from the heat-killed organisms showed a significant decrease in secretion of CCL2 compared to cells stimulated with killed Schu S4 bacteria alone (Fig. 4).

C. Inhibitory Effect of *F. tularensis* on Endothelial Cells is Dose-Dependent and Rapid

To determine the optimal number of bacteria needed for suppression, the MOI of the living *F. tularensis* LVS was varied. Activation of the endothelial cells by material released from killed *F. tularensis* LVS, as measured by secretion of CCL2, was inhibited by live organisms in a dose-dependent manner. Incubations using 75 bacteria per cell significantly suppressed secretion of CCL2, and production of the chemokine was reduced to basal levels at a MOI of 300 or greater (Fig. 5). In contrast, living LVS organisms at a MOI of ~200 had no effect on secretion of CCL2 by HUVEC in response to *E. coli* LPS (Fig. 6). This observation is critical in that it indicates that the suppression of HUVEC by the living bacteria was not due to death of the endothelial cells nor to the global inhibition of their ability to react to proinflammatory stimuli.

Two different approaches were used to investigate the kinetics of this suppressive effect. First, HUVEC were incubated with living LVS organisms added at the same time as or 30, 60, or 120 min before challenge with material released from heat-killed bacteria. Secretion of CCL2 was down-regulated to the same degree in all cases (Fig. 7). Second, production of CCL2 was measured with time after living and killed bacteria were added simultaneously to HUVEC. As shown in Fig. 8, the presence of living LVS halted secretion of CCL2 almost completely within 8 h, whereas amounts continued to rise throughout the 24-h assay period when HUVEC were stimulated with killed organisms alone. Together, these results indicate that suppression of the proinflammatory response of endothelial cells by living *F. tularensis* is relatively rapid. Moreover, living LVS rapidly blunted secretion of CCL2 by HUVEC that were pre-incubated with heat-killed LVS up to 9 h before the live bacteria were added (data not shown).

We next examined whether pre-exposure to living *F. tularensis* LVS was sufficient to down-regulate the secretion of CCL2 when HUVEC were challenged with heat-killed bacteria. Endothelial cells were incubated with the live organisms for 2 h, washed with medium containing streptomycin, and challenged with heat-killed *F. tularensis* LVS. The HUVEC that were only pre-exposed to the living LVS secreted levels of CCL2 equivalent to cells stimulated with heat-killed LVS alone (Fig. 9). Pre-exposure to *F. tularensis* LVS thus is not sufficient to suppress activation of the endothelial cells, at least within the time-frame tested.

D. *F. tularensis* LVS Requires Contact to Inhibit the Proinflammatory Activation of Endothelial Cells

To assess whether *F. tularensis* LVS must have contact with HUVEC to exert its suppressive effect, the live bacteria were separated from the endothelial cells using a porous insert. When live bacteria were kept apart from the HUVEC, they no longer inhibited the secretion of CCL2 (Fig. 10). Additionally, conditioned medium from HUVEC exposed to *F. tularensis* LVS was added to a second set of HUVEC to see whether it contained a factor that could inhibit activation of the endothelial cells. For this study, HUVEC were incubated with medium only or living *F. tularensis* LVS for 24 h. The conditioned media were harvested, filtered to remove bacteria, and added to a second set of HUVEC in the absence or presence of heat-killed *F. tularensis* LVS. Conditioned medium from HUVEC exposed to live LVS organisms did not inhibit the response of HUVEC to the heat-killed LVS, as shown in Figure 11. Collectively, these results indicate that inhibition of endothelial activation by live *F. tularensis* is not mediated by a stable, soluble factor.

E. *F. tularensis* LVS Must be Viable and Actively Produce Proteins in Order to Suppress Activation of Endothelial Cells

Based on the need for contact between the bacteria and endothelial cells, we investigated whether there is a component in the OM of *F. tularensis* that causes the inhibition of the proinflammatory response of endothelial cells. To this end, we treated HUVEC with formalin-fixed (Fig. 12A) or UV-inactivated (Fig. 12B) bacteria, or an OM preparation from the LVS (Fig. 13). HUVEC incubated with the formalin- or UV-inactivated bacteria were not stimulated to secrete CCL2 above the level induced by the living organisms. Even so, these inactivated

bacteria did not suppress stimulation of the endothelial cells by the heat-killed bacteria, even when the MOI was increased to account for any replication that occurred during the course of experiments with live bacteria (Fig. 12B). In the case of the OM preparation, the sarkosyl used in isolating the membrane appeared inflammatory or toxic to the cells. Even after an additional wash step, the sham preparation (used at the highest dilution of OM tested) activated secretion of CCL2 to the same degree as the heat-killed LVS (Fig. 13). CCL2 levels dropped as the OM preparation was diluted; however, this was likely due to a reduction in sarkosyl. This effect was not due to contamination from the sonicator, as a sham preparation did not stimulate secretion of CCL2. Given that the control conditions were activating when they should not be, no conclusive results can be drawn from the experiments using OM preparations of the LVS.

To test whether synthesis of new proteins is required for the inhibitory effect of *F. tularensis* on HUVEC, the bacteria were treated with 25 μg/ml of chloramphenicol and added to the endothelial cells at MOI of ~200, 500, or 700 (Fig. 14). As in the prior experiments, the inactivated bacteria alone did not stimulate the HUVEC; however, they also were not able to suppress secretion of CCL2 by HUVEC when co-incubated with heat-killed LVS. These results suggest that the bacteria need to be viable and synthesizing proteins in order to inhibit the proinflammatory response of endothelial cells.

F. Mutants of *F. tularensis* LVS Lacking Pilus Components, TolC, or IglC Prevent Proinflammatory Activation of Endothelial Cells

In continuing to assess the role of outer-membrane structures of *F.tularensis*, we tested several mutant strains deficient for surface-related components. Of three pilus mutants tested,

only Δ*pilT* activated secretion of CCL2 slightly, yet significantly, above control levels (Fig. 15A). When combined with heat-killed LVS, all of the mutants reduced the amount of CCL2 produced by HUVEC compared to the heat-killed bacteria alone. However, Δ*pilT* and Δ*pilF* were slightly, but significantly, less effective at blocking the secretion of CCL2 compared to wild-type LVS (Fig. 15A). This difference in suppression may have been due to the presence of more dead bacteria in those wells. The MOI was initially estimated using the OD_{600}. The cultures were then retroplated to determine the actual MOI for each strain; generally these two numbers were similar. In the case of this experiment, the Δ*pilT* and Δ*pilF* mutants were added at MOI of 79 and 85, respectively, roughly half of what should have been present based on the OD_{600}. If preparations of those mutants contained greater numbers of dead bacteria in the starting cultures, it is probable that the dead organisms would activate secretion of CCL2 by HUVEC. A LVS Δ*tolC* mutant was also tested in this manner. The Δ*tolC* strain did not elicit secretion of CCL2 by the endothelial cells (Fig. 15A). In contrast, the Δ*tolC* strain induces secretion of CXCL8 by HUVEC to levels greater than wild-type (140), suggesting that *F. tularensis* induces these chemokines differently. In addition, human monocyte-derived macrophages infected with the Δ*tolC* mutant secrete more chemokines than do macrophages infected with wild-type LVS (140).

The last LVS mutant tested was Δ*iglC*. IglC is necessary for escape from the phagosome and, consequently, replication of *F. tularensis* in macrophages (105, 175). The Δ*iglC* mutant does not block the proinflammatory activation of leukocytes (104). When HUVEC were incubated with this mutant strain, they secreted less CCL2 than cells exposed to the wild-type strain or, surprisingly, even unstimulated endothelial cells (Fig. 15B). This is an interesting result on its own, as the mutant was either hyper-inhibitory or rapidly toxic to the HUVEC to account for there being less CCL2 present than in the control conditioned medium. Importantly, this

finding also indicates that the method of suppression by *F. tularensis* for endothelial cells is distinct from that for leukocytes.

G. AcrA is a Necessary Bacterial Factor to Suppress the Activation of Endothelial Cells

Our laboratory created a collection of 5,542 transposon mutants in the LVS. For the purposes of this work, the mutants were screened in order to find those that triggered secretion of either CCL2 or CXCL8 by HUVEC. As our results thus far indicate, the bacteria are actively suppressing the proinflammatory response of endothelial cells; loss of the bacterial suppressive factor would then result in a strain that could stimulate HUVEC. After establishing parameters for detecting strains that activate the endothelial cells (see Materials and Methods), 580 mutants were screened, and the pooled conditioned media were tested for CXCL8. One pool of conditioned medium with elevated levels of CXCL8 was identified. The individual strains that contributed to the pool were tested to determine which was responsible for the activating phenotype. Once identified, the transposon insertion was sequenced, and it mapped to the gene encoding AcrA, a membrane fusion protein conserved across all subspecies of *F. tularensis* sequenced to date (143, 144).

The AcrA gene is a part of the *dsbB* operon and functions with AcrB as a part of a multi-drug efflux pump, where AcrB is the pump protein and AcrA is the membrane fusion protein that can associate with various pore proteins, including TolC (143, 176). The role of this operon was probed further using Schu S4 strains containing individual, targeted deletions for each of these genes. The Δ*acrA* Schu S4 strain confirmed the LVS transposon mutant results, in that it stimulated secretion of significantly more CXCL8 and CCL2 by HUVEC than did the wild-type

strain (Fig. 16). Additionally, the strains deficient for DsbB or AcrB behaved like the wild-type Schu S4 strain (Fig. 16), indicating that AcrA may be acting independently of AcrB in this process.

We also investigated whether TolC or its homologues, FtlC and SilC, may function with AcrA to prevent activation of endothelial cells, as TolC and AcrA have been shown to interact in *E. coli* (143, 176, 177). HUVEC were treated with Δ*tolC*, Δ*ftlC*, or Δ*silC* Schu S4 strains. Of the three mutants, only Δ*tolC* triggered a proinflammatory response from the endothelial cells (Fig. 16). This result is in contrast to what was seen using the Δ*tolC* LVS mutant (Fig. 15A) and may indicate that TolC plays a role in suppressing the proinflammatory activation of endothelium, but only in the virulent Schu S4 strain. However, it is also possible that these results are due to increased death of the Δ*tolC* Schu S4 mutant during the course of the experiment. Both the initial inoculum and the wells sampled at the end of the assay contained half the amount of bacteria (~91 bacteria/cell) estimated by the OD_{600}, whereas the other mutants and the wild-type Schu S4 strain were close to the estimated MOI of 200. As for some of the pilus mutants, a greater amount of dead bacteria in the inocula could account for the enhanced activation of the HUVEC by the Schu S4 Δ*tolC* mutant.

FIGURE 3. Living *F. tularensis* LVS suppresses the proinflammatory response of endothelial cells to killed *F. tularensis* LVS.

HUVEC were incubated for 24 h with 20% medium alone (Control), *F. tularensis* LVS at a MOI of ~75 (Live), a 1:10 dilution of material released from heat-killed LVS (Killed), or a combination of live and killed LVS. Live bacteria were added to the HUVEC 1 h before the killed LVS and remained present throughout the assay. Amounts of CCL2 (A) or CXCL8 (B) in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ when compared to the heat-killed bacteria. This experiment was performed five times (A) or two times (B) with similar results.

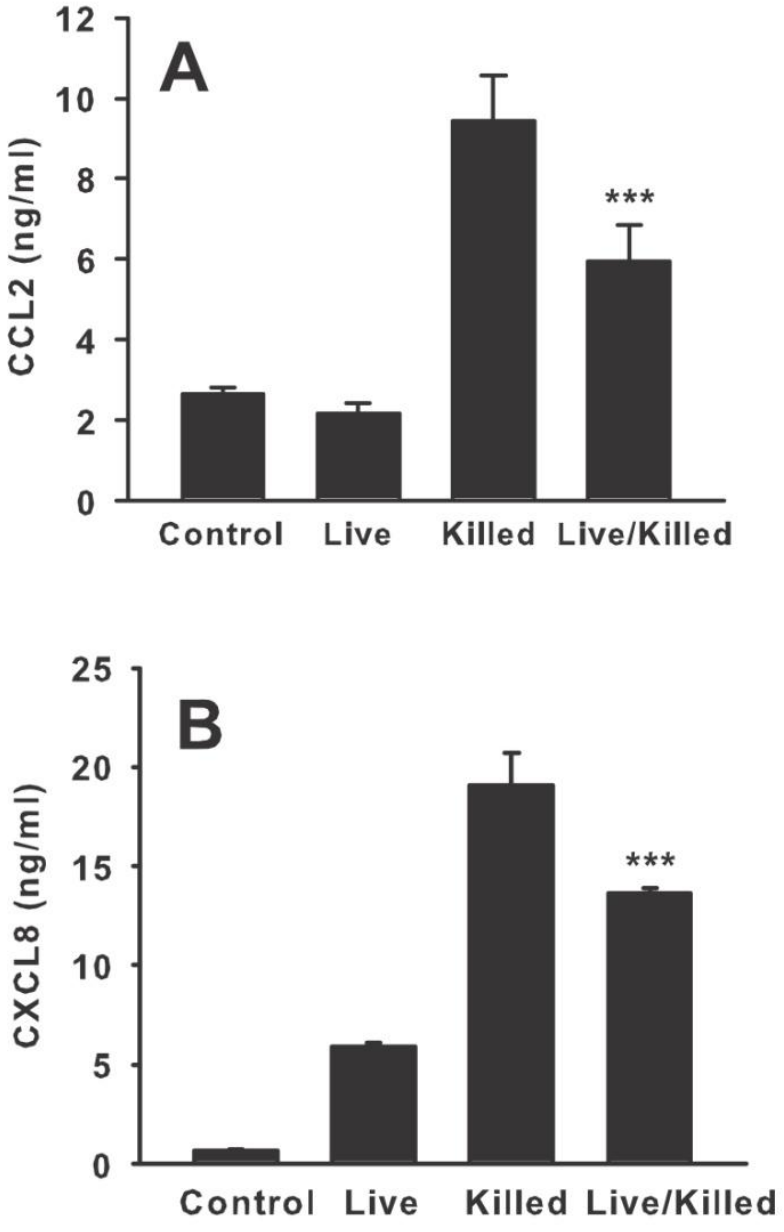

A
CCL2 (ng/ml)
12
10
8
6
4
2
0
***
Control
Live
Killed
Live/Killed
B
CXCL8 (ng/ml)
25
20
15
10
5
0
***
Control
Live
Killed
Live/Killed

FIGURE 4. Living *F. tularensis* Schu S4 suppresses the proinflammatory response of endothelial cells to killed *F. tularensis* Schu S4.

HUVEC were incubated for 24 h with 20% medium alone (Control), *F. tularensis* Schu S4 at a MOI of ~200 (Live), a 1:10 dilution of material released from heat-killed Schu S4 (Killed), or a combination of live and killed Schu S4. Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ when compared to the heat-killed bacteria.

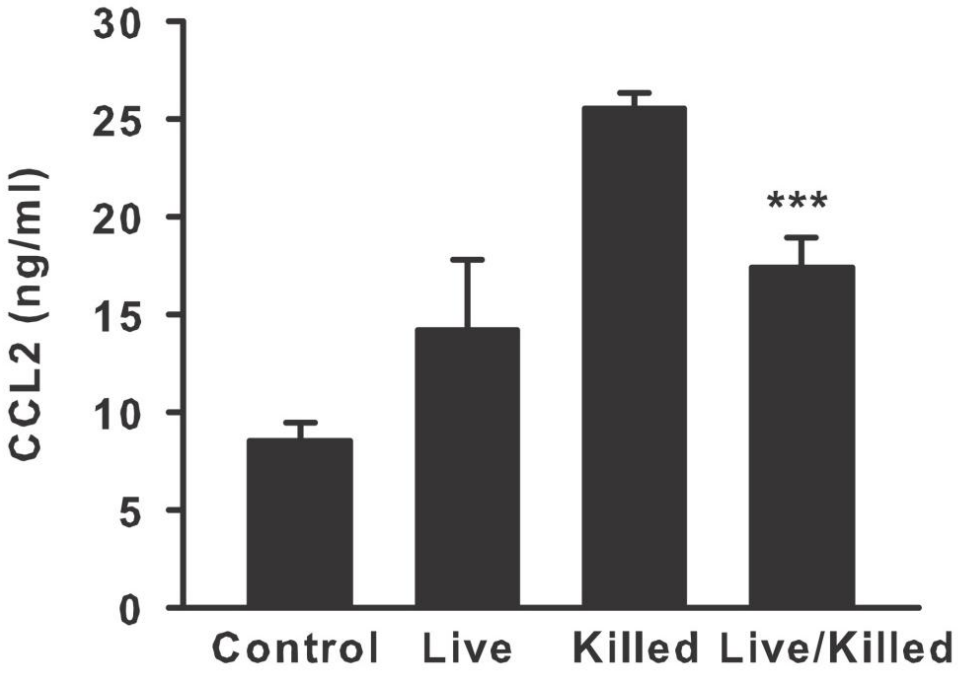

30
25
20
15
10
5
0
CCL2 (ng/ml)
***
Control
Live
Killed
Live/Killed

FIGURE 5. Activation of endothelial cells by killed *F. tularensis* LVS is suppressed by living *F. tularensis* LVS in a dose-dependent manner.

HUVEC were incubated for 24 h with 20% medium alone (Control), *F. tularensis* LVS at a MOI of ~75 (Live), a 1:10 dilution of material released from heat-killed LVS (Killed), or a combination of living and killed LVS added simultaneously where the MOI of the live bacteria varied from ~1 to 500 bacteria/endothelial cell. Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ as compared to the heat-killed bacteria. This experiment was performed two times with similar results.

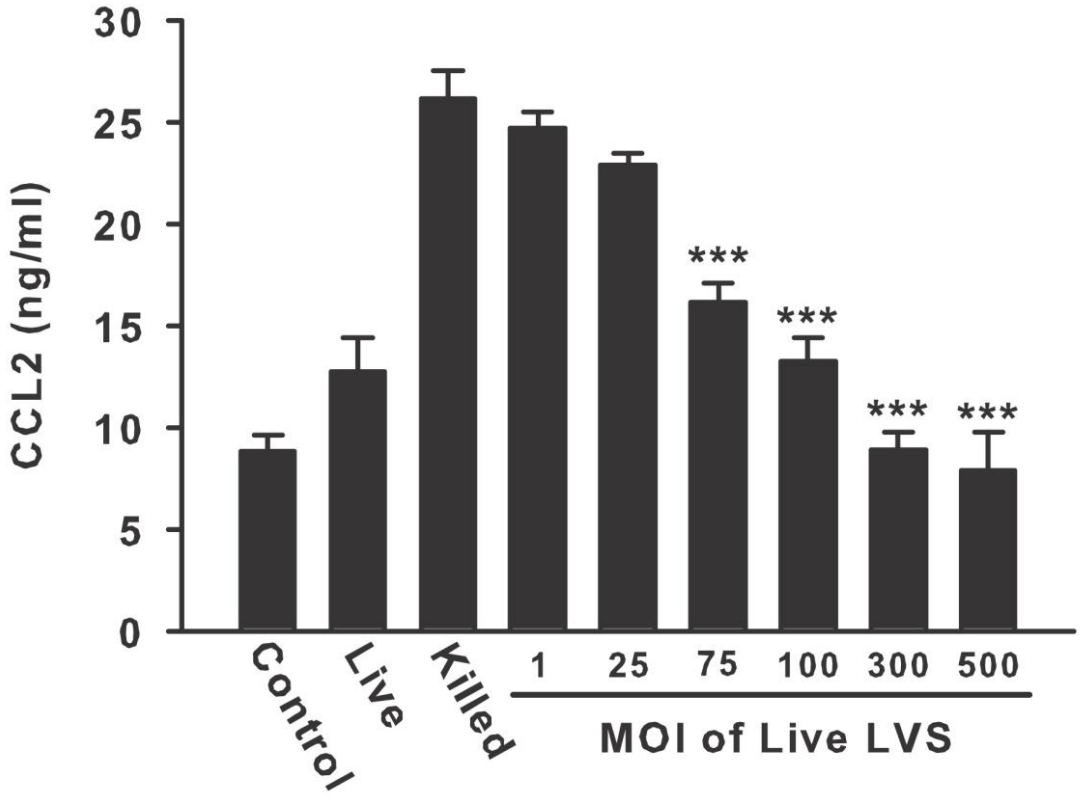

CCL2 (ng/ml)
30
25
20
15
10
5
0
***
***
***
***
Control
Live
Killed
1
25
75
100
300
500
MOI of Live LVS

FIGURE 6. Down-regulation of the proinflammatory response of endothelial cells by *F. tularensis* is stimulus-specific.

