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Francisella tularensis Inhibits the Intrinsic and Extrinsic Pathways to Delay Constitutive Apoptosis and Prolong Human Neutrophil Lifespan

Justin T. Schwartz,*† Jason H. Barker,*† Justin Kaufman,*† Drew C. Fayram,*† Jenna M. McCracken,*† and Lee-Ann H. Allen*†‡

Francisella tularensis is a facultative intracellular bacterium that infects many cell types, including neutrophils. We demonstrated previously that F. tularensis inhibits NADPH oxidase assembly and activity and then escapes the phagosome to the cytosol, but effects on other aspects of neutrophil function are unknown. Neutrophils are short-lived cells that undergo constitutive apoptosis, and phagocytosis typically accelerates this process. We now demonstrate that F. tularensis significantly inhibited neutrophil apoptosis as indicated by morphologic analysis as well as annexin V and TUNEL staining. Thus, ~80% of infected neutrophils remained viable at 48 h compared with ~50% of control cells, and ~40% of neutrophils that ingested opsonized zymosan. In keeping with this finding, processing and activation of procaspases-8, -9, and -3 were markedly diminished and delayed. F. tularensis also significantly impaired apoptosis triggered by Fas crosslinking. Of note, these effects were dose dependent and could be conferred by either intracellular or extracellular live bacteria, but not by formalin-killed organisms or isolated LPS and capsule, and were not affected by disruption of wbtA2 or FTT1236/FTL0708—genes required for LPS O-antigen and capsule biosynthesis. In summary, we demonstrate that F. tularensis profoundly impairs constitutive neutrophil apoptosis via effects on the intrinsic and extrinsic pathways, and thereby define a new aspect of innate immune evasion by this organism. As defects in neutrophil turnover prevent resolution of inflammation, our findings also suggest a mechanism that may in part account for the neutrophil accumulation, granuloma formation, and severe tissue damage that characterizes lethal pneumonic tularemia. The Journal of Immunology, 2012, 188: 000–000.

Polymorphonuclear leukocytes (PMNs; neutrophils) represent the largest leukocyte population in human blood and are rapidly mobilized to sites of infection (1). These cells phagocytose microbes and use a combination of reactive oxygen species (ROS) and cytotoxic granule components to generate a highly lethal intraphagosomal environment for killing of ingested microorganisms (2). In circulation, human PMNs have a relatively short lifespan (<24 h) and undergo rapid constitutive (spontaneous) apoptosis (1). However, upon recruitment to inflammatory foci, PMN lifespan is modulated by cytokines, microbial components, and the local environment (1). In particular, phagocytosis of microbes and subsequent NADPH oxidase-dependent ROS production accelerate the apoptotic program, functioning to target spent neutrophils to tissue macrophages for removal from sites of infection (3–5). Timely apoptosis and clearance of PMNs are critical for resolving the inflammatory response, minimizing tissue damage by downregulating the phagocytic and proinflammatory capacity of neutrophils, preventing the release of cytotoxic PMN components into the extracellular compartment, and delivering dying cells to macrophages for disposal (6–8). Some pathogens manipulate PMN function, including apoptosis, to avoid killing and cause disease. For example, Anaplasma phagocytophilum, Chlamydia pneumoniae, and Neisseria gonorrhoeae inhibit PMN apoptosis as a mechanism to protect their intracellular replicative niche (9–11). In contrast, Streptococcus pyogenes, Pseudomonas aeruginosa, Burkholderia cenocepacia, and Staphylococcus aureus markedly accelerate PMN apoptosis or redirect cell death toward necrosis to evade intracellular killing and eliminate neutrophils from sites of infection (3, 12–14).

Francisella tularensis is a facultative intracellular, a Gram-negative bacterium, and the causative agent of the zoonotic disease tularemia (15, 16). The clinical presentation and severity of tularemia depends on the bacterial strain, dose, and route of infection (17). Human infection most commonly occurs after inoculation into the skin by infected arthropods (including ticks, mosquitoes, and deer flies) or through skin breaks when handling infected animal carcasses (15). However, a distinguishing feature of this organism is its extreme virulence when acquired via the respiratory route, whereby inhalation of as few as 10 CFU can cause severe pneumonic disease, sepsis, and death in humans (17). Consequently, F. tularensis was stockpiled by several countries for use as a bioweapon agent and is currently classified as a Category...
A select agent by the Centers for Disease Control and Prevention (15, 16).

The two subspecies of *F. tularensis* that account for nearly all cases of human tularemia differ in both geographic distribution and virulence. *F. tularensis* subspp. *tularensis* is found almost exclusively in North America and is highly virulent, whereas *F. tularensis* subspp. *holarctica* is distributed throughout the Northern Hemisphere and causes milder disease that is rarely fatal (16, 17).

The attenuated live vaccine strain (LVS) of *F. tularensis* subspp. *holarctica* retains many of the pathogenic mechanisms of virulent *F. tularensis* strains during in vitro infections of eukaryotic cells, and for this reason it has been studied widely (15, 16, 18).

The ability of *F. tularensis* to cause rapid overwhelming disease or death at low inocula suggests that this organism has developed effective mechanisms to disrupt the innate immune response. Indeed, *F. tularensis* evades killing by macrophages, monocytes, dendritic cells, and neutrophils, and it resists the lytic effects of serum complement (16, 18–21). Specifically, we and others have shown that killing of virulent *F. tularensis* strains by human PMNs is inefficient in vitro (19, 22, 23) and that *F. tularensis* disrupts oxidant production and escapes the phagosome to the cytosol (19, 22). Of note, several studies have demonstrated that *F. tularensis*-PMN interactions are important in the pathogenesis of tularemia. In both simian and murine models, infected neutrophils accumulate in the airways and alveoli, yet cannot eliminate the organism, and bacterial burden increases throughout the course of infection (24–27). Furthermore, blockade of PMN migration into the lung diminishes disease severity and enhances survival of mice infected with *F. tularensis* strain Schu S4, suggesting that PMN microbicidal mechanisms are not just ineffective, but are dysregulated and harmful to the host (28, 29). As toxic NADPH oxidase-derived ROS are key regulators of PMN apoptosis and this aspect of host defense is impaired by *F. tularensis*, we predicted that PMN lifespan may also be affected. We used a multifaceted approach to test this hypothesis, and our findings demonstrate that this pathogen not only failed to accelerate human neutrophil death; cell lifespan was instead profoundly prolonged via effects on the intrinsic and extrinsic apoptotic pathways.

