Temporal Transcriptional Response during Infection of Type II Alveolar Epithelial Cells with Francisella tularensis Live Vaccine Strain (LVS) Supports a General Host Suppression and Bacterial Uptake by Macropinocytosis

Christopher E. Bradburne, Anne B. Verhoeven, Ganiraju C. Manyam, Saira A. Chaudhry, Eddie L. Chang, Dzung C. Thach, Charles L. Bailey, and Monique L. van Hoek

From the Center for Bio/Molecular Science and Engineering, United States Naval Research Laboratory, Washington, D. C. 20375, the Asymmetric Operations Department, Johns Hopkins University, Applied Physics Laboratory, Laurel, Maryland 20723-6009, the School of Systems Biology, George Mason University, Manassas, Virginia 20110, the Department of Bioinformatics and Computational Biology, University of Texas MD Anderson Cancer Center, Houston, Texas, and the National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, Virginia 20110

Background: The mechanism of Francisella LVS entry into A549 cells is unknown. Microarrays were performed at early infection time points, supported by phenotypic observations and inhibition experiments. Francisella LVS may enter A549 cells by macropinocytosis and quiets the host infection response. Francisella LVS induces significant host cell signaling at very early time points after the bacteria’s interaction with the cell.

Results: Pneumonic tularemia is caused by inhalation of Francisella tularensis, one of the most infectious microbes known. We wanted to study the kinetics of the initial and early interactions between bacterium and host cells in the lung. To do this, we examined the infection of A549 airway epithelial cells with the live vaccine strain (LVS) of F. tularensis. A549 cells were infected and analyzed for global transcriptional response at multiple time points up to 16 h following infection. At 15 min and 2 h, a strong transcriptional response was observed including cytokskeletal rearrangement, intracellular transport, and interferon signaling. However, at later time points (6 and 16 h), very little differential gene expression was observed, indicating a general suppression of the host response consistent with other reported cell lines and murine tissues. Genes for macropinocytosis and actin/cytoskeleton rearrangement were highly up-regulated and common to the 15 min and 2 h time points, suggesting the use of this method for bacterial entry into cells. We demonstrate macropinocytosis through the uptake of FITC-dextran and amiloride inhibition of Francisella LVS uptake. Our results suggest that macropinocytosis is a potential mechanism of intracellular entry by LVS and that the host cell response is suppressed during the first 2–6 h of infection. These results suggest that the attenuated Francisella LVS induces significant host cell signaling at very early time points after the bacteria’s interaction with the cell.

Francella tularensis is a zoonotic, facultative intracellular bacterium that causes tularemia in a wide variety of small and large mammals, including humans (1). Although Francisella is not normally a respiratory pathogen, the most severe infections by Francisella species occur via inhalation or direct inoculation of the lungs, leading to pneumonic tularemia (1). Francisella is a particularly virulent pathogen, requiring inhalation of as few as 10–100 cells to cause a potentially life-threatening pulmonary infection (1, 2). It is an unusual bacterium in that it is taken up by phagocytic cells, such as macrophages and dendritic cells, yet does not cause a large activation of the antimicrobial activity of the cell and therefore has been called a “stealth pathogen” (3, 4). Proinflammatory and immune mediators may take days to become detectable; TNF-α, IL-1β, and IFN-γ do not appear until 3 days after infection in a murine respiratory tularemia model (5).

Macrophages and dendritic cells are not the only cell types in the respiratory mucosa that may be infected by this bacterium. Type II alveolar epithelial cells are integral components of the lung mucosa, are non-phagocytic, and are able to detect and respond to the presence of pathogens and pathogen-associated molecules. They modulate the majority of the epithelial cellular response during infections (compared with Type I), and they may play an important role in Francisella infection.
In these studies, we have focused on *F. tularensis* live vaccine strain (LVS), a live, attenuated vaccine strain derived from Type B *F. tularensis holarctica* (6). This vaccine was administered to vaccinate human volunteers in the United States and in the former Soviet Union (7, 8). LVS was even administered via aerosol delivery in some studies (9, 10). This strain is avirulent for humans but retains lethality for mice. However, *in vitro*, LVS infects and replicates within A549 cells to the same extent as other *Francisella* strains tested. Due to critical differences in the host response, studies on LVS may not fully represent infection by virulent strains of *Francisella* but will provide important insights into the host response to the vaccine strain (11–14). Hall et al. (15) showed that the *F. tularensis* LVS could infect and replicate within a human airway epithelial cell line, A549, and *F. tularensis* LVS has been shown to be internalized by Type II epithelial cells both *in vitro* (A549, TC-1, and MLE 12) and *in vivo* (C57BL/6 mice) (15–18). Thus, we chose to study the molecular changes in lung epithelial cells following infection by *F. tularensis* LVS.

The mechanism of entry of *F. tularensis* LVS into these cells is unknown, but it is thought to be dependent on an active cell process involving cytoskeletal rearrangement. Both live and dead *F. tularensis* LVS are able to be internalized by epithelial cells with identical kinetics, and this internalization can be blocked by inhibition of actin polymerization (16, 18).

