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Francisella Acid Phosphatases Inactivate the NADPH Oxidase in Human Phagocytes

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Francisella tularensis contains four putative acid phosphatases that are conserved in Francisella novicida. An F. novicida quadruplet mutant (AcpA, AcpB, AcpC, and Hap [ΔABC]) is unable to escape the phagosome or survive in macrophages and is attenuated in the mouse model. We explored whether reduced survival of the ΔABC mutant within phagocytes is related to the oxidative response by human neutrophils and macrophages. F. novicida and F. tularensis subspecies failed to stimulate reactive oxygen species production in the phagocytes, whereas the F. novicida ΔABC strain stimulated a significant level of reactive oxygen species. The ΔABC mutant, but not the wild-type strain, strongly colocalized with p47phox and replicated in phagocytes only in the presence of an NADPH oxidase inhibitor or within macrophages isolated from p47phox knockout mice. Finally, purified AcpA strongly dephosphorylated p47phox and p40phox, but not p67phox, in vitro. Thus, Francisella acid phosphatases play a major role in intramacrophage survival and virulence by regulating the generation of the oxidative burst in human phagocytes. The Journal of Immunology, 2010, 184: 000–000.

Francisella tularensis has been classified by the Centers for Disease Control and Prevention as a category A pathogen. Inhalation of <10 CFU of F. tularensis subspecies tularensis (hereafter F. tularensis) can have fatal consequences (1). F. tularensis can enter and multiply in a wide range of host cell types (2–9); however, in vivo, its primary target is the phagocyte (10). F. tularensis enters host macrophages by asymmetric pseudopod loops, and this uptake is dependent on serum complement and host cell receptors, including C3, mannose, and scavenger (3, 11–13). After entering the host cell, the bacterium arrests maturation of the phagosome, and the phagosome is transiently acidified. This acidification is reported to be essential for the subsequent escape of F. tularensis into the cytosol of the macrophage (14, 15), but more recent work has contradicted this (15, 16). Eventually, the phagosomal membrane is compromised by unknown mechanisms, and the bacteria escape into the cytoplasm and replicate (15, 17, 18). Subsequently, it was reported that the bacteria re-enter the endocytic pathway by an autophagy-like process, residing in large multiple-membrane bound vesicles (17, 19). Bacterial release from host cells is thought to occur following Francisella-induced apoptosis (20–22) and pyroptosis (21). However, the final stages of the intracellular cycle are not well understood.

The intracellular life cycle of F. tularensis is complex, and the genes involved with all stages are not well understood. However, the key genes involved in Francisella survival in host cells are found on the Francisella pathogenicity island (FPI). This island contains 19 genes, and bioinformatic analysis revealed that the FPI encodes a putative type VI secretion system, similar to the systems involved in the virulence of Pseudomonas aeruginosa (23) and Vibrio cholerae (24). Genes within the FPI are regulated by MglA, SspA, PrnA, MigR, Hfq, and FevR (25–31). Transcriptional analysis indicated that many genes outside of the FPI are also affected by these regulators (27, 30).

In addition to the FPI genes, Francisella acid phosphatase AcpA was shown to play a key role in intracellular survival in macrophages (19, 32, 33). Bioinformatic analysis revealed that Francisella carries four or five acid phosphatases in its genome, depending on the species. Deletion of acpA resulted in a strain that was more susceptible than wild-type (WT) Francisella tularensis subspecies novicida (F. novicida) to killing by human and murine macrophages and had decreased phosphatase activity (32). Additionally, the mutant showed a decreased ability to escape from the phagosome (32). Deletion of three additional acid phosphatases (AcpA, AcpB, AcpC, and Hap [ΔABC]) in F. novicida resulted in an attenuated strain that was 100% protective against homologous challenge in the mouse model. This mutant did not escape from the macrophage phagosome (19). Transcriptional analysis

Abbreviations used in this paper: ΔABC, deletion of acid phosphatases AcpA, AcpB, AcpC, and Hap; Acp, acid phosphatase; BMDM, bone marrow-derived macrophage; DPI, diphenylene iodonium; FN, Francisella novicida; FKFN, formalin-killed Francisella novicida; FPI, Francisella pathogenicity island; LVS, live vaccine strain; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; OPZ, serum-opsonized zymosan; PKC, protein kinase C; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; SchuS4, F. tularensis subspecies tularensis SchuS4; WT, wild-type.

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demonstrated that AcpA and Hap expression are increased during the early stages of macrophage infection (19).