Materials and Methods

Reagents

Cysteine heart agar was obtained from Becton Dickinson (Sparks, MD). Defibrinated sheep blood was from Remel (Lenexa, KS). Endotoxin-free Dulbecco PBS, HBSS, and HEPES buffer were from Mediatech Incorporated (Herndon, VA). Clinical-grade dextran (molecular mass, 500,000 Da) was purchased from Pharmacosmos A/S (Holbaek, Denmark). Ficol-Paque Plus was obtained from GE Healthcare Biosciences (Uppsala, Sweden). Endotoxin-free HEPES-buffered RPMI 1640 (with and without phenol red) was from Lonza (Walkersville, MD). Staurosporine was obtained from Sigma-Aldrich (St. Louis, MO). PROTOCOL HEMA-3 staining kit was purchased from Fisher Scientific (Kalamazoo, MI). Annexin V-FTTC conjugate was obtained from Invitrogen (Camarillo, CA). APO-BRDU kit was obtained from BD Biosciences (San Jose, CA). The CytoTox-ONE Homogeneous Membrane Integrity Assay and the Caspase-Glo 3/7, -8, and -9 assay kits were purchased from Promega (Madison, WI). Mouse anti-Fas antiserum was obtained from Bio-Optica (Milan, Italy). Staurosporine was obtained from Sigma-Aldrich (Milwaukee, WI) and has been described (32). FTL0708 was disrupted in LVS by group II intron retargeting using Sigma Targetron reagents as we described previously for disruption of the homologous gene FTT1236 in Schu S4 (33). All studies of the virulent type A strain were performed in a biosafety level 3 (BSL-3) facility with select agent approval and in accordance with all Centers for Disease Control and Prevention and National Institutes of Health regulatory and safety guidelines. Wild-type and mutant bacteria were inoculated onto cysteine heart agar supplemented with 9% defibrinated sheep blood (CHAB) and grown for 48 h at 37°C in 5% CO₂ Bacteria were harvested from the plates and washed twice with HBBS (containing Ca²⁺ and Mg²⁺). Formalin-killed (5%) LVS were prepared by incubating washed bacteria in 10% buffered formalin (Sigma-Aldrich) for 30 min at room temperature. Killed bacteria were subsequently washed two additional times with HBBS (containing Ca²⁺ and Mg²⁺) and sterility was confirmed by plating aliquots on CHAB.

Opsonization and infection of neutrophils

Washed bacteria were quantified by measuring absorbance at 600 nm. Unless otherwise indicated, wild-type *F. tularensis* (1 × 10⁹/ml) and yeast zymosan particles were opsonized in 50% pooled human serum for 30 min at 37°C, followed by two washes with HBBS prior to infection of PMNs. Conversely, the FTL0708 and wbtA2 mutants are exquisitely serum sensitive, and both mutant and wild-type bacteria were left not opsonized for studies of these strains. PMNs (5 × 10⁶/ml) were diluted in HEPES-buffered RPMI 1640 (without serum) and mixed with zymosan at multiplicity of infection (MOI) 5:1 or with *F. tularensis* at MOIs ranging from 5:1 to 200:1 as indicated. One-milliliter aliquots of each suspension were transferred into 5-ml polypropylene tubes and subsequently incubated at 37°C with 5% CO₂ for 0–48 h.

Immunofluorescence microscopy

PMNs in polypropylene tubes were washed twice with cold PBS and then cytospun and centrifuged onto acid-washed coverslips. Cells were fixed with 10% formalin, permeabilized in cold (1:1) methanol-aceton, and blocked in PBS supplemented with 0.5 mg/ml NaN₃ and 5 mg/ml BSA using our established methods (19, 34). Bacteria were detected using anti-*F. tularensis* antiserum and secondary Abs conjugated to rhodamine, and DAPI was used to stain PMN nuclei. For studies of infection efficiency, 300 PMNs per coverslip were scored for the presence of 0, 1–5, 6–10, 11–20 or >20 bacteria per cell. In addition, differential staining was performed as we described previously to distinguish bound and internalized cell-associated LVS (34).

Quantitation of phagocytosis and intracellular growth by measurement of CFU

LVS phagocytosis and growth in PMN were quantified as we described (19) with minor modifications. PMNs (5 × 10⁶/ml in RPMI) were infected with LVS at an MOI of 200:1. At the indicated time points, an aliquot of the suspension was removed, and PMNs were lysed using 1% saponin. This sample was used for enumeration of total viable bacteria. The rest of the suspension was centrifuged at 300 × g to separate PMNs from extracellular bacteria, and the cell pellet was washed extensively with PBS to remove unengested LVS. After lysis in 1% saponin, cell pellets and supernatants were serially diluted in PBS and plated on CHAB. Viable intracellular and extracellular bacteria were determined by enumeration of CFUs after 48–72 h at 37°C. In some cases, quantifying intracellular *F. tularensis* was complicated by growth as described above. After 12 h, PMNs were pelleted and washed extensively with PBS. The medium was collected and depleted of bacteria by centrifugation and passage through a 0.2-μm filter, and its sterility was confirmed by

Neutrophil isolation

Heparinized venous blood was obtained from healthy adult volunteers in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. PMNs were isolated using dextran sedimentation followed by density gradient separation as described (31). Neutrophils were suspended in HBBS without divalent cations, counted, and diluted to 2 × 10⁶ cells/ml. Purity of the each preparation was assessed by HEMA-3 staining followed by microscopic analysis, and the suspensions were routinely 95–98% PMNs. In all cases, replicate experiments were performed using PMNs from different donors.

