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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: 3351–3363.

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 pneumo- niae, 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 pulmonary disease, sepsis, and death in humans (17). Consequently, F. tularensis was stockpiled by several countries for use as a biowarfare 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 widely used (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 pathway 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). Ficoll-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-FITC conjugate was obtained from Invitrogen (Camarillo, CA). APO-BRDU kit was obtained from BD Biosciences (San Jose, CA). The CytoTox-ONE Homogenous Membrane Integrity Assay and the Caspase-Glo 3/7, -8, and -9 assays were obtained from Promega (Madison, WI). Mouse anti-Fas (human, activating) mAb (clone CH-11) was obtained from Millipore (Temecula, CA). Rabbit anti-F; *F. tularensis* antiserum was obtained from BD Diagnostics (Sparks, MD). Mouse anti-caspase-3 mAb (clone C33) and rabbit anti-active caspase-9 polyclonal Ab were from BioVision Research Diagnostics (Sparks, MD). Mouse anti-caspase-8 mAb (clone IC12) was obtained from Cell Signaling Technologies (Danvers, MA). Mouse anti-actin mAb (clone JLA20) was obtained from Calbiochem (Darmstadt, Germany). Mouse mAb FB11, specific for *F. tularensis* LPS, was obtained from QED Biosciences (San Diego, CA), and mouse mAb 11B7 to *F. tularensis* capsule (30) was a gift from Michael Apicella (University of Iowa, Iowa City, IA). Rhodamine-conjugated donkey-anti-rabbit F(ab')2 was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP-conjugated goat-anti-mouse IgG (H+L) was from Bio-Rad Laboratories (Hercules, CA). DAPI and Pierce SuperSignal West Pico ECL substrate were purchased from Thermo Scientific (Rockford, IL).

**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 HBSS without divalent cations, counted, and diluted to $2 \times 10^7$ 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* subsp. *tularensis* (type A) strain Schu S4 and the attenuated *F. tularensis* subsp. *holarctica* LVS (ATCC 29684) have been described (22). An LVS HImar transposon mutant lacking wba2 was a gift from Dara Frank (Medical College of Wisconsin, Milwaukee, WI) and has been described (32). FTL0708 was disrupted in LVS by group II intron retargeting using Sigma Targetrogen 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 HBSS (containing Ca2+ and Mg2+). Formalin-killed (fk) 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 HBSS (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* ($1 \times 10^{9}$/ml) and yeast zymosan particles were opsonized in 50% pooled human serum for 30 min at 37°C, followed by two washes with HBSS prior to infection of PMNs. Conversely, the FTL0708 and wba2 mutants were exquisitely serum sensitive, and both mutant and wild-type bacteria were left not opsonized for studies of these strains. PMNs ($5 \times 10^6$/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 cytocoentrifuged onto acid-washed coverslips. Cells were fixed with 10% formalin, permeabilized in cold (1:1) methanol-acetone, 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 ($5 \times 10^6$/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 $\times$ g to separate PMNs from extracellular bacteria, and the cell pellet was washed extensively with PBS to remove uningested 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 and quantified using a QBC Pro 2 counting system (West Grove, PA). Results were calculated using the formula 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...
plating on CHAB. Washed PMNs were resuspended in the sterile infection medium at 5 × 10⁶ 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 zymosan (MOI, 5:1) as described above. Aliquots (100 μl) containing 2.5 × 10⁵ 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 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 cytospun onto coverslips at the indicated time points, fixed with 10% formalin for 15 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 quantity 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 co-cultured with annexin V-FITC and PI in 10 μM HEPE/SNaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂ prior to analysis using a FACS-Calibur flow cytometer (Becton Dickson). Twenty thousand events were collected for each sample, and the data were analyzed using CellQuest (Becton Dickson) and FlowJo software (Tree Star).

**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 FACS-Calibur flow cytometer (Becton Dickson) and 20,000 events were collected for each sample. Data were analyzed using CellQuest software.

