Identification of a Novel *Francisella tularensis* Factor Required for Intramacrophage Survival and Subversion of Innate Immune Response

Manish Mahawar1,2, Maninjay K. Atianand3,1, Rachel J. Dotson1, Vanessa Mora1, Seham M. Rabadi2, Dennis W. Metzger1, Jason F. Huntley1, Jonathan A. Harton1, Meenakshi Malik1,2,3, and Chandra Shekhar Bakshi4,5

From the 1Center for Immunology and Microbial Disease, Albany Medical College, Albany, New York 12208, the 2Department of Medical Microbiology and Immunology, University of Toledo, College of Medicine, Toledo, Ohio, 43615, 3School of Arts and Sciences, Albany College of Pharmacy and Health Sciences, Albany, New York 12208, and the 4Department of Microbiology and Immunology, New York Medical College, Valhalla, New York 10595

**Background:** The mechanism of immune suppression caused by *Francisella tularensis* SchuS4 is not yet understood. Our results establish that FTL_0325/FTT0831c is a key virulence factor and functions as an immunosuppressant.

**Results:** FTL_0325/FTT0831c genes of *F. tularensis* suppress proinflammatory cytokines by preventing activation of NF-κB signaling.

**Conclusion:** FTL_0325/FTT0831c of *Francisella* is a key virulence factor and functions as an immunosuppressant.

**Significance:** Understanding of such pathogenic mechanisms will define vaccine candidates to prevent tularemia acquired naturally or through an act of bioterrorism.

*Francisella tularensis*, the causative agent of tularemia, is one of the deadliest agents of biological warfare and bioterrorism. Extremely high virulence of this bacterium is associated with its ability to dampen or subvert host innate immune response. The objectives of this study were to identify factors and understand the mechanisms of host innate immune evasion by *F. tularensis*. We identified and explored the pathogenic role of a mutant interrupted at gene locus FTL_0325, which encodes an OmpA-like protein. Our results establish a pathogenic role of FTL_0325 and its ortholog FTT0831c in the virulent *F. tularensis* SchuS4 strain in intramacrophage survival and suppression of proinflammatory cytokine responses. This study provides mechanistic evidence that the suppressive effects on innate immune responses are due specifically to these proteins and that FTL_0325 and FTT0831c mediate innate immune subversion by interfering with NF-κB signaling. Furthermore, FTT0831c inhibits NF-κB activity primarily by preventing the nuclear translocation of p65 subunit. Collectively, this study reports a novel *F. tularensis* factor that is required for innate immune subversion caused by this deadly bacterium.

*Francisella tularensis* is a Gram-negative facultative intracellular bacterium capable of causing lethal disease called tularemia in various species including humans. *F. tularensis* exists as two clinically relevant strains; the highly virulent Type A (*F. tularensis* ssp. *tularensis* (SchuS4)) strains that are often associated with severe clinical course and pneumonic tularemia in North America (1) and Type B (*F. tularensis* ssp. *holarctica*) strains that cause acute but mild self-limiting infections in the Eurasian and American continents (2). The live vaccine strain (LVS) is a derivative of *F. tularensis* ssp. *holarctica*. The other two subspecies, *Francisella novicida* and *Francisella mediasiatica*, are not associated with human disease. In the past, bioterror threats have renewed interest in understanding the pathogenesis of *Francisella*. The Centers for Disease Control has classified *F. tularensis* as a category A agent based on its high virulence and potential use in a terrorist attack. Use of *F. tularensis* as a bioterrorism agent arises from its high infectivity, ease of aerosolization, and dissemination to cause severe pulmonary disease (1, 3, 4). The control of pneumatic tularemia in a large population is difficult due to a lack of a licensed vaccine and ineffective therapies against antibiotic-resistant strains (5). Another critical characteristic of *F. tularensis* is its ability to actively suppress host innate immune responses (6). However, the factors and mechanisms that *F. tularensis* utilizes to interfere with innate immune development are yet unknown.

Activation of NF-κB and MAPK signaling play a central role in immune-dependent bacterial clearance. To dampen the host innate immune response, it is not surprising that several bacterial pathogens have evolved mechanisms to circumvent these signaling events. To inhibit NF-κB, bacterial pathogens adopt...
mechanisms that either involve secretion of effectors with inhibitory TLR-like domains or use type III or IV secretion systems to inject their effectors directly into the host cells (7, 8). *F. tularensis* lacks type III and IV secretion systems; however, it does contain a type IV pilus biogenesis system that secretes soluble proteins via type II-like secretion machinery (9–12). Additionally, a type VI secretion system encoded by *Francisella* pathogenicity island has recently been identified in *F. tularensis*, which is required for intracellular survival and modulation of host cell signaling (13, 14). *F. tularensis* also encodes a functional type I secretion system that is required for pathogenesis (11). A recent study has shown that an unknown *Francisella* factor suppresses proinflammatory cytokine production from infected as well as uninfected bystander cells (6). Another report has speculated that this factor may be secreted in a TolC-dependent fashion to cause immune suppression (15). To date, a limited number of *F. tularensis* factors including intracellular growth locus C, RipA, and antioxidant enzyme catalase (KatG) of *F. tularensis* LVS have been shown to cause innate immune subversion through inhibition of MAPK and NF-κB signaling (16–19). In contrast, *F. tularensis* SchuS4-mediated cytokine suppression is independent of intracellular growth locus C (20), and the roles of RipA or KatG in the immune subversion have not been fully established.

The objectives of this study were to identify factors and understand the mechanisms of host innate immune evasion by *F. tularensis* LVS (Type B) and the highly virulent *F. tularensis* SchuS4 (Type A) strain. We characterized mutants in the FTL_0325 gene encoding OmpA-like protein, which was identified in a transposon screen of *F. tularensis* LVS and its ortholog FTT0831c in the virulent *F. tularensis* SchuS4 for their role in innate immune subversion. We report that OmpA-like proteins encoded by FTL_0325/FTT0831c genes of *F. tularensis* LVS and SchuS4 strains, respectively, are required for intramacrophage survival and suppression of proinflammatory cytokines. We further demonstrate that FTL_0325/FTT0831c proteins of LVS and SchuS4 interfere with NF-κB signaling to restrict proinflammatory cytokines. This study provides an understanding of the innate immune subversion mechanisms of *F. tularensis* especially with reference to the highly virulent SchuS4 strain.

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Culture—** *F. tularensis* LVS (ATCC 29684; American Type Culture Collection) was provided by Dr. K. Elkins (U. S. Food and Drug Administration, Bethesda, MD). *F. tularensis* SchuS4, originally isolated from a human case of tularemia (21), was obtained from the U. S. Army Medical Research Institute for Infectious Diseases (Frederick, MD). All experiments using SchuS4 were conducted within the Centers for Disease Control-certified Biosafety level-3 (BSL-3) facility at Albany Medical College. *F. novicida* was obtained from the Microbiology Core Facility at Albany Medical College. All the *Francisella* strains were cultured in brain heart infusion, Mueller Hinton broth, or Mueller Hinton chocolate agar plates. Brain heart infusion was supplemented with 10% heat inactivated fetal bovine serum (FBS), whereas Mueller Hinton broth was supplemented with calcium chloride, magnesium chloride, ferric pyrophosphate, glucose, and isovitalex (BD Biosciences). For the selection of transposon and gene deletion mutants and transcomplemented strains, kanamycin was included at a concentration of 10–20 μg/ml in Mueller Hinton broth culture or chocolate agar medium (in the case of *F. tularensis*) or 35 μg/ml (in the case of *Escherichia coli*). The FTL_0325 mutant was identified by screening a transposon mutant library in murine alveolar macrophage cell line, MH-S. An in-frame gene deletion mutant of FTL_0325 ortholog, FTT0831c, in SchuS4 and its transcomplemented strain were generated in this study (supplemental Table S1). Wild type (WT) *F. tularensis* LVS and FTL_0325 mutant were killed by exposing to UV light as described earlier (22).