Human neutrophils play a key role in host defense against invading pathogens and are major effectors of the acute inflammatory response. In response to a variety of agents, neutrophils produce a large amount of superoxide anion (O$_2^-$), which is essential for bacterial killing and amplifies the inflammatory response (34, 35). The NADPH oxidase complex is responsible for O$_2^-$ production, and its activation is induced by receptor ligation and uptake of microbial pathogens or chemical or particulate stimuli, such as PMA, fMLF, or zymosan. NADPH oxidase induction in neutrophils or macrophages requires the translocation of phosphorylated p47$^{phox}$, p67$^{phox}$, and Rac1/2 from the cytoplasm to the phagosomal membrane (34). However, F. tularensis live vaccine strain (LVS) enters neutrophils without triggering the respiratory burst and inhibits NADPH oxidase assembly by an unknown mechanism (36). Earlier studies showed that purified AcpA isolated from Francisella inhibited the respiratory burst of fMLF-induced porcine neutrophils in vitro (33). This suggests that Francisella may alter the function of the NADPH oxidase complex in a manner involving acid phosphatase dephosphorylation of key cellular components. We show in this study that the Francisella acid phosphatases are required for inhibiting NADPH oxidase dase assembly and function and may do so by dephosphorylation of NADPH oxidase components. This process aids the intracellular survival and proliferation of the bacterium.

Materials and Methods

Isolation of human monocyte-derived macrophages and neutrophils for infection with Francisella spp

Using an Ohio State University-approved Institutional Review Board protocol, heparinized blood samples were collected from normal human donors. The heparinized blood samples from normal human donors were diluted 1:1 with normal saline, and the fraction containing the PBMCs was obtained following centrifugation at 800 x g for 40 min at 20°C over a Ficoll-Hypaque density cushion (Amersham Biosciences, Piscataway, NJ). This fraction was diluted 1:1 with RPMI 1640, and the PBMCs were collected again by centrifugation. The cells were washed twice with RPMI 1640, resuspended in the same media, and counted in a hemocytometer. The cell numbers were adjusted to 2 x 10^6 cells/ml in RPMI 1640 containing 20% autologous human serum. The monocytes were allowed to differentiate into monocyte-derived macrophages (MDMs) by incubation for 5 d at 37°C in 5% CO$_2$ in sterile screw cap Teflon wells. PBMCs (MDMs plus lymphocytes) were recovered and resuspended in RPMI 1640 with 10% autologous serum, and MDMs were plated in 24-well tissue culture plates or on coverslips at a density of 2 x 10^5 cells/well in 0.5 ml culture media, resulting in 2 x 10^3 MDMs/monolayer. For the respiratory burst experiments, the MDMs were placed in monolayer culture at 10^5 cells/well in 0.2 ml culture media in 96-well plates. After 2 h of incubation at 37°C in 5% CO$_2$, nonadherent cells were removed by washing the monolayers three or four times with prewarmed RPMI 1640 and replated with fresh culture medium with 10% autologous serum. For intramacrophage survival assays, MDMs were incubated for an additional 7 d with bacteria containing 20% autologous serum to stabilize the MDM monolayer prior to infection (37). Macrophages were incubated with Francisella at a multiplicity of infection (MOI) ~50:1, as described earlier (12). At various time points, macrophages were lysed with 0.05% SDS and plated on Chocolate II plates to enumerate the CFU. This lysis protocol does not result in death of the bacteria (38).

After PBMC separation on Ficoll-Hypaque, the RBC-containing pellet was further fractionated by dextran sedimentation to separate the neutrophils. Briefly, RBCs were diluted 1:1 with normal saline, and an equal amount of 3% dextran was added to sediment the RBCs. After 30 min incubation on ice, the top layer of cells was transferred to a new tube and centrifuged at 800 x g for 15 min to enrich for the neutrophils. The erythrocytes that remained in the cell pellet were lysed with sterile distilled water for 15 s, and an equal amount of HBSS (without calcium and magnesium ions) containing 0.9% normal saline was added to prevent the lysis of neutrophils. Neutrophils were separated by centrifugation at 800 x g for 5 min. The cell pellet was washed, resuspended in HBSS media, and kept on ice for experimentation.

Growth and opsonization of Francisella spp

F. novicida U112, AcpA, and AABCH mutants were routinely cultured, as previously described (19). For phagocyte infection studies, Francisella strains were grown on Chocolate II plates overnight at 37°C and collected in HBSS buffer or RPMI 1640 (without phosphate). Cell density was determined spectrophotometrically at 600 nm; bacteria were opsonized with 50% autologous serum for 30 min at 37°C and subsequently washed three times with HBSS buffer to remove excess serum. F. tularensis subspecies tularensis (Schu S4, Type A) and F. tularensis subspecies holarctica (OR96-0246 from Biodense and Emerging Infections Research Resources Repository, Type B, Manassas, VA) were cultivated and opsonized similarly to F. novicida strains. Opopsonized Francisella were resuspended in appropriate buffer and kept on ice for experiments. Formalin-killed Francisella were prepared as described earlier (11). Zymosan beads were opsonized with 50% autologous serum, washed twice in HBSS without divalent cations, and kept on ice for the experiments.

