Virulence Factors Involved in Passage of Francisella tularensis subsp. novicida Through an Air-Blood Barrier Model

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Abstract

Francisella tularensis subsp. novicida (F. novicida) is a facultative intracellular pathogen that when inhaled causes respiratory infection in mice; it is widely used as a model to study tularemia caused by F. tularensis. F. novicida is able to infect different cell types including macrophages and dendritic cells. In the present study we examined F. novicida interactions with human lung epithelial cells and determined the role of established virulence determinants in these processes. A549 type II lung epithelial cells and murine LA-4 bronchial epithelial cells were used to examine the ability of wild type F. novicida and the mutant F. novicida strains deficient in IgIC, Tu4, MglA, 58kDa membrane lipoprotein, and RipA to adhere, invade, replicate within, and translocate through an in vitro transwell system. Using this systematic approach, we determined that different virulence factors play cell-site specific roles during infection: Tu4 is important for adhesion to lung cells; MglA, 58kDa protein, and Tu4 are important for cell invasion; IgIC and its transcriptional regulator MglA are important for intracellular replication; and the function of MglA is required for migration across cell barriers. In addition we also determined that F. novicida infection results in upregulation of matrix metalloprotease 9 (MMP-9) by lung epithelial cells and the subsequent disruption of cell adherens junctions as characterized by loss of cadherin, alpha and beta catenin, and the basal membrane protein laminin.

We conclude that F. novicida is able to attach, invade, and cross through lung epithelial cells and that these properties are determined by individual virulence determinants.

Keywords: Francisella; Air-blood barrier; Lung; Epithelium; Adhesion; Invasion.

Introduction

Francisella tularensis is a facultative intracellular pathogen that is the causative agent of tularemia. Francisella has a broad host range as it is able to infect amoeba, arthropods, rodents and higher mammalian species [1-4]. In humans the primary replication site appears to be species [1-4]. In humans the primary replication site appears to be the macrophage although other cell types have been implicated [5-7]. Infection is established through contact with infected tissues, arthropod bites, inhalation and ingestion, leading to various clinical manifestations [2,8]. The route of infection is a key factor in determining disease severity with inhalation being the most dangerous route [2,9,10]. Two subspecies of F. tularensis are capable of causing disease in humans [9,10]. F. tularensis (type A) is the most virulent subspecies and is primarily found in North America. It is capable of causing respiratory disease with as few as 10 organisms. The highly virulent nature of type A bacteria combined with its ability to be aerosolized has led the Centers for Disease Control and prevention (CDC) to designate it as a category A biological warfare agent [2,11]. F. tularensis subsp. holarctica (type B) is less virulent but more widespread. It is found across the Northern hemisphere, as well as Europe, and Asia. The attenuated F. tularensis live vaccine strain (LVS) was derived from a Type B strain [12]. F.tularensis subsp. novicida (F. novicida) is an important laboratory strain, as it remains highly virulent in mice causing respiratory disease with severe lung pathology and death within 3-5 days after infection; it remains highly virulent in mice causing respiratory disease with severe lung pathology and death within 3-5 days after infection; it is able to infect amoeba, arthropods, rodents and higher mammalian species [1-4]. In humans the primary replication site appears to be the macrophage although other cell types have been implicated [5-7]. Infection is established through contact with infected tissues, arthropod bites, inhalation and ingestion, leading to various clinical manifestations [2,8]. The route of infection is a key factor in determining disease severity with inhalation being the most dangerous route [2,9,10]. Two subspecies of F. tularensis are capable of causing disease in humans [9,10]. F. tularensis (type A) is the most virulent subspecies and is primarily found in North America. It is capable of causing respiratory disease with as few as 10 organisms. The highly virulent nature of type A bacteria combined with its ability to be aerosolized has led the Centers for Disease Control and prevention (CDC) to designate it as a category A biological warfare agent [2,11]. F. tularensis subsp. holarctica (type B) is less virulent but more widespread. It is found across the Northern hemisphere, as well as Europe, and Asia. The attenuated F. tularensis live vaccine strain (LVS) was derived from a Type B strain [12]. F.tularensis subsp. novicida (F. novicida) is an important laboratory strain, as it remains highly virulent in mice causing respiratory disease with severe lung pathology and death within 3-5 days after infection; it is principally used to model Type A infections outside of a Biosafety level 3 (BSL-3) facility [13].

