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Francisella Targets Cholesterol-Rich Host Cell Membrane Domains for Entry into Macrophages[1]

Batcha Tamilselvam and Simon Daefler[2]

***Francisella tularensis* is a pathogen optimally adapted to efficiently invade its respective host cell and to proliferate intracellularly. We investigated the role of host cell membrane microdomains in the entry of *F. tularensis* subspecies *holarctica* vaccine strain (*F. tularensis* live vaccine strain) into murine macrophages. *F. tularensis* live vaccine strain recruits cholesterol-rich lipid domains ("lipid rafts") with caveolin-1 for successful entry into macrophages. Interference with lipid rafts through the depletion of plasma membrane cholesterol, through induction of raft internalization with choleratoxin, or through removal of raft-associated GPI-anchored proteins by treatment with phosphatidylinositol phospholipase C significantly inhibited entry of *Francisella* and its intracellular proliferation. Lipid raft-associated components such as cholesterol and caveolin-1 were incorporated into *Francisella*-containing vesicles during entry and the initial phase of intracellular trafficking inside the host cell. These findings demonstrate that *Francisella* requires cholesterol-rich membrane domains for entry into and proliferation inside macrophages. *The Journal of Immunology,* 2008, 180: 8262–8271.**

Francisella tularensis is a category A biothreat because of its high infectivity and high case fatality rate when untreated (1). As *F. tularensis* appears to have devised efficient ways of entering its host cell and replicating intracellularly (2), this presents a primary target for prevention through vaccine and novel treatment strategies. The pathway of entry that *Francisella* chooses is also likely to determine its intracellular fate and thus the overall outcome of the infection. However, the pathway by which *Francisella* enters its host cells while meeting little resistance by the host cell is not well understood.

The ability of *F. tularensis* to cause disease is closely correlated with its ability to enter into and survive inside of what is currently thought to be its permissive host cell, the macrophage (2–4). *Francisella* strains that are deficient for intramacrophage survival cannot elicit disease in the mouse model. Histopathological examinations of tissue specimens from primates infected with *F. tularensis* have consistently shown that the bacterium resides inside the cell (5).

Most studies that have examined *Francisella* entry have used murine macrophages and the attenuated *Francisella* live vaccine strain (LVS)[3] or *Francisella novicida.* Clemens and coworkers have described the morphology of *Francisella* entry into human macrophages (THP-1) in unique forms of "spacious asymmetric pseudopod loops" (6), which are distinct from conventional or coiling phagocytosis or ruffling/triggered macropinocytosis. This uptake process is strongly dependent on complement receptors and serum with intact complement factor C3. Microfilaments are thought to be involved in this uptake process, which can also be observed when formalin-killed bacteria are used. The latter findings suggest a preformed bacterial cell-surface complex such as capsular elements or LPS to be involved in the uptake process. Protease treatment of bacteria does not affect this entry pathway, whereas peroxidation and crosslinking of bacterial surface carbohydrates and/or lipopolysaccharides lead to conventional phagocytosis of *Francisella*. Using *F. novicida* and specifically looking at primary human monocytes, contributions from complement receptor 3, Fcγ receptors, mannose receptors, and surfactant protein A have been described (7–9). Interestingly, these authors also described a 60% reduction of association of *Francisella* LVS with primary human monocytes in comparison to *F. novicida*. This suggests host cell-specific surface interactions beyond the ubiquitous complement and mannose receptor. Multiplicities of infection (MOI) $>10-60$ were required to achieve efficient infection. A role for the mannose receptor was further confirmed in an infection model using *F. tularensis* LVS and J774 macrophages, where induction of expression of the mannose receptor on human monocyte-derived macrophages by IL-4 led to a significant increase in the uptake of *Francisella* (9). The authors also acknowledged that because neither blockage of the mannose receptor nor of the complement receptor could ablate infection, there are probably more receptors involved. An additional important role has been described for the scavenger receptor class A (SRA), which mediates adhesion and subsequent host cell entry in the presence of an unknown serum component (10). Heterologous expression of SRA in HEK cells allowed efficient entry of *Francisella*.

The contribution of specific membrane domains required for *Francisella* entry, which play a major role for the infectious process by other intracellular organisms (11), has not been addressed. *Brucella*, *Rickettsia*, and *Mycobacteria* are prominent examples of intracellular bacteria that interact with specific cholesterol-rich plasma membrane domains of the host cell and thus negotiate their entry into the host (12–17). In all of these cases cholesterol is required for cellular uptake and for successful intracellular survival or proliferation.

In this study we sought to investigate the significance of specialized host cell membrane domains for entry and intracellular

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[3] Abbreviations used in this paper: LVS, live vaccine strain; Cav1-GFP, fusion of GFP to caveolin-1; MβCD, methyl-β-cyclodextrin; MOI, multiplicity of infection; PI-PLC, phosphatidylinositol phospholipase C; SRA, scavenger receptor class A.

survival of *F. tularensis* subspecies *holarctica* vaccine strain (*F. tularensis* LVS) in macrophages.

Materials and Methods

Bacterial strains, cell lines, and growth conditions

F. tularensis LVS (army lot 11) was generously provided to us by Dr. Karen Elkins (U.S. Food and Drug Administration). *F. tularensis* LVS constitutively expressing GFP (SD833) was transformed with plasmid pFNLTP6 *gro-gfp*. *Francisella* was grown on chocolate II agar enriched with IsoVitaleX (BD Biosciences) for 40–48 h at 37°C. As liquid medium we used Mueller-Hinton broth supplemented with IsoVitaleX. *Escherichia coli* XL-1 was grown at 37°C with shaking in Luria-Bertani broth without glucose (18). When indicated, antibiotics were present at: kanamycin, 50 μg/ml; chloramphenicol, 50 μg/ml; when used for *Francisella*, kanamycin was used at 10 μg/ml.

Murine macrophage cells, J774A.1 (TIB-67), and RAW 264.7 (TIB-71) were obtained from the American Type Culture Collection. DMEM (Cellgro from Mediatech) was supplemented with 10% FBS (HyClone, not heat-inactivated) and penicillin (100 IU/ml) and streptomycin (100 μg/ml). When cells were used for *Francisella* infection assay, no antibiotics were added 24 h before infection. Cells were grown at 37°C and 5% CO_2.

Plasmids

A plasmid expressing a fusion of GFP to caveolin-1 (Cav1-GFP) was generously provided to us by Dr. Ari Helenius (19). The backbone of this plasmid is pEGFP (Clontech Laboratories) and carries a kanamycin antibiotic resistance marker.

A shuttle plasmid, which encodes GFP under the control of the groE promoter (pFNLTP6 *gro-gfp*), was kindly provided to us by Dr. T. C. Zahrt (20). It carries a kanamycin antibiotic resistance marker.