**Macrophages and Cell Lines—**MH-S (murine alveolar macrophage cell line of BALB/c origin) (23) and human THP-1 cells were maintained and cultured in RPMI 1640 medium containing 25 mM L-glutamine, 25 mM HEPES, 10% heat inactivated FBS, 1% sodium pyruvate, and penicillin-streptomycin. The THP-1 cells were differentiated by treating with 100 nM phorbol 12-myristate 13-acetate overnight. The bone marrow-derived derived macrophages (BMDMs) from WT C57BL/6 and TLR2−/− mice and human embryonic kidney 293 (HEK293) T cells were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose, 10% FCS, 1% HEPES, 1% sodium pyruvate, and 1% L-glutamine.

**Site-directed Mutagenesis and Transcomplementation—**An allelic replacement method was used to construct an in-frame FTT0831c gene deletion mutant (ΔFTT0831c) of *F. tularensis* SchuS4 (24). For construction of the ΔFTT0831c mutant, the entire coding region of the FTT0831c gene was deleted by employing an approach described earlier (25, 26). For transcomplementation, the full-length FTT0831c gene was amplified by PCR and cloned in pKK214 vector downstream of GroEL promoter of *F. tularensis* following our recently published protocol (26, 27). The plasmid constructs, bacterial strains, and the primer sequences used in this study are shown in supplemental Table S1.

**Macrophage Invasion and Replication Assays—** Gentamicin protection assays were performed as described earlier (26). In macrophage assays involving live or UV-killed WT *F. tularensis* LVS, SchuS4, FTL_0325, or ΔFTT0831c mutant and its transcomplemented strain, a multiplicity of infection (m.o.i.) of 100 was used. After infection, the culture supernatants were collected at various time points for quantification of cytokines. The macrophages were lysed at various time points, and lysates were diluted 10-fold and plated on Mueller Hinton chocolate agar plates to enumerate the intracellular bacterial replication. The colonies were counted after 48 h, and the results were expressed as cfu/ml. The cell lysates were also used for Western blot analysis and quantification of NF-κB luciferase activity. To quantitate the intramacrophage lysis, *F. tularensis* LVS, *F. novicida*, and FTL_0325 mutant were transformed with pFNLTp6: luciferase construct (kindly provided by Dr. Denise Monack, M. Mahawar, M. K. Atianand, R. J. Dotson, V. Mora, S. M. Rabadi, D. W. Metzger, J. F. Huntley, J. A. Harton, M. Malik, and C. S. Bakshi, unpublished data.)
Subversion of Innate Immune Responses by F. tularensis

Stanford University) and used in an assay as recently published (28).

Cytokine Measurement—A mouse inflammation cytometric bead array kit (BD Biosciences) was used for the measurement of TNF-α, IL-6, and IL-1β. Data were acquired on a FACSAarray instrument (BD Biosciences) and analyzed using cytometric bead array software Version 1.1 (BD Biosciences). The cytokine levels were expressed as pg/ml.

Protein Expression, Cell Culture, DNA Transfections, and Luciferase Assay—FTT0831c open reading frame was cloned into the eukaryotic expression vector pcDNA3.1 (under the control of the CMV promoter). HEK293T cells were transfected with pcDNA3.1-expressing FTT0831c using empty vector as a control. The expression of FTT0831c in transfected cells was validated by Western blot analysis using anti-FTT0831c primary and goat anti-rat HRP-conjugated secondary antibodies.

For luciferase gene reporter assays, HEK293T cells (2 × 10⁵ cells/well) were transfected with 100 ng of plasmid encoding 3xNF-κB luciferase reporter (29) (a generous gift from Dr. Albert S. Baldwin, Lineberger Comprehensive Cancer Center Chapel Hill, NC) in FTT0831c-expressing or control cells transfected with 1000 ng of plasmid:FTT0831c using FuGENE 6 transfection kit (Roche Applied Science) as per the manufacturer’s instructions. The amount of transfected DNA was kept constant by using pcDNA3.1 empty vector. The cells were activated either by treating with TNF-α (20 ng/ml × 3 h) or co-transfecting with a vector containing NF-κB p65 (50 ng). For ISRE-luciferase reporter assays, Cignal ISRE reporter ([Luc] kit (Qiagen)) was used, and the transfections were performed as per the manufacturer’s instructions. After 24 h of transfection, the cells were treated with 1000 units/ml human IFN-β (PBL Biosciences) for 18 h. The cells were lysed using a passive reporter lysis buffer (Promega), and luciferase activity was monitored using a Dual-Luciferase assay kit (Promega) according to the manufacturer’s protocol and read in a Victor Lumimeter (Wallac). The luciferase activity was normalized to Renilla levels.

Western Blot Analysis—HEK293T cells were transfected with empty or pcDNA3.1 expressing FTT0831c (1000 ng) as described above. The cells were lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40 (v/v), 2 mM EDTA, 2 mM DTT supplemented with protease inhibitors (Roche Applied Science)). The protein concentrations were measured by BCA assay. Equal amounts of proteins (~30 μg) were resolved on a SDS-PAGE gel (4–20%; Bio-Rad), transferred to nitrocellulose membrane (0.2 μm), and probed with anti-FTT0831c or anti-β actin antibodies (Santa Cruz Biotechnology). For IL-1β blots, BMDMs were infected with WT F. tularensis LVS or the FTL_0325 mutant at 100 m.o.i., and the cells were lysed, resolved on SDS-PAGE gels, and blotted using anti-IL-1β antibodies (Santa Cruz). The blots were developed using a chemiluminescent substrate and visualized either by autoradiography or on a chemiluminescent imager.

Immunofluorescence—HEK293T cells were transfected with pcDNA3.1:FTT0831c or empty vector as described above. After TNF-α stimulation (20 ng/ml × 30 min), the cells were washed with PBS and fixed with a 3:2 ratio (v/v) of acetone:PBS for 5 min. After 3 washes with 1% bovine serum albumin fraction V (BSA), the cells were blocked with 10% normal goat-serum in 1% (BSA), PBS for 30 min. The cells were stained for endogenous NF-κB p65 using rabbit anti-p65 IgG (1:300) in blocking solution for 1.5 h followed by Alexa 594-conjugated goat anti-rabbit IgG (1:500) for 1 h. The cells were washed three times with 1% BSA, followed by PBS and distilled H₂O. The cells were stained with DAPI to visualize nucleus, mounted with a coverslip, and visualized using an Axio Observer Z1 fluorescence microscope (Zeiss). Images were taken at 20× magnification. Fields were chosen randomly to count the number of cells showing nuclear, cytoplasmic, or diffused staining. More than 200 cells were counted for each transfection condition for quantification of p65 localization.

Binding of anti-FTT0831c antibodies to the cell surface of F. tularensis was detected by immunofluorescent staining. Bacterial cells (1 × 10⁹) untreated or treated with proteinase-K were suspended in 500 μl of PBS containing 5 % BSA and then incubated for 2 h with anti-FTT0831c polyclonal monospecific antibodies, anti-F. tularensis LPS monoclonal, or polyclonal anti-KatG antibodies at a dilution of 1:50 in PBS containing 5 % BSA. After washing twice with PBS, the pellets were incubated for 2 h with 50 μl of Alexa Fluor 488-conjugated goat anti-rat IgG antibodies for FTT0831c or goat anti-mouse antibodies (Invitrogen) at a 1:100 dilution. Cell pellets were washed 3 times with PBS and resuspended in 50 μl of PBS. One drop of the cell suspension was smeared onto a microscope slide, mounted using slowfande gold antifade reagent (Invitrogen), covered with a coverslip, and viewed under a fluorescence microscope. Bacterial cells stained with anti-F. tularensis LPS and another outer membrane protein, anti-KatG, antibodies were used as positive and negative controls, respectively.