HUVEC were incubated for 24 h with 20% medium alone (Control), living *F. tularensis* LVS at a MOI of ~200 (Live), a 1:10 dilution of material released from heat-killed LVS (Killed), live and killed LVS organisms together (Live/Killed), 1 ng/ml of *E. coli* LPS, or living LVS organisms and *E. coli* LPS combined (Live/*E. c.* LPS). Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ when compared to the heat-killed bacteria. This graph is representative of three experiments yielding similar results.

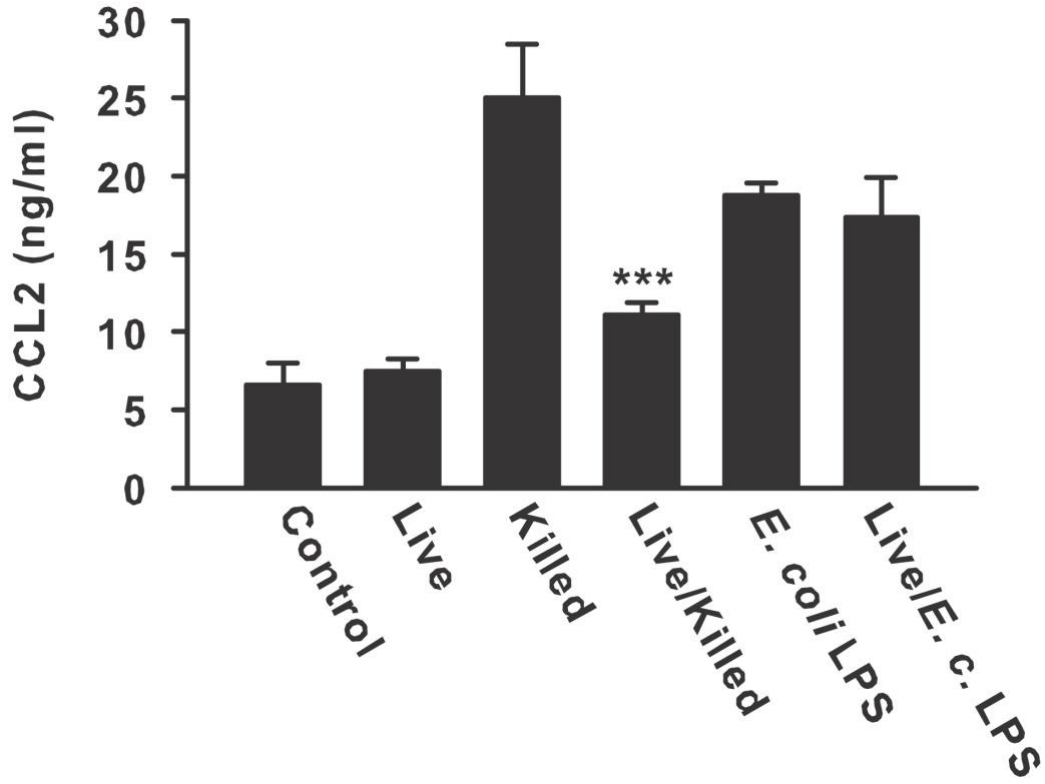

CCL2 (ng/ml)
30
25
20
15
10
5
0
***
Control
Live
Killed
Live/Killed
E. coli LPS
Live/E. c. LPS

FIGURE 7. Suppression of the endothelial cell proinflammatory response by living *F. tularensis* LVS is initiated rapidly.

HUVEC were incubated for 24 h with 20% medium alone (Control), *F. tularensis* LVS at a MOI of ~75 (Live), or a 1:10 dilution of material released from heat-killed LVS (Killed). In other samples, HUVEC were pre-incubated with living bacteria for 0, 30, 60, or 120 min prior to challenge with killed LVS. The live organisms remained present for the duration of the assay. Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ as compared to the heat-killed bacteria. This graph is representative of three experiments with similar results.

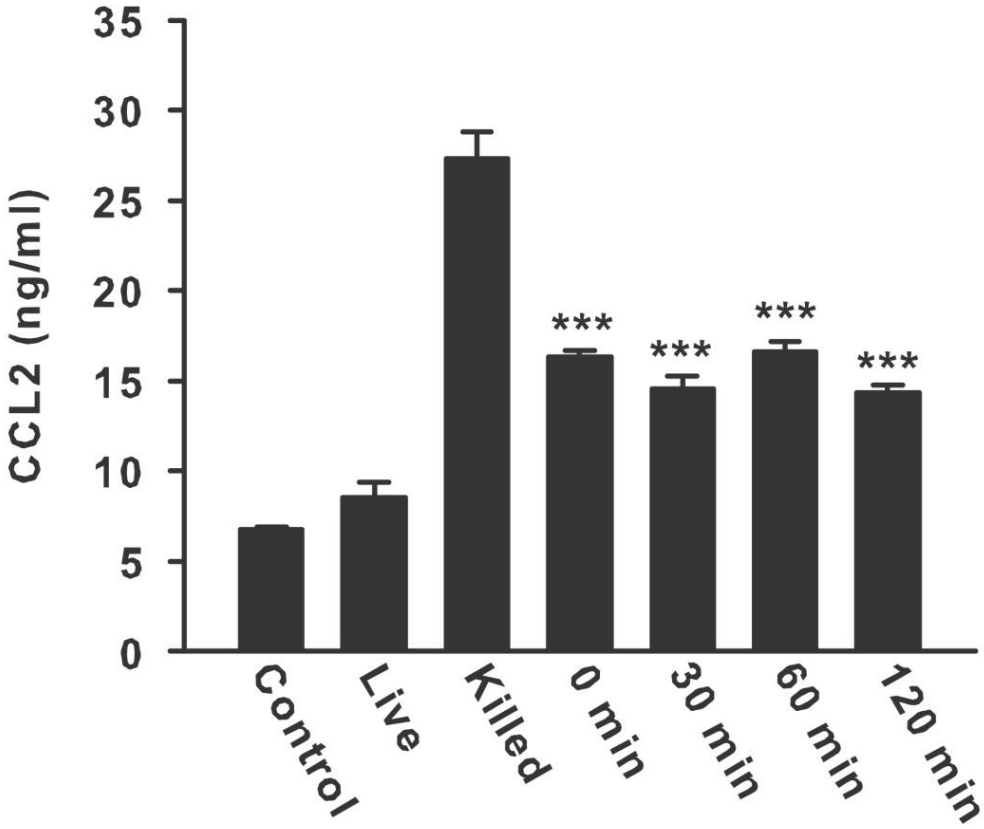

CCL2 (ng/ml)
35
30
25
20
15
10
5
0
***
***
***
***
Control
Live
Killed
0 min
30 min
60 min
120 min

FIGURE 8. Secretion of CCL2 by HUVEC exposed to living *F. tularensis* is suppressed within 8 h.

HUVEC were activated with material released from heat-killed LVS at a dilution of 1:10 (closed circles) or living LVS at a MOI of ~200 in combination with material released from heat-killed LVS (open circles). Conditioned media were collected at 4, 8, 12, 16, 20, and 24 h and assayed for CCL2 by ELISA. Points represent the means ± SD of triplicate samples. The difference between samples with or without living organisms was significant at 8 h ($P < 0.05$) or longer ($P < 0.001$).

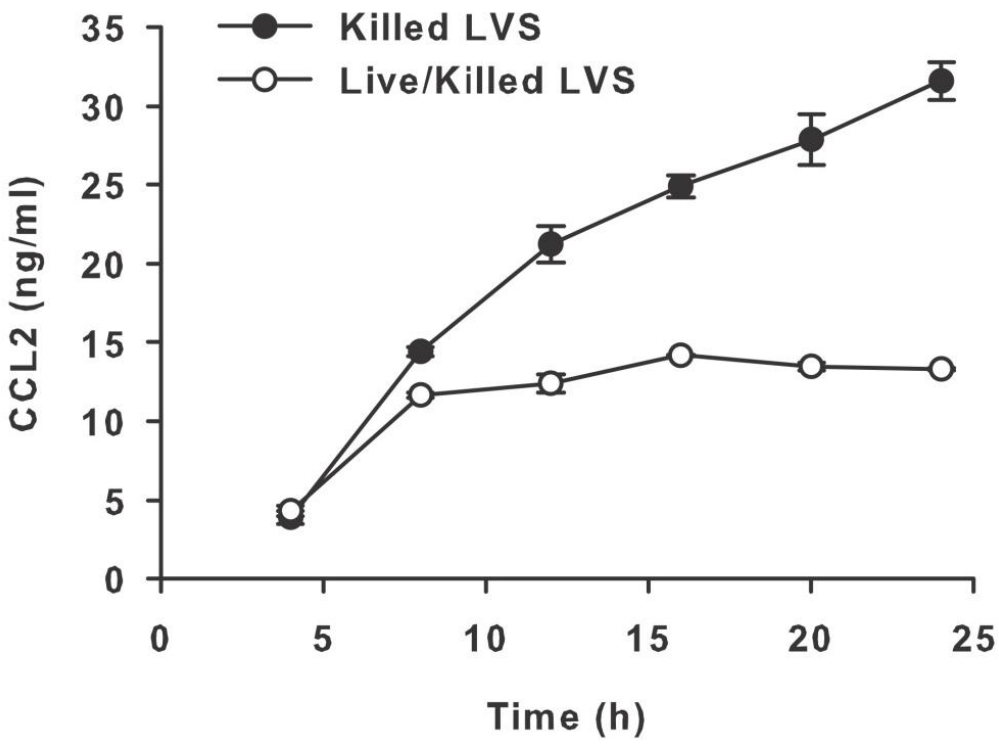

Killed LVS
Live/Killed LVS
CCL2 (ng/ml)
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Time (h)

FIGURE 9. Pre-exposure to *F. tularensis* LVS is not sufficient to suppress the activation of endothelial cells.

HUVEC were incubated for 24 h with 20% medium alone (Control) or live *F. tularensis* LVS at a MOI of ~200 for 2 h. After 2 h, the HUVEC incubated with live LVS were either left alone (Live), challenged with a 1:10 dilution of material released from heat-killed LVS (Live/Killed), or washed three times with 20% medium containing 10 µg/ml of streptomycin before challenge with killed LVS (Pre-exposure) for an additional 22 h. HUVEC were also exposed to killed LVS alone (Killed) for 24 h. Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ as compared to the heat-killed bacteria. This experiment was performed three times with similar results.

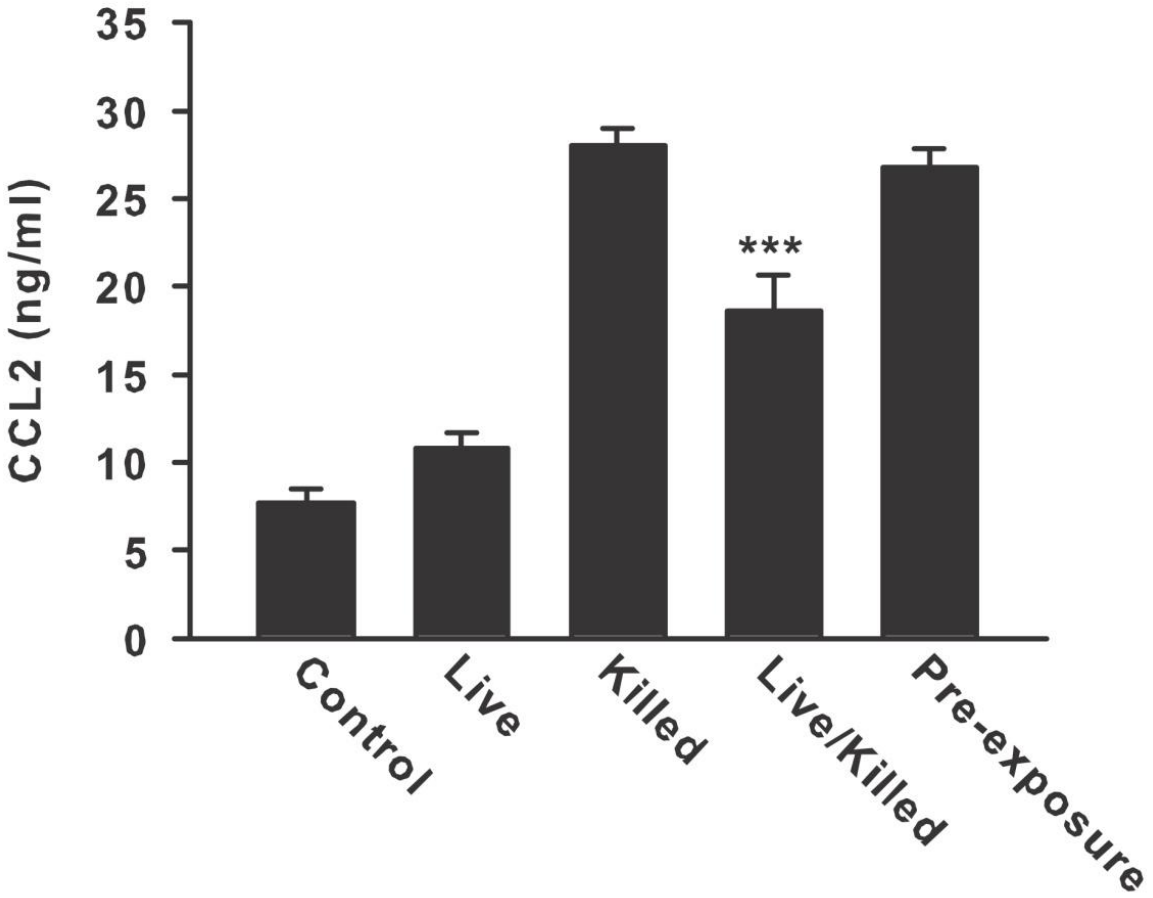

CCL2 (ng/ml)
35
30
25
20
15
10
5
0
***
Control
Live
Killed
Live/Killed
Pre-exposure

FIGURE 10. Living *F. tularensis* LVS requires contact to inhibit the activation of endothelial cells.

HUVEC were incubated for 24 h with 20% medium alone (Control), *F. tularensis* LVS at a MOI of ~200 (Live), or a 1:10 dilution of material released from heat-killed LVS (Killed). In other samples, live and killed LVS were combined under circumstances where the live bacteria were separated from the HUVEC using a Transwell® insert (Well) or allowed to contact the cells (No Well). Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ as compared to the heat-killed bacteria. This graph is representative of three experiments with similar results.

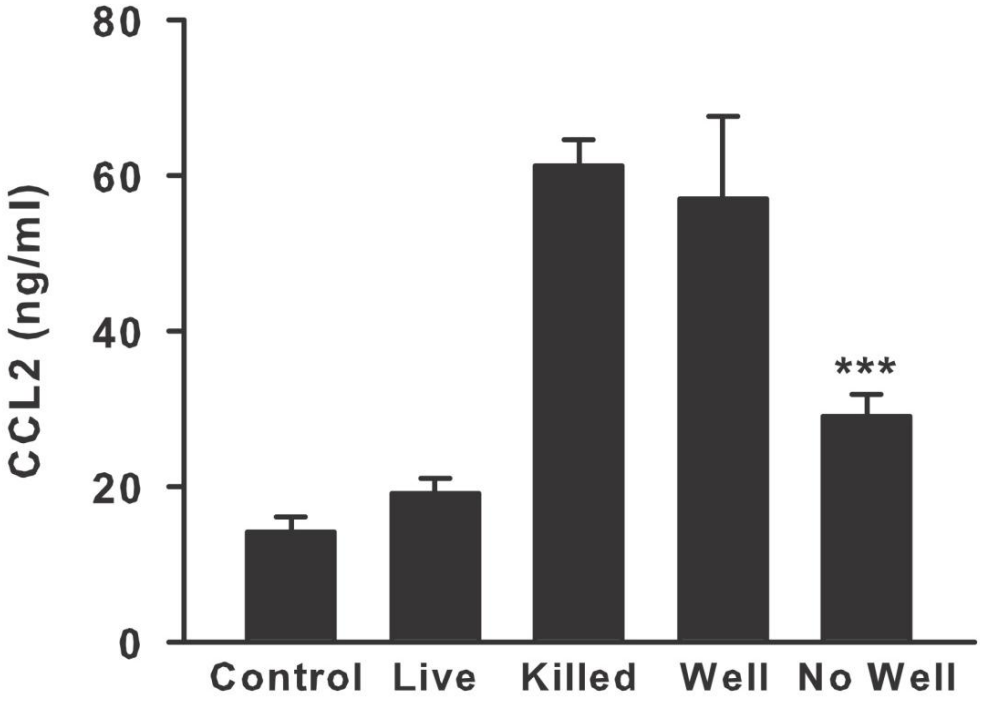

80
60
40
20
0
CCL2 (ng/ml)
***
Control
Live
Killed
Well
No Well

FIGURE 11. Conditioned medium from endothelial cells exposed to *F. tularensis* LVS does not down-regulate the proinflammatory response of HUVEC.

One set of HUVEC was incubated for 24 h with medium alone or *F. tularensis* LVS at a MOI of ~200. The conditioned media were then filtered to remove bacteria. A second set of HUVEC was incubated for 24 h with the conditioned medium from HUVEC alone (Control CM) or conditioned medium from HUVEC exposed to the LVS (Live CM) from the first set of HUVEC, or with fresh 20% medium (Control). Other samples were challenged with a 1:10 dilution of material released from heat-killed LVS alone (Killed), as well as combined with the 20% conditioned medium (Control CM/Killed) or the LVS conditioned medium (Live CM/Killed) from the first set of HUVEC. Amounts of CCL2 from the second set of HUVEC were determined by ELISA. Bars represent the means ± SD of triplicate samples. This graph is representative of three experiments with similar results.

