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A Conserved and Immunodominant Lipoprotein of *Francisella tularensis* is Proinflammatory but not Essential for Virulence

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Abstract

Francisella tularensis is a highly virulent bacterium that causes tularemia, a disease that is often fatal if untreated. A live vaccine strain (LVS) of this bacterium is attenuated for virulence in humans but produces lethal disease in mice. *F. tularensis* has been classified as a Category A agent of bioterrorism. Despite this categorization, little is known about the components of the organism that are responsible for causing disease in its hosts. Here, we report the deletion of a well-characterized lipoprotein of *F. tularensis*, designated LpnA (also known as Tul4), in the LVS. An LpnA deletion mutant was comparable to the wild-type strain in its ability to grow intracellularly and cause lethal disease in mice. Additionally, mice inoculated with a sublethal dose of the mutant strain were afforded the same protection against a subsequent lethal challenge with the LVS as were mice initially administered a sublethal dose of the wild-type bacterium. The LpnA-deficient strain showed an equivalent ability to promote secretion of chemokines by human monocyte-derived macrophages as its wild-type counterpart. However, recombinant LpnA potently stimulated primary cultures of human macrophages in a Toll-like receptor 2-dependent manner. Although human endothelial cells also were activated by recombinant LpnA, their response was relatively modest. LpnA is clearly unnecessary for multiple functions of the LVS, but its inflammatory capacity implicates it and other *Francisella* lipoproteins as potentially important to the pathogenesis of tularemia.

Keywords

lipoprotein; inflammation; *Francisella*; virulence; mice

Introduction

Francisella tularensis is a small, non-motile, aerobic, Gram-negative coccobacillus, first isolated by G.W. McCoy in 1911 [1]. There are two main subspecies of *F. tularensis* that are

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pathogenic for humans. *F. tularensis* subsp. *tularensis* (also known as type A) is highly virulent in humans and causes the majority of tularemia cases in North America. *F. tularensis* subsp. *holarctica* (type B) causes a milder form of tularemia and is responsible for the bulk of tularemia cases reported throughout Eurasia [2]. A live vaccine strain (LVS) of *F. tularensis* was developed from a type B isolate [3]. Although this strain is attenuated for virulence in humans, it remains highly virulent in mice, making it a useful model for the study of the pathogenesis of tularemia [4].

Despite the high degree of virulence of *F. tularensis*, little is known about how this facultative intracellular pathogen causes lethal disease in its hosts; only recently have some of the factors involved in the pathogenesis of tularemia been identified. The ability of this organism to replicate to high numbers in murine macrophages, as well as to cause fatal disease in mice, is associated with IglC, a 23-kDa protein that is highly expressed during intramacrophage growth [5–8]. Mutation of the *pdpA* and *pdp*D genes of *F. tularensis* subsp. *novicida* also results in defects in virulence and growth within macrophages [9]. The *pdpA* and *pdpD* genes, along with *iglC* and several others, are thought to represent a *Francisella* pathogenicity island regulated by MglA, another protein that is required for virulence [9,10]. Type IV pili play a major role in the pathogenicity of many bacteria [11]. The genes required for the production of type IV pili exist in *Francisella*, and ultrastructural analysis of the LVS revealed the presence of long protrusions with the appearance of type IV pili [12]. Deletion of one of the putative pilin genes in a type B strain of *F. tularensis* results in attenuation of virulence [13], further implicating type IV pili in the pathogenesis of tularemia. Finally, deletion of the *tolC* or *ftlC* genes in the LVS decreases the resistance of the organism to anti-bacterial agents, and, in the case of the former, causes attenuation of virulence in mice [14].

The lipopolysaccharide (LPS) of *F. tularensis* possesses an atypical lipid A moiety and core structure [15]. Mice deficient in Toll-like receptor (TLR) 4, which binds LPS, do not gain increased resistance to *F. tularensis*, succumbing to disease as rapidly as do wild-type mice [16]. The purified LPS of *F. tularensis* LVS does not stimulate production of tumor necrosis factor-α, interferon-γ, interleukin (IL)-12, IL-10, or nitric oxide by murine macrophages [17, 18]. Nonetheless, *F. tularensis* LVS induces inflammation in mice *in vivo* [19,20] and can elicit the production of proinflammatory mediators by cultured murine macrophages and dendritic cells [21,22]. Further, human cells of innate immunity respond to culture with the LVS by producing an array of chemokines, cytokines, and adhesion molecules [23,24]. Together, these data suggest that *F. tularensis* possesses components other than LPS that are capable of initiating inflammation and regulating virulence. Recent findings have implicated TLR2, a receptor for lipoproteins, as important in the host response to infection with *Francisella* [21, 22,25,26]. Therefore, the lipoproteins of *F. tularensis* may be important in the pathogenesis of tularemia.

LpnA (also called Tul4) is an immunodominant 17-kDa lipoprotein [27] that is conserved throughout *Francisella* strains [28]. LpnA is associated with the outer membrane [29], stimulates T cells from *F. tularensis*-primed individuals [30,31], confers partial protective immunity to tularemia in mice when administered in recombinant form [32], and is predominantly expressed relative to other *F. tularensis* lipoproteins [27]. Herein, we set out to determine what role LpnA plays in the pathogenesis of tularemia. We show that absence of LpnA does not affect the intracellular replication of *F. tularensis* LVS or its virulence in mice, yet the recombinant lipoprotein induces production of proinflammatory mediators by primary human endothelial cells and macrophages. Our results clearly demonstrate that LpnA is not required for virulence of *F. tularensis*. However, its ability to stimulate cells of innate immunity implicates it and other *F. tularensis* lipoproteins as important mediators of the host response during tularemia.

Results

F. tularensis LVS *lpnA* was deleted by allelic exchange

To generate a strain of *F. tularensis* LVS with a deletion of *lpnA*, we utilized the conjugation method of Golovliov *et al.* [7], with changes as described in Materials and Methods. Briefly, *Escherichia coli* was transformed with pPV/Δ*lpnA*, a plasmid harboring a deleted version of *lpnA*, the *sacB* gene (which confers resistance to sucrose), and a chloramphenicol-resistance cassette. *F. tularensis* LVS was conjugated with this strain and plated on agar supplemented with chloramphenicol. A primary recombination event yielded an intermediate strain of the LVS with pPV/Δ*lpnA* incorporated into its genome. The intermediate strain then was plated on sucrose-containing agar, leading to a secondary recombination event that eliminated genomic *lpnA* along with the plasmid components of pPV.