Infection assay

Several colonies of *F. tularensis* LVS were collected, washed twice with sterile phosphate buffer at pH 7.0 (PBS, Mediatech 46-013-CM), and dispersed in cell culture complete medium for 15 min. MOI was adjusted to 50 using a standardized calibration curve of OD_{600}/CFU. Bacteria were added to host cells at 60–80% confluency in 12-well dishes. At a given time point after the infection, host cells were washed repeatedly with warm PBS. Remaining extracellular bacteria were killed by the addition of 10 μg/ml of gentamicin (37°C, 5% CO_2) for 30 min. Gentamicin was removed by washing in DMEM. Infected cells were resuspended in complete tissue culture medium without addition of antibiotics. After a given time of infection, cells were lyzed in 0.5% *N*-octyl-β-glucopyranoside (eBioscience). Serial dilutions of cell lysates were plated on chocolate II agar and incubated at 37°C for 2 days.

For assaying infection by microscopy, host cells were grown on sterile coverslips to 60–80% confluency in 6- or 12-well tissue culture dishes. Infection was conducted as above, except that no gentamicin was used for killing of extracellular bacteria. Coverslips were washed with PBS at given time points of infection and then directly processed for microscopy. As control, we measured uptake of fluorescent zymosan A (*Saccharomyces cerevisiae*) particles and fixed *E. coli* (K-12) particles, which were labeled with Alexa Fluor 488 (Molecular Probes E-13231 and Z-2841). Zymosan A particles were opsonized using Molecular Probes (Z-2850) opsonizing reagents and protocol. Particles were used at a ratio of 50:1 (particles-cell). Uptake of particles was measured by fluorescence microscopy as described below.

Immunostaining

Macrophage cell lines were grown on sterile coverslips in petri dishes or 6- or 12-well plates. Cells on coverslips were fixed in 4% paraformaldehyde solution (Polysciences, 18814) for 10 min, washed with PBS, and permeabilized in 0.1% Triton X-100 (IBI Scientific, IB07100) in PBS for 15 min. Reaction with antisera was performed in 0.05% Tween 20/PBS for 1 h at room temperature unless stated otherwise. Stained and dried coverslips were mounted on glass slides using Gold antifade medium (Invitrogen, P36930).

Extracellular bacteria were stained with *Francisella*-specific antiserum (BD Biosciences, 240939) conjugated to Alexa Fluor 594 using Zenon Ab labeling kit (Invitrogen, Z25370). Conjugated antiserum was used at a dilution of 1/1000. This staining procedure was conducted before permeabilization step in 0.1% Triton X-100 to prevent staining of intracellular bacteria. After washing in PBS, coverslips were further processed as described above.

Two units of phalloidin conjugated to Alexa 594 (Invitrogen, A12381) was used per coverslip for 20 min. Filipin III (Sigma, F4767) was used at a concentration of 5 μg/ml for 30 min. Staining with choleratoxin (Invitrogen, V34405) was done at a concentration of 1 μg/ml of cholera toxin subunit B for 10 min at 4°C, followed by reacting with a 200-fold dilution of anti- cholera toxin B rabbit serum coupled with Alexa 594 for 15 min.

Mouse mAbs to Cav1 (Abcam, ab17052) were used at a dilution of 1/500. Visualization was with staining with a goat-anti-mouse IgG conjugated to Alexa 594 (Invitrogen. A11020). When indicated, bacteria were prestained with SYTO 62 red fluorescent nucleic acid stain (Invitrogen, S11344), washed in PBS, and then used for infection assays.

Microscopy

An Olympus IX81 fluorescent microscope with motorized Z-stage was used. An XCite120 illumination system served as the light source. Electronic filter wheels (Ludl Electronic Products) were used for excitation and emission with appropriate dichroic beam splitters. For green fluorescence (GFP), the excitation filter was 494/20 nm and the emission filter was 531/22 nm; for red fluorescence (Alexa 594, Syto 62), the excitation filter was 575/25 nm and the emission filter was 624/40 nm; for red fluorescence (FM4–64), the excitation filter was 494/20 nm and the emission filter was 632/22 nm; and for blue fluorescence (filipin III), the excitation filter was 387/11 nm and the emission filter was 422/30 nm. A fully automated *x*-*y*-stage equipped with a temperature-controlled heating stage was used. Image acquisition was controlled by software (Volocity 4.1 software, Improvision). Images were acquired with a monochrome digital camera (Hamamatsu ORCA-ER, unbinned pixel size). In general, each series consisted of a minimum of 40 Z-stacks at 0.2-μm intervals. Images were deconvolved using iterative algorithms (Volocity 4.1 software) based on measured point-spread functions. A maximum of 25 cycles of iteration was performed and was constrained at a 99.2% confidence limit.

Overlap of individual fluorescence pixels from separate channels for each optical plane was determined with the Volocity 4.1 colocalization module. When results were quantified, 200 cells from randomly selected fields were evaluated. All cells found in a given field were analyzed, except for cells with obvious signs of cell death (detachment, ballooning), which were excluded (in general <5%). Results are reported as the percentage of 200 cells analyzed or as the means of a given data set.

Phospholipase C treatment of macrophages

Phosphatidylinositol phospholipase C (PI-PLC) was purchased from Sigma-Aldrich (P5542). Host cells were treated at final concentrations of 0.01, 0.05, 0.1, and 0.5 U/ml before the infection with *Francisella*. One hour and 24 h after the infection, cells were washed repeatedly with warm PBS, and remaining extracellular bacteria were killed by the addition of 10 μg/ml of gentamicin (37°C, 5% CO_2, 30 min). Cells were lysed in 0.5% *N*-octyl-β-glucopyranoside in PBS for 5 min. Serial dilutions of cell lysates were plated on chocolate II agar and incubated for 2 days. CFUs were determined after infections for 1 and 24 h. When uptake of zymosan particles or fixed *E. coli* particles was measured, host cells were pretreated with PI-PLC as described above. Cells were exposed to particles for 1 h. Cells were washed and intracellular particles measured by fluorescence microscopy.

Four-dimensional microscopy of Francisella *entry into macrophages*

Macrophage cells were seeded in glass bottom culture dishes (Willco Wells, GWST-3522) and grown to 60–80% confluency. Cells were washed with CO_2 independent cell culture medium (Invitrogen, 18045-088) enriched with 10% FBS and incubated in this medium. The glass bottom culture dish was placed on a heated microscopy stage (Harvard Apparatus). Incubation was conducted for 2 h at 37°C without CO_2. Treatment with methyl-β-cyclodextrin (MβCD) was conducted a described above, except that all manipulations were conducted with the culture dish in situ on the microscopy stage. Five minutes before infection, FM 4–64 (Invitrogen, T13320) was added to the culture dish at a final concentration of 1 μg/ml. FM 4–64 stains plasma membrane and endocytic vesicles (21). Infection was initiated by addition of *Francisella* suspended in tissue culture medium at a calculated MOI of 100. Image acquisition was automated at intervals of 10 min (t_i) with 40 Z-stacks at each *x*-*y*-coordinate for each fluorescence channel.