Localization of FTT0831c—Spheroplasting and sucrose density gradient centrifugation for localization of FTT0831c was essentially performed as previously reported (30). Sequential fractions were collected from gradients, and densities (g/ml) were calculated based upon refractive indices. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect outer and inner membrane proteins in fractionated sucrose density gradients. Anti-FTT0831c polyclonal monospecific antibodies were used to determine FTT0831c localization in the various membrane fractions. Polyclonal monospecific antibodies against FopA and Mip were used as localization controls for outer membrane, whereas SecY antibodies were used as inner membrane controls.

RNA Isolation and Quantitative RT-PCR—HEK293T cells were transfected with pcDNA3.1:FTT0831c or empty vector as described above. Total RNA was isolated using RNasy RNA purification columns (Qiagen) following the manufacturer’s protocol and treated with DNase I to remove any residual genomic DNA. Quantitative real-time PCR was performed using the SuperScript III Platinum SyBR Green One-Step qRT-PCR kit (Invitrogen) and a CFX96 Real-time PCR Detection System instrument (Bio-Rad). Intron spanning primer sequences were used to amplify: IL-8 (forward, 5’-GCT CTG TGT GAA GGT GCA GT-3’, reverse, 5’-CCA GAC AGA GCT CTC TTC CA-3’); and β-actin (forward, 5’-CCC CCA TGC
CAT CCT GCG TCT G-3'; reverse, 5'–CTC GGC CGT GGT GGT GAA GC-3'). All reactions were run in triplicate, and the specificity of PCR amplification was analyzed by melting curve analysis. C_\text{t} \text{ values were normalized to } \beta\text{-actin, and relative copy number was calculated by the standard } 2^{-\Delta\Delta C_{\text{t}}} \text{ method. Results are represented as fold change over untreated.}

**Statistical Analysis**—All results were expressed as means ± S.E. or S.D. Statistical comparisons between the groups were made using one-way ANOVA followed by Bonferroni’s correction, non-parametric Mann-Whitney test, or unpaired Student’s t test. Differences between the experimental groups were considered statistically significant at a p < 0.05 level.

**RESULTS**

**FTL_0325 Mutant of F. tularensis Induces Significantly Increased Proinflammatory Cytokines in Macrophages**—In our transposon mutant screen, a mutant in FTL_0325 gene of *F. tularensis* was identified that exhibited a 7-fold reduction in the number of bacteria recovered at 24 h post-infection (PI) as compared with the WT *F. tularensis*. We further investigated the role of FTL_0325 in intramacrophage survival by performing in vitro assays. Infection of MH-S cells or primary BMDMs with FTL_0325 mutant resulted in attenuated growth of the mutant (Fig. 1, A and B). Analysis of proinflammatory cytokines revealed significantly elevated TNF-α levels in culture supernatants of FTL_0325 mutant as compared with those observed for WT *F. tularensis* LVS-infected macrophages (Fig. 1, C and D). Similar results were obtained when human undifferentiated or phorbol 12-myristate 13-acetate-differentiated THP1 cells were used (Fig. 1E). These results suggested that FTL_0325 is required for intramacrophage survival and participates in restricting TNF-α production.

Our observation that the FTL_0325 mutant of *F. tularensis* LVS induced proinflammatory cytokine production prompted us to investigate if this feature was specific to the FTL_0325 mutant or is common to other attenuated mutants of *F. tularensis* LVS. It was found that infection of macrophages with FTL_0557 (∆pmrA) or other gene mutants that are as attenuated for intramacrophage growth as FTL_0325 mutant (Fig. 1F) did not induce significant amounts of TNF-α and that the quantitated cytokine levels in the culture supernatants were similar to those observed after infection with WT *F. tularensis* LVS. By comparison, as observed earlier, significantly elevated levels of TNF-α were observed in macrophages infected with FTL_0325 mutant (Fig. 1G). Collectively, these results demonstrate that FTL_0325 is specifically required for the suppression of cytokine responses, and its loss results in significantly increased induction of proinflammatory cytokine TNF-α.

**Subcellular Localization of FTL_0325/FTT0831c of F. tularensis**—Immunofluorescence staining was performed using polyclonal anti-FTT0831c antibodies to determine if FTL_0325 is exposed on the bacterial surface. The anti-FTT0831c antibodies positively stained the bacterial surface, and the staining was lost after treatment with proteinase-K, indicating that the protein is surface-exposed. No staining was observed when the bacteria were stained with antibodies against KatG, indicating the specificity of the surface staining with anti-FTT0831c antibodies. The surface staining of bacterial cells with anti-*F. tularensis* LPS antibodies either untreated or treated with proteinase-K indicated that the loss of anti-FTT0831c staining after proteinase-K treatment is not due to the removal of antibodies by residual protease activity (Fig. 2A). Furthermore, sucrose density gradient fractionation and immunoblotting localized FTT0831c to the outer membrane fractions in *F. tularensis* SchuS4 strain (Fig. 2B), indicating that FTL_0325/FTT0831c proteins localize to the bacterial outer membrane with surface-exposed regions.

**Induction of Proinflammatory Cytokines TNF-α and IL-6 by FTL_0325 Mutant Is not Due to Increased Release of TLR Ligands**—Toll-like receptor-2 (TLR2) plays a major role in innate immunity and induction of proinflammatory cytokines after *Francisella* infection (31). Because FTL_0325 is localized to the outer membrane with surface-exposed structures, we investigated whether its loss results in induction of proinflammatory cytokines due to enhanced TLR2 signaling. The BMDMs derived from WT or TLR2−/− C57BL/6 mice were infected with FTL_0325 mutant or WT *F. tularensis* LVS at an m.o.i. of 100. The results showed that significantly lower numbers of FTL_0325 mutant bacteria were recovered from WT BMDMs at 48 h PI as compared with the WT LVS (Fig. 3A). Contrary to this, FTL_0325 mutant replicated similarly as WT *F. tularensis* LVS in TLR2−/− BMDMs (Fig. 3B). As observed earlier, FTL_0325 mutant induced higher TNF-α and IL-6 levels than WT *F. tularensis* LVS in BMDMs derived from WT C57BL/6 mice. However, both TNF-α and IL-6 were not observed in TLR2−/− BMDMs infected with either *F. tularensis* LVS or FTL_0325 mutant (Fig. 3, C and D). These results indicate that TLR2 signaling is required to control the growth of the FTL_0325 mutant and to drive TNF-α and IL-6 production. These results also led us to hypothesize that loss of FTL_0325 may affect the outer membrane structure, thereby exposing a higher proportion of TLR2 ligands, which may result in an enhanced TLR2-dependent proinflammatory cytokine response. To address this, MH-S cells or the BMDMs derived from C57BL/6 mice were infected with ultraviolet (UV) killed WT LVS (UV LVS) or FTL_0325 mutant (UV FTL_0325), and TNF-α levels were measured in culture supernatants 24 h later. No differences in the levels of TNF-α were observed between UV FTL_0325 and UV LVS-infected macrophages (Fig. 3E). These results indicate that elevated levels of proinflammatory cytokines are not due to an increase in TLR2-dependent recognition of bacteria because of an alteration in the outer membrane structures caused by the loss of FTL_0325. Furthermore, these results also suggest that live *Francisella* and phagosome escape is required for suppression of proinflammatory cytokines and that FTL_0325-mediated suppression of proinflammatory cytokines may not be due to the inhibition of TLR2 *per se*, but rather, the likely targets are TLR2-dependent inflammatory pathways.