The first and second recombination events were verified by polymerase chain reaction (PCR) (Fig. 1) and by growth on selective media. Primers specific to *sacB* generated the expected product of approximately 500 base pairs (bp) from the DNA of the intermediate colonies that had undergone a first recombination event, as well as from the pPV vector. Primers flanking *lpnA* (LpnA1; LpnA4) produced amplicons of approximately 3000 bp from the pPV vector, a selected intermediate colony, and the resulting final deletion strain, as well as a product of approximately 3500 bp from the wild-type LVS. The intermediate should have yielded both 3000- and 3500-bp products; however, we suspect that the primers preferentially amplified the smaller product. Verification of the deletion of *lpnA* was carried out using a primer specific to the deleted region of the gene and primer LpnA4. These primers generated amplicons from the LVS and the intermediate but not from the final deletion strain, indicating successful elimination of *lpnA* (Fig. 1). *F. tularensis*-specific DNA microarray analysis was performed to determine whether any transcriptional alterations occurred as a result of the *lpnA* deletion. Comparison of mRNA from wild-type and LpnA-deficient (Δ*lpnA*) *F. tularensis* LVS showed no change in transcript levels for any gene other than, as expected, *lpnA* itself (data not shown).

F. tularensis LVS Δ*lpnA* replicates to the same extent in murine macrophages as does wild-type LVS

F. tularensis LVS lacking *lpnA* grew in Mueller-Hinton (MH) broth at the same rate and to the same extent as the wild-type bacteria over a 48-h time period (data not shown). The ability of *F. tularensis* LVS to proliferate within murine macrophages has been well documented [5, 24,33,34]. To determine whether a lack of LpnA affected intracellular replication, murine bone marrow-derived macrophages (muBMDM) were cultured with either wild-type or LpnA-deficient *F. tularensis* LVS for 2 h, after which the extracellular bacteria were killed with gentamicin. The *F. tularensis* strains then were allowed to multiply intracellularly for an additional 22 h. In three independent experiments, numbers of wild-type LVS increased 622 ± 66 fold in this span, and the mutant grew comparably, increasing 638 ± 370 fold over the same time period. Immunofluorescence microscopy demonstrated that similar percentages of muBMDM were infected with either wild-type or LpnA-deficient bacteria after 2 h and confirmed the extensive intracellular replication of these two strains over a subsequent 22-h period (Fig. 2).

Deletion of *lpnA* from *F. tularensis* LVS does not alter its virulence in mice

Others have previously shown that when administered to C3H/HeN mice intradermally, *F. tularensis* LVS is lethal at doses of 10^6 and 10^7 bacteria but not when 10^5 or fewer organisms are given [4]. For this reason, we used infectious doses ranging from 10^5 to 10^7 bacteria to compare the abilities of wild-type and LpnA-deficient *F. tularensis* LVS to cause illness in mice. *F. tularensis* LVS Δ*lpnA* was no different than wild-type LVS in its ability to cause lethal disease (Table 1). At an infectious dose of 10^7 bacteria, one of 20 mice infected with the wild-

type LVS survived, whereas three of 20 mice infected with the LpnA-deficient LVS survived infection. The median times to death for each strain were 4 and 5 days, respectively. Following infection with 10^6 total organisms, one of 15 mice survived from each of the wild-type- and mutant-infected groups, with a median time to death of 5 days for both. Mice infected with 10^5 organisms of *F. tularensis* LVS or *F. tularensis* LVS Δ*lpnA* were less susceptible to lethal disease, with seven of ten and eight of ten animals surviving, respectively (Table 1).Three days after inoculation with 10^6 organisms, mice harbored on average similar numbers of wild-type or mutant bacteria in the lung, liver, and spleen, although variability was greater among the animals that received the Δ*lpnA* strain (data not shown). To determine whether our above observations held true irrespective of the route of inoculation, C3H/HeN mice, in groups of five, were inoculated intranasally with a lethal dose of either wild-type *F. tularensis* LVS or the LpnA-deficient mutant strain. All mice from both groups died within 8 days of infection, with comparable times-to-death for each group (Table 1).

LpnA is not necessary to confer immunity to *F. tularensis* LVS in mice

Mice surviving intradermal challenge with sublethal doses of *F. tularensis* LVS become immune to subsequent challenges with otherwise lethal inocula of the organism [4]. To determine whether LpnA is necessary to confer this immunity, C3H/HeN mice were administered a sublethal dose of wild-type LVS, LpnA-deficient LVS, or phosphate-buffered saline (PBS), followed by a challenge with an ordinarily lethal dose of *F. tularensis* LVS two months later. LpnA was not needed to confer immunity, as all ten mice from the group initially administered the Δ*lpnA* strain survived the secondary challenge. Similarly, eight of nine mice that were initially infected with wild-type LVS survived the challenge. All five mice that were initially given PBS, rather than bacteria, died within 6 days of the second inoculation (Fig. 3).

Recombinant LpnA activates human macrophages and endothelial cells

Our laboratory previously demonstrated that human monocyte-derived macrophages (huMDM) and human umbilical vein endothelial cells (HUVEC) respond to stimulation with live or killed preparations of *F. tularensis* LVS by producing a wide array of proinflammatory mediators [23,24]. These mediators include the chemokines CXCL8 and CCL2 and the adhesion molecule E-selectin. E-selectin is expressed on the surface of activated endothelium and participates in the initial capture of circulating leukocytes [35]. Subsequently, chemokines induce the transendothelial migration of captured leukocytes and thus recruit these cells to extravascular sites of inflammation. CXCL8 and CCL2 are produced by both HUVEC and huMDM that have been exposed to the LVS and are attractants for neutrophils and mononuclear leukocytes, respectively [36].

Purified LPS from the LVS activates cells of innate immunity poorly [37] (C. E. Noah and M. B. Furie, unpublished results), indicating that other components mediate the response elicited by live or killed bacteria. LpnA is potentially one such component. Because we have found that human macrophages have a far greater inflammatory response to *F. tularensis* LVS than do murine macrophages [24], we tested the capacity of LpnA to stimulate cells of human origin. There was no difference in the ability of the LpnA-deficient and wild-type strains of *F. tularensis* LVS to stimulate production of the chemokines CXCL8 and CCL2 by huMDM (Fig. 4). However, preparations of purified, recombinant LpnA ranging from 10 ng/ml to 1 μg/ml of protein induced huMDM to secrete both CXCL8 (Fig. 5A) and CCL2 (Fig. 5B), but not IL-1β (data not shown). LpnA was a potent stimulus, as even the lowest doses of LpnA tested elicited greater production of chemokines than did 10 ng/ml of *E. coli* LPS (Figs. 5A and 5B). All samples of LpnA were cleared of potential contaminating *E. coli* LPS prior to experimentation, as discussed in Materials and Methods. When TLR2 was blocked by antibody, a significant decrease in the production of both chemokines occurred, whereas no change was seen when the cells were pre-incubated with a control antibody of the same isotype

(Fig. 6). As expected, the antibody to TLR2 inhibited secretion of CXCL8 by huMDM exposed to tripalmitoyl-Cys-Ser-$(Lys)_4$, a known agonist for this receptor, but had no effect on cells stimulated by *E. coli* LPS (data not shown).