Bacterial strains and growth conditions

Fully virulent, wild-type *F. tularensis* subspp. *tularensis* (type A) strain Schu S4 and the attenuated *F. tularensis* subspp. *holarctica* LVS (ATCC 29268) have been described (22). An LVS Himar transposon mutant lacking *wbtA2* was a gift from Dana Frank (Medical College of Wisconsin, Milwaukee, WI) and has been described (32). FTL0708 was disrupted in LVS by group II intron retargeting using Sigma Targetron reagents as we described previously for disruption of the homologous gene FTT1236 in Schu S4 (33). All studies of the virulent type A strain were performed in a biosafety level 3 (BSL-3) facility with select agent approval and in accordance with all Centers for Disease Control and Prevention and National Institutes of Health regulatory and safety guidelines. Wild-type and mutant bacteria were inoculated onto cysteine heart agar supplemented with 9% defibrinated sheep blood (CHAB) and grown for 48 h at 37°C in 5% CO₂. Bacteria were harvested from the plates and washed twice with HBBS (containing Ca²⁺ and Mg²⁺). Formalin-killed (5%) LVS were prepared by incubating washed bacteria in 10% buffered formalin (Sigma-Aldrich) for 30 min at room temperature. Killed bacteria were subsequently washed two additional times with HBBS (containing Ca²⁺ and Mg²⁺) and sterility was confirmed by plating aliquots on CHAB.
plating on CHAB. Washed PMNs were resuspended in the sterile infection medium at 5 × 10^5 cells/ml and returned to 37°C. At the indicated time points, PMNs were washed with PBS, lysed in 1% saponin, diluted in PBS, and then plated on CHAB for enumeration of CFUs.

**Cytotoxicity assay**

PMNs were left untreated or mixed with *F. tularensis* (MOI, 200:1) or yzymosan (MOI, 5:1) as described above. Aliquots (100 μl) containing 2.5 × 10^5 PMNs were transferred into white-walled, clear-bottom 96-well plates, and PMN viability was assessed using the CytoTox-ONE Homogenous Membrane Integrity Assay (Promega) according to the manufacturer’s instructions. This fluorometric assay measures activity of cytosolic lactate dehydrogenase (LDH) released from cells with damaged plasma membranes. LDH activity was assayed in triplicate wells using cytosolic lactate dehydrogenase (LDH) released from cells with damaged plasma membranes. LDH activity was assayed in triplicate wells using a FLUOstar OPTIMA Microplate fluorimeter (BMG LabTech) with subtraction of the background signal obtained at time zero.

**Morphologic assessment of apoptosis**

Aliquots of PMNs were cytocrifuged onto coverslips at the indicated time points, fixed with 10% formalin for 5 min, and stained using the HEMA 3 kit (Fisher Scientific) according to the manufacturer’s instructions. PMNs were examined by light microscopy and were scored as apoptotic when they exhibited nuclear condensation (4, 35). To quantify the percentage of apoptotic cells in the population, at least 300 cells per coverslip and condition were analyzed in each experiment.

**Detection of externalized phosphatidylserine using annexin V**

Phosphatidylserine (PS) externalization was determined by flow cytometric analysis using an annexin V-FITC conjugate (Invitrogen) according to the manufacturer’s instructions. Propidium iodide (PI) staining was included to distinguish early apoptotic cells from late apoptotic and necrotic PMNs. PMNs were costained with annexin V-FITC and PI in buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) prior to analysis.

**Fas-stimulated apoptosis**

PMNs were treated with 500 ng/ml mouse anti-Fas IgM Ab for 6 h prior to analysis of nuclear morphology or staining with annexin V and PI as described above. Where noted, PMNs were infected with LVS or Schu S4 (MOI, 200:1) for 1 h at 37°C prior to Fas crosslinking.

**Assessment of DNA fragmentation**

PMNs were stimulated with bacteria (MOI 200:1) or zymosan (MOI 5:1) or were left untreated and DNA fragmentation was determined using the Apo-BRDU apoptosis detection kit (BD Biosciences), a modified TUNEL assay. Samples were labeled according to the manufacturer’s instructions with minor modifications. PMNs were fixed with 4% paraformaldehyde for 60 min at 4°C, washed, and stained for 90 min. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickson) and 20,000 events were collected for each sample. Data were analyzed using CellQuest software (Tree Star).

**Caspase activity assays**

PMNs were left untreated or treated with 1 μM staurosporine, 500 ng/ml anti-Fas mAb, zymosan (MOI, 5:1), or *F. tularensis* (MOI 200:1) as described above. The activities of caspases 3, 8, and 9 were measured using Caspase-Glo 3/7, 8, and 9 Assay Kits (Promega) in accordance with the manufacturer’s instructions. In each case, caspase activity was assessed by quantifying the luminescence generated upon cleavage of a caspase-specific proluminogenic instructions. In each case, caspase activity was assessed by quantifying the luminescence generated upon cleavage of a caspase-specific proluminogenic...
that uptake of LVS under these conditions was inefficient at low doses, and an MOI of 200:1 was used to ensure that the majority of PMNs were infected. Representative confocal images are shown in Fig. 1A with quantitation of infection efficiency in Fig. 1B. Specifically, we showed that ~40% of PMNs were infected with one to five LVS each by 3–6 h postinfection (hpi). Between 12 and 48 hpi, we observed a marked increase in both the percentage of infected cells and the bacterial load per cell. At 24 hpi, 80% of PMNs were infected, but the number of bacteria per cell was heterogeneous as indicated by the mixture of lightly, moderately, and heavily infected cells. By 36 hpi, at least 50% of PMNs were heavily infected (>20 LVS per cell), and by 48 hpi the vast majority of PMNs contained large numbers of bacteria. Quantitation of total, PMN-associated, and extracellular CFUs (Fig. 1C) revealed that LVS was viable in serum-free RPMI but did not replicate in this media, whereas the number of PMN-associated bacteria increased 19-fold over the time course examined, and differential staining demonstrated that >97% of PMN-associated LVS were intracellular (not shown). These data indicate that LVS accumulated in PMNs over 48 h, but do not distinguish intracellular growth from continued phagocytosis of extracellular organisms. To quantify intracellular replication directly, we infected PMNs with LVS for 12 h and then removed extracellular bacteria by extensive washing of the cells and sterile filtration of the media. Samples were returned to 37°C, and viable intracellular LVS were quantified by plating PMN lysates for enumeration of CFU at 12, 24, 36, and 48 hpi. By this assay, intracellular LVS replicated 8-fold between 12 and 36 hpi, with no further increase at 48 hpi (Fig. 1D).