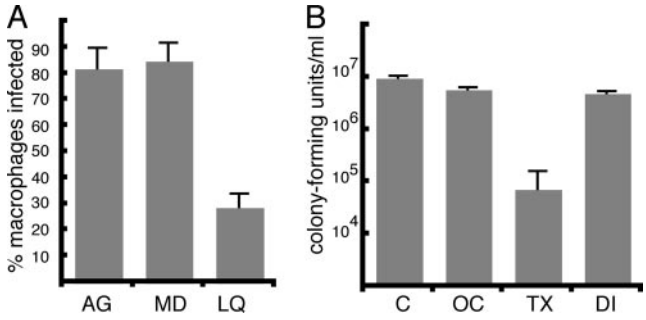

FIGURE 1. *A*, *Francisella* LVS derived from agar plates or macrophages are more infective than *Francisella* grown in liquid culture. Murine macrophages (J774A.1) were infected with *Francisella* expressing GFP grown for 24 h on chocolate II agar plates (AG), with *Francisella* derived from infected murine macrophages (J774A.1) (MD), or with *Francisella* harvested from enriched Mueller-Hinton broth at mid-logarithmic growth phase (LQ). Percentage of infected macrophages was determined by fluorescence microscopy. *B*, Effect of detergents on the viability of *Francisella* LVS. CFUs (10^7) of *Francisella* were kept in PBS (C), 0.5% *N*-octyl-β-glucopyranoside (OC), 0.5% Triton X-100 (TX), or 1% digitonin (DI) for 15 min. Bacteria were then washed three times in PBS and plated for CFUs on chocolate II agar plates.

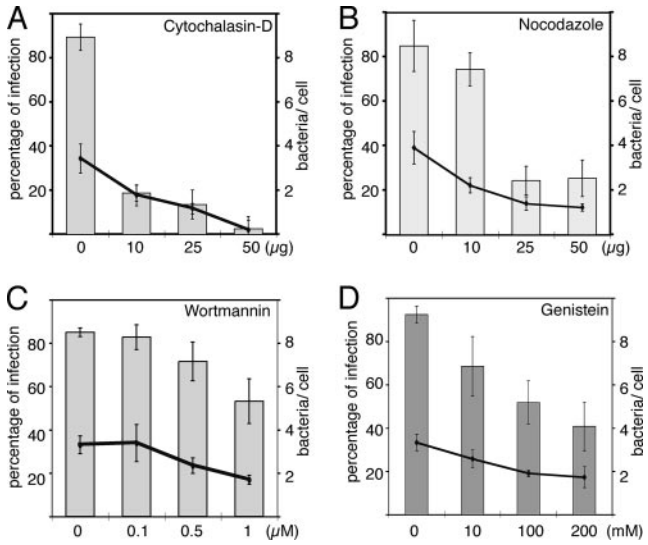

FIGURE 2. Effect of inhibitors on the entry of *Francisella* LVS into murine macrophages (J774A.1). Macrophages were pretreated for 60 min with (*A*) cytochalasin D (0, 10, 25, 50 μg/ml), (*B*) nocadazole (0, 1, 25, 50 μg/ml), (*C*) genistein (0, 10, 100, 200 μM), and (*D*) wortmannin (0, 0.1, 0.5, 1 μM). After removal of the agents by washing with PBS, cells were infected with *Francisella* LVS (MOI of 50) expressing GFP for 60 min. Percentage of infected macrophages and average number of intracellular bacteria per infected cell were determined by fluorescence microscopy as described in detail in *Materials and Methods*.

Results

Infection of macrophages by Francisella

In the studies described herein we have used the *F. tularensis* subspecies *holarctica* vaccine strain (*F. tularensis* LVS) for infection of murine macrophages (J774A.1). In contrast to some previous reports, we found that macrophages were quite efficiently (80–90%) infected (Fig. 1*A*). For infection experiments, we have used bacteria grown on chocolate agar plates rather than after a passage in liquid culture. The latter decreases the infection rate of host cells by at least 50% (Fig. 1*A*). Infection of macrophages by agar-derived *Francisella* (80–90%) after 1 h is comparable to rates of infection if *Francisella* is passaged through macrophages first and then used for infection (Fig. 1*A*). The changes *Francisella* might undergo during growth on agar plates and after a passage in macrophages are not understood and are being investigated, but our results agree with previously reported findings for macrophage-derived *Francisella* (22). Infection of macrophages by *F. tularensis* LVS expressing GFP was measured by fluorescence microscopy. This avoided using detergents such as Triton X-100, which are commonly used to selectively lyse eukaryotic host cells to determine CFUs and which affect the viability of *Francisella* by 2–3 logs (Fig. 1*B*). *N*-octyl-β-glucopyranoside (0.5%) and digitonin (1%) were found not to affect the viability of *Francisella* after a 10-min exposure (Fig. 1*B*). Rates of infectivity obtained by fluorescence microscopy agreed with findings from plating for intracellelular bacteria for CFUs when host cells were lysed with 0.5% *N*-octyl-β-glucopyranoside (data not shown). No gentamicin was used to kill extracellular bacteria for these entry assays, because even low concentrations of gentamicin (5 μg/ml) were detrimental to *Francisella* and lower CFU counts by 1–2 logs (data not shown). Our studies have used non-heat-inactivated serum, which has been shown to lead to a more efficient uptake (7, 10).

Actin and microtubules are required for entry of Francisella

Cytochalasin D prevents actin filament polymerization, whereas nocodazole exerts its effect on microtubule polymerization. We determined the infection of murine macrophages (J774A.1) by *F. tularensis* subspecies *holarctica* vaccine strain (*F. tularensis* LVS) after pretreatment of host cells with various concentrations of the respective agent. The percentage of macrophages and the number of intracellular *Francisella* expressing GFP were measured by fluorescence microscopy. Consistent with previous reports (23–24), we found that cytochalasin D and nocodazole can block uptake of *Francisella* by macrophages (Fig. 2, *A* and *B*). This confirms that microtubules and actin cytoskeleton play a role during entry.

PI3K and tyrosine kinases do not play a role in the entry of Francisella *into macrophages*

PI3K has been implicated in the uptake process of a variety of bacteria. We tested the effect of Wortmannin, a specific inhibitor of PI3K (25). Wortmannin irreversibly inhibits the catalytic subunit of PI3K at concentrations up to 100 nM (26). At this concentration it affects late steps of macrophagocytosis and phagocytois, presumably by preventing proper membrane closure and maturation of the phagosome, but has little effect on receptor-mediated endocytosis (27). Inhibtion of PI3K affects entry of *Francisella* entry into macrophages only slightly (Fig. 2*C*). Viability of the host cell was not significantly affected as determined by LDH release assays and trypan blue exclusion at concentrations up to 1 μM (data not shown). Genistein specifically inhibits tyrosine kinases (28). Pretreatment of cells with this inhibitor led to a significant decrease of infection by 40–50% at levels that are nontoxic to the host cell (Fig. 2*D*).

These findings demonstrate that PI3K does not play a major role in the uptake process of *Francisella* by macrophages, but they implicate a role for tyrosine kinases. Our experiments cannot address the possibility that these pathways are induced by bacterial entry and may govern downstream events.