HUVEC responded to recombinant LpnA, but the amounts of lipoprotein required were 10- to 100-fold greater than those needed to stimulate macrophages. LpnA at 1 μg/ml induced secretion of modest levels of CXCL8 and CCL2 in five of eight independent experiments; stimulation was insignificant in the remaining three studies. Representative results are shown in Fig. 7, panels A and B. Upregulation of surface expression of the adhesion molecule E-selectin was observed in response to 100 ng/ml of LpnA (Fig. 7C). In contrast, stimulation of huMDM was reproducibly achieved with 10 ng/ml of LpnA (compare Fig. 5 and Fig. 7).

Discussion

We regarded LpnA as potentially important in the pathogenesis of tularemia based on a number of previously published observations. These include the relatively high expression of LpnA in the outer membrane of *F. tularensis* [27], its high conservation among subspecies of *Francisella* [28], its immunogenicity [32], and its identity as a lipoprotein [27]. Bacterial lipoproteins can be predicted from genomic sequences by the presence of a lipobox, a consensus signal sequence at their N-terminus that is recognized and subsequently cleaved by the enzyme signal peptidase II [38]. The resultant lipid modification, N-acyl-S-diacylglyceryl cysteine, occurs at the N-terminus and is conserved throughout bacterial lipoproteins of many different species [39]. LpnA contains palmitic acid [27], which is the predominant amide-linked fatty acid in the murein lipoprotein of *E. coli* [38]. Moreover, all enzymes responsible for the N-acyl-S-diacylglyceryl modification, which include prolipoprotein diacylglyceryl transferase, signal peptidase II, and apolipoprotein N-acyltransferase [40], are encoded by the genome of the LVS (GenBank accession number AM23362). Nonetheless, we cannot be certain that the lipid modification of recombinant LpnA, expressed in *E. coli*, is identical to that of native LpnA.

Lipoproteins are important factors in the pathogenesis of bacterial diseases. For example, lipoproteins from *Borrelia burgdorferi*, the causative agent of Lyme disease, provoke a proinflammatory response in endothelial cells [41] and leukocytes [42] and play an essential role in transmission of the infection [43]. The proinflammatory nature of lipoproteins has been attributed to their attached lipid, and not the protein itself. Lipoproteins from *Borrelia burgdorferi* and *Brucella abortus* are able to induce inflammation in host cells only after they have been processed and modified with lipid [41,42,44]. However, lipoproteins display significant functional diversity that likely can be attributed to the variability in their associated protein moieties. They can serve structural purposes in the bacterial membrane, function as transporters across membranes, or work as toxins in some bacterial species [39]. Thus, it can be expected that lipoproteins of all bacteria display both unique and common functions.

Our observation that LpnA-deficient *F. tularensis* LVS could induce secretion of chemokines by human macrophages to the same extent as the wild-type LVS was not entirely surprising. A search of the annotated *F. tularensis* subsp. *tularensis* Schu S4 genome [45] reveals 38 potential lipoproteins, all of which are also present in the genome of the LVS. If LpnA were involved in inflammation, it would likely be due to its lipid attachment, which should be the same in all *F. tularensis* lipoproteins. Ablation of only one of these putative lipoproteins, even if predominantly expressed, may be insufficient to affect the inflammatory capacity of the organism. Our finding that recombinant LpnA elicits production of proinflammatory factors by host cells strongly supports this possibility. Consequently, it is plausible that the proinflammatory capacity of LpnA is shared by all *Francisella* lipoproteins. Compared to huMDM, HUVEC were relatively resistant to activation by LpnA. Recently, we observed that

mice infected with the LVS contain substantial numbers of extracellular organisms in the blood throughout the course of their illness [46]. Perhaps a poor response of endothelium to LpnA permits these bacteria to persist in the circulation without provoking overwhelming, systemic inflammation.

We did anticipate a role for LpnA in the pathogenesis of tularemia. *F. tularensis* is known for its ability to proliferate within the macrophages of mice and humans. In fact, this is one of the few established properties that contribute to the virulence of the organism, as mutants lacking a gene required for intra-macrophage growth are avirulent in mice [7,8,10]. Others have shown that an LpnA-deficient strain of *F. tularensis* subspecies *novicida* replicates 10-fold less in the murine macrophage-like J774 cell line than does its parental strain [47]. As a consequence, one might expect this decreased growth rate to correspond to a decrease in virulence of the mutant strain. Although this assumption was not tested with the LpnA-deficient strain of *F. tularensis* subspecies *novicida*, we show here that it does not hold true for the LVS lacking LpnA; LpnA-deficient *F. tularensis* LVS did not display any changes in virulence in mice when administered via either the intradermal or intranasal route. Moreover, we did not observe any deficiency in growth of the mutant strain within muBMDM relative to the wild-type LVS. It is possible that LpnA is more essential to *F. tularensis* subsp. *novicida* than it is to the LVS, leading to the observed decrease in intracellular replication of the LpnA-deficient strain of the former [47]. In *F. tularensis* LVS, however, LpnA clearly makes no indispensable contribution to virulence, despite its predominant expression compared to other *F. tularensis* lipoproteins [27] and its ability to elicit an immune reaction [30,32,48].

Others have shown previously that immunity to *F. tularensis* can be attained after exposure to sublethal doses of the organism [4]. In addition, mice can be immunized against *F. tularensis* LVS by administration of its purified LPS alone [49,50]. The observations that LpnA stimulates T cells of individuals previously exposed to *F. tularensis* [31] and that it can confer partial resistance to challenge with the LVS when given to mice in recombinant form [32,48] suggested a potentially important role for the lipoprotein in acquired immunity to *F. tularensis*. Despite these previous findings, we determined that mice immunized with either wild-type or LpnA-deficient *F. tularensis* LVS are equally resistant to subsequent challenge with a typically lethal dose of the bacterium. Thus, LpnA is not required for full immunoprotection of mice against infection with *F. tularensis* LVS.