Next, we quantified the viability of control and LVS-infected PMNs using the LDH cytotoxicity assay to detect loss of plasma membrane integrity and cell death. As a positive control, neutrophils were stimulated with serum-opsonized zymosan (OpZ), the phagocytosis of which accelerates PMN apoptotic death (10). Unstimulated neutrophils undergo constitutive apoptosis following 12–24 h of in vitro incubation (4, 5, 35). Consistent with this finding, we show that untreated PMNs began losing membrane integrity ~12 h after isolation, and the fraction of dead cells increased progressively over the time course examined (Fig. 2). As expected, death of PMNs that engulfed OpZ was accelerated significantly relative to untreated controls. Conversely, the vast majority of neutrophils incubated with LVS remained viable, and LDH release was significantly diminished at 48 h compared with both control and OpZ-treated cells. These data demonstrate that LVS not only failed to induce PMN death, but also prolonged cell viability.

Infection with *F. tularensis* LVS delays human neutrophil apoptosis

As the LDH release assay does not discriminate between different forms of cell death, and LDH activity can be affected by media composition (41), we used several experimental approaches to assess directly the extent to which LVS modulated the kinetics of PMN apoptosis. Neutrophils undergo distinctive morphologic changes during apoptosis, including cell shrinkage, cytoplasmic vacuolation, and nuclear condensation, that are amenable to single-cell analysis and can be quantified by light microscopy (4, 35). Using this approach, we demonstrated that LVS significantly delayed the progression of neutrophils toward an apoptotic mor-

**FIGURE 1.** *F. tularensis* LVS infects human neutrophils in serum-free media. (A) Representative confocal images of PMNs incubated with LVS at an MOI of 200:1 for 6, 12, 24, 36, or 48 h at 37°C. Bacteria are shown in red and PMNs were detected by differential interference contrast optics and DAPI-staining of nuclear DNA (blue). Arrows indicate infected cells. (B) Infection efficiency was quantified by confocal microscopy. Data indicate the percentage of PMNs containing 0, 1–5, 6–10, 11–20, or >20 bacteria per cell at each time point and are the mean ± SEM (n = 3). Original magnification 3×1000. (C) Total, extracellular (media), and cell-associated (PMN pellet) LVS were quantified by CFU measurement. Data are the mean ± SEM (n = 3). (D) Intracellular growth. PMNs were infected with LVS at an MOI of 200:1 for 12 h, washed extensively to remove extracellular bacteria, and returned to 37°C in sterile media. At the indicated time points, PMNs were lysed with saponin and viable intracellular bacteria were quantified by enumeration of CFU. Data are the mean ± SEM (n = 3).
FIGURE 2. *F. tularensis* LVS prolongs neutrophil viability. PMNs were left untreated or were mixed with OpZ (MOI, 5:1) or LVS (MOI, 200:1) at 37°C. Loss of neutrophil plasma membrane integrity was quantified at 0, 12, 24, and 48 h by measuring release of cytosolic lactate dehydrogenase into the culture medium. Data are the mean ± SEM from three or more independent experiments. *p < 0.05, **p < 0.01, versus PMNs alone.

FIGURE 3. *F. tularensis* LVS delays the progression of human neutrophils to an apoptotic morphology. Untreated PMNs or cells infected with LVS (MOI, 200:1) for the indicated times were fixed and stained with HEMA-3 reagents, and nuclear morphology was analyzed by light microscopy. (A) Representative images of untreated and LVS-infected PMNs. Arrows indicate PMNs with condensed, apoptotic nuclei. Arrowheads indicate intracellular LVS that also stain with HEMA-3. Original magnification ×1000. (B) Pooled data indicate the percentage of cells with apoptotic nuclei determined by microscopic evaluation of >300 cells per time point and are the mean ± SEM (n = 4). **p < 0.01, ***p < 0.001.

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Of note, processing of procaspases to their mature forms is essential but not sufficient for activity, as enzyme function is modulated further by direct association with cytoplasmic regulatory factors (41). We therefore used a luminescence assay to measure caspase-3 activity (Fig. 6B). In agreement with the Western blotting data, caspase-3 activity in untreated neutrophils increased steadily beginning 12 h after isolation. This pattern was conserved using PMNs from different donors and was accelerated by both staurosporine and OpZ (compare Fig. 6B and Supplemental Fig. 1A). Specifically, staurosporine-triggered caspase-3 activity routinely peaked at 6 or 12 h and then declined, whereas OpZ-stimulated activity peaked at 12 or 18 h. In all cases, caspase-3 activity in PMNs undergoing constitutive apoptosis increased progressively 12–24 h after isolation, and analyses of later time points suggest that caspase-3 activity in these cells peaked at ∼30 h and began to decline by 36 h (Supplemental Fig. 1B, 1C). In contrast, caspase-3 activity was consistently and profoundly inhibited by LVS at all time points examined from 12 to 30 hpi (the longest time point examined; Fig. 6B; Supplemental Fig. 1A, 1C). Marked inhibition was also observed using LVS at an MOI of 50:1 (Supplemental Fig. 1A, 1B). Our data demonstrate that the ability of LVS to delay PMN apoptosis is due, at least in part, to defects in processing and activation of executioner caspase-3.