**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, Fas mAb, zymosan (MOI, 5:1), or *F. tularensis* (MOI 200:1) as described above. At the indicated time points, PMNs were pelleted and then resuspended in ice-cold protease inhibitor mixture (TBS containing aprotinin, leupeptin, PMSF, sodium orthovanadate, AEBSF, levanoside, bivalent, E-64, and pepstatin A) and incubated on ice for 10 min. PMNs were subsequently lysed using 1% NP-40 and clarified by centrifugation. Protein concentrations were determined using the BCA Protein Assay (Pierce). Twenty micrograms of each sample were separated using NuPAGE 4–12% Bis-Tris gradient gels and then transferred to polyvinylidene fluoride (PVDF) membranes. Western blot analyses for processing of caspases 3, 8, and 9 were performed using mouse anti-caspase-3 mAb (1 μg/ml, BioVision), mouse anti-caspase-8 mAb (1:1000 dilution; Cell Signaling Technologies), and rabbit anti-active caspase-9 polyclonal Ab (1 μg/ml, BioVision). Mouse anti-actin mAb (1:10,000 dilution; Calbiochem) was used as the loading control. Bands were detected using HRP-conjugated secondary Abs and Pierce Supersignal West Pico ECL reagents.

**CXCL8 ELISA**

Where noted, tissue culture media from control or infected PMNs were collected, sterile filtered (0.2 μm), and assayed for the presence of CXCL8 according to the manufacturer’s directions (R&D Systems, Minneapolis, MN).

**Isolation of F. tularensis LPS and capsule**

LVS was grown on CHAB for 48 h at 37°C in 5% CO₂. LPS and capsule were isolated using a modification of a recently described method, but omitting the final steps in which LPS and capsule are separated from one another (30). Specifically, bacteria were scraped into PBS, pelleted, and resuspended in 2% SDS containing 10 mM EDTA and 60 mM Tris (pH 6.8). This suspension was brought to 50 μg/ml of proteinase K, heated to 65°C for 45 min, and then incubated at 37°C overnight. Samples were washed three times by precipitation in 0.3 M sodium acetate and three volumes of ethanol at −20°C; this was followed by freeze treatment, hot phenol extraction, and then ethanol precipitation. To remove any remaining DNA and ethanol, this final pellet was subjected to two rounds of ultracentrifugation in 14 ml of water at 147,000 × g for 75 min at room temperature. The resulting clear pellet was raised in HPLC-grade water, lyophilized, and weighed. The final product lacked detectable protein as determined by colloidial gold staining and was devoid of DNA as indicated by lack of absorbance at 260 nm (not shown).

**Immunoblot analysis of bacterial lysates and isolated LPS/capsule**

Bacterial whole cell lysates (from 5 × 10⁹ cells in 200 μl) were heated to 72°C for 10 min in SDS loading buffer. Fifteen microliters of each bacterial lysate or 5 μl of the LPS/capsule preparation (1.0 mg/ml) were separated on NuPAGE 4–12% Bis-Tris gradient gels and then transferred to polyvinylidene fluoride. Blocked membranes were probed with mAb11B7 (1:1,000) to detect capsule or mAb FB11 (1:10,000) to detect LPS. Bands were visualized using HRP-conjugated secondary Abs and Pierce Supersignal West Pico ECL reagents.

**Statistical analysis**

Data from experiments containing a control group and one experimental group were analyzed using an unpaired two-sided Student *t* test. Data from experiments containing multiple experimental groups were analyzed with one-way ANOVA followed by a Tukey posttest. All analyses were performed using GraphPad Prism version 4.0 software; *p* < 0.05 was considered statistically significant.

**Results**

*F. tularensis LVS prolongs human neutrophil lifespan*

Neutrophils are intrinsically short-lived phagocytes that undergo constitutive apoptosis (1, 4, 5). This inherent death program is accelerated markedly by ROS production that typically accompanies phagocytosis (3–5) and does not occur in PMNs from persons with chronic granulomatous disease (CGD) (1, 36). As neither virulent strains of *F. tularensis* nor LVS trigger NADPH oxidase activation in PMNs (19, 22), we hypothesized that *F. tularensis* alters PMN lifespan.

To begin to address this question, purified human neutrophils were incubated in suspension with preosposorized *F. tularensis* LVS in serum-free RPMI 1640 media. This established approach for studies of PMN apoptosis (37) is preferred because it avoids confounding effects of growth factors and other serum components on cell viability (38–40). Microscopic analysis demonstrated...
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 observed 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 PMN 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 ≥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).
In independent experiments, data were the mean ± SEM from three or more independent experiments. *p < 0.05, **p < 0.01, versus PMNs alone.

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.

Using HEMA-3 reagents, and nuclear morphology was analyzed by light microscopy. (Fig. 3A). By 12 h, the percentage of apoptotic cells in the control and LVS-treated populations diverged, with control PMNs exhibiting condensed nuclei with significantly faster kinetics than the infected cells at later time points (Fig. 3B). Thus, 90–100% of control neutrophils appeared apoptotic by 36 h compared with ~40% of LVS-treated PMNs. These data confirm and extend the data shown in Fig. 2 and strongly suggest that LVS modulates neutrophil apoptosis to extend cell lifespan.