The airway epithelium constitutes the primary barrier that microorganisms encounter when they enter the body through the aerosol route. The close proximity of alveolar epithelial cells to the bloodstream make them a critical cell barrier that prevents microorganisms from entering the bloodstream and disseminating throughout the body. In addition to the barrier function, the airway epithelium produces pro-inflammatory molecules, immune regulators, as well as a variety of antimicrobial factors such as lysozyme, defensins and reactive nitrogen species [14,15]. Therefore, the interaction of pathogenic microorganisms with the airway epithelial cells and the resulting response determines whether bacteria disseminate and cause disease in the host.

Recent studies have reported the ability of F. tularensis LVS to invade and replicate within alveolar type II epithelial cells [5,16,17]. In this study we show that F. novicida is also able to adhere, invade and replicate within lung epithelial cells enabling it to cross the air-blood barrier. Importantly, we observed that several known virulence factors play distinct roles in F. novicida adhesion, invasion, replication and migration through the air-blood barrier. Together, these observations provide new insights into the pathogenic mechanisms of Francisella.

Materials and Methods

Bacterial strains and cell lines

Wild type F. novicida (U112) strain was obtained from Dr. Bernard Arulanandam (University of Texas at San Antonio, San Antonio, TX, 78249). 210-458-7024; Fax: 210-458-7025; E-mail: judy.teale@utsa.edu

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bacteria was determined as previously described. were washed and lysed with Triton X-100. The number of intracellular media containing 2 μg/mL of gentamicin. After 2, 6 and 24 hrs, the cells with Triton X-100, plating and bacterial colony counting the intracellular bacteria were determined by washing with PBS, lysing of 0.1% Triton X-100. The lysate was serially diluted, spread on agar 37°C, 5% CO2 incubator for 1 hour. After the incubation, unattached were grown at 37°C on trypticase soy agar plates supplemented with 0.1% cysteine (TSAcsy). Mutant strains ΔiglC, ΔmglA, and Δtul4 were grown in the presence of 100 μg/mL of erythromycin. The Δ58kdA mutant was grown on trypticase soy agar plates supplemented with 0.1% ceynine (TSAcys). The mutant strain ΔripA was grown on TSAcsy in the presence of 50 μg/mL of kanamycin. INV110 is a laboratory strain of Escherichia coli that is normally used for cloning [22]. INV110 was cultured on Luria Bertani (LB) broth and agar at 37°C; broth cultures were grown in an orbital shaker. A549 cells (ATCC CCL-185) are a type II pneumocyte cell line derived from a human lung adenocarcinoma [23]. LA-4 cells (ATCC CCL-196) are a bronchial epithelial cell line derived from a murine lung adenoma [24]. Both cell lines were low passage derivatives. Cell lines were cultured in F-12 media supplemented with 10% fetal bovine serum and an antibiotic-antimycotic (1 unit of penicillin, 100 μg of streptomycin and 0.25 μg/mL of amphotericin B) (Gibco, Carlsbad, CA) in a humidified atmosphere containing 5% CO2 at 37°C. Human umbilical vein endothelial cells (HUVECs) were acquired from Lifeline Technology, Part#: FC-0003, lot # 00213, manufactured 09/19/07 and cultured in Endothelial Basal Media (EBM) supplemented with 2% fetal bovine serum, 0.4% bovine brain extract, 0.1% human epidermal growth factor, 0.1% hydrocortisone and 0.1% gentamicin sulphate amphotericin B.

Adhesion, Invasion and Replication Assays

Cells were grown in 24-well tissue culture treated polystyrene plates; all assays were performed using confluent monolayers of cells (10% cells per well). Prior to each experiment, bacteria were streaked onto fresh agar plates and grown overnight. The next day bacteria were scraped from the plates and used to create a bacterial suspension corresponding to approximately 1 x 10⁸ colony forming units (CFU)/mL in F-12 media. For adhesion assays, cell monolayers were washed to remove antimicrobials and 250 μL of the bacteria suspensions added to the wells at a Multiplicity of infection (MOI) of 1. Plates were cenrifuged at 1000 cgf for 15 min and then transferred to a 37°C, 5% CO₂ incubator for 1 hour. After the incubation, unattached bacteria were removed by washing the cells 3 times for 5 minutes with phosphate buffer saline (PBS; pH 7.4). Cells were lysed using 100 μL of 0.1% Triton X-100. The lysate was serially diluted, spread on agar plates, and the plates incubated overnight. Adhered bacteria were extrapolated from the number of colonies counted the next day on the plates. For invasion assays, following the 1 hr incubation, bacteria infected cells were washed and then incubated for 2 hrs with F-12 culture media containing 50 μg/mL of gentamicin. The invasion rate was calculated dividing the number of bacterial cells invading by the number of bacterial cells attaching. Subsequently, the numbers of intracellular bacteria were determined by washing with PBS, lysing the cells with Triton X-100, plating and bacterial colony counting the next day. To assess intracellular replication, after treatment with 50 μg/mL gentamicin for 2 hours, cells were washed and incubated with F-12 media containing 2 μg/mL of gentamicin. After 2, 6 and 24 hrs, the cells were washed and lysed with Triton X-100. The number of intracellular bacteria was determined as previously described.

