


RESEARCH ARTICLE

Bacterial lipoproteins and other factors released by *Francisella tularensis* modulate human neutrophil lifespan: Effects of a *TLR1* SNP on apoptosis inhibition

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Abstract

Francisella tularensis infects several cell types including neutrophils, and aberrant neutrophil accumulation contributes to tissue destruction during tularaemia. We demonstrated previously that *F. tularensis* strains Schu S4 and live vaccine strain markedly delay human neutrophil apoptosis and thereby prolong cell lifespan, but the bacterial factors that mediate this aspect of virulence are undefined. Herein, we demonstrate that bacterial conditioned medium (CM) can delay apoptosis in the absence of direct infection. Biochemical analyses show that CM contained *F. tularensis* surface factors as well as outer membrane components. Our previous studies excluded roles for lipopolysaccharide and capsule in apoptosis inhibition, and current studies of [¹⁴C] acetate-labelled bacteria argue against a role for other bacterial lipids in this process. At the same time, studies of isogenic mutants indicate that ToIC and virulence factors whose expression requires FevR or MglA were also dispensable, demonstrating that apoptosis inhibition does not require Type I or Type VI secretion. Instead, we identified bacterial lipoproteins (BLPs) as active factors in CM. Additional studies of isolated BLPs demonstrated dose-dependent neutrophil apoptosis inhibition via a TLR2-dependent mechanism that is significantly influenced by a common polymorphism, *rs5743618*, in human *TLR1*. These data provide fundamental new insight into pathogen manipulation of neutrophil lifespan and BLP function.

KEYWORDS

apoptosis, lipoproteins, neutrophils, SNP, TLR2, tularaemia

1 | INTRODUCTION

Francisella tularensis is a Gram-negative, facultative intracellular bacterium and the causative agent of zoonosis tularaemia. Infection

can ensue following inhalation of bacteria into the lungs, ingestion of contaminated food or water, direct contact with an infected animal carcass, or inoculation into the skin via the bite of an infected arthropod vector (Kinkead & Allen, 2016). As few as 10 organisms can result

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in severe disease with mortality rates up to 60% in untreated infections (Dennis et al., 2001). *F. tularensis* subspecies *tularensis* (Type A) is exclusive to North America, whereas *F. tularensis* subspecies *holarctica* (Type B) is found throughout the northern hemisphere (Kinkead & Allen, 2016). Both Type A and Type B strains of *F. tularensis* replicate in phagocytes at sites of infection and disseminate to distal organs, including liver, spleen, and lymph nodes, leading to tissue destruction that may progress to sepsis or death, often prior to development of an adaptive immune response (Dennis et al., 2001; Kinkead & Allen, 2016).

F. tularensis utilises a variety of strategies to modulate the innate immune response and its effectors, as exemplified by its ability to elicit severe illness at low inocula. This organism infects several cell types including macrophages and neutrophils, interferes with oxidative host defence mechanisms by disrupting reduced nicotinamide adenine dinucleotide phosphate oxidase assembly and activity, escapes the phagosome to replicate in host cell cytosol, and produces an atypical lipopolysaccharide (LPS) that lacks endotoxic activity yet synergises with capsular polysaccharides for protection against complement-mediated lysis (J. H. Barker, Weiss, Apicella, & Nauseef, 2006; Kinkead & Allen, 2016; Lindemann et al., 2011; McCaffrey & Allen, 2006; McLendon, Apicella, & Allen, 2006; Schulert et al., 2009). Moreover, we discovered that both Type A and Type B *F. tularensis* strains modulate the major apoptotic pathways of human neutrophils and significantly extend cell lifespan (McCracken, Kinkead, McCaffrey, & Allen, 2016; Schwartz et al., 2013, 2012).

Neutrophils (also called polymorphonuclear leukocytes [PMNs]) are critical innate immune cells that are responsible for the rapid detection and eradication of invading bacteria and fungi. Phagocytosis of these microbes triggers a cascade of antimicrobial mechanisms that act in concert for microbe destruction and clearance. At the same time, neutrophils are inherently short-lived and are programmed to undergo apoptosis within 24 hr of release into circulation. At sites of infection, this cell death programme is significantly accelerated by phagocytosis and is coupled to removal of dying cells by macrophages, both critical points required for control of infection and resolution of inflammation. Thus, if apoptosis does not occur in a timely manner, there are detrimental consequences for the host that exacerbate disease by favouring pathogen dissemination and enhancing inflammation and tissue destruction (Kennedy & DeLeo, 2009).

F. tularensis-infected tissues are notable for an accumulation of neutrophils, and there is clear evidence that neutrophils contribute to disease severity and lethality in vivo (Bosio & Elkins, 2001; Dennis et al., 2001; Kinkead & Allen, 2016; Malik et al., 2007). As defects in neutrophil turnover favour the release of histotoxic cell contents, the ability of *F. tularensis* to inhibit apoptosis is one mechanism that contributes to tissue destruction (Kennedy & DeLeo, 2009; Schwartz et al., 2012). At the molecular level, processing and activation of intrinsic and extrinsic apoptosis pathway caspases are impaired, and this is coupled to global transcriptional reprogramming of infected neutrophils, leading to differential expression of a subset of apoptosis regulatory factors (McCracken et al., 2016; Schwartz et al., 2013, 2012). Additional experiments identified a possible role for factors secreted or released by *F. tularensis*, as modulation of neutrophil lifespan is most efficient when coupled to phagocytosis but is still significantly influenced when neutrophils and bacteria are separated by a Transwell membrane (Schwartz

et al., 2012). To date, we have excluded roles for LPS and capsule in this process, and the bacterial factors that mediate this aspect of virulence remain unidentified and uncharacterised (Schwartz et al., 2012).

Pattern recognition receptors, both surface and intracellular, allow phagocytes to quickly recognise invading microbes. There are 10 toll-like receptors (TLRs) in humans, and all except TLR3 are expressed by neutrophils (Hayashi, Means, & Luster, 2003). Most of these receptors detect a certain type of ligand, such as TLR4 that binds LPS/MD-2 complexes, whereas TLR2 detects a variety of different ligands when heterodimerised with TLR1 or TLR6 (Akira, Uematsu, & Takeuchi, 2006; Shimazu et al., 1999). In addition to cytokine production, TLR ligation can result in priming, a process that allows neutrophils to respond more robustly to a subsequent activating stimulus, with enhanced reactive oxygen species (ROS) production and degranulation increasing the efficiency of microbe killing (Prince, Whyte, Sabroe, & Parker, 2011; Singh, Zarembek, Kuhns, & Gallin, 2009; Whitmore et al., 2016). Nevertheless, excessive TLR signalling can be detrimental, exacerbating inflammation and tissue destruction during sepsis (Prince et al., 2011; Whitmore et al., 2016).

F. tularensis LPS does not bind MD-2 or other LPS binding proteins and for this reason is not a TLR4 agonist (J. H. Barker et al., 2006). Instead, TLR2 is an important receptor in the recognition and response to this Gram-negative bacterium (Abplanalp, Morris, Parida, Teale, & Berton, 2009; Dai, Rajaram, Curry, Leander, & Schlesinger, 2013; Katz, Zhang, Martin, Vogel, & Michalek, 2006; Malik et al., 2006). There is evidence that both TLR2/1 and TLR2/6 are activated in mouse models of infection (Abplanalp et al., 2009; Katz et al., 2006), and *F. tularensis* lipoproteins are detected by TLR2/1 in vitro (Thakran et al., 2008). Studies of macrophages show that during Schu S4 or live vaccine strain (LVS) infection, TLR2 signalling leading to cytokine production is diminished (Dai et al., 2013; Medina, Morris, & Berton, 2010). However, similar studies of neutrophils have not been performed, and the significance of this receptor in delayed apoptosis during *F. tularensis* infection is unknown.

The central objective of this study was to identify and characterise *F. tularensis* factors that contribute to apoptosis inhibition. On the basis of the results of prior studies, we predicted a role for secreted or released bacterial components and tested our hypothesis using biochemical and genetic approaches. Our findings are significant as they identify *F. tularensis* bacterial lipoproteins (BLPs) as antiapoptosis mediators in conditioned medium (CM). In addition, we demonstrate that BLP bioactivity required TLR2/1 and was significantly influenced by a polymorphism in human *TLR1* that is known to influence susceptibility to infection as well as sepsis severity (Hawn et al., 2009; Schumann & Tapping, 2007; Thompson et al., 2014; Whitmore et al., 2016). Taken together, our data significantly advance understanding of BLP function, neutrophil apoptosis, and tularaemia pathogenesis.

2 | RESULTS

2.1 | Apoptosis inhibition does not require *Francisella* regulatory factors or TolC-dependent secretion

F. tularensis LVS (Type B) and Schu S4 (Type A) strains significantly inhibit human neutrophil apoptosis and thereby prolong cell lifespan, but how this is achieved is only partially understood (McCracken et al., 2016; Schwartz et al., 2013, 2012). We previously excluded roles

for capsule and LPS in apoptosis inhibition (Schwartz et al., 2012), and the bacterial factors required for this aspect of virulence are unknown. *FevR* and *MigR* are major regulatory factors that act via *MglA* and *SspA* to control expression of more than 100 genes in *Francisella*, including genes that are required for disruption of the respiratory burst and phagosome escape (Brotcke et al., 2006; Buchan, McCaffrey, Lindemann, Allen, & Jones, 2009; Charity et al., 2007; McCaffrey et al., 2010). Nevertheless, we show here that isogenic mutants lacking functional *fevR*, *migR*, *mglA*, or *sspA* (Buchan et al., 2009; Charity et al., 2007) inhibited neutrophil apoptosis at least as well as wild-type LVS (Figure 1a), as indicated by annexin V-fluorescein isothiocyanate (AV) and propidium iodide (PI) staining.

Unlike neutrophils, macrophages are long-lived, can survive for months or more in tissues, and are self-renewing. However, extensive replication of *F. tularensis* in macrophage cytosol culminates in cell death 36–48 hr postinfection. Thanassi and colleagues have shown that disruption of *tolC*, which is required for Type I secretion, slightly, but significantly, accelerates apoptosis of *F. tularensis*-infected human or murine macrophages (Platz et al., 2010). To examine whether *TolC* is required for sustained viability of infected neutrophils, we constructed a *tolC* deletion mutant in LVS and confirmed its effects on the rate of human macrophage death following infection (Figure S1). Yet at the same time, we also found that loss of *tolC* did not alter the ability of *F. tularensis* to extend neutrophil lifespan (Figure 1b).

The AV/PI staining data were validated by analysis of neutrophil nuclear morphology as an independent indicator of progression to apoptosis (McCracken et al., 2016; Schwartz et al., 2012). Cells with condensed, apoptotic nuclei were quantified using light microscopy at 24 hr after infection, and these results confirm the ability of all LVS strains tested to significantly delay PMN death (Figure 1c). Taken together, these data underscore differences between phagocyte types and indicate that neither *FevR*, *MigR*, *MglA*, *SspA*, nor *TolC* is required for sustained viability of *F. tularensis*-infected neutrophils.