Cholesterol is required for entry of Francisella *into macrophages*

Entry through cholesterol-enriched membrane-domains ("lipid rafts") into host cells has been demonstrated for several intracellular pathogens (11). MβCD preferentially depletes plasma membrane cholesterol (29) by forming soluble inclusion complexes

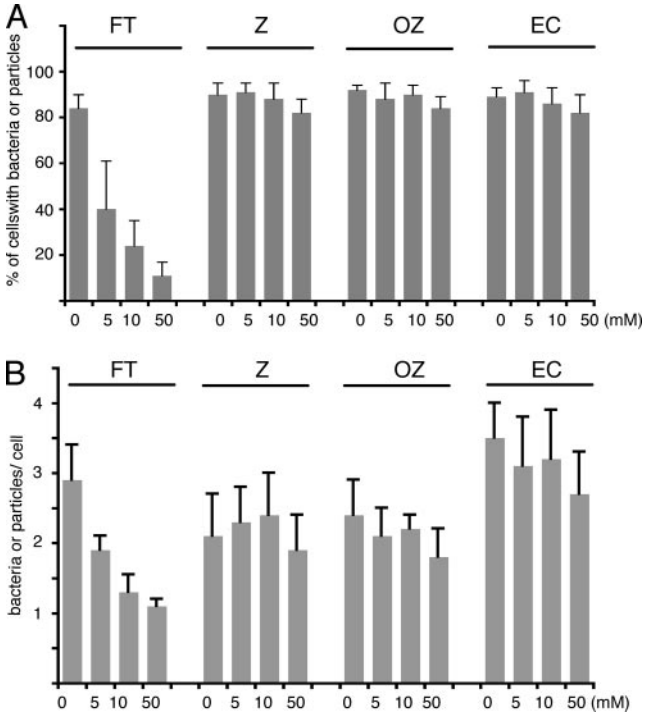

FIGURE 3. Depletion of plasma membrane cholesterol prevents entry of *Francisella* into macrophages (J774A.1). Cells were treated with various concentrations (0, 5, 10, and 50 mM) of MβCD for 60 min. MβCD was removed by washing cells in PBS. Macrophages were infected with *Francisella* (FT) for 1 h or exposed to fluorescent (Alexa 488) zymosan A particles (Z), opsonized fluorescent (Alexa 488) zymosan A particles (OZ), or fluorescent (Alexa 488) fixed *E. coli* (EC) for 1 h. Percentage of cells with intracellular bacteria or particles (*A*) and number of intracellular bacteria or particles per cell (*B*) were determined by fluorescence microscopy as described in detail in *Materials and Methods*.

(30). MβCD removes 70–86% of raft cholesterol and 44–47% of non-raft cholesterol (31). To test whether cholesterol is required for *Francisella* to enter into host cells, macrophages (J774.1) were treated with MβCD at varying concentrations and then infected with *Francisella*. Decrease of membrane cholesterol was verified by staining with filipin III (data not shown). Whereas 90% of macrophages take up *Francisella* after 1 h, only 10% of MβCD-treated cells are infected with a concomitant decrease in the number of intracellular bacteria when a maximum concentration of 50 mM of MβCD was used (Fig. 3). At a concentration of 50 mM of MβCD we could observe significant toxicity to the host cells, which was not observed at a concentration of 10 mM. To exclude nonspecific phagocytosis defects due to the treatment with MβCD, we measured uptake of fluorescent zymosan A (*S. cerevisiae*) particles, opsonized zymosan particles, and fixed fluorescent *E. coli* (K-12). Uptake of these particles was not impaired by cholesterol depletion (Fig. 3). Only at the toxic MβCD concentration of 50 mM was a slight decrease in the number of particles per cell observed (Fig. 3*B*).

In an in vivo observation experiment (*x-y-z-t* microscopy), *Francisella,* which constitutively expressed GFP, attached to the cell surface of cholesterol-depleted cells, but did not enter them (Fig. 4*A*). In contrast, *Francisella* entered efficiently into macrophages with intact cholesterol-rich lipid rafts (Fig. 4*A*). Of interest is also the finding that we could find up to three bacteria in a single vesicle after cell entry (Fig. 4*A*, "control", which depicts two bacteria in one vesicle after 60 min (white arrows)). The inefficient entry of *Francisella* into macrophages whose membranes were depleted of cholesterol was further confirmed by in vivo observations of 10 separate events for each 10-min time point during a 1-h infection (Fig. 4*B*).

Francisella *colocalizes with lipid rafts*

Filipin III is a cholesterol-binding agent, which displays blue fluorescence after intercalation into the cell membrane (32). It also

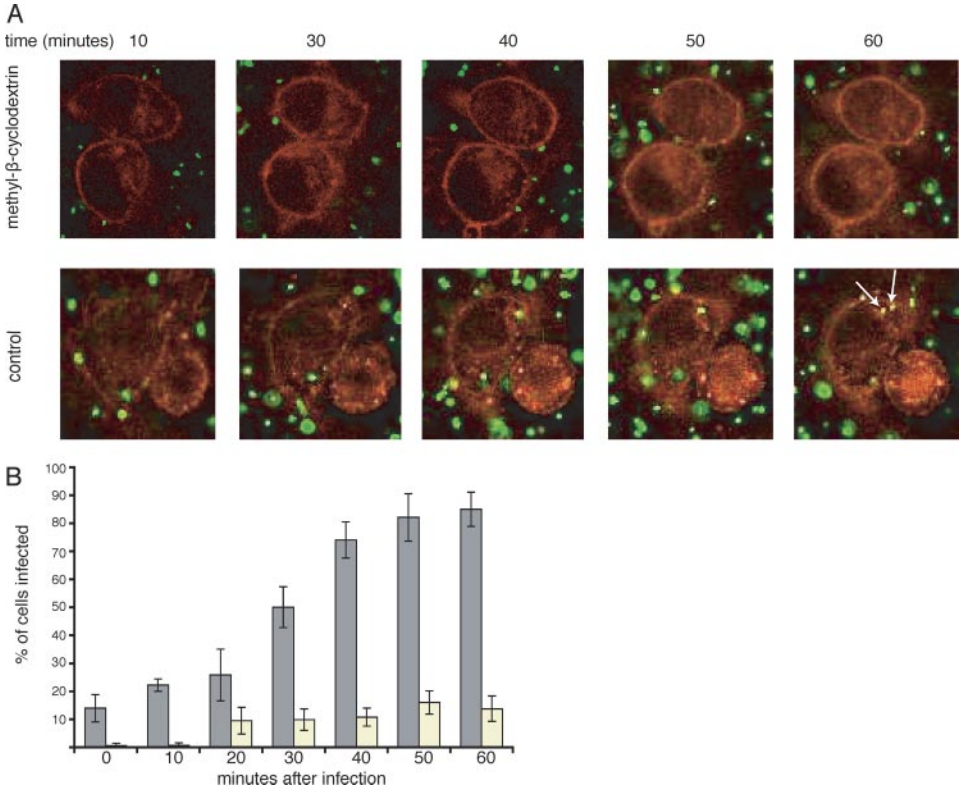

FIGURE 4. Cholesterol content of the host cell membrane determines infection with *Francisella*. *A*, Macrophages were treated with MβCD (10 mM) for 60 min or left untreated (control). Membranes were visualized with FM-4–64 (red fluorescence). Infection was conducted with *Francisella* constitutively expressing GFP in CO_2-independent medium in glass-bottom petri dishes on a heated microscopy stage at 37°C. Forty Z-stacks (*x-y-z*) were acquired for each fluorescence channel over time (t_i). Representative deconvolved images (single *x-y* optical plane) for each time point are shown. *B*, Observations as described in *A* were quantified for 10 independent events for each time point and expressed as the percentage of macrophages infected. Intracellular location of bacteria was verified by analysis of deconvolved stacks in the presence of counterstaining of membranes and endocytic vesicles with FM-4–64 (not shown).