Neutrophils (2 x 10^6 cells/well) were seeded onto human serum-coated 24-well plates in RPMI 1640, and Francisella were added at an MOI ~50:1. At time points ~2 h postinfection, cells were washed and treated with 50 μg/ml gentamicin for 30 min, followed by washing with HBSS to remove the extracellular bacteria. Neutrophils were lysed by the addition of 0.05% SDS, and lysates were diluted in HBSS and plated on Chocolate II plates to enumerate the CFU.

Microscopy of Francisella association with and uptake by polymorphonuclear leukocytes

The association with and uptake of F. novicida and acid phosphatase mutants by polymorphonuclear leukocytes (PMNs) were performed as described earlier (12). In brief, neutrophils (2 x 10^6 cells/well) were seeded onto human serum-coated 24-well plates in RPMI 1640, and opsonized Francisella was added at an MOI ~50:1 and incubated for 10 min at 37°C in 5% CO$_2$. After incubation, the cells were washed extensively with RPMI 1640 to remove nonadherent bacteria and fixed in 3.5% paraformaldehyde without permeabilization or permeabilized after parafomaldehyde fixation with chilled 100% methanol for 15–30 s. The coverslips were washed and allowed to dry. Phagocyte-associated bacteria were visualized by indirect immunofluorescence microscopy (34). In this assay, monolayer cultures were infected, incubated with a monoclonal mouse anti-F. novicida LPS primary Ab (Immunoprecise Antibodies Limited, Victoria, British Columbia, Canada) and diluted 1:100 in blocking buffer composed of 20% human AB serum (Cambrex, East Rutherford, NJ) and 5% BSA (Sigma-Aldrich, St. Louis, MO) for 4 h at room temperature with gentle rotation. After being washed extensively, PMNs were incubated with Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Molecular Probes, Eugene, OR) (diluted 1:1000 in blocking buffer) for 90 min at room temperature. Coverslips were mounted on glass slides. In all assays, the average number of bacteria per PMN on each coverslip was determined by counting a minimum of 200 cells per coverslip. The number of associated bacteria was assessed by scoring permeabilized PMNs, and total associated bacteria were assessed by scoring permeabilized PMNs. The number of bacteria taken up (internalized) was calculated by subtracting the number of attached bacteria from the number of associated bacteria.

Respiratory burst assays

Neutrophils (10^6/well) were added to human serum-coated microtiter wells containing 5% human AB serum, and 50 μm luminol (Invitrogen, Carlsbad, CA) and left on ice for 15 min. Subsequently, serum-opsonized Francisella spp. were added at an MOI ~50:1. The microtiter plate was centrifuged at 400 x g for 2 min at 12°C to synchronize the infection. The relative amount of reactive oxygen species (ROS) generated by neutrophils over time was detected by measuring the luminescence by addition of luminol as a substrate in an ELISA reader (Spectramax M5, Molecular Devices, Sunnyvale, CA) after warming the cells to 37°C. In similar fashion, MDMs (10^5/well) were added in 0.2 ml culture media in 96-well plates. After 2 h of incubation at 37°C in 5% CO$_2$, nonadherent cells were removed by washing the monolayers three or four times with prewarmed RPMI 1640 and replated with fresh culture medium containing 10% autologous serum, and the ROS production in MDMs in response to serum-opsonized Francisella (MOI ~50:1) was determined by measuring luminescence over time using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) equipped with 96-well plate detection (National Diagnostics, Atlanta, GA) with lucigenin as the substrate. Human serum-opsonized zymosan particles (MOI of 20:1) and PMA (200 nM) were used as positive controls for ROS production in
neutrophils and MDMs. The inhibition of ROS production by Francisella spp. was tested by incubating phagocytes with Francisella for 10 min at 37°C prior to adding opsonized zymosan. ROS production was detected, as described above, in an ELISA reader.