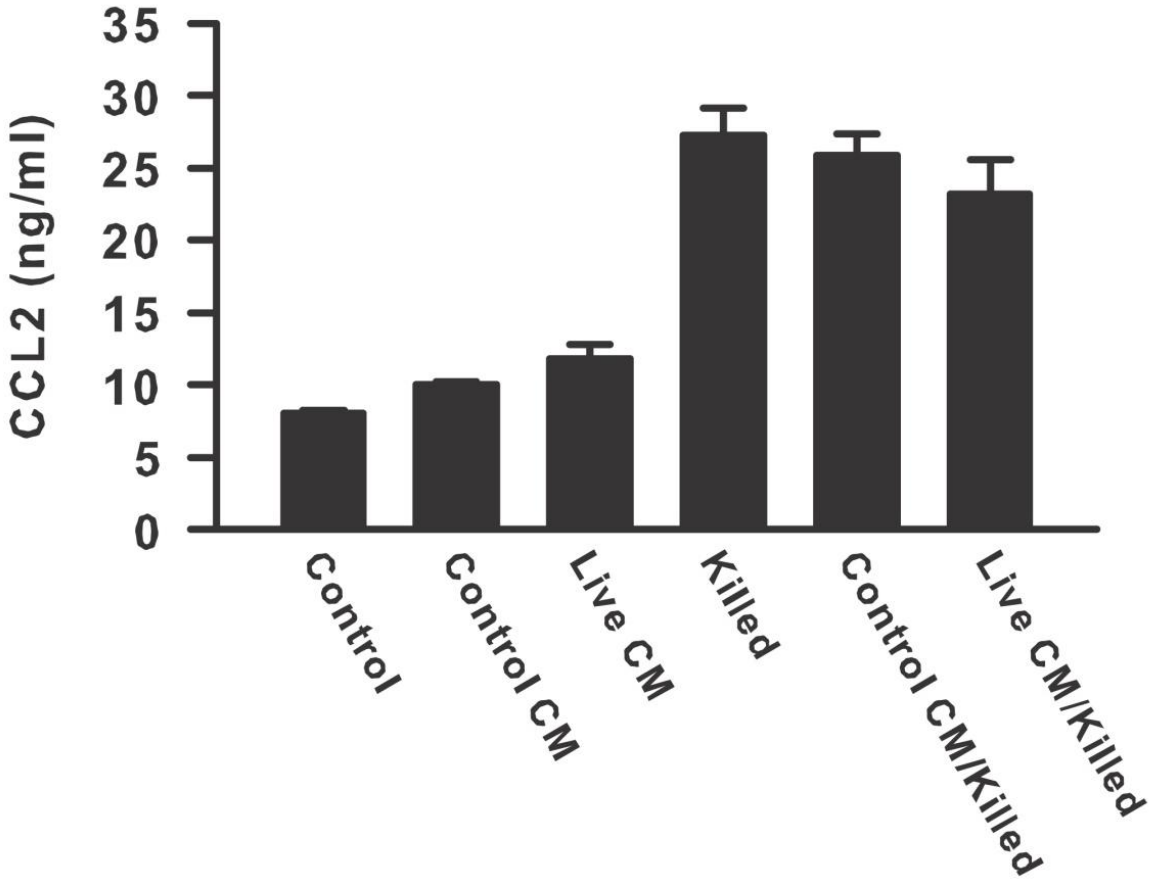
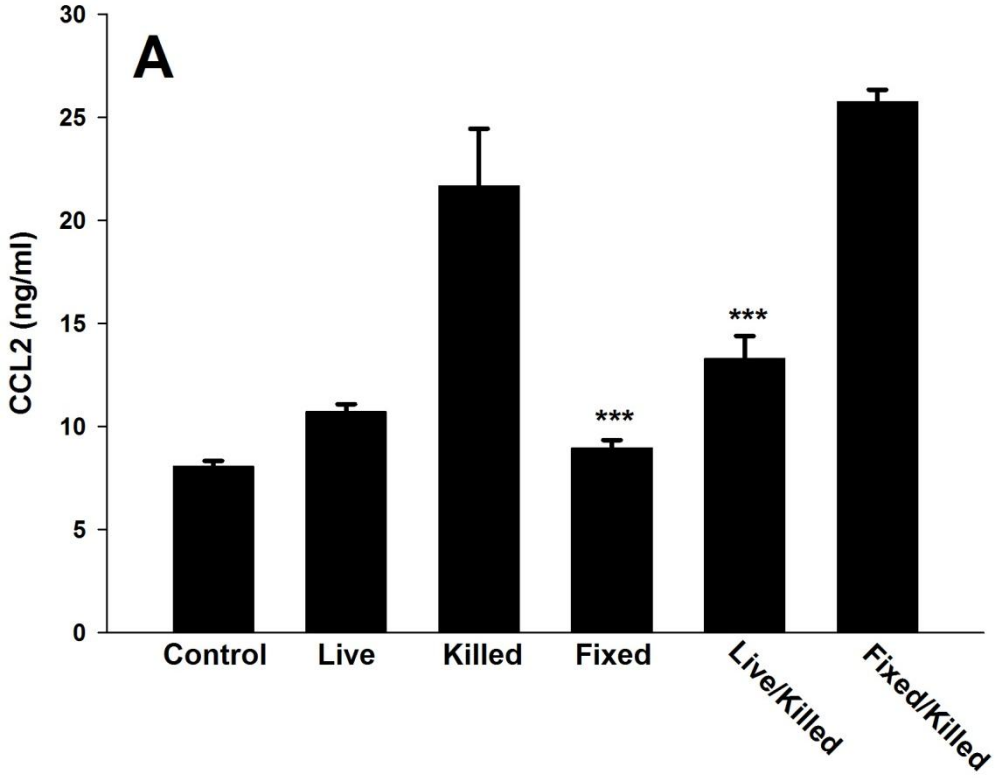

CCL2 (ng/ml)
35
30
25
20
15
10
5
0
Control
Control CM
Live CM
Killed
Control CM/Killed
Live CM/Killed

FIGURE 12. Formalin- and UV-inactivated *F. tularensis* neither activate nor suppress the proinflammatory response of endothelial cells.

HUVEC were stimulated with cell medium (Control), live LVS at a MOI of ~200 (Live), a 1:10 dilution of heat-killed LVS (Killed), or a combination of the live and heat-killed LVS (Live/Killed). In Panel A, HUVEC also were incubated with LVS that was fixed with formalin for 1 h before use either alone (Fixed) or with heat-killed LVS (Fixed/Killed). In Panel B, HUVEC were exposed to UV-inactivated LVS at a MOI of ~200 or 500 (UV 200 or UV 500), alone or in combination with heat-killed LVS (UV 200/Killed, UV 500/Killed). The HUVEC were exposed to the various stimuli for 24 h at which point the conditioned media were collected and assayed by ELISA for CCL2. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ compared to the heat-killed bacteria. There is no significant difference between Fixed, UV 200, or UV 500 samples and their respective controls. These experiments were performed three times with similar results.


A
CCL2 (ng/ml)
30
25
20
15
10
5
0
***
***
Control
Live
Killed
Fixed
Live/Killed
Fixed/Killed

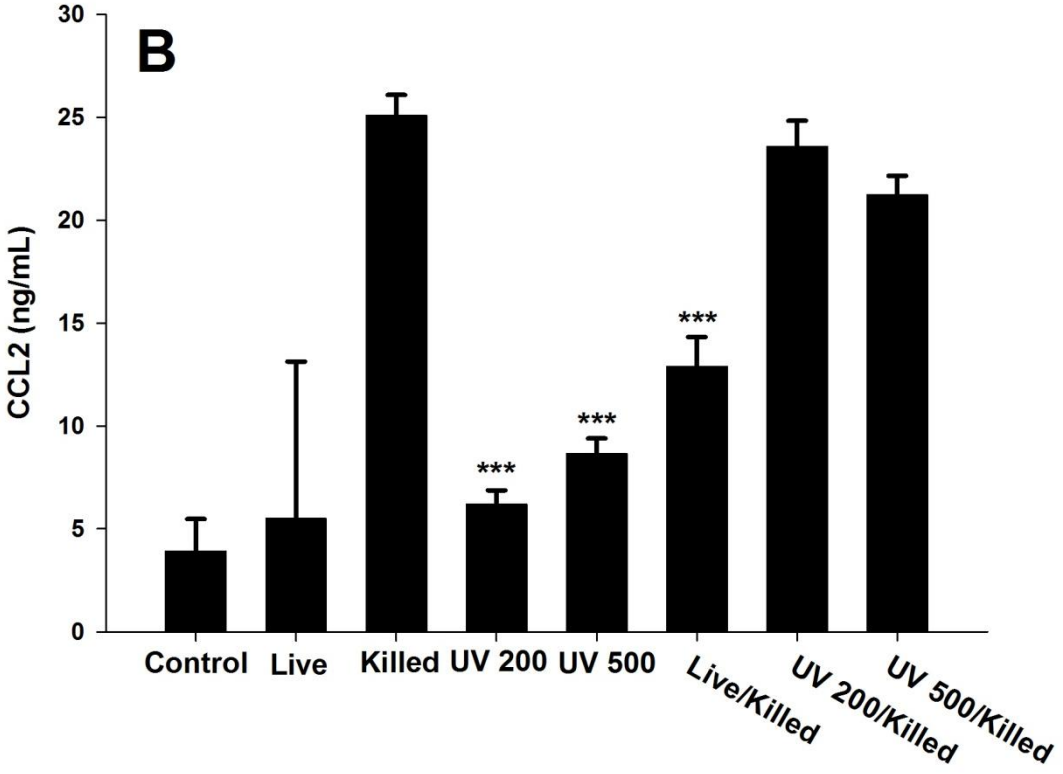

B
CCL2 (ng/mL)
30
25
20
15
10
5
0
***
***
***
Control
Live
Killed
UV 200
UV 500
Live/Killed
UV 200/Killed
UV 500/Killed

FIGURE 13. Outer-membrane preparations from *F. tularensis* LVS contain non-bacterial components that activate HUVEC.

HUVEC were incubated with 20% medium (Unstim), 20% medium that had been subjected to all the same procedures as the outer-membrane preparation itself (Sham), live LVS at a MOI of ~200 (Live), a 1:10 dilution of heat-killed LVS (Killed), or various dilutions of an outer-membrane preparation from *F. tularensis* LVS (OM) for 24 h. After incubation, the conditioned media were collected and assayed by ELISA for CCL2. Bars represent the means ± SD of triplicate samples. These experiments were performed twice with similar results.

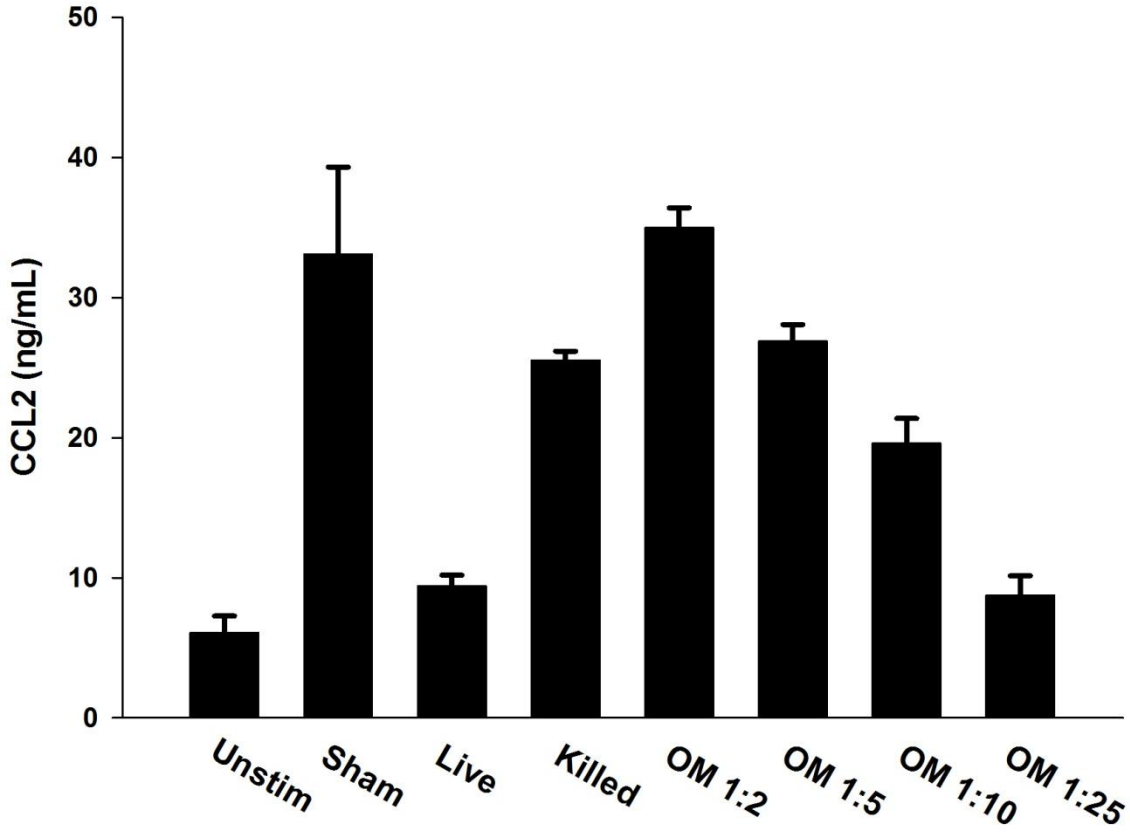

CCL2 (ng/mL)
50
40
30
20
10
0
Unstim
Sham
Live
Killed
OM 1:2
OM 1:5
OM 1:10
OM 1:25

FIGURE 14. Protein synthesis is necessary for the inhibitory effect of *F. tularensis* LVS on endothelial cells.

HUVEC were incubated with 20% medium (Control), 20% medium containing 25 µg/ml of chloramphenicol, live LVS at a MOI of ~200 (Live), LVS that had been treated for 1 h with 25 µg/ml of chloramphenicol (Chlor/LVS), or a 1:10 dilution of heat-killed LVS (Killed). Heat-killed LVS treated with 25 µg/ml of chloramphenicol (Chlor/Heat-Killed) was used as a control. The ability to suppress activation of HUVEC was tested using a combination of the live and heat-killed LVS (Live/Killed) or the chloramphenicol-treated LVS with the heat-killed bacteria (Chlor/LVS/Heat-Killed). After a 24 h incubation, the conditioned media were collected and assayed by ELISA for CCL2. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ compared to the heat-killed bacteria. This assay is representative of three with similar results.

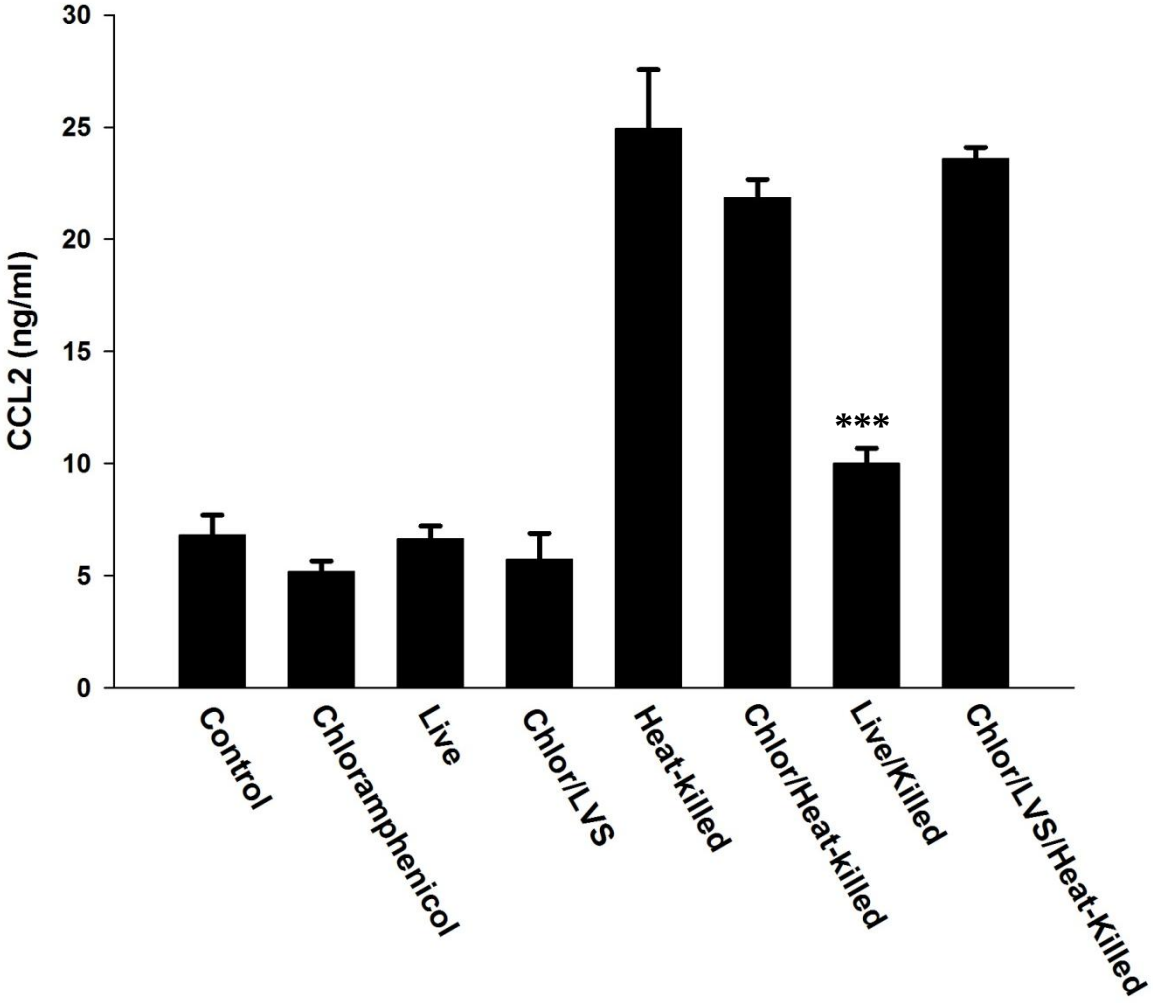
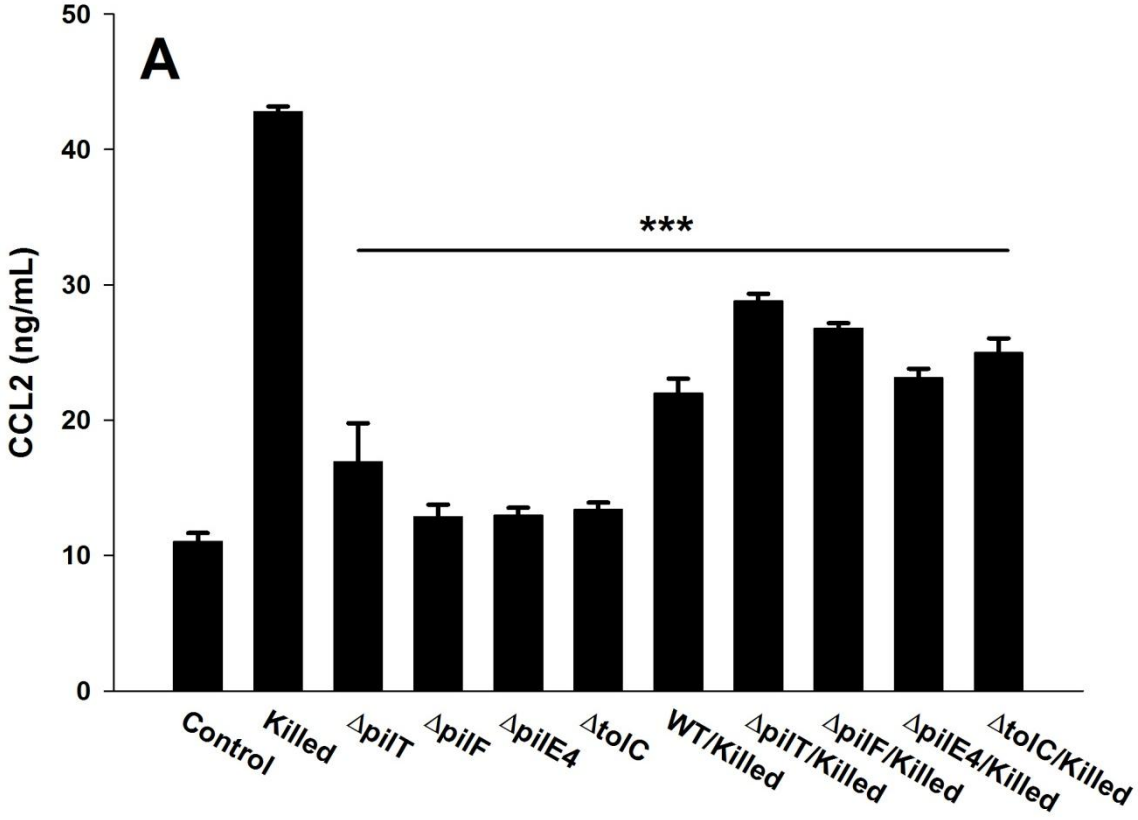

CCL2 (ng/ml)
30
25
20
15
10
5
0
***
Control
Chloramphenicol
Live
Chlor/LVS
Heat-killed
Chlor/Heat-killed
Live/Killed
Chlor/LVS/Heat-Killed

FIGURE 15. Pilus, TolC, and IglC mutants of *F. tularensis* LVS suppress activation of endothelial cells similarly to the wild-type strain.