Visual inspection of an infected A459 monolayer shows the “stealth” of the pathogen. Imaging of cells with a 100 MOI infectious dose (15, 17, 18), shows a heterodisperse infection, with some cells in the monolayer becoming infected, whereas others are not. At 24–25 h, A549 cells that are infected show a large, diffuse distribution of bacteria within their cytoplasm (15, 18) and induce little epithelial cell death (17). The mechanism by which *Francisella* LVS are entering the A549 cells may play contribute to this uneven distribution of infected cells.

For clues to the dynamics of the early steps of infection, we investigated the genomic and phenotypic response of A549 cells. The host cell response was evaluated within the first few hours of infection (15 min, 2 h, 6 h, and 16 h), during which time bacteria infect and proliferate within the cytoplasm but do not induce significant secretion of inflammatory mediators or the induction of an apoptotic phenotype. Our results suggest one mechanism of *Francisella* LVS entry into lung epithelial cells and may reveal regulation by *Francisella* LVS of important host pathways in human Type II alveolar epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Epithelial Cells, Bacterial Cultures, and Infection Conditions—** A549 Type II alveolar epithelial cells (American Type Culture Collection, Manassas, VA) were maintained in complete, Ham’s F-12 medium (10% FBS, 1% penicillin/streptomycin). Prior to infection, cells were incubated overnight in antibiotic-free, complete medium. *F. tularensis holarctica* LVS FSC155, NR-646, was obtained from the Biodefense and Emerging Infections Research Resources Repository (Manassas, VA).

Infections were carried out at 1:100 MOI for microarray, qPCR, and amiloride studies and MOI as indicated for microscopy studies.

For microarray and qPCR studies, a spinfection protocol was performed. A549 cells in 6-well plates (1 × 10⁶ cells/ml) were infected by the addition of the suspension of bacteria in complete, antibiotic-free medium onto the cells. Plates were then spun for 5 min at 1200 rpm (290 relative centrifugal force; Eppendorf 5810R). Following spin and after a subsequent incubation of 5 min, the 15 min time point was removed from the incubator and washed three times with PBS. Trypsin/EDTA (0.25%, 0.2 ml) was then added, and the plates were incubated for an additional 5 min (total of 15 min). Cells were collected from the plates by adding 0.8 ml of medium to inactivate the trypsin and placed in sterile DNA/RNA-free Eppendorf tubes and spun at 300 × g for 5 min. RNA was then purified using the RNeasy minikit (Qiagen). RNA quality was confirmed (1% agarose gel and quantified at 260 nm). For subsequent time points, medium was removed and replaced with complete medium containing 50 μg/ml gentamycin in order to kill any extracellular bacteria and model an intracellular infection. At additional time points of 2, 6, and 16 h, cells were similarly collected.

**Microscopy and Immunofluorescence Staining—** A549 cells (1 × 10⁵) were seeded onto each well of an 8-chamber, Lab-Tek II, RS-treated, 8-well glass microscopy slide (Nunc, Inc. (subsidiary of ThermoFisher), Rochester, NY). The following day, 500 MOI *F. tularensis* LVS was suspended in complete, antibiotic-free cell culture medium and added to appropriate wells for infection following the standard 3-h infection protocol, as described previously (20). This represented the t₀ time point. Following an 18-h infection with 2 μg/ml gentamycin, cells were washed and then fixed (ice-cold 3.5% paraformaldehyde, 15 min), washed, and permeabilized (10 min, 0.25% Triton X-100). Blocking (1% BSA, 30 min) was followed by the addition of anti-*Francisella* rabbit IgG antibody (Tetracore, Inc., Rockville, MD) (1:50 dilution in 1% BSA/Tween solution, 1 h at room temperature). R-Phycerythrin goat anti-rabbit IgG (H + L) secondary antibody (Invitrogen) (1:100 dilution, 1 h, room temperature, in the dark) was used. Cells were then washed, stained with DAPI, and imaged using epifluorescence on a Nikon Eclipse C1 confocal laser-scanning microscope system (Nikon Eclipse 90i), using an excitation at 488 nm and an emission at 578 nm.

**Macropinosome Formation Studies—** For amiloride inhibition studies, A549 cells were seeded on 6-well plates (1 × 10⁶ cells/ml), and 3 wells were preincubated with 1 mM amiloride for 2 h. A549 cells were spininfected using *F. tularensis* LVS at a 1:10 MOI in culture medium with or without 1 μM amiloride (5 min, 1200 rpm). After 10 min, cells were washed three times with 50 μg/ml gentamycin to kill extracellular bacteria. Fresh medium was added with or without 1 μM amiloride as appropriate, and the cells were incubated for the required time (2, 6, 16, and 24 h; 37 °C; 5% CO₂). Cells were washed and lysed with sterile water. cfu were determined after 24 h at 37 °C by serial dilutions on TSB-C agar plates in triplicates.

