Intracellular survival mechanisms of Francisella tularensis, a stealth pathogen

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Abstract

Research on the highly virulent and contagious, facultative intracellular bacterium Francisella tularensis has come into the limelight recently, but still little is known regarding its virulence mechanisms. This review summarizes recent studies on its intramacrophage survival mechanisms, some of which appear to be novel.

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1. Introduction: the bacterium Francisella tularensis and the disease tularemia

F. tularensis is a highly virulent and contagious, facultative intracellular bacterium. It causes the zoonotic disease tularemia in a large number of mammals. Rabbits, hares and small rodents play an important role in its natural life cycle and through intermediate vectors such as flies, mosquitoes, and ticks, it infects mammals [1]. Several pieces of evidence indicate that F. tularensis may have a yet not identified reservoir in nature separate from mammals and arthropods. Amoebae have been suggested to be one potential reservoir. There are four subspecies of F. tularensis subsp. tularensis and holarctica, subsp. mediasiatica, and subsp. novicida [2]. The former two subspecies are clinically important. Subspecies tularensis is confined to North America, whereas subspecies holarctica is found in many countries of the Northern Hemisphere. Subspecies novicida has a strong association with water but is also a rare, opportunistic human pathogen [1]. Also outbreaks caused by strains of subsp. holarctica show a strong predilection for lakes and rivers. Thus, the natural reservoir for several of the subspecies seems to be related to water. Strains of subsp. tularensis and holarctica are contagious since the infectious dose even in humans is very low, at most 10 CFU, and they can infect via several routes and give rise to various clinical forms such as respiratory, ulceroglandular, or oropharyngeal tularemia [1]. There is, however, a distinct subspecies-dependent difference in virulence insofar as strains of subsp. tularensis cause a very aggressive form of human disease with a mortality as high as 30% if untreated, whereas holarctica strains give rise to a non-fatal disease [1].

Several attenuated F. tularensis strains were developed in the 1950’s and the most successful candidate was the so called live vaccine strain, F. tularensis LVS, which has been given to tens of thousands of individuals and affords good albeit not complete protection against laboratory-acquired forms of tularemia (reviewed in [3]). The strain was originally developed by attenuation of a Russian strain of the subsp. holarctica but subsequently transferred to the US and there produced as a vaccine.

2. Little is known regarding virulence mechanisms of F. tularensis but there has been some recent progress

At present, essentially nothing is known that explains the high virulence of subsp. tularensis, however, the genome sequence of the prototypic strain of the subspecies, SCHU S4 has been published recently [4]. The 1892 kb genome contains 1804 genes. A few notable features were revealed from the genome sequence; a set of genes encoding type IV pili, a type II secretion system, a surface polysaccharide, a putative poly-D-glutamic acid capsule, an iron acquisition systems, and a conspicuously high proportion, > 10%, of genes con-
taining insertions/deletions or substitution mutations. The latter is also reflected in a considerable proportion of disrupted metabolic pathways, explaining the fastidious nutritional requirements of the bacterium. Although there is evidence for a secretion system in the genome, no secreted bacterial products have been identified. Moreover, there is no evidence for any exotoxin production by *F. tularensis*.

The genome sequence did not reveal any other obvious explanations for the high virulence of the bacterium. One notable feature is the presence of a duplicated 33.9 kb region containing 25 genes lacking homologs in other characterized bacterial species. A variable GC content, between 26.6% and 31.2% vs. 33.2% for the whole genome, and lack of bilateral flanking elements makes the origin of the duplicated region unclear. There is accumulating evidence that genes of this genomic island are essential for intracellular survival even for non-virulent strains of *F. tularensis*, such as *F. tularensis* LVS and strains of subsp. *novicida* [5–8]. When genes of the region have been inactivated, for example the *iglC* gene, the mutants do not escape from the phagosome and do not multiply intracellularly as the wild type strains do [9,10]. Besides the *igl* genes, mutants of subspecies *novicida* defective in expression of the *pdpD* and *pdpA* genes, both of which are located in the genomic island, are markedly attenuated in their replication in mouse bone marrow-derived macrophages (BMM) [8]. The phenotypes of the mutants of the genome island have summarized in Table 1.