2.2 | Prokaryotic and eukaryotic protein syntheses are required for *F. tularensis*-mediated apoptosis inhibition

Having excluded roles for several virulence factors in our system, we sought to determine if protein synthesis was required. To this end, we utilised the bacteriostatic antibiotic, chloramphenicol (CAM), which targets the 50S ribosomal subunit to prevent prokaryotic protein synthesis. The data in Figure 2a show that CAM had no effect on neutrophils in the absence of bacteria but ablated the ability of LVS to delay apoptosis at low multiplicity of infection (MOI) and significantly diminished its efficacy at high MOI. In parallel experiments, we confirmed the ability of the eukaryotic protein synthesis inhibitor cycloheximide (CHX) to induce neutrophil death (Ozaki, Kato, Kitagawa, Fujita, & Kitagawa, 2008; Whitmore, Weems, & Allen, 2017) and also show that CHX treatment abrogated the antiapoptotic effects of *F. tularensis* (Figure 2b). These data indicate that host and bacterial protein syntheses are required for delayed apoptosis of *F. tularensis*-infected neutrophils.

2.3 | Delayed infection curtails apoptosis inhibition

As neutrophils are inherently programmed to die by constitutive apoptosis, we hypothesised that the ability of LVS to extend neutrophil

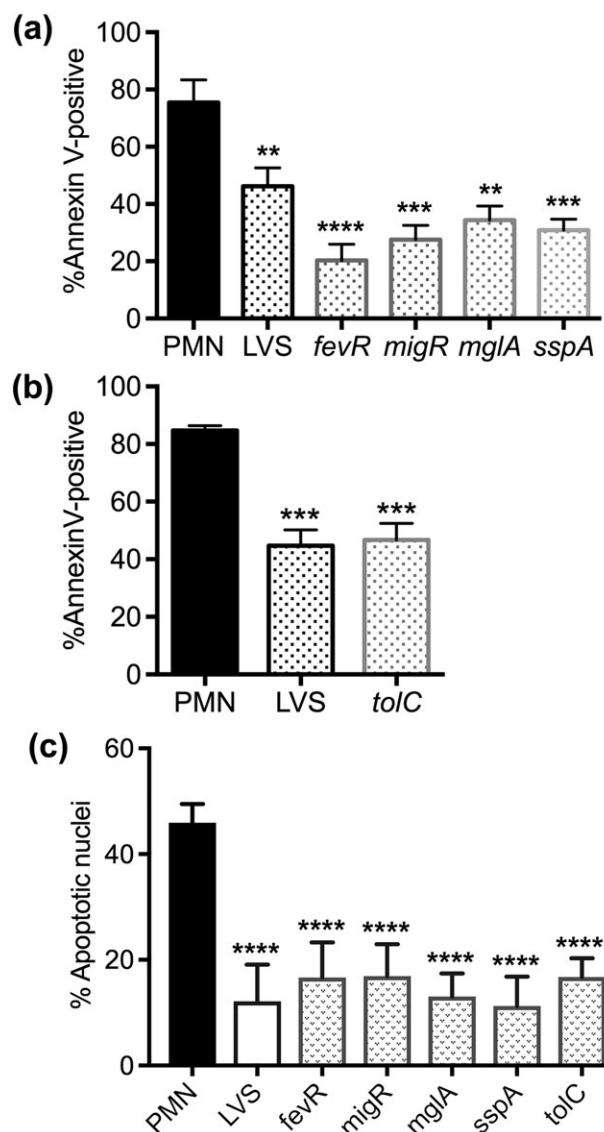


FIGURE 1 Apoptosis inhibition does not require major *Francisella* regulatory factors or *TolC*-dependent secretion. Apoptosis of control (uninfected) and infected polymorphonuclear leukocytes (PMNs) was assayed at 24 hr using annexin V and propidium iodide staining and flow cytometry. (a) PMNs were infected with wild-type live vaccine strain (LVS) or isogenic mutant strains lacking functional *fevR*, *migR*, *mglA*, or *sspA*, as indicated. Data are the mean + standard error of the mean (SEM) of at least three independent experiments. The *p* values were calculated using one-way analysis of variance (ANOVA) and Dunnett's multiple-comparison posttest. ***p* < .01; ****p* < .001 versus uninfected control. (b) Neutrophils were infected with LVS or the Δ *tolC* mutant. Data are the mean + SEM of five independent experiments. The *p* values were calculated using one-way ANOVA and Tukey's multiple-comparison posttest. ****p* < .001 versus uninfected control. (c) Neutrophils were infected with wild-type or mutant strains of LVS as indicated. The percentage of cells that had acquired an apoptotic nuclear morphology at 24 hr was quantified using light microscopy. Data are the mean + SEM of three independent experiments. The *p* values were calculated using one-way ANOVA and Dunnett's multiple-comparison posttest. *****p* < .0001 versus uninfected control

lifespan may wane as cells aged in culture. To test this hypothesis, bacteria were added to neutrophils after 0, 4, 10, 18, and 24 hr of incubation at 37 °C, and apoptosis was quantified at 24 hr following the time of infection using dual AV/PI staining and flow cytometry. Our

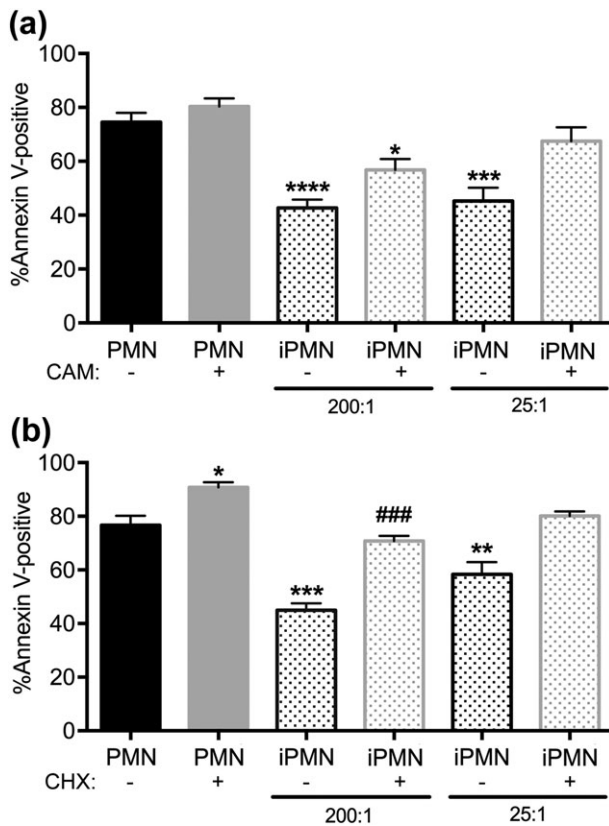


FIGURE 2 Prokaryotic and eukaryotic protein syntheses are required for *Francisella tularensis*-mediated apoptosis inhibition. (a) Live vaccine strain (LVS) was pretreated with chloramphenicol (CAM) for 1 hr and then used to infect neutrophils at a high multiplicity of infection (MOI; 200:1) and a low MOI (25:1). Data are the mean + standard error of the mean of five independent experiments. The p values were calculated using one-way analysis of variance and Dunnett's multiple-comparison posttest. (b) Neutrophils were pretreated with cycloheximide (CHX) for 1 hr and then infected with LVS at a high MOI (200:1) and a low MOI (25:1). Apoptosis was quantified at 24 hr using annexin V/propidium iodide staining and flow cytometry. Data are the mean + standard error of the mean of five independent experiments. The p values were calculated using one-way analysis of variance and Tukey's multiple-comparison posttest. * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$ versus the uninfected, untreated control. ### $p < .001$ versus uninfected CHX-treated polymorphonuclear leukocytes (PMNs). Neutrophils infected with LVS are indicated by iPMN. Other comparisons were not significant

data demonstrate that neutrophils aged for 10 hr or less could be rescued by *F. tularensis*, whereas cells aged for 18 hr or more could not (Figure S2). The data also show that LVS was more effective when added at 0–4 hr than when added at 10 hr. Our findings strongly suggest that a critical window for apoptosis inhibition by *F. tularensis* exists, beyond which cells may be irreversibly committed to death.

2.4 | Media conditioned by *Francisella tularensis* prolongs neutrophil lifespan

Previous experiments using Transwells suggested that factors secreted or released by *F. tularensis* significantly modulate neutrophil lifespan (Schwartz et al., 2012). Herein, we obtained further support for this hypothesis by analyses of CMs that were prepared from

neutrophils alone (PMN-CM), neutrophils infected with *F. tularensis* LVS (iPMN-CM), or bacteria that were incubated in tissue culture medium in the absence of neutrophils (LVS-CM). In each case, medium was collected after 24 hr, and neutrophils and bacteria were removed using centrifugation and sterile filtration. Each CM was then added to newly isolated autologous neutrophils, and apoptosis was analysed by AV/PI staining and flow cytometry at 24 hr posttreatment. The data in Figure 3a indicate that media conditioned by infected neutrophils or LVS alone significantly diminished neutrophil apoptosis, whereas medium conditioned by neutrophils alone did not, confirming results obtained using the Transwell approach (Schwartz et al., 2012).

Having excluded a requirement for *Francisella* regulatory factors and ToIC in apoptosis inhibition during direct infection (Figure 1), we

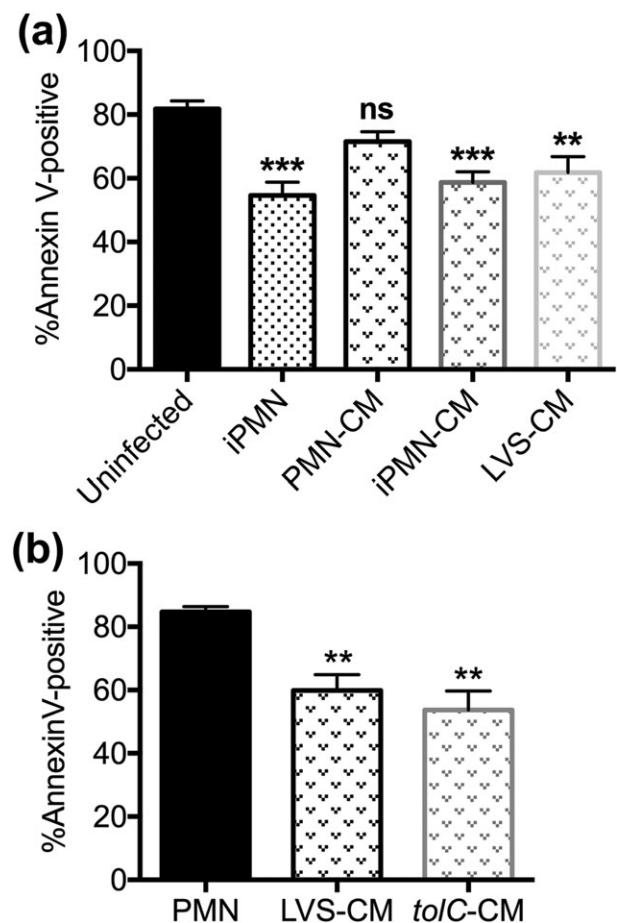


FIGURE 3 Conditioned media prolong neutrophil lifespan. Apoptosis was quantified at 24 hr using annexin V/propidium iodide staining and flow cytometry. (a) Effects of media that had been conditioned by neutrophils alone (PMN-CM), infected polymorphonuclear leukocytes (PMNs; iPMN-CM), or bacteria alone (LVS-CM) on PMN apoptosis. Untreated PMNs and cells directly infected with LVS (iPMN) were used as controls. Data are the mean + standard error of the mean of six independent experiments, and p values were calculated using one-way analysis of variance and Dunnett's multiple-comparison posttest. ** $p < .01$; *** $p < .001$; ns, not significant, when compared to the uninfected, untreated control. (b) Comparison of LVS-CM and Δ toIC-CM. Data are the mean + standard error of the mean of five independent experiments. The p values were calculated using one-way analysis of variance and Tukey's multiple-comparison posttest. ** $p < .01$ versus uninfected, untreated control

next asked if these molecules contributed to apoptosis inhibition by CM. Both LVS-CM and $\Delta tolC$ -CM significantly diminished neutrophil apoptosis and were indistinguishable from one another when directly compared (Figure 3b). Apoptosis was also delayed by iPMN-CM prepared using the *mgIA*, *sspA*, *migR*, and *fevR* mutants (data not shown).

