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A *Francisella tularensis* pathogenicity island protein essential for bacterial proliferation within the host cell cytosol

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Summary

Francisella tularensis **is an intracellular bacterial pathogen, and is a category A bioterrorism agent. Within quiescent human macrophages, the *F. tularensis* pathogenicity island (FPI) is essential for bacterial growth within quiescent macrophages. The *F. tularensis*-containing phagosome matures to a late endosome-like stage that does not fuse to lysosomes for 1–8 h, followed by gradual bacterial escape into the macrophage cytosol. Here we show that the FPI protein *IglD* is essential for intracellular replication in primary human monocyte-derived macrophages (hMDMs). While the parental strain replicates robustly in pulmonary, hepatic and splenic tissues of BALB/c mice associated with severe immunopathologies, the isogenic *iglD* mutant is severely defective. Within hMDMs, the *iglD* mutant-containing phagosomes mature to either a late endosome-like phagosome, similar to the parental strain, or to a phagolysosome, similar to phagosomes harbouring the *iglC* mutant control. Despite heterogeneity and alterations in phagosome biogenesis, the *iglD* mutant bacteria escape into the cytosol faster than the parental strain within hMDMs and pulmonary cells of BALB/c mice. Co-infections of hMDMs with the wild-type strain and the *iglD* mutant, or super-infection of *iglD* mutant-infected hMDMs with the wild-type strain show that the mutant strain replicates robustly within the cytosol of hMDMs coinhabited by the wild strain. However, when the wild-type strain-infected hMDMs are super-infected by the *iglD* mutant, the mutant fails to replicate in the cytosol of communal macrophages. This is the first demonstration of a *F. tularensis* novel protein essential for proliferation in the macrophage cytosol. Our data indicate that *F. tularensis* transduces signals to the macrophage cytosol to remodel it into a proliferative niche, and IglD is essential for transduction of these signals.**

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Introduction

Francisella tularensis is a facultative intracellular bacterium that causes tularaemia in many mammalian species including humans (Francis, 1925; Morner, 1992; Tarnvik *et al*., 1992; Ellis *et al*., 2002). Naturally, it can initiate human infection following ingestion of contaminated food or water, through dermal microabrasions when handling infected animal carcasses, via bites from various arthropods, and by inhalation of contaminated air (Choi, 2002; Ellis *et al*., 2002). Disease manifestation depends on the route of infection and may take many forms including respiratory, oropharyngeal, ulceroglandular or oculoglandular. (Francis, 1925; Morner, 1992; Tarnvik *et al*., 1992; Ellis *et al*., 2002). Because of its high infectivity and morbidity and mortality, *F. tularensis* has been classified as category A bioterrorism agent.

There are four closely related subspecies of *F. tularensis* (*tularensis, holarctica, mediasiatica* and *novicida*), and subspecies *tularensis* is the most virulent to humans (Forsman *et al*., 1990; 1994; Santic *et al*., 2006). The subspecies *holarctica* can produce similar disease symptoms in humans but are less virulent than the *tularensis* subspecies. The attenuated live vaccine strain (LVS) of *F. tularensis* is of the *holarctica* subspecies derived from an attenuated vaccine strain but its use as an attenuated vaccine has been discontinued in the USA because of its uncertain genetic history (Saslaw *et al*., 1961). Therefore, there is an urgent need for an attenuated LVS to protect against tularaemia. *F. tularensis* ssp.

novicida is attenuated in humans but causes disease in mice similar to that caused by ssp. *tularensis*, and is an attractive model to study pathogenesis of tularaemia (Sjostedt, 2003; Lauriano *et al*., 2004; Santic *et al*., 2006). Comparison of two *Ft* genomes of the Schu4 (type A) and LVS strains indicates that these organisms are almost identical. Analyses of unidirectional genomic deletions and single nucleotide variations have shown that the four closely related subspecies of *F. tularensis* have evolved by vertical descent, and the subspecies *novicida* is evolutionarily the oldest (Johansson *et al*., 2004; Svensson *et al*., 2005). Recent studies have shown that the increased virulence of the *tularensis* and *holarctica* subspecies may be due to the loss of genes encoding secreted virulence proteins (Hager *et al*., 2006).

Ingested particles are normally processed by macrophages through the 'default' endosomal lysosomal degradation pathway, which is one of the first lines of defence against microbial infection (Duclos and Desjardins, 2000; Hackstadt, 2000; Kahn *et al*., 2002; Santic *et al*., 2006). This process is very rapid and is completed within few minutes by formation of the phagolysosome (Santic *et al*., 2006). Therefore, many intracellular pathogens have evolved with distinct strategies to avoid a fatal fate within the phagolysosomes. The intracellular fate of three *F. tularensis* subspecies (*tularensis, holarctica*-derived LVS and *novicida*) within quiescent macrophages is similar but unique when compared with other intracellular pathogens (Golovliov *et al*., 2003a; Clemens *et al*., 2004; Santic *et al*., 2005a,b; 2006). The *Francisella*-containing phagosome (FCP) matures into a late endosomal-like stage that acquires the late endosomal marker LAMP-2 but does not fuse to lysosomes. Within a few hours of this unique modulation of phagosome biogenesis, the organism disrupts the phagosome and escapes into the cytoplasm where it replicates (Golovliov *et al*., 2003a; Clemens *et al*., 2004; Santic *et al*., 2005a,b; 2006). However, within IFN-γ-activated primary human macrophages, the FCP matures into a phagolysosome and the organism fails to escape into the cytoplasm (Santic *et al*., 2005a). The 30-Kb *F. tularensis* pathogenicity island (FPI) is essential for phagosome biogenesis and escape into the cytoplasm (Nano *et al*., 2004; Santic *et al*., 2005b). Within the FPI, the two proteins IglA and IglB, which are encoded within the *iglABCD* gene cluster, have some homology to two *Rhizobium leguminosarum* proteins ImpBC, which are thought to be involved in protein secretion. Interestingly, *iglA* and *iglC* are regulated by MglA, which is encoded outside the FPI (Baron and Nano, 1998; Gray *et al*., 2002; Golovliov *et al*., 2003b; Lindgren *et al*., 2004). The FPI protein IglC and its regulator, MglA, play essential roles in the ability of *F. tularensis* to modulate biogenesis of the phagosome (Santic *et al*., 2005b) and escape into the cytoplasm (Lindgren *et al*., 2004; Santic *et al*., 2005b). The phagosomes harbouring the *mglA* and *iglC* mutants rapidly mature into phagolysosomes, and the mutants fail to escape into the cytoplasm (Lindgren *et al*., 2004; Santic *et al*., 2005b). Both mutants are severely defective in intracellular replication and are attenuated in the mice model of tularaemia (Lauriano *et al*., 2004). The mechanisms by which *F. tularensis* blocks fusion of its phagosome to lysosomes, and then escapes into the cytoplasm are not known.

In this paper, we show that the IglD FPI protein is essential for intracellular growth of *F. tularensis* in the cytoplasm of primary human macrophages, and in pulmonary, hepatic and splenic tissues of BALB/c mice. IglD is essential for pulmonary, hepatic and splenic histopathological manifestations of tularaemia in BLAB/c mice. We show that some phagosomes harbouring the *iglD* mutant acquire the late endosomal marker LAMP-2 but not the lysosomal enzyme Cathepsin D, while the others acquire both markers. Despite this heterogeneity in phagosome biogenesis, the *iglD* mutant bacteria escape into the cytoplasm faster than the parental strain but fail to replicate in the cytoplasm of human monocyte-derived macrophages (hMDMs) and pulmonary cells of BALB/c mice. Defect of the *iglD* mutant to grow within the macrophage is rescued *in trans* by the parental strain coinhabiting the macrophage.

