Importance of PdpC, IglC, IglI, and IglG for modulation of a host cell death pathway induced by *Francisella tularensis* LVS

Marie Lindgren, Kjell Eneslätt, Jeanette E. Bröms, and Anders Sjöstedt*

Department of Clinical Microbiology, Clinical Bacteriology and Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå University, SE-901 85 Umeå, Sweden

**Running title.** Modulation of a cell death pathway by *F. tularensis*

**Key words.** *Francisella tularensis*, host cell death, PdpC, IglG, IglI

*Corresponding author.*

Mailing address: Department of Clinical Microbiology, Clinical Bacteriology, Umeå University, SE-901 85 Umeå, Sweden.

Phone: +46 90 785 1120

Fax: +46 90 785 2225

Email: Anders.Sjostedt@climi.umu.se
Modulation of host cell death pathways appears to be a prerequisite for the successful life styles of many intracellular pathogens. The facultative intracellular bacterium *Francisella tularensis* is highly pathogenic and effective proliferation in the macrophage cytosol leading to host cell death is a requirement for its virulence. To better understand the prerequisites of this cell death, macrophages were infected with the *F. tularensis* live vaccine strain (LVS) and the effects were compared to those resulting from infections with deletion mutants lacking expression of either of the *pdpC*, *iglC*, *iglG*, or *iglI* genes, which encode components of the *Francisella* pathogenicity island (FPI), a type VI secretion system. Within 12 h, a majority of the J774 cells infected with the LVS strain showed production of mitochondrial superoxide and after 24 h, marked signs of mitochondrial damage, caspase-9 and caspase-3 activation, phosphatidylserine expression, nucleosome formation, and membrane leakage. In contrast, neither of these events occurred after infection with the Δ*iglI* or Δ*iglC* mutants, although the former strain replicated. The Δ*iglG* mutant replicated effectively but induced only marginal cytopathogenic effects after 24 h and intermediate effects after 48 h. In contrast, the Δ*pdpC* mutant showed no replication, but induced marked mitochondrial superoxide production and mitochondrial damage, caspase-3 activation, nucleosome formation, and phosphatidylserine expression, although the effects were delayed compared to LVS. The unique phenotypes of the mutants provide insights regarding the roles of individual FPI components for the modulation of the cytopathogenic effects resulting from the *F. tularensis* infection.
Intracellular bacteria have evolved distinct strategies to survive and multiply within host cells, the most challenging of which is to evade the antibacterial immune mechanisms of the phagocytic cells. This can be achieved either by preventing the oxidative burst, by manipulating the phago-lysosome fusion, by having sufficient physical stability to withstand the antimicrobial mechanisms within the phagosome, or by escaping from the phagosome (1). Such evasion strategies can be counteracted by the host by induction of cell death, thereby efficiently removing the intracellular pathogen’s habitat and exposing it to extracellular antibacterial mechanisms. However, induction of host cell death can also be advantageous for pathogens since it may avoid phagocytosis or allow escape from a habitat depleted of nutrients. Thus, manipulation of host cell death pathways is a critical step in the ever continuing host-parasite battle. Host cell death occurs via several mechanisms, either without inflammation, apoptosis, or with accompanying inflammation, pyroptosis or necrosis, and all of the three pathways have been found to result from various types of infections (2).

Francisella tularensis is a Gram-negative, facultative intracellular bacterium, which is able to infect many mammalian species, and the etiological agent of the zoonotic disease tularemia (3). The pathogenicity of the bacterium is not fully understood, but replication in the macrophage appears to play an essential role. It rapidly escapes from the phagosome before fusion with the lysosome occurs, allowing it to multiply within the cytosol (4, 5). The initial encounter with the host cell leads to a rapid proinflammatory response, but this is repressed after internalization of the bacterium (6-8). The infected cells, regardless of whether they are dendritic or macrophages, are unable to secrete cytokines in response to secondary stimuli (9-11). Francisella novicida is a closely related species and it has become a prototypic agent for studies of AIM2-mediated inflammasome activation, which leads to pyroptosis, a form of programmed cell death characterized by inflammasome formation resulting in caspase-1-dependent secretion of IL-1β and IL-18 (12, 13). Also infection with the LVS strain leads to secretion of IL-1β but it has previously been observed that in addition, the infection leads to several cytopathogenic features characteristic of apoptosis but not pyroptosis, such as release of cytochrome c, caspase-3 activation, and nucleosome formation (12, 14-16). Thus, the cytopathogenic effects do not fully conform to either apoptosis or pyroptosis and it is clear that F. tularensis is able to modulate many essential host cell pathways to facilitate its intracellular survival.
Much work aimed at understanding the bacterial factors responsible for modulation of the host cell signaling has been focused on the components constituting the *Francisella* Pathogenicity Island (FPI), a large genomic region duplicated in all strains of the highly virulent subspecies *tularensis* and *holarctica* and in the live vaccine strain (LVS) (17, 18). The latter is an empirically attenuated strain of subspecies *holarctica* that has been used for many decades as a human vaccine, but also in experimental models of tularemia since it still shows marked virulence in, e.g., mice (19). The FPI encodes a type VI secretion system (T6SS), containing some 17 proteins, most of which have been found to be essential for the intramacrophage replication and virulence of the bacterium (20-23). When mutants have been generated in the FPI, many of them show a uniform phenotype characterized by lack of phagosomal escape, no intracellular replication, and a loss of virulence in vivo. Examples of such mutants include those of the *iglABCD* operon and the core components VgrG and DotU (20-25). In contrast, we have observed that the Δ*iglI* and Δ*iglG* mutants showed aberrant intracellular replication and much diminished cytopathogenic effects (26). The Δ*pdpC* mutant of the LVS and SCHU S4 strains shows no intracellular replication but other phenotypes distinct to those of the T6SS core component mutants (27, 28). To understand the contribution of T6SS components to the cytopathogenic effects of the *F. tularensis* infection, we analyzed how cell death pathways of infected macrophages were affected by infection with the LVS strain or the T6SS mutants Δ*pdpC*, Δ*iglC*, Δ*iglG*, and Δ*iglI*.
Materials and Methods