Caspase 3 is the primary effector caspase in human neutrophils and is responsible for the cleavage of cellular components leading to the biochemical and morphological changes that are characteristic of apoptotic cells. We demonstrated previously that *F. tularensis* significantly diminishes and delays caspase 3 activation (Schwartz et al., 2012). Herein, we used a pro-luminogenic caspase-3-specific substrate to determine if LVS-CM had a similar effect. Indeed, the data in Figure 4a indicate that LVS-CM significantly diminished, but did not ablate, neutrophil caspase 3 activity assayed at 24 hr. At later stages of apoptosis, caspase-activated DNases cleave nuclear DNA into fragments, exposing 3-hydroxy termini that can be detected using terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling (TUNEL) reagents and flow cytometry (Schwartz et al., 2012). We show here that this aspect of PMN apoptosis was also

significantly curtailed by LVS-CM (Figure 4b). Thus, the ability of LVS-CM to significantly delay PMN apoptosis has been demonstrated by quantitation of phosphatidylserine (PS) externalisation and caspase 3 activation, as well as DNA fragmentation (Figures 3a and 4).

2.5 | Parameters for optimal medium conditioning by *Francisella tularensis*

To determine the optimal parameters for medium conditioning by *F. tularensis* LVS, we varied the concentration of bacteria (mock MOI), as well as the incubation time and temperature. Consistent with our previous data, optimal conditioning occurs at a mock MOI of 25:1 or more (Figure S3a). Temperature and time were also important variables, as significant apoptosis inhibition was achieved using media conditioned at 37 °C for 4–24 hr, but not by medium conditioned at lower temperatures (Figures S3b,c). Additional studies showed that the activity of CM was sensitive to dilution yet was not altered by storage for 6 weeks at –20 °C (data not shown) and was only partially diminished by heating at 65 °C for 30 min or 100 °C for 10 min (Figure S3d).

2.6 | Bacterial lipids are present in conditioned medium but are not transferred to neutrophils

To address the possible involvement of bacterial lipids in apoptosis inhibition by CM, we used our established methods to biosynthetically label fatty acids of *F. tularensis* phospholipids and LPS to high specific activity by growth in medium containing [¹⁴C]acetate (J. H. Barker, Kaufman, Apicella, & Weiss, 2016; J. H. Barker, Kaufman, Zhang, & Weiss, 2014). Although radioactivity was readily detected in CM prepared using these organisms, only background levels of radioactivity were transferred to neutrophils over 24 hr in two independent experiments (Figure S4). These data argue that sizeable amounts of *F. tularensis* lipids are not transferred to neutrophil as a means of modulating apoptosis.

2.7 | Conditioned medium is complex and contains major surface proteins of *Francisella tularensis*

Having established that bacterial protein synthesis contributed to apoptosis inhibition and that a direct role for lipids was unlikely, we sought to characterise the bacterial proteins in LVS-CM. To this end, proteins were concentrated by trichloroacetic acid precipitation, separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and detected with silver stain (Figure 5a). These data demonstrate that the composition of LVS-CM is complex.

Next, we used ultrafiltration as one approach to determine the relative size(s) of the antiapoptotic factors in LVS-CM; but this strategy was not successful as we were unable to identify fractions that differed in bioactivity despite testing filters over a range of molecular weight cut-offs. Subsequent analysis showed that passage through the 50-kDa filter did not eliminate proteins of smaller molecular mass from the retentate when compared to unfiltered LVS-CM (data not shown). Considered together, these data suggest that multiple proteins in CM may contribute to apoptosis inhibition and that at least some of

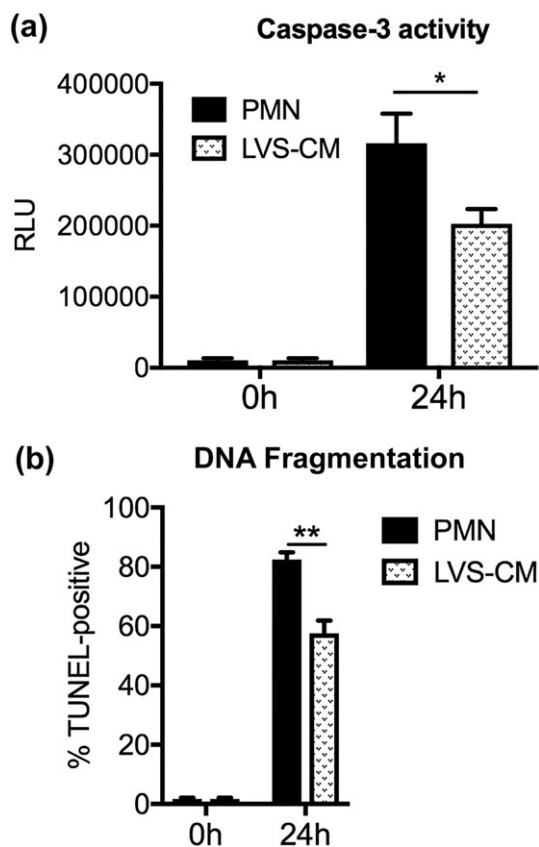


FIGURE 4 *Francisella tularensis* live vaccine strain conditioned medium (LVS-CM) impairs caspase 3 activity and DNA fragmentation. Polymorphonuclear leukocytes (PMNs) were left untreated or were incubated with LVS-CM for 24 hr. (a) Caspase 3 activity was quantified using a luminogenic DEVD peptide substrate at 0 and 24 hr. RLU, relative luminescence unit. (b) DNA fragmentation was quantified by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling staining and flow cytometry at 24 hr. In each case, data are the mean + standard error of the mean from four independent experiments, and *p* values were determined using a two-tailed paired *t* test. **p* < .05 and ***p* < .01 as indicated

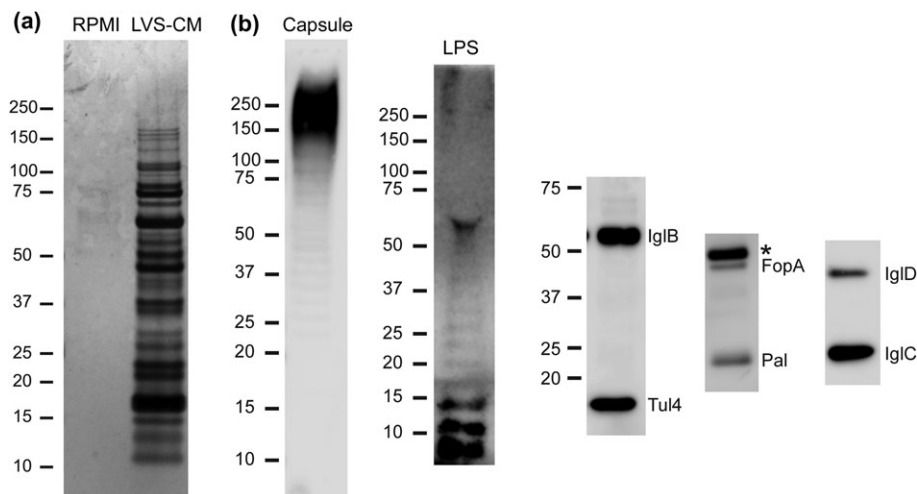


FIGURE 5 Major surface components of *Francisella tularensis* are present in conditioned media. (a) Proteins in RPMI (5 ml) or medium conditioned with live vaccine strain (LVS-CM; five culture equivalents) were precipitated, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and visualised using silver stain. (b) Immunoblots prepared from one to two culture equivalents of trichloroacetic-acid-concentrated LVS-CM were probed to detect capsule, lipopolysaccharide (LPS); IglB, IglC, and IglD; Tul4, FopA, and Pal as indicated. The asterisk indicates a nonspecific band detected by anti-FopA antibodies (Dr. Jason Huntley, personal communication)

these molecules may form large complexes or be part of other structures such as outer membrane vesicles (OMVs).

As an alternative strategy to further define the composition of LVS-CM, we probed immunoblots prepared using concentrated CM with antibodies specific for major surface components of *F. tularensis* and identified LPS, capsule, components of the *Francisella* pathogenicity island-encoded Type VI secretion system (IglB, IglC, and IglD), outer membrane proteins (FopA), and lipoproteins (Tul4, Pal; Figure 5 b; Huntley, Conley, Hagman, & Norgard, 2007; Rowe & Huntley, 2015; Schwartz et al., 2012). Evaluation of relative lipoprotein and LPS abundance in LVS-CM demonstrated that these molecules were present at a concentration roughly equal to 10^7 bacteria per millilitre, 100-fold less than the concentration of bacteria used for media conditioning (Figure S5). As our previous (Schwartz et al., 2012) and current studies (Figure 1) indicate that neither capsule, LPS, nor the FevR/MigR-regulated Type VI secretion components IglB, IglC, and IglD extend neutrophil lifespan, we focused our studies on BLPs.

2.8 | Effects of isolated *Francisella tularensis* bacterial lipoproteins on neutrophil apoptosis

To test directly the role of BLPs in *F. tularensis*-mediated neutrophil apoptosis inhibition, we used established methods to enrich BLPs from a total membrane fraction of *F. tularensis* LVS (Jones, Sampson, Nakaya, Pulendran, & Weiss, 2012) and assessed its composition using SDS-PAGE and silver stain (Figure 6a). The efficacy of BLP isolation was demonstrated by enrichment for the lipoprotein Tul4 and the absence of IglB, results that distinguish this preparation from unfractionated LVS-CM (Figure 6b). Lipoproteins Mip and Pal were also detected by immunoblotting (Figure 6b), and the silver-stained band at ~14 kDa (Figure 6a) is likely FTL_0645/FTT1416c, an abundant palmitoylated BLP that is also known as Flpp3 (Parra et al., 2010). Hence, we added increasing doses (10–500 ng) of BLPs

to neutrophils and assayed apoptosis at 24 hr. The data in Figure 6c indicate a dose-dependent reduction in neutrophil PS externalisation by BLPs but also demonstrate that the results for neutrophils from individual blood donors did not cluster around the mean and instead segregated into two groups above and below the mean, which was most apparent at the highest tested BLP dose. The bimodal nature of the data suggested that BLPs may have delayed neutrophil apoptosis in a subset of donors. Testing this hypothesis and elucidation of the mechanism was the focus of subsequent experiments.