**FTL_0325 Mutant Escapes from the Phagosomes**—Escape of *Francisella* from the phagosomes and its subsequent replication in the host cell cytosol is associated with activation and secretion of IL-1β as a result of inflammasome activation (28, 32). To investigate if FTL_0325 mutant of *F. tularensis* escapes the phagosomes similar to its WT counterpart, we performed Western blot analysis to detect proteolytic cleavage and activa-
Subversion of Innate Immune Responses by *F. tularensis*

It was observed that higher levels of bioactive IL-1β were observed in *FTL_0325* mutant-infected macrophages as compared with the WT *F. tularensis* LVS-infected cells 24 h PI (Fig. 4A). Because the cleavage of the pro-form of IL-1β results in active IL-1β, which is secreted in the culture supernatants, levels of secreted IL-1β were also measured in the culture supernatants of infected BMDMs at 12 and 24 h PI. Similar to the Western blot analysis results, significantly higher
Subversion of Innate Immune Responses by *F. tularensis*

Activation of NF-κB Signaling—Our observation that *FTL_0325* likely inhibits signaling pathways downstream of TLR2 led us to investigate downstream signaling events. Because activation of TLR2 signaling leads to activation of NF-κB, we monitored the effects of *FTL_0325* on NF-κB activity by using a NF-κB-responsive firefly luciferase reporter system in HEK293T cells. We first established that both the *F. tularensis* LVS and *FTL_0325* mutant were equally capable of infecting HEK293T cells (not shown). Infection of HEK293T cells transfected with NF-κB-luciferase reporter construct with WT *F. tularensis* LVS suppressed NF-κB-dependent luciferase activity. However, infection with *FTL_0325* mutant induced significantly higher NF-κB-dependent luciferase activity (Fig. 5). These results indicated that *FTL_0325* likely suppresses the induction of cytokines by impairing NF-κB activation.

*FTT0831c* Protein Inhibits NF-κB Activity by Preventing Nuclear Translocation of p65 Subunit—To further establish the role of *FTL_0325* in mediating the suppression of cytokines, we generated an in-frame deletion (Δ*FTT0831c*) and transcomplemented strain (Δ*FTT0831c*+p*FTT0831c*) of its ortholog *FTT0831c* in the highly virulent *F. tularensis* SchuS4 strain. These bacterial strains were analyzed in a macrophage cell culture assay using MH-S cells. The cell lysates were quantified for bacterial replication, whereas the cell culture supernatants were quantified for TNF-α levels. As observed for the *FTL_0325* mutant, Δ*FTT0831c* was attenuated for intramacrophage survival at 48 h PI (Fig. 6A). Furthermore, a significantly decreased TNF-α production was observed in MH-S cells infected with the WT SchuS4 strain as compared with Δ*FTT0831c*-infected macrophages (Fig. 6B). The phenotype of the Δ*FTT0831c* was restored to its WT *F. tularensis* SchuS4 counterpart by transcomplementing the *FTT0831c* gene, confirming that the suppression of cytokine response is mediated specifically by *FTT0831c*.

We observed that *FTL_0325* or *FTT0831c* mutants were able to induce significantly elevated levels of proinflammatory cytokines in infected macrophages. To rule out pleiotropic affects associated with gene deletion in these mutants, the role of *FTT0831c* was examined in the absence of any other *Francisella* protein. To investigate the direct effects of *FTT0831c* protein on NF-κB inhibition, HEK293T cells were co-transfected with plasmids expressing the NF-κB-luciferase and *FTT0831c*-expressing pcDNA3.1 vector. The expression of *FTT0831c* in the transfected cells was confirmed by Western blot analysis (Fig. 6C) and immunofluorescence staining. It was observed that *FTT0831c* was expressed in the cytoplasm of the transfected cells (not shown). The transfected cells were stimulated with TNF-α to induce NF-κB-dependent luciferase gene expression. The luciferase activity increased after TNF-α stimulation in cells transfected with the empty pcDNA3.1 vector, but this level was reduced significantly in the cells co-transfected with the *FTT0831c*-expressing pcDNA3.1 vector. It was observed that transfection with graded amounts of pcDNA expressing *FTT0831c* reduced luciferase activity in a dose-dependent fashion (Fig. 6D). These results corroborated the findings observed with *FTL_0325* mutant of LVS (Fig. 5) and demonstrated that *FTT0831c* can restrict NF-κB-regulated gene levels of secreted IL-1β levels were detected in culture supernatants from *FTL_0325*-infected macrophages 24 h PI (Fig. 4B).

To further investigate if the activation of IL-1β was due to an enhanced intramacrophage lysis of *FTL_0325* mutant, a recently described luminescence assay was performed (28). No differences in the levels of intramacrophage lysis were observed between WT *F. tularensis* and *FTL_0325* mutant (Fig. 4C). These results indicated that similar to the WT *F. tularensis* LVS, *FTL_0325* mutant escapes from the phagosomes into the cytosol and activates the inflammasome that results in the activation and secretion of proinflammatory cytokine IL-1β. Furthermore, the IL-1β activation/secretion is not in response to the excessive release of ligands due to intracellular bacterial lysis in *FTL_0325*-infected macrophages.

Hyper-induced Proinflammatory Cytokine Response in *FTL_0325* Mutant-infected Macrophages Is Due to Enhanced...
expression in the absence of any other Francisella gene products.

To validate the specificity of FTT0831c-induced attenuation of NF-κB activity and to avoid any bias linked to the luciferase assay, effects on IL-8 mRNA expression were also examined by quantitative real-time PCR. Expression of FTT0831c in HEK293T cells suppressed TNF-α-mediated IL-8 expression by nearly 40–50% at 1 and 6 h post-TNF-α treatment (Fig. 6E), demonstrating that FTT0831c acts specifically on NF-κB to attenuate its activity and also affects IL-8 expression. Specificity of attenuation of NF-κB activity by FTT0831c was validated further by performing an interferon-stimulated response ele-
ment (ISRE)-luciferase assay. The HEK293T cells were transfected with ISRE-luciferase reporter plasmid, FTT0831c-expressing, or an empty pcDNA plasmid, the transfected cells were treated with IFN-β/H9252 for 18 h, and luciferase activity was measured. In this assay, FTT0831c did not inhibit IFN-β/H9252-mediated activation of ISRE-luciferase activity, suggesting that FTT0831c has no effect on ISRE-dependent transcription factors (Fig. 6F).

Taken together, these data suggest that FTT0831c selectively inhibits NF-κB activation. We next tested whether the expressed FTT0831c protein acts at the level of p65/RelA subunit of NF-κB and prevents its nuclear translocation. HEK293T cells were transiently transfected with plasmids expressing NF-κB-dependent luciferase reporter and p65 subunit in the presence or absence of FTT0831c-expressing pcDNA3.1. The luciferase activity was quantified as a measure of NF-κB activity. In the cells overexpressing p65 subunit, the FTT0831c protein expression blocked p65-mediated luciferase activity (Fig. 7A), whereas a robust luciferase activity was observed in the control cells. Consistently, HEK293T cells transfected with empty or FTT0831c-expressing pcDNA3.1 plasmid showed a marked reduction in the nuclear translocation of endogenous p65 after TNF-α treatment as detected by immunofluorescence staining with anti-p65 antibodies (Fig. 7, B and C). These results demonstrate that FTT0831c protein exerts its inhibitory effect on NF-κB primar-

**FIGURE 4.** *FTL_0325* mutant escapes from the phagosomes. A, shown is detection of IL-1β activation. Lysates from BMDMs infected with 100 m.o.i. of LVS or *FTL_0325* mutant at 24 h PI were run on a SDS-gel, transferred, and blotted with anti-IL-1β antibodies. Blotting with anti-β actin antibodies was used as the loading control. B, levels of secreted IL-1β were measured in culture supernatants from infected BMDMs at the indicated times. C, quantification of intramacrophage bacterial lysis. Macrophages were infected with an m.o.i. of 100 with the indicated strains carrying pFNLTP6:luciferase construct. The macrophages were lysed at the indicated times, and luminescence was measured in a luminometer (PerkinElmer Life Sciences). *F. novicida* was used as a positive control. The results are representative of two to three independent experiments, expressed as the mean ± S.D., and the p values were determined using one-way ANOVA. In C, comparisons are shown between *F. novicida* and LVS or *FTL_0325* mutant strain. ND, not determined.