Immunofluorescence staining

A549 cells were grown on poly-L-lysine-coated coverslips. Cells were infected with F. novicida as indicated above. At the designated time points, single immunofluorescence staining was performed. Cells were washed 3 times with PBS. The cells were fixed using 70% ethanol for 5 min at room temperature (RT), washed twice again with PBS and then washed with TNT buffer (Tris 0.1M, NaCl 0.15M and Tween 0.2% 0.05%). To block unspecific antibody binding the cells were incubated 30 min at RT with 10% serum from a naïve mouse, followed by a 40 min RT incubation with an antibody specific for for F. novicida lipopolysaccharide (LPS) conjugated with Alexa 488 (1:300). After that, the cells were washed three times with TNT, one wash with 1% Triton X-100 and 3 more washes with TNT. The slides were mounted with Cytoseal (Calbiochem) containing nuclear staining 4,6-diamidino-2-phenylindole (DAPI, 0.3 μM). Double immunofluorescence staining was performed as described above but using first a set of primary antibodies specific for pan-cadherin, α-catenin and β-catenin (R&D Systems, Minneapolis, MN), basal membrane protein laminin (Sigma-Aldrich, St. Louis, MO), and the pro and active form of matrix metalloproteinase 9 (MMP-9) (Sigma-Aldrich, St. Louis, MO) which were purified rabbit anti-mouse and as secondary antibody rhodamine red X (RRX)-conjugated AffiniPure anti rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). This procedure was then followed by incubation with a specific antibody for F. novicida as described above.

Generation and infection of an in vitro air-blood barrier

The air-blood barrier was constructed on 0.4 μm pore swannell membranes (CORNING, Corning, NY 14831 USA) first seeded on the bottom (membrane) upside down with 2.5 X 10⁵ cells/mL of HUVECs in EBM media supplemented as described above. HUVECs were allowed to grow for 48 hrs at 37°C and 5% CO₂. Then, the swannell membranes were inverted and the A549 cells were seeded on the top side of the membrane at a concentration of 2.5 X 10⁵ cells/mL in F-12 media supplemented with 10% FBS and antibiotic-antimycotic as described above. The media was changed in the top and lower chamber the next day. The confluence of the bilayer was monitored by changing the media present in both chambers for fresh media and by measuring the volume present in the top chamber 5 minutes after fresh media had been added; if 10% or less volume of fresh media was lost from the top chamber the bilayer was considered to be ready for infection. Once the air-blood barrier was ready for infection, cells were washed three times with F-12 media, and each well was infected with approximately 1 X 10⁶ CFU/mL of bacteria in 250 μL of media. Immediately after infection, the content of the lower chamber in each well was collected, spun down, suspended in 120 μL of F-12, and then plated to determine the number of bacteria crossing the air-blood barrier (bilayer). Following this procedure, plates of bilayers were incubated at 37°C and 5% CO₂. Collection of the lower chamber contents was performed at 2, 6 and 24 hrs after infection following the procedure described above. 2.5 mL of media contained in the lower chamber were refilled after the collection at each time point.

Statistical Analysis

Results were expressed as means and the error bars indicate the standard error of the mean. In all the figures, data were pooled from at least three independent experiments with each experiment having 3 or more replicates. Data obtained for U112 was compared to E. coli and F. novicida mutant strains ΔiglC, Δtul4, ΔmglA, ∆58kdA and ΔripA by
using a homoscedastic two-tailed Student’s t-test. Statistical significance was established at P < 0.05. Invasion data were analyzed statistically comparing *F. novicida* against *E. coli* infected cells for each cell type separately. Replication data were analyzed statistically comparing *F. novicida* against each mutant strain for each time point, 2 hrs, 6 hrs and 24 hrs of replication. A one-way ANOVA and a Dunn’s multiple comparison post-test were used to analyze the infection of the air-blood barrier.