For FITC-dextran uptake studies, A549 cells (1 × 10⁵ in 0.5 ml) were seeded onto each well of an 8-chamber, Lab-Tek II, RS-treated, 8-well glass microscopy slide. The next day 1:10
MOI of *F. tularensis* LVS was added to some wells for infection, and 0.1 mg/ml FITC-dextran (Sigma) was added to all wells, incubated or co-incubated (1 h, 37 °C, 5% CO₂). Cells were washed (PBS), fixed (15 min, 3.5% (w/v) paraformaldehyde), and washed again. Cells were permeabilized (10 min, 0.25% (v/v) Triton X-100 plus PBS) and washed, and Image-it Signal Enhancer (catalog no. 126933, Invitrogen) was added to reduce nonspecific binding. After 30 min, cells were washed and blocked (30 min, 1% (w/v) BSA). A 1:500 dilution of anti- *Francisella* rabbit IgG antibody (Tetracore TC7005) was applied for 1 h. Cells were washed and incubated with a 1:1000 dilution of AlexaFluor 568 goat anti-rabbit IgG (emission at 603 nm) containing the blue actin stain phalloidin (AlexaFluor 350 Phalloidin) (emission at 442 nm) at 5 μl of 6.6 μM phalloidin/ml secondary antibody for 30 min. Following a final wash step, ProLong Gold Antifade Reagent was used to mount the coverslip. Images were obtained using Nikon Eclipse 90i confocal laser-scanning microscopy with three lasers (405, 488, and 568 nm) using a ×60 oil immersion objective.

### Microarrays and Bioinformatics Analysis

Affymetrix HGU133plus2 chips were run using Affymetrix reagents following the manufacturer’s instructions. Briefly, the mRNA was converted into double-stranded cDNA and then to cRNA, biotin-labeled, and fragmented. Fragmented cRNA was hybridized and added to the Affymetrix chip. The CEL files obtained by scanning the Affymetrix HGU133plus2 chips were analyzed using R along with Bioconductor (21). R packages “affy” and “simpleaffy” were used to extract the probe level data and perform quality control analysis, respectively (22). Chips were filtered from the data set based on the following quality control metrics: (a) average background; (b) scale factor; and (c) percentage present. Filtering criteria included a minimum of three chips per group of the data set, with special emphasis for the scaling factor. The RMA algorithm is used for normalization and background correction of the data set (23). The summarized expression data set had eight groups representing infected and uninfected (mock-infected) groups at four time points (15 min, 2 h, 6 h, and 16 h). The statistical analysis for differential expression was performed using the limma package of R (24). The contrasts are defined to extract the differentially expressed transcripts at each time point specified. The p values obtained from the t statistic are adjusted for multiple testing using the Benjamini-Hochberg method (25). Significant transcripts (p value < 0.05) obtained after multiple-test correction were used to perform the functional analysis. Principal component analysis was performed on the significant transcript subsets over all time points to verify the unsupervised clustering pattern between the infected and uninfected sample groups (26). The first three principal components amount to at least 99.95% of the proportion of variance in all of the time points (supplemental Fig. S1). The first principal component represents the similarity between the cell tissue types, whereas the second component acts as a marker for the infection separating the infected and uninfected groups at every time point. In order to select genes in various pathways, an in-house tool was developed to represent the significant transcripts in the corresponding KEGG pathways using KEGG API (data not shown) (27). Pathways obtained from this tool were ranked based on the number of significant genes in the microarray data set, and the complete set of significantly under-/overexpressed transcripts was extracted for each of these pathways. A detailed network-based analysis was performed using the MetaCore GeneGo Knowledge base to further explore the interlaying molecular interactions (28).

### Quantitative PCR

Gene expression was confirmed by quantitative PCR on a Bio-Rad iCycler. Briefly, cDNA was generated from each time point by RT-PCR using the Cells-to-Signal lysis kit (Applied Biosystems/Ambion, Austin, TX), using an equivalent of 2 μg of RNA per 20 μl of RT-PCR. Individual genes were then evaluated by real-time PCR using custom TaqMan array standard 96-well plates (Applied Biosystems, Inc., Carlsbad, CA), which contained Taqman primer/probe sets for 30 unique gene expression arrays (spotted in triplicate). Applied Biosystems Taqman gene expression arrays unique to each gene are shown in Table 1. Genes picked represented 20 genes from all time points that were determined in the microarray study to be significantly changing (Table 1) and 10 genes used for housekeeping (Table 2). qPCR for all conditions evaluated was performed three times. For each housekeeping gene, cDNA from all four uninfected time points was equally pooled, and cDNA from each infected time point was also equally pooled. The resulting two pooled samples were each run in respective Taqman plates in order to biologically normalize each housekeeping gene over multiple time points. The DataAssist software package (Applied Biosystems/Ambion) was used to calculate gene expression using the 2^(-ΔΔCt) method. This package uses the Genorm algorithm to determine the best combination of housekeeping genes and then normalizes them using the method of Vandesompele et al. (29).