**FIGURE 5.** Hyper-induced proinflammatory cytokine response in *FTL_0325* mutant-infected macrophages is due to enhanced activation of NF-κB. MH-S cells were transfected with NF-κB-luciferase construct and infected with *FTL_0325* mutant and *F. tularensis* LVS at 100 m.o.i. for 4 h. The cells were lysed, and luciferase activity was monitored using the Dual Luciferase Assay kit (Promega). The data are expressed as percent luciferase activity and represent the mean ± S.D. from one of the three independent experiments. The statistical significance of the results was examined with Student’s t test, and p values were recorded.
ily by preventing the translocation of p65 subunit to the nucleus.

**DISCUSSION**

OmpA, a major protein component of the *E. coli* outer membrane, has been very well characterized. OmpA in *E. coli* serves a multitude of functions including being a structural protein, a phage, and colicin receptor, mediating serum resistance, playing a role in F-factor-dependent conjugation, and contributing to immune evasion (33). OmpA is expressed at a very high level in *E. coli* but regulated tightly at posttranscriptional level (34). OmpA-expressing *E. coli* prevents macrophage apoptosis by inducing the anti-apoptotic factor, Bcl-xl (35), and suppresses cytokine and chemokine induction by the infected monocytes through inactivation of both NF-κB and MAP kinases (36). Furthermore, OmpA from a meningitis-causing strain of *E. coli* subverts dendritic cell maturation and expression of co-stimulatory molecules CD40, HLA-DR, and CD86. This inhibitory
effect on dendritic cell maturation is also associated with an increase in TGF-β and a decrease in TNF-α, IL-6, and IL-12 (37). Based on the homology to the OmpA domain of E. coli OmpA protein, several proteins have been classified as OmpA-like proteins, and their role in virulence has been very well established in several bacterial pathogens (38–42). In the virulent E. coli strain K1 and other bacterial pathogens such as Pasteurella, Klebsiella, and Neisseria, OmpA-like protein is required for entry and survival within macrophages (43–45).

The OmpA-like protein of F. tularensis is encoded by FTL_0325 gene in LVS and by FTT0831c in the virulent SchuS4 strain. This protein is a 417-amino acid sequence that encodes a 45.75-kDa protein. The annotation of FTL_0325 and FTT0831c as an OmpA-like protein is based on the homologies of their conserved OmpA domains to the C-terminal domain of E. coli OmpA protein (46–48). The OmpA domain of F. tularensis LVS and SchuS4, similar to E. coli OmpA domain, possesses a periplasmic peptidoglycan-associating motif. However, the remainder of OmpA-like protein of Francisella appears to be structurally unique as it does not bear any sequence homology to E. coli OmpA or OmpA-like proteins from other Gram-negative bacteria. The surface localization studies confirmed

FIGURE 7. FTT0831c protein inhibits NF-κB activity by preventing nuclear translocation of p65 subunit. A, HEK293T cells expressing FTT0831c were co-transfected with vector expressing p65 subunit. p65-induced NF-κB luciferase reporter activity was measured. B, HEK293T cells were transfected as in A and treated with TNF-α for 30 min. Immunofluorescence staining was performed to detect the cellular localization of p65 subunit of NF-κB (magnification 20× and 100×; red, p65; blue, nucleus). C, quantification of nuclear localization of p65 subunit of NF-κB is shown. At least 200 cells were counted in randomly chosen fields for each condition and expressed as percent cells with p65 staining. Data presented in A are derived from quadruplicate samples and are cumulative of five independent experiments and are represented as the mean ± S.E. The p values were determined by one-way ANOVA.
that the OmpA-like protein of *Francisella* is localized to the outer membrane, suggesting that surface exposed regions exist. Similar features have been reported for OmpA-like protein of *Leptospira interrogans* (41). *In vivo*, the FTL_0325 mutant of *F. tularensis* LVS was found to be highly attenuated for virulence in mice and induced an early inflammatory response.6 We report here that FTL_0325 and FTT0831c of *F. tularensis* LVS and SchuS4, respectively, mediate their immune subversive effects by interfering with NF-κB signaling. Overall, this study describes a novel virulence factor of *F. tularensis* that is required for intramacrophage survival and innate immune subversion.

The innate immune response represents the first line of defense during infection and uses a number of pathogen-associated molecular pattern recognizing TLRs that converge in the activation of downstream signaling pathways. NF-κB is a key factor downstream of TLRs, and its activation is responsible for controlling the expression of multiple genes involved in the inflammatory response such as TNF-α, IL-1, IL-6, IL-8, and IL-12. Several bacterial pathogens have evolved sophisticated mechanisms to subvert the host immune response by suppressing NF-κB activity either at the level of IκB phosphorylation or nuclear translocation. *Mycobacterium*, *E. coli*, and *Brucella* secrete effector proteins homologous to Toll/IL-1R domains (7, 8, 50, 51), whereas *Salmonella* (52, 53) and *Chlamydia* (54) utilize their secretion systems to inject effector proteins that interfere with the host ubiquitin signaling pathways to suppress IκB ubiquitination and NF-κB activity. In addition to NF-κB, some bacterial pathogens have evolved mechanisms to suppress MAPKs, another key antimicrobial signaling pathway involved in bacterial clearance. *Yersinia*, *Shigella*, and *Bacillus anthracis* inhibit MAPK signaling to suppress inflammatory response that permits bacterial growth and dissemination (55–58). It has been reported that *F. tularensis* LVS suppresses MAPK signaling via intracellular growth locus C and RipA (16, 19). This study reports that the FTL_0325/FTT0831c proteins of both the *F. tularensis* LVS and SchuS4 suppress proinflammatory cytokine response by inhibiting NF-κB signaling. The beneficial effects of NF-κB activation appeared to be related to the increased production of TNF-α as observed in the culture supernatants of macrophages infected with either FTL_0325 or FTT0831c mutants as compared with WT *F. tularensis* LVS or SchuS4. It was also observed that infection of FTL_0325 mutant induced significantly elevated levels of IL-1β. It is expected that this may be due to higher pro-IL-1β levels induced in response to enhanced NF-κB signaling observed in FTL_0325 mutant-infected macrophages. Alternatively, it is also possible that FTL_0325 interferes with activation of inflammasome and is a subject of ongoing investigation.