Results

Defect of the IglD *mutant to replicate within hMDMs*

A mutation in *iglD*, which is the most downstream gene in the *iglABCD* operon was constructed. We examined intracellular replication of the *iglD* mutant in primary hMDMs. The hMDMs were infected with the parental strain U112 of *F. tularensis* ssp. *novicida*, and the *iglD* isogenic mutant at a multiplicity of infection (moi) of 10 for 1 h followed by wash of extracellular bacteria, and treatment with gentamicine for 1 h. At different time points after infection (1, 24, 48 and 72 h) the cells were lysed and the bacteria were grown on agar plates to determine the number of colony-forming units (cfu). The data showed that the *iglD* mutant was severely defective in intracellular replication within hMDMs. The number of the *iglD* mutant bacteria remained relatively unchanged through the 72 h period after infection (Fig. 1). In contrast, the parental strain of *F. tularensis* exhibited robust replication and the bacterial numbers increased by more than 1000-fold by 48 h after infection (Fig. 1). Similar results were obtained within U937 macrophages (data not shown). We have attempted to complement the *iglD* mutant with *iglCD* without success, although the complementing plasmid was successfully introduced into the mutant, which was confirmed by PCR. This is reminiscent of the failure to complement the *iglC* mutant, which is likely due to hyper-expression by

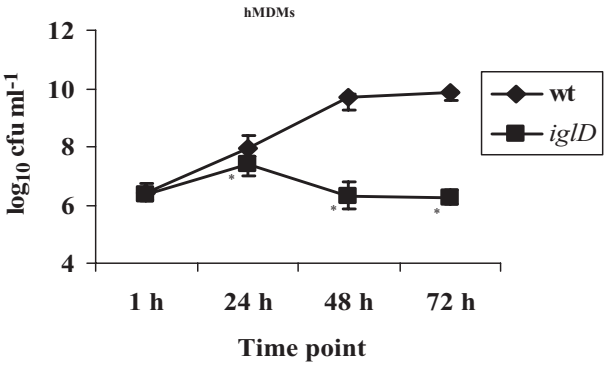

Fig. 1. Intramacrophage defect of the *iglD* mutant. Growth kinetics of the wild-type strain of *F. tularensis* and its *iglD* mutant in the hMDMs. Macrophages were infected for 1 h at an moi of 10, treated with gentamicine to kill extracellular bacteria, and the number of intracellular bacteria was determined at the indicated time points. The error bars represent standard deviations of triplicate samples and the results shown are representative of three independent experiments. The asterisks represent statistically significant difference.

the heat shock promoter regulating expression of the complementing gene. Thus, the FPI novel protein IglD is essential for intracellular proliferation within primary human macrophages and macrophages cell lines.

IglD *is required for intracellular replication of* F. tularensis *in mice*

To study the role of IglD during experimental tularaemia in mice, five BALB/c mice per group were infected intratracheally with 10^4 cfu of the parental strain or its isogenic *iglD* mutant. At different time points after infection, liver, lungs and spleen were removed to determine the organ bacterial loads (see *Experimental procedure*). Our results showed that the wild-type strain proliferated robustly in the organs of mice with the peak of infection at 48 h after infection, where the number of bacteria reached 2×10^{10}, $> 1 \times 10^{10}$ and 10^9 cfu, in the liver, lungs and spleen respectively (Fig. 2). At 48 h after infection with the wild-type strain, the number of bacteria in all organs gradually declined over the following few days.

In contrast, the *iglD* mutant did not replicate in the organs of BALB/c mice (Fig. 2). Between 24 and 48 h after infection, the number of bacteria in the organs of mice reached a plateau. Our results clearly show that IglD is essential for proliferation of *F. tularensis* in the lungs, liver and spleen of BALB/c mice.

IglD *is involved in pulmonary immunopathology of experimental tularaemia*

Examination of pulmonary tissues of wild type-infected BALB/c mice showed that there was prominent immunopathological changes compared with the *iglD* mutant. Compared with uninfected BALB/c mice, 2 h after inoculation by the parental strain there were minor histopathological changes in the pulmonary parenchyma (Fig. 3), which was not observed in the mice infected with the *iglD* mutant (Fig. 3).

At 24 h after infection with the parental strain, the pathological changes of the bronchiolar cells were characterized by a vacuolar degeneration (Fig. 3). The peribronchiolar spaces were infiltrated with mononuclear cells. The infiltration was also observed within bronchiole and alveoli but with less intensity than in peribronchiolar spaces. At 48 h after infection with the wild-type strain, the lungs showed acute bronchopneumonia, which involved one or more lobes (Fig. 3). There were large numbers of neutrophils and macrophages in the bronchial lumen (Fig. 3). The pulmonary tissue surrounding blood vessels showed infiltration of macrophages, and fibrinous exudates in the alveolar spaces were detected (Fig. 3). The pulmonary changes at 72 h post infection with the wild-type strain were similar to those at 48 h after infection. (Fig. 3). Restoration of pulmonary parenchyma was observed at 14 days after infection with the parental strain of *F. tularensis.*

There were minimal pulmonary immunopathological changes in mice infected with the *iglD* mutant for all time

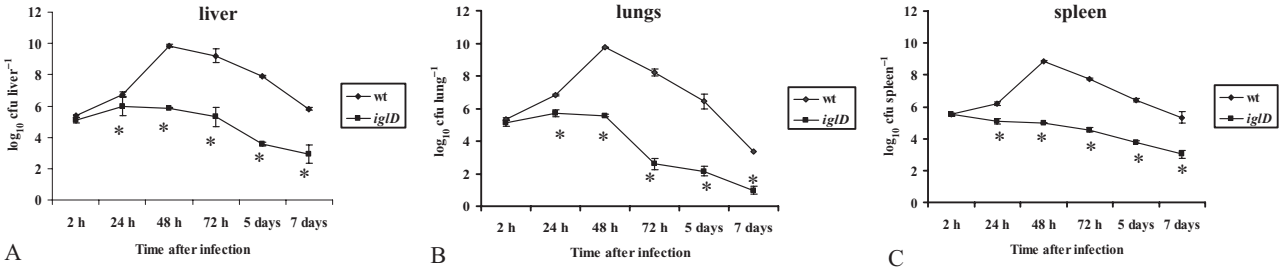

Fig. 2. The *iglD* mutant is defective in replication in internal organs of BALB/c mice. Growth kinetics of the wild-type strain of *F. tularensis* and its *iglD* mutant in (A) liver, (B) lungs and (C) spleen of BALB/c mice after intratracheal inoculation. At different time points after infection of BALB/c mice, organs were removed for determination of the number of bacteria (cfu) by plating serial dilutions on agar plates. The error bars represent standard deviations of triplicate samples and the results shown are representative of three independent experiments. The asterisks represent statistically significant difference.