### Determination of Significant Gene Ontology and Pathway Analysis

Comparative analysis of gene pathways in this study was done using the Enrichment Analysis program of the GeneGo software package. Genes from all time points (15 min, 2 h, 6 h, and 16 h) were first “enriched” (or selected) based on the following parameters. Genes were picked for comparison if they contained a p value threshold of 0.05 and a program threshold of 0.001 and if signals were detected in both control and experimental samples. Using these parameters, a gene list was generated, which was then compared with known gene pathway ontologies. Each experimentally generated ontology was scored by a p value, representing the probability of a random, hypergeometric intersection of the generated gene list with genes of the known ontology. In this way, the lowest p values represent the highest probabilities that a particular ontology is relevant to the experimental conditions. Relevant ontologies selected from the GeneGo-curated database were then reported as (a) canonical pathways and (b) process networks.

### RESULTS

**F. tularensis LVS Establishes an Intracellular Infection of A549 Cells**—In order to confirm the establishment of a stable intracellular infection by *F. tularensis* LVS over the time line evaluated in this microarray study, we performed immunofluorescence microscopy to detect intracellular bacteria. Fig. 1 shows the intracellular, stable, polydisperse infection of A549...
cells at 18 h (Fig. 1, A–E). Bacteria are localized in the cytoplasm and excluded from the nucleus (stained blue with DAPI), and there was virtually no loss or change in gross morphology of A549 cells by differential interference contrast microscopy over the time course of infection (data not shown). Stable, active infection was confirmed by MOI counts of *F. tularensis* LVS at each time point evaluated by microarray, indicating steadily increasing bacterial numbers over 24 h (Fig. 2).

The number of intracellular *F. tularensis* LVS bacteria over these time points is lower than the number reported for a standard 3-h infection protocol (15, 18), probably due to our highly abbreviated infection process (30). Briefly, bacterial cells are “spun” down onto the epithelial cells (“spinfection”), and gentamycin is added after only 15 min of uptake to kill extracellular bacteria. Other investigators allow 2 h for bacterial uptake before applying the gentamycin for an additional 1 h (2 + 1), designating time 0 (\( t = 0 \)) at 3 h after the initial application of the bacteria. The spinfection approach allows us to study the establishment of a stable, intracellular, cytoplasmic infection at early time points under 3 h.

An Initial Genomic Response Is Followed by Suppression at Later Time Points—The microarray data set consists of a total of 31 Affymetrix chips representing the infected and uninfected cells from four different time points: 15 min, 2 h, 6 h, and 16 h postinfection. Principal component analysis over all time points shows the expected grouping of infected and uninfected samples (supplemental Fig. S1). In addition, qPCR was performed for selected genes that were differentially expressed in our microarray analysis (Tables 1 and Table 2; housekeeping genes used for qPCR). Table 1 demonstrates agreement...
between our qPCR and microarray data, both in direction and general -fold change. Transcripts determined to be differentially expressed were selected from the significance analysis at every time point (supplemental Table 1) and are illustrated in the Venn diagram (Fig. 3). Interestingly, differential expression decreases with time; the earlier time points (15 min and 2 h) show many changes upon initial interaction with bacteria, but at later time points (6 and 16 h), there is little difference between the infected and uninfected groups. This indicates an initial activation of cellular response, followed by a general suppression of response by 6 h postinfection. The suppressed transcriptional response that we observe is in agreement with phenotypic observations for these cells, in which no significant morphological changes or innate immune mediators appear in the first 24 h following infection (data not shown). Effects from spinning during spinfection on actin cytoskeleton rearrangements are not observed in phenotypic experiments and are controlled for in microarray experiments from direct comparison with controls that received the same treatment.

Pathway Activation Analysis Implicates Macropinocytosis as a Novel Entry Mechanism for F. tularensis LVS in A549 Cells—Pathways that were the most highly activated were selected and scored using the MetaCore (GeneGo) Enrichment Analysis program. Table 3 lists the activated pathways determined from the differentially expressed genes in this study. Cytoskeleton/actin rearrangement and integrin binding (focal adhesion kinase signaling) are among the significant pathways, consistent with the reported importance of actin polymerization during epithelial cell and macrophage invasion by F. tularensis LVS (16, 18, 31).

Our analysis indicates with high confidence activation of the macropinocytosis pathway (Table 3). This is a cellular process of engulfment of particles, apoptotic bodies, antigens, macromolecules, and even pathogens into a cell. Whereas endocytosis and phagocytosis display uniform and regular engulfment of small particles, macropinocytosis involves the irregular blebbing of the cellular surface and an intake of large amounts of extracellular material. This mechanism was investigated phenotypically through two experimental approaches, FITC-dextran uptake and inhibition with amiloride, which are described below.