TLRs are a group of pattern-recognition receptors that play important roles in host responses to invading pathogens. Bacterial lipoproteins and atypical LPS of certain bacteria signal via TLR2, whereas LPS from most bacteria signals through TLR4 [51]. The weak proinflammatory activity of LPS from *F. tularensis* has been well described [17,18], and mice deficient in TLR4 are as susceptible to lethal tularemia as are naïve mice of the same background [16,52]. In contrast, recent reports have indicated an important role for TLR2 in the pathogenesis of tularemia [21,22,25,26]. The fact that recombinant LpnA induced inflammation in human macrophages in a manner that was dependent on TLR2 implicates it and other *F. tularensis* lipoproteins as important mediators of the host response in tularemia. Lipoproteins from several different bacterial species are released during growth of the bacteria and upon killing of the organisms with antibiotics, and they subsequently stimulate the production of proinflammatory mediators *in vitro* [53]. These data support the notion that biogenesis of lipoproteins can be an important factor in microbial pathogenesis. Our findings clearly rule out LpnA as an essential component in the pathogenesis of tularemia in the murine model. However, the strong proinflammatory capacity of LpnA indicates that it and other lipoproteins of *F. tularensis* may play a key role in the host response to infection.

Materials and Methods

Culture of bacteria

F. tularensis LVS (American Type Culture Collection No. 29684) was maintained and cultured as previously described [23]. For each experiment, a frozen stock of bacteria was thawed and streaked on solid medium composed of MH II agar supplemented with 1% bovine hemoglobin and 1% IsoVitaleX Enrichment (all from BD Biosciences, Lincoln Park, NJ). The bacteria were grown for 2 to 3 days at 37°C in a 5% CO_2 /95% air environment to allow for formation of colonies. A single colony was inoculated into supplemented MH broth [23] and grown to late-log phase for 16 to 18 h at 37°C with shaking at 100 rpm in a 5% CO_2 atmosphere. Aliquots of bacterial culture were centrifuged, and the pellets were resuspended in culture medium appropriate to the experiment being performed. *E. coli* strains S17-1 and DH5α were grown on Luria-Bertani (LB) plates or in LB broth at 37°C. Stocks of these two strains were maintained at −80°C in LB broth containing 10% glycerol.

Generation of LpnA-deficient *F. tularensis* LVS

The oligonucleotides 5'-ATTT**GCGGCCGCGTCGAC**TTGTTCTGGTGTTTCAGCC-3' (LpnA1), 5'-AGGT**CTGCAG**TCATCAGAGCCACCTAACCC-3' (LpnA2), 5'GCAA**CTGCAG**AACTTCTGTAGCGGTAAGTGGAC-3' (LpnA3), and 5'ATTT**GCGGCCGCGTCGAC**ATCAATTTCATGCGTCGTAG-3' (LpnA4) were used to amplify the upstream (LpnA1 and LpnA2) or downstream (LpnA3 and LpnA4) flanking regions of *lpnA* in *F. tularensis* LVS by PCR. Primers LpnA1 and LpnA4 incorporated both *Not*I and *Sal*I restriction sites into their products (shown in bold). Primers LpnA2 and LpnA3 generated *Pst*I restriction sites in their products. The amplification size for each set of primers was approximately 1500 bp, and the resulting products incorporated the first 98 bp and the last 87 bp of *lpnA*. These products were ligated directly into the pGEM-T Easy vector (Promega, Madison, WI) and electroporated into DH5α. Transformants were selected on LB agar supplemented with 100 μg/ml of ampicillin. Plasmid DNA then was isolated from the transformants using a Spin Miniprep Kit (Qiagen, Valencia, CA) and digested with *Not*I and *Pst*I. The 1500-bp fragments corresponding to the upstream and downstream regions of *lpnA* were recovered with the Qiaquick Gel Extraction Kit (Qiagen) and ligated simultaneously into pPCR-Script (Stratagene, La Jolla, CA) that had been previously digested with the same two enzymes. The resultant plasmid, pPCR-Script*ΔlpnA*, was recovered as described above. This protocol culminated in the joining of the upstream and downstream regions of *lpnA*, with the central region of the gene being absent. This insert then was digested out of pPCR-Script with *Sal*I and ligated into *Sal*I-digested pPV [7], which was provided by Anders Sjöstedt (Umeå University, Umeå, Sweden). The resultant mutagenesis plasmid was designated pPV*ΔlpnA*. This plasmid then was used to generate an LpnA-deficient strain of *F. tularensis*, designated *F. tularensis* LVS Δ*lpnA*, by following a previously described conjugation protocol [7], with some modifications [14].

F. tularensis DNA microarray

RNA was prepared from overnight cultures of LpnA-deficient or wild-type *F. tularensis* LVS using an RNeasy Midi Kit (Qiagen). All subsequent steps were performed following protocols provided by The Institute for Genomic Research (TIGR) [54]. *F. tularensis*-specific microarray chips were obtained through the National Institute of Allergy and Infectious Diseases' Pathogen Functional Genomics Research Center, managed and funded by the Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS and operated by TIGR.

Preparation of murine bone marrow-derived macrophages

Murine BMDM were obtained from the femurs of female C3H/HeN mice [55]. Bone marrow was collected from femurs flushed with Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Inc., Gaithersburg, MD) containing 5% heat-inactivated (HI) (56°C for 30 min) fetal bovine serum (FBS; HyClone, Logan UT) and washed with the same medium. After the final wash, the cells obtained were resuspended in DMEM containing 2 mM L-glutamine, 1 mM sodium pyruvate (both from Life Technologies), 20% HIFBS, and 30% medium previously conditioned by L929 cells. The conditioned medium was obtained by plating 2×10^5 L929 cells in 75-cm^2 culture flasks in Minimal Essential Medium (Life Technologies) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids (all three from Life Technologies), and 10% FBS and collecting the medium after 10 days. The bone marrow cells were then seeded in 100 mm-diameter dishes (Nunc, Naperville, IL) at a concentration of 4×10^6 cells per plate. After 5 days at 37°C, muBMDM were detached with ice-cold PBS, resuspended in culture medium, and seeded in 24-well plates at a concentration of 1.5×10^5 cells per well. The muBMDM were used for experiments the following day.

Infection of muBMDM

Murine BMDM grown in 24-well plates were exposed to *F. tularensis* LVS at a multiplicity of infection (MOI) of approximately 50 bacteria per cell. After 2 h of co-culture at 37°C, leukocytes were washed extensively and incubated with 5 μg/ml of gentamicin for 1 h to kill extracellular bacteria [23]. To measure intracellular content of viable *F. tularensis* LVS, some samples were lysed with 1.0 ml of water for 10 min. Serial dilutions were plated to determine the numbers of intracellular bacteria. Other infected cultures were incubated for an additional 21 h in antibiotic-free medium before lysis.