**Bacterial strains, plasmids, and growth conditions.** The live vaccine strain (LVS) was originally obtained from the United States Army Medical Research Institute for Infectious Diseases (USAMRIID), Frederick, MD, USA. The ∆iglC, ∆pdpC, ∆iglG, and ∆iglI mutants, and complemented strains have been described earlier (20, 26, 27). *F. tularensis* strains were grown on modified GC-agar at 37°C, 5% CO2. When required, kanamycin (10 μg/ml) was added to the medium.

**Cultivation and infection of macrophages.** The murine macrophage-like cell line J774A.1 was used in all cell infection assays. Macrophages were cultured and maintained in DMEM (GIBCO BRL, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum, FBS, (GIBCO). For all experiments, cells were seeded either in tissue culture plates, or 50 ml Falcon tubes, incubated overnight, and reconstituted with fresh culture medium 30 min prior to infection. A multiplicity of infection (MOI) of 200 was used and bacteria, grown on agar plates, were suspended in PBS and kept on ice prior to infection. Intracellular replication experiments were performed essentially as described earlier (20).

**Detection of cleaved caspase-9.** One million J774 cells were seeded in 6-well plates and incubated overnight. Infection with *F. tularensis* strains was allowed for 2 h before extracellular bacteria were removed by washing and fresh medium containing 5 μg/ml gentamicin was added. At indicated time points, plates were placed on ice and ice-cold PBS supplemented with complete EDTA-free protease inhibitor (Roche, Basel, Switzerland) was used to wash the monolayers once before Laemmli buffer was added. Cells were scraped and transferred to pre-cooled microcentrifuge tubes. After 10 min of boiling, samples were separated on SDS-PAGE and transferred to nitrocellulose membrane. Mouse specific anti-caspase-9 antibody (Cell Signaling Technologies) was used to detect pro-caspase-9 and cleaved caspase-9, followed by a secondary horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (GE Healthcare, UK). As a loading control, antibodies against β-actin were used.
Flow cytometry analysis. For all flow cytometry experiments, cells were grown overnight, and reconstituted with fresh medium the next day. Cells were allowed to recover for 30 min before being infected at an MOI of 200. After 2 h, the cells were once again pelleted and extracellular bacteria were removed by washing. Then, cells were resuspended in fresh medium and incubated for indicated time points. A Vi-cell-XR cell viability analyzer (Beckman Coulter, Fullerton, CA, USA) was used to estimate cell viability at the time of sampling. Irrespective of type of analysis, a minimum of 10,000 events were acquired for each analysis using a LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) with FACSDiva software (BD Biosciences). Results were analyzed using FlowJo (Tree Star) software.

To measure the levels of reactive oxygen species, MitoSOX red mitochondrial superoxide indicator kit (Molecular probes) was used according to manufacturer’s instructions. Briefly, cells were incubated with medium containing 5 μM of the MitoSOX reagent for 10 min at 37°C and washed two times in PBS before analysis.

The changes in mitochondrial membrane potential (Δψ) were detected using the Flow Cytometry mitochondrial membrane potential detection kit, MitoScreen (BD Biosciences), according to manufacturer’s instructions. In live cells with normal, polarized Δψ, JC-1 is taken up leading to the formation of JC-1 aggregates, which results in high levels of red fluorescence. In mitochondria with depolarized Δψ, JC-1 remains in the cytoplasm as monomers resulting in green fluorescence.

For measurement of phosphatidylserine (PS)-positive cells, the FITC Annexin-V apoptosis detection kit II (BD Biosciences) was used according to manufacturer’s instructions. Briefly, cells were stained with Annexin-V-FITC and propidium iodide (PI). Early apoptosis was defined by Annexin-V'/PI' staining and late apoptosis by Annexin-V'/PI' staining.

For staining for active caspase-3, cells were transferred to 96-well plates and centrifuged for 3 min at 500 × g. Supernatants were removed and cells were washed once in PBS. After fixation and permeabilization, Cytofix/Cytoperm (BD Biosciences) for 20 min at RT, cells were pelleted and washed with Perm/Wash (BD Biosciences) and subsequently stained with PE conjugated rabbit anti-human active caspase-3 antibody (clone C92-605, BD Biosciences). Cells were then washed and resuspended in Perm/Wash buffer. Staurosporin (Sigma) was used as a positive control for cell death.
TUNEL staining was carried out using the In Situ Cell Death Detection Kit (Roche), according to the manufacturer’s instructions.

**Multiplex cytokine analysis.** Supernatants from infected cells were collected and stored at -80°C until analyzed using the Bio-Plex Pro Mouse cytokine 23-plex kit and a Bio-Plex 200 system (BioRad Laboratories) according to manufacturer’s instructions. Samples were analyzed in duplicates. For statistical analysis, samples below standard range were set to 0.5 times the detection limit for the respective cytokine, and samples above the standard range were set to the highest standard value for the respective cytokines.