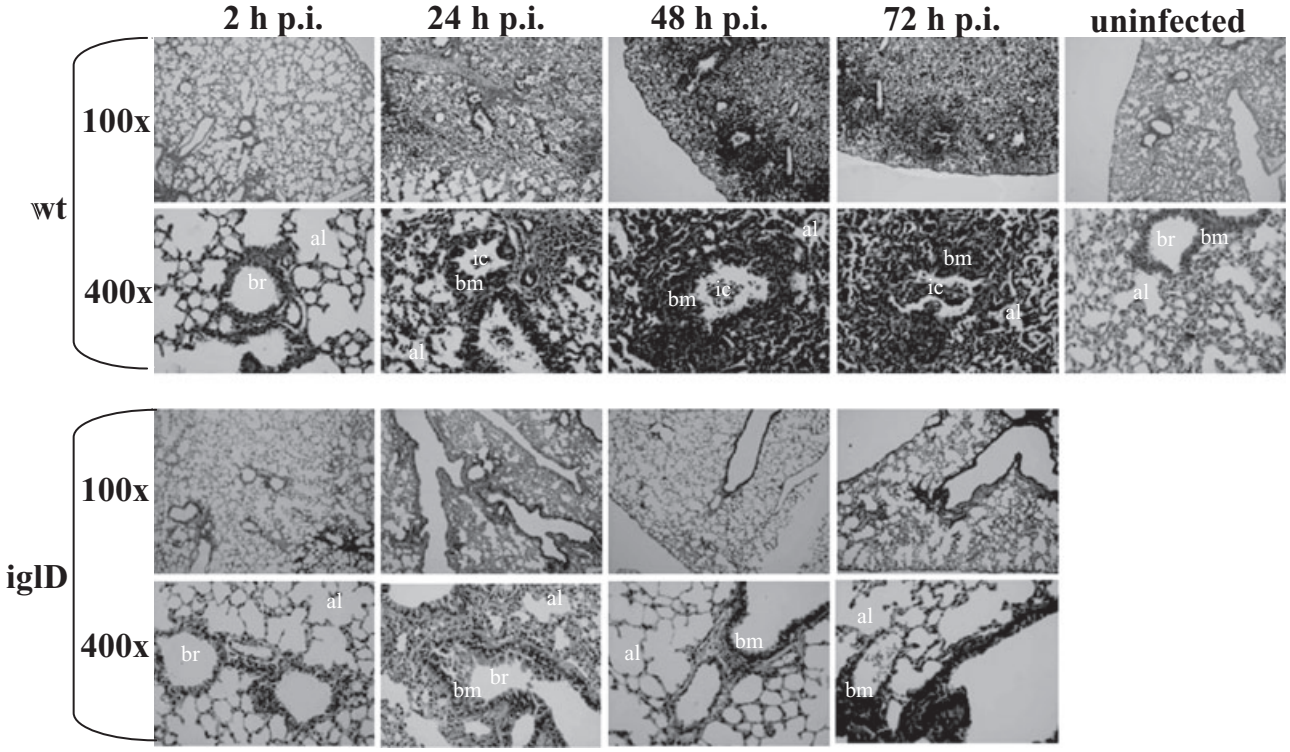

Fig. 3. Lung histopathology of BALB/c mice infected with the wild-type strain of *F. tularensis* and the *iglD* mutant strain. At 2, 24, 48 and 72 h after infection of BALB/c, pulmonary tissue sections were stained with H&E. The experiments were done in triplicate using three mice for each time point, and the images are representative of 20 microscopic fields from each animal. The results are representative of three independent experiments. as, alveolar spaces; br, bronchioles; be, bronchial epithelium; bm, basal membrane; ic, inflammatory cells.

points examined after infection (Fig. 3). As a negative control, BALB/c mice injected with saline showed no detectable pulmonary changes in at any time point (data not shown). Taken together, IglD is essential for pulmonary pathology of BALB/c mice infected with *F. tularensis.*

The iglD *mutant does not elicit hepatic and splenic immunopathology*

Infected mice were also examined at various time points for hepatic and splenic immunopathology. At 2 h after infection, no hepatic pathological changes were observed in mice infected with the wild-type strain (data not shown). However, after 24 h infection, the liver showed occasional presence of small foci of mononuclear cells and neutrophils (data not shown). By 48 h after infection, numerous small to medium-size inflammatory infiltrates were detected throughout the liver (data not shown). By 72 h after infection with the wild-type strain, inflammatory cells were mainly within portal vein. In contrast, no hepatic histopathological changes were detected in mice infected with the *iglD* mutant strain at all time points after infection (data not shown).

At 2 h after infection, there were no remarkable splenic changes in mice infected with either the wild-type strain or the *iglD* mutant (data not shown). At 24 h after infection, the spleen of mice infected with the wild-type strain showed mild depletion of lympocytes in the medular areas and few neutrophils were detected (data not shown). By 48 and 72 h after infection with the wild-type strain signs of splenitis were clear (data not shown). In contrast, the spleen of mice infected with the *iglD* mutant strain showed only mild degeneration of lymphoid follicles and neutrophils infiltrations in the white pulp by 48 and 72 h after infection (data not shown). Restoration of hepatic and splenic parenchyma was observed at 14 days after infection. Our data show that IglD is necessary for hepatic and splenic pathology caused by the parental strain.

Intracellular trafficking of the iglD *mutant in hMDMs*

Because of the severe defect of the *iglD* mutant in intracellular replication, we hypothesized that biogenesis of the *iglD* mutant-containing phagosomes was altered. To test this hypothesis, we examined colocalization of the *iglD* mutant containing-phagosomes with the late endosomal/lysosomal marker LAMP-2 and the lysosomal enzyme Cathepsin D. As a positive control, we used the *iglC* mutant, because the *iglC* mutant-containing phagosomes colocalize with both LAMPs and Cathepsin D due