Immunofluorescent staining of intracellular bacteria

F. tularensis LVS organisms that had been internalized by murine leukocytes were visualized by indirect immunofluorescence. Murine BMDM were plated on 12 mm-diameter glass coverslips in 24-well dishes and incubated with *F. tularensis* LVS at a MOI of approximately 50 for 2 h. Some samples were washed and immediately fixed. Others were incubated for 1 h in medium containing 5 μg/ml of gentamicin, followed by an additional 21-h incubation in antibiotic-free medium before fixation. The cells were fixed with 2.5% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in PBS, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich), and incubated with a polyclonal rabbit anti-serum to *F. tularensis* (BD Biosciences) for 30 min. Cells then were washed with PBS and incubated with fluorescein isothiocyanate-labeled goat anti-rabbit Ig (BD Biosciences) for 30 min. Coverslips were inverted onto slides containing Slowfade® mounting medium (Molecular Probes, Eugene, OR) and viewed by epifluorescence and phase contrast microscopy with a Zeiss Axioplan2 microscope equipped with a 40X objective. Images were captured using a Spot camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and processed using Adobe Photoshop 6.0.

Infection of mice with *F. tularensis*

All studies with mice were approved by the Institutional Animal Care and Use Committee of Stony Brook University. Murine infections used 6 to 8 week-old C3H/HeN mice (Taconic Laboratories, Germantown, NY) infected via the intradermal or intranasal route. Mice infected intradermally were inoculated in groups of five with infectious doses of *F. tularensis* LVS or *F. tularensis* LVS Δ*lpnA* ranging from 10^5 to 10^7 total bacteria in a volume of 100 μl. An inoculum of 10^6 bacteria was used to compare dissemination of bacteria in mutant and wild-type mice. After 3 days, the liver, spleen, and one lung were removed from each mouse. Organs were weighed and homogenized in a Stomacher 80 Biomaster laboratory blender (Seward Ltd.,

Worthing, West Sussex, UK), and dilutions of the homogenates were plated on MH II agar to determine the number of bacteria per gram of tissue.

Mice inoculated intranasally were anaesthetized with a mixture of 100 mg/ml of Ketamine and 20 mg/ml of Xylazine (0.01 ml of mixture/gram of body weight, both from Henry Schein, Melville, NY), placed into groups of five, and infected with *F. tularensis* LVS or *F. tularensis* LVS Δ*lpnA*. Inocula of 10^6 wild-type or mutant *F. tularensis* organisms per 20 μl of PBS (Life Technologies) were generated, and 10 μl was applied to each nare for each mouse.

Immunoprotection of mice by wild-type and mutant *F. tularensis*

Groups of ten C3H/HeN mice were infected intradermally with a sublethal inoculum [4] consisting of 10^4 *F. tularensis* LVS or *F. tularensis* LVS Δ*lpnA* bacteria. As a control, five mice were administered a solution of sterile PBS. After 2 months, all mice received a second intradermal challenge with a normally lethal dose of 10^7 *F. tularensis* LVS organisms. Animals were monitored for an additional 4 weeks.

Preparation of recombinant LpnA

LpnA was amplified from the genome of *F. tularensis* LVS using the oligonucleotides 5'GACT**GTCGAC**CGTAATGTTAGCTGTATCATC 3' (LpnA5) and 5'GACT**GCGGCCGC**TTAGTGATGGTGATGGTGATGAATATTTATTGAATCAGAA GCGATTACTTC 3' (LpnA6). LpnA5 incorporated an N-terminal *Sal*I restriction site (in bold), and LpnA6 incorporated a C-terminal *Not*I restriction site (in bold) and a 6X-histidine tag (underlined). Amplified products were ligated into the pGEM-T Easy vector and transformed into *E. coli* DH5α, followed by purification of the LpnA-containing plasmid as described above. Next, the region corresponding to LpnA was excised using *Sal*I and *Not*I and ligated into the pET30a vector (Novagen, Madison, WI). Ligation products were expressed in DH5α followed by purification of the recombinant vector, designated pET30a-LpnA. This vector then was sent to the Northeast Biodefense Center Protein Expression Core (Wadsworth Center, Albany, NY) for purification of LpnA by Triton X-114 phase partitioning [56]. This method has been previously employed to isolate functional, acylated proteins [44,56]. Potential contaminating *E. coli* LPS was removed from the LpnA preparations using Endotoxin Removal Affinity Resin (Cape Cod Associates, East Falmouth, MA). The QCL-1000 Chromogenic Limulus Amebocyte Lysate Endpoint Assay (Cambrex, East Rutherford, NJ) confirmed that only a trace amount of LPS (4 pg per μg of LpnA) remained. This amount of LPS was too small to stimulate HUVEC or macrophages (data not shown). Nonetheless, 20 μg/ml of the LPS inhibitor polymyxin B (Sigma-Aldrich, St. Louis, MO) was included in all samples containing recombinant LpnA in all experiments.

Preparation of human monocyte-derived macrophages

Monocytes were obtained from the venous blood of healthy adult volunteer donors, using 0.12% disodium EDTA as an anticoagulant. Leukocytes were separated by density gradient centrifugation using Accu-Prep™ Lymphocytes (Accurate Chemical Co., Westbury, NY). Monocytes were negatively selected by immunomagnetic sorting using the Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA) according to the instructions of the manufacturer. Isolated monocytes were resuspended in RPMI medium (Life Technologies) containing 10% HIFBS, added to 24-well plates at a concentration of 2.0×10^5 cells per well, and cultured for 5 to 7 days in the presence of 10 ng/ml of recombinant human macrophage colony-stimulating factor (Sigma-Aldrich) to induce formation of huMDM.

Culture of endothelial cells

Endothelial cells were isolated from human umbilical veins via collagenase perfusion and grown to confluence in 60-mm dishes in a 37°C, 5% CO_2 /95% air environment in Medium 199 (M199; Life Technologies) supplemented with 20% HIFBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 μg/ml of amphotericin B [57]. Cells were cultured for 3 to 5 days, trypsinized, and passaged onto 48- or 96-well plates (both from BD Biosciences) at a concentration of 2.3×10^5 cells/ml.

Quantitation of cytokines

To assess production of cytokines by huMDM or HUVEC, cells were treated for 24 h with preparations of live or gentamicin-killed (50 μg/ml for 1 h) *F. tularensis*, recombinant LpnA, *E. coli* LPS (serotype 0111:B4, Sigma-Aldrich), a sham preparation of bacteria consisting of uninoculated MH broth processed in the same fashion as the bacterial culture, or control medium. Conditioned media were collected and clarified by centrifugation. Amounts of CXCL8, CCL2, or IL-1β in the supernatants were quantitated using commercial enzyme-linked immunosorbent assay (ELISA) kits (Antigenix America, Inc., Franklin Square, NY).