Several previous studies have reported the requirement of *F. tularensis* structural components for virulence and immune modulation. FopC protein of *F. novicida* and *F. tularensis* SchuS4 is an important virulence factor (59, 60). Moreover, it has been demonstrated that FopC suppresses IFN-γ signaling and thus interferes with generation of an effective innate immune response (59). Several other structural components of both the LVS and *F. novicida* such as MviN, a lipid II flippase (61), RipA, a cytoplasmic membrane protein (19), or the LPS O-antigen (62) and capsular components have been shown to be required for virulence, suppression of proinflammatory cytokines, and cell death in infected macrophages. Results from the present study indicate that FTL_0325 serves to play a role different than these structural proteins. First, unlike FopC, FTL_0325 is not required for maintaining the structural integrity of the bacteria; second, its loss does not result in as severe attenuation of intramacrophage growth as observed for mviN, ripA, or the LPS O-antigen-deficient mutants. A recent report by Peng et al. (28) has challenged the existing paradigm by demonstrating that the hyperinflammatory nature of *F. tularensis* LVS and *F. novicida* mutants deficient in membrane components is due to compromised structural integrity and excessive release of bacterial ligands resulting from bacterial lysis in macrophages and not due to the loss of gene in question. In this study we took three different approaches to rule out that the hyperinflammatory nature of FTL_0325 or FTT0831c mutants of LVS and SchuS4 is not due to 1) compromised membrane integrity, 2) release/exposure of surface TLR ligands, and 3) excessive intramacrophage lysis that releases TLR ligands in the macrophage cytosol to cause NF-κB activation. Our data demonstrate that FTL_0325 mutant does not exhibit any *in vitro* growth defect and increased sensitivity to antibiotics, detergents, or serum, indicating that the membrane integrity is not compromised in this mutant (supplemental Figs. S1 and S2). Furthermore, an identical cytokine response of the MH-S cells and BMDMs to UV-killed LVS and FTL_0325 mutant also suggests that loss of OmpA-like protein does not lead to increased surface-exposed TLR ligands. The activation and secretion of IL-1β in FTL_0325-infected macrophages suggests that the mutant escapes the phagosomes, indicating that the mutant is similar to WT *F. tularensis* in every other respect. Furthermore, intramacrophage lysis studies revealed that both the WT LVS and FTL_0325 mutant exhibit an increased intramacrophage lysis at 24 h; however, the extent of lysis did not differ between the two strains and, thus, does not account for the differences in the proinflammatory cytokine profiles. When comparisons were made with other mutants deficient in membrane components or a hypothetical membrane protein, it was observed that all mutants including FTL_0325 were attenuated for intramacrophage growth (Fig. 1F). However, enhanced innate immune response was observed only in macrophages infected with FTL_0325 mutant, indicating that loss of structural components does not necessarily result in hyper cytokine response (Fig. 1G). Several recent studies have highlighted the differences between the pathogenesis of *F. novicida*, LVS, and SchuS4 strain (63–65) and have shown that infection of mice or macrophages with the former strain results in rapid induction of proinflammatory cytokines, whereas these responses are extremely muted after infection with the latter two strains. *F. novicida*, which was used as a positive control in intramacrophage lysis studies, underwent significantly higher lysis as compared with *F. tularensis* LVS or FTL_0325 mutant. This observation strongly supports the notion that extensive lysis of *F. novicida* may be a major contributor to its hyperinflammatory nature. Collectively, our data demonstrate that the enhanced inflammatory response after infection with mutants of *F. tularensis* deficient in FTL_0325/FTT0831c is not due to
altered membrane integrity, surface exposed ligands, or enhanced intracellular lysis but more specifically to the loss of these proteins.

Our data support the notion that in the absence of OmpA-like protein, the mutant bacteria escape the phagosomes and replicate for a while without undergoing extensive lysis. However, these data do not exclude the possibility that this may enhance blebbing and shedding of outer membrane material in the host cell cytosol resulting in a hypercytokine response. We, therefore, performed functional expression studies with FTT0831c protein of SchuS4 (Figs. 6, D, E, and F, and 7) to eliminate any bias associated with the excessive release of bacterial ligands in macrophages infected with mutants and resulting in a hyperinflammatory phenotype. Our cell-based assays showed that overexpression of FTT0831c suppressed the canonical NF-κB pathway by inhibiting nuclear translocation of the p65 protein, resulting in blockade of NF-κB-dependent activation of inflammatory cytokines. Reduced IL-8 expression and no effect on IFN-β-dependent activation of ISRE further established that FTT0831c-mediated inhibition is relatively specific for NF-κB. Our data suggest that the FTL_0325/FTT0831c proteins of F. tularensis LVS and SchuS4 are targeted into the host cells to specifically inactivate NF-κB to dampen the innate immune response. We speculate that FTL_0325/FTT0831c protein mediates this effect either by direct physical interaction with p65 subunit or by preventing its phosphorylation and subsequent nuclear translocation. Therefore, targeting p65 subunit in the host cell cytoplasm and preventing its nuclear translocation may represent an efficient strategy that F. tularensis adopts to inhibit NF-κB transactivating activity. A similar role has recently been reported for OmpA protein of Klebsiella pneumoniae (66, 67). Collectively, these results demonstrate that FTT0831c of F. tularensis SchuS4 offers this pathogen a selective advantage by allowing immune evasion that is independent of any other Francisella protein. Understanding the molecular mechanism of action of FTT0831c of F. tularensis requires additional studies.

Our initial software predictions indicate that OmpA-like protein of F. tularensis may not span the membrane several times as has been shown for E. coli (47), and thus, it is likely that majority of the protein is surface-exposed. Results of our surface localization and immunofluorescence staining experiments support these predictions and demonstrate that FTT0831c/FTL_0325 localizes to the outer membrane and is exposed on the surface of Francisella. However, in the context of the Francisella infection, the mechanism through which OmpA-like protein on the bacterial surface comes in contact with the NF-κB signaling components remained an unanswered question in this study. Our hypothesis is that Francisella sheds OmpA-like protein during its residence in the mildly acidic environment of the phagosomes and during its rapid replication in the macrophage cytosol. The support for our hypothesis comes from a recent report demonstrating that Francisella produces outer membrane vesicles that may serve as a “vesicle-mediated secretion” system to deliver bacterial proteins in the extracellular milieu (68). Outer membrane vesicles are spherical fragments of bacterial outer membrane that are produced continuously without the concomitant bacterial lysis by several Gram-negative bacteria to mediate inflammatory response and virulence in vivo (49, 69). Interestingly, proteome characterization of outer membrane vesicles from F. novicida revealed the presence of FTN_0346, an ortholog of FTT0831c/FTL_0325 proteins of F. tularensis LVS and SchuS4, respectively (68). Based on these observations, we speculate that outer membrane vesicles containing surface-exposed OmpA-like protein shed abundantly by Francisella in the macrophage cytosol may interact with cytosolic NF-κB-signaling components to mediate its suppressive effect. Additionally, OmpA-like protein may also be released from the dying bacterial cells. A comprehensive understanding of how OmpA-like protein interacts with p65 to subvert NF-κB signaling and induction of proinflammatory cytokines remains a matter of continued investigation in our laboratory.

In conclusion, this study establishes FTL_0325/FTT0831c protein of Francisella as a key virulence factor that functions as an immunosuppressant to facilitate bacterial persistence. Stimulation of the inflammatory response at mucosal surfaces is an essential antimicrobial defense mechanism, and NF-κB plays a central role in the transcriptional regulation of proinflammatory cytokine genes. Suppression of this key signaling pathway via FTT0831c protein by virulent F. tularensis SchuS4 explains one of the mechanisms adopted by this pathogen to down-modulate the host innate immune responses. This study has identified one of the structural proteins of F. tularensis playing a role in the modulation of innate immune response. However, keeping in view the extreme virulence of F. tularensis SchuS4, there might be several other unknown factors that may have a similar role. A better understanding of such pathogenic mechanisms will lead to the identification of defined subunit vaccine candidates for the prevention of tularemia acquired naturally or through an act of bioterrorism.

Acknowledgment—We thank Dr. Zhongtao Zhang, Assistant Professor, New York Medical College, Valhalla for bioinformatics analysis.