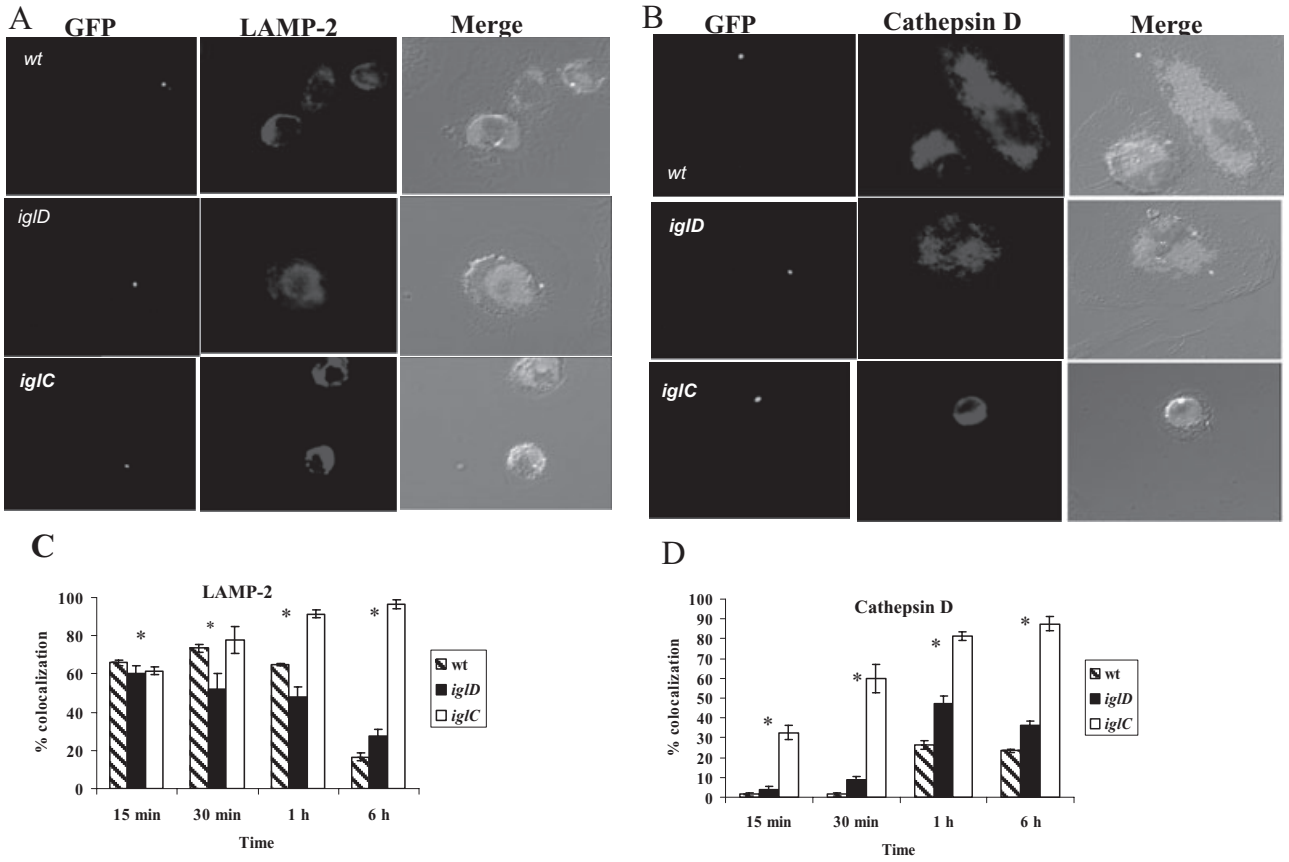

Fig. 4. Acquisition of late endosomal/lysosomal markers LAMP-2 and Cathepsin D by the FCP in infected hMDMs. The hMDMs were infected with the wild-type strain, the *iglD* or the *iglC* mutants at an moi of 10, followed by wash of extracellular bacteria.
A and B. Representative confocal microscopy images that show colocalization of LAMP-2 and Cathepsin D with the phagosomes harbouring the *iglD* or *iglC* mutants and the wild-type strain.
C and D. Quantification of colocalization of phagosomes harbouring the *iglD* or *iglC* mutants and the wild-type strain with LAMP-2 (C) and Cathepsin D (D) was based on examination of 100 infected cells from different coverslips. The error bars represent standard deviations of triplicate samples and the results shown are representative of three independent experiments. The asterisks represent statistically significant difference.

to fusion of its phagosomes to the lysosomes (Santic *et al*., 2005b). At 15 min post infection of hMDMs, ~85% of the phagosomes harbouring the wild-type strain, the *iglC* or the *iglD* mutants colocalized with the LAMP-2 marker (Fig. 4A and C). However, colocalization of the wild-type strain FCP with LAMP-2 began to decline at further time points after infection (Fig. 4), which is an indication of initiation of gradual bacterial escape from phagosomes into the cytoplasm. At 30 min after infection, ~75% of the phagosomes harbouring the wild-type strain colocalized with the LAMP-2 (Fig. 4A and C). Interestingly, there was a faster decrease in colocalization of the *iglD* mutant-containing phagosomes with LAMP-2 compared with the wild-type strain at 30 min (Student's *t*-test, $P < 0.001$) and 60 min post infection (Student's *t*-test, $P < 0.03$), suggesting faster escape of the *iglD* mutant into the cytoplasm compared with the parental strain. Most of the phagosomes harbouring the *iglC* mutant, as a control, were persistently positive for LAMP-2 (Fig. 4A and C), consistent with previous observations of the defect of this mutant (Santic *et al*., 2005b).

By 6 h after infection, only 15% and 28% of the phagosomes harbouring the wild-type strain and the *iglD* mutant respectively, were still positive for the LAMP-2 marker (Student *t*-test, $P < 0.01$), while more than 90% of the *iglC* mutant containing-phagosomes colocalized with LAMP-2 (Fig. 4A and C). We conclude that the gradual decrease in colocalization of the *iglD* mutant phagosomes with the LAMP-2 marker was faster than the wild-type strain during the first 60 min after biogenesis of the phagosomes.

We also examined colocalization of the FCP of the three strains with the lysosomal enzyme Cathepsin D. Phagosomes harbouring the *iglC* mutant showed gradual increase in colocalization with Cathepsin D from 25% at 15 min to ~80% at 1 h after infection (Fig. 4B and D), which is consistent with previous observations of fusion of the *iglC* mutant-containing phagosomes to lysosomes (Santic *et al*., 2005b). In contrast, the wild-type and *iglD*

mutant-containing phagosomes showed lower levels of colocalization with Cathepsin D. At early time points (15 and 30 min), there was very little colocalization (< 5%) of the wild-type and *iglD* mutant-containing phagosomes with Cathepsin D (Fig. 4B and D), which increased to 30% and 50% respectively, by 1 h after infection. The difference in phagosome–lysosome fusion of the *iglD* mutant-containing phagosomes compared with those containing either the wild type (lower) or the *iglC* mutant (higher) was statistically significant (Student's *t*-test, $P < 0.01$). Our data show that the *iglD* mutant phagosomes fuse to lysosomes at much higher frequency than the wild-type strain but lose their endosomal and lysosomal markers more rapidly than phagosomes harbouring the wild-type strain.

Rapid escape of the iglD *mutant into the macrophage cytoplasm*

Our data above suggested that the *iglD* mutant-containing phagosomes were disrupted more rapidly than the wild-type strain by 30–60 min post infection. Therefore, we examined integrity of the phagosomal membranes harbouring the *iglD* mutant within hMDMs by transmission electron microscopy (TEM) at several time intervals after the infection (Fig. 5). At 15 min after infection, most of the bacteria were enclosed in intact phagosomes but ~7% of the phagosomes harbouring the wild-type strain were disrupted. Approximately, 10% of phagosomes harbouring the *iglD* mutant were disrupted, which was significantly different from the wild-type strain (Student's *t*-test, $P < 0.01$) (Fig. 5).

By 30 min after infection, only ~20% of the phagosomes harbouring the wild-type strain were disrupted. In contrast, 35% of the phagosomes harbouring the *iglD* mutant were disrupted, which was significantly different from the parental strain (Student's *t*-test, $P < 0.007$) (Fig. 5). By 1 h after infection, only 35% of the wild-type strain phagosomes were disrupted, while more than 65% of the phagosomes harbouring the *iglD* mutant were disrupted (Student's *t*-test, $P < 0.001$) (Fig. 5). At 6 h after infection most of the wild-type or the *iglD* mutant bacteria were free in the cytoplasm with no visible limiting membrane surrounding them (Fig. 5).

To confirm the ultrastructural studies, we developed a fluorescence microscopy-based phagosomal integrity assay based on differential labelling of intracellular bacteria that are cytoplasmic/or within compromised phagosomes and those enclosed within intact phagosomes impermeable to large molecules (such as antibodies) loaded into the macrophage cytoplasm. In this assay, we utilized the glass beads 'loading technique' (Muder *et al.*, 1984; McNeil and Warder, 1987) to load the cytoplasm of live hMDMs with antibacterial antibodies to examine their ability to bind the bacteria once integrity of the FCP membrane is compromised. At 15, 30 and 60 min post infection, 25%, 30% and 50% of the cells harbouring the wild-type strain respectively, the bacteria bound the loaded antibodies (Student's *t*-test, $P < 0.05$) (Fig. 6). In contrast, at 15, 30 and 60 min post infection in 15%, 50% and 60% of cells, respectively, harbouring the *iglD* mutant, the mutant bacteria bound the antibodies, and the difference