Endothelial cells were incubated with cell medium (Control), wild-type LVS (WT), heat-killed LVS at a 1:10 dilution (Killed), wild-type LVS combined with heat-killed (WT/Killed), or the various mutants alone or combined with heat-killed bacteria. The Δ*pilE4*, Δ*pilT*, Δ*pilF*, and Δ*tolC* mutants were evaluated in (A) and the Δ*iglC* mutant in (B). The MOI for wild-type, Δ*pilE4*, and Δ*tolC* were ~200 but only 79 and 85 for Δ*pilT* and Δ*pilF*, respectively. After 24 h, the conditioned media were collected and assayed by ELISA for CCL2. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ when compared to the heat-killed bacteria. In Panel B, the Δ*iglC* mutant sample was significantly reduced compared to the control and wild-type samples. These results are representative of two experiments with similar results.


A
CCL2 (ng/mL)
50
40
30
20
10
0
***
Control
Killed
ΔpilT
ΔpilF
ΔpilE4
ΔtolC
WT/Killed
ΔpilT/Killed
ΔpilF/Killed
ΔpilE4/Killed
ΔtolC/Killed

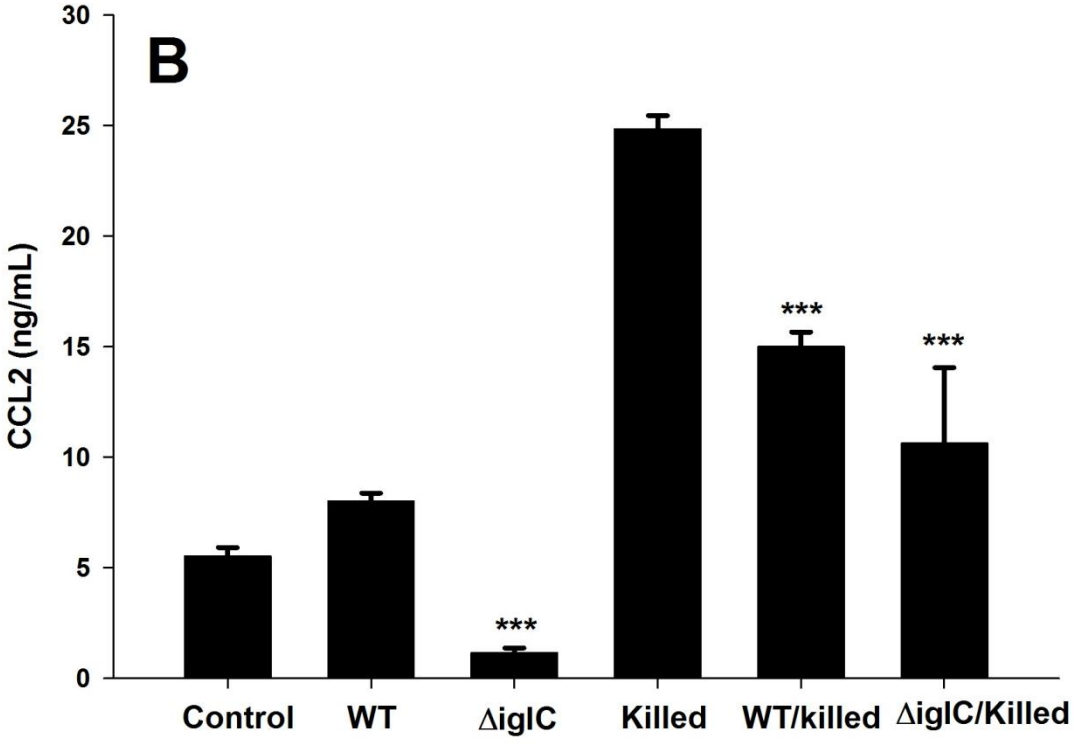
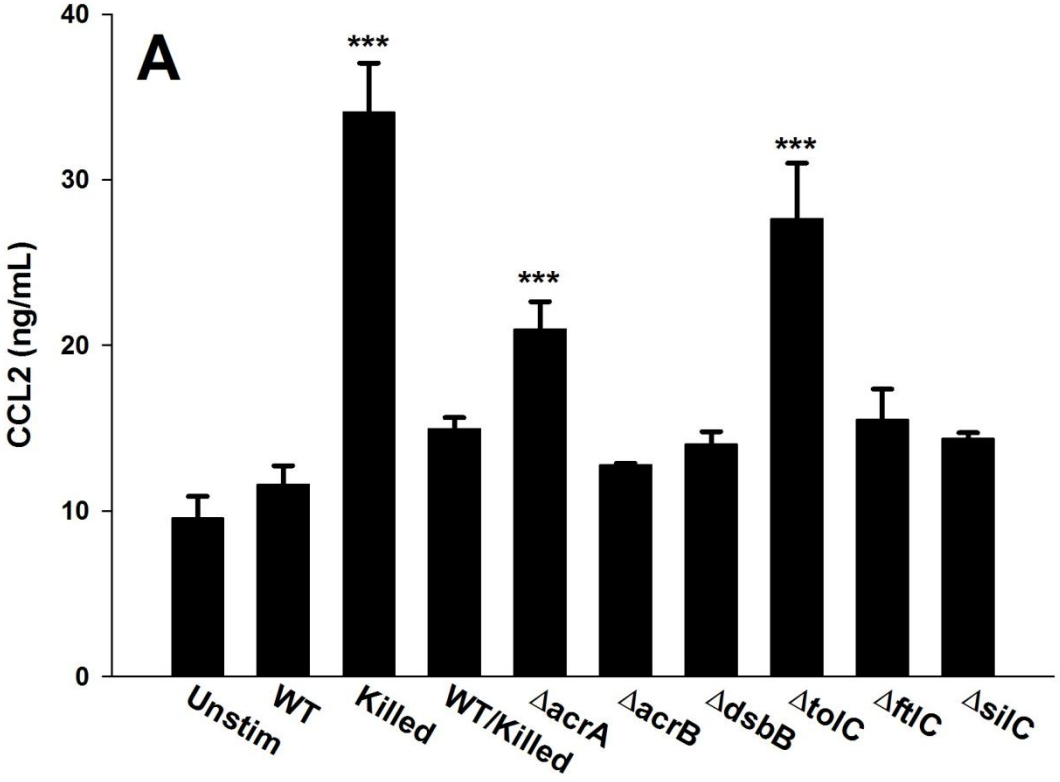
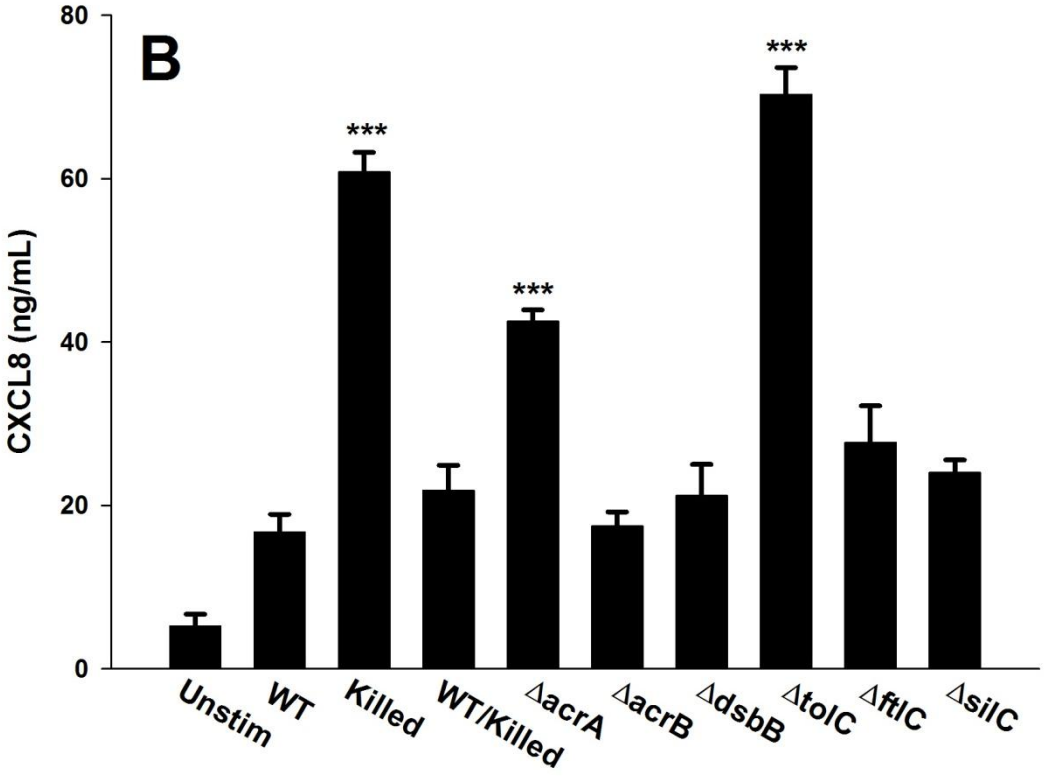

B
CCL2 (ng/mL)
30
25
20
15
10
5
0
***
***
***
Control
WT
ΔiglC
Killed
WT/killed
ΔiglC/Killed

FIGURE 16. A *F. tularensis* Schu S4 Δ*acrA* mutant activates endothelial cells.

HUVEC were incubated for 24 h with 20% medium alone (Unstim), *F. tularensis* Schu S4 wild-type (WT) or mutants Δ*acrA*, Δ*acrB*, Δ*dsbB*, Δ*tolC*, Δ*ftlC*, and Δ*silC* at estimated MOI of ~200, a 1:10 dilution of material released from heat-killed Schu S4 (Killed), or a combination of wild-type and killed Schu S4 (WT/Killed). Amounts of CCL2 (A) or CXCL8 (B) in the conditioned media were determined by ELISA. The actual MOI for Δ*tolC* was only 91, approximately half that expected by estimation using the OD_{600}. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ when compared to the wild-type bacteria. This experiment was performed three times for the Δ*acrA*, Δ*acrB*, and Δ*dsbB* mutants and two times for the remaining three mutants with similar results.


A
CCL2 (ng/mL)
40
30
20
10
0
***
***
***
Unstim
WT
Killed
WT/Killed
ΔacrA
ΔacrB
ΔdsbB
ΔtolC
ΔftlC
ΔsilC


B
CXCL8 (ng/mL)
80
60
40
20
0
***
***
***
Unstim
WT
Killed
WT/Killed
ΔacrA
ΔacrB
ΔdsbB
ΔtolC
ΔftlC
ΔsilC

II. Involvement of the Endothelial Protein C Receptor in the Response of Endothelial Cells to *F. tularensis*

A. *F. tularensis* Acts Through the EPCR to Suppress Proinflammatory Activation of Endothelial Cells

Collectively, the previous results suggest that *F. tularensis* is acting directly on a surface component of endothelial cells to produce suppression. The aPC/EPCR complex exerts effects on endothelial cells (158) that are similar to the response of HUVEC to *F. tularensis*. To test the role of the EPCR in suppression mediated by *F. tularensis,* the receptor was blocked with a well-characterized mAb (162, 178, 179). When the EPCR was blocked, living *F. tularensis* LVS was no longer capable of inhibiting the response of HUVEC to material released from killed LVS organisms, whereas an isotype-matched control mAb had no effect (Fig. 17A). Remarkably, living *F. tularensis* LVS acquired the ability to stimulate endothelial secretion of CCL2 on its own when the EPCR was rendered non-functional. Similarly, HUVEC became responsive to living Schu S4 organisms in the presence of the mAb to the EPCR (Fig. 17B). The antibody alone, like living LVS, had little effect on HUVEC, and it did not reduce the viability of the bacteria (Fig. 17A and data not shown). These data indicate that the EPCR plays an essential role in suppression of endothelial activation by both an attenuated and a virulent strain of *F. tularensis.*

B. PAR-1 Antibodies Do Not Reverse the Inhibitory Effect of *F. tularensis*

The majority of the literature concerning the EPCR and signal transduction implicates PAR-1 as the responsible co-receptor (149, 156, 180). However, two different antibodies characterized as preventing activation of PAR-1 by aPC and thrombin (156, 181) did not change the response of HUVEC to the LVS (Fig. 18). In an attempt to confirm the efficacy of these antibodies, we tested their ability to block the response of HUVEC to human recombinant thrombin, which is known to act through PAR-1 (155). However, the antibodies did not prevent secretion of CCL2 by HUVEC stimulated with the thrombin (Fig. 18). Given that we could not determine the efficacy of the blocking antibodies, no firm conclusions can be drawn regarding the involvement of PAR-1 in blocking the proinflammatory response of HUVEC by *F. tularensis*.

C. *F. tularensis* Alters the Localization of the EPCR on Endothelial Cells

To better understand the role of the EPCR in the context of tularemia, we characterized its expression patterns in the presence of *F. tularensis* LVS. Membrane-bound EPCR is only one form of the receptor. The EPCR can be cleaved and released from the endothelial cell surface, forming sEPCR. Though the function of sEPCR is not clear, its levels are elevated during certain disease states, including sepsis (153, 182, 183). We examined the effects of *F. tularensis* on release of sEPCR by endothelium. HUVEC that were exposed to live LVS, either alone or with heat-killed LVS, shed less sEPCR into the medium as compared to HUVEC incubated with either heat-killed LVS or cell medium alone (Fig. 19). The finding that there is less sEPCR in the conditioned medium from HUVEC exposed to the live bacteria indicated that the receptor might

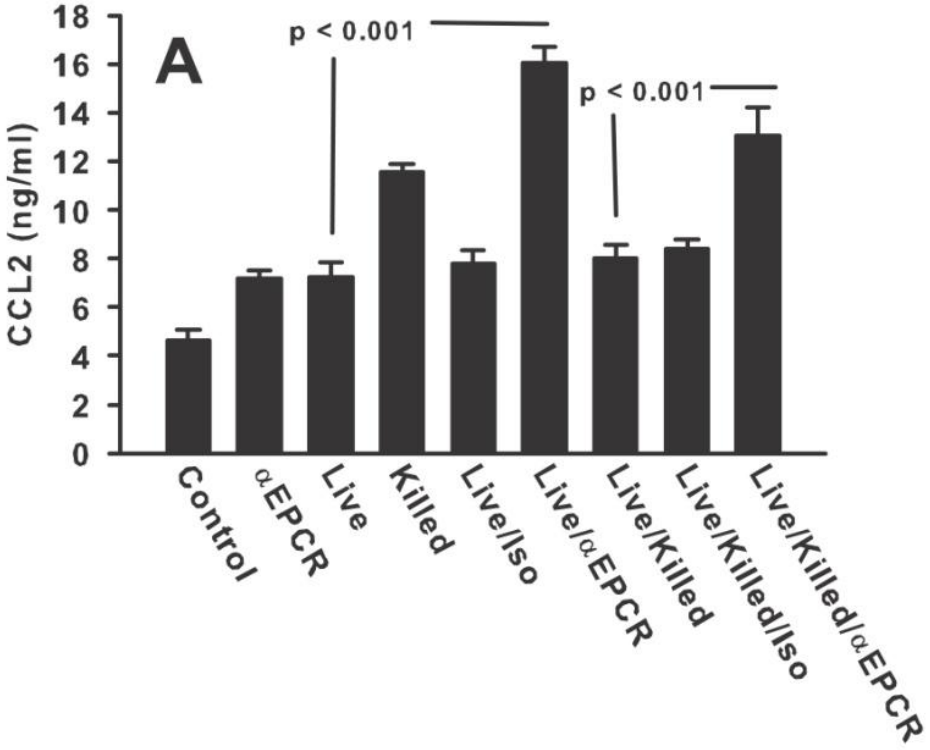
be retained in greater numbers on the surface of these cells. To assess surface expression of the EPCR, endothelial cells were exposed to cell medium, live LVS, heat-killed LVS, or both the live and heat-killed bacteria for 24 h, followed by flow cytometric analysis of the EPCR. Just as HUVEC exposed to the live bacteria released less sEPCR, they also had less surface-expressed EPCR as compared to HUVEC incubated with the heat-killed LVS or cell medium alone (Fig. 20). The difference, however, was more modest compared to what was seen in the sEPCR ELISA assay. When comparing HUVEC exposed to the live and killed LVS to cells incubated only with the heat-killed organisms, the average reduction in surface-bound EPCR (from three experiments) was ~20% vs. ~30% for the sEPCR. Given these results, the reduced amount of sEPCR released from HUVEC incubated with the live bacteria cannot be accounted for by enhanced retention on the endothelial cell surface.

As a result of the previous assays, it seemed that the EPCR might be internalized by endothelial cells in the presence of *F. tularensis.* Using confocal microscopy, we found a clear difference in the expression pattern of the EPCR in control cells vs. HUVEC incubated with living bacteria for 2 h. When examining non-permeabilized cells, HUVEC exposed only to medium or heat-killed LVS had a distinct halo of red along the perimeter of the cell, whereas those incubated with living LVS lacked this border (Fig. 21). To determine if the halo coincided with the plasma membrane, some samples were treated with wheat germ agglutinin conjugated to Alex Fluor 488. This agent binds sialic acid and N-acetylglucosaminyl residues and is meant to stain the plasma membrane of mammalian cells. Staining with the tagged wheat germ agglutinin can be seen in the non-permeabilized control cells in Figure 21. Unexpectedly, the staining for the EPCR did not overlap with that of the wheat germ agglutinin. However, the fact that these cells were not permeabilized indicates that the halo represents EPCR on the cell surface.

When the HUVEC were permeabilized, cells under all conditions stained internally for the EPCR. This observation is in accordance with published work showing that the EPCR is endocytosed and recycled by endothelial cells (184). However, a distinction is that untreated HUVEC maintained a peripheral halo, whereas those incubated with living LVS had only a diffuse intracellular distribution of the EPCR (Fig. 21, 22A and B). To further confirm this phenotype, a series of 34 images of endothelial cells exposed to cell medium, live bacteria, or heat-killed LVS were coded. Blind scoring was conducted on these images, describing the pattern of EPCR staining as either having a halo or not. Of the 34 images, 85% were scored correctly, with those being judged as having mostly cells with the halo phenotype matched back to control cells or cells exposed to heat-killed LVS, and those lacking a halo matched to HUVEC treated with living LVS. In some of these studies, GFP-expressing *F. tularensis* was used to look for possible co-localization of bacteria and the EPCR. Though there was no clear co-localization, at 2 h post-infection, some of the bacteria appeared to have been taken up by the endothelial cells (Fig. 22C and 23). It is also possible that the bacteria remained external but were located in invaginations on the surface of HUVEC. Even if the bacteria were internalized, it is unlikely that they survived for very long, given that at 24 h post-infection, fewer than 1 in 50 HUVEC contain viable bacteria (97). Whether the bacteria are internal or tucked into caveolae, these data are consistent with our previous results, which indicated that inhibition of the proinflammatory response of HUVEC by *F. tularensis* was contact-dependent (Figs. 9, 10, and 11).

FIGURE 17. Blocking the EPCR ablates the ability of *F. tularensis* to suppress the activation of endothelial cells.