**Data analysis and statistical methods.** Statistical significances were determined using paired, two-tailed Student’s *t*-tests, or Pearson’s chi-squared test. To compare the cytokine and chemokine data, principal component analysis was used. All data were log-transformed and analyzed by principal component analysis using the SIMCA program, version 13.

**Results**

**∆iglG and ∆iglI but not ∆pdpC or ∆iglC are able to replicate in J774 cells.** Previously, we and others have shown that the FPI mutants display a spectrum of intracellular phenotypes since some replicate as well as the wild-type, whereas others show compromised or no growth (20, 23-26, 28-30). We followed bacterial numbers of the ∆pdpC, ∆iglC, ∆iglG, and ∆iglI mutants and the LVS strain in J774 cells after infection. In agreement with previously published data (20, 23-26, 28-30), the ∆iglC and ∆pdpC mutants showed very marginal net replication, whereas the ∆iglG and the ∆iglI mutants replicated as well as LVS (Error! Reference source not found.). In separate experiments, we observed that the complemented strains showed the same degree of replication as did LVS (not shown).

**Mitochondrial-specific superoxide is induced upon LVS, ∆pdpC, or ∆iglG infection.** The mitochondrial production of ROS increases in response to various stimuli and is indicative of
mitochondrial dysfunction related to many pathophysiological processes, such as inflammation, infection, cancer, and aging (31). Accumulation of mitochondrial ROS occurs concomitantly to redistribution of cytochrome \( c \) from the mitochondria into the cytoplasm, where it causes activation of caspases and triggering of the intrinsic pathway of apoptosis (32). We measured the amount of mitochondrial superoxide in J774 cells using the indicator dye MitoSOX, which penetrates cell and mitochondrial membranes and, upon contact with mitochondrial superoxide, emits red fluorescence. We observed that the number of superoxide-positive cells was drastically increased upon infection with LVS (Fig. 1 and S1). Within 12 h, around 50 % of the cell population was MitoSOX-positive in LVS-infected cells, in comparison to < 4 % of uninfected cells. At this time point, no cells infected with the LVS mutants differed markedly from uninfected cells, whereas all the complemented strains had a phenotype more or less identical to LVS. After 24 h, however, 42 % and 25 % of the \( \Delta pdpC \) and \( \Delta iglG \)-infected cells, respectively, but < 15 % of the \( \Delta iglI \) - and \( \Delta iglC \)-infected and uninfected cells were MitoSOX-positive (Figs. 1 and S1).

Thus, infection with LVS, \( \Delta pdpC \), or \( \Delta iglG \) leads to mitochondrial superoxide production, albeit with delayed kinetics for the latter two infections. In contrast, very little or no production was seen in cells infected with \( \Delta iglC \) or \( \Delta iglI \).

Mitochondrial membrane instability is induced in cells infected with LVS or \( \Delta pdpC \).

Among the first events of the mitochondria-triggered intrinsic apoptotic pathway is the perturbation of the mitochondrial transmembrane potential (32). We measured it by utilizing the JC-1 dye, the fluorescence of which will be affected by the mitochondrial membrane potential; as the dye accumulates inside the mitochondria with intact membrane potential, it forms aggregates with red fluorescence. In cells with depolarized mitochondrial membranes, this kind of aggregates is not formed and the JC-1 dye exists as green fluorescent monomers. Previously, we have demonstrated that the LVS infection resulted in no significant change of the potential during the first 12 h, whereas a significant decrease occurred at 18 h and onwards (16). Therefore, the membrane potentials were determined at 24 and 48 h. After this time point, very few of the LVS-infected cells are intact and total cell numbers decrease drastically; therefore, we did not follow the effects beyond 48 h. At both time points, only minimal changes were observed in the \( \Delta iglC \), \( \Delta iglI \), and \( \Delta iglG \)-infected cells compared to uninfected cells, whereas already at 24 h, 72 % and 56 %, respectively, of the LVS- and \( \Delta pdpC \)-infected cells demonstrated significant decreases of the potential (Fig. 2 and S2).
effects were even more marked at 48 h as similar proportions, > 83 %, of both LVS- and
\( \Delta pdpC \)-infected cells were affected (Fig. 2 and S2). Thus, the \( \Delta pdpC \) infection resulted in
marked changes in the mitochondrial potential, albeit with slightly delayed kinetics compared
to the LVS infection. In contrast, very little or no changes in the potential were observed in
cells infected with \( \Delta iglC \), \( \Delta iglG \), or \( \Delta iglI \). All complemented strains had a phenotype similar
to that of LVS.

Cells infected with LVS or \( \Delta pdpC \) become markedly Annexin-V positive. Surface-exposed
exposure of phosphatidylserine (PS) is considered to be an early event of apoptosis, preceding
the full sequence of morphological changes at the ultrastructural level (33). At this initial
stage of cell death, the barrier function of the plasma membrane remains intact. At a later
stage of apoptosis, the plasma membrane becomes leaky for compounds like propidium
iodide (PI). Thus, we monitored the presence of surface-exposed PS by staining with
Annexin-V, a phospholipid-binding protein with high affinity for PS, and concomitantly the
integrity of the plasma membrane by staining with PI.