F. tularensis LVS modulates both the intrinsic and extrinsic apoptotic pathways in PMNs

The initiator caspases-8 and -9 are upstream activators of caspase-3. Caspase-8 is the initiator caspase of the extrinsic apoptotic pathway and is activated upon stimulation of surface death receptors (47). The intrinsic apoptotic pathway requires caspase-9, which is activated following release of cytochrome c from permeabilized mitochondria and assembly of the apoptosome (47). We therefore determined whether LVS impaired caspase-3 processing and activity via effects on either initiator caspase.

The extrinsic pathway was initiated using Abs to crosslink and activate Fas at the PMN surface. Similar to caspase-3, activation of caspase-8 can be followed by Western blotting, as procaspase-8 (57 kDa) is cleaved into intermediate p43/p41 forms and subsequently cleaved further to generate a p18 subunit. Consistent with previous reports (48), robust processing of procaspase-8 was
observed within 6 h in PMNs treated with anti-Fas IgM (Fig. 7A). As expected, procaspase-8 processing was slower in PMNs undergoing spontaneous apoptosis, with a marked increase in mature caspase-8 18 h after cell isolation. In contrast, only trace amounts of mature caspase-8 were detected in LVS-infected PMNs at 18–24 hpi (Fig. 7A). Consistent with the blotting data, caspase-8 activity peaked 12–18 h after Fas crosslinking, increased progressively over 24 h in PMNs undergoing spontaneous apoptosis, and it was markedly lower in cells infected with LVS (Fig. 7B).

The intrinsic (mitochondrial) apoptotic pathway plays a critical role in the constitutive turnover of human neutrophils and is also activated by exposure to staurosporine (1, 47, 49–51). Using immunoblotting and luminescence assays, we examined the time course of caspase-9 activation in either control, staurosporine-treated, or LVS-infected PMNs (Fig. 7C, 7D). We observed a time-dependent increase in caspase-9 processing and activity in control neutrophils between 12 and 24 h after isolation that was accelerated by staurosporine. Thus, caspase-9 activity in control PMNs at 24 h was similar to cells treated with staurosporine for only 6 h. Infection with LVS had the opposite effect, as accumulation of mature caspase-9 was delayed relative to both control and staurosporine-treated cells. Moreover, caspase-9 activity remained low in LVS-infected neutrophils and was significantly reduced compared with control cells at all time points examined over 3–24 h. These data demonstrate that, relative to control PMNs, activation of both caspases-8 and -9 was significantly impaired by LVS, and as such suggest that this organism acts at multiple points in the apoptotic cascade to curtail execution of caspase-3 and extend cell lifespan.

**F. tularensis LVS inhibits Fas-mediated PMN apoptosis**

The data shown above suggest that *F. tularensis* can inhibit the extrinsic pathway. To address this in more detail, we tested the ability of LVS to affect apoptosis triggered by Fas crosslinking. PMNs were left untreated or were infected with LVS for 1 h prior to the addition of Fas-activating Abs, and apoptosis was quantified using annexin V-staining and flow cytometry. Consistent with the data shown in Fig. 7A and 7B, 71% of PMNs externalized PS within 6 h of Fas crosslinking, compared with only 10% of control neutrophils or cells exposed to LVS alone (Fig. 8). At the same time, prior exposure to LVS significantly inhibited Fas-stimulated PS externalization, as the percentage of annexin V-positive cells was reduced by 36% (*p < 0.05*). These data strongly suggest that LVS inhibits or overrides the Fas-mediated apoptotic pathway in neutrophils.

**Live and killed bacteria differentially affect PMN apoptosis and CXCL8 secretion**

For certain pathogens, such as *A. phagocytophilum*, killed bacteria retain the ability to prolong PMN lifespan (9), but it is unknown whether this is also true for *F. tularensis*. For this study, LVS was killed by exposure to 10% formalin, washed, opsonized, and then incubated with PMNs. We now show, as judged by LDH release, that fkLVS had a distinct phenotype, because these organisms lost the ability to significantly prolong neutrophil viability compared with untreated control cells (Fig. 9A), yet also failed to accelerate or induce PMN death, as do most particles including OpZ (Figs. 2, 5A). In marked contrast to live *F. tularensis*, fkLVS also failed to alter the constitutive rate of PS externalization detected by annexin V staining (Fig. 9B), indicating an inability of killed bacteria to delay the onset of apoptosis. At the same time, the ability of fkLVS to affect caspase-3 activity was diminished but not ablated. Thus, caspase-3 activity increased more slowly in fkLVS-infected cells than in untreated controls, yet more rapidly than in cells infected with live bacteria (Fig. 9C, Supplemental Fig. 1B). Control experiments indicated that live and fkLVS infected PMNs to a similar extent (data not shown).
Live *C. pneumoniae* stimulates secretion of the antiapoptotic cytokine CXCL8 as a means to prolong PMN lifespan (11). In this study, we show that fkLVS stimulated CXCL8 secretion that was detected as early as 12 hpi, and increased sharply 24–48 hpi, whereas live bacteria did not (Fig. 9D). Our data reinforce the notion that live and killed LVS have distinct effects on PMNs (19) and indicate that, although fkLVS stimulated secretion of CXCL8 and had some capacity to impair caspase-3 activity, this was not sufficient to delay spontaneous PMN apoptosis.