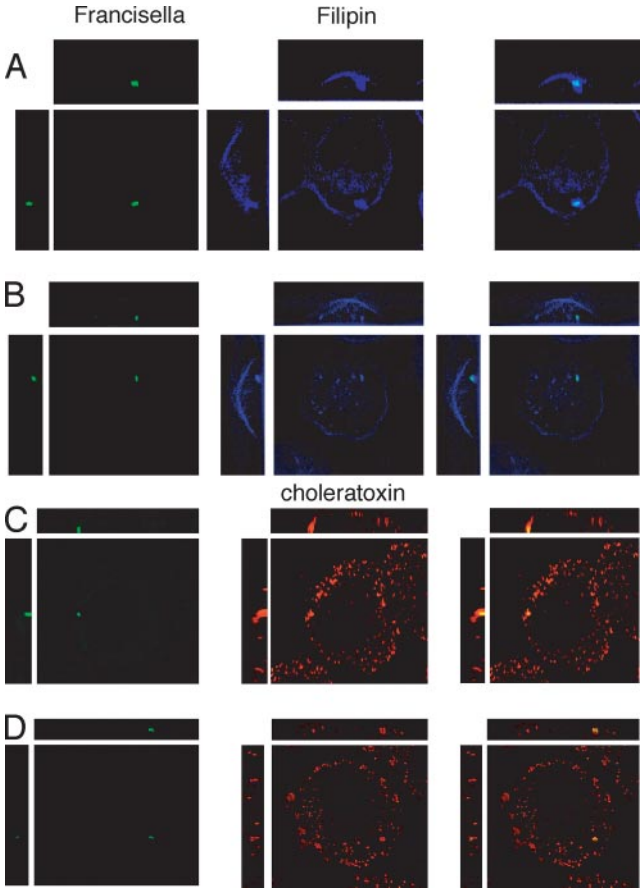

FIGURE 5. *Francisella* associates with cholesterol-dense membrane domains. Macrophages (J774A.1) were pulsed with filipin III (blue fluorescence) while cells were infected with *Francisella* LVS constitutively expressing GFP (green fluorescence). Infected cells were maintained on glass-bottom petri dishes on a heated microscopy stage at 37°C. Images were continuously acquired from various fields as 30 Z-stacks (*x-y* at 0.2-μm intervals). Representative deconvolved images are shown 14 min after start of infection (*A*) and 25 min after infection (*B*). Representative single *x-y* plane is shown with *x-z* plane above and *y-z* plane at the left for each fluorescence channel. For detection of cholesterol rafts by binding of cholera toxin, macrophages (J774A.1) were maintained on coverslips and infected with *Francisella* LVS constitutively expressing GFP. After 15 (*C*) and 30 min (*D*), coverslips were processed for immunostaining with Alexa 594-conjugated choleratoxin (red fluorescence). Representative single optical planes from deconvolved images are shown. Representative single *x-y* plane is shown with *x-z* plane above and *y-z* plane at the left for each fluorescence channel.

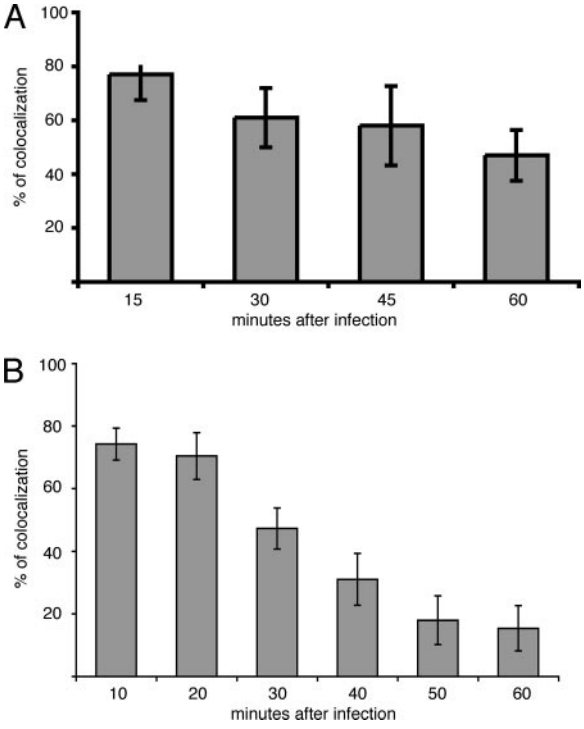

FIGURE 6. Association of lipid rafts and *Francisella* declines as infection progresses. Macrophages (J774A.1) were infected with *Francisella* constitutively expressing GFP. Lipid rafts were visualized with filipin III (*A*) or by staining with choleratoxin (*B*) as described for Fig. 5. One hundred images from each infection experiment were analyzed. Colocalization of green fluorescence channel (*Francisella* LVS) and blue fluorescence channel (filipin III) or red fluorescence channel (Alexa 594-conjugated choleratoxin) was assessed using the Improvision Volocity colocalization module.

interferes with lipid rafts. To circumvent this interference and to be able to use filipin III for visualization of cholesterol-rich membrane domains, we pulsed macrophages with filipin III while at the same time infecting them with *Francisella* expressing GFP. At early time points, during interaction of the bacterium with the host membrane, there is extensive colocalization of cholesterol-rich domains (intercalating filipin III) with *Francisella* (Fig. 5*A*). Cholesterol-rich domains are maintained in vesicles surrounding *Francisella* as it traffics away from the membrane (Fig. 5*B*).

Cholera toxin subunit B binds GM1 gangliosides clustered within lipid rafts and triggers caveolae-mediated internalization of the toxin (33). This depletes caveolae from the surface. Choleratoxin can therefore be used as a specific marker for cholesterol-rich lipid rafts or as an agent to interfere with rafts. Treatment of macrophages with cholera toxin (at a concentration of 10 μg/ml) concomitantly with ongoing infection with *Francisella* prevented entry of bacteria, while not affecting the uptake of fluorescent zymosan A or fixed *E. coli* particles (data not shown). Because binding of cholera toxin leads to a disruption of lipid rafts, this finding further supports the idea that intact rafts are required for entry of *Francisella* into macrophages. When macrophages were infected with *Francisella* and then stained with cholera toxin conjugated to Alexa 594 (red fluorescence), there was a significant colocalization of lipid raft-containing membrane domains with *Francisella* (Fig. 5, *C* and *D*). This association occurred during time of membrane contact (Fig. 5*C*) and was maintained during early vesicle trafficking (Fig. 5*D*).

We sought to further quantify the degree of association of *Francisella* with lipid rafts over time. At early time points nearly all of the intracellular or membrane-attached bacteria colocalized with cholesterol-enriched membranes, which were measured by staining with filipin III (Fig. 6*A*) or by reaction with cholera toxin Fig. 6*B*). As the time of infection progresses and the vacuole matures or disintegrates with ensuing escape of *Francisella* into the cytosol, the colocalization with lipid rafts diminishes (Fig. 6). Kinetics and degree of association of choleratoxin-labeled lipid domains or membrane domains visualized with filipin III and bacteria are remarkably similar.

