Title

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Uptake and Intracellular Fate of *Francisella tularensis* in human macrophages

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Abstract

F. tularensis is an intracellular pathogen that survives and multiplies within host mononuclear phagocytes. We have shown that uptake of the bacterium by human macrophages occurs by a novel process, "looping phagocytosis", in which the bacterium is engulfed in a spacious, asymmetric, pseudopod loop. Whereas looping phagocytosis is resistant to treatment of the *F. tularensis* with formalin, proteases, or heat, the process is abolished by oxidation of the bacterial carbohydrates with periodate, suggesting a role for preformed surface carbohydrate molecules in triggering looping phagocytosis. Following uptake, *F. tularensis* initially resides in a spacious vacuole at the periphery of the cell, but this vacuole rapidly shrinks in size. The nascent *F. tularensis* vacuole transiently acquires early endosomal markers, but subsequently exhibits an arrested maturation, manifest by only limited amounts of lysosome-associated membrane glycoproteins (consistent with limited interaction with late endosomes), non-fusion with lysosomes, and minimal acidification. In ultrastructural studies, we have observed that the *F. tularensis* phagosome displays a novel feature in that many of the phagosomes acquire an electron dense fibrillar coat. This fibrillar coat forms blebs and vesicles, and with time, is seen to be fragmented and disrupted. With increasing time after infection, increasing numbers of the *F. tularensis* are found free in the macrophage cytoplasm, such that by 14 hours after infection, less than 15% of the bacteria are surrounded by any discernible phagosomal membrane. Further research is needed to determine the mechanisms underlying looping phagocytosis, and the maturational arrest, fibrillar coat formation, and disruption of the phagosome.

Introduction

F. tularensis is a facultative intracellular bacterial pathogen that is divided into two main biogroups: subspecies *tularensis* (Type A) and subspecies *holarctica* (Type B). Type A is found only in North America and is highly virulent. Type B is found both in North America and in Europe and is of a lower virulence. A partially attenuated "live vaccine strain" (LVS) was developed from Type B and has been used as a vaccine with some success.[1] *F. tularensis* has a relatively broad host-range and is capable of multiplying intracellularly in insects as well as in a broad range of mammals, including rabbits, rodents, beavers, and man. For mammals, *F. tularensis* is the most infectious bacterial pathogen known; the LD_{50} in mice for a subcutaneous dose of the virulent Schu strain is 1 to 4 organisms.[2] In humans, as few as 10 organisms delivered subcutaneously or 25 organisms delivered by inhalation can lead to life-threatening infection.[3, 4] Because of its high infectivity and its capacity to cause serious and potentially life-threatening disease in man, *F. tularensis* is classified as a category A potential agent of bioterrorism.[5] Concerns over its use as a biological weapon have engendered a renewed interest in the cell biology of *F. tularensis*. Nevertheless, the pathogenic mechanisms that underlie its remarkable infectivity and its capacity to cause disease in a broad range of hosts are not understood.

Macrophages as host cells for *F. tularensis*

While recent studies have shown that other cell types, such as type II alveolar epithelial cells[6] and neutrophils[7], can serve as host cells for *F. tularensis*, it is clear that mononuclear phagocytes are an important host cell for *F. tularensis*. It is well established that *F. tularensis* infects host mononuclear phagocytes during natural infections. Early

studies of infected guinea pigs[8] and chick embryos[9] documented extensive infection of mononuclear phagocytes as well as other cell types. In an immunofluorescent histopathologic study of monkeys infected with the virulent Schu strain, the bacterium was described within macrophages of respiratory bronchioles.[10] In a study using the attenuated LVS strain of *F. tularensis*, the bacterium was also found within mononuclear phagocytes.[11] Mononuclear phagocytes are important both as a site of bacterial replication and as a site of host defense against *F. tularensis*. Accordingly, in our research on the cell biology of *F. tularensis*, we have chosen to study human cells because of their clear relevance to human disease. We have focused on *F. tularensis* interaction with macrophages, and we have examined the interaction of *F. tularensis* with human peripheral blood monocytes, monocyte-derived macrophages (MDM), and a human monocyte-like cell line, THP-1.

Uptake of *F. tularensis* by macrophages: Ultrastructure and Receptor Mechanisms

The remarkably high infectivity of *F. tularensis* suggests that the bacterium possesses a mechanism for efficient invasion of host cells. However, the ultrastructural morphology and receptor mechanisms that mediate the uptake of *F. tularensis* by host cells have only recently begun to be elucidated.

Ultrastructure of the uptake process

Defining the ultrastructure of the uptake process is an important step in understanding the mechanism underlying entry of a pathogen into a host cell. The ultrastructure may

suggest potential uptake mechanisms, and any model of the uptake process should account for the observed uptake morphology.

We have examined the interaction of human macrophages with two strains of *F. tularensis*: the attenuated type B LVS strain and a recent clinical isolate (RCI) of the highly virulent type A strain. To examine the uptake process, we mixed the bacteria and macrophages (either in suspension or monolayer culture) at 4°C, centrifuged the sample to enhance the contact between the bacteria and macrophages, warmed the cells to 37°C for 3 – 5 min to permit phagocytosis, and fixed them for transmission electron microscopy (TEM) ultrastructural analysis.

For both the LVS and the RCI strains, and both for MDM and THP-1 cells, the uptake profiles that we observe are highly unique.[12] The majority of the *F. tularensis* bacteria are engulfed by the macrophages within asymmetric, exuberant pseudopod loops (Fig. 1A). Fusion of the pseudopod loop with the plasma membrane results in enclosure of the *F. tularensis* bacteria within spacious vacuoles at the surface of the macrophage (Fig. 1B). Within seconds to minutes, the vacuole shrinks dramatically in size as it moves towards the center of the host cell (Fig. 1C). This rapid and dramatic shrinkage in size of the *F. tularensis* vacuole indicates that the spaciousness of the pseudopod loop is not attributable to a large, invisible capsule surrounding *F. tularensis*. Moreover, we have excluded the possibility that large capsules or pili might account for the spaciousness of the pseudopod loops by examining negatively-stained preparations of *F. tularensis* that we employ in our infection experiments. We have found that the dimensions of the

negatively stained bacteria are comparable to the dimensions of the bacteria observed in the thin sections of our TEM uptake profiles and that the bacteria used in our studies, which are agar plate grown, do not have pili that can account for the spaciousness of the loops. Long, thin type IV pili have been reported for *F. tularensis* grown in liquid culture,[13] but not for agar plate-grown *F. tularensis*. We do observe that a small percentage of our agar plate-grown bacteria have sex pili, but the relatively low percentage of bacteria (less than 25%) with these structures and their arrangement on the bacteria cannot account for the spaciousness of the pseudopod loops that we observe uniformly for *F. tularensis*.

Prior to our work with *F. tularensis*, the ultrastructural profiles that have been demonstrated to mediate the uptake of other bacterial pathogens included conventional phagocytosis (exemplified by *M. tuberculosis,*[14] Fig. 2A), coiling phagocytosis (exemplified by *L. pneumophila*,[15] Fig. 2B), and ruffling or triggered macropinocytosis (exemplified by *Salmonella typhimurium* and *Shigella flexneri*, Fig. 2C-D). However, the uptake process that we observe for *F. tularensis*, which we term "looping phagocytosis", differs dramatically from each of these.

Looping vs. Conventional phagocytosis

Conventional phagocytosis, a process that has been demonstrated for a large variety of microorganisms and inert particles, represents a zipper-like process involving sequential interaction between ligands on the surface of the particle and phagocytic receptors leading to engulfment of the particle within tightly fitting pseudopodia that move circumferentially and symmetrically around the particle.[16-18] Obviously, the

ultrastructural morphology of looping phagocytosis of *F. tularensis* is markedly dissimilar to that of conventional phagocytosis in that the former but not the latter involves highly asymmetric pseudopod extension and lacks any close juxtaposition between the pseudopodia and the bacterium.

Looping vs. coiling phagocytosis

Coiling phagocytosis, as exemplified by the uptake of *L. pneumophila*[15] (Fig. 2B), differs from looping phagocytosis of *F. tularensis* in that the former involves uptake within multiple coils of pseudopodia that maintain tight juxtaposition to the bacterium. In contrast, for looping phagocytosis, the pseudopod loops do not maintain close contact with the *F. tularensis* and the loops do not coil repeatedly around the bacterium.

Looping vs. ruffling/triggered macropinocytosis

In the case of ruffling/triggered macropinocytosis[19], bacteria such as *Shigella flexneri* or *Salmonella typhimurium* trigger a rearrangement of the host cell cytoskeleton that results in ruffling of the surface of the eukaryotic cell and macropinocytotic uptake of the bacteria within the ruffles of the host cell. This process allows the bacteria that employ this mechanism to enter eukaryotic cells that lack phagocytic receptors (e.g. epithelial cells). The TEM uptake profiles that we observe for *F. tularensis* uptake by human macrophages differ from those that we have observed under the same conditions for uptake of *S. flexneri* and *S. typhimurium* (Fig. 2C - 2D) in that the *F. tularensis* are engulfed within highly asymmetric, exuberant, spacious loops, whereas the *S. flexneri* and *S. typhimurium* are engulfed within symmetrically arranged, shorter pairs of

pseudopodia. In three dimensions, the triggered macropinocytotic process has been described as a "splash pattern", a pattern that is not consistent with the asymmetric, exuberant spacious pseuodopod loops we observe mediating uptake of *F. tularensis*. The differences that we observe between looping phagocytosis and triggered macropinocytosis are not attributable to different planes of section since in our examination of hundreds of uptake profiles of *S. flexneri* or *S. typhimurium*, we have not observed any profiles that resemble the highly asymmetric and spacious profiles that we observe for *F. tularensis* (Fig. 1A).