**Interferon Pathways and Some Inflammatory Mediators Are Induced, whereas Other Innate Immune Response Pathways Are Mostly Silent**—The interferon α/β signaling pathway is activated in our system, as is the expression of IL-8 (CXCL8), a major mediator of inflammatory response (Table 3). In addition, we identified the up-regulation of three chemokine genes as a result of Francisella LVS infection: CXCL1, CXCL2, and CXCL3 (Table 1). These CXCR2 ligands (CXCL1 and CXCL2/3) are chemokines thought to be important for neutrophil mobilization and migration (32), are functional homologs of human CXCL8 (IL-8) (11, 33), and may be involved in various forms of lung injury (33, 34). Interestingly, other innate immune pathways are conspicuously absent. Francisella LVS interaction with the Toll-like receptors (TLRs) has been well studied, with LPS binding and activating TLR2 in conjunction with TLR1 and TLR6 (35–39). In this study, we found that TLR1 had a 2-fold increase in gene expression following a 2-h *F. tularensis* LVS infection (*p* = 0.02; supplemental Table 1); however, no significant change in gene expression was observed for TLR2, TLR6, or other TLRs under these assay conditions and at these early time points. TLR2 is up-regulated in macrophages and DCs during LVS infection (36, 39, 40), suggesting that there may be cell type-specific differences.

An interesting note is the activation of pathways for selenium and selenoamino acid incorporation into proteins during translation (Table 3). The role of selenoamino acids in *Francisella* pathogenesis is currently unaddressed but may reflect the facultative intracellular lifestyle of this bacterium. Selenoamino acids were found to be important for the metabolism of other bacteria (41, 42). Several key genes in selenocompound metabolism are found in LVS (KEGG pathway f000450).

**Cell Survival and Pro-/Antia apoptotic Pathways Are Affected**—We have previously reported a lack of any significant A549 cytotoxicity during the first 24 h of *Francisella* infection (17), but it is not clear how the bacteria subverts or evades the induction of apoptotic signaling pathways. This is particularly important for the establishment of tularemia because *Francisella* requires 2–4 h to escape the phagosome, followed by replication in the cytosol until it can establish an infection.

Caspase signaling is a classical pathway modulating apoptosis. We observed that gene expression of caspase 9 (CASP9), an apoptosis initiator that responds to mitochondrial cytochrome c leakage, was up-regulated somewhat (1.6-fold, *p* = 0.035) at 2 h, whereas expression of caspase 6 (CASP6), an effector downstream of other caspases was down-regulated (0.68-fold, *p* = 0.013) following 15 min of LVS infection. CFLAR (CASP8 and FADD-like apoptosis regulator), an antiapoptosis regulator whose suppression has been suggested to prevent apoptosis (data not shown) (28), was shown to be down-regulated early (15 min) with high confidence (0.63-fold, *p* = 0.015). These proapoptosis programs could prevent bacterial “hijacking” of
the intracellular environments of cells, but this response is lost by the 6 h time point.

The regulation of apoptosis related to MAPK gene expression (MPK1, MPK2, and MPK3) is more difficult to interpret. In our study, the majority of MPK1, -2, and -3 genes that we detected (supplemental Table 1) are down-regulated at early time points and then return to normal expression at later time points, illustrating the suppression of cell signaling and blocking of a potential apoptotic phenotype. However, other studies and cell types have indicated a proapoptotic effect of Francisella-induced MAPK disruption. Hrstka et al. (43) have shown that p38 and p44 are down-regulated, which causes murine macrophages to undergo apoptosis. Lai et al. (44, 45) demonstrated that during infection with Francisella, murine macrophages begin to signal stress events and eventually the apoptotic signaling cascade. During a 48-h infection time course, macrophage cell detachment, morphological changes, and an increase in the activity of the proapoptotic caspases 3 and 9 was observed (46). This differs from our study, in which A549 cells after 16–24 h of infection do not show an indication of an apoptotic phenotype. Type II epithelial cells may therefore provide a foothold in the mucosal superstructure for the infection to propagate.

Consistent with other Francisella studies was the effect on Akt, a serine/threonine kinase that is recruited to the cell membrane upon cell surface stimulation. In our study, we found a...
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TABLE 2
Genes evaluated and/or used as housekeeping genes in this study
For each replicate, the housekeeping genes whose Ct values were the most stable (i.e. changed the least) as determined by M value were picked for normalization and used as housekeeping genes. The M value represents the measure of internal control gene stability, which is the average pairwise variation of a particular gene with all other control genes. Genes with the five lowest M values were chosen: YWAHZ, UBC, POLR2A, 18S, and GAPD. Normalization was done using the GeNorm algorithm of Vandesompele et al. (29).