2.9 | A TLR1 single-nucleotide polymorphism affects TLR1 surface expression on human neutrophils

A known single-nucleotide polymorphism (SNP) in *TLR1* (designated rs5743618, T1805G) affects TLR1 trafficking and consequently its abundance in the neutrophil plasma membrane. This SNP results in a substitution of isoleucine for serine at position 602, which, in turn, alters TLR1 association with ER chaperones responsible for receptor trafficking to the cell surface (Hart & Tapping, 2012). TLR1 heterodimerises with TLR2, and this complex recognises triacylated lipoproteins. Hence, we sought to examine if this SNP altered TLR1 surface expression in our two subsets of neutrophils, consequently influencing neutrophil responses to BLPs. We isolated genomic DNA from each donor and sequenced *TLR1* to determine the nucleotide identity at position 1805 in each allele. Of the 10 donors tested, five were homozygous for the G allele, three were homozygous for the T allele, and two were GT heterozygotes (Table 1). We subsequently quantified TLR1, TLR2, and TLR6 surface expression on neutrophils isolated from each genotyped donor. Our data demonstrate that surface expression of TLR1 was significantly reduced in neutrophils from GG homozygous donors as compared with cells from TT or GT donors (Figure 7), confirming published data (Johnson et al., 2007; Whitmore et al., 2016). In contrast, TLR2 and

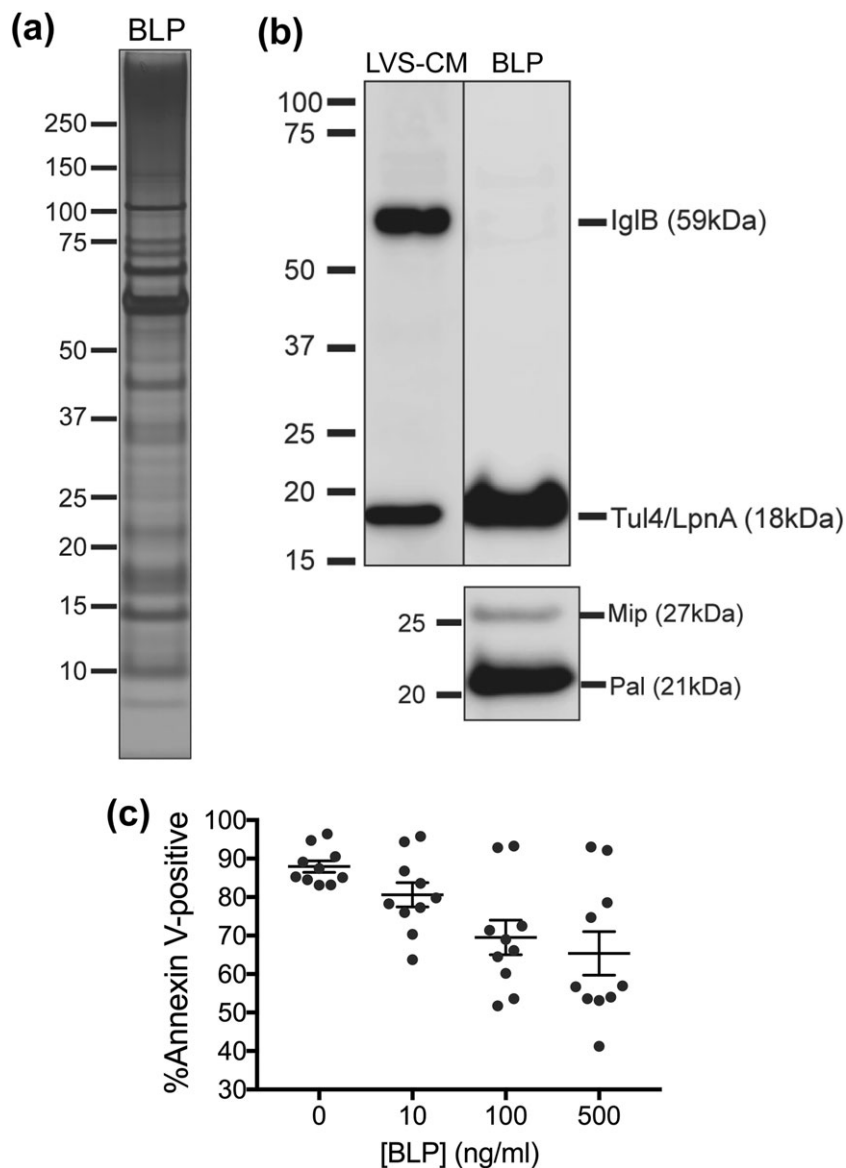


FIGURE 6 *Francisella tularensis*-derived bacterial lipoproteins (BLPs) delay apoptosis in a dose-dependent manner. BLPs were enriched from a total membrane fraction of live vaccine strain (LVS). (a) Isolated BLPs were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualised by silver stain. (b) Immunoblots show that BLP preparations are enriched in Tul4 and also contain Mip and Pal, but not IgB. (c) Neutrophils were incubated with increasing concentrations of LVS BLPs. Apoptosis was examined at 24 hr using annexin V/propidium iodide staining and flow cytometry. Graph shows the data from each polymorphonuclear leukocyte donor ($n = 10$) as an individual dot along with the mean and standard error of the mean. Note that the data stratify into two groups above and below the mean for the highest BLP doses tested. LVS-CM, medium conditioned by LVS

TABLE 1 TLR1 single-nucleotide polymorphism genotype among neutrophil donors

Donor type	T1805G (<i>rs5743618</i>)		
	GG	GT	TT
TLR1 low ($n = 5$)	5 (100%)	0 (0%)	0 (0%)
TLR1 high ($n = 5$)	0 (0%)	2 (40%)	3 (60%)

Data show the number and percentage of donors in each group.

TLR6 showed similar surface abundance on neutrophils from all donors (Figure 7).

2.10 | Neutrophils are differentially primed by TLR2 agonists

As the T1805G substitution affects surface TLR1 abundance and has been linked to differential neutrophil priming (Whitmore et al., 2016), we sought to confirm this phenotype in each of the subsets of donors in our study. To this end, we used the luminol assay to quantify ROS production by neutrophils that were primed with a TLR2/1

agonist (Pam₃CSK₄), a TLR2/6 agonist (FSL-1), or a TLR-independent priming agent (tumour necrosis factor alpha [TNF α]), and subsequently stimulated with fMLF. Neutrophils isolated from donors categorised in the TLR1-low GG group exhibited more robust fMLF-triggered oxidant production after priming with FSL-1 or TNF α but when pretreated with Pam₃CSK₄ did not significantly differ from the unprimed, fMLF-treated controls (Figure 8a,b). Conversely, neutrophils isolated from donors categorised as TLR1-high (GT and TT) were primed by all three agents and in each case exhibited more robust ROS production following fMLF stimulation than the unprimed controls (Figure 8c,d). These data confirm that the SNP *rs5743618* selectively alters neutrophil priming by TLR2/1 agonists (Whitmore et al., 2016).

2.11 | Different TLR2 agonists inhibit neutrophil apoptosis in a TLR1-dependent manner

It is established that TLR signalling can influence neutrophil lifespan, and although the majority of studies have focused on LPS and TLR4,

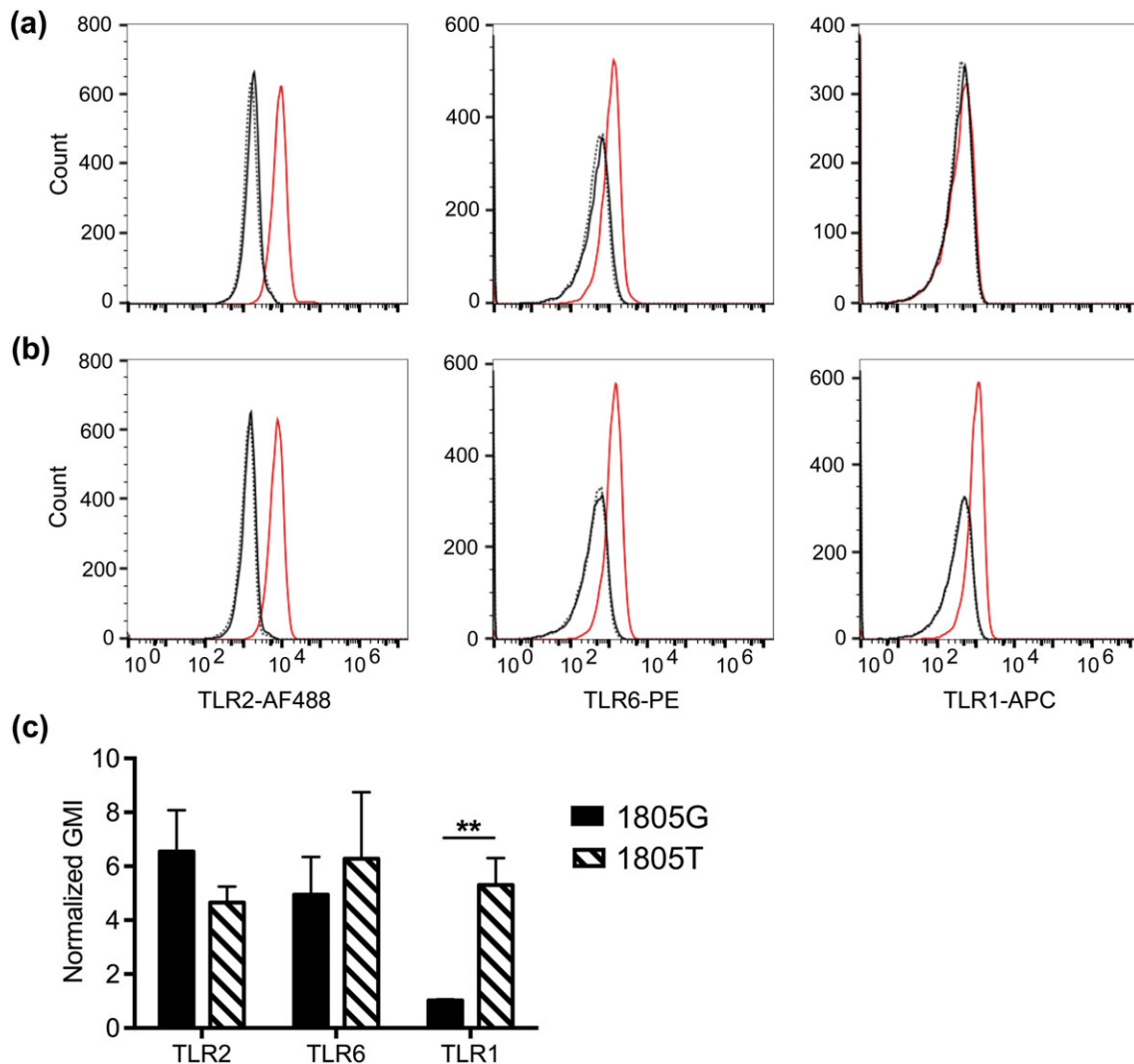


FIGURE 7 A human *TLR1* single-nucleotide polymorphism affects *TLR1* surface expression on neutrophils. Surface expression of *TLR2*, *TLR6*, and *TLR1* on neutrophils from genotyped donors was quantified by flow cytometry (red lines). Unstained cells (black lines) and isotype antibody stained cells (dotted lines) were included as negative controls. (a) Representative histograms of *TLR2*, *TLR6*, and *TLR1* expression on neutrophils from homozygous 1805G donors. (b) Representative histograms of *TLR2*, *TLR6*, and *TLR1* expression on neutrophils from donors with at least one copy of the 1805T allele. (c) Pooled data are the mean + standard error of the mean of five donors per group. The *p* values were calculated using a multiple *t*-test analysis and the Holm-Sidak method to correct for multiple comparisons. ***p* < .01 as indicated

there is some evidence that FSL-1 can significantly delay neutrophil apoptosis and that Pam₃CSK₄ can delay apoptosis of neutrophils that express high levels of *TLR1* (Whitmore et al., 2016). Thus, we added each *TLR2* agonist to neutrophils from donors in each subset and assayed apoptosis at 24 hr. Compared to the uninfected controls, FSL-1 delayed apoptosis to a similar extent in cells from both *TLR1*-low (Figure 8e) and *TLR1*-high (Figure 8f) groups. In contrast, Pam₃CSK₄ significantly delayed neutrophil apoptosis exclusively in the *TLR1*-high group. Thus, both *TLR2/1* and *TLR2/6* agonists can modulate neutrophil lifespan.