The genes of the region do not explain the high virulence of subspecies *tularensis* or *holarctica* but rather appear to be essential for *F. tularensis* to survive intracellularly. It should be noted that the mechanisms executed by the *igl* operon and regulated by MglA are critical in other experimental models as well, since the *AiglC* and *ΔmglA* strains of subsp. *novicida* are markedly attenuated in an amoeba model [6] and the *AiglC* mutant of *F. tularensis* LVS is avirulent in mice [7]. A few other genes, summarized in Table 1, have been identified that contribute to intracellular macrophage growth since inactivation of *F. tularensis* genes encoding homologues of glutamine phosphoribosyl-pyrophosphate amidotransferase, alanine racemase, and the heat shock-inducible ClpB protease resulted in attenuated phenotypes in vitro [12]. Altogether, the genome information suggests that virulence mechanisms different from those described in other intracellular bacteria are operative in *F. tularensis*.

### 3. *F. tularensis*-a facultative intracellular bacterium

*F. tularensis* is a bacterium capable of infecting and multiplying in a wide variety of phagocytic cells such as human, rat, rabbit, guinea pig, and mouse mononuclear cells as well as non-phagocytic cells such as hepatic cells, fibroblasts, tick epithelial cells, endothelial cells, and HeLa cells (reviewed in [13]). There is virtually no available data on the receptors involved in its uptake and if the bacterium engages specific TLRs.

#### 3.1. Mechanisms controlling the *F. tularensis* infection in murine macrophages

Most modern studies on *F. tularensis* have employed various forms of mononuclear cells of human or mouse origin. Resident or elicited macrophages from mice, rats and guinea pigs, bone marrow-derived mouse macrophages, mouse macrophages cell lines, such as J774, human peripheral blood mononuclear cells support the growth of *F. tularensis* [14–19]. Generally, virulent strains and LVS proliferate efficiently intracellularly for 24–48 h in non-activated cells. Notably, bacterial replication does not start until 6–12 h after invasion [20]. IFN-γ has been shown to be crucial to activate the macrophages and execute control of the infection [14,15,18,19,21,22]. In most cell types, the IFN-γ-mediated effect is partially reversed in the absence of iNOS, by NO inhibitors, or by neutralizing anti-TNF antibodies [21,23], the exception being murine alveolar macrophages [19]. In the latter case, there was no effect of inhibitors of NO or of reactive oxygen metabolites. In a recent study, employing mouse peritoneal exudate cells (PEC), it was observed that iNOS-gene-deficient PEC showed compromised killing of LVS whereas...
PEC from phox gene-deficient (p47-phox–/–) mice were capable to kill the bacteria [21]. A decomposition catalyst of ONOO\(^-\), FeTPPS, completely reversed the IFN-\(\gamma\)-induced killing of LVS [21]. Thus, the data strongly suggested that the reversal of the cidal effects by inhibition or the absence of iNOS was due to effects on peroxynitrite formation. The cidal molecule appeared to be formed even in the absence of phox, explaining the non-essential role of the latter. Additional support of the critical role of peroxynitrite was the finding that exposure of \(F. tularensis\) LVS under host cell free conditions to S-nitroso-acetyl-penicillamine (SNAP), which generates NO, led to no killing during a 6 h period whereas exposure to 3-morpholinosydnonimine hydrochloride (SIN-1), which generates NO and superoxide and then formation of peroxynitrite, resulted in effective killing [21].