Finally, heat-killed *F. tularensis* are ingested by looping phagocytosis; however, heat-killed or antibiotic killed *Salmonellae* are evidently not able to enter cultured epithelial cells which live *Salmonellae* invade by triggered macropinocytosis.[20] Moreover, macropinocytosis is associated with a Type III secretion system, absent from the *F. tularensis* genome.[21]

Uptake of *F. tularensis* requires rearrangement of actin, is sensitive to cytochalasin, and requires Phosphatidyl Inositol 3 Phosphokinase (PI3K) signaling

Conventional phagocytosis of microorganisms requires signal transduction through PI3K.[22] PI3K activation leads to the formation of PI(3,4,5)P3, which in turn recruits proteins involved in rearrangement of the actin cytoskeleton. Accordingly, conventional phagocytosis is sensitive to wortmannin (a potent PI3K inhibitor) and cytochalasin B (an inhibitor of microfilament formation). We have found that the uptake of live and killed *F. tularensis* subsp. *tularensis* of the RCI strain are equally sensitive to inhibition by

wortmannin (Fig. 3), indicating that uptake of these bacteria via looping phagocytosis is dependent upon signal transduction through the host cell PI3K. We have also found that uptake of *F. tularensis* RCI and LVS, whether live or dead, is equally sensitive to cytochalasin B, consistent with a requirement for actin microfilaments in the dramatic pseudopod extensions that mediate looping phagocytosis.

Possible molecular determinants of looping phagocytosis

We have observed that formalin-killed *F. tularensis* are internalized by the same morphologic process of looping phagocytosis as live bacteria. This indicates that preformed molecules on the surface of the *F. tularensis* are capable of triggering the rearrangements of the host cell cytoskeleton that mediate the dramatic pseudopod extensions of looping phagocytosis. We have also observed *F. tularensis* are still internalized by looping phagocytosis even after heat treatment and protease treatment of the bacteria. On the other hand, we have found that oxidation of bacterial carbohydrates by treatment of the *F. tularensis* with periodate followed by cross-linking with lysine abolishes looping phagocytosis and results in uptake of the bacteria almost exclusively by conventional phagocytosis (Fig. 4). This suggests that the bacterial molecules that trigger looping phagocytosis are preformed molecules that are heat stable and protease resistant, but sensitive to periodate oxidation. This makes it likely that they are surface carbohydrates, such as lipopolysaccharide or capsular material. To examine this possibility further, we have examined the uptake morphology of O-antigen mutants of *F. tularensis* and found that these bacteria are engulfed within pseudopod loops that are less spacious and tighter than those of the parental strains. Nevertheless, the uptake of these

bacteria does involve multiple layers of pseudopodia and much more vigorous pseudopod extensions than we observe for other bacteria, indicating that *F. tularensis* possesses molecules other than the LPS O-antigen that are important in triggering the dramatic, asymmetric pseudopod extensions.

Receptor mechanisms underlying the uptake process

At low multiplicities of infection, efficient uptake of *F. tularensis* by human macrophages is critically dependent on the presence of serum in the culture medium or on pre-opsonization of the bacteria with serum. When we began our studies of the cell biology and uptake of *F. tularensis* in human macrophages, we observed remarkably low levels of infection when using culture media supplemented with commercially available AB human sera, but we found very efficient levels of infection when using serum freshly obtained from volunteer blood donors. Furthermore, we found that the adherence and uptake of the bacteria by the macrophages increases in a dose-dependent fashion as the serum concentration in the medium is increased from 1% to 10% (Fig. 5A). Heat-inactivated human serum and heat-inactivated fetal bovine serum did not support efficient uptake of *F. tularensis* by human macrophages. This suggested to us that efficient uptake of *F. tularensis* by macrophages requires an intact complement pathway with sufficient levels of C3 complement. Indeed, when we examined the uptake of *F. tularensis* by macrophages in C3-deficient AB serum, we observed negligible uptake of the bacteria. Addition of C3 to the serum restored efficient uptake of the bacteria in a dose-dependent fashion (Fig. 5B). Furthermore, antibodies to complement receptors CR3 and CR4 inhibited uptake of *F. tularensis* by the macrophages. These results indicate that uptake

of *F. tularensis* by macrophage requires interaction of macrophage complement receptors CR3 and possibly CR4 with complement factor C3 fixed to molecules on the surface of the bacteria.

Our data indicate that complement receptor – complement interactions are of pivotal importance to the uptake of *F. tularensis* by human macrophages. The fact that we observe negligible uptake in C3 deficient serum and that uptake is restored by the replenishment of the serum with C3 indicates that other phagocytic receptors, such as Fc receptors and mannose receptors, cannot take the place of complement receptors in non-immune individuals when the complement pathway is inactive.

Nevertheless, it is possible that the fixation of complement on the surface of the *F. tularensis* might be mediated by natural antibody to the *F. tularensis*. To determine the role of natural antibody in mediating uptake of the bacteria by human macrophages, we examined the capacity of agammaglobulinemic serum to support uptake of *F. tularensis*. We found that human serum totally deficient in gamma globulin supports the uptake of the bacteria with an efficiency nearly indistinguishable from that observed in the presence of normal human serum (Fig. 6). Furthermore, the addition of immunoglobulin to the agammaglobulinemic serum did not further enhance the uptake of *F. tularensis* Schu and only slightly enhanced the uptake of the *F. tularensis* RCI strain (Fig. 6). These results indicate that natural antibody is not necessary for complement fixation on the bacteria or for uptake of the bacteria.

We have also examined the uptake of *F. tularensis* by human cell lines that lack professional phagocytic receptors – HeLa cells (an epithelial cell line) and HepG2 cells (a liver cell line), and found that uptake and adherence of *F. tularensis* by these cells is extremely inefficient. Less than 0.5% of the HepG2 or HeLa cells are infected under conditions that lead to infection of more than 80% of human MDM. Nevertheless, *F. tularensis* has been reported to infect mouse hepatocytes *in vivo* in mouse models of tularemia, suggesting that *F. tularensis* may possess additional mechanisms for invasion of eukaryotic cells that lack phagocytic receptors. Our immortalized cell lines may not adequately reflect normal epithelial cells and hepatocytes, as the cell lines may lose receptors and properties present in the primary cells.

Role of mannose receptors and class A scavenger receptors in mediating adherence and uptake of *F. tularensis*

In the absence of an intact complement pathway, the uptake of *F. tularensis* by macrophages is relatively inefficient. However, additional ligand-receptor interactions may play a role in promoting the adherence and uptake of the bacteria by macrophages. Schulert and Allen[23] have shown that in the absence of complement, mannose receptor (MR) can play a role in mediating uptake of the LVS strain by human monocyte-derived macrophages, and that in the presence of complement, increased expression of MR promotes more efficient uptake of the LVS bacteria. Pierini[24] has examined the pathways involved in uptake of *F. tularensis* by mouse J774 cells and reported a role for the types I and II class A scavenger receptors (SRA), showing that treatment of J774 macrophages with fucoidan and polyinosinic acid inhibited uptake of LVS, and that SRA-/- J774 macrophages ingest significantly fewer LVS than do wild-type J774 cells. Interestingly,

Pierini found that the SRA did not mediate binding or uptake of LVS in the absence of serum or in heat-inactivated serum[24]. Thus, while complement receptor-complement interactions play a dominant role in mediating adherence and uptake of the bacteria by macrophages, the mannose receptor and scavenger receptors may contribute to this process.

Significance of complement-dependent looping phagocytosis of *F. tularensis* and potential mechanisms underlying it

Complement and/or complement receptors have been shown to play a role in the internalization of many other intracellular pathogens, including *L. pneumophila*,[25] *M. tuberculosis*,[14] *M. leprae*,[26, 27] *M. avium*,[28] *Leishmania donovani*,[29] *Leishmania major*,[30] *Listeria monocytogenes*,[31] *Histoplasma capsulatum*,[32] and *Trypansoma cruzi*.[33] Although all of these pathogens are internalized via complement and complement receptors, the ultrastructural process of their internalization and their subsequent intracellular compartments show great variability. For example, whereas *M. tuberculosis* is internalized via "conventional phagocytosis", with the bacterium sinking into the macrophage between tightly juxtaposed pseudopodia, *L. pneumophila* is internalized via coiling phagocytosis, with the bacterium being engulfed within a tightly fitting pseudopod that coils repeatedly around the bacterium. Clearly, there are additional molecular interactions between the bacteria and the macrophage that modulate the uptake process. The morphology of the uptake may depend on factors such as the abundance of complement fixed to the surface of the phagocytic target and the strength of the intracellular signaling events that trigger the macrophage pseudopod extension. In this

model, abundant complement-complement receptor interactions combined with relatively weak intracellular signaling for pseudopod extension would lead to conventional phagocytosis as the pseudopodia are guided by the complement-complement receptor interactions around the circumference of the target. On the other hand, a particularly strong stimulus for pseudopod extension combined with a paucity of complement fixed on the surface of the phagocytic target (or complement fixed to molecules that are easily shed from the bacterium) would lead to the phagocyte pseudopod overshooting its target and maintaining only loose contact with the target, as is the case in looping phagocytosis of *F. tularensis*. According to this model, periodate oxidation combined with lysine cross-linking of the surface carbohydrates of *F. tularensis* might abolish looping phagocytosis and cause exclusively conventional phagocytosis by destroying the *F. tularensis* molecules that trigger vigorous pseudopod extension and possibly also by increasing the amount of complement that can be fixed to the surface of the bacteria and/or by preventing the shedding of the molecules that have covalently bound to complement. Extending this model further, coiling phagocytosis of *L. pneumophila* may represent a case in which the bacterium elicits an extremely strong signal for pseudopod extension in combination with a relatively high number of complement molecules on the surface of the bacterium, thus resulting in a pseudopod extension that "overshoots" the target, but nonetheless retains intimate contact with the target, thereby causing the bacterium to roll multiple times within a pseudopod coil.