Quantitation of E-selectin

The ability of recombinant LpnA to induce expression of E-selectin on HUVEC was evaluated by whole-cell ELISA. HUVEC were incubated with 100 μl of control medium (M199 with 20% HIFBS), LpnA, or *E. coli* LPS at 37°C for 4 h. ELISAs to detect surface expression of E-selectin on living endothelial monolayers then were performed as described previously [58].

Blocking of TLR2

To assess whether LpnA stimulates cells via TLR2, a murine neutralizing monoclonal antibody to human TLR2 (clone TLR2.1; eBioscience, San Diego, CA) was used in some experiments. Human MDM were cultured with medium alone, 20 μg/ml of antibody, or 20 μg/ml of an isotype-matched control antibody (mouse IgG2a, κ; eBiosciences) for 30 min. An equal volume of medium containing stimuli then was added, so that 10 μg/ml of antibody remained throughout the experiment. Measurement of the production of E-selectin, CXCL8, or CCL2 was performed as described above. A similar protocol was used to determine the ability of the antibody to inhibit stimulation of huMDM by 1 ng/ml of tripalmitoyl-Cys-Ser-$(Lys)_4$ (EMC Microcollections, Tübingen, Germany), a known agonist for TLR2, or 10 ng/ml of *E. coli* LPS, which signals through TLR4 [51].

Statistics

To determine statistical significance, data were subjected to an unpaired analysis of variance and the Tukey-Kramer multiple-comparison test using Instat version 3.01 (GraphPad Software, San Diego, CA).

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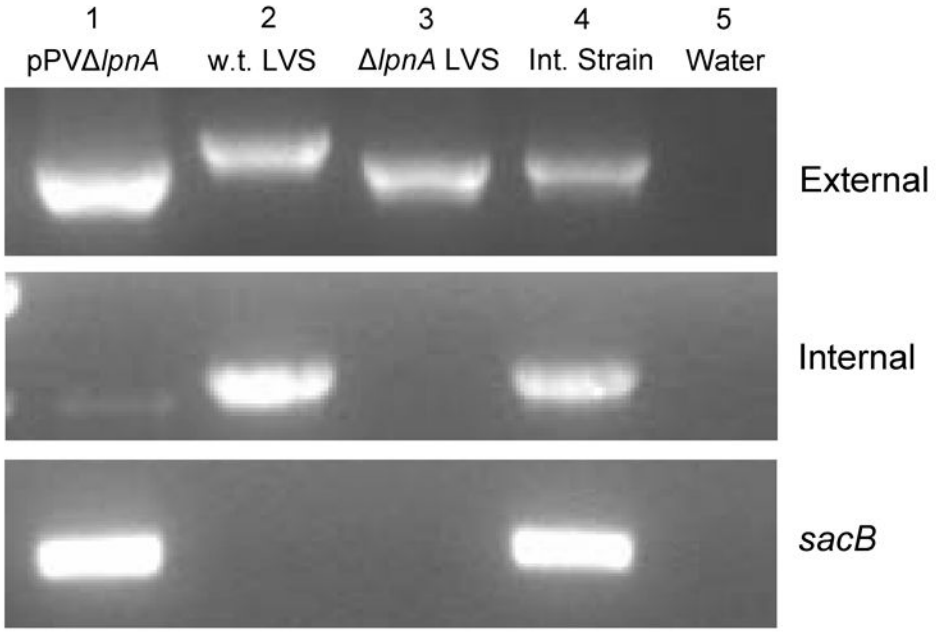

Figure 1.

Verification of the *lpnA* deletion mutant. PCR amplification of the mutant strain (lane 3) with primers external to *lpnA* yielded a smaller-sized amplicon than that produced when the wild-type LVS (lane 2) was amplified with the same primers. Primers internal to *lpnA* did not generate an amplicon from the mutant strain or the mutagenesis vector pPVΔ*lpnA* (lane 1) but did amplify the wild-type LVS and the intermediate strain (lane 4). Amplification of *sacB* occured in both the mutagenesis vector and the intermediate strain, but not the wild-type or mutant strain, indicating exclusion of the vector from the mutant strain. Lane 5: water control.

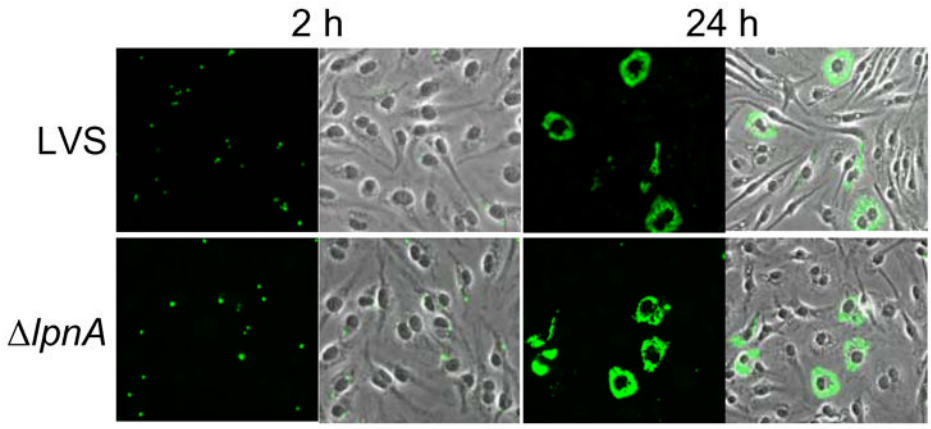

Figure 2.

F. tularensis LVS lacking LpnA replicates to the same extent in muBMDM as does its wild-type counterpart. Cultured muBMDM were incubated with *F. tularensis* LVS or *F. tularensis* LVS Δ*lpnA* for 2 h at a MOI of ~50. Two-hour samples were washed and fixed immediately, whereas 24-h samples were washed, incubated for 1 h in the presence of 5 μg/ml of gentamicin, cultured in antibiotic-free medium for 21 h more, and fixed. *F. tularensis* LVS then was detected by indirect immunofluorescence. Samples were visualized by epifluorescence (columns 1 and 3) and phase microscopy. Columns 2 and 4 depict epifluorescent microscopic images superimposed on the phase microscopic images of the same field.