An in vitro system has been established to elucidate the cellular and cytokine requirements for the macrophage-mediated control of an \(F. tularensis\) infection [17,24]. It is based on the use of BMM and to the BMM cultures purified T cell subsets are added. It was observed that even when the \(F. tularensis\)-infected BMM were IFN-\(\gamma\)R\(^-\), the addition of purified CD4 T cells from LVS-immune spleens, and even more so of CD8 T cells, were sufficient to control the infection [24]. Moreover, also addition of a population of Thy1\(^+\)CD4\(^+\)CD8\(^-\) cells afforded effective control of the intracellular growth of LVS. When IFN-\(\gamma\) was not operative, TNF-\(\alpha\) appeared to be critically required for the control [24]. Although this study identified an important role of IFN-\(\gamma\) in the control of the LVS infection, it demonstrated that IFN-\(\gamma\)-independent, TNF-\(\alpha\)-dependent mechanisms also are effective. Using the same in vitro system, it was demonstrated that B cells did not contribute to the control of the intramacrophage LVS infection [17].

The targets for the \(F. tularensis\)-specific immune responses are in part characterized. It was concluded, based on the use of purified or fractionated proteins, that LVS-vaccinated individuals and former tularemia patients demonstrated a CD4\(^+\) and CD8\(^+\) T cell-response to multiple proteins and that each individual recognized a unique mosaic of proteins (reviewed in [25]). It was concluded that the mosaic likely was dependent on the HLA haplotype of the individual. The specificities of \(F. tularensis\)-specific human and mouse sera have been characterized in detail in recent studies [26,27].

3.2. The role of neutrophils for controlling the \(F. tularensis\) infection

The role of human neutrophils in the control of an \(F. tularensis\) infection has been studied previously. It was found that a virulent strain of subsp. holarcticus was more resistant than \(F. tularensis\) LVS to the neutrophil-mediated killing [28]. Both strains efficiently activated the respiratory burst of the neutrophils but the virulent strain was more resistant to bactericidal products formed from oxygen and, in particular, LVS was much more susceptible to hypochlorous acid than to other oxygen products. Thus, a concentration of 0.03 mM of hypochlorous acid killed more than 99.9% of the LVS bacteria, whereas the virulent strain was completely resistant to this treatment.

A critical role for neutrophils has been identified also in the experimental LVS mouse model using a protocol with depletion of the cells in vivo before bacterial challenge [29]. However, it is unclear whether this can be attributed to similar cidal effects as executed by the human neutrophils since studies using peritoneal cell populations highly enriched in neutrophils have not disclosed any significant killing of \(F. tularensis\) LVS (Lindgren, unpublished). Thus, it is possible that the role of neutrophils in the murine model is due to other mechanisms. In this regard, a recent study has identified a critical requirement for neutrophils in early granuloma formation during \(M. tuberculosis\) infection. This regulation was dependant on chemokine signaling through CXCR3, in particular MIG [30]. Analogously, the data available on the role of neutrophils in the control of the LVS infection favor more indirect effects, possibly via production of cytokines or chemokines, rather than direct killing of the bacteria, since the effects were not observed before the third day of infection [29].

3.3. The localization of \(F. tularensis\) in the macrophage

Recent studies have elucidated the early host–parasite interaction during the intramacrophage \(F. tularensis\) infection [22,31,32]. Studies on \(F. tularensis\) LVS, \(F. tularensis\) subsp. novicida, as well as a clinical isolate of \(F. tularensis\) subsp. \(tularensis\) showed that, initially, bacteria colocalized with the late endosomal/lysosomal markers LAMPs, but not with cathepsin D. Moreover, the phagosomes containing \(F. tularensis\) bacteria were not significantly acidified [32]. Irrespective if the macrophages were of human or mouse origin, within 2–4 \(h\), bacteria started to egress from the phagosome and after 4–8 \(h\), a large majority of bacteria appeared to be free in the cytoplasm [22,31,32]. Thus, the findings indicate that \(F. tularensis\) bacteria are capable to alter the maturation of the phagosome, as evidenced by the exclusion of cathepsin D, lysosomal tracers, and the lack of acidification, and then, by an unknown mechanism, disrupt the phagosomal membrane.