Membrane trafficking and intracellular fate of *F. tularensis* in human macrophages

We have used confocal immunofluorescence microscopy, conventional electron microscopy, and immunoelectron microscopy to follow the membrane trafficking and intracellular fate of *F. tularensis* LVS and RCI within human macrophages [THP-1 cells and MDM (Figs. 7 and 8)].[34] A study prior to our work had identified *F. tularensis* as residing within membrane-bound vacuoles in host cells.[35] Phagosomes containing inert particles, such as latex beads, have been shown to interact sequentially with the compartments of the endosomal-lysosomal pathway: early endosomes, late endosomes, and lysosomes.[36] The compartments of the endosomal-lysosomal pathway each contain their own distinctive markers, or they can be labeled kinetically by exogenously added tracers, such as Texas-red dextran, which is endocytosed and accumulates in lysosomal compartments (Fig. 8). The extent to which a phagosome interacts with the various compartments of the endosomal-lysosomal pathway can be assessed by examining the extent to which the phagosome acquires the markers of the various compartments of the endosomal-lysosomal pathway. Therefore, to characterize the intracellular biology of *F. tularensis*, we examined the interaction of the phagosome with the endo-lysosomal pathway. As noted above, we have confirmed by electron microscopy that *F. tularensis* resides in a phagosome initially after infection (e.g. Fig. 1C), and we have found by immunofluorescence microscopy that this phagosome interacts transiently with early endosomes, as reflected by its transient acquisition of early endosomal antigen 1 (EEA-1). The association of EEA-1 with the *F. tularensis* vacuole reaches its maximum within 15 minutes of infection and declines to very low levels within 60 minutes of infection. The kinetics of acquisition and loss of EEA-1 are the same for vacuoles containing live and dead *F. tularensis*. The *F. tularensis* phagosome subsequently interacts with late

endosomes, as reflected by its acquisition of limited amounts of lysosome-associated membrane glycoproteins (CD63, LAMP-1 and LAMP-2; markers that are present on both late endosomes and lysosomes). However, the amounts of these markers that associate with vacuoles containing live *F. tularensis* are consistently lower than the amounts associating with vacuoles containing either dead *F. tularensis* or latex beads (Fig. 9). Furthermore, whereas vacuoles containing dead *F. tularensis* and latex beads acquire abundant staining for lysosomal markers, such as cathepsin D (Fig. 10) and fluorescent dextran, vacuoles containing live *F. tularensis* do not acquire these lysosomal markers, indicating that the *F. tularensis* vacuoles do not interact with lysosomes. Whereas vacuoles containing inert particles, such as latex beads and killed *F. tularensis*, show a uniform and intense staining for lysosome-associated membrane glycoproteins even 1 – 2 days after phagocytosis, the maximal colocalization of live *F. tularensis* with LAMPs is achieved at 2 – 4 hours after infection of the macrophages. With additional time after infection, a steady decline in the degree of immunofluorescent colocalization of the bacteria with LAMPs is seen, such that by 16 hours after infection of the macrophages with either *F. tularensis* RCI or LVS, less than 15% of the *F. tularensis* colocalize with LAMPs (Fig. 9). As noted below, this progressive loss of immunofluorescent staining for LAMP coincides with progressive loss of phagosomal membrane and escape of the bacteria into the macrophage cytoplasm.

We also examined the extent to which the *F. tularensis* vacuole becomes acidified, both by employing the lysosomotropic fluorescent marker Lysotracker red DND-99 and by employing the lysosomotropic agent DAMP (3-(2,4-dintroanalino)-3'-amino-N-

methyldipropylamine) and immunoelectron microscopy. We found that, in contrast to vacuoles containing killed *F. tularensis*, vacuoles containing live *F. tularensis* did not acquire Lysotracker red fluorescence, indicating that vacuoles containing dead but not live bacteria are acidified. Likewise, we found that vacuoles containing killed *F. tularensis* acquire abundant staining for DAMP, whereas vacuoles containing live *F. tularensis* acquire minimal amounts of DAMP. DAMP immunogold staining can be quantitated and used to calculate the pH of the labeled compartments. Using this technique, we found that the pH of vacuoles containing killed *F. tularensis* is 5.5, and the pH of vacuoles containing live *F. tularensis* is 6.7.

Ultrastructural analysis of the *F. tularensis* vacuole within infected macrophages

We have assessed the ultrastructure of *F. tularensis* LVS and RCI within human MDM and THP-1 cells by transmission electron microscopy (Fig. 7 and 11). Immediately after infection, the bacteria reside within membranes containing discrete, easily identifiable lipid bilayers (Fig. 1C). A large percentage (25% - 50%) of the vacuoles containing live *F. tularensis*, acquire a densely staining coat of fibrillar material on their cytoplasmic surface (Fig. 11 A). The fibrils measure 25 – 34 nm in length and resemble clathrin coats of the plasma membrane, except that they appear coarser, more electron dense, and, when present, usually coat the entire circumference of the *F. tularensis* vacuole. The phagosomal coats often appear to be in the process of forming buds and vesicles, and fragmenting (Fig. 11 B). The formation of fibrillar coats on the vacuoles containing live *F. tularensis* is unique. Using identical electron microscopy fixation and staining techniques, we have not observed fibrillar coats on phagosomes containing killed *F.*

tularensis, or on phagosomes containing either live or killed *M. tuberculosis*, *L. pneumophila*, or *Escherichia coli.*

Our ultrastructural analysis of macrophages infected with *F. tularensis* RCI and LVS revealed that the percentage of bacteria surrounded by an identifiable membrane bilayer decreases rapidly with increasing time after infection (Fig. 11-12). While 80-90% of the bacteria were surrounded by discrete membrane bilayers at the conclusion of a 90 minute incubation with macrophages, by 6 h after infection, less than 50% of the bacteria were surrounded by a vacuolar membrane (Fig. 11C and Fig. 12), and by 14 h, fewer than 20% of the bacteria were surrounded by a vacuolar membrane (Fig. 11D and Fig. 12). Fragmented membranes with fibrillar coats and small vesicles with fibrillar coats were often apparent in the vicinity of *F. tularensis* that otherwise lacked vacuolar membranes (Fig. 11C). We observed that *F. tularensis* subsp. *tularensis* of the Schu strain also disrupted its phagosomal membrane and escaped into the cytoplasm in human monocyte-derived macrophages and in THP-1 cells (Fig. 13). While no vacuolar membrane was apparent around the live *F. tularensis* bacteria at later time points, the bacteria were surrounded by electron lucent zones measuring approximately 0.3 microns in width (Fig. 11D and Fig. 13). We believe that this electron lucent zone is attributable to bacterial lipopolysaccharide or capsular material, as we have recently observed that it is completely absent from O-antigen deficient mutants of *F. tularensis* in human macrophages.

We observe the acquisition of fibrillar coats and the loss of vacuolar membrane only in macrophages infected with live *F. tularensis*, and not in macrophages that have ingested

killed *F. tularensis*. The loss of vacuolar membranes around *F. tularensis* is not attributable to a generalized destruction and loss of ultrastructural integrity of the macrophages. While many macrophages do exhibit necrosis or apoptosis at late times after infection (e.g. at 12 - 14 hours post-infection), we readily observe bacteria free in the cytoplasm of lightly infected macrophages within the first 7 hours of infection. Golovliov *et al.* have also demonstrated that disruption of phagosomal membranes can be seen in cells lightly infected with *F. tularensis* LVS.[37]

Role of autophagy in the life cycle and intracellular trafficking of *Francisella tularensis*

Checroun *et al.*[38] have observed a much more rapid escape of *F. tularensis* LVS in mouse bone marrow macrophages, with the majority of the bacteria escaping into the host cytoplasm by 1 hour post-infection. In addition, these investigators report that after an initial 20 hour period of intracytoplasmic replication in mouse primary macrophages, the majority of the bacteria re-entered large vacuoles that stained positively for LAMP-1 and cathepsin D. Checroun *et al.*[38] found that these large *F. tularensis* containing vacuoles fused with secondary lysosomes, as evidenced by their acquisition of endocytosed fluorescent dextran, and that they were acidified, as evidenced by their acquisition of Lysotracker red. These investigators describe the compartment ultrastructurally as being enclosed within the double-membrane that is characteristic of ER derived autophagosomes. In support of the concept that these structures represent autophagosomes, Checroun *et al.*[38] showed that the compartments acquire the autophagosome markers dansylcadaverine and LC3-GFP.