At 24 h, 21 % of the LVS-infected cells were both Annexin-V and PI negative, 15 % were
Annexin-V-positive only, and 53 % both Annexin-V- and PI-positive. In contrast, the
corresponding percentages for the \( \Delta iglC \)-infected cells were 78 %, 11 %, and 10 %, clearly
distinct to LVS-infected cells but similar to uninfected cells (Fig. 3). Cells infected with either
the \( \Delta iglG \) or \( \Delta iglI \) mutants were similar to uninfected cells, whereas \( \Delta pdpC \)-infected cells and
those infected with the complemented strains had phenotypes more similar to that of LVS. Of
the \( \Delta pdpC \)-infected cells, 36 % were both Annexin-V and PI negative, 31 % were Annexin-V-
positive only, and 23 % both Annexin-V- and PI-positive (Fig. 3).

The differences became more marked at 48 h, as only 3 % of the LVS-infected cells were
negative for both Annexin-V and PI, 29 % positive for Annexin-V, and 67 % positive for both
PI and Annexin-V. In contrast, the proportions seen in \( \Delta iglC \)-infected cells and uninfected
cells did not differ much from those seen at 24 h, whereas cells infected with either the \( \Delta iglG \)
or \( \Delta iglI \) mutants showed intermediate phenotypes. Of the latter, 45 % and 38 %, respectively,
were Annexin-V-positive only and 24 % and 15 %, respectively, both Annexin-V- and PI-
positive (Fig. 3). The \( \Delta pdpC \)-infected cells were distinct since a large majority, 88 %, was
only Annexin-V positive and 7 % were positive for both Annexin-V and PI. The
complemented strains had a phenotype similar to LVS infected; at 24 h the proportion of
Annexin-V- and PI-positive cells were 38-48 % and at 48 h 58-66 % (data not shown).

These results indicate that upon an LVS infection, a significant increase in the relative
numbers of Annexin-V-positive cells occurs within 24 h, and that the proportion of cells that
are both Annexin-V- and PI-positive, presumably dying cells, increased over time. In contrast,
very few of the ΔiglC-infected cells became Annexin-V- and/or PI-positive within 48 h,
whereas the ΔpdpC infection resulted in higher proportions of Annexin-V-positive, PI-
negative cells at both time points than did the LVS infection but very few double-positive
cells. Cells infected with the ΔiglG or ΔiglI mutants showed intermediate phenotypes with
larger proportions of Annexin-V-positive cells than for ΔiglC, but much lower numbers than
for LVS- or ΔpdpC-infected cells.

Detection of caspase-9 in infected J774 cells. As a response to the mitochondrial membrane
destabilization, cytochrome c is released into the cytoplasm and interacts with ApaF1 to
activate caspase-9 which in turn will activate caspase-3 and other effector caspases (34). The
proform and mature form of caspase-9 were distinguished using Western blot analysis.
Infection with LVS or the complemented strains led to distinct cleavage already within 24 h,
whereas none of the four mutants showed any distinctly cleaved caspase-9 at 24 or 36 h (Fig.
4). Not even at 48 h was marked cleavage of caspase-9 detected, although weak cleavage in
cells infected with the ΔiglG mutant was observed (Fig. 4).
Thus, infection with LVS and ΔiglG, although the latter much less prominently, led to
activation of caspase-9.

Caspase-3 cleavage occurs in LVS, ΔpdpC, or ΔiglG-infected cells. Caspase-3 is a crucial
effector in many forms of caspase-dependent cell death and is activated both through the
extrinsic and intrinsic pathways and required for condensation of the nucleus and DNA
fragmentation (35). The infected cell populations were analyzed using flow cytometry using
an antibody specific to the activated form of caspase-3 at 24 or 48 h; uninfected cells or cells
treated with the apoptosis-inducing substance staurosporin were used as controls. Within 24 h
there were clear differences among the cell populations; infection with LVS and ΔpdpC
resulted in at least 35 % positive cells, whereas less than 10 % of uninfected cells, or cells
infected with the ΔiglC, ΔiglG, or ΔiglI mutants were caspase-3-positive (Fig. 5 and S3).
After 48 h, the proportions of caspase-3-positive cells were approximately 20% for the ∆iglC- and ∆iglG-infected cells, whereas all other infected cells showed only small changes compared to 24 h (Fig. 5 and S3). Cells infected with the complemented strains were caspase-3-positive almost to the same extent as LVS-infected cells, with the exception of the complemented ∆iglI mutant that showed intermediate values. In staurosporin-treated, uninfected cells, 86% were caspase-3 positive.

Nucleosome formation occurs within 24 h in cells infected with LVS but is delayed in cells infected with ∆pdpC or ∆iglG. At the terminal stage of apoptosis, DNA fragmentation occurs, leading to nucleosome formation (33). Here, it was assessed by following the appearance of TUNEL-positive cells. At 12 h, there were very low numbers of such cells even after infection with LVS (data not shown). At 24 h, and in repeated experiments, infection with either of the mutants resulted in no significant fragmentation, < 4%, whereas the corresponding number for LVS-infected cells was 20% (Fig. 6 and S4). At 48 h, also the ∆pdpC infection resulted in significant fragmentation and 22% of the cells were positive vs. 17% of the LVS-infected cells, whereas < 5% of the cells infected with either of the other mutants were TUNEL-positive (Fig. 6 and S4). It should be noted that staining of bacterial DNA may also occur with this method. However, since the infection with the replicating ∆iglI mutant consistently resulted in very low values, < 5%, the data indicate that the fragmentation occurred only in the eukaryotic DNA.

