Invasion and replication assays were performed using the hu-
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tularemia acquired by inhalation, we sought to address this
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Francisella, the etiological agent of tularemia, is a
zoonotic pathogen with a broad host range, spanning from
prostis to humans. The organism is transmitted to humans
through handling of contaminated material, insect bites, or
inhalation (6, 10). It has been determined that contact with 25
or fewer bacteria is sufficient to cause disease (16, 17). Follow-
ing contact, the bacteria disseminate to the liver, spleen, and
lungs regardless of the initial transmission route (7, 10).

The low infectious dose, myriad of transmission routes,
host cells for growth of Francisella in humans and other ani-
Francisella led a number of nations to develop and stockpile this organism
maurine as a biological weapon (9), yet little is known about the basis of
its pathogenesis or virulence. It has been reported that mac-
rophages and, potentially, dendritic cells serve as the primary
host cells for growth of Francisella in humans and other ani-
mals (5, 18). This conclusion is supported by the observations
that F. tularensis and other closely related Francisella species
survive and replicate in human and rodent macrophages (1, 4)
and that many of the identified attenuating mutations impair
intramacrophage growth (2, 11, 12, 14, 15).

Clearly, intramacrophage survival is an important virulence
property of Francisella. However, apart from one report of
hepatocyte association in infected liver (8), the potential con-
tribution of other host cell interactions to F. tularensis viru-
ence and pathogenesis remains untested. Given the severity of
tularemia acquired by inhalation, we sought to address this
possibility by using in vitro and in vivo models to examine whether
Francisella invades and replicates within epithelial
cells of the lung.

Francisella tularensis LVS invades and replicates within lung
epithelial cells in vitro. To determine whether Francisella
could invade and replicate within lung epithelial cells, in vitro
invasion and replication assays were performed using the hu-
man alveolar type II (ATII) epithelial cell-derived cell line
A549 (ATCC), the mouse ATII cell-derived cell line MLE-12
(ATCC), and the mouse lung epithelial cell-derived cell line
Tc-1 (ATCC). For comparison, the mouse macrophage-like
cell line J774A.1 (ATCC) was also used. Intracellular bacteria
were enumerated by a gentamicin protection assay. The cell
lines were grown to confluence in 24-well plates and inoculated
with F. tularensis LVS at a multiplicity of infection of 100:1.
Four hours after inoculation, the cells were washed with phos-
phate-buffered saline (PBS), and medium containing gentami-
cin (25 µg/ml) was added to kill extracellular bacteria.

Intracellular organisms were recovered from all cell lines 6
and 24 h postinoculation (Fig. 1A). The number of intracellular
organisms recovered 6 h postinoculation and the percent-
age of infected cells varied among the lung epithelial cell lines,
with the fewest intracellular Francisella organisms recovered
from the human ATII cell line, A549. The percentage of in-
fected epithelial cells ranged from 0.2% of A549 cells to 17%
of Tc-1 cells (Fig. 1A). The number of intracellular bacteria
recovered from each lung epithelial cell line at 6 h was smaller
than the number recovered from J774A.1 cells, as was the
percentage of infected cells, indicating that entry into epithe-
rial cells may be a less efficient process than uptake by macro-
phages.

The number of intracellular bacteria increased by an average
of 100-fold during the following 18 h in each cell line, repre-
senting an intracellular doubling time of roughly 3 h (Fig. 1A).
Intracellular localization and proliferation were confirmed us-
ing fluorescence microscopy and digital image deconvolution
(Fig. 1B to E). From 6 to 24 h postinoculation, the relative
increase of intracellular bacteria within lung epithelial cells was
similar to that observed within J774A.1 cells, indicating that F.
tularensis LVS replicated to the same degree within lung epither-
ilial cells as in macrophages.

There are many reports of Francisella replicating within
macrophages in vitro, and here we report its ability to replicate
within cultured lung epithelial cells. Whereas some work has
been done to examine localization of Francisella in the liver
(8), little is known about the localization of inhaled Francisella
in the lung. Bosio and Dow recently reported that Francisella
was associated primarily with dendritic cells isolated from

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† Published ahead of print on 6 November 2006.
FIG. 1. *F. tularensis* LVS invades and replicates within ATII cell lines in vitro. (A) Intracellular bacteria recovered from A549, MLE-12, Tc-1, and J774A.1 cells 6 and 24 h postinoculation. The percentages above the bars represent percentages of infected cells. (B and C) Fluorescence imaging of A549 cells inoculated with GFP-expressing LVS 6 h (B) and 24 h (C) following inoculation. Cell borders were visualized by rhodamine-phalloidin (Molecular Probes) staining (red), and nuclei were visualized with 4',6'-diamidino-2-phenylindole (DAPI; blue). (D and E) Fluorescence imaging of J774A.1 cells inoculated with GFP-expressing LVS (green) 6 h (D) and 24 h (E) postinoculation. Cell borders were visualized using biotinylated lectin from *Lens culinaris* and streptavidin-conjugated Alexa Fluor 647 (Molecular Probes) (red), and nuclei were stained with DAPI (blue). Intracellular replication experiments were carried out in triplicate; error bars represent standard deviations of the means.
bronchioalveolar lavage fluid of mice 1 hour after inhalation (5). However, the localization of bacteria in lung tissue throughout the course of infection has not been addressed.