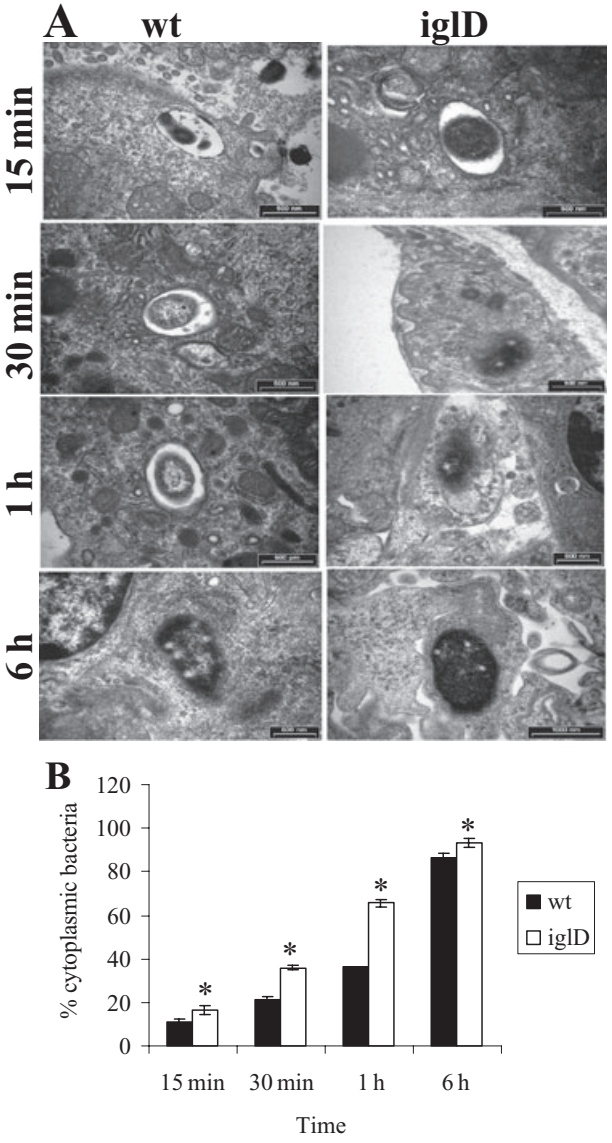

Fig. 5. Rapid escape of the *iglD* mutant into the cytoplasm of hMDMs.
A. Representative electron micrographs of hMDMs infected with the wild-type strain of *F. tularensis* or the *iglD* mutant at different time points after 1 h infection at an moi of 10.
B. Quantification of cytoplasmic bacteria shown was based on examination of 100 infected cells from different sections. The error bars represent standard deviations of triplicate samples and the results shown are representative of three independent experiments. The asterisks represent statistically significant difference.

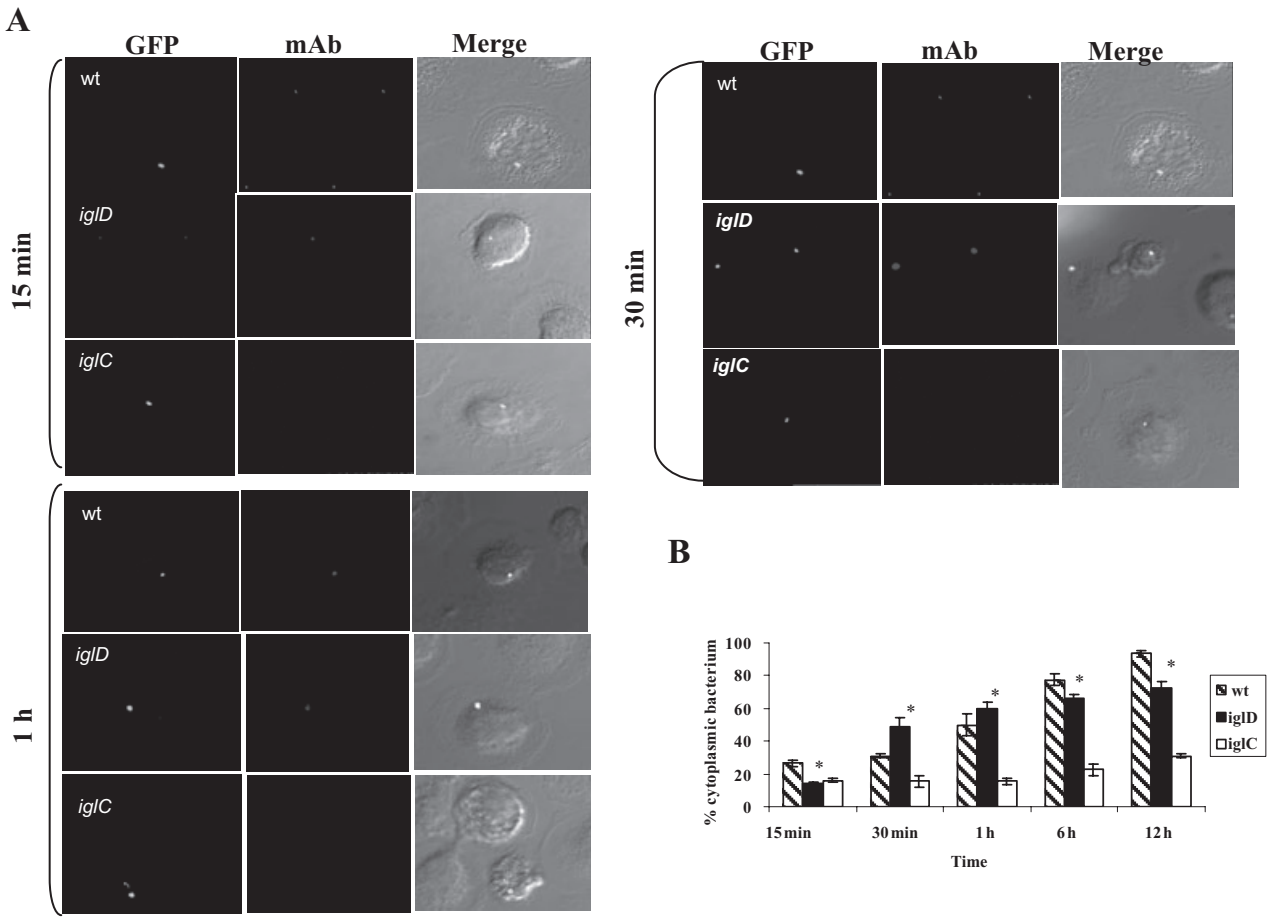

Fig. 6. Representative confocal microscopy images of binding of specific antibacterial antibodies loaded into the cytoplasm of live hMDMs.
A. After infection for 1 h at an moi of 10, specific antibacterial Mabs and glass beads were added to load cytoplasm of live macrophages. After washing and 1 h incubation, the cells were fixed and processed for confocal microscopy. All the bacteria expressed GFP.
B. Quantification of the results shown was based on examination of 100 infected cells from different coverslips. The error bars represent standard deviations of triplicate samples and the results shown are representative of three independent experiments. The asterisks represent statistically significant difference.

from the wild-type strain was significant at all these time points (Student's *t*-test, $P < 0.03$–0.008) (Fig. 6). At all time points after infection by the *iglC* mutant, only approximately in 20% or less of the cells harbouring the *iglC* mutant, the bacteria bound the antibodies, consistent with its failure to escape from the phagosome. For a negative control, infected cells were treated with monoclonal antibody without permeabilization of the plasma membrane by the glass beads, which showed no binding of the antibody to intracellular bacteria for any of the strains tested (data not shown). For the positive control, the cells were permeabilized by Triton-X100, which allowed the antibodies to bind to all intracellular bacteria for all the strains (data not shown). Taken together, we conclude that the *iglD* mutant of *F. tularensis* escapes from the phagosomes of hMDMs much more rapidly than the wild-type strain within the first 1 h after infection. In addition, the *iglD* mutant escapes from late endosome-like phagosomes and from phagosomes that acquire lysosomal enzymes.