Results

**F. novicida** adheres and invades lung epithelial cells in vitro

It has been previously reported that *F. tularensis* LVS invades and replicates within TC-1, MLE 12, A549, HEP-2 and HBE rodent and human lung epithelial cells [5,17,25]. In contrast it remains unknown if *F. novicida* is able to replicate within lung epithelial cells. To determine whether *F. novicida* could adhere and invade lung epithelial cells we performed adhesion and invasion assays in vitro using A549 lung cells and LA-4 bronchial epithelial cells. For comparison purposes we included a non-invasive, non-pathogenic *E. coli* strain, INV100. Wild type (WT) *F. novicida* (U112) adhered to both cell lines at levels equivalent to INV100 (Figure 1A). In contrast, U112 invaded A549 and LA-4 cells at levels 5-fold (p=0.016) and 14-fold (p=0.0013) greater than INV100, respectively (Figure 1B). Extrapolation of the invasion rate (invasion/adherence) indicated that approximately 20% of U112 that attached to a cell were able to invade; in contrast only 2-5% of INV100 were detected as intracellular (Figure. 1C). Importantly, no cell line specific differences were observed between A549 or LA-4 cells for U112 adhesion or invasion, suggesting that bronchial and alveolar epithelial cells are equally susceptible to these processes. Overall, similar levels of adhesion by U112 and INV100 indicate a passive binding to lung cells. However, *F. novicida* seems to have the ability to invade these cells, whereas this property is absent in non-pathogenic *E. coli*.

**Differential adhesion and invasion of *F. novicida* and mutant strains to lung epithelial cells**

We next sought to determine the role of established Francisella virulence genes in the adhesion and invasion process of epithelial cells. Five different mutant derivatives of U112 were used: 1) ∆*iglC* (∆*iglC*:ermC) which lacks the *iglC* gene that is present in the Francisella...
pathogenicity Island (FPI) and is necessary for intramacrophage survival and growth [21, 26]. 2) ΔmglA (ΔmglA::ermC) that lacks the mglA gene which is the transcriptional regulator of the FPI genes [21, 26]. 3) Δtul4 (Δtul4::ermC) that lacks a 17-kDa membrane lipoprotein that is known to be recognized by T cells from a majority of F. tularensis primed individuals and has been described as a ligand for the human TL2R/TLR1 heterodimer [21, 27, 28]. ΔiglC, ΔmglA and Δtul4 mutants were generated by utilizing universal priming of optimized antibiotic resistance cassettes and splicing by overlap extension (SOE) [21], mutations generated through this method have characteristics of non-polar mutations [29]. 4) Δ58kDa that lacks a 58kDa membrane lipoprotein reported to contribute to virulence in vitro and in vivo [30, 31]; this mutant has shown non-polar effects [30]. Finally, 5) ΔripA that lacks a cytoplasmic membrane protein required for intracellular replication of F. tularensis LVS within macrophages and lung epithelial cells [32]; this is a transposon mutant with disruption of the ripA gene [20].

Following adherence and invasion assays we determined that mutant strain ΔiglC adhered and invaded lung cells at levels greater than U112 (Figures 2A and 2B), however analysis of the invasion rate suggested that cell surface attached ΔiglC mutants had an equal propensity to invade lung cells as U112 (U112 (U112 invasion rate = 16%; ΔiglC = 15%) suggesting that mutation of iglC only affected adhesion (Fig. 2C). In contrast Δtul4 adhered and invaded lung cells at significantly lower levels than U112 (Figures 2A and 2B). Despite this, invasion rate analyses suggest that adhered Δtul4 mutants had a greater capacity to invade cells than U112 (invasion rate = 46%) (Figure 2C). The ΔmglA mutant adhered to A549 cells at a significantly higher level than U112 (p < 0.0005) (Figure 2A). However, a significant decrease in the number of intracellular ΔmglA was observed versus U112 (Figure 2B). Of note the invasion rate of ΔmglA was 10-fold lower than U112 (ΔmglA invasion rate < 1%) (Figure 2C). This finding suggests that ΔmglA mutant strain improperly regulates the expression of protein(s) involved in both adhesion and invasion. The mutant strain Δ58kDa showed similar levels of adhesion when compared to U112; however, it too had significantly diminished invasion levels (p < 0.05) (Figure 2B) with an invasion rate of 6% (Figure 2A). However, it appeared to have decreased levels of invasion of lung cells although the final value was not statistically significant p = 0.1466 (Figure 2B) and its invasion rate was only 1% (Figure 2C). Collectively, these findings suggest that F. novicida adherence and invasion are distinct pathogenic processes and that deletion of established virulence determinants may have unexpected outcomes on these complex processes.