HUVEC were incubated for 24 h with 20% medium alone (Control), living *F. tularensis* LVS (A) or Schu S4 (B) at a MOI of ~200 (Live), a 1:10 dilution of material released from heat-killed bacteria of the same strain (Killed), or 20 µg/ml of an anti-EPCR mAb (αEPCR). HUVEC also were incubated with living bacteria mixed with 20 µg/ml of an isotype-matched control mAb (Live/Iso), anti-EPCR mAb (Live/αEPCR), or heat-killed bacteria (Live/Killed). In panel A, HUVEC additionally were exposed to living and killed *F. tularensis* LVS in the presence of the isotype-matched control mAb (Live/Killed/Iso) or to live and killed *F. tularensis* with the anti-EPCR mAb (Live/Killed/αEPCR). Antibodies were added to the HUVEC 1 h before challenge with *F. tularensis*. Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. These graphs are representative of three (A) or two (B) experiments with similar results


A
CCL2 (ng/ml)
18
16
14
12
10
8
6
4
2
0
p < 0.001
p < 0.001
Control
αEPCR
Live
Killed
Live/Iso
Live/αEPCR
Live/Killed
Live/Killed/Iso
Live/Killed/αEPCR

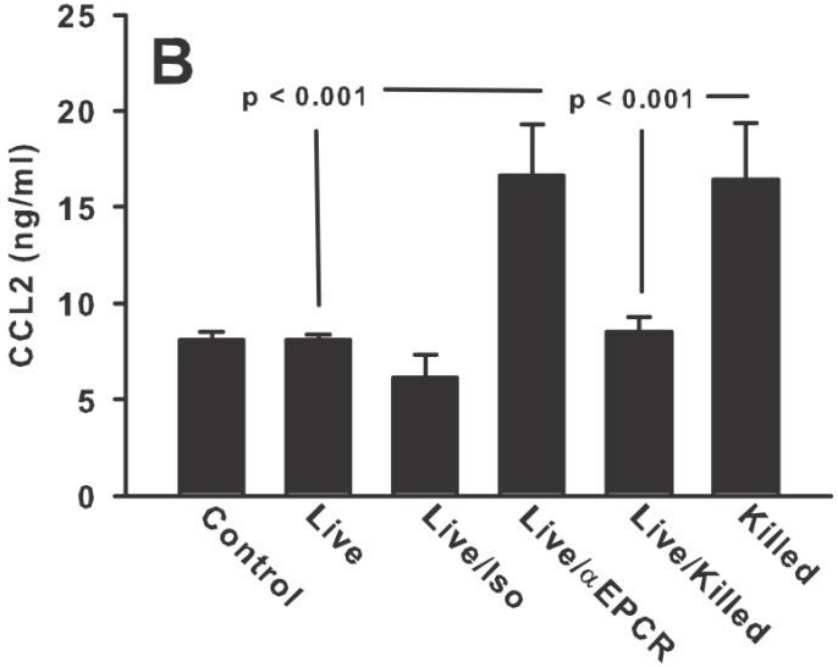

B
CCL2 (ng/ml)
25
20
15
10
5
0
p < 0.001
p < 0.001
Control
Live
Live/Iso
Live/αEPCR
Live/Killed
Killed

FIGURE 18. Antibodies to PAR-1 do not alter the proinflammatory response of HUVEC to *F. tularensis* LVS.

HUVEC were incubated for 24 h with 20% medium alone (Control), living *F. tularensis* LVS at a MOI of ~200 (Live), a 1:10 dilution of heat-killed LVS (Killed), 10 µg/ml of human alpha thrombin (Thrombin), or the PAR-1 blocking antibodies WEDE15 and H-111 at 25 and 20 µg/ml, respectively (Blocking Antibodies). HUVEC were also stimulated with the living bacteria combined with heat-killed LVS (Live/Killed) or the blocking antibodies (Live/Antibodies), whereas other HUVEC were exposed to thrombin with the blocking antibodies (Thrombin/Antibodies). Antibodies were added to the HUVEC 1 h before challenge with *F. tularensis* and remained present throughout the assay. Amounts of CCL2 in the conditioned media were determined by ELISA. Bars represent the means ± SD of triplicate samples. This graph is representative of two experiments with similar results.

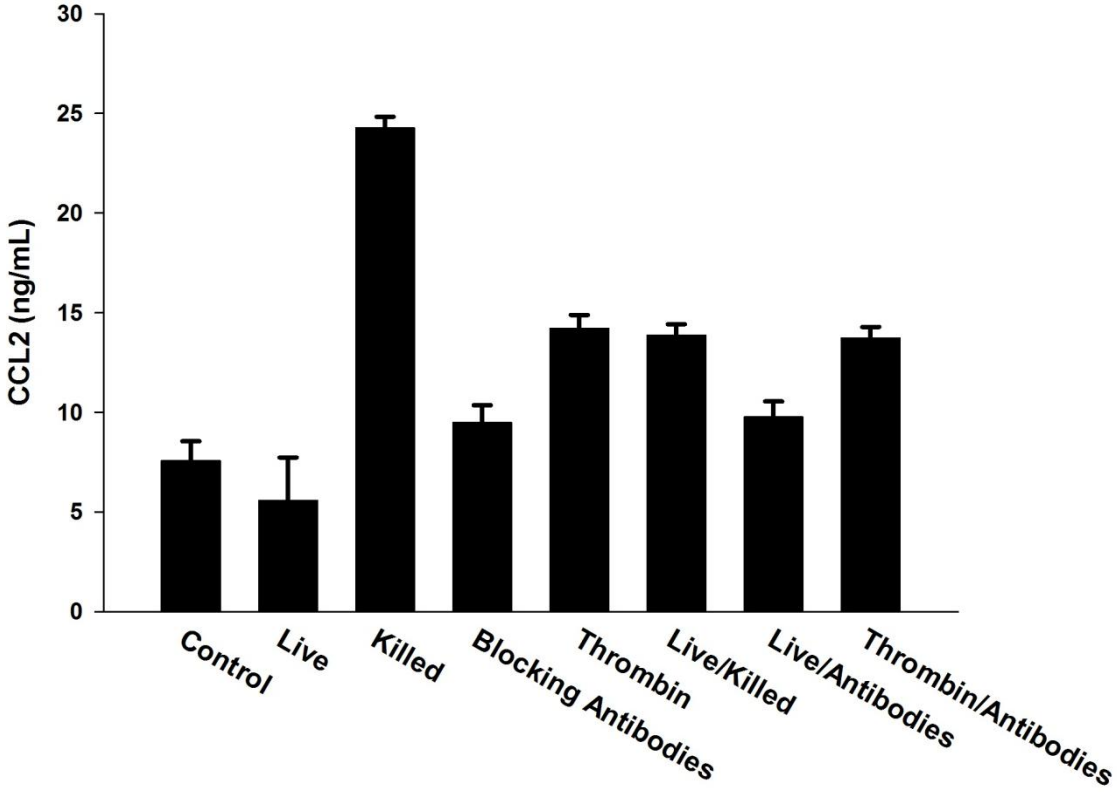

CCL2 (ng/mL)
30
25
20
15
10
5
0
Control
Live
Killed
Blocking Antibodies
Thrombin
Live/Killed
Live/Antibodies
Thrombin/Antibodies

FIGURE 19. Less soluble EPCR is released from HUVEC exposed to live *F. tularensis* LVS.

HUVEC were incubated with cell medium (Control), live LVS at a MOI of ~200 (Live), a 1:10 dilution of heat-killed LVS (Killed), or the live and heat-killed LVS combined (Live/Killed) for 24 h. The conditioned media were collected and assayed by ELISA for soluble EPCR. Bars represent the means ± SD of triplicate samples. ***, $P < 0.001$ when compared to the heat-killed bacteria. This assay was conducted three times with similar results.

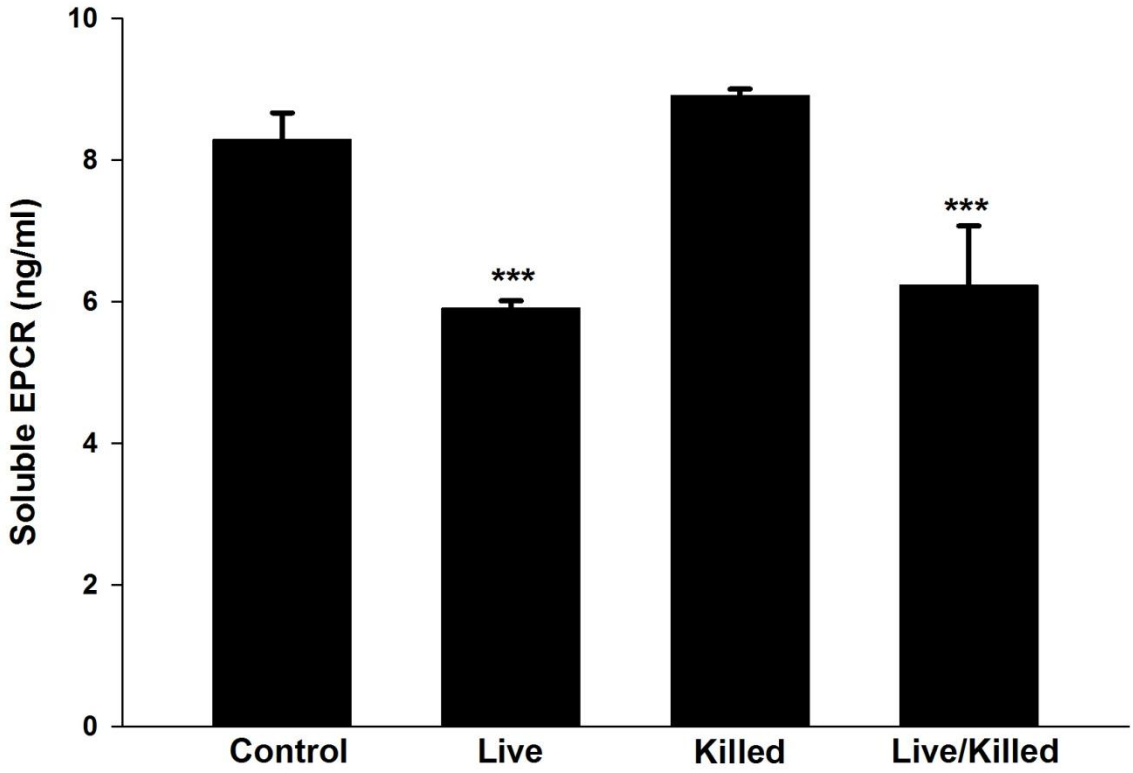

Soluble EPCR (ng/ml)
10
8
6
4
2
0
***
***
Control
Live
Killed
Live/Killed

FIGURE 20. Endothelial cells incubated with *F. tularensis* have less surface-expressed EPCR.

HUVEC were incubated with cell medium (Green), live LVS at a MOI of ~200 (Yellow), a 1:10 dilution of material released from heat-killed LVS (Blue), or the combination of live and killed LVS (Pink). After 24 h, the cells were harvested and stained for the EPCR. Surface expression of the EPCR was measured by flow cytometry.

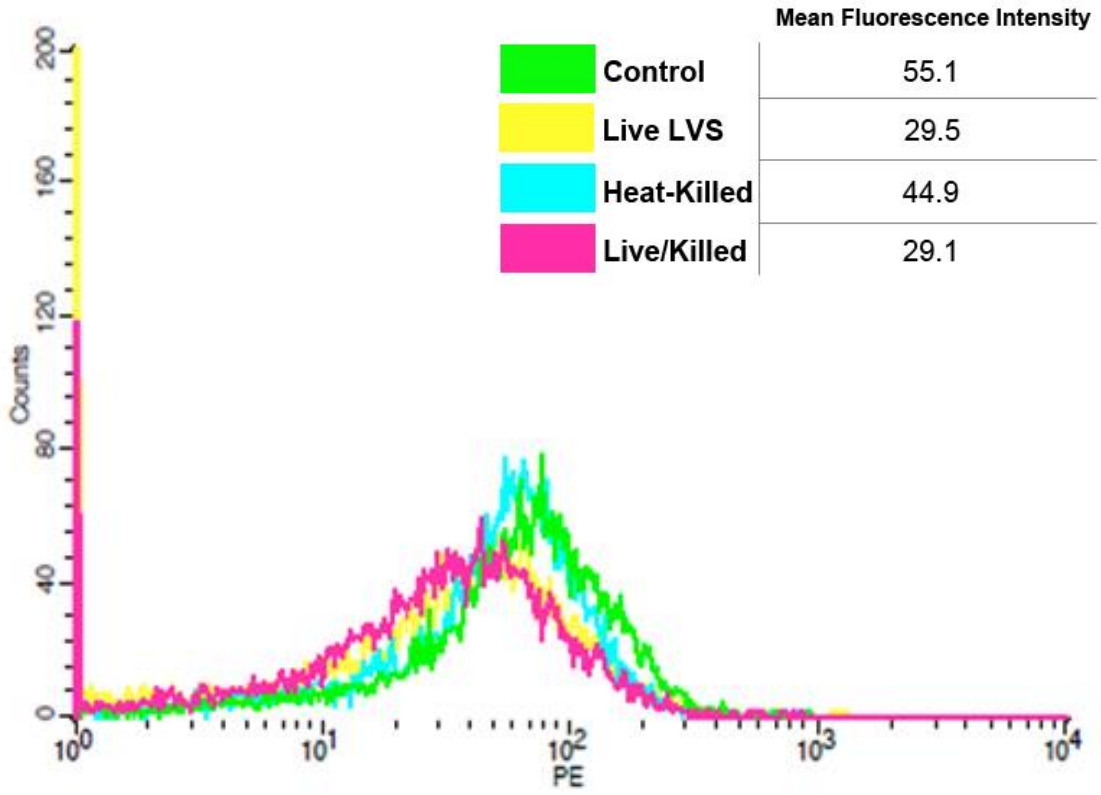

Mean Fluorescence Intensity
Control
55.1
Live LVS
29.5
Heat-Killed
44.9
Live/Killed
29.1
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
PE

FIGURE 21. The EPCR appears to be internalized by endothelial cells exposed to live *F. tularensis* LVS.

HUVEC were incubated for 2 h with 20% medium (Control), *F. tularensis* LVS (Live), or heat-killed LVS (Killed). Some of the endothelial cells were permeabilized prior to being fixed and stained for the EPCR (Red). The HUVEC nuclei were stained with DAPI (Blue) and, for some samples, the plasma membrane was stained with fluorescently-tagged wheat germ agglutinin (Green). In all experiments, HUVEC were deposited on slides by cytospin centrifugation and then visualized on a Nikon E600 microscope, and images were processed using Autoquant 2.0.

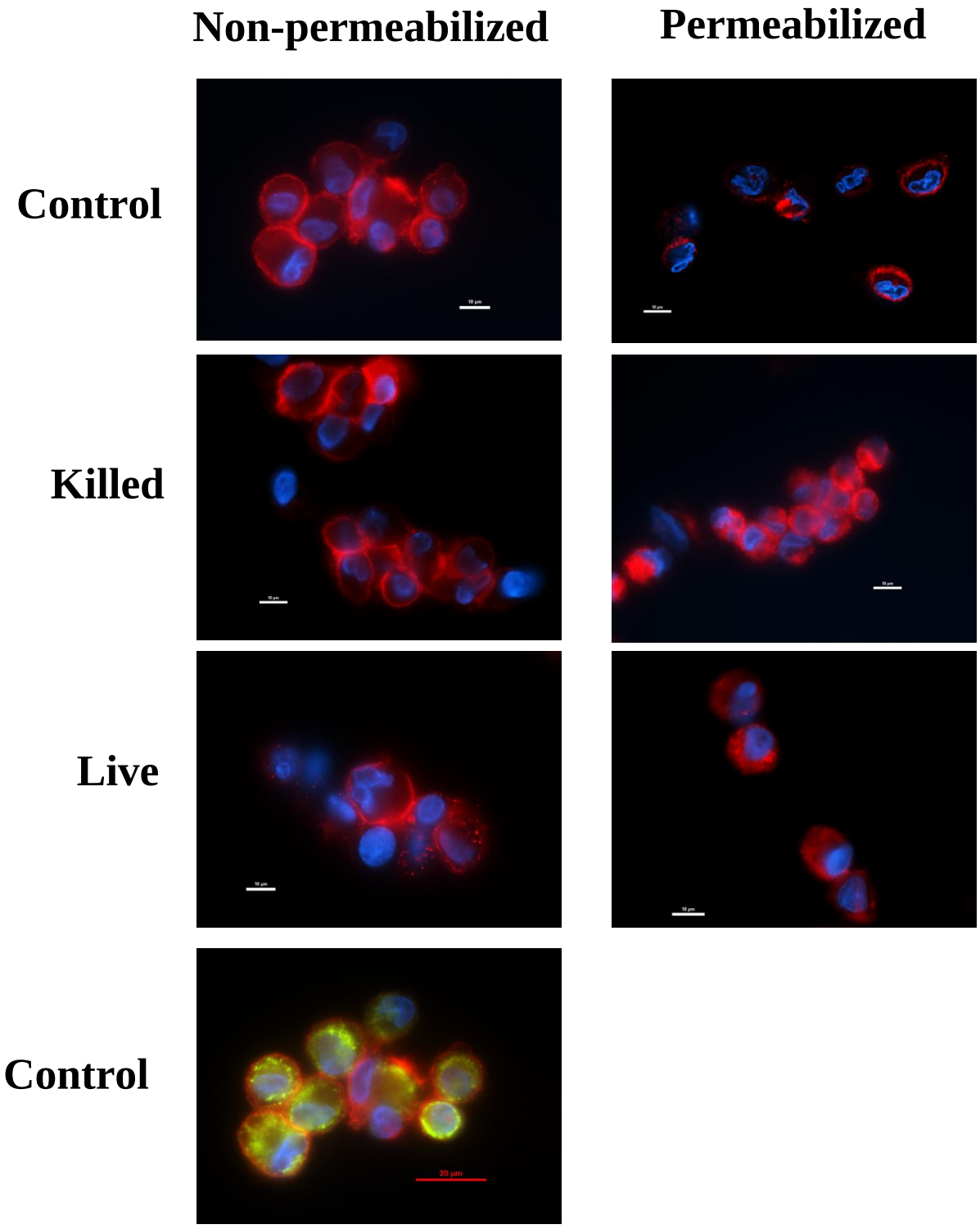

Non-permeabilized
Permeabilized
Control
Killed
Live
Control

FIGURE 22. Surface staining for the EPCR is drastically reduced when endothelial cells are exposed to *F. tularensis.*

HUVEC were incubated for 2 h with 20% medium (A), *F. tularensis* LVS (B), or GFP-expressing *F. tularensis* LVS (C; Green). The endothelial cells were permeabilized prior to being fixed and stained for the EPCR (Red), and the nuclei were stained with DAPI (Blue). For A and B, the image borders depict a cross-section of the endothelial cells showing that the red staining appears to go through the entire cell, indicating that the EPCR is internal. Panel C is a 100X image depicting LVS associated with the HUVEC, as well as the internalized EPCR. In all experiments, HUVEC were deposited on slides by cytospin centrifugation and then visualized on a Nikon E600 microscope, and images were processed using Autoquant 2.0.