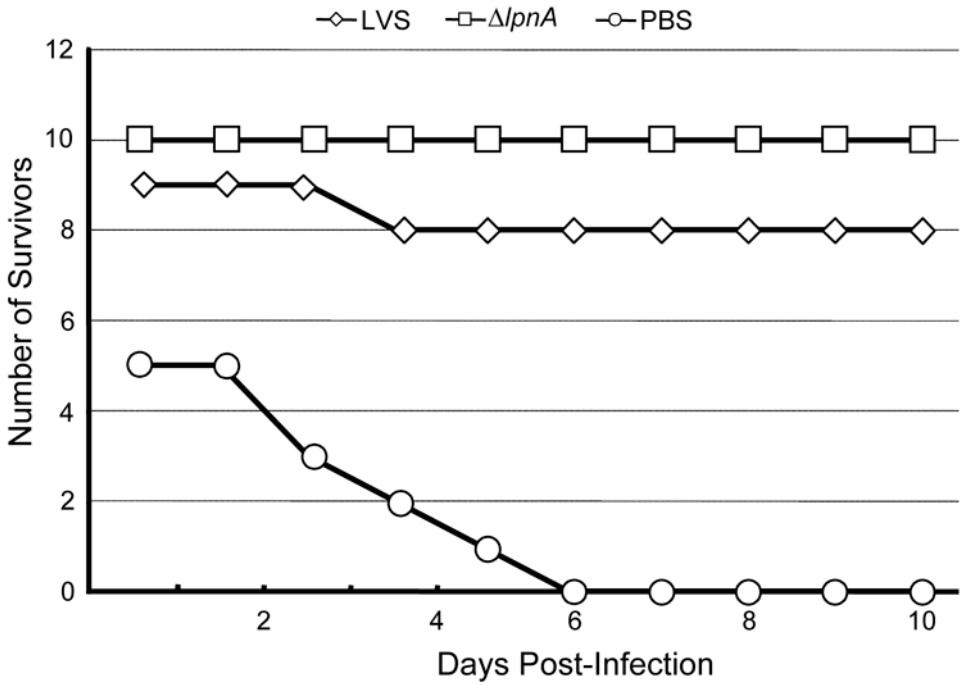

Figure 3.
LpnA is not required for immunoprotection of mice against *F. tularensis* LVS in mice. C3H/HeN mice were inoculated with a sublethal dose of *F. tularensis* LVS or *F. tularensis* LVS Δ*lpnA*, or with PBS. After 2 months, all mice were challenged with a normally lethal dose of *F. tularensis* LVS and monitored for survival for an additional 4 weeks.

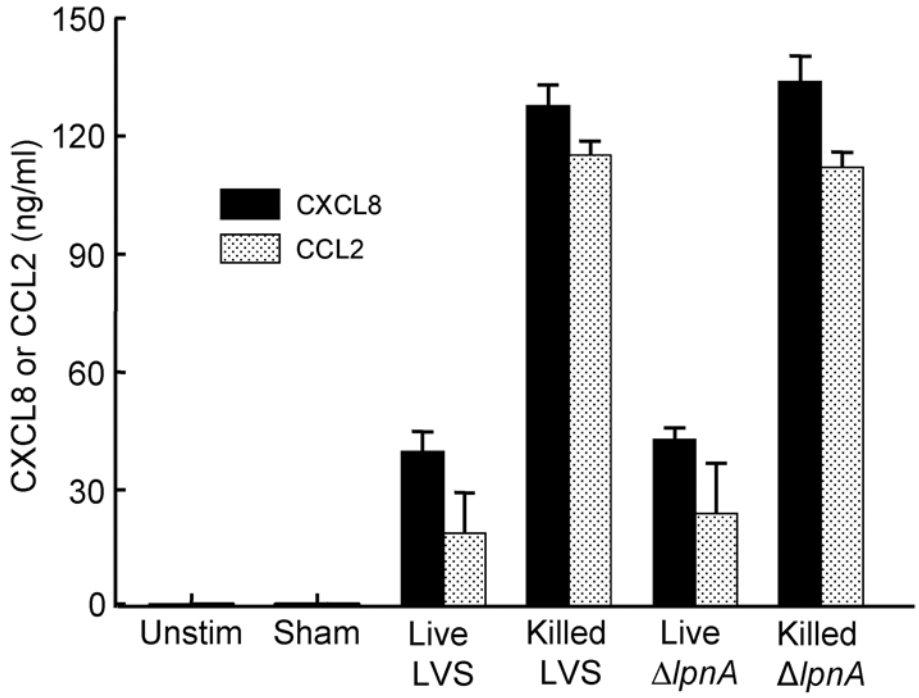

Figure 4.

Wild-type and LpnA-deficient *F. tularensis* LVS promote similar secretion of the chemokines CXCL8 and CCL2 by huMDM. HuMDM were incubated for 24 h at 37°C with medium alone (Unstim), a sham preparation of bacteria, live or killed *F. tularensis* LVS at a MOI of ~25, or live or killed *F. tularensis* LVS Δ*lpnA* at a MOI of ~25. Cell-free conditioned media were collected, and amounts of CXCL8 or CCL2 were measured by ELISA. Bars represent the means ± SD of three replicate samples. This experiment was repeated twice, yielding similar patterns of chemokine secretion.

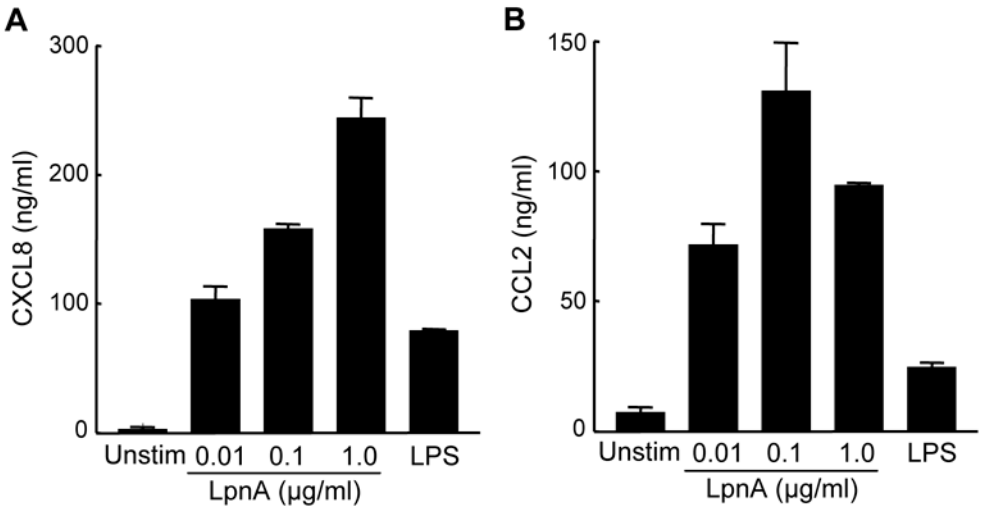

Figure 5.