F. novicida and mutant strains replicate in lung epithelial cells

One major aspect of F. tularensis lethality is its ability to interact with the airway epithelium, enter the bloodstream and rapidly disseminate to distant organs such as liver and spleen [30]. To assess the ability of F. novicida to cross the air-blood barrier, we established an in-vitro cell co-culture composed of a top layer of A549 human airway epithelial cells grown on a transwell membrane and a bottom layer of HUVEC human endothelial cells grown on the opposite side of the membrane. This air-blood barrier model was infected through the apical side with F. novicida at an MOI of 1. The ability of F. novicida to cross the air-blood barrier was assessed by collecting all the volume present in the lower chamber of the transwell system, refilling with fresh media for the next time point, and by plating immediately after (0 hrs), 2 hrs, 6 hrs and 24 hrs of infection. Experimental results obtained at time 0 and 2 hrs revealed that the barrier was tight, as no bacteria were isolated from the lower chamber despite intracellular bacteria (Figure 5). By 6 hrs of infection, 101 CFU/ml of bacteria had crossed the air-blood barrier and by 24 hrs of infection the amount of bacteria

\[ \Delta \text{iglC} \]

replicates contrasted with its increased ability to adhere and invade cells, whereas the inability of \( \Delta \text{mglA} \) to replicate was consistent with more global defects including those observed for invasion. These findings were confirmed by immunofluorescent microscopy where very few bacteria were observed in A549 cells after 24 hrs for \( \Delta \text{iglC} \) and \( \Delta \text{mglA} \) (Figures 4B and 4D respectively). Importantly no defects were found for intracellular replication for \( \Delta \text{tul4}, \Delta58kDa \) or \( \Delta \text{ripA} \) mutants, thus indicating that these genes did not impact intracellular survival and/or replication.

Ability of F. novicida and mutants to cross in vitro model of air-blood barrier

One of the key aspects of F. tularensis lethality is its ability to interact with the airway epithelium, enter the bloodstream and rapidly disseminate to distant organs such as liver and spleen [30]. To assess the ability of F. novicida to cross the air-blood barrier, we established an in-vitro cell co-culture composed of a top layer of A549 human airway epithelial cells grown on a transwell membrane and a bottom layer of HUVEC human endothelial cells grown on the opposite side of the membrane. This air-blood barrier model was infected through the apical side with F. novicida at an MOI of 1. The ability of F. novicida to cross the air-blood barrier was assessed by collecting all the volume present in the lower chamber of the transwell system, refilling with fresh media for the next time point, and by plating immediately after (0 hrs), 2 hrs, 6 hrs and 24 hrs of infection. Experimental results obtained at time 0 and 2 hrs revealed that the barrier was tight, as no bacteria were isolated from the lower chamber despite intracellular bacteria (Figure 5). By 6 hrs of infection, 101 CFU/ml of bacteria had crossed the air-blood barrier and by 24 hrs of infection the amount of bacteria

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present in the lower chamber had increased to 10^4 CFU/ml (Figure 5).

We also studied the role of the proteins IglC, MglA, Tul4, 58kDa and RipA in the capacity of F. novicida to cross this modeled air-blood barrier. By 2 hrs of infection, in contrast to the WT strain, the ∆58kDa mutant was the only strain capable of crossing the air-blood barrier, although the number of bacteria did not show a significant increase over time (Figure 5). On the other hand, by 6 hrs of infection, in addition to the WT strain, mutant strains ∆58kDa, ∆tul4 and ∆ripA were able to cross the air-blood barrier. Mutant strain ∆58kDa was isolated from the lower chamber at higher levels than the rest of the strains used (Figure 5) but statistical significance was not achieved. By 24 hrs of infection, mutant strains ∆58kDa, ∆iglC, ∆ripA and ∆tul4 crossed air-blood barrier, but statistical analyses revealed that the WT strain crossed the bilayer at significantly higher levels than the mutant strains ∆mglA, ∆tul4 and ∆ripA (p< 0.0005, p< 0.005 and p< 0.05 respectively) (Figure 5). These data suggests that 1) mglA is necessary for F. novicida in order to cross the air-blood barrier; 2) the absence of the 58kDa protein increases the ability of F. novicida to cross the bilayer; 3) the absence of IglC, Tul4 and RipA does not seem to have a major effect in Francisella's ability to cross the air-blood barrier.