Escape of the iglD *mutant from pulmonary cells* in vivo

To confirm our *in vitro* findings of rapid phagosomal escape of the *iglD* mutant into the cytoplasm, we examined integrity of the phagosomal membranes harbouring the *iglD* mutant in pulmonary tissues of infected BALB/c mice. At 15, 30 and 60 min after infection by the parental strain, ~15%, 30% and 45% of the phagosomes, respectively, were disrupted. At 30 min after infection, only 30% of the phagosomes harbouring the wild-type strain were disrupted, while 67% of the phagosomes harbouring the *iglD* mutant in pulmonary cells were disrupted (Student's *t*-test, $P < 0.0003$) (Fig. 7). In contrast, there was more rapid phagosomal disruption by the *iglD* mutant, where at

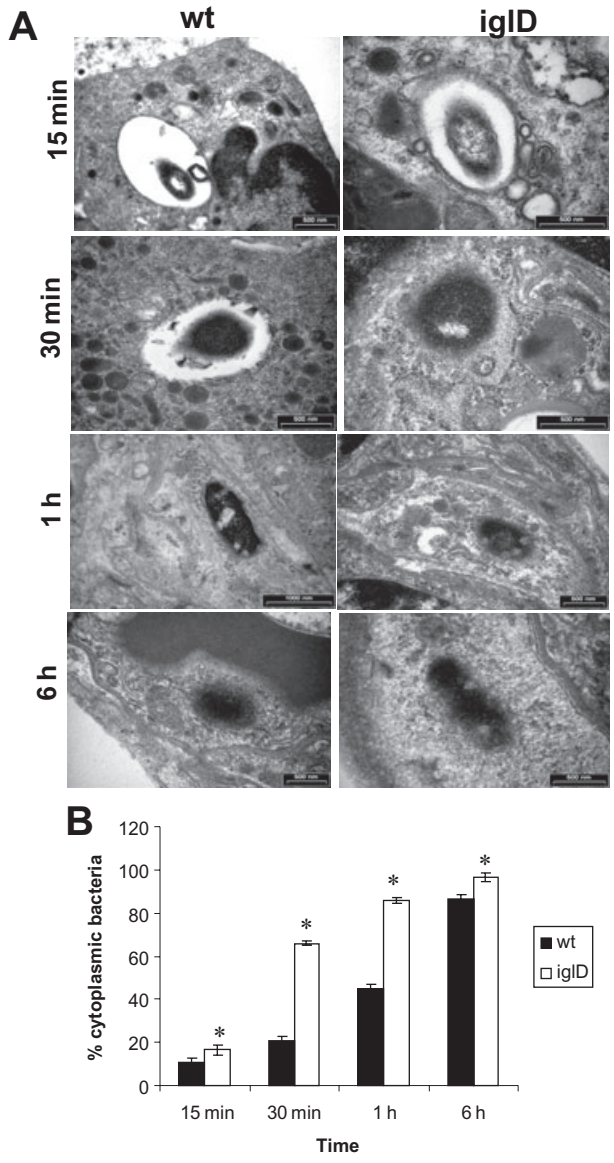

Fig. 7. Rapid escape of the *iglD* mutant into the cytoplasm of pulmonary cells of BALB/c mice.
A. Transmission electron micrographs of pulmonary tissues of BALB/c mice infected with the wild-type strain or the *iglD* mutant at 15 min, 30 min, 1 h and 6 h after inoculation.
B. Quantification of cytoplasmic bacteria shown was based on examination of 100 infected cells from different coverslips. The experiments were done in triplicate using three mice for each time point, and the images are representative of cells in 10 ultrathin sections from each animal. The asterisks represent statistically significant difference.

15, 30 and 60 min after infection, ~15%, 67% and 85% of the phagosomes, respectively, were disrupted. The faster escape of the *iglD* mutant compared with the wild-type strain was significant at 30 min and 60 min (Student's *t*-test, $P < 0.0003$). By 6 h after infection most of the phagosomes harbouring either wild-type strain or the *iglD* mutant were disrupted and the bacteria were free in the cytoplasm (Fig. 7). The above data confirm the *in vitro* findings that the *iglD* mutant bacteria escape into the cytoplasm more rapidly than the wild-type strain within pulmonary cells during experimental tularaemia in BALB/c mice.

In trans *rescue of the* iglD *mutant for intramacrophage growth by the wild-type strain*

Inability of the *iglD* mutant to replicate within the macrophage cytosol indicated that the wild-type strain of *F. tularensis* transduces signals into the macrophage cytosol to remodel it into a permissive replicative niche, and these signals are not transduced by the *iglD* mutant. Here we asked the question whether the *iglD* mutant would replicate in a communal macrophage harbouring the wild-type strain, which is able to remodel the cytosol into a replicative niche. We coinfected hMDMs with both the wild-type strain expressing GFP and the *iglD* mutant at an moi of 10 for each strain for 1 h, followed by gentamicine treatment to kill extracellular bacteria. To differentiate the two strains by microscopy at different time points after infection, the cells were fixed and both strains were labelled with anti-*F. tularensis* antibody followed by an Alexa-red-conjugated secondary antibody. At 1 h post coinfection, more that 50% of the infected cells harboured two organisms, one from each strain (data not shown). The rest of the infected cells harboured one organism from either strain but few cells harboured two organisms of the same strain (data not shown). As expected, in single infections where the *iglD* mutant-infected cells were examined at 18 h post infection, all the infected cells contained one bacterium, while cells infected by the parental strain contained > 20 organisms (Fig. 8). Remarkably, the *iglD* mutant exhibited robust replication in ~55% of the communal macrophages harbouring the wild-type strain (Fig. 8).

We also performed super-infection, in which we subjected *iglD* mutant-infected cells to super-infection by the wild-type strain, and vice versa, and examined potential rescue of the *iglD* mutant for its intramacrophage growth defect. In either case of the sequence of infections, immediately after the super-infection, more that 50% of the infected hMDMs harboured two organisms, one from each strain (data not shown). When examined at 18 h post infection, ~50% of the cells infected by the *iglD* mutant first then super-infected by the wild-type strain, the *iglD* mutant was rescued for intracellular replication (Fig. 8). Surprisingly, when the sequence of infections were switched and the wild-type strain-infected hMDMs were super-infected by the *iglD* mutant, less than 5% of the dually infected cells exhibited rescue of the *iglD* mutant (Fig. 8). For both the coinfections and super-infection studies, similar results were obtained