REFERENCES

1. Oyston, P. C., Sjostedt, A., and Titball, R. W. (2004) Tularemia. Bioterrorism defense renews interest in Francisella tularensis. Nat. Rev. Microbiol. 2, 967–978
2. Tärnvik, A., Priebe, H. S., and Grunow, R. (2004) Tularemia in Europe. An epidemiological overview. Scand. J. Infect. Dis. 36, 350–355
3. Dennis, D. T., Inglis, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eiten, E., Fine, A. D., Friedlander, A. M., Hauer, J., Layton, M., Lillibridge, S. R., Mcdade, J. E., Osterholm, M. T., O’Toole, T., Parker, G., Perl, T. M., Russell, P. K., and Tonat, K. (2001) Tularemia as a biological weapon. Medical and public health management. JAMA 285, 2763–2773
4. Gallagher-Smith, M., Kim, J., Al-Bawardy, R., and Josko, D. (2004) Francisella tularensis. Possible agent in bioterrorism. Clin. Lab. Sci. 17, 35–39
5. Salslaw, S., Eigelsbach, H. T., Prior, J. A., Wilson, H. E., and Carhart, S. (1961) Tularemia vaccine study. II. Respiratory challenge. Arch. Intern. Med. 107, 702–714
6. Bosio, C. M., Bielefeldt-Ohmann, H., and Belisle, J. T. (2007) Active suppression of the pulmonary immune response by Francisella tularensis Schu4. J. Immunol. 178, 4538–4547
7. Alvarez, J. I. (2005) Inhibition of Toll-like receptor immune responses by microbial pathogens. Front. Biosci. 10, 582–587
8. Noss, E. H., Pai, R. K., Sellati, T. J., Radolf, J. D., Belisle, J., Golenbock, D. T., Boom, W. H., and Harding, C. V. (2001) Toll-like receptor 2-dependent
inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. J. Immunol. 167, 910–918

9. Chakraborty, S., Monfett, M., Maier, T. M., Benach, J. L., Frank, D. W., and Thanassi, D. G. (2008) Type IV pili in *Francisella tularensis*. Roles of pilF and pIlF in fiber assembly, host cell adherence, and virulence. Infect. Immun. 76, 2852–2861

10. Forslund, A. L., Kuoppa, K., Svensson, K., Salomonsson, E., Johansson, A., Byström, M., Oyston, P. C., Michell, S. L., Titball, R. W., Noppa, L., Frithz-Lindsten, E., Forsman, M., and Forsberg, A. (2006) Direct repeat-mediated deletion of a type IV pilin gene results in major virulence attenuation of *Francisella tularensis*. Mol. Microbiol. 59, 1818–1830

11. Gil, H., Benach, J. L., and Thanassi, D. G. (2004) Presence of pilin on the surface of *Francisella tularensis*. Infect. Immun. 72, 3042–3047

12. Hager, A. J., Bolton, D. L., Pelletier, M. R., Brittnacher, M. J., Gallagher, L. A., Kaul, R., Skerrett, S. J., Miller, S. I., and Guina, T. (2006) Type IV pili-mediated secretion modulates *Francisella virulence*. Mol. Microbiol. 62, 227–237

13. Bröms, J. E., Sjöstedt, A., and Lavander, M. (2010) The Role of the *Francisella tularensis* pathogenicity island in type VI secretion, intracellular survival, and modulation of host cell signaling. Front. Microbiol. 1, 136

14. Bröms, J. E., Lavander, M., and Sjöstedt, A. (2009) A conserved α-helix essential for a type VI secretion-like system of *Francisella tularensis*. J. Bacteriol. 191, 2431–2446

15. Platz, G. J., Bublitz, D. C., Mena, P., Benach, J. L., Furie, M. B., and Thanassi, D. G. (2010) A tolC mutant of *Francisella tularensis* is hypercytotoxic compared to the wild type and elicits increased proinflammatory responses from host cells. Infect. Immun. 78, 1022–1031

16. Telepnev, M., Golovilov, I., Grundström, T., Tärnvik, A., and Sjöstedt, A. (2003) *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signaling and secretion of TNF-α and IL-1 from murine macrophages. Cell Microbiol. 5, 41–51

17. Telepnev, M., Golovilov, I., and Sjöstedt, A. (2005) *Francisella tularensis* LVS initially activates but subsequently down-regulates intracellular signaling and cytokine secretion in mouse mononuclear and human peripheral blood mononuclear cells. Microb. Pathog. 38, 239–247

18. Melillo, A. A., Bakshi, C. S., and Melendez, J. A. (2010) *Francisella tularensis* antioxidants harness reactive oxygen species to restrict macrophage signaling and cytokine production. J. Biol. Chem. 285, 27553–27560

19. Huang, M. T., Mortensen, B. L., Taxman, D. J., Craven, R. R., Faust, S., Michaud, S. A., and Norgard, M. V. (2012) Elevated AIM2-mediated pyroptosis triggered by hypercytotoxic *Francisella mutal* mutants is attributed to increased intracellular bacteriolysis. Cellular Microbiology 13, 1586–1600

20. Bedoya, F., Sandler, L. L., and Harton, J. A. (2007) Pyrin-only protein 2 modulates NF-κB and disrupts ASC-CLR interactions. J. Immunol. 178, 3837–3845

21. Hunley, J. F., Conley, P. G., Hagman, K. E., and Norgard, M. V. (2007) Characterization of *Francisella tularensis* outer membrane proteins. J. Bacteriol. 189, 561–574

22. Malik, M., Bakshi, C. S., Sahay, B., Shah, A., Lotz, S. A., and Sellati, T. J. (2006) Toll-like receptor 2 is required for control of pulmonary infection with *Francisella tularensis*. Infect. Immun. 74, 3657–3662

23. Sanapala, S., Yu, J. J., Murthy, A. K., Li, W., Guentzel, M. N., Chambers, J. P., Klose, K. E., and Arulanandam, B. P. (2012) Perforin- and granzyme-mediated cytotoxic effector functions are essential for protection against *Francisella tularensis* following vaccination by the defined *F. tularensis* subspp. novicida opC vaccine strain. Infect. Immun. 80, 2177–2185

24. Prasadarao, N. V., Wiss, C. A., Weiser, J. N., Stins, M. F., Huang, S. H., and Kim, K. S. (1996) Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. Infect. Immun. 64, 146–153

25. Koebnik, R., Locher, K. P., and Van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins. Barrels in a nutshell. Mol. Microbiol. 37, 239–253

26. Sukumar, S. K., Selvaraj, S. K., and Prasadarao, N. V. (2004) Inhibition of apoptosis by *Escherichia coli* K1 is accompanied by increased expression of BclXL and blockade of mitochondrial cytochrome c release in macrophages. J. Immunol. 170, 6012–6022

27. Selvaraj, S. K., and Prasadarao, N. V. (2005) *Escherichia coli* K1 inhibits proinflammatory cytokine induction in monocytes by preventing NF-κB activation. J. Leukoc. Biol. 78, 544–554

28. Mittal, R., and Prasadarao, N. V. (2008) Outer membrane protein A expression in *Escherichia coli* K1 is required to prevent the maturation of myeloid dendritic cells and the induction of IL-10 and TGF-β. J. Immunol. 181, 2672–2682

29. Hu, Q., Han, X., Zhou, X., Ding, C., Zhu, Y., and Yu, S. (2011) OmpA is a virulence factor of *Riemerella anatipestifer*. Vet. Microbiol. 150, 278–283

30. Nair, M. K., Venkitanarayanan, K., Silbart, L. K., and Kim, K. S. (2009) Outer membrane protein A of *Orobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. Foodborne Pathog. Dis. 6, 495–501

31. Nicholson, T. F., Watts, K. M., and Hunstad, D. A. (2009) OmpA of uropathogenic *Escherichia coli* promotes postinvasion pathogenesis of cystitis. Infect. Immun. 77, 5245–5251

32. Ristow, P., Bourhy, P., da Cruz McBride, F. W., Figueira, C. P., Huere, M., Ave, P., Girons, I. S., Ko, A. I., and Picardeau, M. (2007) The OmpA-like protein Loa22 is essential for leptomeningeal virulence. PLoS Pathog. 3, e97

33. Martin, S., Leuzzi, R., Ghisetti, V., De Francesco, M. A., Cusini, M., Impara, G., Galluppi, E., Pizza, M., and Stefanelli, P. (2010) Molecular analysis of two novel *Neisseria gonorrhoeae* virulent components. The macrophage infectivity potentiator and the outer membrane protein A. New Microbiol. 33, 167–170