Collectively, the data indicate that infection with the LVS and the ∆pdpC strains, but not the other mutants, resulted in significant levels of nucleosome formation.

Cytokine secretion patterns reveal clear distinctions of the effects from infections with different strains of F. tularensis. In view of the distinct phenotype of several of the mutants in the aforementioned assays, we performed a comprehensive multiplex analysis of the secretion of 22 cytokines and chemokines from J774 cells infected with either of the four mutants or the LVS strain. At 24 h, levels were much higher in ∆iglC-infected than LVS-infected cells, and higher or identical to uninfected cell. The differences between ∆iglC-infected cells and LVS-infected were 2-4-fold for GM-CSF, IFN-γ, IL-12p70, IL-13, IL-17,
IL-1α, IL-2, IL-3, IL-4, IL-5, IL-9, KC, and TNF-α, or > 4-fold for Eotaxin, G-CSF, IL-10, IL-12p40, IL-6, MCP-1, MIP-1α, MIP-1β, and RANTES (Table). The secretion patterns from ΔiglG- or ΔiglI-infected cells were intermediate. In contrast, the secretion pattern from ΔpdpC-infected cells was much more similar to LVS-infected cells than ΔiglC-infected cells; all cytokine levels were < 2-fold different compared to LVS.

In conclusion, the secreted cytokines indicate that the ΔpdpC mutant-infected cells showed the most similar phenotype to the LVS-infected cells, whereas the other types of infected cells demonstrated distinct patterns. This was also confirmed with principal component analysis (PCA) and the analysis resulted in a model that explained 91% of the variation of the data (Fig. 7). The secretion pattern of the ΔiglC-infected cultures was separated from the other infections and the uninfected cultures were clearly separated from all infected cultures. The secretion patterns of LVS-, ΔpdpC-, ΔiglI-, and ΔiglG-infected cultures were each distinct and their distances to the ΔiglC-infected cultures were in the corresponding order with the LVS-infected culture being the most distant.

Discussion

The virulence of *F. tularensis* is intimately linked to its ability to replicate in macrophages since mutants defective for intramacrophage replication are rendered avirulent. A prerequisite for the intracellular replication is a functional type VI secretion system, encoded by the FPI. Many of the FPI components are presumed to be essential core components since they appear critically required and in their absence, a lack of phagosomal escape, no intracellular replication, and lack of virulence are manifested (17). The best characterized example of such mutants is the ΔiglC mutant. Not all of the investigated mutants so far fit into this uniform pattern, however, since the ΔiglI and ΔiglG mutants of LVS show delayed cytopathogenicity and lack of virulence, although more or less intact intracellular replication in J774 cells, whereas only the latter mutant replicated in peritoneal exudates cells and bone marrow-derived macrophages (26). In addition, in a recent study, we observed that the ΔpdpC mutant of LVS showed a very unique phenotype, characterized by a lack of intracellular replication, incomplete phagosomal escape, and marked attenuation in the mouse model, however, unlike a phagosomally contained FPI mutant, it triggered secretion of IL-1β and an MOI-dependent...
release of LDH, a marker of membrane damage (27). Another recent publication demonstrated likewise that the ΔpdpC mutant of the highly virulent subspecies tularensis strain SCHU S4 displays incomplete phagosomal escape and lacks intracellular replication (28).

We wanted to understand how the unique FPI mutant phenotypes were related to the marked cytopathogenic effects that are hallmarks of the F. tularensis infection. This will provide important information regarding the roles of individual FPI components for the modulation of the cellular pathways required for the successful intracellular life style of F. tularensis as well as provide a more complete understanding of its T6SS.

A number of publications have demonstrated that F. novicida triggers cell death via release of its DNA into the cytosol leading to recruitment of absent in melanoma 2 (AIM2) and apoptosis-associated specklike protein (ASC), which result in inflammasome activation, cleavage of caspase-1, and release of IL-1β, a form of cell death designated pyroptosis (12, 13, 36, 37). Although a variant of programmed cell death, it is distinct from the immunologically silent cell death taking place during apoptosis and it occurs independently of proapoptotic caspases (38). On the cellular level, it is characterized by plasma membrane rupture, water influx, cellular swelling, osmotic lysis, release of proinflammatory cellular content, and DNA cleavage (39). The DNA degradation is executed by an unknown nuclease and is distinct from the nucleosome formation characteristic of apoptosis and, conversely, caspase-1 is not involved in apoptosis (38). As demonstrated in many studies, not only the F. novicida infection, but also the F. tularensis macrophage infection is characterized by secretion of IL-1β, indicating a pyroptotic mechanism (26, 36, 37, 40-44), however, at the same time also by activation of proapoptotic caspases and other characteristics of the mitochondria-triggered intrinsic apoptotic pathway (14-16). In fact, studies in vivo using virulent strains demonstrated very little activation of caspase-1 and normal pathology in caspase-1-deficient mice, whereas cell death strongly correlated to caspase-3 activation (14). Thus, the evidence indicates that the effects of the F. tularensis infection may not unambiguously fit with the characteristics of either apoptosis or pyroptosis. This conclusion was corroborated by our recent findings that ΔigI G and ΔpdpC-infected cells secrete IL-1β (26, 27), but, as we show in this study, also display many features characteristic of activation of the intrinsic apoptotic pathway, such as mitochondrial damage, caspase-3 activation and nucleosome formation. The reasons for these dichotomous findings are unclear, but may be due to the presence of simultaneous signals that trigger both types of pathways or,
alternatively that the unusual form of inflammasome activation triggered by \textit{F. tularensis} also leads to engagement of the intrinsic apoptotic pathway, however, there is no direct evidence for the latter hypothesis. It should be noted that regardless of stimulus, IL-1β secretion from J774 cells is very low (26) indicating that the inflammasome activation may be defective and, therefore, alternative cell death pathways may be more easily discernible in this cell type. An interesting finding on \textit{F. novicida} was recently published demonstrating that an AIM2/ASC-dependent, caspase-3-mediated apoptosis, that also involves caspase-8 and caspase-9, occurs in caspase-1-deficient macrophages (45). If these findings also are relevant for the LVS strain, then they would support the hypothesis that the inflammasome activation triggered by \textit{Francisella} may lead to several types of terminal events and the ultimate cause of the cell death will depend on both bacterial and host factors. An example of this is our previous demonstration that the \textit{F. novicida} U112 strain more potently induces release of IL-1β than does LVS (26). Thus, the lower potency of LVS to induce IL-1β may suggest that also other cell death pathways than pyroptosis are important for the cell death occurring during the LVS infection.