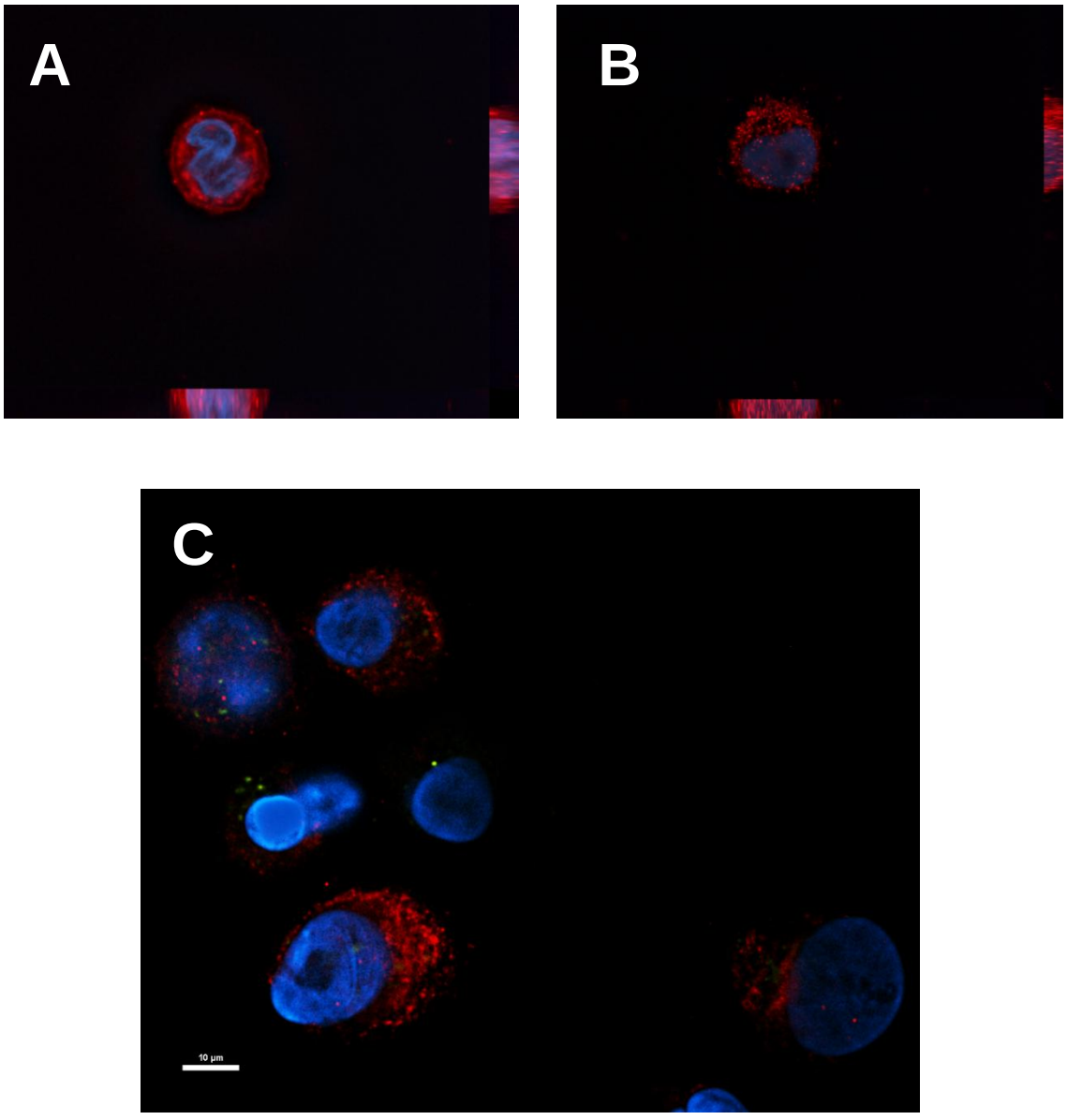
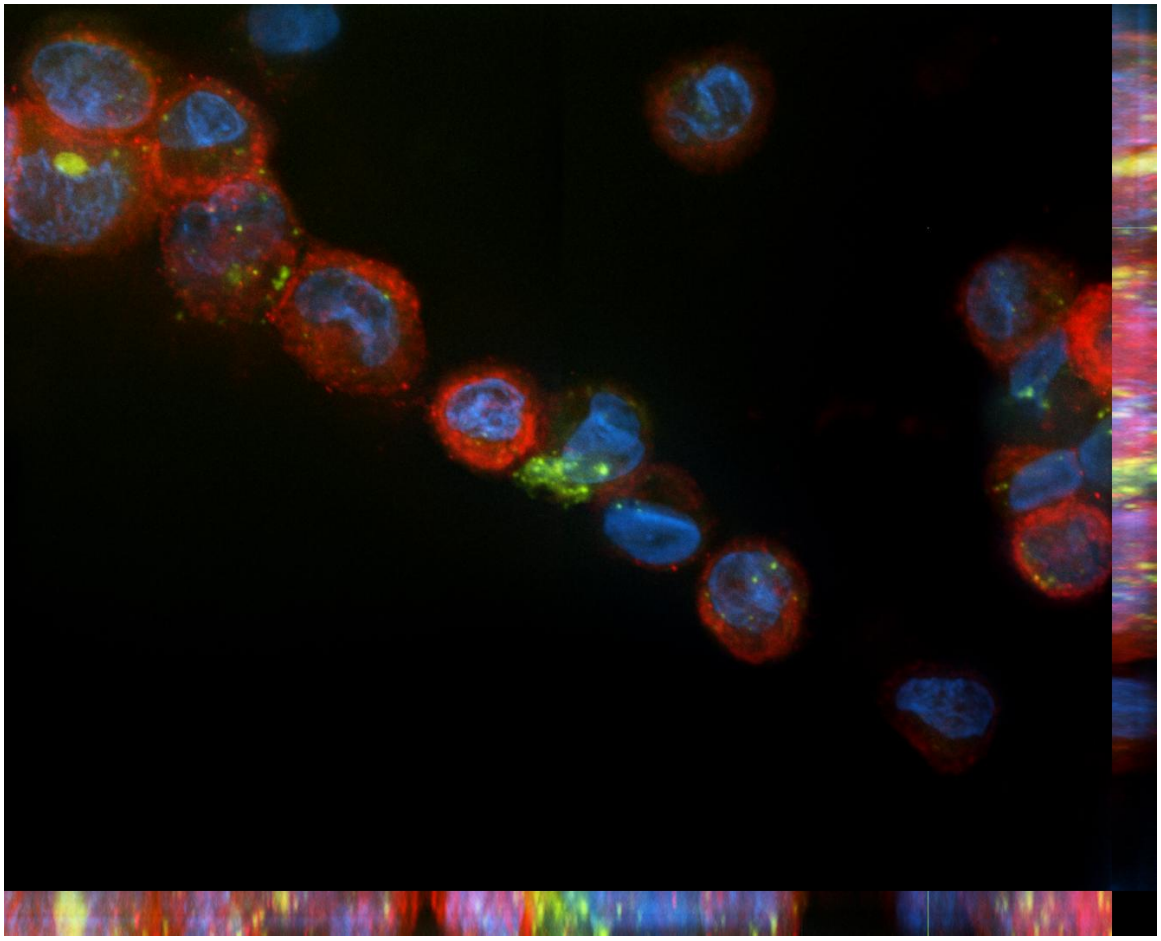

A
B
C
10 µm

FIGURE 23. *F. tularensis* may be rapidly internalized by endothelial cells.

HUVEC were incubated for 2 h with GFP-expressing *F. tularensis* LVS (Green), permeabilized, fixed, and stained for the EPCR (Red). The HUVEC nuclei were also stained with DAPI (Blue). HUVEC were deposited on slides by cytospin centrifugation and then visualized on a Nikon E600 microscope, and images were processed using Autoquant 2.0. The image is a composite of different Z-axes combined into a 3D image and flattened for presentation. The borders depict a cross-section of the endothelial cells from this composite and show that the red and green staining appears to go through the entire cell, suggesting that the EPCR and bacteria are internal.



III. Characterization of Endothelial Cell Signaling Pathways Disrupted by *F. tularensis*

It is generally understood that *F. tularensis* manipulates the host to down-regulate the proinflammatory response of a number of cell types (91, 97-99, 106, 185). What is less understood are the pathways that the bacteria alter to gain this end. Some studies have pointed to the bacteria blocking activation of NF-κB and MAPK pathways (101, 186). Other groups have implicated activation of the inflammasome *in vitro* and *in vivo* (187-189). These last studies, however, were conducted using *F. tularensis* spp. *novicida*, which is noninfectious in humans (75) and activates endothelial cells (data not shown). The gap in knowledge regarding where these proinflammatory pathways are disrupted is magnified when moving from leukocytes to endothelial cells, which have been little-studied in the context of tularemia. As such, the following experiments were conducted to elucidate the mechanisms underlying inhibition of endothelium by *F. tularensis*.

A. Microarray Analysis Highlights a Number of Endothelial Proteins Affected by *F. tularensis* LVS

In order to gain a broader picture of the endothelial cell processes affected by *F. tularensis*, HUVEC were stimulated with cell medium, live LVS, heat-killed LVS, or a combination of live and heat-killed bacteria for 8 h, and the RNA from these cells was then subjected to microarray analysis. Using WebGestalt (Web-based gene set analysis toolkit), created by Vanderbilt University, the microarray results were grouped into functional pathways. Of interest, a number of genes involved in TLR signaling and activation of NF-κB and AP-1 were affected by the presence of the live LVS. Notably, TGFβ-activated kinase1/MAP3K7

binding protein 3 (TAB3) was the gene most highly down-regulated by the live bacteria as compared to the control cells (34.4-fold). TAB3 interacts with TAB2, TRAF6, and TAK1 to activate NF-κB signaling (Fig. 24) (190-192). However, TAB3 transcript levels were not altered to a significant degree under the other conditions. In particular, there was no difference in levels in HUVEC incubated with live bacteria vs. live and killed bacteria combined. This result argues against a role for TAB3 in suppression.

Of greater interest are two genes that were upregulated by the live LVS alone and in the presence of killed organisms. These encoded mothers against decapentaplegic homolog 7 (Smad7) and prolyl hydroxylase 3 (PHD3). Smad7 interacts with TAB1, which blocks the recruitment of TAK1, thereby inhibiting the activation of NF-κB (193). PHD3 is involved in the negative regulation of hypoxia-inducible factor-1 alpha (HIF1α). Under normoxic conditions, PHD3 hydroxylates HIF1α, thus targeting it for proteosomal degradation (194). Independent of its enzyme activity, PHD3 has recently been shown to bind IKKβ, thereby blocking its phosphorylation. This interaction inhibits the activation of NF-κB in colorectal cells, and it has the potential to also block activation of AP-1 (195, 196).

Genes that could be transcribed downstream of activation of NF-κB or AP-1 include those encoding CCL2, CXCL8, and IL-6. CXCL8 was the most highly up-regulated gene when HUVEC were exposed to the heat-killed bacteria alone (19.7-fold). However, addition of live organisms did not reduce the levels significantly. IL-6, a proinflammatory cytokine, also had increased transcript levels in endothelial cells in response to the heat-killed bacteria. Moreover, expression of IL-6 was down-regulated when the live bacteria were also present. Expression of CCL2, the other chemokine of interest in this study, was not significantly affected by the bacteria at the transcriptional level under any condition. Table 1 and Figure 24 summarize the genes that

were up- or down-regulated in endothelial cells, comparing cells exposed to the heat-killed organisms alone or in combination with the live bacteria. The genes noted are those that would be expected to have an impact on the proinflammatory response.

Real-time RT-PCR was performed and confirmed a 92-fold and 4-fold up-regulation of PHD3 and Smad7 levels, respectively, by the combination of the live and killed bacteria vs. the killed LVS alone. Real-time RT-PCR also confirmed down-regulation of the transcript for IL-6 by further addition of live bacteria (5-fold down-regulation vs. heat-killed organisms alone). Moreover, it revealed a substantial decrease in CXCL8 transcripts when live organisms were added to the killed bacteria (128-fold upregulation by heat-killed vs. 43-fold increase by live with heat-killed). We also probed expression of the genes for CCL2 and the EPCR, even though they were not altered at the time-point used for the microarray. The live bacteria alone down-regulated the CCL2 gene transcript 6-fold compared to control cells. Additionally, when combined with the heat-killed bacteria, living LVS reduced CCL2 transcript levels 2.6-fold in comparison to the heat-killed LVS alone. On the other hand, there was no significant change in levels of the EPCR transcript under any of the conditions.

B. Knockdown of PHD3 by siRNA Does Not Alter the Response of Endothelial Cells to *F. tularensis*

To follow up on results from the microarray analysis, we investigated involvement in suppression of PHD3, a molecule that can block activation of NF-κB and is highly up-regulated in HUVEC by the live bacteria. Knockdown of 50-75% of PHD3 transcript in HUVEC was achieved using 100 nM siRNA but had no effect on the amount of CCL2 secreted by the

endothelial cells in response to the live LVS. The experiment was repeated using 100, 150, and 200 nM PHD3 siRNA (Fig. 25A). Knockdown of as much as 80% was achieved, compared to untransfected HUVEC stimulated with live bacteria. However, there was still no change in the amount of CCL2 secreted by the HUVEC in the presence of the live bacteria (Fig. 25B). These results argue against a role for PHD3 in suppressing the proinflammatory activation of HUVEC by *F. tularensis*.

C. *F. tularensis* LVS inhibits activation of c-Jun but not NF-κB

NF-κB is an important transcription factor that regulates the expression of a multitude of proinflammatory genes, including those encoding CXCL8 and CCL2 (197, 198). The ability of *F. tularensis* to inhibit the activation of NF-κB has been demonstrated in macrophages (101), so we wanted to test whether the same was true for endothelial cells. Using an ELISA-based assay, where activated NF-κB binds an immobilized oligonucleotide followed by detection of the p65 subunit with a monoclonal antibody, we determined relative activation levels at early (10, 15, 20, and 60 min) and later (3.5, 4, 4.5, 5, 6, 8, 18, and 24 h) time points. The greatest differences in activation were seen at 15 min (data not shown), 4.5 h (Fig. 26A), and 8 h (data not shown). In general, the live bacteria activated substantially less NF-κB than did the heat-killed organisms. However, when compared to control cells, HUVEC incubated with live LVS had significantly more activated NF- κB. Moreover, live LVS did not block activation of NF-κB in response to the heat-killed bacteria (Fig. 26A).

AP-1 regulates transcription for many of the genes of interest that came to light as a result of the microarray, including caspase-1, IL-1β, and CCL7. Furthermore, according to the

SABiosciences transcription factor database, DECODE, there are 22 AP-1 binding sites in the promoter of the gene encoding CCL2 as opposed to eight for NF-κB (www.sabiosciences.com/chipqpcrsearch.php). As result, we also looked at the activation of AP-1 by *F. tularensis* in HUVEC. These experiments were conducted in the same manner as the NF-κB assays, except that time points were limited to 4.5 and 7 h. Of the six AP-1 transcription factors tested, only c-Jun was consistently down-regulated by the live bacteria, both alone and when combined with heat-killed organisms (Fig. 26B). Together, these data suggest that *F. tularensis* targets both the AP-1 and NF-κB signaling pathways, yet living *F. tularensis* suppresses only activation of c-Jun in response to the heat-killed bacteria. Given this finding, it seems more likely that the c-Jun signaling pathway is involved in blockade of the proinflammatory response of endothelial cells *F. tularensis*.

TABLE 1. Selected endothelial cell gene transcripts with altered expression after exposure to live and heat-killed *F. tularensis* LVS as compared to heat-killed *F. tularensis* alone

GENE	FOLD CHANGE	PRODUCT
DATF1	-3.25	Death Inducer-Obliterator-1: Pro-apoptotic factor
CASP1	-2.1	Caspase 1: cleaves and activates IL-1β
IL1B	-3.48	IL-1β: proinflammatory cytokine
CXCL10	-4.0	CXCL10: proinflammatory chemokine; recruits macrophages, dendritic cells, and T cells
RGS4	-3.48	Regulator of G-protein Signaling 4: GTPase activating protein (GAP); acts on G_i and G_q G-coupled receptors such as PAR-1
CCL7	-2.3	CCL7: proinflammatory chemokine; recruits monocytes
IL6	-3.25	IL-6: proinflammatory cytokine; mediates fever and acute phase response
SMAD7	+2.8	Mothers against decapentaplegic homolog 7: interacts with TAB1 to block recruitment of TAK1, thereby inhibiting the activation of NF-κB
EGLN3/PHD3	+17.1	Prolyl hydroxylase : negative regulator of HIF1α; blocks phosphorylation of IKKβ which inhibits activation of NF-κB
TRAF1	-2.46	TNF receptor-associated factor 1: complexes with TRAF2 to form a complex that activates proinflammatory signaling; required for TNF-alpha-mediated activation of NF-κB

FIGURE 24. Selected endothelial cell gene transcripts with altered expression after exposure to live *F. tularensis* LVS.

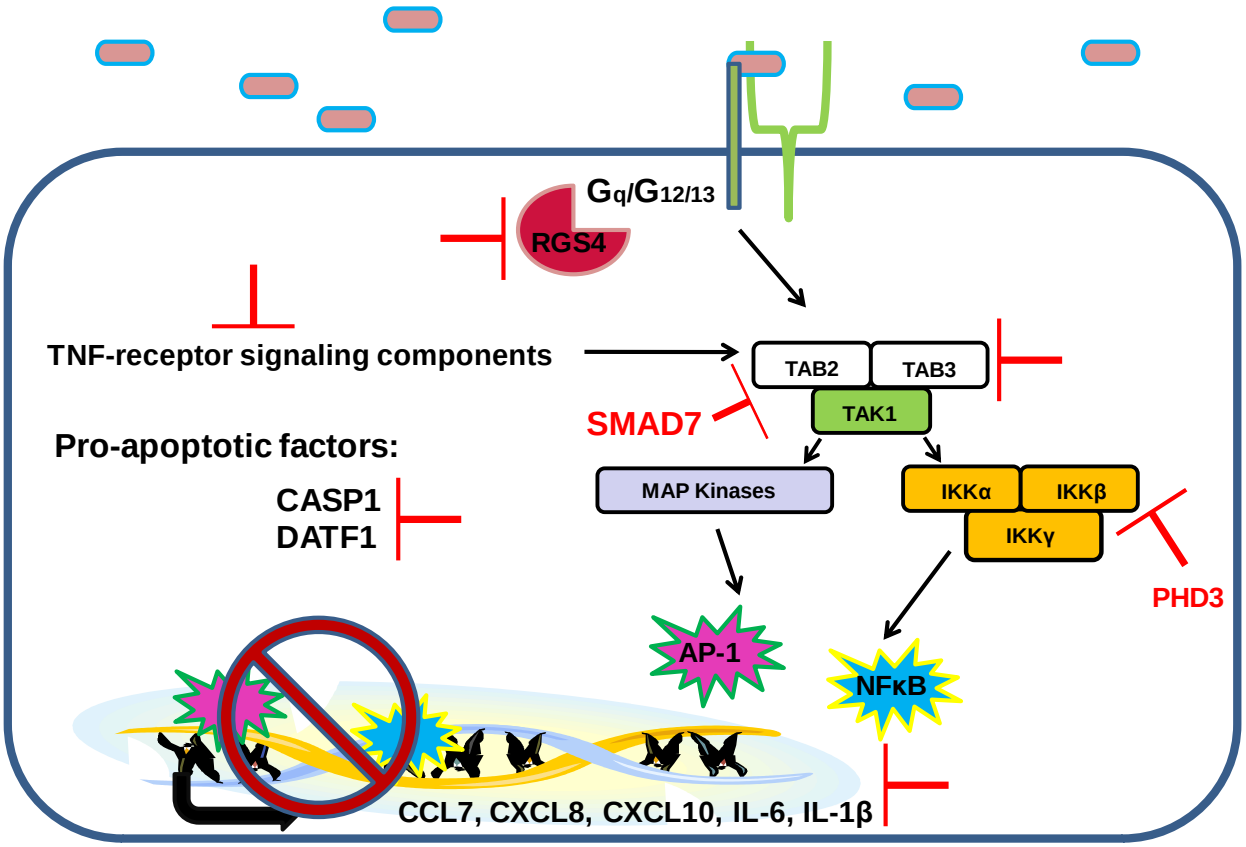
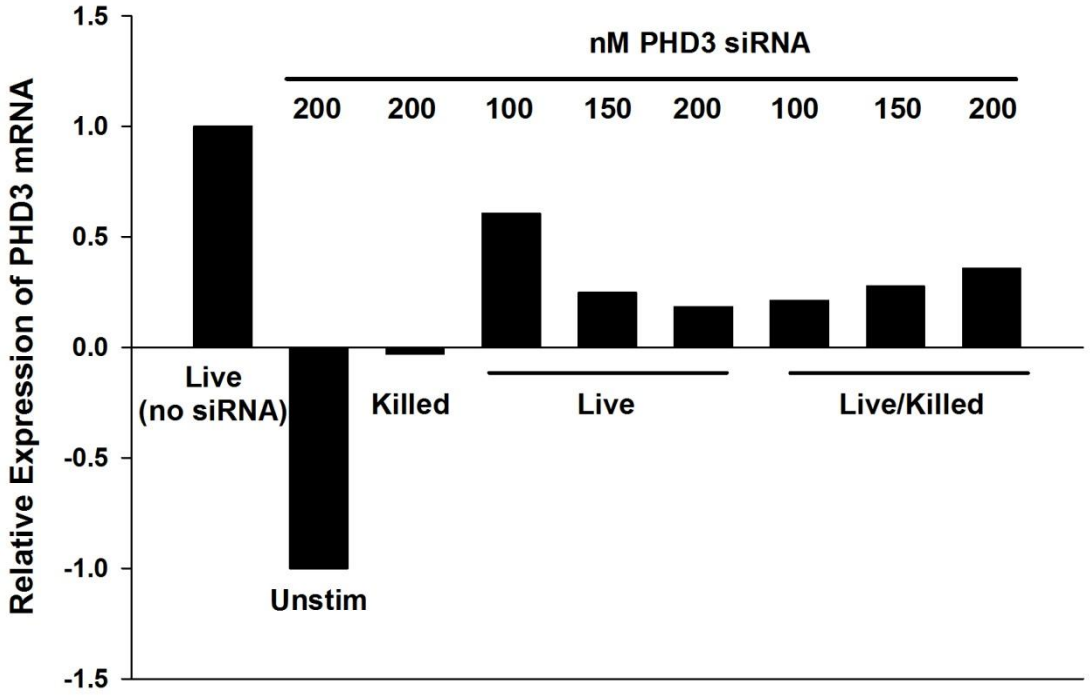