2.12 | A *TLR1* single-nucleotide polymorphism affects the ability of *Francisella tularensis* bacterial lipoproteins to modulate neutrophil lifespan

With this new information in hand, we reanalysed the data in Figure 6c, which suggested *F. tularensis* BLPs may influence

apoptosis of neutrophils from a subset of donors, and clustered our datasets according to donor genotype, surface level of *TLR1*, and priming responses. Our in-depth characterisation of neutrophil phenotypes allowed us to be confident in our classification of donor-specific neutrophil responses to *F. tularensis* BLPs, and the data in Figure 9 definitively demonstrate that *F. tularensis* lipoproteins significantly inhibit neutrophil apoptosis in a dose-dependent and *TLR1*-dependent manner.

2.13 | *Francisella novicida* bacterial lipoproteins have limited capacity to influence neutrophil apoptosis

We recently demonstrated that *Francisella novicida*, an environmental organism closely related to *F. tularensis*, can also delay neutrophil apoptosis but achieves this by a different mechanism that is CXCL8 dependent (Kinkead et al., 2017). Thus, we sought to determine whether *F. novicida* BLPs also had the capacity to modulate human

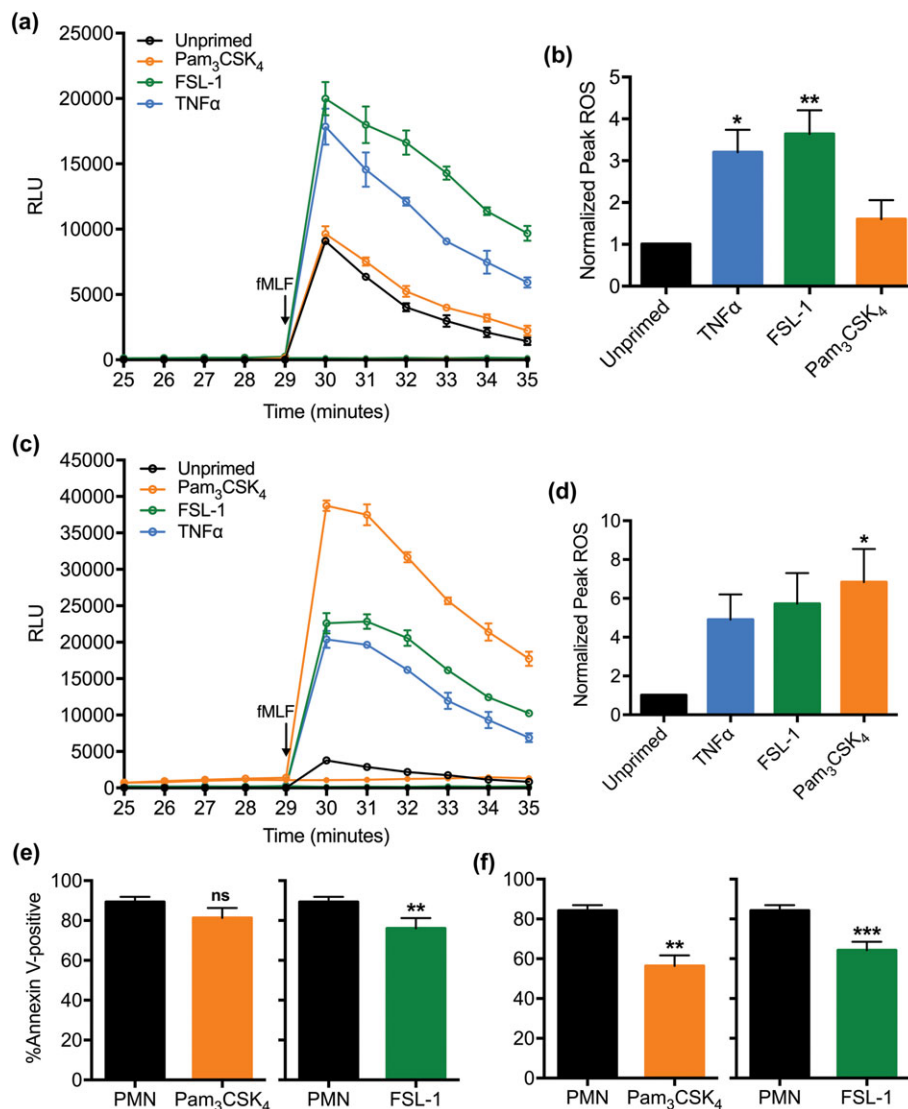


FIGURE 8 Differential effects of TLR2/6 and TLR2/1 agonists on polymorphonuclear leukocyte (PMN) priming and apoptosis. (a–d) Reduced nicotinamide adenine dinucleotide phosphate oxidase activity was measured using the luminol assay. PMNs from the low- and high-TLR1-expressing groups were left untreated or were primed with either the TLR2/6 agonist FSL-1, the TLR2/1 agonist Pam₃CSK₄, or the TLR-independent agent tumour necrosis factor (TNF α) for 29 min at which point fMLF (10 μ M) was added. (a, c) Rate and extent of oxidant production by PMNs from the TLR1-low and TLR1-high donors respectively. Each dataset is the mean \pm standard deviation of triplicate samples from one representative experiment. RLU, relative luminescence unit. (b, d) Pooled data indicate normalised reactive oxygen species (ROS) production triggered by fMLF for PMNs from the low- and high-TLR1-expressing donors respectively. Data were normalised to the peak luminescence obtained for unprimed neutrophils stimulated with fMLF in each experiment and are the mean + standard error of the mean of five donors per group. The p values were calculated using a one-way analysis of variance and Dunnett's multiple-comparison posttest. * p < .05; ** p < .01 versus unprimed controls. (e, f) PMNs from the TLR1-low or TLR1-high donors were treated with FSL-1 or Pam₃CSK₄. At 24 hr posttreatment, apoptosis was assessed using annexin V/propidium iodide staining and flow cytometry. (e) TLR1-low PMNs. (f) TLR1-high PMNs. Data are the mean + SEM of five donors per group. The p values were calculated using Student's t test. ns, not significant; ** p < .01; *** p < .001 versus untreated controls

neutrophil lifespan. To this end, we isolated BLPs from *F. novicida* strain U112 (Figure 10a). Neutrophils from our cohort of genotyped donors (Table 1) were treated with increasing amounts of U112 BLPs and assayed for apoptosis at 24 hr (Figure 10b–d). The data demonstrate that BLPs derived from *F. novicida* can also delay neutrophil apoptosis in a TLR1-dependent manner but were markedly less potent, as the pooled data for all donors did not clearly segregate into two groups (Figure 10b) and only the highest concentration (500 ng/ml) of *F. novicida* BLPs reached statistical significance in the TLR1-high group, which resembled neutrophils treated with

10 ng/ml of *F. tularensis* BLPs (compare Figures 9a,b to Figures 10c,d respectively).

2.14 | High surface TLR1 is not essential for apoptosis inhibition by *Francisella tularensis* and medium conditioned by live vaccine strain

Having demonstrated the influence of TLR1 abundance on apoptosis inhibition by *F. tularensis* BLPs, we sought to determine if the rs5743618 SNP significantly influenced apoptosis of neutrophils that

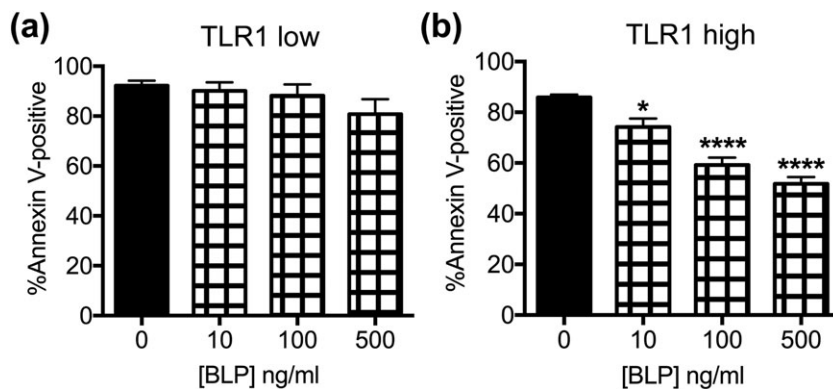


FIGURE 9 A TLR1 single-nucleotide polymorphism determines the ability of *Francisella tularensis* bacterial lipoproteins (BLPs) to modulate neutrophil lifespan. Neutrophils from the TLR1-low or TLR1-high groups were treated with increasing concentrations of *F. tularensis* live vaccine strain BLPs. At 24 hr, apoptosis was assessed using annexin V/propidium iodide staining and flow cytometry. (a) Data for TLR1-low PMNs. (b) Data for TLR1-high PMNs. Each graph shows data for five donors as the mean + standard error of the mean. The *p* values were calculated using a one-way analysis of variance and Dunnett's multiple-comparison posttest. **p* < .05; *****p* < .0001 versus untreated controls

were directly infected with bacteria or treated with LVS-CM. Because of turnover in our donor pool over the course of this study, information regarding TLR1 abundance on many of the PMNs used in early experiments is unknown. However, reanalysis of the data from Figure 2 using scatter plots suggested that LVS-mediated apoptosis inhibition may not be strictly TLR1 dependent (Figure S6). Therefore, we compiled data for cells from donors of known TLR1 status that had been infected with LVS or treated with LVS-CM, using filled circles and open circles of different colours to designate results for each TLR1-high donor and TLR1-low donor, respectively. With respect to LVS infection, data for cells from six donors were available, some of which were assayed on more than one occasion (Figure 11a). In this case, distinct subpopulations were not observed, and the data clustered around the mean regardless of TLR1 status (Figure 11a). Similar results were obtained when the effects of LVS and LVS-CM were directly compared (Figure 11b) and when apoptosis was quantified using TUNEL (Figure 11c) or measurements of caspase 3 activity (not shown) rather than AV/PI staining. Note that the data in Figure 11c are a scatter plot of the data in Figure 4c. Thus, in marked contrast to BLPs, LVS and LVS-CM inhibited apoptosis of TLR1-high and TLR1-low PMNs to a similar extent. As such, these data demonstrate that BLPs do not entirely account for the ability of *F. tularensis* to extend human neutrophil lifespan.

3 | DISCUSSION

Neutrophils are important components of the innate immune response that perform an essential function of bacterial recognition and clearance. However, pathogen perturbation of neutrophil lifespan can sustain infection and enhance tissue destruction (Kennedy & DeLeo, 2009). Our published data demonstrate that Type A and Type B strains of *F. tularensis* significantly prolong neutrophil lifespan by interfering with multiple apoptotic pathways, but the bacterial factors that mediate this aspect of virulence are unknown (Schwartz et al., 2012). Addressing this knowledge gap was the central objective of this study, and our results are noteworthy for the identification of *F. tularensis* BLPs as modulators of

neutrophil lifespan, acting via a TLR2-dependent mechanism that is significantly influenced by an SNP in human *TLR1*.