Virulent *F. tularensis* subsp. *tularensis* Schu S4 inhibits caspase-3 activation and prolongs PMN viability

To determine whether the ability to delay PMN apoptosis was conserved in human pathogenic strains of *F. tularensis*, we infected PMNs with virulent *F. tularensis* subsp. *tularensis* strain Schu S4. Parallel samples were left untreated, infected with LVS, or treated with staurosporine. Similar to LVS, Schu S4 prolonged PMN viability as judged by LDH release (Fig. 10A). As expected, staurosporine accelerated PMN apoptosis relative to untreated controls as judged by analysis of nuclear morphology (Fig. 10B) and quantitation of caspase-3 activity (Fig. 10C) over 30 h at 37°C. In marked contrast, both these apoptotic parameters were profoundly diminished and delayed by Schu S4 and LVS. In particular, the fraction of PMNs exhibiting condensed nuclei was reduced more than 5-fold at 30 h (Fig. 10B), and caspase-3 activity was markedly inhibited by both *F. tularensis* strains at 12, 18, 24, and 30 hpi (Fig. 10C). Reproducibility of Schu S4-mediated caspase-3 inhibition was confirmed using PMNs from another donor (Supplemental Fig. 1C). Finally we show that, similar to LVS, Schu S4 also inhibited apoptosis triggered by Fas crosslinking (Fig. 10D).

Delayed apoptosis does not require direct contact between neutrophils and LVS

In this study, we show that infection of human PMNs by *F. tularensis* in serum-free media was inefficient during the first several hours of infection (Fig. 1A, 1B). Nevertheless, exposure of neutrophils to LVS or Schu S4 for only 1 h prior to Fas crosslinking was sufficient to inhibit neutrophil apoptosis initiated via the extrinsic pathway (Figs. 8, 10D). These data suggest that extracellular bacteria have a role in modulating PMN apoptosis in our infection model. To test this hypothesis, we used Transwells equipped with 0.4-μm pore membranes to prevent contact between LVS and PMNs. For each experiment, neutrophils were added to the lower chamber of the Transwell, and LVS was added either to the upper chamber (to prevent direct contact with PMNs) or to the lower chamber (which allowed direct contact and resembled our typical experimental conditions), and PMNs incubated in the absence of LVS were used as controls. We also confirmed by measuring CFUs that LVS did not cross the Transwell membrane (data not shown). Consistent with data shown above, conditions that allowed direct contact between LVS and PMNs markedly delayed neutrophil apoptosis as judged by

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**FIGURE 7.** LVS inhibits processing and activation of the initiator caspases-8 and -9. Untreated PMNs or cells treated with staurosporine (1 μM), anti-Fas IgM (500 ng/ml), or LVS (MOI, 200:1) were incubated at 37°C. (A) Immunoblots of cell lysates obtained at the indicated time points show full-length procaspase-8 (57 kDa), cleavage intermediates p43/p41, and mature caspase-8 (p18). Actin immunoblots demonstrate equal loading. Data shown are from one representative experiment of two. (B) Caspase-8 activity was assessed using a caspase-8–specific proluminogenic substrate. Data indicate relative luminescence units (RLU) and are the mean ± SEM of triplicate samples from a representative experiment (n ≥ 3). **p < 0.01, ***p < 0.001, for LVS-infected versus control PMNs. (C) Mature caspase-9 was detected in PMN lysates using an Ab specific for the 37-kDa processed enzyme. Actin served as a loading control. Data shown are representative of two independent experiments. (D) Caspase-9 activity was measured using a caspase-9–specific proluminogenic substrate. Data indicate RLU and are the mean ± SEM of triplicate samples from one representative experiment (n ≥ 3). *p < 0.05, **p < 0.01, ***p < 0.001, for LVS-treated versus control PMNs as indicated.

**FIGURE 8.** *F. tularensis* LVS inhibits Fas-induced apoptosis. Neutrophils were preincubated with LVS (MOI, 200:1) for 1 h and then treated with 500 ng/ml anti-Fas IgM for an additional 6 h. Apoptosis was measured using annexin V staining and flow cytometry. Data indicate the percentage of annexin V-positive cells and are the mean ± SEM (n = 3). *p < 0.05.

**FIGURE 9.** LVS inhibits Fas-induced apoptosis. Neutrophils were preincubated with LVS (MOI, 200:1) for 1 h and then treated with 500 ng/ml anti-Fas IgM for an additional 6 h. Apoptosis was measured using Annexin V staining and flow cytometry. Data indicate the percentage of Annexin V-positive cells and are the mean ± SEM (n = 3). *p < 0.05.
annexin V-staining (Fig. 11A). However, LVS on the opposite side of the Transwell filter also significantly delayed PMN apoptosis relative to untreated controls (Fig. 11A). Thus, these results extend our findings to show that direct contact enhances but is not essential for the ability of LVS to delay PMN apoptosis.

*F. tularensis* LPS and capsular polysaccharides are neither sufficient nor required for delayed apoptosis

The data in Fig. 11A suggest that one or more factors released or secreted by live *F. tularensis* delay PMN death. Major surface components of this organism include LPS and capsule (30), and most gram-negative bacteria can shed LPS and other surface components via release of outer membrane vesicles or by other mechanisms. We isolated a fraction containing both LPS and capsule from LVS using established procedures (30, 33) (see Materials and Methods), and confirmed sample composition by Western blotting (Fig. 11B). This material was then tested directly for its ability to modulate PMN lifespan. Data shown in Fig. 11C demonstrate that, in marked contrast to the effects of whole *F. tularensis*, annexin V staining was not affected by our capsule and LPS-enriched samples at any of the concentrations tested. Thus, under these conditions, isolated LPS and capsule were not sufficient to delay PMN apoptosis.