FIGURE 25. Knockdown of PHD3 with siRNA does not alter the proinflammatory response of HUVEC exposed to *F. tularensis***.**

HUVEC were transfected with 100, 150, or 200 nM siRNA for PHD3. After 48 h, the HUVEC were exposed to cell medium (Unstim), live LVS (Live), a 1:10 dilution of heat-killed LVS (Killed), or both live and killed LVS (Live/Killed). (A) At 24 h, RNA was isolated and used to make cDNA for real-time RT-PCR to measure the amount of PHD3 transcripts. The relative fold change of the transcripts was calculated with respect to the amount of PHD3 transcript in unstimulated cells. The data then were normalized to the Live no siRNA sample, which showed the greatest increase in PHD3 transcripts. (B) The corresponding conditioned media were assayed for CCL2 by ELISA.


nM PHD3 siRNA
200
200
100
150
200
100
150
200
Relative Expression of PHD3 mRNA
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Live
(no siRNA)
Unstim
Killed
Live
Live/Killed

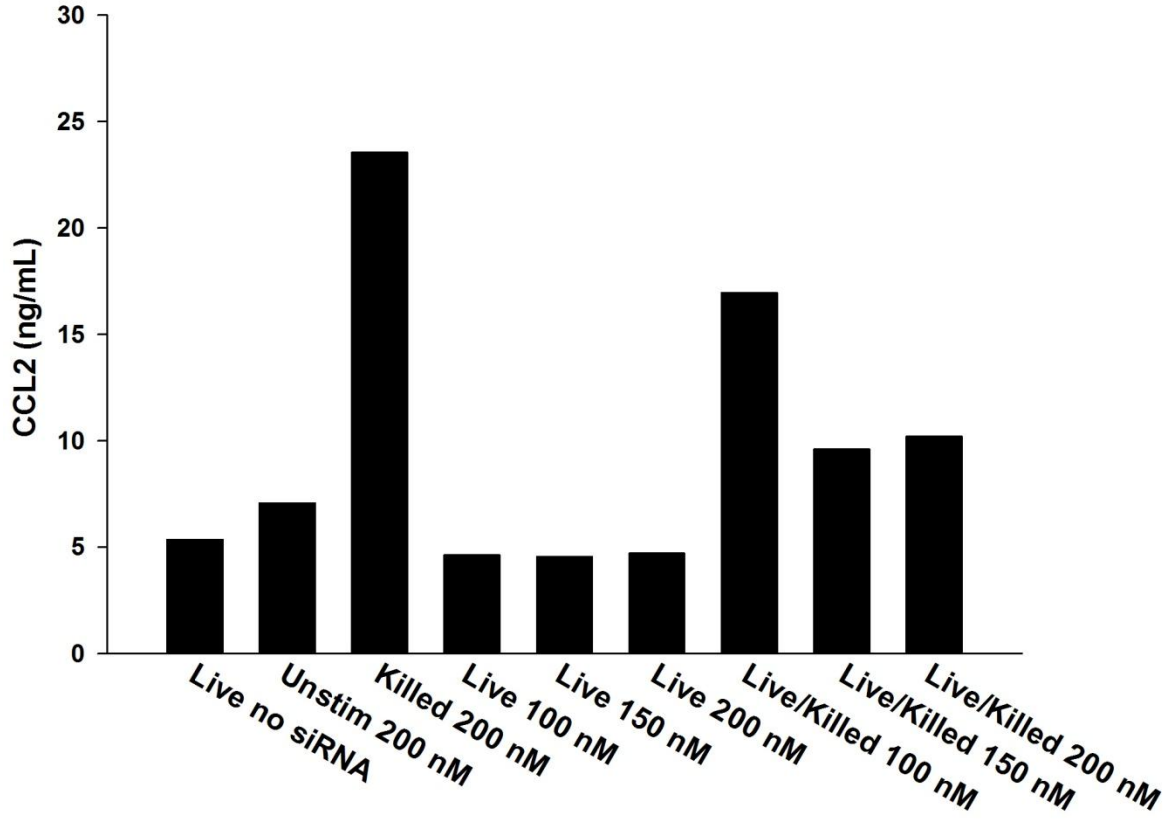
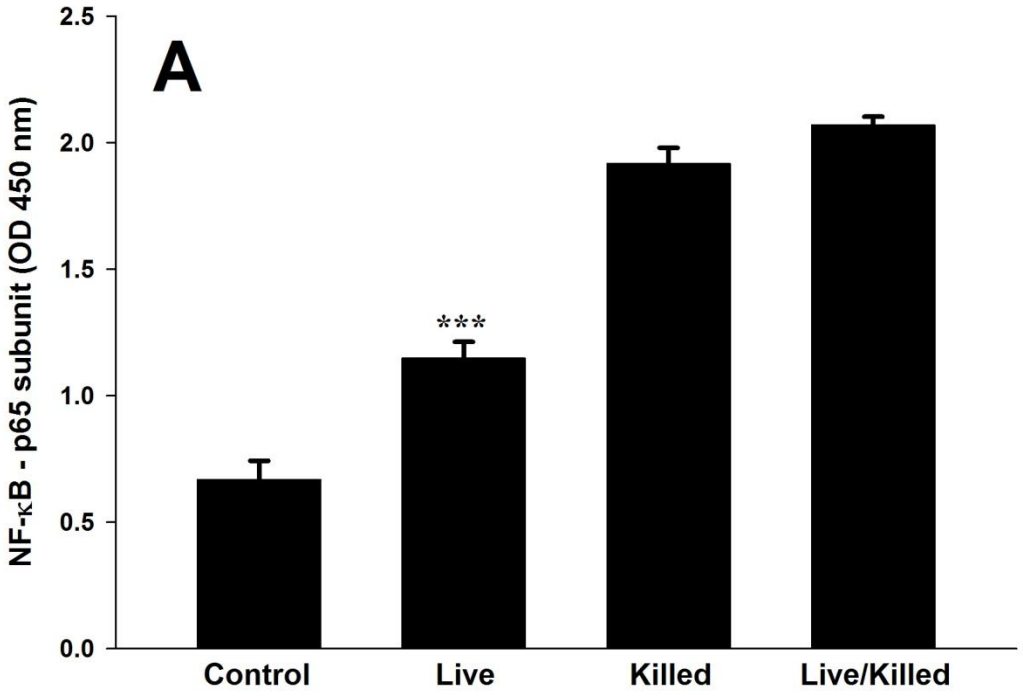
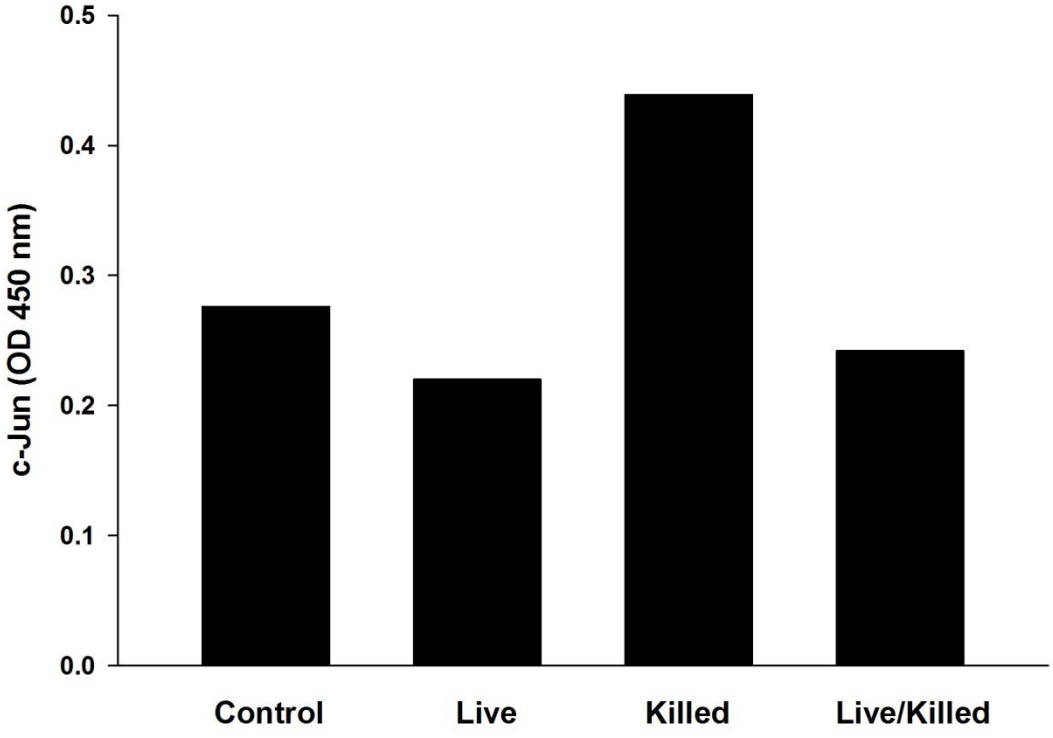

CCL2 (ng/mL)
30
25
20
15
10
5
0
Live no siRNA
Unstim 200 nM
Killed 200 nM
Live 100 nM
Live 150 nM
Live 200 nM
Live/Killed 100 nM
Live/Killed 150 nM
Live/Killed 200 nM

FIGURE 26. *F. tularensis* LVS activates AP-1 and NF-κB transcription factors differently.

HUVEC were incubated with 20% medium (Control), living *F. tularensis* LVS at a MOI of ~200 (Live), heat-killed LVS (Killed), and living with killed LVS (Live/Killed) for 4.5 h. Nuclei of the endothelial cells were collected, and total protein for each sample was assessed by Bradford assay. Ten µg of protein for each sample were used to detect activation of NF-κB (A) or c-Jun (B) using an ELISA-based assay. In Panel A, bars represent the means ± SD of triplicate samples and ***, $P < 0.001$ when compared to the heat-killed organisms. In Panel B, each bar represents a single sample. These data are representative of five assays for each transcription factor.


A
NF-κB - p65 subunit (OD 450 nm)
2.5
2.0
1.5
1.0
0.5
0.0
***
Control
Live
Killed
Live/Killed


c-Jun (OD 450 nm)
0.5
0.4
0.3
0.2
0.1
0.0
Control
Live
Killed
Live/Killed

DISCUSSION

I. *F. tularensis* Inhibits Proinflammatory Activation of Endothelial Cells

Throughout its life cycle in the mammalian host, *F. tularensis* evades immune detection and clearance. Furthermore, this bacterium often proves lethal before an adaptive immune response can be mounted (79, 199). This rapid lethality makes deciphering the innate immune response to *F. tularensis* imperative for understanding the pathogenesis of tularemia. During the course of infection, *F. tularensis* suppresses the proinflammatory response normally triggered by cells of innate immunity. Upon exposure to a mammalian host, the bacteria invade macrophages and dendritic cells (106, 136, 200), which can then serve as a protected niche for growth and dissemination. Once inside, living *F. tularensis* does not trigger the secretion of the proinflammatory cytokines IL-1β, TNFα, and IL-6 by dendritic cells (91, 106), or TNFα and IL-1β by macrophages (99, 101). Moreover, mice exposed to *E. coli* LPS after infection with a low dose of the Schu S4 strain have near-basal expression of CD86, a co-stimulatory molecule, on their dendritic cells and reduced recruitment of monocytes to the site of infection (111). *F. tularensis* also inhibits the secretion of cytokines by dendritic cells or macrophages exposed *in vitro* to secondary stimuli, such as *E. coli* LPS, zymosan, and bacterial lipopeptides. Blocking the secretion of proinflammatory cytokines is an active process by *F. tularensis,* as the bacteria must be alive to inhibit the stimulation of these cells (91, 99, 106). Indeed, only the living LVS can suppress the respiratory burst of polymorphonuclear leukocytes upon challenge with zymosan, a known activator of the oxidative burst (94). As demonstrated by our data, endothelial cells exposed to living *F. tularensis* had little to no proinflammatory response, while those incubated

with heat-killed bacteria were triggered to secrete CCL2 (Fig. 3). This inhibitory effect was the same for the attenuated LVS and the virulent Schu S4 strain (Fig. 4), indicating that it is a conserved trait across subspecies. When *F. tularensis* was inactivated, but intact, it also did not elicit secretion of CCL2 by HUVEC; however, only living *F. tularensis* blunted the activation of the endothelial cells by heat-killed bacteria (Fig. 12). Moreover, this inhibitory effect required synthesis of new proteins (Fig 14), indicating that immune suppression of endothelial cells by *F. tularensis* is an active process similar to what is seen in macrophages and dendritic cells (91, 99, 101).

Since the suppressive effect on HUVEC was dose-dependent and occurred rapidly (Fig. 5, 7, and 8), there could be a concern that the endothelial cells were globally compromised by incubation with *F. tularensis*. However, the HUVEC remained responsive to other stimuli, as living *F. tularensis* did not inhibit secretion of CCL2 due to *E. coli* LPS (Fig. 6). These data rule out cell death as well as global inhibition of function as explanations for the inhibitory effect. Additionally, bacteria contained in a microporous insert lost the ability to suppress the activation of HUVEC (Fig. 10), an observation that eliminates depletion of nutrients by *F. tularensis* as a cause of the phenomenon.

While the outcome in suppressing the secretion of proinflammatory cytokines by host leukocytes and endothelial cells is similar, the mechanisms may be different. *F. tularensis* LVS lacking the gene *iglC* can no longer inhibit the proinflammatory response of macrophages (104). This gene is a part of the *Francisella* pathogenicity island and is necessary for *F. tularensis* to escape from the phagosome and grow within macrophages (105, 200, 201). This observation suggests that suppressing the activation of these host cells requires intracellular replication of the bacteria. However, unlike macrophages, HUVEC do not support replication of *F. tularensis*

LVS. Our previous results show that after 24 h of co-incubation at an initial MOI of 180, fewer than one viable intracellular LVS organism was recovered for every 50 HUVEC (97). In addition, the Δ*iglC* LVS mutant inhibited activation of endothelial cells by heat-killed LVS to the same degree as the wild-type bacteria (Fig. 15). As such, the suppressed proinflammatory of endothelial cells response is not dependent on intracellular replication of the bacteria. Furthermore, as discussed above, macrophages and dendritic cells infected with *F. tularensis* are refractory to *E. coli* LPS (91, 99, 106, 111), whereas HUVEC remained responsive (Fig. 6). In sum, it appears that the suppression of activation of endothelium by *F. tularensis* may be more specific and the mechanism distinct when compared to what has been observed in leukocytes.

Numerous bacterial species have been shown to modulate the host immune response by secretion of virulence factors. For instance, *Yersinia pestis* secretes various *Yersinia* outer proteins that inhibit the release of IFNγ and TNFα by macrophages, as well as their activation of NF-κB (202). Likewise, ESAT-6, a protein secreted by the ESX-1 system of *Mycobacterium tuberculosis*, interacts directly with TLR2 on host macrophages to dampen the proinflammatory response (203). Regarding endothelial cells, *Staphylococcus aureus* produces β-hemolysin, which inhibits endothelial production of CXCL8, a chemoattractant for neutrophils (204). These bacteria manipulate the host via secretion systems that are not known to exist in *F. tularensis* (127, 130, 138). Nevertheless, a factor shed from *F. tularensis* recently has been implicated in the suppression of activation of dendritic cells (91). We therefore investigated the possibility that a soluble factor might be responsible for inhibiting the proinflammatory activation of endothelial cells. However, when living *F. tularensis* organisms were separated from HUVEC by a microporous insert, they no longer blocked the secretion of CCL2 triggered by the heat-killed bacteria (Fig. 10). In addition, neither pre-exposure to the living bacteria nor conditioned

medium from HUVEC exposed to *F. tularensis* was sufficient to block the activation of endothelial cells by the heat-killed bacteria (Fig. 9 and 11). Together, these results indicate that it is unlikely that the suppressive effect is exerted by a secreted factor, either bacterial or host-derived, released into the extracellular environment**.** As such, the mechanism for suppression of endothelial cells by *F. tularensis* remains unique from what has been observed in dendritic cells (91). Rather, it seems probable that *F. tularensis* mediates the proinflammatory response via a bacterial outer membrane component that binds to a host cell receptor or a factor that is induced and secreted only upon contact with the host cell.

F. tularensis possesses several characterized outer-membrane structures, including Type IV pili that function in host cell adhesion, and biofilm formation (131-133). These outer-membrane structures are required for virulence in *F. tularensis* (132), yet LVS mutants with deletions of *pilT*, *pilF*, or *pilE4* retained the ability to suppress activation of endothelial cells in response to heat-killed LVS. In addition, a LVS mutant lacking TolC, a component of the Type I secretion system, also blunted secretion of CCL2 by HUVEC (Fig. 15). In contrast, the Δ*tolC* LVS elicits secretion of this chemokine by infected macrophages at greater levels than wild-type LVS (140). These data further support the idea that *F. tularensis* uses different mechanisms to block activation of endothelial cells vs. leukocytes.

Although the LVS Δ*tolC* mutant prevented endothelial production of CCL2, it did trigger HUVEC to secrete greater amounts of CXCL8 than did the wild-type strain (140). One explanation of these seemingly contradictory results could be differential regulation of these genes by endothelial cells. It is possible that TolC plays a greater role in dampening the production and/or release of CXCL8 than CCL2. The genes for CXCL8 and CCL2 are both regulated by NF-κB and AP-1 transcription factors but in a subunit-specific manner (205-207).

In this way, transcription of CXCL8 could be induced by a particular NF-κB dimer that does not efficiently promote transcription of CCL2. For instance, alprazolam, a tranquilizer, triggers production of CXCL8 but not CCL2, based on different NF-κB subunits being activated by the drug in a human glioblastoma cell line (208). Moreover, production of CCL2 and CXCL8 depend on different NF-κB dimers in corneal cells (205). This differential activation also appears to be cell-specific, where a dimer that activates CCL2 in one cell type may activate CXCL8 in another (205-207). In addition, the experiments measuring CXCL8 elicited by Δ*tolC* LVS did not include heat-killed LVS. As such, it is not known what the level of activation is in comparison to the heat-killed organisms or if the mutant suppresses secretion of CXCL8 by the heat-killed LVS. Regardless, the triggering of CXCL8, but not CCL2, in endothelial cells by the Δ*tolC* LVS potentially could be attributed to activation and/or suppression of different transcription factor subunits.

To further complicate matters, a Δ*tolC* mutant in the Schu S4 strain triggered secretion of both CCL2 and CXCL8 by endothelial cells to levels near those of heat-killed organisms (Fig. 16). As mentioned in the Results, the deletion of this gene may be more deleterious to the Schu S4 strain than to the LVS. Both the initial MOI and the number of Δ*tolC* bacteria enumerated from wells at the end of the assay were roughly half the number expected from the OD_{600} of the starting culture. Elevated numbers of dead bacteria present during the assay could explain the increase in CCL2 elicited by the Schu S4 Δ*tolC* strain. Alternatively, TolC may play a more prominent role in immune evasion in the virulent Schu S4 strain than for the attenuated LVS, perhaps highlighting a factor that distinguishes these strains.