Remarkably, the aforementioned findings were identical for both a highly strain of subsp. \(tularensis\) and the LVS strain in human macrophages [32]. Thus, the attenuation of LVS does not affect its ability to survive and multiply inside non-activated macrophages. A recent study on \(F. tularensis\) LVS demonstrated that activation of PEC by addition of IFN-\(\gamma\) rendered the bacteria less capable, but did not prevent the escape from the phagosome [9] and in IFN-\(\gamma\)-activated human-derived macrophages, the escape of \(F. tularensis\) subsp. \(novicida\) was completely inhibited [22]. No study has addressed how the intracellular localization of virulent strains of \(F. tularensis\) is affected when macrophages are activated. Other possible differences between attenuated and virulent strains of \(F. tularensis\) may relate to the efficiency of cell-to-cell spread of bacteria at late stages of infection [32].
3.4. The fate of F. tularensis-infected macrophages

In non-activated cells, virulent F. tularensis strains as well as LVS continue to grow until the viability of the host cells is compromised. The mechanisms of this cytopathogenic effect have been elucidated in a series of studies and it was observed that the F. tularensis LVS-infected cells of the mouse macrophage cell line J774 are killed by apoptosis within 24–48 h [20,33,34]. The apoptosis is mediated via the intrinsic pathway with critical involvement of the mitochondria, as evidenced by a change of the mitochondrial potential and release of cytochrome c, followed by activation of caspase-9, formation of the apoptosome, and subsequent activation of caspase-3 [33]. These events are first observed 12–18 h after start of infection, thus, since bacterial replication starts at 6 h, it appears to be required for the induction of apoptosis. Further corroborating the requirement for bacterial replication, it was observed that killing of bacteria by addition of ciprofloxacin at 12 h after start of infection prevented the apoptosis [20].

The apoptotic nature of the macrophage death was further substantiated by the demonstration that infected cells became TUNEL-positive and that the degraded DNA showed the ladder pattern typical of apoptosis-specific nucleosomes. It remains to be determined if the host cell death resulting from infection with virulent strains also is mediated via apoptosis. The scarce data that exist indicate that infection with the SCHU S4 strain of subsp. tularensis induces more rapid cytopathogenicity than does LVS [35].

A recent study confirmed the findings of Lai et al. by showing that apoptosis developed in infected J774 cells within 10 h and also it was demonstrated that the infected cells after 30 h developed signs of necrosis [36]. Moreover, the study identified that concomitant with the development of apoptosis, phosphorylation of p42/p44 MAPK (Erk1/2) occurred and it was demonstrated that levels of apoptosis were markedly diminished if cells were treated with an inhibitor of MEK1/2 and that this inhibitor also abrogated phosphorylation of p42/p44 MAPK. In agreement with the study by Telepnev et al. [37], it was also noted that a significant inhibition of p38 MAPK activity occurred in infected cells and, on the basis of results with an inhibitor of p38 MAPK, SB203580, it was proposed that the latter inhibition is an important component in the pro-apoptotic activity of F. tularensis [36].

The apoptosis induced by the F. tularensis infection presumably favors the pathogen since it allows the bacteria to escape from host cells deplete of nutrients without generating an inflammatory response and thereby activating neighboring monocytes. The latter cells, replete of nutrients, may serve as new hosts for F. tularensis.