It remains to be determined what role re-entry into autophagic vacuoles plays in the cell biology of *F. tularensis*. In our studies of *F. tularensis* LVS and of virulent type A *F. tularensis* RCI and Schu in human monocyte-derived macrophages and in human THP-1 macrophages, we have found only a small percentage of the bacteria within autophagic vacuoles at 24 – 48 hours post-infection. This difference between our results and those of Checroun *et al.* with respect to the extent of autophagy could reflect differences in the host cell studied (human *vs.* mouse) or other technical differences in the culture conditions. In our observations of *F. tularensis* interaction with human macrophages, it is clear that the bacteria are able to replicate to extremely high numbers in the cytoplasm of the infected cells, to destroy the infected macrophages, and to begin new rounds of infection without passing through an autophagosomal stage. It is possible that the autophagosomal pathway described by Checroun *et al.*[38] in mouse bone marrow macrophages represents a host response mechanism for control of the *F. tularensis* infection. In broth culture, *F. tularensis* grows poorly under acidified conditions. Therefore, the acidified environment of the autophagosome would not favor bacterial replication. In addition, the acid hydrolases within the autophagic vacuole would permit antigen processing and presentation of *F. tularensis* antigens to promote control of the infection by the host immune system.

Intracellular trafficking of *F. tularensis* in interferon-gamma activated macrophages

Interferon-gamma (IFN-gamma) has been shown to be important in the host control of many infections, including tularemia.[39, 40] Lindgren *et al.*[41] have examined the interactions of *F. tularensis* LVS in quiescent and IFN-gamma activated mouse peritoneal exudate cells

(PECs) and found that IFN-gamma treatment of the PECs abolished their permissiveness to growth of the LVS, and decreased but did not abolish the capacity of the *F. tularensis* LVS to escape into the macrophage cytoplasm. Indeed, Lindgren *et al*. found that the majority of the LVS continued to escape into the cytoplasm of the PECs, but, nevertheless, did not grow intracellularly. [41] Santic *et al.*[42] examined the interaction of *F. tularensis* subsp. *novicida* (strain U112) with quiescent and IFN-gamma activated human MDM and observed that, as is the case with virulent type A *F. tularensis* and the attenuated LVS strain, *F. tularensis* subsp. *novicida* initially entered a late-endosomal like compartment that acquired LAMP-2 but not cathepsin D and that failed to acidify or to fuse with lysosomes. By 8 hours after infection in non-activated macrophages, as is the case with fully virulent *F. tularensis*, the *F. novicida* were observed to disrupt their phagosomal membranes and to replicate in the host cell cytoplasm. In contrast, in interferon activated macrophages, Santic et al.[42] observed that *F. tularensis* subsp. *novicida* entered an acidified phagolysosomal compartment (staining positive for cathepsin D, markers of secondary lysosomes, and Lysotracker red), failed to escape into the host cell cytoplasm, and failed to grow intracellularly. Thus, for both *F. tularensis* LVS and *F. novicida*, IFN-gamma treatment alters the intracellular trafficking of the bacteria. The mechanism for the restricted intramacrophage growth and altered intracellular trafficking has not been determined, but could be due to restriction of iron availability to the bacteria, induction of reactive oxygen and nitrogen intermediates, or modulation of intracellular signaling pathways.[43, 44]

Intracellular trafficking and the *Francisella* pathogenicity island

The genome of highly virulent type A *F. tularensis* contains a *Francisella* pathogenicity island (FPI) containing several genes essential for growth of *F. tularensis* within macrophages.[45] This FPI is also present in *F. tularensis* subsp. *novicida*, and in the type B strains of *F. tularensis*, including the attenuated LVS strain, except that the type B strains lack one of the genes (*pdpD*) and have an extra copy of the remainder of the FPI.[45] The FPI includes four genes, *iglABCD*, that are organized into an operon.[45] IglC has been shown to be induced during growth of *F. tularensis* in macrophages and to be required for intracellular multiplication in macrophages and for virulence in mice for both *F. tularensis* LVS and supbsp. *novicida*. [41, 46-49]. The expression of IglC is dependent on MglA, which is thought to act as a global regulator of virulence factors in *F. tularensis*.[50] It has recently been shown that the *iglC* and *mglA* genes are required for proper trafficking of *F. tularensis* LVS and subsp. *novicida* within macrophages. Lindgren *et al.* have shown that inactivation of the *iglC* gene of *F. tularensis* LVS abolishes its capacity to escape into the cytoplasm of mouse peritoneal exudate macrophages.[41] Santic *et al.* have shown that inactivation of the *mglA* or *iglC* genes in *F. tularensis* subsp. *novicida* abolishes the capacity of the mutants to avoid fusion with lysosomes or to escape into the cytoplasm.[49] While the pathogenic mechanisms underlying the role of the FPI proteins in modulating intracellular trafficking of *F. tularensis* within macrophages is unknown, it is speculated that the FPI encodes genes involved in secretion of virulence factors into the macrophage.[51, 52]

Significance of the *F. tularensis* lifestyle

Intracellular parasites have been shown to subvert the host cell's normal membrane trafficking pathways to achieve intracellular compartments that are more hospitable to

their growth and multiplication. There are three major pathways that are followed by intracellular parasites: (a) an intraphagolysosomal pathway in which there is residence in an acidified phagolysosome, as is the case for *Coxiella burnetti*, (b) an intraphagosomal pathway in which there is residence in a phagosome that does not fuse with lysosomes, either because of arrested maturation, as is the case for *M. tuberculosis*, or because of avoidance of the endo-lysosomal pathway, as is the case for *L. pneumophila*; and (c) an extraphagosomal pathway in which there is escape into the host cell cytoplasm, as is the case for *Listeria monocytogenes*. Within each of these three general pathways, there are additional variations that are unique to each pathogen. The intracellular life-style of *F. tularensis* is a combination of the arrested phagosomal maturation pathway and the extraphagosomal pathway, but it is clearly unique.

Our studies have demonstrated that *F. tularensis* is internalized by a novel process of looping phagocytosis and that the bacterium initially resides in a membrane-bound vacuole that briefly interacts with early endosomes and has limited interaction with late endosomes. The *F. tularensis* vacuole exhibits arrested maturation in that it only acquires limited amounts of LAMPs, it is only minimally acidified to a pH of 6.5, and it does not fuse with lysosomes or acquire acid hydrolases, such as cathepsin D. The *F. tularensis* vacuole acquires a unique fibrillar coat, which subsequently disintegrates, and the bacteria reside and multiply within the macrophage cytoplasm. *F. tularensis* subverts the host cell's membrane trafficking events to achieve an environment that is optimal for its growth and multiplication. In liquid culture, the bacterium grows optimally at neutral pH, which is compatible with the pH we have measured for its intracellular niche.

Furthermore, the arrested maturation of the phagosome and subsequent escape of the bacterium into the host cytoplasm eliminates exposure of the *F. tularensis* to degradative hydrolases of the host cell, while providing it access to a rich source of nutrients available in the host cell cytoplasm.

The mechanisms that allow *F. tularensis* to arrest the maturation of its phagosome and to escape into the cytoplasm remain to be elucidated. Our observation that live and dead *F. tularensis* acquire and lose the early endosomal antigen EEA1 with similar kinetics suggests that important differences in membrane trafficking between dead and live *F. tularensis* phagosomes occur subsequent to EEA1 acquisition, i.e. after the limited acquisition of late endosomal markers. Permeabilization of the phagosomal membrane is an event of pivotal importance in the intracellular life of *F. tularensis* and could account for additional aspects of the apparent arrested maturation of the phagosome. For example, permeabilization of the membrane (i.e. creation of pores), even without full disruption of the membrane, would prevent acidification by equilibrating the pH of the *F. tularensis* vacuole with the pH of the host cell cytoplasm, i.e. 6.5. Failure of the phagosome to acidify would also prevent the delivery of acid hydrolases such as cathepsin D from the Golgi to the phagosome via the mannose-6-phosphate receptor, since the release of cathepsin D from the mannose-6-phosphate receptor requires an acidic environment. Permeabilization of the vacuolar membrane with pores of sufficient size would also allow macromolecules that were delivered to the phagosome by other pathways to diffuse away. The *F. tularensis* genome is not known to code for any porins or hemolysins that could permeabilize the phagosome; however, *F. tularensis* does have an acid phosphatase

(acpA) with phospholipase activity that could play a role in escape of the bacterium from the phagosome. Mohapatra *et al*.[53] studied *F. novicida* bearing knock-out mutations of these phospholipases and showed that the mutants exhibited delayed escape into the host cell cytoplasm. The fibrillar coat formation that we have documented could also play a role in disruption of the phagosomal membrane. For example, the bacteria may trigger recruitment of host coat proteins and cytoskeletal components to the vacuole that subsequently lead to the vacuole pinching off, forming blebs, and becoming physically disrupted, as evident in our electron micrographs. If so, then agents that prevent the formation of this fibrillar coat or prevent the blebbing and budding of vesicles from the coat would also prevent escape of the bacterium into the cytoplasm. Thus, the ultrastructural morphology and the membrane trafficking events that we have observed suggest a testable pathogenic mechanism by which *F. tularensis* might arrest the maturation of its phagosome and escape into the cytoplasm.