**F. novicida infection of lung epithelial cells disrupts intraepithelial junction proteins**

In an attempt to fully examine the mechanisms by which Francisella may cross the lung epithelial cell barrier, we also tested the effects of F. novicida infection on intraepithelial junction proteins. Adherens are members of junction protein complexes on the lateral membrane that occur at points of cell-to-cell contact [35]. A confluent monolayer of A549 cells on poly-L-lysine coated coverslips was infected with F. novicida for 24 hrs. Subsequently coverslips were fixed and stained for adherens junction proteins pan-cadherin, α-catenin and β-catenin and the basement membrane protein lamin. Our results showed that infected cells had a significant decrease in the expression of adherens junction proteins as well as laminin when compared with uninfected cells (Figure 6), suggesting a disruption of the cell-to-cell contacts. Recent studies report that MMP-9 activity enhances host susceptibility to pulmonary infection with Francisella [36]. In addition, in vivo assays
have shown that MMP-9 is one of the few proteins that are expressed early during respiratory tularemia [30]. Furthermore, recent studies show the ability of MMP9 to modulate tight junction integrity and cell viability in the human airway epithelia [37]. For these reasons, we decided to determine the presence of MMP-9 in infected cells. Our data showed that in fact MMP-9 expression is significantly increased in cells that contain intracellular F. novicida (Figure 6); however, cells that apparently do not have intracellular bacteria showed a decrease in the expression of MMP-9 when compared to uninfected cells. These data suggest that intracellular F. novicida could induce the expression of MMP-9.

**Discussion**

This study used and in vitro model to understand the interaction between the respiratory pathogen F. novicida and the pulmonary epithelium. Our goal was to systematically examine the epithelial cell interaction with F. novicida in a stepwise manner. To characterize these interactions, we first investigated the ability of organisms to adhere and invade lung epithelial cells. We determined that U112 adheres to both cell types at similar levels than the non-pathogenic E. coli INV110, suggesting a passive adhesion to lung epithelial cells during infection. In contrast, we observed that U112 showed a higher invasion level to both cell lines suggesting that despite the passive mechanism it uses to attach to lung epithelial cells it is able to invade this cell type at relatively high rates. The results also showed that F. novicida is capable of replicating within A549 cells at an exponential rate. This is consistent with previous studies indicating that F. tularensis LVS has the ability to invade and replicate within alveolar epithelial cells [5, 25]. In addition, recently Hall et al. [38], described the presence of F. novicida in lung epithelial cells as well as its replication within A549 cells.

The present study expanded on these findings by determining the role of known Franciscella virulence factors including igIC, mglA, tul4, 58kDa and ripA in adhesion, invasion and intracellular replication. Our results showed that the F. novicida mutant strain ∆tul4 adhered to A549 cells at significantly lower levels than the WT strain; since Tul4 is a membrane lipoprotein it could be serving not only as an antigen that can be recognized by T cells [27], but also as a ligand that facilitates the binding of F. novicida to lung epithelial cells. This proposed role is consistent with the finding that Tul4 stimulates the TLR2/TLR1 heterodimer [28]. In contrast, mutant strains ΔigIC, ΔmglA and ΔripA adhered to A549 cells at higher levels than the U112 strain suggesting that none of these proteins are required for F. novicida adhesion to A549 cells. These findings also suggests that adhesion to lung cells is a process tightly regulated by F. novicida, that the bacteria must gain an advantage by limiting its contact with epithelial cells, and that adhesion is a pathogenic mechanism that is distinct from invasion.