3.5. The F. tularensis infection affects intramacrophage signaling

Today, there are more than 10 TLRs described in mice and humans and there are more than 20 microbial ligands described for these receptors. However, the downstream signaling cascades of the TLRs have an hourglass shape and disruption of only two adaptor proteins, MyD88 and TRIF, will result in inhibition of signaling from all TLRs important for sensing microbial components [38] and there are only two primary kinases further downstream in the NF-κB pathway. Thereafter, there is a rapidly increased complexity in the pathways and activated NF-κB is responsible for regulation of hundreds of genes. Due to the small numbers of these factors involved in the intermediate parts of these signaling pathways, they are attractive targets for pathogenic bacteria since modulation of their expression will have profound effects on the host cells. The NF-κB pathway may be inhibited by for example Salmonella and Yersinia species. Non-virulent Salmonella species inhibit degradation of IkB by preventing its ubiquitination [39]. The YopJ protein of Y. enterocolitica, a component of a type III secretion system, blocks phosphorylation and subsequent activation of the NF-κB regulating kinases and the YopP protein blocks both NF-κB and MAPK kinase pathways by disrupting the ubiquitination of host cell proteins [40,41]. MAP kinases are also targets for other infectious organisms and, for example, the mycobacterial virulence factor Man-LAM appears to limit macrophage activation by reducing ERK 1/2 phosphorylation [42].

A similar targeting of intracellular pathways by F. tularensis was illustrated by the demonstration that F. tularensis LVS-infected cells are not capable to respond to TLR-agonists by secretion of proinflammatory cytokines such as TNF-α or IL-1β [37]. Using a model where F. tularensis bacteria were added to a cell culture with human or mouse monocyctic cells, the effects of both intracellular and extracellular bacteria, or intracellular bacteria alone, on the cells have been characterized. Altogether, a rather complicated picture has appeared, involving both activation and inhibition of TLR-mediated signaling during the various phases of infection. In a study employing four types of monocyctic cells; J774 cells, mouse PEC, human peripheral blood mononuclear cells, and the human macrophage cell lines THP-1, it was found that the addition of LVS bacteria to the cell cultures, with the exception of J774 cells, resulted in secretion of TNF-α [43]. In parallel, cells were also infected with the ΔiglC strain. At 300 min after addition of bacteria, levels of TNF-α were significantly higher in cultures with the latter strain. Concomitantly, the phosphorylation of the inhibitor of NF-κB, IκB, and of p38 and c-Jun, both of which serve as important checkpoints in distinct MAPK pathways, were investigated. Initially, phosphorylation of the factors occurred but at the final time point, 300 min, levels were low or undetectable in LVS-infected cells, whereas infection with the ΔiglC strain induced phosphorylation during the whole study period. The inhibitory effects of the F. tularensis LVS infection were less prominent in human as compared to mouse cells [43].

By use of another infection protocol, when cells were infected for 120 min and then extensively washed before addition of TLR stimuli such as E. coli lipopolysaccharide (LPS) or bacterial lipoprotein (BLP), it was found that phosphory-
lation of all three transcription pathway factors was observed in cultures with J774 cells infected with the ΔIglC strain whereas no phosphorylation occurred in cell cultures infected with LVS [37]. Addition of killed LVS bacteria resulted in no modulation of the response to E. coli LPS or BLP.

A recent publication analyzing the inflammatory response in human and murine cells infected with F. tularensis LVS concluded that replication occurred to a similar degree in the cells but that levels of proinflammatory responses were significantly higher in human cells [44]. Although this conclusion appears not to fully agree with that of the study by Telepnev et al. [43], a reason may be that the cytokine levels were measured after 24 h in the study by Bolger et al. [44]. Therefore, the initially vigorous inflammatory response was not assayed and since Telepnev et al. observed that the subsequent down-regulation was more pronounced in murine cells, this could explain the markedly lower levels at 24 h. The study by Bolger et al. compared the levels in cell cultures with both intracellular and extracellular bacteria versus the levels in cultures with extracellular bacteria only and found significantly higher levels in the former cultures, which also agreed with the findings by Telepnev et al. [37,43].