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Figure Legends

Fig. 1. Uptake of *F. tularensis* virulent RCI by human peripheral blood monocyte-derived macrophages (MDM). Bacteria were pre-opsonized in 10% fresh human serum, pelleted onto human MDM at 4°C, warmed to 37°C for 5 min, fixed, and processed for electron microscopy. Uptake profiles show engulfment within a spacious pseuodpod loop (A), closure of the loop to form a spacious vacuole (B), and a less spacious vacuole within the macrophage (C). Size bars indicate 1 µm. Reprinted from reference 12.

Fig. 2. Morphology of uptake of various bacterial intracellular pathogens by human macrophages. Shown are the uptake of *M. tuberculosis* via conventional phagocytosis (A), *L. pneumophila* via coiling phagocytosis (B), and *S. typhimurium* (C), and *S. flexnerii* (D) via macropinocytosis. Panel (A) reprinted with permission of The Rockefeller Press from reference,[14] panel (B) reprinted with permission of Cell press from reference,[15] and panels (C-D) reprinted from reference 12.

Fig. 3. Uptake of both live and killed *F. tularensis* RCI by human monocyte-derived macrophages is sensitive to wortmannin. Human peripheral blood MDM were incubated with 0 – 1 μM wortmannin for 30 min prior to incubation with *F. tularensis* RCI at an MOI of 35:1 in the presence of 10% human AB serum and the same concentration of wortmannin for 60 min. The monolayers were washed to remove non-adherent bacteria, fixed without permeabilization, and incubated with a red fluorescent antibody to stain adherent but non-internalized *F. tularensis* bacteria. The monolayers were permeabilized and the internalized bacteria were stained with a green fluorescent antibody. The number of internalized (green but not red) *F. tularensis* RCI per MDM was determined by fluorescence microscopy. Values shown represent the mean ± standard errors of the numbers of internalized *F. tularensis* RCI per macrophage at each concentration of wortmannin.

Fig. 4. Periodate-treated *F. tularensis* are internalized by conventional phagocytosis. The micrograph shows human MDM ingesting 3 periodate-treated *F. tularensis* RCI. Size bar 1 μm. Reprinted from reference 12.

Fig. 5. Adherence and uptake of *F. tularensis* by human macrophages requires serum (A), and complement factor C3 (B). (A) Monolayers of human MDM on glass coverslips were incubated with *F. tularensis* LVS or RCI in culture medium containing 10% heat-inactivated fetal bovine serum and 0 – 10% autologous human serum at an MOI (multiplicity of infection) of 35:1 for 1 h at 37°C. Monolayers were washed, fixed, and stained by immunofluorescence for adherent or internalized bacteria. The number of adherent or internalized bacteria per MDM and the percentage of MDM infected was determined by fluorescence microscopy. (B) Monolayers of human MDM were incubated with *F. tularensis* LVS or RCI at an MOI of 35:1 in culture medium containing 10% C3-deficient serum supplemented with 0, 32.5, or 65 μg/ml of purified C3, and the number of bacteria per MDM and the percentage of infected MDM were determined as above. For both panels, values shown are the mean ± standard error for triplicate determinations of at least 100 macrophages counted for each condition at each time point. Reprinted from reference 12.

Fig. 6. Uptake of *F. tularensis* by human macrophages does not require natural antibody. Monolayers of human THP-1 cells were incubated with *F. tularensis* RCI or Schu at an MOI of 35:1 in the presence of heat-inactivated human AB serum from a normal donor (HI-NS), agammaglobulinemic serum [with or without either purified human IgG (1

mg/ml final concentration) or HI-NS (10% final concentration)], or normal human AB serum (NS) for 60 min. Monolayers were washed to remove non-adherent bacteria, fixed without permeabilization, and incubated with a red fluorescent antibody to stain adherent but non-internalized *F. tularensis* bacteria. The monolayers were permeabilized and the internalized bacteria were stained with a green fluorescent antibody. The number of adherent (red) bacteria and internalized (green but not red) bacteria per THP-1 cell was determined by fluorescence microscopy. Values shown represent mean ± standard error of adherence (dotted bars) and uptake (black bars) for both *F. tularensis* Schu (left side of figure) and *F. tularensis* RCI (right side of figure).

Fig. 7. Life cycle of *F. tularensis* in human macrophages. *F. tularensis* enters human macrophages by looping phagocytosis and quickly comes to reside in a membrane-bound phagosome. Many of these phagosomes acquire a unique densely staining fibrillar coat on their cytoplasmic surface. The fibrillar coat and phagosome membrane subsequently fragment, releasing the bacterium to the cytoplasm of the host cell. Subsequently, the bacterium multiplies intracytoplasmically. Copyright 2006 by Marcus A. Horwitz and Daniel L. Clemens. Reproduced by permission.

Fig. 8. Membrane trafficking of *F. tularensis* in human macrophages. The interaction of the *F. tularensis* phagosome with the endosomal-lysosomal pathway can be assessed by examining the distribution of markers of the compartments of the early endosomes, late endosomes, and lysosomes. Following uptake, *F. tularensis* resides in a vacuole that transiently acquires early endosomal markers, including early endosomal antigen 1

(EEA1). This marker is rapidly lost (reaching its peak within 15 minutes of uptake) and the *F. tularensis* vacuole subsequently acquires limited amounts of late endosomal markers – such as the lysosome-associated membrane glycoproteins CD63, LAMP1, and LAMP2. However, the *F. tularensis* phagosome acquires only limited amounts of these markers, and it does not fuse with lysosomes or acquire lysosomal markers such as cathepsin D or Texas Red dextran, which can be used to label lysosomes kinetically. With time, all markers of the endocytic pathway are lost, and *F. tularensis* escapes into the host cell cytoplasm. In contrast, phagosomes containing killed *F. tularensis* have extremely abundant lysosome-associated membrane glycoproteins (CD63, LAMP1, and LAMP2) and fuse extensively with lysosomes, acquiring abundant cathepsin D and Texas Red dextran. Copyright 2005 by Marcus A. Horwitz and Daniel L. Clemens. Reproduced by permission.

Fig. 9. Quantitation of immunofluorescence staining for CD63 in THP-1 cells infected with *F. tularensis* RCI or LVS. Monolayers of THP-1 cells were incubated with latex beads, formalin-killed *F. tularensis*, or live *F. tularensis* LVS or RCI, washed, fixed, and stained for CD63 and *F. tularensis* at 1 – 16 hours post-infection. The number of latex beads or *F. tularensis* colocalizing with CD63 was determined by fluorescence microscopy. Colocalization of live *F. tularensis* LVS and RCI with CD63 reached a maximum at approximately 3 h post-infection and subsequently declined. In contrast, killed *F. tularensis* and latex beads exhibited strong continuous staining for CD63 from 1 through 16 hours. Similar results were obtained for LAMP1 and LAMP2. Reprinted from reference 32.

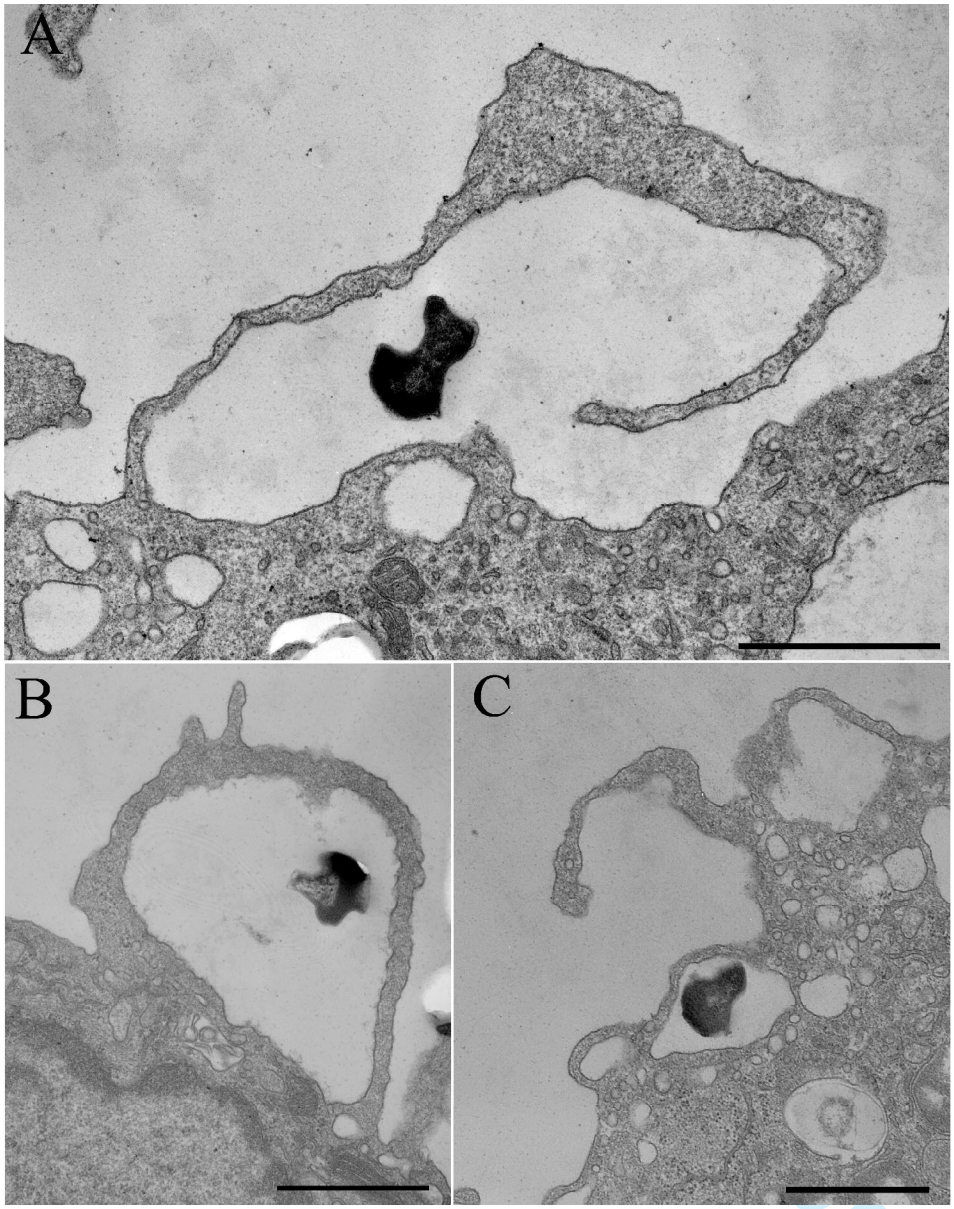
Fig. 10. Cathepsin D is present on latex bead phagosomes but not *F. tularensis* phagosomes. Human MDM were fixed and processed for immunoelectron microscopy 4 hours after coincubation with *F. tularensis* RCI and latex beads. The lysosomal acid hydrolase cathepsin D is identified by staining with 5 nm gold particles (arrowheads) and *F. tularensis* is identified by immunostaining with a larger (15 nm) gold particle (arrows). Size bar 0.5 µm. Reprinted from reference 32.