Besides our findings that the LVS infection resulted in activation of features characteristic of the intrinsic apoptotic pathway, we observed that each of the mutants showed very distinct features in this regard. Although the \textit{ΔiglI} and \textit{ΔiglG} mutants replicated and the \textit{ΔiglC} and \textit{ΔpdpC} mutants did not, this did not correlate to the resulting cytopathogenic effects. In almost all aspects, infection with the \textit{ΔiglC} mutant showed very marginal or no cytopathogenic effects and also infection with the \textit{ΔiglI} mutant resulted in minimal effects. In contrast, the \textit{ΔiglG} mutant showed an intermediate phenotype and in most assays, it showed minimal effects after 24 h but significant effects after 48 h. The most unexpected effects resulted from infection with the \textit{ΔpdpC} mutant since, despite its lack of replication and unlike the other mutants, it resulted in marked activation of most mechanisms analyzed, although with delayed kinetics compared to the LVS infection. Specifically, the \textit{ΔpdpC} mutant markedly triggered mitochondrial damage, caspase-3 activation, PS expression, and DNA fragmentation. In contrast to the LVS infection, infection with the mutant, somewhat unexpectedly, did not lead to caspase-9 cleavage or PI-positive cells.

The \textit{ΔiglC} mutant has served as a prototype for \textit{F. tularensis} T6SS mutants and it has been found to lack phagosomal escape, intracellular replication, and virulence in the mouse model (20-22). In addition, it induces increased expression of a subset of TLR2-dependent, proinflammatory genes (11). In the present investigation, we observed that the cytopathogenic
effects on the ΔiglC-infected cells were very discrete, in most respects not much different
from those of uninfected cells, although the cytokine secretion patterns were clearly distinct.
Thus, the lack of phagosomal escape appears to result in minimal cytopathogenic effects but
distinct secretion of cytokines. Most likely these features are not modulated directly by IgIC,
but result from the lack of a functional T6SS due to the essential role of the protein for the
secretion system. It should be noted, however, that no defined role of IgIC for the secretion
machinery has been demonstrated and, in fact, a recent publication demonstrated that it is
secreted during macrophage infection (46).

The ΔigII and ΔigIG mutants of LVS demonstrate aberrant intramacrophage replication and
much diminished LDH release (26). The former strain replicated only in J774 cells and the
latter showed slightly delayed replication in BMDM but their phagosomal escape in J774
cells was only slightly delayed compared to LVS. Additionally, a recent publication
demonstrated that the SCHU S4 ΔigII mutant did not replicate in BMDM (28). These features
appear to coincide with distinct patterns of activation of the host cell death pathways
investigated since infection with the ΔigII mutant resulted in effects similar to those of the
ΔigIC infection, whereas the ΔigIG mutant infection resulted in an intermediate phenotype
with marginal cytopathogenic effects after 24 h but significant effects after 48 h, although not
as marked as those due to the LVS infection. Still, the infection with ΔigIG led to a secreted
cytokine pattern similar to that of ΔigII and ΔigIC. The findings suggest that IgII and IgIG are
indirectly or directly involved in modulating the cytopathogenic effects of the *F. tularensis*
infection. Although it is possible that their functions are essential for T6SS and thereby, their
effects are indirect as for IgIC, our previous findings that the corresponding mutants can
replicate in certain types of macrophages and escape from the phagosome in J774 cells (26),
show that they are distinct from mutants such as ΔigIC and that it is more likely that they
affect the cytopathogenic effects by other means than the T6SS core components do.