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.

Morphologic changes associated with apoptosis are a late manifestation of this programmed cell death pathway. In contrast, redistribution of PS from the inner leaflet of the plasma membrane to the cell surface occurs early in apoptosis (42). Using FITC-tagged annexin V, which binds with high specificity to externalized PS, we used flow cytometry to identify and quantify the number of apoptotic cells in the PMN population. In addition, cells were costained with the membrane-impermeable dye PI. This double-staining approach allows three subgroups of cells to be distinguished: viable, non-apoptotic cells are annexin V negative and PI negative (lower left quadrant); early apoptotic cells with intact plasma membranes are annexin V positive and PI negative (upper right quadrant); and late apoptotic cells with compromised plasma membranes are annexin V positive and PI positive (lower right quadrant). Our data demonstrate that at 12, 24, and 48 h, the control and LVS-infected neutrophil populations diverged significantly. At 24 and 48 hpi, markedly fewer LVS-treated PMNs were annexin V positive compared with uninfected controls. Representative FACS plots are shown in Fig. 4A, and pooled data from 12 independent experiments are shown in Fig. 4B. PI staining of the infected neutrophil population was also reduced at 24 and 48 hpi (Fig. 4A), confirming that LVS maintained PMN plasma membrane integrity as indicated by the LDH cytotoxicity data shown above. Of note, these effects of LVS were dose dependent, with a profound decline in PS externalization achieved at an MOI of 5:1 and maximum inhibition achieved at an MOI of ∼100:1 (Fig. 4C). During the late stages of apoptosis, caspase-activated DNases cleave nuclear DNA. The TUNEL assay labels with a fluorescent marker the exposed 3-hydroxy termini of cleaved, fragmented DNA, enabling apoptotic cells to be identified and quantified by flow cytometry (42). Using this approach we demonstrate that, relative to untreated control PMNs, DNA fragmentation was significantly delayed by LVS at all time points examined between 12 and 48 hpi (Fig. 5A, 5B). Approximately 60% of the untreated PMNs were TUNEL positive at 24 h, whereas only 30% of LVS-infected PMNs exhibited fragmented DNA. As an additional control, we confirmed that OpZ accelerated PMN apoptosis, as indicated by increased TUNEL labeling at 24 h (untreated versus OpZ-stimulated PMNs: 60% and 77% TUNEL-positive, respectively; Fig. 5A). Collectively, these data indicate that F. tularensis LVS markedly impairs PMN apoptosis as indicated by morphologic analysis as well as annexin V and TUNEL staining.

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 using 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.

Collectively, these data indicate that F. tularensis LVS markedly impairs PMN apoptosis as indicated by morphologic analysis as well as annexin V and TUNEL staining.

F. tularensis LVS inhibits caspase-3 processing and activity in PMNs

Caspase-3 is the primary effector caspase in human neutrophils and is responsible for the proteolytic cleavage of cellular targets that ultimately lead to apoptotic cell death (43, 44). Caspase-3 is synthesized as an inactive proenzyme that is processed into large (17 kDa) and small (12 kDa) subunits that associate to form a mature active enzyme (45). Using Western blotting and densitometric analysis, we followed the time course of procaspase-3 processing in untreated and LVS-infected PMNs, and we used OpZ- and staurosporine-treated treated cells as positive controls (Fig 6A). In untreated PMNs undergoing constitutive apoptosis, trace processing of procaspase-3 to its mature form was detected at 6 h. Procaspase-3 processing increased markedly by 12 h and progressed to near complete depletion of procaspase-3 by 48 h. In PMNs treated with staurosporine, a potent proapoptotic stimulus (46), procaspase-3 processing was rapid, with substantial amounts of the mature enzyme present by 6 h, and near complete depletion of procaspase-3 by 12 h. Uptake of OpZ also accelerated procaspase-3 processing relative to untreated PMNs as indicated by increased mature caspase-3 at 6 h, and more rapid depletion of procaspase-3 at later time points, which is consistent with the ability of this particulate stimulus to accelerate PMN apoptotic death. In stark contrast, procaspase-3 processing was markedly diminished and delayed by LVS, relative to untreated control PMNs as well as cells exposed to control stimuli, and did not go to completion by 48 h.