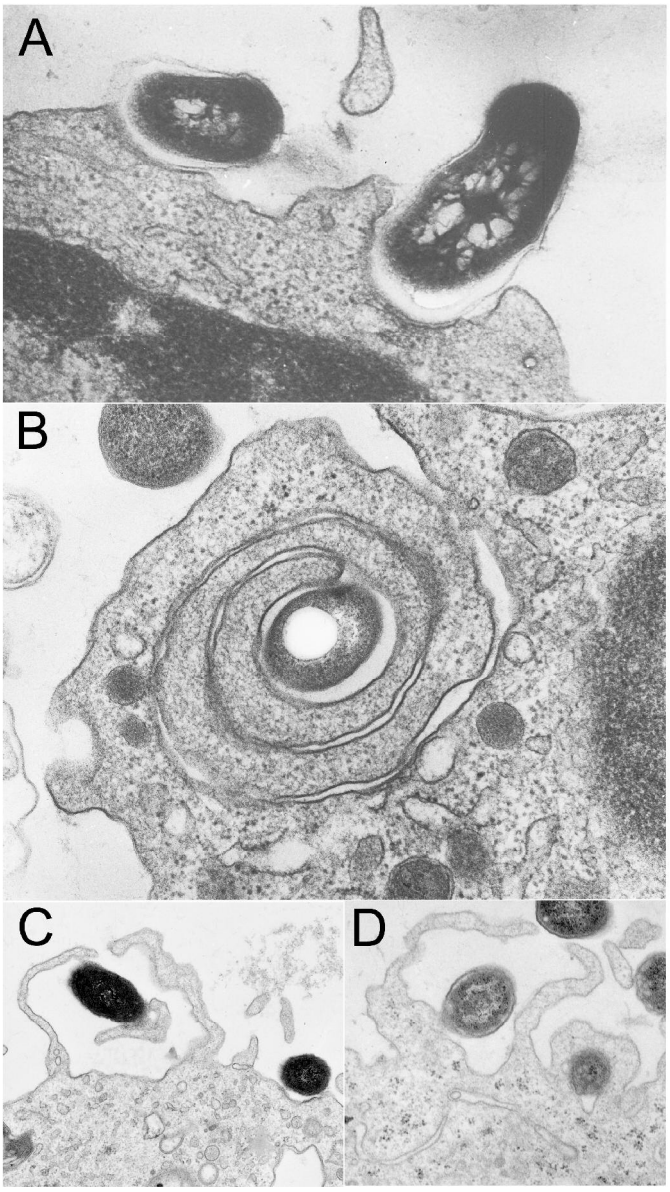
Fig. 11 Ultrastructural evaluation of the interaction of *F. tularensis* LVS (A-C) and RCI (D) with human macrophages immediately after a 90 min incubation (A), and at 3 h (B), 6 h (C) and 14 h (D) post-infection. Bacteria are indicated by asterisks. Many bacterial phagosomes exhibited a densely staining fibrillar coat radiating 30 nm from the cytoplasmic aspect of the phagosomal membrane (A-B, solid black arrowheads). The coated membranes appeared to form buds (B, open arrow heads), pinch off, and form vesicles (B-C, arrows). The *F. tularensis* LVS in (C) are partially surrounded by fragmented membranes (open arrows) and the *F. tularensis* RCI in (D) are not surrounded by phagosomal membranes and are free in the host cell cytoplasm. Nucleus (Nu) is indicated in (D). Size bars indicate 0.5 µm in all panels. Reprinted from reference 32.

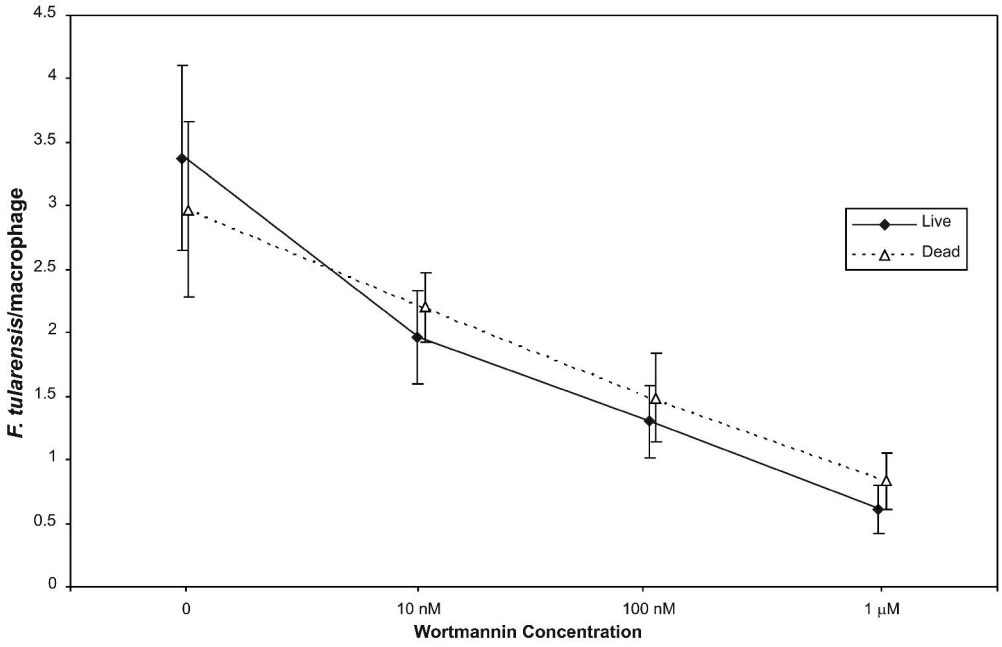
Fig. 12. Quantitation of the percentage of *F. tularensis* surrounded by discrete lipid bilayers in thin sections of human THP-1 cells. Monolayers of human THP-1 cells were incubated for 90 min with *F. tularensis* LVS or RCI and prepared for transmission

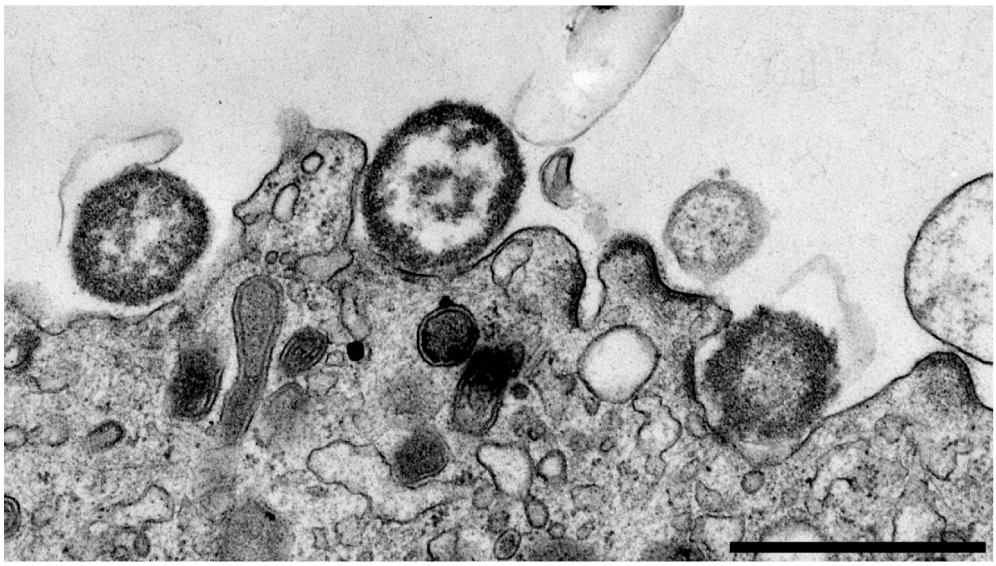
electron microscopy at 0 h to 14 h post-infection. Thin section profiles of bacteria within THP-1 cells were scored as being within membrane vacuoles if a discrete host membrane bilayer was present around at least 50% of the bacterial circumference. Reprinted from reference 32.