LpnA stimulates secretion of CXCL8 and CCL2 by huMDM. HuMDM were treated with medium alone (Unstim), recombinant LpnA ranging from 0.01 μg/ml to 1.0 μg/ml, or 0.01 μg/ml of *E. coli* LPS for 24 h at 37°C. All samples of LpnA caused significant increases in the production of CXCL8 (A) and CCL2 (B) relative to that of unstimulated macrophages as measured by ELISA of cell-free conditioned media ($P < 0.001$). Bars represent the means ± SD of three replicate samples. Although the magnitude of the response differed from donor to donor, LpnA at concentrations of 0.01 μg/ml or above significantly increased secretion of these chemokines in two additional experiments.

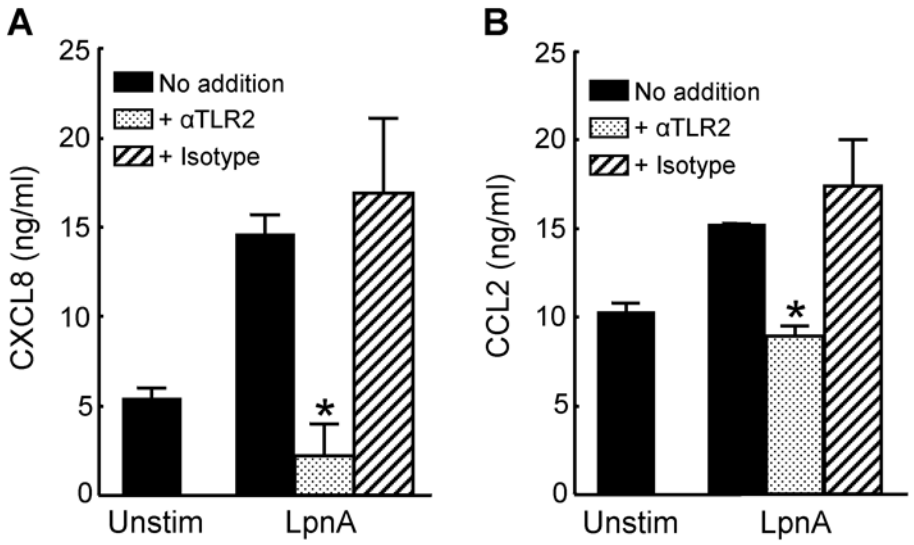

Figure 6.

Secretion of CXCL8 and CCL2 by huMDM in response to LpnA is TLR2-dependent. Addition of anti-human TLR2 antibody to huMDM subsequently stimulated with recombinant LpnA (0.01 μg/ml) resulted in the production of only basal levels of CXCL8 (A) or CCL2 (B), whereas macrophages incubated with an isotype-matched control antibody were unaffected. Bars represent the means ± SD of three replicate samples. *, Significantly different from samples stimulated with LpnA but not treated with anti-TLR2, $P < 0.001$. This experiment was repeated twice with similar results.

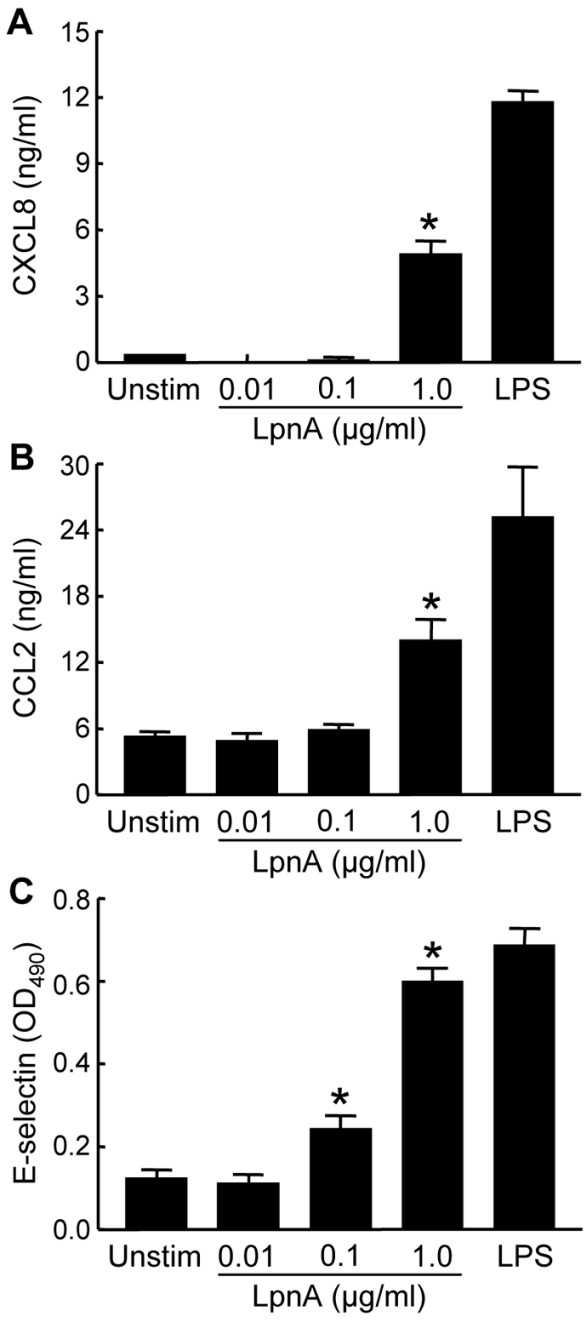

Figure 7.

HUVEC respond relatively poorly to LpnA. (A, B) HUVEC were cultured for 24 h with medium alone (Unstim), recombinant LpnA (0.01 μg/ml to 1.0 μg/ml), or *E.coli* LPS (0.5 ng/ml). Conditioned media then were collected and analyzed by ELISA. Significant increases in the levels of CXCL8 (A) and CCL2 (B) were observed in response to 1.0 μg/ml of LpnA. (C) HUVEC were incubated for 4 h with the same samples as described above. Whole-cell ELISA performed on living monolayers of endothelial cells showed significant upregulation of E-selectin in response to 0.1 μg/ml and 1.0 μg/ml of LpnA. Bars represent the means ± SD of three (A, B) or four (C) replicate samples. *, Significantly different from unstimulated controls, $P < 0.001$. These experiments were repeated once, yielding similar results.

Table 1

Virulence of wild-type and LpnA-deficient *F. tularensis* LVS in C3H/HeN mice

Strain	Number of surviving/infected mice and median time-to-death (days) at an infectious dose of:			
	10^7 CFU, i.d.[a]	10^6 CFU, i.d.[a]	10^5 CFU, i.d.[a]	10^6 CFU, i.n.[b]
F. tularensis LVS	1/20; 4	1/15; 5	7/10; 6	0/5; 7
F. tularensis LVS Δ*lpnA*	3/20; 5	1/15; 5	8/10; 5	0/5; 7

[a] Mice were infected intradermally (i.d.).

[b] Mice were infected intranasally (i.n.).