| Symbol | Gene name | Taqman primer/probe set | M value |
|--------|-----------|-------------------------|---------|
| 18S    | 18S ribosome | Hs9999901_s1 | 0.7989  |
| B2M    | Human B2 M (β-2-microglobulin) | Hs9999907_m1 | 1.1522  |
| GAPD   | Human GAPD (GAPDH) | Hs9999905_m1 | 0.8386  |
| HMBS   | Hydroxymethylbilane synthase | Hs00609297_m1 | 0.9714  |
| HPRT1  | Hypoxanthine phosphoribosyltransferase 1 | Hs9999909_m1 | 0.8962  |
| POLR2A | Polymerase (RNA) II (DNA-directed) polypeptide A | Hs01108291_m1 | 0.7231  |
| SDHA   | Succinate dehydrogenase complex, subunit A, flavoprotein (Fp) | Hs00417200_m1 | 0.9791  |
| TRP    | Human TRP (TATA-box binding protein) | Hs9999910_m1 | 2.2045  |
| UBC    | Ubiquitin C | Hs00824723_m1 | 0.6857  |
| YWAHZ  | Monoxygenase activation protein, ζ polypeptide | Hs00237047_m1 | 0.6777  |

TABLE 2 (continued)

### FIGURE 3. Comparison of transcripts.
Venn diagram showing the up- and down-regulated transcript quantifications (represented in red and blue, respectively) at each of the time points. Differentiation between infected samples and uninfected controls is significant at the earlier time points (15 h and 2 min) and then falls by 6 h, at which point very little difference from the uninfected control is detectable.

significant down-regulation of AKT3 expression after 15 min of infection (Table 1). The AKT pathway influences actin polymerization/dem polymerization, which allows the cell to migrate throughout the body; however, it can also regulate cell cycle, cell survival, and NF-κB-dependent gene transcription. Interestingly, Akt has been studied in other tularemia model systems, appearing to negatively regulate NF-κB while up-regulating MAPK, ERK, p38, JNK (47–52). Interestingly, Akt is implicated in Francisella phagosomal escape (52), and Akt expression was also substantially down-regulated in Francisella novicida- and SchuS4-infected human monocyes (53). Akt plays a central role in regulating the host response to Francisella infection (47–52), so this finding serves as an internal validation for our results.

Genes for Orphan Nuclear Receptors and Ubiquitination Are Affected—Interestingly, the expression of a group of NR4A type orphan nuclear receptors was strongly up-regulated following F. tularensis LVS infection (Table 1 and supplemental Table 1). The expression of NR4A receptors has been shown to be highly induced in macrophages exposed to bacterial LPS and cytokines, such as INF-γ and TNF-α (54, 55). Up-regulation of these orphan nuclear receptors has been found to be proinflammatory (56), and they are known to be early response genes to multiple stimuli. Specifically, we identified up-regulation of NR4A2 (Nurr1; 40-fold by qPCR), NR4A3 (NOR1; 2.6–3.62-fold), and a related orphan nuclear receptor gene NR1H2 (LXR-B; 1.5-fold) following 2-h F. tularensis infection. LVS infection (Table 3 and supplemental Table 1). Thus, the up-regulation of these orphan nuclear receptors may reflect a mechanism by which F. tularensis LVS induces the later proinflammatory response in host cells.

Host cell recognition of intracellular pathogens via ubiquitination is another important aspect of Francisella infection (57–59). For Salmonella typhimurium, Perrin et al. (60) have demonstrated that ubiquitinatation is one way that host cells can recognize the presence of bacterial pathogens in their cytosol. In our study, we identified many ubiquitin proteases that were down-regulated at 15 min following F. tularensis LVS infection. These include USP-1, -9X-33, and -37, a group of ubiquitin-specific peptidases that are regulated by phosphorylation and cleave ubiquitin from ubiquitinated proteins. Andersson et al. (61) and Akimana et al. (58) identified the related Usp-22 as down-regulated in their studies. We also identified down-regulation of UBE2H (ubiquitin-conjugating enzyme E2H), a member of the E2 ubiquitin-conjugating enzyme family that is capable of ubiquitinating histone H2A and probably catalyzes the covalent attachment of ubiquitin to other proteins. This suggests that A549 cells lose the ability to recognize intracellular F. tularensis.

Cytoskeleton and Actin Rearrangement and Extracellular Matrix Genes Are Affected but Do Not Result in Observable Disruption of the Cell Monolayer—Here we report the high activation of networks for cytoskeleton rearrangement, remodeling (e.g. focal adhesion kinase signaling), and mitosis (Table 3 and supplemental Table 1) at early time points. This is followed by a general deactivation of these genes/pathways at 6 and 16 h, which is also consistent with our observations of cell morphology (Fig. 1) (16, 18). In addition, we also observed a significant down-regulation of a collection of collagen genes associated with the extracellular matrix (ECM): COL4A4 and COL4A4BP at 15 min and COL4A1 and COL4A5 at 2 h postinfection (supplemental Table 1). All of this is despite the little changes in overall monolayer morphology observed over 16 h.

Experimental Support for Macropinocytosis as One Mechanism of F. tularensis LVS Internalization—The microarray and MetaCore pathway analysis findings suggested that macropi-
nocytosis may be one mechanism by which *Francisella* is taken up into the non-phagocytic A549 epithelial cells. To further investigate this result, we performed experiments using a known actin rearrangement inhibitor, amiloride, which has been previously used to demonstrate macropinocytosis in A549 and other cell types (62, 63). A549 epithelial cells preincubated with or co-incubated with amiloride showed greatly reduced *F. tularensis* LVS internalization (Fig. 4A). Additionally, amiloride was not cytotoxic to the bacteria (data not shown). In further support of this mechanism, it had been previously published that other actin cytoskeleton inhibitors, such as cytochalasin D, also interfere with the uptake of *Francisella* into macrophages and A549 epithelial cells (31, 62, 64, 65), supporting our results.