Next, we used mutants with defects in LPS O-antigen and capsule biosynthesis to define better the role of surface sugars in modulation of PMN lifespan by LVS. The ΔwbtA2 mutant (32) was a gift from Dara Frank (Medical College of Wisconsin) and is devoid of capsule and O-antigen as judged by Western blotting of bacterial lysates (Fig. 11B), confirming published data (30, 32). FTL0708 was disrupted by group II intron retargeting as described in the Materials and Methods and is the LVS homolog of Schu S4 FTT1236 (33). Disruption of FTL0708 also prevented O-antigen synthesis; however, lysates prepared from this mutant retained weak reactivity with the anti-capsule mAb 11B7, whereas ΔwbtA2 lysates did not (Fig. 11B).

As both mutants are serum sensitive (32 and our unpublished data), studies of these strains used unopsonized wild-type and mutant bacteria. We demonstrate that both mutant strains retained the ability to delay PMN apoptosis to a similar extent as wild-type LVS, as indicated by annexin V staining performed 24 hpi (Fig. 11D). These results are consistent with the data shown in Fig. 11C and further suggest that LPS O-antigen and capsular polysaccharides are dispensable for the ability of LVS to delay PMN apoptosis.

**Discussion**

Innate immune defense against invading bacteria relies heavily on the aggressive response of neutrophils at sites of infection. These phagocytes are equipped with potent antimicrobial systems that collaborate to create a highly lethal intraphagosomal environment. However, effective neutrophil function at infection sites extends beyond containment and killing of bacterial invaders. Neutrophils also have a critical role in orchestrating the resolution phase of inflammation, undergoing controlled cellular demolition through apoptosis to downregulate their proinflammatory capacity and target spent cells to macrophages for disposal (4, 6, 7). Therefore, neutrophil apoptosis at sites of infection is a characteristic feature of an effective immune response and is essential for resolution of the inflammation following bacterial infection (52).

In the current study, we examined the extent to which the facultative intracellular pathogen *F. tularensis* modulates human neutrophil apoptosis. We used a serum-free assay system developed by Gardai et al. (37) to avoid confounding effects of growth factors and other serum components on the PMN lifespan, and we followed established guidelines to quantify the rate and extent of cell death and apoptosis (41). In our hands, untreated control PMNs began to exhibit signs of apoptosis at 12 h, and 60–70% of cells were apoptotic by 24 h, confirming published data (4, 5, 35, 39). As reported previously, we show that procaspases-8, -9 and -3 were processed and activated during constitutive PMN apoptosis (47, 48, 53), and we confirm the ability of OpZ, staurosporine, and anti-Fas IgM to significantly accelerate the onset of apoptosis relative to untreated controls (10, 46, 54).

In regard to neutrophils and *F. tularensis*, we used the LDH release assay to quantify cell death as indicated by loss of plasma membrane integrity, and we show for the first time, to our knowl-
edge, that *F. tularensis* significantly prolongs human neutrophil lifespan. Thereafter, we used multiple complementary biochemical assays to demonstrate definitively that the constitutive apoptosis program of human neutrophils was impaired for at least 48 h. Specifically, our data indicate that, relative to control cells, *F. tularensis* profoundly diminished the fraction of PMN exhibiting morphologic signs of apoptosis, such as nuclear condensation. Consistent with this finding, DNA fragmentation detected by TUNEL staining was also significantly impaired, as was the rate of PS externalization detected using annexin V-FITC. Of particular note, the rate and extent of executioner caspase-3 processing and activation were markedly affected both the extrinsic and intrinsic apoptotic pathways. Finally, we demonstrate that in our assay system uptake of *F. tularensis* was inefficient, yet PMNs accumulated large numbers of bacteria over the 48 h time course examined. Additional experiments revealed that neutrophil bacterial load increased 19-fold overall, and direct measurement of intracellular growth revealed 8-fold replication of LVS between 12 and 36 hpi. In contrast, bacteria were viable but did not replicate in the tissue culture medium, which is consistent with the absence of cysteine, a critical nutrient for *Francisella* (55), in serum-free RPMI 1640. These data are of interest as live *N. gonorrhoeae* and heat-killed *Escherichia coli* can delay PMN apoptosis at a low MOI (0.01:1 to 1:1), but this effect is rapidly negated and reversed by ROS at higher bacterial loads (5, 10, 57). In contrast, our published data demonstrate that *F. tularensis* prevents oxidant production via effects on NADPH oxidase assembly and activity (19, 22), and we show in this study that this bacterium inhibits PMN apoptosis in a dose-dependent manner (Fig. 4C) and under conditions in which intracellular bacterial burdens were high (Fig. 3A). Thus, disruption of the oxidative burst likely accounts in large part for the fact that *F. tularensis* does not induce PMN apoptosis. However, chronic granulomatous disease neutrophils exhibit no apparent defects in constitutive apoptosis despite their profound inability to accelerate the apoptotic program upon stimulation (1, 36). Therefore, defects in oxidant production likely cannot account for the ability of *F. tularensis* to inhibit basal PMN turnover.

Although the majority of neutrophils were infected with *F. tularensis* by 12 h under the conditions used in this study (Fig. 1A, 1B), and cells containing large numbers of bacteria did not exhibit signs of apoptosis at 24 or 36 hpi (Fig. 3A), we found to our surprise that inhibition of apoptosis could be uncoupled from phagocytosis and that bacterial uptake was not essential for this process. This was suggested first by the ability of *F. tularensis* to impair apoptosis at...
a markedly lower MOI (EC₅₀ of 5:1; Fig. 4C), although only a subset of cells were infected. Furthermore, using Transwell supports to prevent direct contact between neutrophils and bacteria, we observed that PMN lifespan was significantly prolonged, albeit with somewhat diminished efficiency (Fig. 11A). We favor a model in which intracellular and extracellular bacteria collaborate to extend PMN viability, but it remains to be determined whether intracellular and extracellular F. tularensis have independent, sequential or synergistic effects on neutrophil apoptosis.