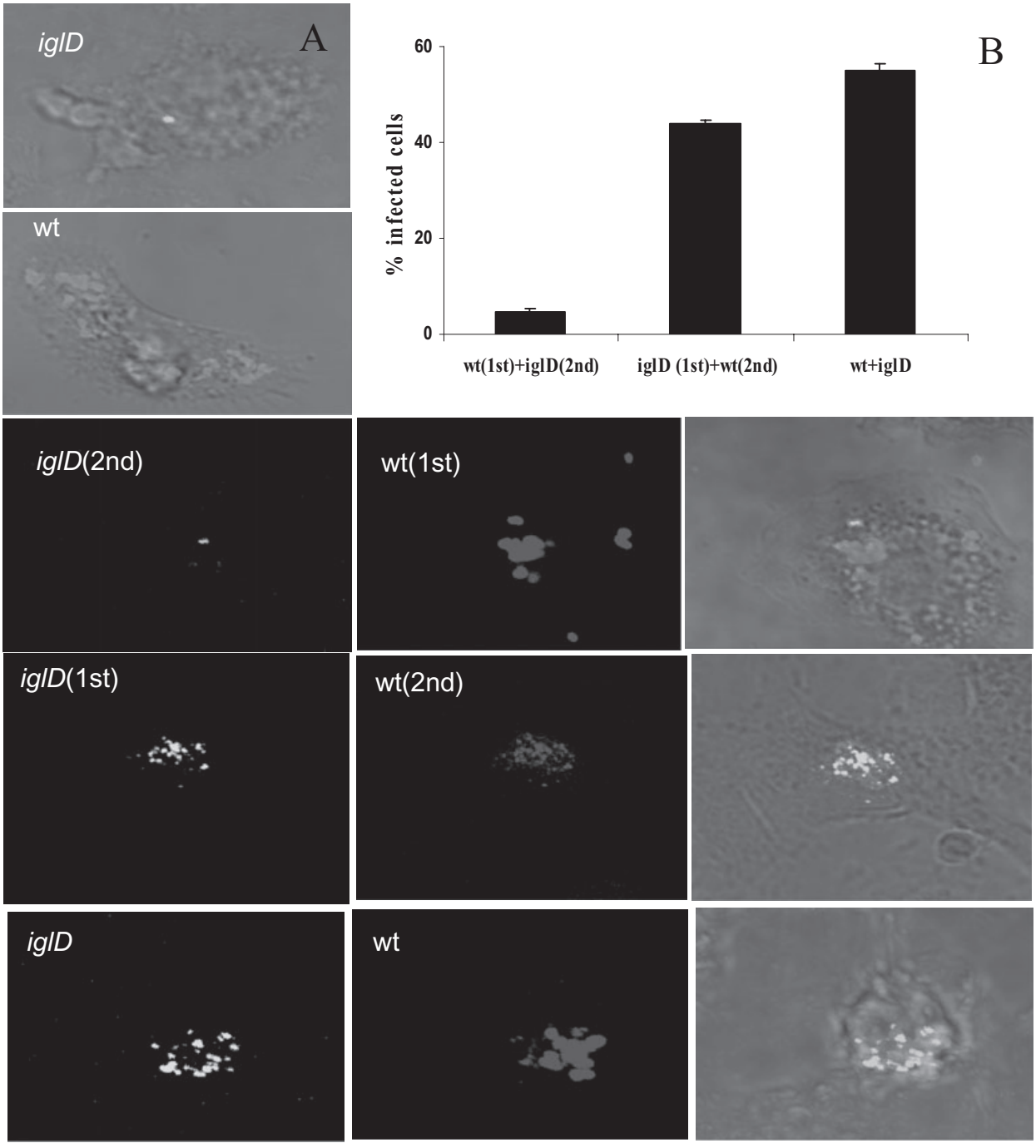

Fig. 8. *In-trans* rescue of the *igID* mutant by the wild-type strain in communal macrophages. The hMDMs were infected with the wild-type strain or the *igID* mutant separately, coinfected with both strains, or infected with one strain first (1st) followed by a super-infection by the second (2nd) strain.
A. Representative confocal microscopy images of the infected cells at 18 h post infection are shown.
B. Quantification of the percentage of hMDMs harbouring replicating *igID* mutant shown was based on examination of 100 infected cells from different coverslips. The error bars represent standard deviations of triplicate samples and the results shown are representative of three independent experiments.

when GFP expression was reversed from the wild-type strain to the *iglD* mutant strain used for infections (data not shown). In addition, similar results were obtained for both the coinfections and super-infection studies when bacteria that did not express GFP were used, and the cytoplasmic bacteria were labelled with monoclonal antibodies while all the bacteria were labelled with polyclonal antisera after permeabilization of the macrophage membranes with Triton X-100 (data not shown). We conclude that the wild-type strain of *F. tularensis* transduces signals to the macrophage cytosol to render it permissive for bacterial proliferation and IglD is essential for these signals.

Discussion

Francisella tularensis is a highly contagious, facultative intracellular bacterium that causes tularaemia in humans and animals. Although it is considered a potential biological weapon (Dennis *et al*., 2001; Santic *et al*., 2006), little is known about the mechanisms of its pathogenesis. Understanding intracellular trafficking and the molecular and cellular processes manipulated by *F. tularensis* is crucial to our understanding of the pathogenic mechanisms by which this pathogen subverts host cell defences. Several genes necessary for intramacrophage survival have been identified, including *mglA* and *iglC* (Baron and Nano, 1998; Gray *et al*., 2002; Golovliov *et al*., 2003b; Lindgren *et al*., 2004). The MglA global regulator of *F. tularensis* has been shown to control transcription of a number of virulence genes, including *iglC* (Lauriano *et al*., 2004). The MglA and IglC proteins are essential for evasion of fusion of the FCP to lysosomes and for subsequent bacterial escape into the cytoplasm (Santic *et al*., 2005b). In this paper, we show that the *iglD,* another MglA-regulated FPI gene, is indispensable for intracellular replication in primary human macrophages and for replication within the lungs, liver and spleen of BALB/c mice during experimental tularaemia.

Our results indicate that the dissemination of tularaemia in BALB/c mice following intratracheal infection with the wild-type strain of *F. tularensis* is similar to that previously reported for aerosol or intranasal inoculations (Woodward *et al*., 1969). BALB/c mice infected with the *iglD* mutant all survived the infection even those infected with 10^7 cfu, while mice inoculated by 10^7 cfu of the wild-type strain all succumbed to infection (data not shown). The *iglD* mutant is severely defective in intracellular replication in the lungs, liver and spleen of BALB/c mice, while the wild-type strain replicates robustly in all these organs. The histopathological changes of pulmonary, hepatic and splenic tissues upon infection by the *iglD* mutant strain show minimal signs of tularaemia. In contrast, mice infected with the wild-type strain developed sever bronchopneumonia with systemic spread of the disease in the liver and spleen, associated with dramatic histopathological changes. Our studies clearly show that IglD plays an important role in the pathogenesis of experimental tularaemia in mice.

Few facultative intracellular bacteria, such as *Listeria monocytogenes,* escape the phagosomal compartment and replicate in the host cell cytoplasm (Santic *et al*., 2006). Within human macrophages, the FCP of three subspecies of *F. tularensis* (*tularensis, holarctica*-derived LVS and *novicida*) matures to a late endosomal-like stage that does not fuse to lysosomes for the first few hours, followed by a gradual escape of the organisms into the cytoplasm, where they replicate (Golovliov *et al*., 2003a; Clemens *et al*., 2004; Santic *et al*., 2006). Remodelling biogenesis of the FCP to evade fusion to the lysosomes and subsequent bacterial escape into the cytoplasm is central to the mechanisms of manipulation of host cell processes and disease manifestation by *F. tularensis* (Santic *et al*., 2006). Our recent studies have shown that the *iglC* and *mglA* mutants fail to escape into the cytoplasm, and thus, they are defective in intramacrophage growth (Santic *et al*., 2005a,b; 2006). Remarkably, our data show that the *iglD* mutant escapes from the phagosome into the cytosol faster than the wild-type strain, but is completely defective in replication within the cytoplasm of hMDMs and pulmonary cells of BALB/c mice. The mutant cytoplasmic bacteria remain viable within the cytosol as evident from intracellular growth kinetics, which show persistent number of intracellular bacteria. The cytoplasm of eukaryotic cells was long thought to be rich in nutrients, and permissive for proliferation of any bacteria. However, studies involving microinjection of bacteria into the cytoplasm of mammalian cells demonstrate that this environment is not conducive for intracellular proliferation of all bacterial species (Goetz *et al*., 2001). Our studies with the *iglD* mutant indicate that escape of *F. tularensis* into the cytoplasm is not sufficient for intracellular proliferation.

Approximately half of the phagosomes containing the *iglD* mutant colocalize with the lysosomal enzyme Cathepsin D by 1 h after infection. This is significantly higher than the wild-type strain but is much less than the *iglC* mutant, because a majority of its phagosomes fuse to lysosomes, and the bacteria fail to escape into the cytoplasm (Santic *et al*., 2005b). Despite this, the *iglD* mutant bacteria escape from the phagosome faster than the wild-type strain within the first 60 min post infection, and by 6 h post infection, more than 90% of the *iglD* mutant bacteria escape into cytoplasm, similar to the parental strain. This suggests that the mutant bacteria as well as the wild-type strain can escape from both the late endosome-like compartment and from the phagosome that acquires lysosomal enzymes. However, regardless of the maturation stage of the phagosome from which the *iglD* mutant bacteria escape into the cytoplasm, the mutant fails to repli-

cate in any cell examined *in vitro* or *in vivo* in the lungs, spleen or liver of BALB/c mice.