FevR, MigR, MglA, and SspA control expression of many genes in *F. tularensis*, including genes in the *Francisella* pathogenicity island that encode an unusual Type VI secretion system, and are critical for phagosome escape and intracellular growth (J. R. Barker et al., 2009; Brotcke et al., 2006; Buchan et al., 2009; Charity et al., 2007; Clemens, Ge, Lee, Horwitz, & Zhou, 2015; Eshraghi et al., 2016). As isogenic mutants lacking each of these regulatory factors delayed neutrophil apoptosis at least as well as wild-type LVS, our data demonstrate that manipulation of neutrophil lifespan does not require Type VI secretion or bacterial access to neutrophil cytosol. In contrast, as TLR2 accumulates on phagosomes (Ozinsky et al., 2000; Underhill et al., 1999) and the mutant organisms cannot escape these compartments, it is conceivable that under these conditions TLR2-driven prosurvival signalling may be enhanced or prolonged. Relevant downstream targets of this pathway likely include the antiapoptosis factors MCL-1 and XIAP, which sustain mitochondrial integrity and directly prevent caspase 3 activation, respectively (McCracken & Allen, 2014). Both these proteins are short-lived but are maintained at high levels following *F. tularensis* infection (McCracken et al., 2016; Schwartz et al., 2013), results that are in keeping with the ability of CHX to accelerate neutrophil death (Figure 2b).

ToIC is the major apparatus component of Type I secretion systems that mediate drug efflux and can also deliver virulence factors into host cells. Type I secretion contributes to *F. tularensis* virulence and sustains viability of infected macrophages (Doyle, Pan, Mena, Zong, & Thanassi, 2014). We confirmed the ability of LVS $\Delta toIC$ mutants to accelerate human macrophage apoptosis following infection yet also demonstrate that this effect did not extend to neutrophils, as data obtained for PMNs infected with wild-type or mutant bacterial strains were nearly identical. As neutrophils are short-lived and are inherently programmed to undergo apoptosis whereas macrophages are long-lived and self-renewing, our data are consistent with fundamental differences between these phagocyte types. Thus, the mechanisms that allow *F. tularensis* to override the constitutive apoptosis programme of neutrophils are distinct from those that allow macrophages to tolerate an infection that will eventually hasten cell death.

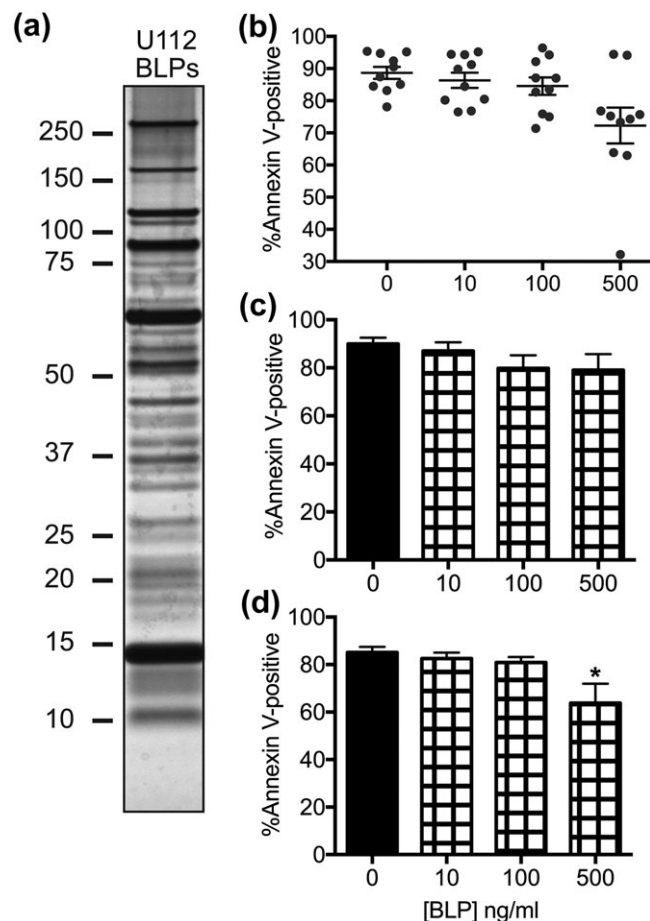


FIGURE 10 *Francisella novicida* bacterial lipoproteins (BLPs) modulate neutrophil apoptosis but are less potent than *Francisella tularensis* BLPs. *F. novicida* U112 BLPs were enriched from a total membrane fraction of U112. (a) Isolated BLPs were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualised by silver stain. (b–d) Neutrophils were incubated with increasing concentrations of U112 BLPs, and apoptosis was examined at 24 hr using annexin V/propidium iodide staining and flow cytometry. Pooled data from all 10 genotyped donors are shown as individual dots and as the mean + standard error of the mean (b). Pooled data for the TLR1-low (c) and TLR1-high (d) groups shown as the mean + standard error of the mean of five donors per group. The p values were calculated using a one-way analysis of variance and Dunnett's posttest. * $p < .05$ versus untreated controls

Several lines of evidence support a role for extracellular bacteria and factors that are released or secreted by these bacteria in prolongation of PMN lifespan. Previous studies indicate that *F. tularensis* can act at a distance to modulate neutrophil function and phenotype (Moreland, Hook, Bailey, Ulland, & Nauseef, 2009; Schwartz et al., 2012). We hypothesised that this allows *F. tularensis* to influence neutrophils as soon as these cells enter infected tissues, prior to or independent of phagocytosis (Schwartz et al., 2012), and our current studies of LVS-CM further support this model. In optimising parameters for media conditioning, we showed that this process is dose, time, and temperature dependent and also found that CM bioactivity is relatively stable. Subsequent experiments eliminated a direct role for bacterial lipids in modulation of neutrophil lifespan and identified proteins as candidate active factors based on sensitivity to inhibition

by CAM. Indeed, the protein composition of concentrated LVS-CM is complex as indicated by silver stain, and multiple outer membrane proteins and lipoproteins were identified by immunoblotting, as were LPS and capsule. Subsequent studies identified BLPs as active factors in LVS-CM that delay PMN apoptosis in a dose-dependent manner. We confirmed enrichment for Tul4, Mip, and Pal in our BLP preparations, and other BLPs detected by silver stain likely include YapH (FTL_0439) and Flpp3 (FTL_0645), FipB, DsbA (FTT1103), and a *F. tularensis* BLP with homology to *Shigella* VacJ (FTL_1637; Huntley et al., 2007; Parra et al., 2010; Straskova et al., 2009).

The mechanism of BLP release or shedding remains to be determined. In this regard, it is noteworthy that *F. tularensis* BLPs form oligomers (Senitkova, Spidlova, & Stulik, 2015), which likely contributed to our inability to fractionate active factors in LVS-CM using ultrafiltration. In addition, both *F. tularensis* and *Neisseria gonorrhoeae* BLPs utilise an atypical trafficking pathway (LoVullo, Wright, Isabella, Huntley, & Pavelka, 2015), and there is increasing evidence that BLPs from *Neisseria* and other organisms can be surface exposed or secreted, including *Shigella* VacJ (Wilson & Bernstein, 2016; Zuckert, 2014). Notably, surface exposure of Tul4 is enhanced in LVS $\Delta wbtA$ mutants that cannot synthesise capsule or LPS O-antigen (Zarella et al., 2011), and it is attractive to predict that BLP release may be facilitated in the absence of these surface polysaccharides. In keeping with this, we used a *wbtA* mutant strain to show that capsule and O-antigen are dispensable for neutrophil apoptosis inhibition (Schwartz et al., 2012).

Additionally or instead, BLPs may be released as components of OMVs. *F. tularensis* sheds very few OMVs as compared with other Gram-negative bacteria, and their contribution to the pathogenesis of tularaemia is only beginning to be defined (Chen et al., 2017). We did not detect OMVs by scanning electron microscopy analysis of CM or neutrophils treated with CM, and no vesicles were apparent by transmission electron microscopy after ultracentrifugation of LVS-CM at $400,000 \times g$ (our unpublished data), results that are in keeping with the outcome of our [14 C]acetate labelling experiments. Nevertheless, as the amount of material secreted into the medium is relatively low, OMV release may be below the limit of detection of our assays. Further studies of BLP trafficking and release are clearly needed, but whatever the mechanism, the results of this study are additional evidence to demonstrate that BLPs can function as virulence factors that contribute to immune evasion or dysregulation (Huntley et al., 2007; Rowe & Huntley, 2015; Zhang, Niesel, Peterson, & Klimpel, 1998).

As the *F. tularensis* envelope is unusual, it is also of interest that BLPs of other bacteria enhance rather than impair host cell death. For example, a 19-kDa lipoprotein of *Mycobacterium tuberculosis* markedly accelerates neutrophil apoptosis, whereas *Escherichia coli* BLPs trigger rapid death of THP-1 macrophages, and both these outcomes are TLR2 dependent (Aleman et al., 2004; Aliprantis et al., 1999; Persson, Blomgran-Julinder, Eklund, Lundstrom, & Stendahl, 2009). Among organisms in the *Francisella* genus, we show here that *F. tularensis* BLPs significantly delayed PMN apoptosis at concentrations as low as 10 ng/ml, whereas *F. novicida* BLPs did not (compare Figures 9b and 10d). In agreement with this, we find that the ability of *F. novicida* to modulate PMN lifespan relies on its ability to stimulate neutrophil CXCL8 secretion, which does not occur in response to *F. tularensis* (Kinkead et al., 2017; Schwartz et al., 2012). Moreover,

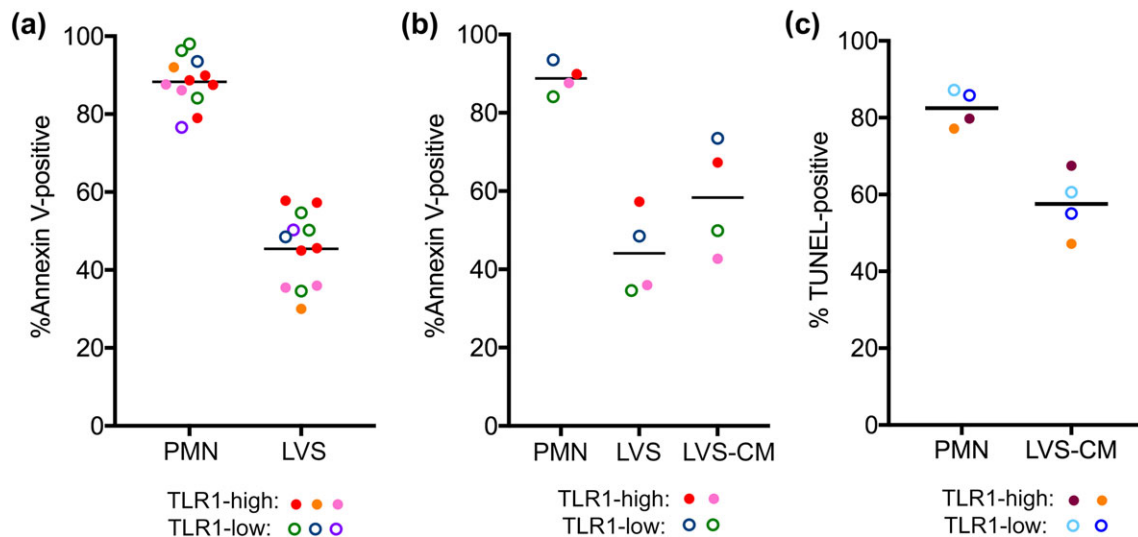


FIGURE 11 Live vaccine strain (LVS) and medium conditioned by LVS (LVS-CM) impair apoptosis of both TLR1-high and TLR1-low neutrophils. Apoptosis of control, LVS-infected, and LVS-CM-treated neutrophils was quantified at 24 hr using annexin V/propidium iodide staining to detect externalised phosphatidylserine (a, b) or using terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling staining to detect DNA fragmentation (c). In each case, scatter plots show data for TLR1-high and TLR1-low polymorphonuclear leukocytes (PMNs) from different donors as filled and open circles of different colours, respectively. As indicated, cells from some donors in Panel a were assayed on more than one occasion. The data in Panel c are from Figure 4c. Black lines indicate the mean of each dataset

F. novicida uses CRISPR/Cas9 to regulate expression of a proinflammatory BLP that is absent in *F. tularensis* (Jones et al., 2012), and it is conceivable that BLPs present in both organisms may differ in structure, as has been shown for LPS (McLendon et al., 2006; Rowe & Huntley, 2015; Schilling, McLendon, Phillips, Apicella, & Gibson, 2007). Whatever the mechanism, these data further support the notion that BLPs contribute to *F. tularensis* virulence.