**Following inhalation, *Francisella* localizes to the alveolus.** To determine the localization of *Francisella* in the airway following inhalation, anesthetized 6- to 8-week-old female C57BL/6 mice were inoculated intranasally with 10^5 CFU of *F. tularensis* LVS expressing green fluorescent protein (GFP) suspended in 50 μl of PBS. (All mouse work was performed according to IACUC-approved protocol.) Immunofluorescence analysis was performed on formalin-fixed and paraffin-embedded tissue sections obtained from nasal turbinates, trachea, and lungs harvested 1, 3, and 7 days postinoculation. No bacteria were observed in turbinates or trachea. In the lung, few bacteria associated with the apical surface of bronchial epithelial cells, while the majority colocalized with alveolar cells (Fig. 2). Fluorescence imaging of sequential vertical planes revealed that most infected alveolar cells contained multiple bacteria throughout the cytoplasm, indicative of intracellular replication (data not shown). Bacterial replication within ciliated or nonciliated (Clara) bronchial epithelial cells was not observed. The absolute number of bacteria (Fig. 2D) and the number of infected alveolar cells increased by day 3 (Fig. 2B). By day 7, *F. tularensis* within the lung was widespread but remained exclusively in the alveolus (Fig. 2C). Throughout the course of infection, the amount of extracellular space observed in the network of alveolar cells decreased, causing the alveolar cells to appear more condensed (Fig. 2).

**Inhaled *Francisella* colocalizes to and replicates within ATII cells in vivo.** Given that *Francisella* invaded and replicated within ATII-derived cell lines in vitro, we probed infected lung tissue sections with antibodies to the nonsecreted surfactant protein precursors proSP-B and proSB-C (Chemicon) to determine whether *Francisella* localized to and replicated within ATII cells in vivo following inhalation. proSP-B is produced by ATII cells and nonciliated bronchiolar Clara cells (13), whereas proSP-C is produced by ATII cells exclusively (3). Three days following inoculation, the majority of alveolar cells associated with *F. tularensis* also bound antibodies to the nonsecreted surfactant protein precursors proSP-B and proSB-C (Fig. 3A to D). Some cells associated with bacteria were not positive for proSP-B or
proSP-C, indicating that Francisella interacts with other cell types in the lung following inhalation.

In addition to the colocalization with proSP proteins, microscopy of sequential vertical planes confirmed that bacteria were inside infected ATII cells (data not shown). Rabbit antibody specific for β-tubulin (Abcam) did not colocalize with F. tularensis (Fig. 3E), indicating that the colocalization of Francisella with rabbit proSP antibodies was not due to bacteria.
ATII cells are not the only alveolar cell type harboring replicating Francisella organisms (1, 4, 5). Due to difficulties in staining for surface macrophage and dendritic cell markers in embedded lung sections, we utilized dispase digestion of infected mouse lungs to stain for surface markers and also to assess our results from the embedded tissue staining.

Three days after inoculation with LVS expressing GFP, lungs were infused and incubated with the neutral protease dispase (BD Biosciences). Digested tissue was washed in PBS and subsequently filtered through 40- and 20-μm-pore-size mesh. Staining with fluorescently labeled antibody specific for the surface markers F4/80 (eBioscience) and CD11c (eBioscience) was performed prior to fixation with 4% paraformaldehyde. Staining for the intracellular markers proSP-B and proSP-C occurred following fixation and required the use of Cytoperm (BD Biosciences) to permeabilize the cells. F. tularensis LVS was observed within cells expressing F4/80, CD11c, proSP-B, and proSP-C (Fig. 4), indicating that following inhalation, bacteria survive and replicate within macrophages, dendritic cells, and ATII cells.

Here we report that in addition to replicating within macrophages and dendritic cells, F. tularensis invades and replicates within alveolar type II epithelial cells, indicating that interaction with these cells following inhalation may be an important component of pneumonic tularemia. Future work will analyze the proportion of various cell types that are infected in the lung as well as attempt to identify the contribution of ATII cell invasion and replication to the disease progression of pneumonic tularemia.

We gratefully acknowledge the technical support and invaluable contributions of Yan Zhao and Kimberly Burns and thank Mats Forsman for sending us the pKK214GFP plasmid prepublication. We also thank Jo Rae Wright for sharing her expertise on lung biology and Robert Fulcher for editing assistance and general advisement.

This work was supported by a Southeast Regional Center of Excellence in Biodefense and Emerging Infections grant (NIH/NIAID U54-AI057157) and by the National Institutes of Health (R21-AI053399).

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Editor: D. L. Burns