Dose-dependent increases in TUNEL labeling and DNA fragmentation were observed at 24 and 48 hpi (Fig. 5A, 5B). Approximately 60% of the untreated PMNs were TUNEL positive at 24 h, whereas only 30% of LVS-infected PMNs exhibited fragmented DNA. As an additional control, we confirmed that OpZ accelerated PMN apoptosis, as indicated by increased TUNEL labeling at 24 h (untreated versus OpZ-stimulated PMNs: 60% and 77% TUNEL-positive, respectively; Fig. 5A). Collectively, these data indicate that F. tularensis LVS markedly impairs PMN apoptosis as indicated by morphologic analysis as well as annexin V and TUNEL staining.

F. tularensis LVS inhibits caspase-3 processing and activity in PMNs

Caspase-3 is the primary effector caspase in human neutrophils and is responsible for the proteolytic cleavage of cellular targets that ultimately lead to apoptotic cell death (43, 44). Caspase-3 is synthesized as an inactive proenzyme that is processed into large (17 kDa) and small (12 kDa) subunits that associate to form a mature active enzyme (45). Using Western blotting and densitometric analysis, we followed the time course of procaspase-3 processing in untreated and LVS-infected PMNs, and we used OpZ- and staurosporine-treated treated cells as positive controls (Fig 6A). In untreated PMNs undergoing constitutive apoptosis, trace processing of procaspase-3 to its mature form was detected at 6 h. Procaspase-3 processing increased markedly by 12 h and progressed to near complete depletion of procaspase-3 by 48 h. In PMNs treated with staurosporine, a potent proapoptotic stimulus (46), procaspase-3 processing was rapid, with substantial amounts of the mature enzyme present by 6 h, and near complete depletion of procaspase-3 by 12 h. Uptake of OpZ also accelerated procaspase-3 processing relative to untreated PMNs as indicated by increased mature caspase-3 at 6 h, and more rapid depletion of procaspase-3 at later time points, which is consistent with the ability of this particulate stimulus to accelerate PMN apoptotic death. In stark contrast, procaspase-3 processing was markedly diminished and delayed by LVS, relative to untreated control PMNs as well as cells exposed to control stimuli, and did not go to completion by 48 h.

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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...
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 between 12 and 24 h after isolation that was accelerated by 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 affect caspase-3 activity was diminished but not ablated. Thus, caspase-3 activity was 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).
PMNs with virulent (RLU) and are the mean substrate. Data indicate relative luminescence units using a caspase-8–specific proluminogenic sub-
ment of two. (Data shown are from one representative experi-
ment). Actin immunoblots demonstrate equal loading. Data shown are from one representative exper-
iment of two. (B) Caspase-8 activity was assessed using a caspase-8–specific proluminogenic sub-
strate. 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.

Live C. pneumoniae stimulates secretion of the antiapoptotic cytokine CXCL8 as a means to prolong PMN lifespan (11). In this study, we show that rF1LVS 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 rF1LVS 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, stauro-
sporine accelerated PMN apoptosis relative to untreated controls as judged by analysis of nuclear morphology (Fig. 10B) and quanti-
tation 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
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 PMN began 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 \textit{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, \textit{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 inhibited. Similar defects in processing and activation of upstream initiator caspases-8 and -9 indicate that \textit{F. tularensis} affected both the extrinsic and intrinsic apoptotic pathways. Finally, we demonstrate that in our assay system uptake of \textit{F. tularensis} was insufficient, 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 \textit{Francisella} (55), in serum-free RPMI 1640. These data are of interest because neutrophils differ from other leukocytes in their ability to synthesize both Fas ligand and Fas (54), and autocrine stimulation of the extrinsic pathway via these molecules may contribute to PMN turnover at sites of infection. Therefore, it is tempting to predict that inhibition of this pathway contributes to the ability of \textit{F. tularensis} to prolong PMN viability.

The ability of NADPH oxidase-derived ROS to accelerate PMN apoptotic death is established (1, 36, 56), and oxidants may also be sufficient to counteract certain antiapoptotic signals. For example, organisms such as live \textit{N. gonorrhoeae} and heat-killed \textit{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 \textit{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 \textit{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 \textit{F. tularensis} to inhibit basal PMN turnover.

Although the majority of neutrophils were infected with \textit{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 \textit{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 lysis and alarmins that amplify inflammation and 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, efferocytosis 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 or U112.

In summary, the results of this study extend previous work to demonstrate for the first time, to our knowledge, that a similar approach would be of therapeutic value in tularemia. We thank Dr. Dara Frank (Medical College of Wisconsin) for sharing the LVS Abs to the *F. tularensis* capsule.

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Disclosures

The authors have no financial conflicts of interest.

References


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.