Typically, TLR2 signalling contributes to effective host defence via cytokine synthesis and priming of oxidant production, but the view of TLR signalling as universally beneficial has been radically altered by the identification of SNPs in the human genome that significantly influence the outcome of infectious and inflammatory diseases in a context-specific manner. Relevant here is the fact that although *F. tularensis* is detected by TLR2 and palmitoylated BLPs are specific ligands for TLR2/1 heterodimers, this pathogen undermines TLR2-driven defence, including cytokine secretion and both direct and primed ROS production (Crane, Ireland, Alinger, Small, & Bosio, 2013; Dai et al., 2013; McCaffrey et al., 2010; Thakran et al., 2008). At the same time, the results of this study strongly suggest that *F. tularensis* exploits TLR2 signalling as one mechanism to prolong neutrophil lifespan.

In testing the ability of isolated BLPs to extend neutrophil lifespan, we noticed that our data had a bimodal distribution, as apoptosis was delayed using cells from only ~50% of donors (Figure 6c). Subsequent studies attributed this heterogeneity to an SNP in human *TLR1*, rs5743618, that influences surface expression of the encoded protein and therefore influences responsiveness of neutrophils to TLR2/1 agonists, including Pam₃CSK₄ (Hart & Tapping, 2012; Whitmore et al., 2016). Specifically, we discovered that 50% of genotyped donors in this study were homozygous for the 1805G allele, which results in substitution of serine for isoleucine in TLR1 at position 602, leading to sequestration of the protein in the ER and a near-complete absence of TLR1 at the cell surface (Hart & Tapping, 2012; Whitmore et al.,

2016). Consequently, cells from TLR1-low donors respond normally to the TLR2/6 agonist FSL-1 or TNF α but cannot be primed by Pam₃CSK₄ (Figure 8); and neither this canonical TLR2/1 agonist (Figure 8e) nor *F. tularensis* BLPs (Figure 9a) significantly influenced constitutive apoptosis of these PMNs. Conversely, neutrophils from donors with at least one copy of the 1805T allele are TLR1-high (Figure 7) and can be primed for activation by Pam₃CSK₄, and when treated with this agonist or with *F. tularensis* BLPs, apoptosis was markedly delayed (Figures 8f and 9b).

Among humans, the 1805T and 1805G alleles of *TLR1* are codominant, with the ancient 1805T allele conferring protection against tuberculosis (Qi et al., 2015), pyelonephritis (Hawn et al., 2009), and *Candida* (Plantinga et al., 2012), yet being detrimental in leprosy (Johnson et al., 2007; Wong et al., 2010), increasing the lethality and severity of sepsis (Thompson et al., 2014; Whitmore et al., 2016), and exacerbating many inflammatory disorders including lupus, atherosclerosis, inflammatory bowel disease, and cancer (Schumann & Tapping, 2007). These data support the notion that both the 1805T and 1805G alleles are maintained at high frequency in the human population by providing protection in the context of some diseases, but not in others (Heffelfinger et al., 2014). Moreover, although both alleles are prevalent, they are not distributed uniformly, as the GG allele is present at high frequency (52–76%) in Europe, including Scandinavia, and in Americans of European descent yet is less prevalent in African Americans (24%) and in Russia, India, and the Middle East and is nearly absent in Africa, Asia, Southeast Asia, and Polynesia (Hawn et al., 2007; Heffelfinger et al., 2014). In this regard, it is of interest that the distribution of the hypomorphic *TLR1* allele among humans is very similar to the geographic range of *F. tularensis* (Ulu-Kilic & Doganay, 2014). The results of this study suggest that the 1805G allele may diminish neutrophil-mediated tissue destruction following *F. tularensis* infection, but to our knowledge, the

possible role of *rs5743618* or other SNPs in tularaemia susceptibility, severity, or vaccine efficacy has not been investigated.

In identifying BLPs as the first *F. tularensis* factors known to significantly delay neutrophil apoptosis, our data also demonstrate that these molecules do not entirely account for the ability of LVS-CM or the bacterium itself to extend PMN lifespan, results that are in keeping with a multifaceted underlying mechanism that is partially TLR1 dependent (Figure 11). Thus, additional studies are needed to identify other active factors in CM and associated with the bacterium and to elucidate their mechanisms of action. Sensitivity to CAM (Figure 2a) strongly suggests a role for other *F. tularensis* proteins that could potentially stimulate neutrophils to secrete prosurvival factors or act by TLR2-independent mechanisms to curtail apoptosis, perhaps at later stages of infection.

In summary, it is established that neutrophils contribute to tularaemia lethality, and we have shown that neutrophil antimicrobial functions and lifespan are dysregulated in the context of *F. tularensis* infection. The results of this study provide fundamental insight into the bacterial factors that modulate neutrophil lifespan. We identified BLPs as active components of CM that delay PMN death, thereby providing definitive evidence of their capacity to function as virulence factors. In demonstrating that BLPs act via TLR2/1 to modulate apoptosis, our data also reveal a previously unappreciated role for this receptor complex in tularaemia pathogenesis that extends beyond diminished cytokine production and aberrant priming. Our data set the stage for in-depth analysis of individual BLPs and their influence on PMNs. At the same time, our identification of the *TLR1* SNP *rs5743618* as important raises fundamental questions regarding the role of human genetics in tularaemia severity and susceptibility that also merit further study.

4 | EXPERIMENTAL PROCEDURES

4.1 | Isolation of human neutrophils

Heparinised venous blood was obtained from healthy, adult volunteers, with no history of tularaemia in accordance with protocols 201609850 and 200307026 approved by the Institutional Review Board for Human Subjects at the University of Iowa. Neutrophils were isolated using sequential dextran sedimentation, density gradient separation with Ficoll-Hypaque (GE Healthcare), and hypotonic erythrocyte lysis as previously described (Nauseef, 2007). With this method, neutrophil purity was routinely > 95%. PMNs were suspended in Hank's balanced salt solution (HBSS) without divalent cations (Fisher Scientific), enumerated, and diluted to 2×10^7 /ml. In all cases, replicate experiments were performed using PMNs from different donors.

4.2 | Cultivation of bacteria

Wild-type *F. tularensis* LVS and isogenic strains with mutations in *fevR*, *migR*, *mglA*, and *sspA* have been previously described (Brotcke et al., 2006; Buchan et al., 2009; Charity et al., 2007; Schwartz et al., 2012). The *mglA* and *sspA* mutant strains were the generous gift of Dr. Simon Dove (Boston Children's Hospital, Harvard Medical School, Boston, MA). *F. novicida* strain U112 was obtained from Dr. Colin Manoil

(University of Washington, Seattle WA) and has been described (Gallagher et al., 2007). All strains were inoculated onto Difco cysteine heart agar (BD Biosciences) supplemented with 9% defibrinated sheep blood (Remel) and grown for 24–48 hr at 37 °C in 5% CO₂. Unless otherwise stated, broth cultures of U112 were started at an OD₆₀₀ of 0.005, cultures of LVS and Δ *tolC* were started at an OD₆₀₀ of 0.01, and cultures of the other mutant strains were started at an OD₆₀₀ of 0.025, in Bacto brain heart infusion (BHI) broth (BD Biosciences) and incubated overnight (14–18 hr), shaking at 200 r.p.m. Overnight cultures were diluted to an OD₆₀₀ of 0.200 in BHI broth and incubated at 37 °C in 5% CO₂, shaking at 200 r.p.m., for 2–4 hr. Midexponential growth phase bacteria were harvested and washed once with HBSS containing divalent cations (Fisher Scientific).

4.3 | Construction of a live vaccine strain Δ *tolC* mutant

Approximately 1 kb of DNA upstream and downstream of the *tolC* locus (*FTL_1865*) was polymerase chain reaction (PCR) amplified and cloned into pCR2.1 and pGEM-T Easy respectively. The upstream fragment was removed from pCR2.1 by restriction digestion with *AscI* and *AvrII* and cloned into the respective sites of the plasmid containing the downstream fragment. The resulting upstream–downstream fragment was removed by digestion with *BamHI* and *BglII* and cloned into the *BamHI* site of the suicide plasmid pJC84. This plasmid was electroporated into LVS (2.5 kV, 25 μ F, and 600 Ω), and the bacteria were plated onto modified Muller Hinton (MMH) agar containing 50 μ g/ml of kanamycin after 2–3 hr of outgrowth. Kanamycin-resistant colonies were grown overnight in MMH broth in the absence of selection and plated onto MMH agar with 8% sucrose for *sacB*-mediated counter selection. Sucrose-resistant, kanamycin-sensitive colonies were screened by colony PCR to detect deletion of *tolC*. Primers were designed to flank the coding sequence of each gene such that an amplicon would be produced regardless of genotype.

4.4 | Infection of neutrophils

Bacteria were washed with HBSS containing divalent cations and were quantified by measurement of absorbance at 600 nm. Unless otherwise stated, PMNs (5×10^6 /ml) were diluted in serum-free HEPES-buffered RPMI-1640 containing L-glutamine and phenol red (Lonza), and infections were performed at an MOI of 200:1. Cultures (1–2 ml) were incubated in 14-ml polypropylene tubes at 37 °C with 5% CO₂ for the indicated times.

4.5 | Assessment of neutrophil apoptosis

Neutrophil apoptosis was quantified first by flow cytometric analysis of PS externalisation after 24 hr at 37 °C. PMNs (5×10^5) were costained with annexin V–fluorescein isothiocyanate (BioVision) and PI (BioVision) in binding buffer (10 mM of HEPES pH 7.4, 140 mM of NaCl, 2.5 mM of CaCl₂) according to the manufacturer's instructions. PI staining was used to differentiate early apoptotic from late apoptotic/necrotic PMNs (Schwartz et al., 2012). Cells were analysed using an Accuri C6 flow cytometer (BD Biosciences). Approximately 10,000 events were collected for each sample, and the data were

analysed using cFlow (BD Biosciences) or FlowJo (TreeStar Inc.) software.