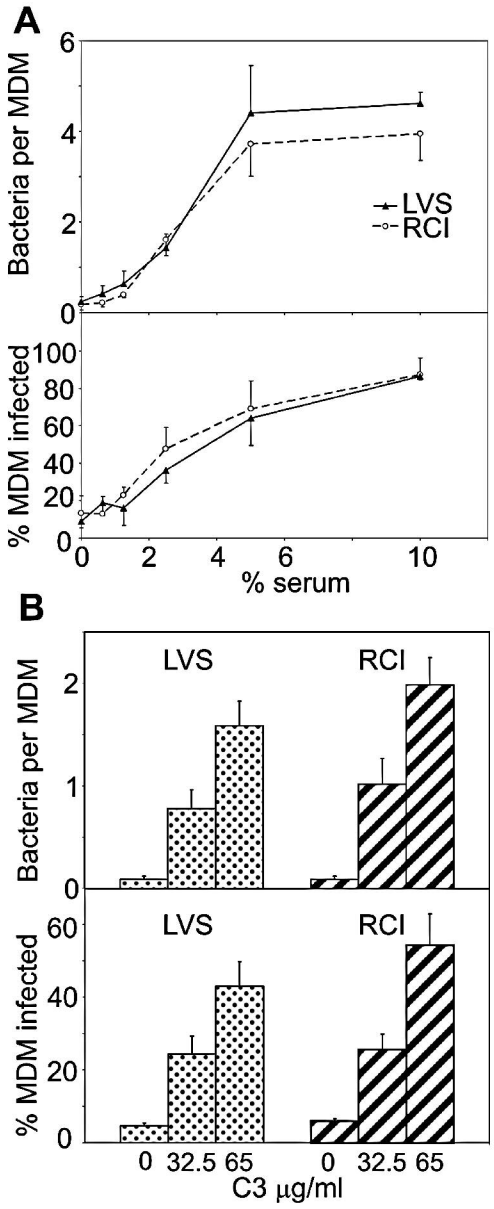
Fig. 13. *F. tularensis* of the Schu strain escapes into the cytoplasm of human macrophages. Human monocyte-derived macrophages were infected with *F. tularensis* Schu, fixed, and prepared for electron microscopy 7 hours post-infection. Three *F. tularensis* Schu bacteria (asterixes) are evident in the host cell cytoplasm, with no phagosomal membrane surrounding them. Size bar 0.5 µm.

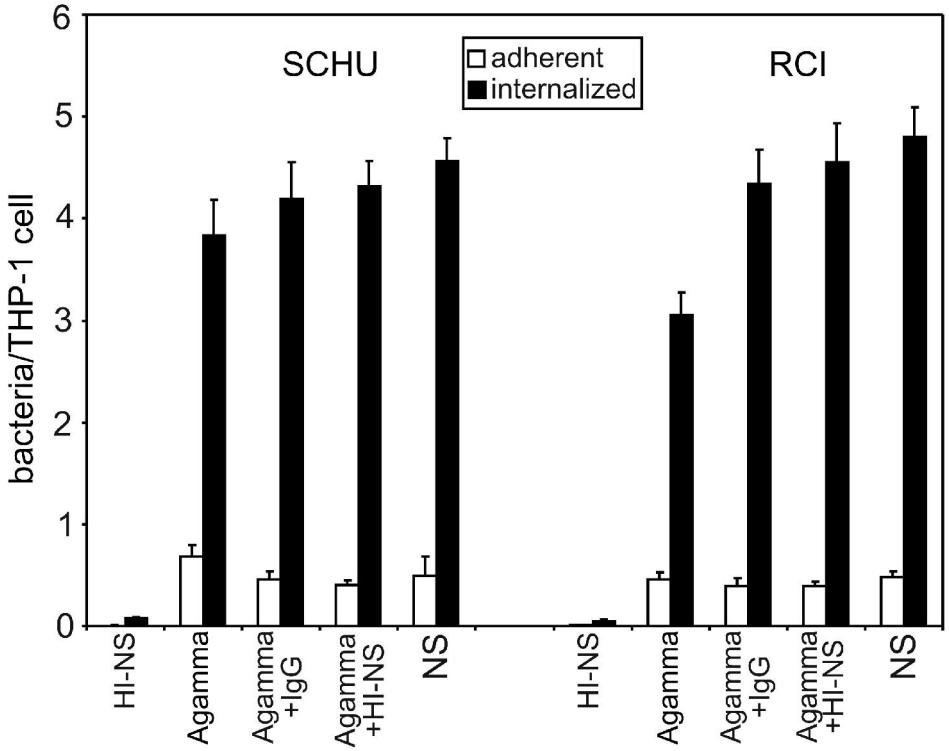

A
B
C

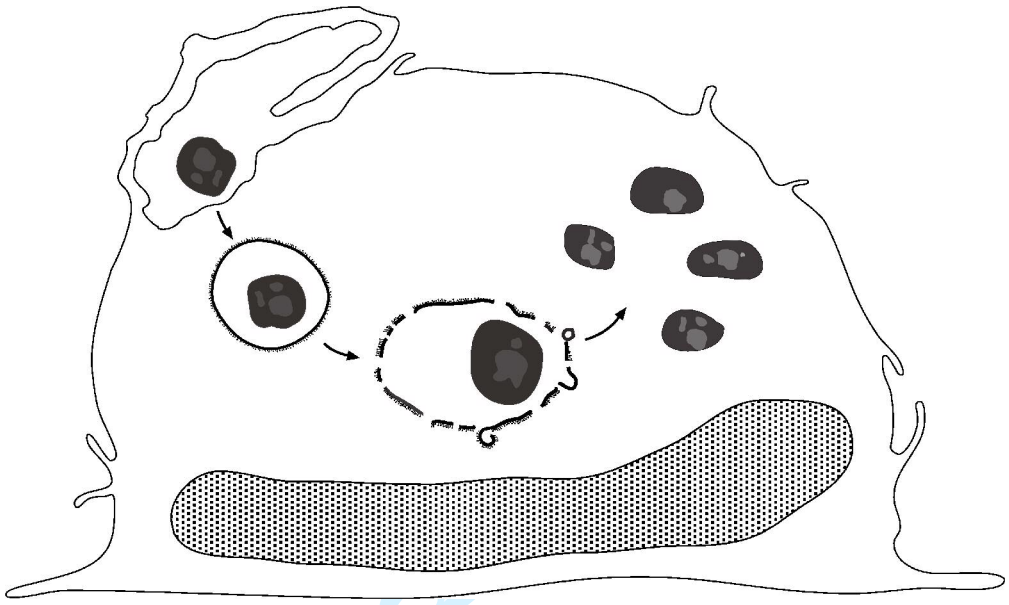

A
B
C
D

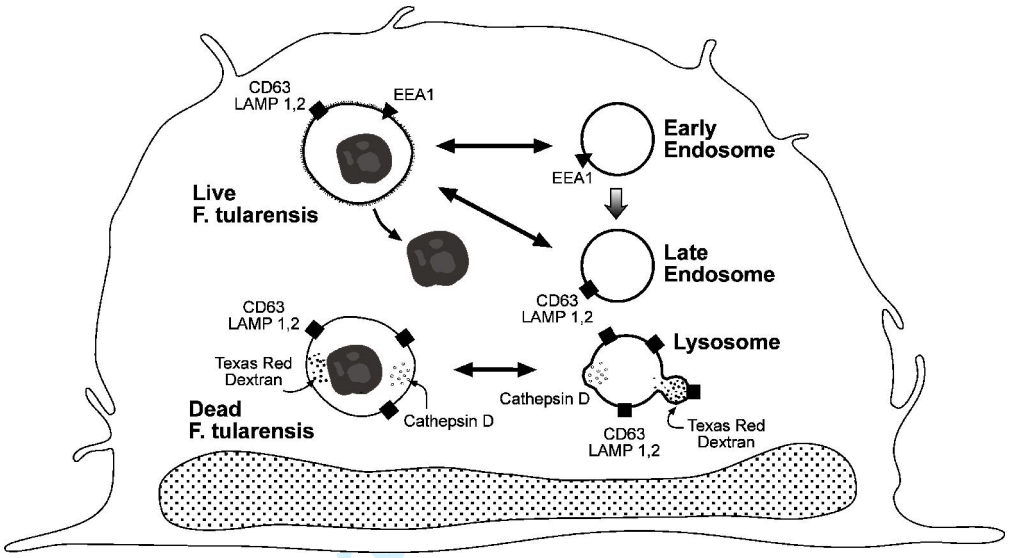

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
F. tularensis/macrophage
0
10 nM
100 nM
1 μM
Wortmannin Concentration
Live
Dead