Despite the increased ability to adhere to A549 cells, mutant strain ΔmglA showed significantly lower levels of invasion. As MglA is the transcriptional regulator of most of the genes included in the FPI [26] and also regulates the transcription of other genes outside FPI (FTT0989, oppb and FTT1209c) [39], it is likely that MglA is regulating the expression of other virulence factors necessary for the invasion of lung epithelial cells. In addition, mutant strains Δ58kDa and ∆tul4 showed significantly lower levels of invasion to A549 cells when compared to U112 strain. This is of interest as both Tul4 and the 58 kDa proteins are membrane lipoproteins and could potentially play synergistic roles for invasion of this cell type. In contrast, consistent with an increased adhesion to A549 cells, mutant strain ΔigIC showed significantly increased levels of invasion suggesting that this gene does not...
not play a role in invasion.

One of the major aspects of *F. novicida* virulence is its ability to replicate within different cell types [5,6,33,34]. The results obtained in our experimental assays, showed an increased replication by U112 strain in A549 cells and that this replication increased in a time dependent manner. These data suggest that the capability of replicating within a non-phagocytic cell type, gives *F. novicida* an advantage to disseminate via the blood to other tissues in the host. It has been demonstrated that Francisella has a significant extracellular phase in blood [40]; therefore, it could disseminate to other tissues not only intracellularly in macrophages or dendritic cells but also extracellularly.

Subsequently, we examined the role of IglC, MglA, Tul4, 58kDa and RipA in intracellular replication. Our data showed that mutant strains ΔIglC and ΔmglA showed significantly lower levels of replication within A549 cells when compared to the WT strain. Moreover, replication levels did not show a significant change from one time point to the next time point tested. This suggests that IglC and its transcriptional regulator MglA are important for intracellular replication within A549 lung epithelial cells, similar to that observed in replication within phagocytic cells [21]. The deletion of RipA protein affected replication slightly (not adherence or invasion). Interestingly, similar results have been previously shown for the replication of the Francisella LVS mutant strain ΔripA in macrophages and murine lung epithelial cell line TC-1 [32].

Several studies have shown that Francisella disseminates to distant organs such as liver and spleen following an aerosol infection [11,30,41]. Other studies have shown that Francisella survives and replicates, among others, within macrophages and dendritic cells [6,33] and others point out the possibility that Francisella could use dendritic cells as a mean to disseminate inside the host [42]. In the present study we tested for the possibility that Francisella could enter the blood stream directly from the lungs and further disseminate. Our results showed that *F. novicida* is able to cross a two cell layer modeling the air-blood barrier as early as 6 hrs after infection, and the number of bacteria crossing the barrier increased with time. The increase in number of bacteria present on the lower chamber after 24 hrs of infection could be explained by the fact that *F. novicida* replicates within alveolar epithelial cells, and that bacteria cells could disrupt this monolayer facilitating the route to possibly continue infecting the endothelial cells and reaching the blood stream.

We also tested whether *F. novicida* could get across the air-blood barrier between the epithelial cells by disrupting the intraepithelial junction proteins. Immunofluorescence staining of adherens junction proteins pan-cadherin, α and β-catenins in A549 cells infected with *F. novicida* after 24 hrs showed a significant decrease in their expression compared to uninfected cells (Figure 6). Recent studies report that MMP-9 activity enhances the host susceptibility to pulmonary infection with Francisella [36]. In addition it has been shown that some bacterial pathogen proteinases can generate the active form of MMP-9 which is then implicated in tissue injury [43]. Furthermore, in vivo assays show that MMP-9 is one of the few proteins whose expression is increased in lung homogenates and bronchoalveolar lavage fluid (BALF) of mice early after infection [30]. Immunofluorescence staining of A549 cells uninfected and infected with *F. novicida* after 24 hrs showed that cells containing intracellular bacteria had an increased expression of MMP-9 compared to uninfected cells (Figure 6). Altogether, these data suggest that a possible mechanism *F. novicida* uses to cross the air-blood barrier is by inducing an increase in the expression of MMP-9 which results in disruption of intraepithelial junction proteins as well as the basal membrane protein. Finally, we propose a model for *F. novicida* passage across an air-blood barrier (Figure 7); following respiratory infection with *F. novicida* and when the bacterium gets in close proximity to the alveolar epithelium, its membrane lipoprotein Tul4 mediates its adherence to this barrier of cells. Once the bacteria are attached, invasion is mediated through the transcriptional regulator MglA and the membrane lipoproteins 58kDa and Tul4. Subsequently, intracellular replication within these cells is mediated by MglA and IglC.
proteins. Then, for translocation to the apical side, the bacteria induces the expression of MMP9 and it also requires MglA.

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