How F. tularensis extends PMN lifespan is unknown, but the ability of the organism to act at a distance suggested a role for factors released into the extracellular milieu. LPS is shed by many bacteria, and C. pneumoniae is thought to extend PMN longevity via the ability of its LPS to stimulate secretion of the antiapoptotic cytokine CXCL8 (11). In contrast, we show that samples containing F. tularensis LPS and capsule had no apparent effect on PMN viability and turnover, and that live bacteria did not trigger release of CXCL8. These results are in keeping with the relatively inert nature of F. tularensis LPS (16) and strongly suggest that F. tularensis and C. pneumoniae inhibit apoptosis by different mechanisms. At the same time, it has long been known that LPS O-antigen and capsular polysaccharides act in concert to render F. tularensis resistant to the lytic effects of serum complement (16, 21, 33). Our studies of isolated capsule as well as mutant strains with defects in capsule and O-antigen synthesis revealed that these surface sugars are neither necessary nor sufficient for delayed PMN apoptosis. These data are noteworthy because we recently reported that a Schu S4 mutant lacking functional FTT1236, the homolog of FTL0708, triggers rapid lysis and death of human macrophages (33). As a result, our findings support a large body of data indicating that pathogens manipulate apoptosis and other mechanisms of death in a cell type-specific manner (58).

Perhaps the best characterized pathogen known to delay neutrophil apoptosis is A. phagocytophilum. This obligate intracellular bacterium uses a multifaceted approach to extend the lifespan of its replicative niche for up to 90 h. Similar to F. tularensis, A. phagocytophilum acts in a dose-dependent manner to disrupt production of ROS by the NADPH oxidase; inhibit caspase-3, -8, and -9 processing and activity; and counteract the effects of Fas crosslinking (9), and this is achieved via effects on multiple intracellular signaling pathways as well as PMN gene expression (9). Unlike F. tularensis, killed A. phagocytophilum also significantly inhibits apoptosis (9, 59), and it has been proposed that surface molecules of live and killed bacteria initiate signaling that impairs apoptosis early in infection, whereas PMN viability is prolonged further by mechanisms specific for live organisms (59).
Consistent with this, recent data indicate that Ats-1, delivered into PMNs by live *A. phagocytophilum*, acts directly on mitochondria to preserve their integrity and prevent release of proapoptotic factors into the cytosol (9). The extent to which *F. tularensis* modulates neutrophil signaling, gene expression, or mitochondrial integrity as a means to prolong cell lifespan is currently under investigation.

Neutrophils are key regulators of the inflammatory response. In addition, granuloma formation, prolonged PMN viability, and tissue necrosis are all hallmarks of an aberrant and defective inflammatory response (52, 56, 60). Studies of primates, rabbits, and mice with tularemia indicate that PMN accumulation, pyogranuloma formation, and tissue necrosis are also prominent histologic features of tissues infected with *F. tularensis* (24–26). Moreover, blockade of PMN migration into the lung diminishes tissue damage and favors survival of mice infected with this organism (28). Because PMN apoptosis is essential to limit tissue injury, particularly in the lung (61), and we demonstrate here that *F. tularensis* profoundly inhibits this process, our findings support a model in which neutrophils have a prominent role in dysregulation of the inflammatory response during tularemia. The effects of delayed PMN apoptosis can be exacerbated by defects in corpse removal by macrophages, which increases the probability that dying neutrophils will progress to secondary necrosis with spilling of toxic cell contents and alarmins that amplify inflammation and tissue damage (6–8, 60, 62). During tularemia, effecrocytosis is directly undermined by intramacrophage bacteria (63) and may be compromised further by local macrophage depletion (64). Moreover, end-stage tularemia is characterized by overwhelming sepsis (63, 65, 66), which is itself associated with decreased neutrophil apoptosis (61). Koedel et al. (67) demonstrated a benefit of inducing neutrophil apoptosis during pneumococcal meningitis, in which tissue damage is associated with increased longevity of neutrophils recruited to the brain. Therefore, one strategy posed to manage infectious diseases in which neutrophils play a role in disease pathology is to combine antibiotic therapy with drugs that induce PMN apoptosis (6). It is unclear whether a similar approach would be of therapeutic value in tularemia.

In summary, the results of this study extend previous work to demonstrate for the first time, to our knowledge, that *F. tularensis* acts at multiple points to disrupt constitutive apoptosis and prolong human neutrophil lifespan. This is achieved by a mechanism that can be uncoupled from phagocytosis, is independent of major surface carbohydrates of the organism, and cannot be recapitulated by isolated LPS. Moreover, as PMN apoptosis is essential for resolution of the inflammatory response, our data define a new mechanism of innate immune evasion by *F. tularensis* and suggest a model to account, at least in part, for the in vivo histopathology that is characteristic of this disease. Although the bacterial components involved in these phenomena remain obscure, the ability of extracellular *F. tularensis* to act at a distance to modulate PMN lifespan at a low MOI may contribute to pathogenicity by altering neutrophil function as soon as these cells enter infected tissues, which is consistent with recent data (68). Collectively, the results of this study substantially advance our understanding of tularemia pathogenesis.

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Disclosures

The authors have no financial conflicts of interest.

References


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SUPPLEMENTAL FIGURE 1. Caspase-3 activity measured using PMNs from additional donors.  

A, Data obtained using PMN from two additional donors demonstrate that staurosporine (1 µM) and OpZ (5:1) accelerate caspase-3 activation relative to untreated controls, whereas LVS (at MOI 50:1 or 200:1) impairs this process. *P< 0.05 and ***P< 0.001 for LVS vs. control PMNs.  

B, Relative effects of live and formalin-killed LVS (each at MOI 50:1) on caspase-3 activation. *P<0.05, ***P<0.001.  

C, Cells from another donor confirm the ability of Schu S4 and LVS at MOI 200:1 to inhibit caspase-3 activation in PMN. In contrast, PMN caspase-3 activation was accelerated by 1 µM staurosporine. ***P<0.001 for Schu S4 and LVS vs. PMN control.