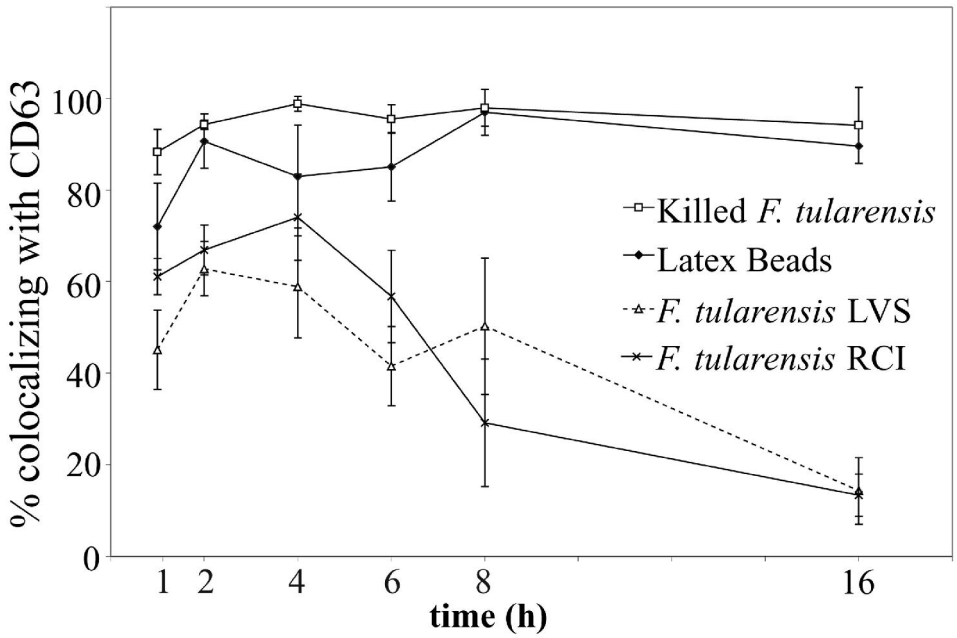


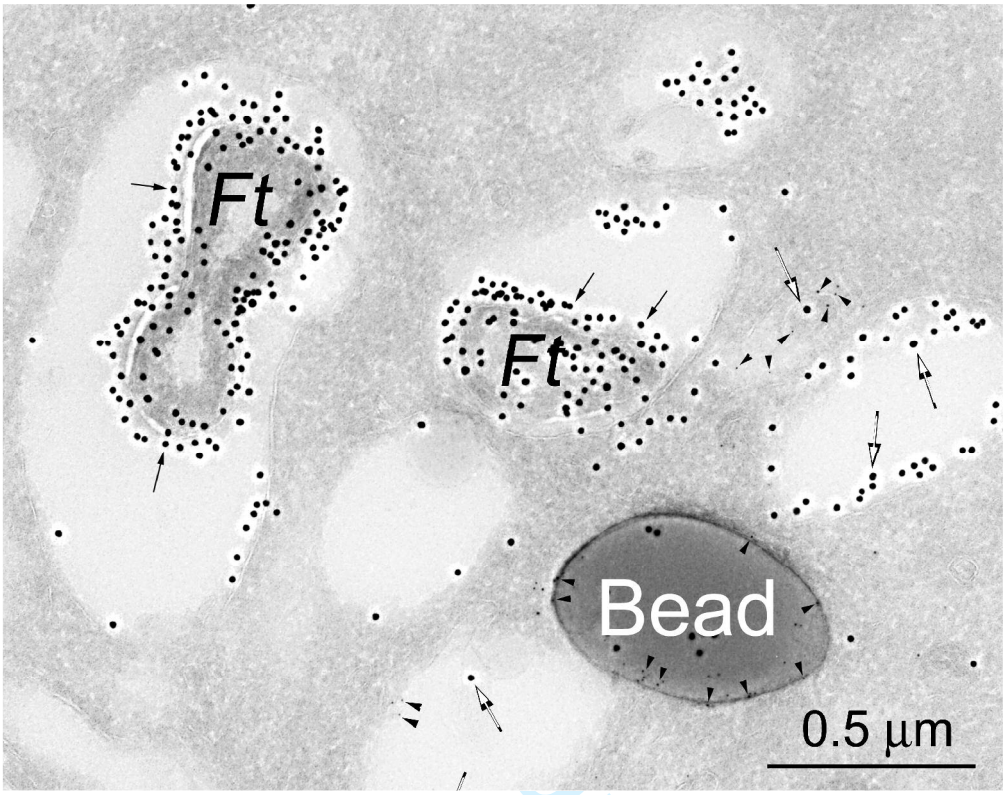

A
Bacteria per MDM
6
4
2
0
LVS
RCI
% MDM infected
100
80
60
40
20
0
0
2
4
6
10
% serum
B
LVS
RCI
Bacteria per MDM
2
1
0
LVS
RCI
% MDM infected
60
40
20
0
0 32.5 65
0 32.5 65
C3 µg/ml

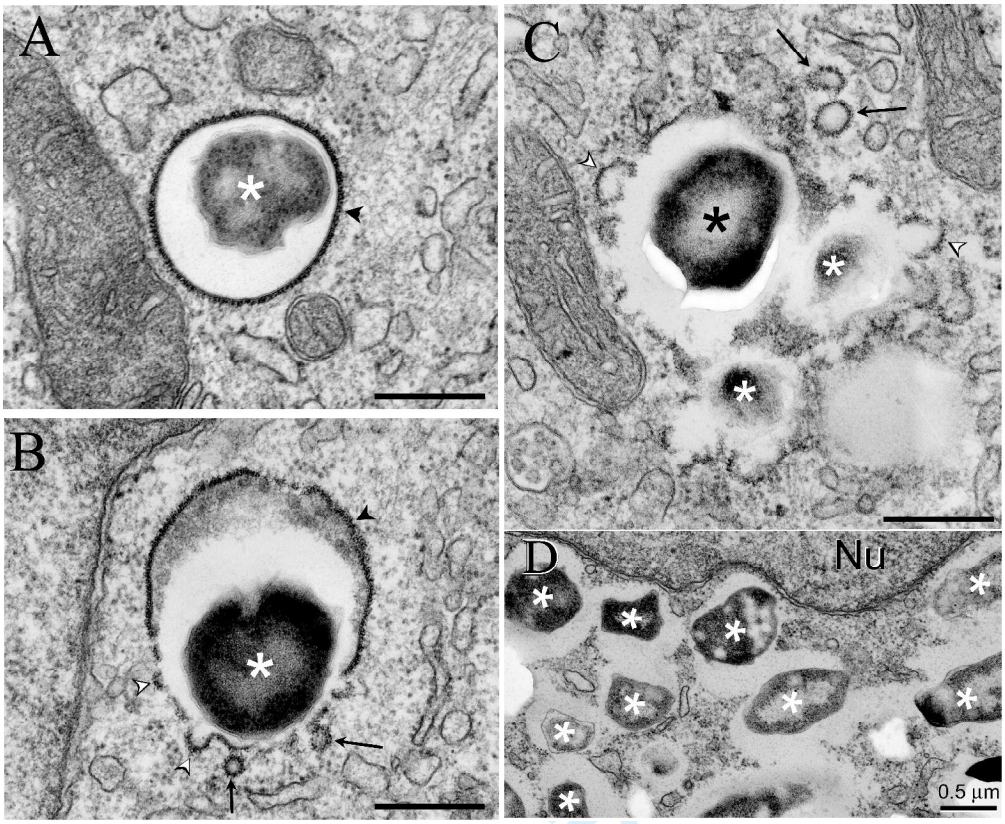

SCHU
RCI
adherent
internalized
bacteria/THP-1 cell
0
1
2
3
4
5
6
HI-NS
Agamma
Agamma +IgG
Agamma +HI-NS
NS
HI-NS
Agamma
Agamma +IgG
Agamma +HI-NS
NS

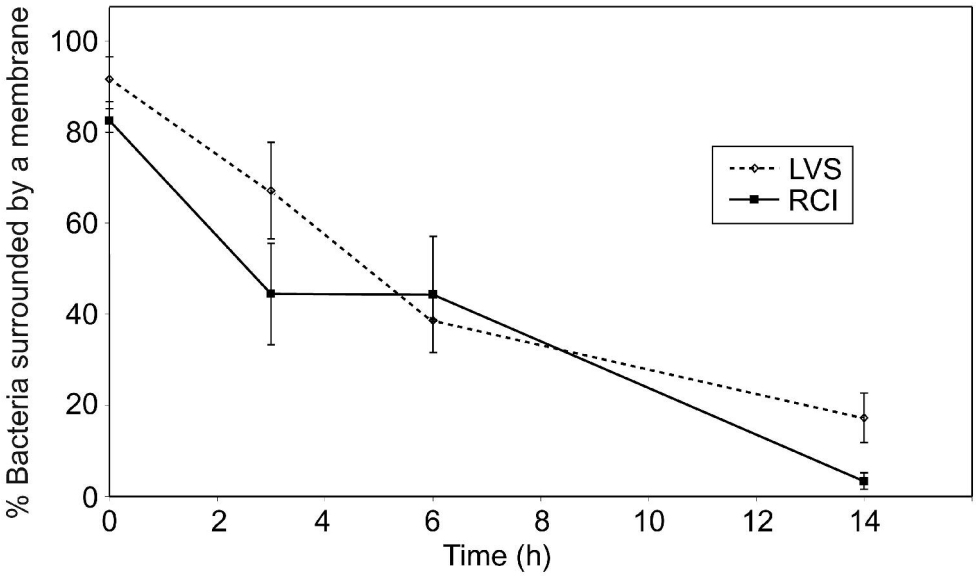





% colocalizing with CD63
100
80
60
40
20
0
1
2
4
6
8
16
time (h)
Killed F. tularensis
Latex Beads
F. tularensis LVS
F. tularensis RCI






% Bacteria surrounded by a membrane
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (h)
LVS
RCI