34. Sukumar, S. K., Shimada, H., and Prasadarao, N. V. (2003) Entry and intracellular replication of *Escherichia coli* K1 in macrophages require expression of outer membrane protein A. Infect. Immun. 71, 9561–9561

35. Ristow, P., Bourhy, P., da Cruz McBride, F. W., Figueira, C. P., Huere, M., Ave, P., Girons, I. S., Ko, A. I., and Picardeau, M. (2007) The OmpA-like protein Loa22 is essential for leptomeningeal virulence. PLoS Pathog. 3, e97

36. Serino, L., Nesta, B., Leuzzi, R., Fontana, M. R., Monaci, E., Mocca, B. T., Cartocci, E., Massignani, V., Jerse, A. E., Rappuoli, R., and Piazza, M. (2007) Identification of a new OmpA-like protein in *Neisseria gonorrhoeae* involved in the binding to human epithelial cells and in vivo colonization. Mol. Microbiol. 64, 1391–1403
Subversion of Innate Immune Responses by F. tularensis

46. Marsh, D. (2000) Infrared dichroism of twisted β-sheet barrels. The structure of E. coli outer membrane proteins. J. Mol. Biol. 297, 803–808
47. Smith, S. G., Mahon, V., Lambert, M. A., and Fagan, R. P. (2007) A molecular Swiss army knife. OmpA structure, function, and expression. FEMS Microbiol. Lett. 273, 1–11
48. Wang, Y. (2002) The function of OmpA in Escherichia coli. Biochem. Biophys. Res. Commun. 292, 396–401
49. Bomberger, J. M., Maceachran, D. P., Coutemarsh, B. A., Ye, S., O’Toole, G. A., and Stanton, B. A. (2009) Long distance delivery of bacterial virulence factors by Pseudomonas aeruginosa outer membrane vesicles. PLoS Pathog. 5, e1000382
50. Yadav, M., Zhang, J., Fischer, H., Huang, W., Lutay, N., Cirl, C., Lum, J., Miethke, T., and Svanborg, C. (2010) Inhibition of TIR domain signaling by TcpC. MyD88-dependent and -independent effects on Escherichia coli virulence. PLoS Pathog. 6, e1001210
51. Cirl, C., Wieser, A., Yadav, M., Duerr, S., Schubert, S., Fischer, H., Stappert, D., Wantia, N., Rodriguez, N., Wagner, H., Svanborg, C., and Miethke, T. (2008) Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. Nat. Med. 14, 399–406
52. Pavlova, B., Wolf, J., Ondrackova, P., Matiasovic, J., Stepanova, H., Crhanova, M., Karasova, D., Faldyna, M., and Rychlík, I. (2011) SPI-1-encoded type III secretion system of Salmonella enterica is required for the suppression of porcine alveolar macrophage cytokine expression. Vet. Res. 42, 16
53. Haraga, A., and Miller, S. I. (2003) A Salmonella enterica serovar typhi-murium translocated leucine-rich repeat effector protein inhibits NF-κB-dependent gene expression. Infect. Immun. 71, 4052–4058
54. Betts, H. J., Wolf, K., and Fields, K. A. (2009) Effector protein modulation of host cells. Examples in the Chlamydia spp. arsenal. Curr. Opin. Microbiol. 12, 81–87
55. Mukherjee, S., and Orth, K. (2008) In vitro signaling by MAPK and NFκB pathways by Yersinia YopJ. Methods Enzymol. 438, 343–353
56. Reiterer, V., Grossniklaus, L., Tschon, T., Kasper, C. A., Sorg, I., and Arrue Romieu, C. (2011) S. flexneri type III secreted effector OsPF reveals new cross-talks of proinflammatory signaling pathways during bacterial infection. Cell. Signal. 23, 1188–1196
57. Chopra, A. P., Boone, S. A., Liang, X., and Duesbery, N. S. (2003) Anthrax lethal factor proteolysis and inactivation of MAPK kinase. J. Biol. Chem. 278, 9042–9046
58. Soundararajan, V., Patel, N., Subramanian, V., Sasisekharan, V., and Sasisekhara, R. (2011) The many faces of the YopM effector from plague causative bacterium Yersinia pestis and its implications for host immune modulation. Innate Immun. 17, 548–557
59. Nallaparaju, K. C., Yu, J. J., Rodriguez, S. A., Zogaj, X., Manam, S., Guntzel, M. N., Seshu, J., Murthy, A. K., Chambers, J. P., Klose, K. E., and Arulananandam, B. P. (2011) Evasion of IFN-γ signaling by Francisella novicida is dependent upon Francisella outer membrane protein C. PLoS ONE 6, e18201
60. Twine, S., Byström, M., Chen, W., Forsman, M., Golovliov, I., Johansson, A., Kelly, J., Lindgren, H., Svensson, K., Zingmark, C., Conlan, W., and Sjöstedt, A. (2005) A mutant of Francisella tularensis strain SCLU54 lacking the ability to express a 58-kDa protein is attenuated for virulence and is an effective live vaccine. Infect. Immun. 73, 8345–8352
61. Ulland, T. K., Buchan, B. W., Ketterer, M. R., Fernandes-Alnemri, T., Meyerholz, D. K., Apicella, M. A., Almenri, E. S., Jones, B. D., Nauseef, W. M., and Sutterwala, F. S. (2010) Cutting edge. Mutation of Francisella tularensis mviN leads to increased macrophage absent in melanoma 2 inflammasome activation and a loss of virulence. J. Immunol. 185, 2670–2674
62. Lindemann, S. R., Peng, K., Long, M. E., Hunt, J. R., Apicella, M. A., Monack, D. M., Allen, L. A., and Jones, B. D. (2011) Francisella tularensis Schu S4 O-antigen and capsule biosynthesis gene mutants induce early cell death in human macrophages. Infect. Immun. 79, 581–594
63. Hall, J. D., Woolard, M. D., Gunn, B. M., Craven, R. R., Taft-Benz, S., Fedlanger, J. A., and Kavula, T. H. (2008) Infected host-cell repertoire and cellular response in the lung following inhalation of Francisella tularensis Schu S4, LV5, or UI12. Infect. Immun. 76, 5843–5852
64. Mortensen, B. L., Fuller, J. R., Taft-Benz, S., Kijek, T. M., Miller, C. N., Huang, M. T., and Kavula, T. H. (2010) Effects of the putative transcriptional regulator IclR on Francisella tularensis pathogenesis. Infect. Immun. 78, 5022–5032
65. Kiefker, T. L., Cowley, S., Nano, F. E., and Elkins, K. L. (2003) Francisella novicida LPS has greater immunobiological activity in mice than F. tularensis LPS and contributes to F. novicida murine pathogenesis. Microbes Infect. 5, 397–403
66. March, C., Moranta, D., Regueiro, V., Llobet, E., Tomás, A., Garmenta, J., and Bengoechea, J. A. (2011) Klebsiella pneumoniae outer membrane protein A is required to prevent the activation of airway epithelial cells. J. Biol. Chem. 286, 9956–9967
67. Regueiro, V., Moranta, D., Frank, C. G., Larrarte, E., Margareto, J., March, C., Garmenta, J., and Bengoechea, J. A. (2011) Klebsiella pneumoniae subverts the activation of inflammatory responses in a NOD1-dependent manner. Cell. Microbiol. 13, 135–153
68. Piersig, T., Matrakas, D., Taylor, Y. U., Manyam, G., Morozov, V. N., Zhou, W., and van Hoek, M. L. (2011) Proteomic characterization and functional analysis of outer membrane vesicles of Francisella novicida suggests possible role in virulence and use as a vaccine. J. Proteome Res. 10, 954–967
69. McBroome, A. J., Johnson, A. P., Vemulapalli, S., and Kuehn, M. J. (2006) Outer membrane vesicle production by Escherichia coli is independent of membrane instability. J. Bacteriol. 188, 5385–5392