To verify the process of macropinocytosis for *F. tularensis* LVS uptake, fluorescence microscopy experiments were performed using FITC-dextran, a large molecule that is known to be taken up in macroinosomes (62). A549 epithelial cells do not spontaneously take up FITC-dextran (Fig. 4B). However, infection of A549 cells with *F. tularensis* LVS strongly induced the formation of FITC-labeled structures on the cell (Fig. 4C). Many FITC-dextran-filled macroininosomes were observed in A549 epithelial cells after a 1-h incubation with *F. tularensis* LVS. *F. tularensis* LVS was also observed to be co-localized within the macroinosome (Fig. 4D). This supports our conclusion that the mechanism of macropinocytosis can be used as a route of internalization of *F. tularensis* LVS into Type II epithelial cells.

**DISCUSSION**

In this study, we examined the infection and global transcriptional response of Type II epithelial A549 cells during *F. tularensis* LVS infection. We applied the bacteria to the cells by performing a “spinning” to quickly place bacteria on top of the cells (30), enabling us to examine very early time points in the infection process. Thus, the overall numbers of bacteria that have replicated within our cells are lower than in other reports (14), but the advantage is that we are able to examine host transcriptional events that occur at the early stages of intracellular infection.

We observe a strong initial transcriptional activation, followed by a return to uninfected control levels. Strong effects are observed on membrane-associated receptors, extracellular matrix, and cytoskeletal signaling genes. We report significant changes in gene expression in pathways for cytoskeleton rearrangement, as well as the ECM genes COL4A4, COL4A4BP, COL4A1, and COL5A1. These Type IV and Type V α-collagen chains form part of the basement membrane, participate in focal adhesions, and interact with integrins, allowing cytoskeleton remodeling due to integrin outside-in signaling from collagen fibers. MMP9 (matrix metalloprotease 9), an ECM-degrading protease, plays an important role in *Francisella* infection. MMP9 is induced by *F. tularensis*, resulting in the overrecruitment of self-destructive leukocytes to the sites of infection (66). Our findings may indicate a cooperative effect of MMP9-mediated degradation of the ECM, coupled with an inability to maintain or reestablish ECM production.

An unexpected finding in our study was the high activation of genes for selenium utilization. Selenium is considered an essential element for most living organisms, either as a cofactor in enzymes or in selenoamino acids (67) and can even be substituted in various enzymes when critical trace elements such as sulfur are lacking. When living as an intracellular pathogen, *Francisella* has to obtain all of its essential elements from the host cell that it has infected (68), so the induction of selenium utilization in the host may be a response to the sequestration of sulfur or other trace elements by *Francisella*. *Francisella* may also require selenium and work to activate its utilization. Interestingly, *Francisella* has the A-subunit of formate dehydrogenase (fdh) within the *Francisella* pathogenicity island (69), which is a selenoamino acid-containing enzyme (70), and the pathogenicity island is required for bacterial replication within the host. *Francisella* does possess some selenoamino acid biosynthetic enzymes (KEGG pathway ftl00450) but appears to lack critical steps in the biosynthetic pathway, suggesting that this

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**TABLE 3**

*GeneGo analysis of networks and pathways*

| Process                              | Network                                                                 | *p* value |
|--------------------------------------|------------------------------------------------------------------------|-----------|
| Cytoskeleton                         | Actin filaments                                                        | 2.167e−03 |
| Cytoskeleton                         | Macropinocytosis and its regulation                                    | 9.721e−03 |
| Cell adhesion                        | Attractive and repulsive receptors                                     | 1.269e−02 |
| Cell cycle                           | Mitosis                                                                | 1.369e−02 |
| Cytoskeleton                         | Regulation of cytoskeleton rearrangement                                | 1.530e−02 |
| Translation                          | Selenium pathway                                                       | 1.897e−02 |
| Transcription                        | Chromatin modification                                                 | 2.883e−02 |
| Translation                          | Translation initiation                                                 | 5.941e−02 |
| Cell adhesion                        | Integrin-mediated cell-matrix adhesion                                 | 1.005e−01 |
| Inflammation                         | Interferon signaling                                                  | 1.127e−01 |

**Process** Pathway                                                                 **p** value
Translation (L)-Selenoamino acid incorporation in proteins during translation 3.478e−04
Cyto...
trace element and its incorporation may be important for Francisella growth.