Because the *iglD* mutant fails to replicate within the macrophage cytosol, it is likely that the macrophage cytosol is modified by the wild-type strain to render it permissive for bacterial proliferation. This would predict that the wild-type strain should be able to rescue the *iglD* mutant when both coinhabit a macrophage. Single cell analyses have shown that when the hMDMs are coinfected by the wild-type strain and the *iglD* mutant, the mutant is rescued for intramacrophage growth. In addition, when the *iglD* mutant-infected cells are super-infected by the wild-type strain, the mutant also proliferates. These data suggest that the wild-type strain of *F. tularensis* modifies the macrophage cytosol to render it permissive for proliferation, and that the IglD protein is essential for this process. We speculate the IglD protein is an effector exported into the host cell cytosol to mediate its function or is a cofactor for a bacterial effector that remodels the host cell cytosol. We have been surprised by non-permissiveness of the wild-type strain-infected cells to rescue the *iglD* mutant during super-infections, because very few super-infected cells are permissive for replication of the *iglD* mutant. It is possible that the bacterial effector(s) involved in transducing the signals by the wild-type strain into the macrophage cytoplasm are labile and therefore the mutant may not be rescued if it enters the cytoplasm at a later time (1–2 h) than the wild-type strain. Future functional, biochemical and cellular studies on the IglD protein should resolve this fascinating phenomenon.

Experimental procedures

Bacteria and macrophages

The wild-type *F. tularensis* ssp. *novicida* strain U112 has been described previously and the *iglD* mutant has been constructed by a deletion/insertion (Δ*iglD*::*ermC*), as we described previously (Lauriano *et al*., 2004). Briefly, 500 bp fragments were amplified from the chromosomal DNA upstream and downstream of the coding region of *iglD*. Overlapping PCR was performed with the two 500 bp PCR fragments containing the deletion as well as restriction sites for insertion of an Erm cassette. The 1 kb PCR product was cloned into pWKS30 and an *erm* cassette was cloned into the deletion site ((Δ*iglD*::*ermC* in pWKS30). The resulting construct was PCR amplified and cryo-transformed into U112. Transformants were selected on tryptic soy agar (TSA) supplemented with 0.1% cystein and erythromycin. In some experiments for microscopy, the bacteria harboured the plasmid pKK214 which encodes *gfp* (Abd *et al*., 2003).

For complementation, a plasmid derivative of the pKK214 plasmid (Abd *et al*., 2003) was constructed in which the Cm^r gene was deleted and replaced with the *Francisella groEl* promoter with the *pET15b* multiple cloning site. This vector was used to clone the *iglCD* operon from *F. novicida* DNA into the NcoI and EcoRI sites, transformed into the *iglD* mutant, and the transformants were selected on TSA supplemented with Tet (10 μg ml^{-1}). In some microscopy experiments, the bacteria harboured the plasmid pKK214 which encodes *gfp* (Abd *et al*., 2003).

To prepare hMDMs, peripheral blood monocytes were isolated from healthy volunteers with no history of tularaemia and hMDMs were prepared as we described previously (Santic *et al*., 2005b). Obtaining blood was approved by the institutional IRB with a consent form according to standard federal laws.

Mouse inoculation

Female pathogen-free BALB/c mice, 8–9 weeks of age, were used in all experiments. Mice were housed in specific pathogen-free conditions within our animal care facility according to standard guidelines, and the use of animals for infection was approved by the institutional IACUC.

BALB/c mice were inoculated intratracheally (i.t.), as we described previously (Molmeret *et al*., 2002). Briefly, the mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (2.5 mg $mouse^{-1}$). A total of 50 μl of the *F. tularensis* suspension (10^4 cfu) in sterile saline was inoculated directly into the trachea using a 26-gauge needle followed by 10–20 μl of air. The skin incision was surgically closed. Control animals were inoculated with saline only. At different time points after inoculation, the mice were humanly sacrificed; the lungs, liver and spleen were aseptically excised, finely minced and homogenized in a tissue homogenizer with 5 ml of sterile distilled water. The number of cfu of *F. tularensis* in the organs was determined by the plate dilution method using TCY agar. After 2 days of incubation at 37°C the colonies were enumerated and the results were expressed as the number of cfu per organ.

Histopathology studies

The histological changes in the organs in the lungs of BALB/c mice in response to *F. tularensis* infection were assessed by light microscopy. At 2, 24, 48 and 72 h after inoculation, the mice were humanely sacrificed. Before organs removal, the pulmonary vasculature was perfused with 10 ml of saline containing 5 mM EDTA, via the right ventricle. The excised organs were fixed in 10% neutral formalin for 24 h, dehydrated and embedded in paraffin. Sections (5 μm) were cut, stained with haematoxylin and eosin (H&E), and analysed by light microscopy. On average of 10 0.2 μm thick serial sections of each image were captured and stored for further analyses, using Adobe Photoshop (Adobe Photoshop).

Confocal laser scanning and electron microscopy

The hMDMs attached to glass coverslips in 24-well culture plates were fixed, permeabilized and blocked as we described previously (Santic *et al*., 2005a). Colocalization of the FCP with LAMP-2 and Cathepsin-D was performed as we described previously (Santic *et al*., 2005a). The anti-LAMP-2 (H4B4) 1:2000 monoclonal antibody (developed by J.T. August and J.E.K. Hildreth) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA) and mAbs anti-Cathepsin D (1:200) was obtained from BD Transduction. Anti-mouse secondary antibodies conjugated to Alexa fluor-594 were obtained from Molecular Probes (Eugene, Oregon).

For TEM, lung tissue of BALB/c mice were removed and fixed in 2.5% gluteraldehyde. Briefly, lungs were post fixed by immersion in 2% osmium tetroxide in 0.1 M sodium Sorenson's buffer for 1 h, followed by dehydration in acetone and infiltration and embedding in Epon 12 epoxy resin. Sections (0.5 μm) were stained with toluidine blue and scanned by light microscopy to define areas containing bacteria for ultrastructural examination. Ultrathin sections (0.1 μm) were then cut, stained with uranyl acetate and lead citrate, and examined in Philips transmission electron microscope (Philips, Morgagni 268D, Netherlands) at 80 kV.

Loading the macrophage cytoplasm with specific monoclonal antibodies

The cytoplasm of live hMDMs was loaded with antibacterial antibodies using the glass bead 'loading technique' to permeabilize the plasma membrane as described previously (McNeil and Warder, 1987; Reddy *et al*., 2001). After 1 h infection, cells were washed three times with PBS and 400 μl of antibacterial monoclonal antibodies was added on the top of the coverslips, along with an aliquot of 0.5 g of acid washed sterile glass beads (Sigma; 425–600 microns). The beads were rolled over the cells 12 times, which had no detectable effect on the viability of cells as confirmed by Trypan Blue exclusion. The glass beads were washed off immediately with PBS and the cells were incubated at 37°C for 1 h to allow a sufficient time for the antibodies to bind. The cells were then fixed and processed with conjugated secondary antibodies. Controls were either cells subjected to the same treatment without the glass beads or cells were fixed and permeabilized with 0.05% Triton X-100 for 15 min on ice.

Statistical analyses

All experiments have been performed at least three times and the data shown are representative of one experiment. To analyse for statistical significant differences between different sets of data, Student's two-tail *t*-test was used and the *P*-value was obtained.

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