The cholesterol-enriched vesicle is also tightly attached with actin fibers (Fig. 7). This resembles more a cuplike formation rather than displaying significant rearrangements such as pod or loop formation. Examination of a series of *Francisella* entry events failed to reveal pedestal-like membrane formations as can, for example, be observed during entry of *Salmonella* (34).

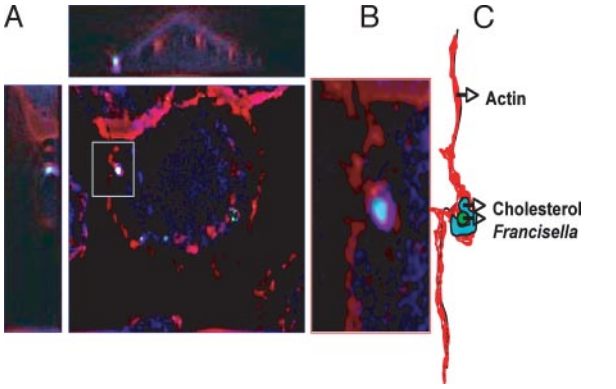

FIGURE 7. Cholesterol-enriched membrane domains and actin filaments engulf the *Francisella*-containing vesicle at the entry site. *A*, Macrophages were infected with *Francisella* expressing GFP for 10 min. Actin was visualized by staining with phalloidin conjugated to Alexa 594 (red fluorescence). Cholesterol was identified by intercalation of filipin III (blue fluorescence). A single deconvolved optical plane with overlay of fluorescence channels is shown. *B*, Magnification of insert in *A*. *C*, Schematic drawing of topology was obtained from intensity plots.

Caveolin colocalizes with Francisella *entry sites*

Caveolin-1 is expressed and associated with lipid rafts in mouse macrophages (31, 35). Caveolae demarcate cholesterol and sphingolipid-rich microdomains of the host cell plasma membrane (36). Agents that deplete plasma membrane cholesterol, such as MβCD, also lead to a disruption of caveolae. We therefore sought to directly address the role of caveolin during *Francisella* entry into macrophages.

Macrophages were infected with *Francisella* expressing GFP and stained with mAbs for caveolin-1. This clearly demonstrated a close association of the *Francisella*-containing vesicles with caveolin-1 (Fig. 8*A*). When we quantified the colocalization of *Francisella* and caveolin, we found dynamics very similar to those of filipin and choleratoxin (75–85% colocalization after 20 min of infection, 30–40% after 60 min of infection; data not shown). In this three-dimensional rendering of a cell infected with multiple bacteria (Fig. 8*A*), one can see the different stages of *Francisella*'s intracellular trafficking during which caveolin is in very close proximity to the bacterium, presumably as part of the *Francisella*-enclosing vesicle. To delineate possible recruitment of caveolin to the *Francisella* entry site, we infected macrophages that expressed a Cav1-GFP fusion product (19) with bacteria stained with Syto62 (red fluorescence). This demonstrated the close proximity of *Francisella* at the plasma membrane with Cav1-GFP in an almost cup-like formation (Fig. 8*B*). These observations suggest that *Francisella* uses lipid raft caveolae for entry into macrophages and maintains decoration of the bacterium-containing vesicle with caveolin during early trafficking events.

Entry of Francisella *through lipid raft is required for intracellular survival*

The previous findings demonstrated a close association of lipid rafts and *Francisella* and a requirement of cholesterol-rich domains for successful entry into macrophages. We sought to determine whether depletion of host cell membrane cholesterol also affected *Francisella* intracellular survival. Disruption of lipid rafts by MβCD still led to entry of some *Francisella*, either through cholesterol and raft-depleted vesicles or other alternative pathways (Figs. 3 and 9). There may also be entry of *Francisella* as the host cell replenishes membrane cholesterol from intracellular pools and resynthesis. If the entry pathway has no bearing on intracellular survival, then one would predict an intracellular proliferation rate of the few bacteria able to enter that is similar to the growth rate after entry in the presence of intact lipid rafts. However, *Francisella* that enter in the presence of depleted host cell membrane cholesterol are unable to proliferate inside macrophages (Fig. 9*A*). Because cholesterol stores are replenished after withdrawal of the raft-disrupting agent, the very little growth observed could represent the growth of bacteria, which might have been released after host cell death or remained attached after washing and which can now enter at a later time point as cholesterol is replenished.

We used *x-y-z-t* fluorescence microscopy of a single macrophage infected with *Francisella* LVS expressing GFP. The number of intracellular bacteria increased more than 8-fold in macrophages not treated with MβCD over a 14-h observation period (Fig. 9*B*). After 14 h there was increased cell damage, presumably due to phototoxicity. When macrophages were treated with MβCD, the

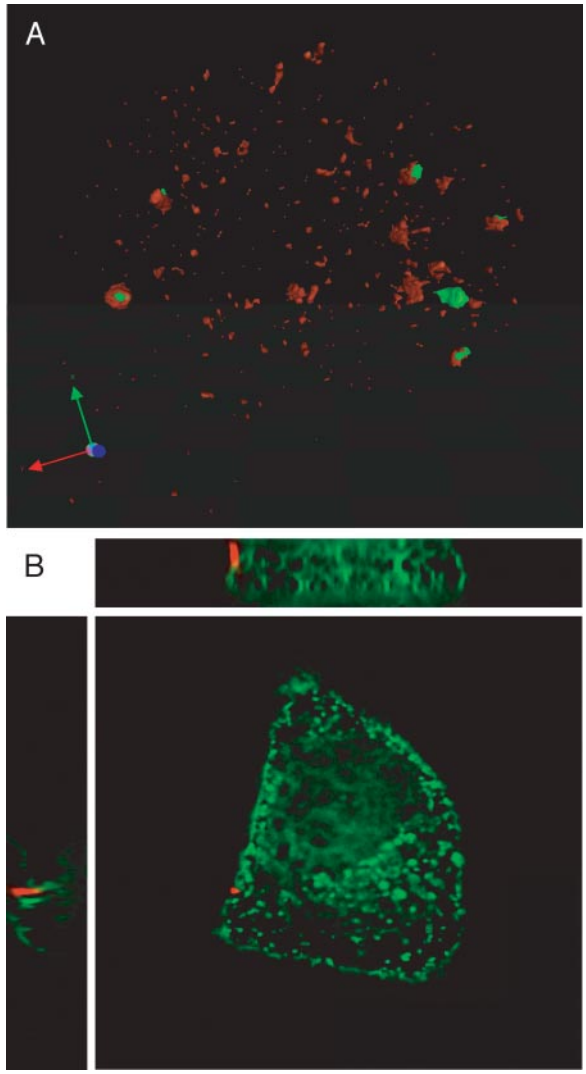

FIGURE 8. Caveolin-1 delineates the membrane attachment site for *Francisella* and is retained during early vesicle trafficking. *A*, Macrophages (RAW264.7) were infected for 30 min with *Francisella* LVS expressing GFP. Caveolin-1 was visualized with Cav-1α- and Alexa 594- (red fluorescence) conjugated secondary Abs. Several optical planes from an infection experiment were rendered in a three-dimensional projection using Improvision Volocity software. *B*, Macrophages (RAW264.7) that express a Cav1-GFP fusion protein were infected with *Francisella* LVS stained with Syto62 (red fluorescence) as in *A*. A single representative optical plane is shown.