Another protein that functions as a part of the drug-efflux machinery, AcrA, also played a role in modulating the proinflammatory response of endothelial cells. As shown in this work, a

Schu S4 strain lacking this gene activated greater secretion of chemokines by HUVEC than did the wild-type Schu S4 strain (Fig. 16). The effect of the Δ*acrA* Schu S4 strain was distinct from mutants lacking the other genes in this operon, as the Δ*acrB* and Δ*dsbB* strains behaved like wild-type bacteria. These data are in contrast to published results, where it was found that Δ*acrB* LVS and Δ*dsbB* Schu S4, but not Δ*acrA* Schu S4, are attenuated *in vivo* (143, 144). The lack of attenuation of the Δ*acrA* Schu S4 strain could indicate that suppression of endothelial cells is not involved in the pathogenesis of tularemia. However, these published studies did not look at chemokine levels or immune cell recruitment elicited by these strains. More importantly, virulence determinants must be considered in a broader context, rather than simply individually. Infection and immunity are complicated systems involving whole-pathogen and whole-host responses. AcrA may not be a major virulence factor but one of several that help *F. tularensis* tip the balance in favor of its successful infection of a host. Blunting the proinflammatory response of endothelial cells would almost certainly be helpful to *F. tularensis* as it disseminates. However, it may not be the most crucial piece of weaponry in its arsenal, considering the number of different cell types with which it must interact. Furthermore, AcrA has been shown to be a somewhat promiscuous protein, whereby the *E. coli* AcrA can functionally interact with a different efflux system in *Pseudomonas aeruginosa* (209, 210). Alternatively, given that the AcrA is a membrane fusion protein, there may be unregulated release of activating factors when it is deleted. Unrestricted secretion of such factors could also account for the phenotype seen with the Δ*acrA* strain. As such, the AcrA may have functions yet to be elucidated that are, perhaps, cell-type specific.

II. The Endothelial Protein C Receptor is Required by *F. tularensis* to Prevent Activation of Endothelial Cells

To further investigate putative outer-membrane interactions, we looked at host-cell receptors that could potentially be engaged by *F. tularensis* to render suppression. Since the endothelial cells were responsive to *E. coli* LPS in the presence of the LVS, it is doubtful that *F. tularensis* acts through TLR4. An alternative candidate was the EPCR. The EPCR is structurally similar to the CD1/MHC I family of antigen-presenting proteins with a cytoplasmic tail of only three amino acids (145). A number of the anti-inflammatory effects that aPC has on endothelial cells when it binds the EPCR are similar to what is observed when endothelial cells are exposed to *F. tularensis* (156-158).

Given the role of the EPCR in reducing inflammation, we assessed whether this receptor was involved in the suppression of endothelial activation by *F. tularensis.* Indeed, when the EPCR was blocked by a mAb, living *F. tularensis* elicited a strong proinflammatory response from HUVEC and no longer inhibited the activation of these cells by the heat-killed bacteria (Fig. 17). These results implicate a role for the EPCR during the course of infection by *F. tularensis.* Interestingly, EPCR occupancy by PC or aPC alters the response of endothelium to PAR-1 cleavage by thrombin from proinflammatory to anti-inflammatory, with reduced activation of NF-κB (Fig. 2) (161, 166), enhanced barrier function, and greater resistance to apoptosis. Ligation of the EPCR by PC or aPC also inhibits migration of human lymphocytes toward chemokines by a mechanism that involves the epidermal growth factor receptor but apparently not PAR-1 (150). Occupancy of the EPCR thus can alter the behavior of other receptors to effect anti-inflammatory changes, independently of the proteolytic activity of aPC. We attempted to assess the role of PAR-1 in our studies using blocking antibodies. Although the

antibodies did not affect the response of HUVEC to *F. tularensis*, we were unsuccessful in showing their efficacy with a positive control. At this point, therefore, the involvement of PAR-1 is not known.

Alternatively, *F. tularensis* may act on the EPCR independent of other host receptors. To further characterize this host-pathogen interaction, we investigated the fate of the EPCR after exposure to the LVS. It is known that in addition to the membrane-bound form of the EPCR, there is also a soluble form. The sEPCR binds PC and aPC, though its function is speculative (153). We determined that when endothelial cells were exposed to live *F. tularensis*, there was less sEPCR in the conditioned medium than in that of cells exposed to either medium or heat-killed organisms alone (Fig. 19). This lack of sEPCR in the medium could not be accounted for by increased retention at the endothelial cell plasma membrane, as surface amounts were also reduced in the presence of live *F. tularensis* (Fig. 20). Further work suggested that the EPCR is internalized by HUVEC exposed to living *F. tularensis* LVS and that the bacteria may be internalized as well (Fig. 21-23). Others have demonstrated endocytosis of the EPCR when bound to aPC (184); in the context of *F. tularensis*, perhaps internalization starts a signaling cascade leading to a diminished proinflammatory response by the endothelium. Interestingly, some receptors in the CD1 family, which are structurally similar to the EPCR, have bound glycolipids, which can be exchanged for pathogen-derived lipids when they are internalized by the presenting cell (147).

Recently, the EPCR has been shown to bind Factor VII/VIIa, which also cleaves PAR-1 (170). Binding of Factor VIIa to the EPCR prevents activation of PC, presumably by preventing PC from binding the EPCR, which facilitates its activation by thrombin. Surprisingly, as it functions in the procoagulant cascade, Factor VIIa bound to the EPCR yields a barrier-protective

response, similar to what is seen with anticoagulant PC/aPC. However, this response may be distinct from that elicited by aPC, as a catalytically inactive form of Factor VIIa does not block thrombin-induced disruption of the endothelial permeability barrier (211). While little is understood about the interaction between Factor VII/VIIa and the EPCR, it is proof-of-principle that the EPCR can bind ligands other than PC, perhaps including a factor from *F. tularensis*. From our data, we hypothesize that a component of *F. tularensis* binds the EPCR, and this somehow triggers the down-stream signaling that blocks the proinflammatory response of endothelial cells. It is tempting to speculate that at early time points, *F. tularensis* is endocytosed along with the EPCR. Intracellular environmental cues may then lead to secretion of virulence factors (Fig. 27). The release of effector molecules may involve the AcrA, which could then alter the host-cell response to *F. tularensis*, thus leading to the blunted proinflammatory response observed in these studies.

III. Endothelial Cell Proinflammatory Signaling Pathways are Disrupted by *F. tularensis*

It is known that *F. tularensis* prevents proinflammatory activation of a number of cell types. What is less well understood is how the bacteria exert this effect. Existing studies demonstrate that there is reduced activation of NF-κB and AP-1 in macrophages infected by *F. tularensis* (101). These transcription factors are master regulators of a multitude of genes including those for CCL2 and CXCL8. In accordance with the work of Telepnev et al. (101), we also found less activated p65 in endothelial cells incubated with the live LVS vs. the heat-killed bacteria. However, the live bacteria did not prevent activation of NF-κB in response to the heat-killed LVS (Fig. 26A). As such, this cannot be the only pathway responsible for inhibiting the proinflammatory response of endothelial cells; however, our results do not rule out a role for NF-κB completely. As mentioned in Part I of this discussion, different subunit dimers of NF-κB regulate different genes. Consequently, further work must be done to determine activation levels of the various NF-κB subunits when endothelial cells are exposed to live and killed *F. tularensis.*

Our work has also implicated AP-1 as being responsible for the suppressive effect of *F. tularensis* on HUVEC. Although the live bacteria did not block activation of NF-κB by heat-killed LVS, they did blunt activation of AP-1. AP-1 consists of a family of related transcription factors, but activation of only c-Jun was inhibited in endothelial cells in the presence of living *F. tularensis* (Fig 26B). Again, these data are in accordance with prior studies demonstrating that living *F. tularensis* could prevent activation of c-Jun in macrophages (101). Interestingly, CCL2, which showed little to no upregulation in HUVEC exposed to *F. tularensis*, has 22 AP-1 binding sites in its promoter vs. eight for NF-κB. On the other hand, CXCL8, whose production is triggered at low levels by *F. tularensis*, has one AP-1 binding site in its gene's promoter vs. four for NF-κB (DECODE database; http://www.sabiosciences.com/chipqpcrsearch.php). Perhaps the

difference in transcription factor binding sites accounts for the differential regulation by *F. tularensis* that we observe with CCL2 and CXCL8 in endothelial cells. These are preliminary results, but they give some insight into what signaling events are manipulated by *F. tularensis* to block the proinflammatory response of endothelial cells. It is possible that the low level of activation of NF-κB and AP-1 by the live LVS is sufficient to produce the modest amount of chemokines secreted by those cells. However, the bacteria did not elicit robust activation of these transcription factors, and production of chemokines was far less than the level seen with heat-killed bacteria.

Involvement of these transcription factors in suppression is supported by the results of our microarray analysis, wherein we saw down-regulation of gene transcripts that are regulated by NF-κB and/or AP-1. The microarray also brought to light additional molecules that may play a role in mediating this suppressive phenotype. PHD3, which has been shown to block activation of NF-κB (195, 196), was highly upregulated by the live LVS. However, when transcription of PHD3 was knocked down by 80% using siRNA, there was no effect on the amount of CCL2 elicitied by the bacteria from HUVEC. Given that the reduced secretion of CCL2 by endothelial cells in response to live *F. tularensis* was not reversed, PHD3 may not play a role in this process. Alternatively, as complete knockdown of the transcript was not achieved, it is possible that there was enough PHD3 present to maintain the suppressive phenotype. Additionally, PHD3 may be stable enough that levels of the protein remained high despite the reduction in transcription. Lastly, PHD1 or PHD2, which function similarly to PHD3 depending on the cell type, environment, and abundance of the other PHD molecules, may compensate for its loss (212, 213).

A second molecule of interest, which we have not yet investigated further, is RGS4. RGS4 is a GTPase-activating protein that can act on G_i and G_q-coupled receptors, and its transcription was down-regulated in endothelial cells exposed to live *F. tularensis*. The GPCR family of molecules can regulate downstream signaling for a multitude of host-cell responses. The current model for signaling via the EPCR/aPC/PAR-1 complex implicates association of PAR-1 with $G_q/G_{12/13}$ for proinflammatory activation and G_i for the inhibitory response (Fig. 2) (166). The role of RGS4 is complicated, as this molecule can act on all of these G-proteins (214, 215). Yet it would be interesting to see if *F. tularensis*-induced down-regulation of RGS4 works to prevent proinflammatory signaling of EPCR via PAR-1 or whatever co-receptor may be utilized in this context. Regardless of whether down-regulation of RGS4 has a direct effect on PAR-1, this molecule interacts with a range of GPCRs, and its inhibition due to *F. tularensis* would likely have downstream effects on the proinflammatory response of endothelial cells.

In addition to its possible effects on GPCRs on endothelial cells, *F. tularensis* may also interfere with caspase signaling. Caspase-1 cleaves pro-IL-1β to activate this proinflammatory cytokine. The microarray results herein show that *F. tularensis* down-regulates the transcripts for both of these molecules. This finding suggests another method by which *F. tularensis* may act directly on endothelial cells to inhibit a proinflammatory response. This result also supports those of other groups, who have demonstrated the ability of *F. tularensis* to block production and/or secretion of IL-1β by macrophages and dendritic cells (91, 98, 99), as well as activation of caspases (123, 216). These molecules are also components of the inflammasome, a complex formed in response to various signals, including cytosolic DNA, that promote cell death and the production of proinflammatory cytokines (217). One group has implicated activation of the inflammasome as critical for the host response to *F. tularensis*. Their studies showed that the

absent in melanoma 2 (AIM2) inflammasome is triggered in murine-derived macrophages infected with *F. tularensis*, leading to activation of caspase-1 and production of IL-1β (187, 188, 218). However, this work was conducted using only the spp. *novicida.* A subsequent study by another group found a greater role for the NLR family, pyrin domain-containing 3 (NLRP3) inflammasome in human macrophages infected with *F. tularensis* (219). Important in the context of our work, they also compared the LVS with ssp. *novicida* and found only modest induction of IL-1β by spp. *holarctica* vs. *novicida*. Our work is in line with that of Atianand et al. (219) showing reduced activation of the factors involved in the inflammasome by *F. tularensis* LVS. Given these disparate results between the ssp. *holarctica* and *novicida*, activation of the inflammsome, caspase-1, and IL-1β, may be another point of distinction between the virulent and avirulent strains of *F. tularensis*.

IV. Conclusions and Future Directions

Use of the EPCR by *F. tularensis* to inhibit the proinflammatory response may aid its dissemination throughout the host. Endothelial cells play a critical role in the clearance of an infection. When activated by a pathogen, these cells upregulate expression of adhesion molecules and secrete various chemokines, events that culminate in recruitment of circulating leukocytes (220). Throughout the course of tularemia in mice, the majority of *F. tularensis* organisms in the blood are extracellular and viable (114). Here the bacteria would directly contact the endothelium. By inhibiting secretion of CCL2 from endothelial cells early during infection, *F. tularensis* might limit the number of immune cells recruited to infected organs, such as lung, liver, and spleen (18). Most likely a balance is struck, whereby early on CCL2 is secreted at a low level so as to attract uninfected macrophages that would allow for replication and dissemination of the bacteria, while protecting against a full immune response that could clear the infection. As the infection progresses and increasing amounts of material are released from dead bacteria, the response may change, leading to the rise in inflammatory cytokines and chemokines that has been reported in mice 48-72 h post-infection (221, 222). However, by the time this inflammatory response is mounted, it may be too late to rescue the infected host (113). The source of the inflammatory cytokines seen late in murine tularemia has not been identified. It is possible that *F. tularensis* suppresses the activation of endothelial cells throughout the course of infection and that the cytokines seen at these later stages derive from other host cells.

This phenomenon warrants further investigation to determine what bacterial component is acting on the EPCR and what host-cell component is recognizing the bacterium when the EPCR is blocked. Another important piece will be confirming the role of the EPCR *in vivo*. Work has been on-going to breed EPCR knockout mice. We are in possession of mice with the

EPCR gene under control of a Cre-Lox system (223). However, we have so far been unsuccessful in generating EPCR-null mice.

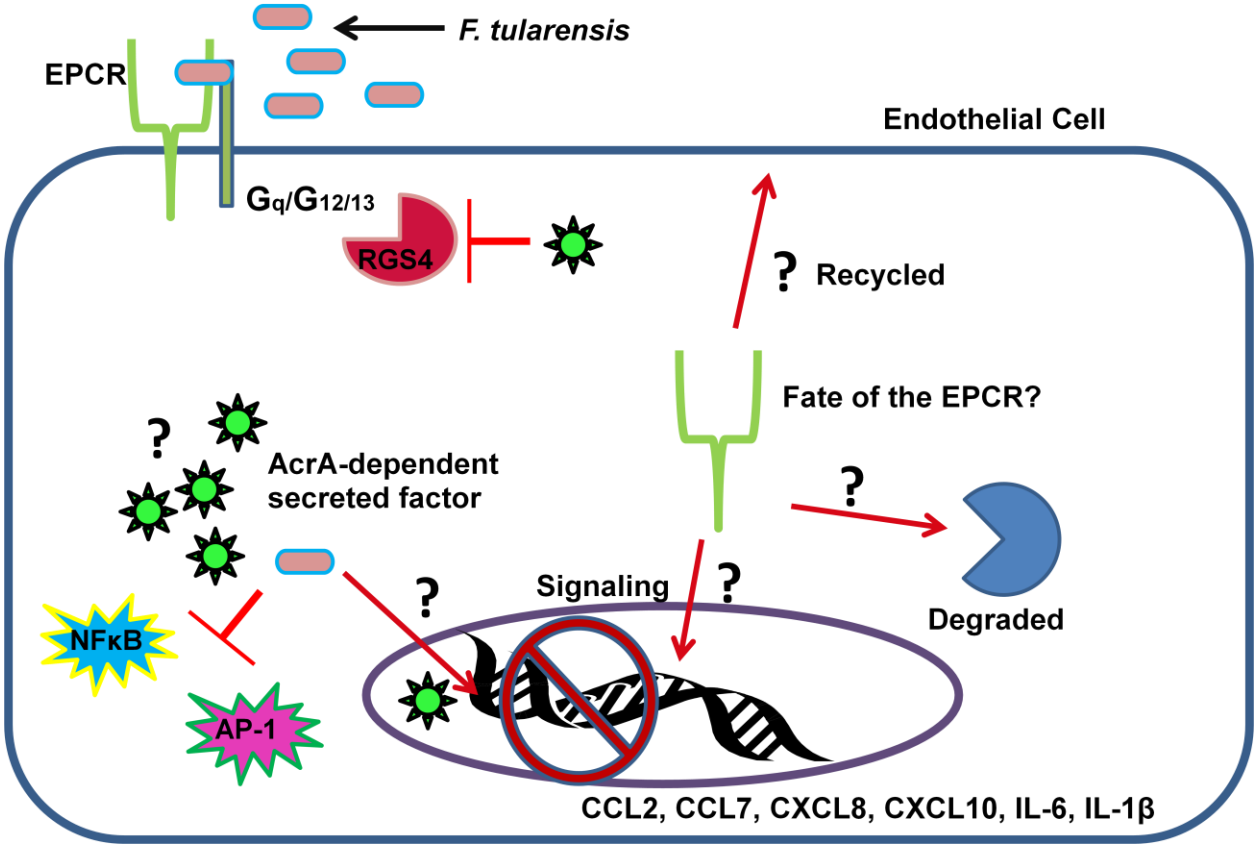
If inhibition of endothelial activation is mediated by a secreted bacterial factor, the secretory machinery could be targeted for novel antibiotics. As the AcrA is present in a range of bacteria, it is possible that any therapy developed for *F. tularensis* would be effective against other pathogens. Moreover, if AcrA or some related molecule blocks a proinflammatory response in endothelial cells, that factor could be marketable as general anti-inflammatory drug. Alternatively, as the AcrA is the membrane-fusion protein, its loss may lead to unregulated secretion of various bacterial proteins via the RND-like efflux pump. These proteins may be what is activating the endothelial cells. There is still much work to be done. First, the Δ*acrA* Schu S4 mutant must be complemented in order to verify that activation of the endothelial cells is due solely to the loss of AcrA and not a secondary effect. It would also be useful to express AcrA recombinantly to determine if it is acting independently and could by itself suppress activation of HUVEC in response to the heat-killed bacteria. In this same vein, we have been trying to express a recombinant version of the EPCR for use in a pull-down assay to elucidate bacterial factor(s) that interact with it. Lastly, it would be worthwhile to define the active subunits and roles of NF-κB and AP-1 upon exposure of endothelial cells to *F. tularensis.* In light of the differential regulation of chemokines by the Δ*tolC* mutants in the LVS vs. the Schu S4 strain, as well as the differences in activation and suppression of NF-κB and AP-1, such a study would be helpful in understanding how *F. tularensis* alters activation of the endothelium.

Given that infection by *F. tularensis* can prove lethal before an adaptive immune response can be mounted, understanding the role of innate immunity is vital. This report is, to our knowledge, the first time that a pathogen has been shown to subvert the host immune

response via the EPCR. Our work has helped shed light on how *F. tularensis* elicits this suppressive effect on endothelial cells and has shown that the mechanism utilized is conserved among *Francisella* strains (Fig. 27).

FIGURE 27. Proposed model for the EPCR-dependent inhibitory effect of *F. tularensis* on endothelial cells.

When in the bloodstream, *F. tularensis* may interact with the EPCR on the surface of endothelial cells, triggering the receptor to be internalized. At early timepoints during infection, the bacteria may also get inside of the endothelial cells. Internal bacteria may secrete virulence factors via the AcrA pump. These bacterial factors could act on the transcription factors AP-1 and NF-κB directly or upstream of molecules like the GTPase activating protein, RGS4, or perhaps the factors enter the nucleus to block activation of proinflammatory genes. Following internalization by the endothelial cells, the fate of the EPCR is unknown. Uptake of the receptor may trigger a signaling cascade of its own that is responsible for the inhibitory phenotypes noted. Alternatively, the EPCR may be degraded or recycled back to the cell surface.


F. tularensis
EPCR
Endothelial Cell
Gq/G12/13
RGS4
? Recycled
Fate of the EPCR?
?
AcrA-dependent
secreted factor
?
?
Signaling
?
Degraded
NFκB
AP-1
CCL2, CCL7, CXCL8, CXCL10, IL-6, IL-1β

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