The role of cytokines and chemokines in Francisella infection has been the subject of intense study. In this study, we confirmed the up-regulation of CXCL1, -2, -3, and -8 (IL-8) in the early stage of infection in A549 cells (14). It has been found that TNF-α, IFN-γ, IL-1β, and many other cytokines and chemokines are produced by macrophages following F. tularensis LVS infection (14). F. novicida infection of J774A.1 murine macrophages for 12 h does not induce CXCL1 or CCL20 expression, although infection with a Δ-pdpA mutant of F. novicida induced a significant increase in CXCL1 and CCL20 gene expression (71). In vivo, Mares et al. (32) determined that CXCL1 and CXCL2 were produced as chemokines in BALF and serum in F. novicida-infected mice but not until 3 days postinfection. Further, at 72 h after intranasal infection, CXCL1 and CXCL2 were found to be elevated in F. novicida-infected mice but less elevated in Δ-p58 protein F. novicida mutant-infected mice (5). In peritoneal macrophages derived from C57BL/6J mice, the gene expression of CXCL1 (KC) was slightly increased following 4 h of F. tularensis LVS expression but was greatly increased by 4 h of infection with the Δ-igC mutant, which cannot escape the phagosome, an event that usually happens at 4 h postinfection, using the standard 3-h preinfection protocol (14).

Francisella infection in host cells has a direct but also an indirect effect on TLR signaling. Our findings (consistent with
other reports) suggest that Francisella appears to disrupt multiple points along the TLR response pathway, including expression of NF-κB, MAPK, ERK, JNK, and p38, causing a decrease in expression of TNF-α, IL-1, and IL-8, all of which are needed to produce an adaptive immune response (73). The disruption of NF-κB signaling is used as an approach by multiple pathogens to reduce the immune response. This suggests that the ability of Francisella may hamper the normal signaling cascade within 2 h after infection (49). The mechanism by which this happens is not yet determined.

Ubiquitination genes involved in intracellular bacteria “recognition” were also affected by Francisella LVS infection, perhaps explaining the “stealth” of Francisella, causing quiescence of gene expression. Interestingly, other pathogens, such as Yersinia pestis, also modulate the ubiquitin pathway in the host (74, 75).

Cellular entry is a key step to establishing intracellular infection. Macropinocytosis is triggered by tyrosine kinase receptor activity either through direct contact with extracellular molecules or through the detection of secreted cytokines. The tyrosine kinase Syk has been shown to be important for Francisella entry into host cells (49, 76). Tyrosine kinase receptor activation results in the actions of Rho guanosine triphosphatases (Rho, Cdc42, and Rac) along with phosphoinositide 4,5-biphosphate. This activity triggers the binding of Wasp/Scar to phosphoinositide 4,5-biphosphate, activating actin and Arp2/3 and resulting in actin polymerization. Unlike the limited actin polymerization in endocytosis and phagocytosis, macropinocytosis actin polymerization occurs in a wide area of the cell membrane around the area of activation. This causes the membrane to branch or “ruffle” over a macromolecule and then fuse back into the cell membrane (77). In this study, macropinocytosis-related genes in A549 cells were strongly affected by Francisella LVS infection, and macropinocytosis was demonstrated by FITC-dextran uptake assays as well as by amiloride inhibition. This differs from a recent report of Law et al. (78) studying a different Francisella species in a different cell type. They demonstrated that F. novicida does not use an amiloride-sensitive pathway for entry into murine hepatocytes but instead utilizes a cholesterol and clathrin-based endocytic mechanism of entry. Whether this difference is due to the cell type (human alveolar epithelial versus murine hepatocyte) or differences between the attenuated vaccine strain F. tularensis LVS and F. novicida will need to be examined in future studies.

Multiple pathogens elicit macropinocytosis in host cells. Salmonella, Legionella, and Shigella all cause macropinocytosis by either Type III or Type IV secretion in epithelial cells, and both pathogenic and non-pathogenic Mycobacterium species can be directly internalized by macropinocytosis (62, 72, 79–81). Adenovirus binding to integrin receptors can also trigger the induction of macropinocytosis, resulting in virus internalization (19). Although macropinocytosis was not explicitly described, Clemens et al. demonstrated that Francisella binding to CR3 (complement receptor 3) receptors in macrophages results in over-sized ruffled phagosomes similar to what is seen during macrophagocytosis (31). Cytochalasin D inhibits macropinocytosis of multiple pathogens, and indeed, cytochalasin D has been reported to inhibit F. tularensis internalization by macrophages and A549 cells (31, 62, 64). For Mycobacterium infection, amiloride has also been shown to inhibit macropinocytosis in A549 cells and therefore greatly decrease mycobacterial entry (62, 63), consistent with our results. Amiloride reduced the number of F. tularensis LVS bacteria inside A549 cells in this study, and macrophagosome formation was strongly induced in the presence of the bacteria, suggesting that macropinocytosis may be one mechanism for entry.

We therefore conclude that macropinocytosis is one possible mechanism of entry during the establishment of F. tularensis LVS infection in human Type II alveolar epithelial cells and that the LVS of Francisella induces significant host cell signaling at very early time points after the bacteria’s interaction with the human Type II alveolar epithelial cells.

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