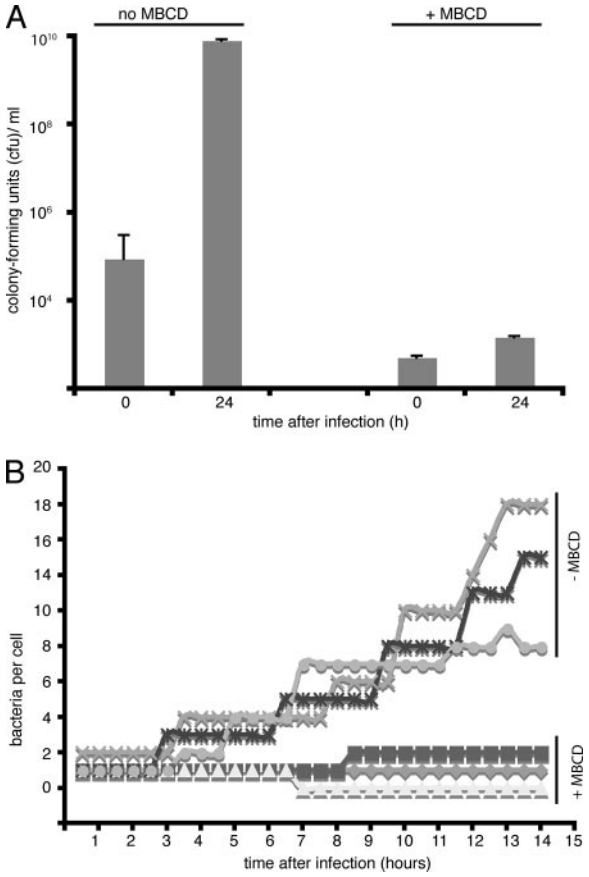

FIGURE 9. Entry of *Francisella* into macrophages through cholesterol-rich membrane domains is required for successful intracellular proliferation. *A*, Macrophages were treated with MβCD (10 mM) for 60 min or were left untreated. Cells were infected with *Francisella*. After 1 (0) and 24 h (24) of infection, the number of intracellular bacteria was determined by plating for CFUs after lysis of macrophages. *B*, Macrophages were maintained in CO_2-independent medium in glass-bottom petri dishes on a heated microscopy stage at 37°C. After treatment with MβCD (10 mM) for 60 min (+MBCD) or no treatment (−MBCD), cells were infected with *Francisella* expressing GFP. Forty Z-stacks (*x-y-z*) were acquired for each fluorescence channel over time (t_i) at 30-min intervals. The number of intracellular bacteria per single macrophage was determined after deconvolution of images and analysis using Volocity Improvision software.

number of intracellular bacteria in a single macrophage did not increase or even went to 0 (Fig. 9*B*).

These findings strongly suggest that entry of *Francisella* through lipid rafts is necessary for successful intracellular proliferation.

GPI-anchored protein are required for Francisella *intracellular survival*

The close association of *Francisella*-containing vesicles with lipid rafts and their associated components suggests that *Francisella*'s intracellular fate is modulated by lipid raft-associated signaling events. This can in part be tested by selectively removing lipid raft-associated GPI-anchored proteins with PI-PLC (37). Treatment of macrophages with PI-PLC (0.5 U/ml) significantly decreased the percentage of cells infected with *Francisella* LVS without affecting the uptake of zymosan A, opsonized zymosan A, or fixed *E. coli* particles (Fig. 10*A*).

Macrophages were also pretreated with various concentrations of PI-PLC and then infected with *Francisella*. After 1 h of infection time, there was a significant decrease of intracellular *Francisella* (Fig. 10*B*). At a concentration of 0.05 U/ml, there is a minor decrease of *Francisella* entering the host cell. However, those bacteria entering were apparently unable to proliferate. As the concentration of PI-PLC is increased, the ability of *Francisella* to proliferate intracellularly is further decreased by almost 2 logs (Fig. 10*B*). Further raising pretreatment with PI-PLC to 0.5 U/ml, which is still four times lower than the concentration used for a similar experiments with *Brucella* (38), completely abolishes *Francisella* entry and intracellular survival. This supports the notion that successful entry and intracellular proliferation by *Francisella* requires lipid raft-mediated signaling through GPI-anchored proteins.

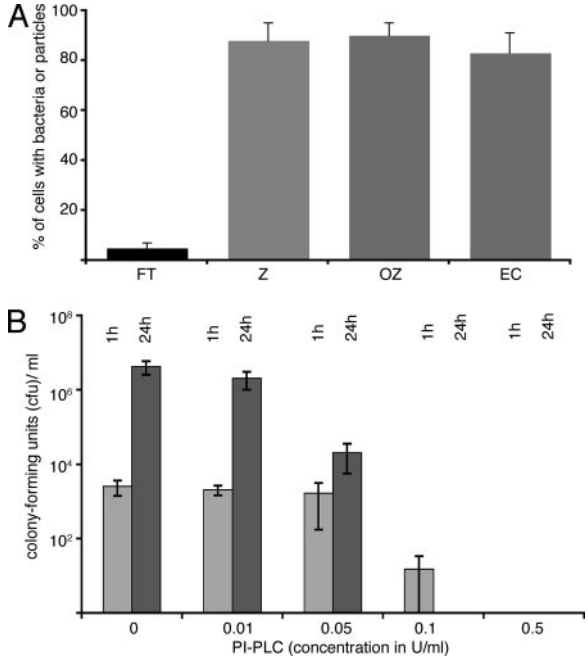

FIGURE 10. Removal of GPI-anchored proteins inhibits entry of *Francisella* into macrophages. *A*, Host cells (J774A.1) were treated with PI-PLC for 1 h at a final concentration of 0.5 U/ml. After removal of PI-PLC, cells were infected with *Francisella* expressing GFP for 1 h or exposed to fluorescent (Alexa 488) zymosan A particles (Z), opsonized fluorescent (Alexa 488) zymosan A particles (OZ), or fluorescent (Alexa 488) fixed *E. coli* (EC) for 1 h. Percentage of cells with bacteria or particles was determined by fluorescence microscopy. *B*, Host cells (J774A.1) were treated with PI-PLC at final concentrations of 0.5 U/ml as indicated. After removal of PI-PLC, cells were infected with *Francisella*. Intracellular bacteria were determined by plating for CFUs after lysis of macrophages.

Discussion

Our studies herein demonstrate that *F. tularensis* subspecies *holarctica* vaccine strain (*F. tularensis* LVS) recruits cholesterol-rich lipid domains (lipid rafts) with caveolin-1 at the host cell membrane for successful entry into macrophages. Interference with these plasma membrane microdomains of the host cell impairs *Francisella*'s intracellular proliferation. Lipid raft-associated components were incorporated into *Francisella*-containing vesicles during entry and the initial phase of intracellular infection of host cells.