Where indicated, apoptosis was also quantified by using light microscopy to detect acquisition of an apoptotic (condensed) nuclear morphology, by measurements of caspase 3 activity, or by using flow cytometry to detect DNA fragmentation. All assays were performed as we previously described (Schwartz et al., 2012). In brief, for analysis of nuclear morphology, control PMNs and cells that had been infected with wild-type or mutant strains of LVS for 24 or 48 hr were attached to coverslips by cyto centrifugation, fixed and stained using Hema-3 reagents (Fisher Scientific), mounted onto glass slides and analysed by light microscopy (ZEISS Axioplan 2, Carl Zeiss, Inc.). In each case, at least 100 cells were examined per condition in each experiment. For analysis of caspase 3 activity, we utilised Caspase-3 Glo reagents (Promega) according to the manufacturer's instructions. In each case, triplicate aliquots of 5×10^6 PMNs were left untreated or were exposed to LVS-CM for 0 or 24 hr. Cleavage of the pro-luminogenic DEVD caspase 3 peptide substrate was quantified using a NOVOstar microplate luminometer (BMG LABTECH). Finally, DNA fragmentation in control and LVS-CM-treated PMNs was assessed at 24 hr using an Apo-BrdU apoptosis detection kit (BD Biosciences), a modified TUNEL assay, according to the manufacturer's instructions. Samples were analysed using an Accuri C6 flow cytometer as described above, and in each case, at least 10,000 events were collected.

4.6 | Infection of primary human macrophages

Heparinised venous blood was obtained from healthy, adult volunteers with no history of tularaemia, in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation using Ficoll-Hypaque, washed twice in HEPES-buffered RPMI-1640 supplemented with L-glutamine and phenol red, and diluted to a final concentration of 2×10^6 PBMCs per millilitre in medium containing 20% autologous serum. PBMCs were differentiated into monocyte-derived macrophages (MDMs) by incubation in Teflon jars for 5–7 days at 37 °C, 5% CO₂ (Schulert et al., 2009). MDMs were collected, washed with RPMI-1640 as outlined above, counted on a haemocytometer, plated in tissue culture dishes, and allowed to adhere overnight at 37 °C, 5% CO₂. MDM monolayers were washed three times with warm Dulbecco's PBS (DPBS) without cations (Fisher Scientific) to remove nonadherent cells prior to infection. Replicate experiments were performed using cells from different donors. Macrophages were infected in triplicate with LVS or the Δ tolC mutant at an MOI of 100:1 and incubated at 37 °C, 5% CO₂.

4.7 | Assessment of macrophage cytotoxicity

Cytotoxicity was assessed at 24 hr postinfection by measuring lactate dehydrogenase (LDH) release using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's instructions. One hundred microlitres of medium from each well was transferred to a 96-well, white-walled, clear-bottom microtitre plate (Costar). An equal volume of LDH reagent buffer and substrate mix

was added to each well for 10 min before the addition of stop solution. Plates were sealed with adhesive foil, and fluorescence was measured on a FLUOstar Optima (BMG LABTECH). Maximum LDH release for each experimental sample was determined by adding 10 μ l of 9% (v/v) Triton X-100 solution for 20 min at 37 °C prior to sample collection. The percent cytotoxicity was calculated by comparing each experimental well to the average maximum LDH release for each sample.

4.8 | Inhibition of protein synthesis

Bacteria were pretreated with 10 or 100 μ g/ml of CAM (Sigma-Aldrich) for 1 hr prior to infection of neutrophils. Alternatively, neutrophils were pretreated with 10 μ g/ml of CHX (Sigma-Aldrich) for 1 hr prior to infection with bacteria (Whitmore et al., 2017).

4.9 | Preparation of conditioned media

CMs were prepared from 5×10^6 /ml uninfected PMNs (PMN-CM), LVS-infected PMNs (iPMN-CM), or 1×10^9 /ml bacteria alone (LVS-CM) after incubation in HEPES-buffered RPMI-1640 containing L-glutamine at 37 °C with 5% CO₂ for 24 hr. After centrifugation to pellet cells and/or bacteria, the supernatants were sterilised using 0.45- μ m syringe filters. CM sterility was confirmed by plating for colony-forming units. Where noted, time and temperature for media conditioning were varied.

4.10 | Concentration and visualisation of proteins in conditioned media

LVS-CM was prepared as described above, and proteins were subsequently concentrated by precipitation in 10% trichloroacetic acid for 1 hr on ice or by drying under a stream of nitrogen. Concentrated proteins were washed twice with cold acetone and then boiled in 1 \times lithium dodecyl sulfate sample buffer (Life Technologies) containing a reducing agent. Proteins (approximately one to five culture equivalents as indicated in the figure legends) were separated by SDS-PAGE using 4–12% NuPAGE bis-tris gradient gels (Life Technologies) and visualised using a Pierce silver stain kit (Thermo Fisher Scientific).

4.11 | Radiolabelling of *Francisella tularensis* lipids

LVS was grown in the presence of [1,2-¹⁴C]sodium acetate as we previously described (J. H. Barker et al., 2016, 2014). Radiolabelled bacteria were used to generate [¹⁴C]LVS-CM as described above, but the conditioning time was 12 hr instead of 24 hr. Radioactivity in the CM was quantified by liquid scintillation counting. PMNs were incubated with [¹⁴C]LVS-CM for 24 hr at which time culture supernates were collected and PMNs were washed twice with HBSS without divalent cations. Radioactivity remaining in the supernatants, in each of the two washes, and the PMN pellets was quantified by liquid scintillation counting.

4.12 | Immunoblotting

Proteins in concentrated CM were separated on NuPAGE 4–12% bis-tris gradient gels and subsequently transferred to polyvinylidene

difluoride membranes (Perkin Elmer). Blocked membranes were probed to detect specific *Francisella* molecules using anti-FopA, anti-Mip, anti-Pal (the generous gift of Dr. Jason Huntley, University of Toledo, Toledo, OH; Huntley, Robertson, & Norgard, 2010), anti-Tul4, anti-IgC, anti-IgD, anti-IgB (BEI Resources, Manassas, VA; Rowe & Huntley, 2015), anti-LPS monoclonal antibody (mAb) FB11 (QED Bioscience Inc., San Diego, CA) or anti-LPS mAb (Meridian Bioscience, Cincinnati, OH; McCaffrey & Allen, 2006, Schwartz et al., 2012), and anticapsule mAb 11B7 (Dr. Michael Apicella, University of Iowa, Iowa City, IA; Schwartz et al., 2012). Bands were detected using horseradish-peroxidase-conjugated secondary antibodies and the Pierce SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific) and the Odyssey Fc imaging system (LI-COR).

4.13 | Whole-cell fractionation and isolation of bacterial lipoproteins from *Francisella*

Midexponential phase broth cultures of *F. tularensis* LVS and *F. novicida* U112 were prepared in BHI broth as described above. Whole-cell fractionation and lipoprotein enrichment were performed as previously described (Jones et al., 2012). In brief, bacteria were harvested by centrifugation at $10,000 \times g$ for 10 min at 4 °C. The bacterial pellets were resuspended in 50 ml of DPBS without cations and lysed via freeze-thaw for four cycles. The cell lysates were subsequently centrifuged at $20,000 \times g$ for 20 min at 4 °C to pellet any remaining intact cells. A protease inhibitor cocktail (Sigma Aldrich) containing AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstatin A was added to the clarified supernate (whole-cell lysate). The whole-cell lysates were centrifuged at $100,000 \times g$ for 90 min at 4 °C to separate the cytoplasmic/periplasmic fraction from the total membrane fraction. Each total membrane pellet was resuspended in 2 ml of cold 1% sarkosyl in DPBS without cations. BLPs were enriched by adding 8 ml of water-saturated *n*-butanol to the total membrane fraction and rocking the sample at 4 °C overnight. The BLP-enriched fraction was centrifuged at $20,000 \times g$ for 90 min at 4 °C. The aqueous phase was extracted and stored at -20 °C. The protein concentration of each BLP preparation was determined using the Pierce BCA protein assay (Thermo Fisher Scientific). BLPs (1 µg) were separated on NuPAGE 4-12% bis-tris gradient gels and visualised by silver staining as described above. BLP composition was analysed by immunoblotting as described above.

4.14 | DNA isolation and *TLR1* sequencing

Genomic DNA was extracted from isolated PBMCs using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Primers flanking *rs5743618* (S602I) have been previously defined (Whitmore et al., 2016). An annealing temperature of 58 °C was used for the PCR. PCR products were sequenced at the Genomics Division at the University of Iowa.

4.15 | Evaluation of toll-like receptor expression by flow cytometry

To assess surface expression of TLR1, TLR2, and TLR6, neutrophils were centrifuged at $300 \times g$ for 5 min and resuspended in blocking buffer (DPBS without cations containing 2% nonfat dry milk and 4%

heat-inactivated fetal bovine serum, HyClone) at 1×10^6 PMNs per millilitre. PMNs were incubated in blocking buffer for 20 min on ice after which TLR1-APC (R&D Systems), TLR2-AF488 (BD Biosciences), TLR6-PE (BioLegend), and their respective IgG controls were each added to PMNs for 1 hr on ice. Neutrophils were then washed with 2 ml of cold DPBS without cations prior to analysis using an Accuri C6 flow cytometer. Ten thousand events were collected for each sample, and data were analysed using FlowJo software.

4.16 | Priming and measurement of reduced nicotinamide adenine dinucleotide phosphate oxidase activity by luminol-enhanced chemiluminescence

PMNs in phenol red-free RPMI-1640 supplemented with L-glutamine (Lonza) and containing 1% human serum albumin (University of Iowa Hospitals and Clinics Pharmacy) were preloaded with 50 µM of luminol (Sigma Aldrich) via incubation at room temperature for 10 min in the dark. PMNs (1×10^6) were plated into each well of a white, flat-bottom, 96-well microtitre OptiPlate (Perkin Elmer; McCaffrey & Allen, 2006). Triplicate samples of PMNs were left untreated or primed with either FSL-1 (100 ng/ml), Pam₃CSK₄ (1 µg/ml), or TNFα (1 ng/ml; R&D Systems) for 29 min, and then activated by exposure to fMLF (10 µM in RPMI containing 1% human serum albumin). Control PMNs were left unstimulated. Luminescence was measured every 60 s for 59 cycles using a NOVostar luminometer (BMG LABTECH), and the data are expressed as relative luminescence units (McCaffrey & Allen, 2006). As noted, data were normalised to the peak luminescence obtained for unprimed neutrophils stimulated with fMLF.

4.17 | Statistical analyses

Data from experiments with one control and one experimental sample were analysed using a paired Student's *t* test. Experiments with one control group and two or more experimental groups were analysed by one-way analysis of variance followed by Dunnett's or Tukey's multiple-comparison posttest as indicated in the figure legends. Grouped analyses were performed using multiple *t* tests and correcting for multiple comparisons using the Holm-Sidak method. All analyses were performed using GraphPad Prism Version 6.0 or 7 software. A *p* value less than .05 was considered statistically significant.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

L.C.K. designed and performed the majority of experiments, analysed data, and wrote the first draft of the manuscript. J.M.M., B.B.K., and L.A.H.A. performed experiments and analysed data. J.W.K. assisted with radiolabelling experiments and preparation of concentrated BLPs. L.C.W., J.R.F., J.H.B., D.S.W., and B.D.J. provided essential expertise, methods, and/or reagents. L.C.W. and J.H.B. analysed data and edited the manuscript. L.A.H.A. conceived of the study, designed and performed experiments, analysed data, and wrote the final version of the manuscript.

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SUPPORTING INFORMATION

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