The present investigation reveals that the ΔpdpC mutant of LVS is another example of an FPI
mutant with a very distinct and paradoxical phenotype, since it in some aspects mimics that of
the LVS strain, for example shows induction of IL-1β and inhibition of LPS-induced TNF-α
release, whereas in other aspects it is very distinct since it does not show normal escape into
the cytosol, lacks intramacrophage replication, and is highly attenuated in the mouse model
(27). Our present findings further corroborate the unusual phenotype of the ΔpdpC mutant
since it was found to be distinct to all other investigated mutants and infection led to marked

15
mitochondrial damage, caspase-3 cleavage, expression of PS and DNA fragmentation, although with delayed kinetics compared to LVS. Moreover, the secreted cytokine pattern was very similar to that of LVS-infected cells. We have previously proposed that the aberrant behavior of the mutant is due to its partial degradation of the phagosomal membrane since this could lead to release of bacterial components into the cytosol that activate pathways that result in cytopathogenic effects and a cytokine pattern like that induced by LVS (27). This hypothesis assumes that PdpC does not directly modulate the cytopathogenic signaling, but rather is involved in the degradation of the phagosomal membrane. The phenotypic differences between the ΔpdpC mutant and the ΔiglG or ΔiglI mutants, both of which also degrade the phagosomal membrane, may be dependent on specific roles of IglG and IglI to modulate the cytosolic signaling that leads to cytopathogenicity.

In contrast to our findings on macrophages, it has recently been demonstrated that the *F. tularensis* infection leads to delay of the host cell death of neutrophils (47). A fundamental difference between the two cell types is that neutrophils are very short-lived cells that undergo constitutive apoptosis (48). Therefore, it may be that the rapid, spontaneous host cell death may not be advantageous for an intracellular bacterium since the life span may not be sufficient for significant replication to occur, thus delay of apoptosis is advantageous for the pathogen, whereas this is not a problem in the more long-lived macrophage. In the latter cell type, however, deprivation of nutrients eventually may be an issue and therefore, induction of host cell death may be beneficial since it allows spread of the pathogen to nutrient-replete cells.

In summary, we have shown that the *F. tularensis* LVS infection leads to induction of host cell death that is similar to the intrinsic apoptotic pathway, although, it is well established that the infection leads to secretion of IL-1β, a hallmark of pyroptosis, whereas infection with each of four FPI mutants results in cytopathogenic effects that are distinct from the LVS infection and, in some instances, with essentially no cytopathogenic effects. The findings provide novel insights regarding the roles of individual FPI components for the modulation of the cytopathogenic effects resulting from the *F. tularensis* infection and contribute to a more thorough understanding of its enigmatic T6SS.

Acknowledgements
We thank Anders Johansson for advice regarding the TUNEL analysis, Nelson Gekara for advice regarding MitoSOX, and Mateja Ozanic for help with the flow cytometry experiments. This work was supported by grant 2009-5026 from the Swedish Research Council and a grant from the Medical Faculty, Umeå University, Umeå, Sweden. The work was performed in part at the Umeå Centre for Microbial Research (UCMR).

References


The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. Nature immunology 11:385-393.


Schwartz JT, Barker JH, Kaufman J, Fayram DC, McCracken JM, Allen LA. 2012. *Francisella tularensis* inhibits the intrinsic and extrinsic pathways to delay

Legends to figures

Fig. 1. Mitochondrial superoxide accumulation in response to infection. J774 cells were infected with the indicated strain of *F. tularensis* for 2 h, after which the cells were washed and incubated for 12 or 24 h. The amount of mitochondrial superoxide was measured by assessing the oxidation of the fluorogenic dye MitoSOX and the percentages of MitoSOX-positive cells are shown. The data shown are representative of one experiment out of two performed. Pearson’s Chi-square test was used to test if the number of gated cells were significantly different from that of LVS-infected cells (*** = *P* < 0.001).

Fig. 2. The effect of *F. tularensis* infection on mitochondrial membrane stability. J774 cells were infected with the indicated strain of *F. tularensis* for 2 h, after which the cells were washed and incubated for 24 or 48 h. They were then stained with MitoScreen and analyzed by flow cytometry. The MitoScreen reagent, JC-1, indicates mitochondrial membrane stability; intact membrane potential leads to red fluorescence and reduced potential to green fluorescence. The data shown are representative of one experiment out of two performed. Pearson’s Chi-square test was used to test if the number of gated cells were significantly different from that of LVS-infected cells (*** = *P* < 0.001).

Fig. 3. Annexin-V- and propidium iodine (PI)-staining of infected J774 cells. Cells were infected with indicated *F. tularensis* strains for 2 h and thereafter washed. Cells were incubated for 24 or 48 h before being subjected to Annexin-V/PI staining and analyzed using flow cytometry. Unaffected cells, lower left quadrant, are both Annexin-V- and PI-negative; apoptotic cells with intact membrane integrity, lower right quadrant, are Annexin-V-positive but PI-negative; apoptotic cells that have lost their membrane integrity, upper right quadrant, are both Annexin-V- and PI-positive. The scatter plots show the entire cell population for each sample and time point. The data shown are from one representative experiment out of three performed.

Fig. 4. Activated (cleaved) caspase-9 in response to *F. tularensis* infection. J774 cells were infected with indicated strains of *F. tularensis* and cell lysates prepared after 24, 36, or 48 h.
A caspase-9 antibody was used to detect pro-caspase-9 and activated caspase-9 by standard Western blot techniques. An antibody against β-actin was used as a loading control. The data shown are from a representative experiment out of two performed.

Fig. 5. Percentage of infected J774 cells containing active caspase-3. Cells were infected with indicated strains of *F. tularensis*, washed and incubated for either 24 or 48 h, before the portion of cells containing active caspase-3 was measured using flow cytometry. The data shown are representative from one out of two experiments performed. Pearson’s Chi-square test was used to test if the number of gated cells were significantly different from that of LVS-infected cells (**P < 0.001**).