Our studies found a good efficiency of entry of *Francisella* into macrophages (80–90%) at a high MOI. Methodological differences, such as avoiding detergents and antibiotics toxic to *Francisella*, use of non-heat-inactivated serum, pregrowth of bacteria on IsoVitaleX-enriched chocolate agar rather than in liquid medium, and assessment of host cell infection by microscopy rather

than by colony count, may have contributed to these findings. Other studies, which have found less efficient entry into macrophages by the *F. tularensis* subspecies *holarctica* vaccine strain (8, 9), also did not use the particular army lot 11 strain used herein, or the derivation of the strain was not detailed (39). Metabolic studies of various lots of the *Francisella* vaccine strain (Ref. (40) and S. Daefler, unpublished observation) revealed significant differences within these strains. This suggests that there might be more heterogeneity than generally assumed and could explain a higher efficiency of infection of the particular strain used here. Nevertheless, the high efficiency of entry into macrophages corresponds with findings from some studies in which non-heat-inactivated serum was used (10) or where *Francisella* was passaged through macrophages before being used in infection assays (22). We propose that the efficiency of cell entry by *Francisella* into macrophages might have been underestimated and that it might depend on minor strain variations and culture conditions, which are ill understood.

PI3K plays a role for the entry process of many bacteria (e.g., (41–44) and viruses (45). PI3K activity effects changes in the cytoskeleton and affects actin polymerization, which is a key element in uptake of bacteria into host cells (41, 44). It has also been shown to play a role in *Francisella* entry (this study and Refs. (6, 23)). In some instances of bacterial uptake, PI3K activity is not required for entry, but the kinase is activated and leads to signaling events that are essential for successful intracellular survival of the invading bacterium or proper closure of the phagosome (27, 46, 47). Infection of macrophages by *F. novicida* leads to an activation of PI3K (48). We show herein that PI3K does not appear to play a role for entry of *Francisella* LVS into macrophages. We cannot rule out that an activation of this kinase is required for entry into other cell types by endocytic processes or that PI3K activation is essential for creation of a protected intracellular environment during infection.

Cholesterol-sphingolipid-enriched microdomains of the plasma membrane (rafts) have emerged as important regulators during infection of host cells by bacteria (11, 49). Lipid raft-associated cell signaling is thought to be involved in important pathways regulating the response of immune cells (50). It has therefore been argued that entry processes through lipid raft compartments allow the incoming bacterium to avoid the phagolysosomal pathway (51). These rafts are thought to concentrate signaling molecules and act as a platform for linking the entry process of the bacterium at the cell membrane to cytoskeleton and intracellular signaling pathways (52). Many examples have now been described in which bacteria such as *Mycobacterium bovis* BCG (53), *Chlamydiae* (54), FimH-expessing *E. coli* (55), or *Brucella* (38), as well as viruses (56), use lipid rafts to subvert ensuing host cell responses to their advantage.

In our studies interference with lipid rafts through the depletion of plasma membrane cholesterol, through induction of raft internalization with choleratoxin, or through removal of raft-associated GPI-anchored proteins by treatment with PI-PLC, significantly inhibited entry of *Francisella*. Visualization of cholesterol-rich membrane domains by microscopy showed a close association of incoming *Francisella* with these domains. As the *Francisella*-containing vesicle traffics from the cell membrane into the cytoplasm, the vesicle retains cholesterol-rich membrane components. We also demonstrated that entry of *Francisella* through lipid rafts is essential for its intracellular proliferation.

Operationally defined lipid rafts serve as an anchoring point for a wide variety of membrane proteins in phagocytic and nonphagocytic cells (31, 50, 52), some of which have been described as possible receptors for entry of *Francisella* into macrophages. The aim of this study was to characterize functional domains of the host cell membrane necessary for the entry of *Francisella* rather than characterizing receptor-bacterium interactions. Membrane receptors described to date might serve as a tethering device for the attaching bacterium or directly mediate translocation and entry of *Francisella*. SRA has previously been implicated as an important receptor for entry of *Francisella* (10). Although SRA and lipid rafts may not colocalize, rafts are necessary for phagocytosis of *E. coli* (57). In contrast, *Brucella abortus* actively induces the association of lipid rafts and SRA (58); intact lipid rafts are a prerequisite for *Brucella* phagocytosis in this setting. complement receptor 3 and Fc receptors, which have been implicated to play an important role during *Francisella* phagocytosis (7, 8), are also associated with lipid rafts (59, 60). Localization of the mannose receptor to lipid rafts and its requirement of cholesterol for signal transduction have not been investigated.

Uptake of bacteria via lipid rafts and incorporation of cholesterol-rich domains into the evolving bacterium-containing vesicle is thought to allow the activation of specific host-cell signaling pathways, which eventually allow he bacterium to establish itself in a membrane-enclosed protected niche inside the cell. However, it is now clear that *Francisella* escapes from the phagosome at an early time point after uptake (22, 61). Interestingly, *Listeria* requires lipid rafts for efficient cell entry (62); it then secretes listeriolysin O, which binds to cholesterol-enriched membrane domains to form a pore and lyse the surrounding vacuole (63). It is thus tempting to speculate that *Francisella* might use a similar mechanism to escape from its phagosome into the cytosol, even though we cannot identify a homolog or paralog to the class of cholesterol-binding cytolysins such as listeriolysin in any of the published *Francisella* genomes.

Francisella has been shown to traffic to autophagosomal structure after uptake (64). There is some evidence that such a transfer may be substantially facilitated by a previous enclosure in a cholesterol-rich vesicle (65).

During entry and early trafficking we also detected a close association of *Francisella* with caveolin-1, which delineates a subset of lipid rafts. Caveolae as specialized lipid raft domains, which are transformed into cuplike structure through the multimerization of the hairpinlike structure of caveolin, have been implicated in endocytic processes in phagocytic and nonphagocytic cells. It has also become clear that caveolin-1 is a necessary component for phagocytosis of parasites and bacteria as a first line of defense (66, 67). It is not clear from the current state of research whether caveolin mediates phagocytosis or whether it is involved in supportive functions, such as membrane recruitment or signal transduction. Our studies do not allow us to conclude that entry is mediated by caveolin, because lipid rafts are sensitive to filipin III and MβCD regardless of whether they contain caveolin. The demonstration of the close association with caveolin during entry and subsequent early trafficking strongly suggests a caveolin-associated process, which could also be coincidental.

From our investigations presented herein we propose that *Francisella* employs cholesterol-enriched caveolin-1-carrying membrane domains for entry into macrophages. This route of entry is essential for its intracellular survival. The recruitment of such lipid rafts to the early *Francisella*-containing membrane enclosure might benefit *Francisella* by at least three possible mechanisms, which remain to be explored. Cholesterol-rich membrane domains might serve as a platform for the induction of yet-to-be-characterized signaling pathways. After entry, lipid rafts might provide a target for a cholesterol-binding toxin produced by *Francisella* to lyse its surrounding membrane structure. At the same time such

membrane microdomains might trigger trafficking of *Francisella* into autophagosomal structures.

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Disclosures

The authors have no financial conflicts of interest.

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