Collectively, the findings indicate that extracellular F. tularensis bacteria engage surface ligands on the monocytes and this leads to activation of MAPK and NF-κB pathways and subsequent secretion of proinflammatory cytokines such as TNF-α (Fig. 1). However, if all extracellular bacteria are removed, or when a sufficient number of extracellular bacteria have been internalized, the signaling is perturbed by the internalized bacteria (Fig. 1) and they also inhibit the specific signaling mediated via TLR2 or TLR4 after addition of agonists such as E. coli LPS or BLP. Possibly, the activation induced by extracellular F. tularensis may be mediated via one or both of these ligands, for example via binding of its lipoproteins, although direct experimental evidence is still lacking. The proinflammatory modulation was not affected by addition of purified F. tularensis LPS [37] indicating that this low toxic and unique LPS [45,46] has no potent effects on the intracellular signaling in monocytes. Intriguingly, after internalization of the ΔIglC strain, the modulation was absent or the stimulatory effects were even enhanced, demonstrating that the IglC protein has a critical role in this inhibition. A ΔmglA strain of F. tularensis LVS, with detectable expression of IglC, as well as ΔiglB and ΔiglD strains, display the same phenotype as the ΔIglC strain in the model (Telepnev et al., unpublished), indicating that the presence of the IglC protein per se is not sufficient for executing the inhibitory effects.

Altogether, the data demonstrate that there is a complex execution of the inhibitory effects, requiring expression of the IglC protein, possibly the other three Igl proteins, and the regulator of the operon, MglA, indicating that the effects are mediated via rapid regulation of the proteins intracellularly. Since the inhibitory effects are observed already within 30 min, at a time when the bacteria presumably are still localized in the phagosome, much remains to be explained regarding this elusive mechanism. Thus, F. tularensis, in common with other pathogenic bacteria, effectively modulates intracellular signaling pathways but the bacterium appears to execute the effects by novel mechanisms.

3.6. Effects of the F. tularensis infection on endothelial cells

A study on the inflammatory changes in endothelial cells following an F. tularensis LVS infection reported findings that to some extent are analogous to those on the monocytic cells. Killed F. tularensis induced a wider spectrum of inflammatory responses than did live F. tularensis LVS [47]. Thus, there are proinflammatory components in the bacterium recognized by both phagocytic and endothelial cells and the most likely explanation for the conspicuous lack of proinflammatory response is active down-regulation by the live bacteria.

3.7. F. tularensis mechanisms required for the intracellular replication

Recent studies employing specific mutants of F. tularensis have revealed that a multitude of effects can be attributed to the four proteins encoded by the igl operon. Using transposon mutants of F. tularensis subsp. novicida, it has been found that the IglA and IglC mutants are incapable of intra-

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Fig. 1. A tentative model for the intracellular lifecycle of F. tularensis.
cellular replication in mouse PEC and human macrophages [5,10,12] and the IglC mutant of growth in amoebae [6]. As aforementioned, the IglC mutant of F. tularensis LVS lacked the inhibitory effects on TLR signaling displayed by the parent strain [37], was incapable of replicating in mouse macrophages and in vivo [7], and unable to escape from the phagosome [9]. Also, a ΔiglC mutant of subspecies novicida was unable to escape from the phagosome [10]. Moreover, no apoptosis of J774 cells was observed after infection with the IglC mutant [34]. It is possible that the two latter observations are connected, i.e. that the escape from the phagosome is a prerequisite for inducing apoptosis. However, the rapid inhibition of TLR signaling observed within 30 min when the bacteria presumably still reside in the phagosome, indicates that certain effects dependent on the expression of IglC can be executed from within this cell organelle.

4. Conclusion

The available genome information has not revealed many apparent virulence traits of F. tularensis but it is obvious that F. tularensis still possesses a number of mechanisms that render it highly adapted to the intracellular habitat. A common theme in the adaptation appears to be to minimize the inflammatory response. Its LPS is a 1000-fold less pyrogenic than enterobacterial LPS [45] and although an initial activation of MAPK and NF-κB pathways and subsequent induction of TNF-α secretion occurs [43], a down-regulation soon follows and during this phase, the infected cells appear to be unresponsive to TLR2 and TLR4 agonists [37]. Moreover, the escape from the phagosome gives access to nutrients necessary for the intracellular replication and when the supply becomes limiting, apoptosis is induced, a non-inflammatory pathway MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm, Cell. Microbiol. 7 (2005) 969–979.

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