Fig. 6. DNA fragmentation in cells infected with *F. tularensis*. J774 cells were infected with indicated strains for 2 h and thereafter washed. They were incubated for 24 or 48 h before lysates were prepared and analyzed using the TUNEL assay and flow cytometry. Shown is the percentage of TUNEL-positive cells for each infection and time point. The data are representative of one experiment out of three performed. Pearson’s Chi-square test was used to test if the number of gated cells were significantly different from that of LVS-infected cells (*P < 0.05; **P < 0.001*).

Fig. 7. Principal component analysis (PCA) of cytokine gene expression or secretion of PECs infected with indicated *F. tularensis* strains. The data are presented in Table 2. The two components shown explained 91% of the variation of the data set.
Table 1. Intracellular replication of *F. tularensis* strains in J774 cells.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Net increase of bacteria (log₁₀ ± SD)/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVS</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>Δ<em>iglC</em></td>
<td>0.7 ± 0.1*</td>
</tr>
<tr>
<td>Δ<em>pdpC</em></td>
<td>1.0 ± 0.2*</td>
</tr>
<tr>
<td>Δ<em>iglG</em></td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Δ<em>iglI</em></td>
<td>3.6 ± 0.1</td>
</tr>
</tbody>
</table>

* The *F. tularensis* strains were added to the J774 cells at an MOI of 200.  
* Data represent the increase of mean log₁₀ CFU ± SD of 3 cultures. A representative experiment out of 3 is shown.  
* Net increase of bacterial CFU from 0 to 24 h was significantly lower (*P* < 0.01) than the increase of LVS by Student’s *t*-test.
Table 2. Cytokines secreted by J774 cells 24 h post infection with *F. tularensis*<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>LVS</th>
<th>ΔiglC</th>
<th>ΔpdpC</th>
<th>ΔiglG</th>
<th>ΔiglI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>105</td>
<td>840</td>
<td>171</td>
<td>437</td>
<td>290</td>
</tr>
<tr>
<td>G-CSF</td>
<td>5.0</td>
<td>32.9</td>
<td>7.1</td>
<td>17.5</td>
<td>15.4</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>36.6</td>
<td>92.9</td>
<td>42.2</td>
<td>68.8</td>
<td>53.5</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.2</td>
<td>3.4</td>
<td>1.7</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td>IL-1α</td>
<td>4.9</td>
<td>13.5</td>
<td>5.5</td>
<td>9.2</td>
<td>8.6</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.8</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-3</td>
<td>0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9</td>
<td>0.8</td>
<td>1.8</td>
<td>0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-4</td>
<td>3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.5</td>
<td>3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.0</td>
<td>3.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.3</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-9</td>
<td>72.8</td>
<td>268</td>
<td>94.6</td>
<td>183</td>
<td>134</td>
</tr>
<tr>
<td>IL-10</td>
<td>11.3</td>
<td>58.5</td>
<td>15.3</td>
<td>32.5</td>
<td>26.2</td>
</tr>
<tr>
<td>IL-12(p40)</td>
<td>3.9</td>
<td>28.3</td>
<td>6.5</td>
<td>17.4</td>
<td>10.3</td>
</tr>
<tr>
<td>IL-12(p70)</td>
<td>12.4</td>
<td>40.7</td>
<td>15.6</td>
<td>26.4</td>
<td>22.4</td>
</tr>
<tr>
<td>IL-13</td>
<td>61.8</td>
<td>194.3</td>
<td>67.6</td>
<td>125</td>
<td>98.4</td>
</tr>
<tr>
<td>IL-17</td>
<td>1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2</td>
<td>1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KC</td>
<td>1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1</td>
<td>1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1709</td>
<td>25520</td>
<td>1980</td>
<td>8491</td>
<td>5125</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>486</td>
<td>28540&lt;sup&gt;c&lt;/sup&gt;</td>
<td>600</td>
<td>3931</td>
<td>2031</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>327</td>
<td>12384&lt;sup&gt;c&lt;/sup&gt;</td>
<td>441</td>
<td>4156</td>
<td>1457</td>
</tr>
<tr>
<td>RANTES</td>
<td>24.9</td>
<td>121</td>
<td>31.9</td>
<td>73.7</td>
<td>49.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>8.5</td>
<td>28.4</td>
<td>9.5</td>
<td>15.4</td>
<td>11.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> J774 cells were infected with an MOI of 200 and incubated for 24 h before supernatants were collected and cytokines were measured.

<sup>b</sup> Value below standard range, set to 0.5 times lowest standard value.

<sup>c</sup> Value above standard range, set to highest standard value.
Fig. 1

% MitoSOX-positive cells

- LVS
- ΔlgC
- ΔpdpC
- ΔlgG
- ΔlgII
- Uninfected
- ΔpdpC/pdpC
- ΔlgG/ΔlgG
- ΔlgII/ΔlgII

12h
24h
Fig. 3

Propidium iodide

Annexin V

Uninfected

ΔigII infected

ΔigIG infected

ΔpdpC infected

ΔigIC infected

LVS infected
Fig. 6

% TUNEL-positive cells

- LVS
- ΔigIC
- ΔpdpC
- ΔigI G
- ΔigII
- Uninfected
- ΔpdpC/pdpC
- ΔigI G/igI G
- ΔigII/igII

24 h
48 h
Fig. 7

LVS

ΔpdpC  ΔiglI

ΔiglC

ΔiglG

Uninfected

R²x[1] = 0.841
R²x[2] = 0.0729