ELISA to detect complement component deposition on Francisella strains
C5-depleted fresh human serum (Complement Tech, Tyler, TX) was used to evaluate complement component C3 deposition on Francisella strains, as described (39). Briefly, after preblocking microcentrifuge reaction tubes for 30 min in PBS with 0.1% HSA (ZLB Plasma, Boca Raton, FL), 3 × 10^6 bacteria/reaction were incubated in 10% or 50% serum for 30 min at 37°C. Reactions were stopped, and samples were washed twice in blocking buffer. A total of 3 × 10^7 bacteria in suspension were added to medium-binding polystyrene wells in triplicate (Costar, Cambridge, MA) and left to dry overnight. Wells were blocked overnight at 4°C with 3% OVA. After extensive washing with PBS, primary Ab (goat anti-rabbit C3 diluted 1:10,000 in 0.3% OVA [Quidel, San Diego, CA]) was added for 1 h at room temperature. HRP-conjugated rabbit anti-goat IgG (Bethyl Laboratories, Montgomery, TX) was added at an MOI ∼50:1, and the infection was synchronized by centrifugation at 400 × g for 2 min at 12°C. At different time intervals, uninfected and infected cells were lysed in TN1 buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM Na_2PO_4, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 10 mM Na_2VO_3, 10 μg/ml each aprotinin and leupeptin). The cell lysates were boiled in Laemmli sample buffer, and equal amounts of proteins in the different test groups were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with a primary Ab against phospho-p47^phox (Cell Signalling Technology, Beverly, MA; 1:500 dilution in Tris-buffer saline with 2% BSA). Western blots were developed with horseradish peroxidase-conjugated secondary Ab (Bio-Rad; 1:10,000 dilution for 2 h at room temperature) and development by ECL (Amersham/GE Healthcare Bio-Sciences, Piscataway, NJ). The ECL signal was quantified using a scanner and densitometry (Scion Image, Frederick, MD), as previously described (45).

In vitro phosphorylation and dephosphorylation of p47^phox, p40^phox, and p67^phox
Recombinant p47^phox, p40^phox, and p67^phox (5 μg each) were expressed in E. coli using pGEX plasmids (gift from Bernard M. Babior, The Scripps Research Institute, La Jolla, CA) and then phosphorylated by active protein kinase C (PKC; Promega, Madison, WI) or AKT (CalBiochem, San Diego, CA) in the presence of 1 μCi [32P]ATP-ATP for 30 min using published protocols (46). The reaction was terminated by adding the same volume of 2× Laemmli sample buffer, boiled, and resolved by SDSPAGE using 10% polyacrylamide gels. The separated proteins were transferred to a nitrocellulose membrane. Proteins were stained by poncTae red (data not shown), and [32P]-labeled p47^phox, p40^phox, and p46^phox were detected by a phosphoimager (Model GS-800, Bio-Rad). Autoradiographs were exposed to X-ray film, and the bands were quantified using a densitometry program (ImageQuant, Molecular Dynamics, Sunnyvale, CA). Bands corresponding to p47^phox were excised from the gel and subjected to phosphatase treatment using an alkaline phosphatase substrate (Sigma). The gel was analyzed by autoradiography. Experiments were performed in duplicate and repeated twice.

Results
Detection of phosphorylated p40^phox and p47^phox in cell lysates of neutrophils and MDMs
Neutrophils (10^5/well) or MDMs (10^5/well) were added to a 12-well plate in RPMI 1640. Serum-opsonized Francisella novicida strains were added at an MOI ∼50:1, and the infection was synchronized by centrifugation at 400 × g for 2 min at 12°C. At different time intervals, uninfected and infected cells were lysed in TN1 buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM Na_2PO_4, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 10 mM Na_2VO_3, 10 μg/ml each aprotinin and leupeptin). The cell lysates were boiled in Laemmli sample buffer, and equal amounts of proteins in the different test groups were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with a primary Ab against phospho-p40^phox (Cell Signalling Technology, Beverly, MA; 1:500 dilution in Tris-buffer saline with 2% BSA). Western blots were developed with horseradish peroxidase-conjugated secondary Ab (Bio-Rad; 1:10,000 dilution for 2 h at room temperature) and development by ECL (Amersham/GE Healthcare Bio-Sciences, Piscataway, NJ). The ECL signal was quantified using a scanner and densitometry (Scion Image, Frederick, MD), as previously described (45).

Production of ROS in human neutrophils and macrophages by F. novicida acid phosphate mutants
Previous work showed that F. tularensis LVS is unable to stimulate an oxidative burst in infected human neutrophils and macrophages (36, 51). In addition, acid phosphatases of organisms, including Francisella spp., have been implicated in respiratory burst inhibition (33, 52–55). To determine whether F. novicida acid phosphate mutants are no longer able to suppress the respiratory burst in human phagocytes, we measured the generation of ROS in infected human neutrophils (30-min time course) and MDMs (60-min time course) using the luminescence probe, luminol or lucigenin, respectively. F. novicida induced minimal amounts of ROS in neutrophils and macrophages (Fig. 1A, 1B), demonstrating that F. novicida, like F. tularensis LVS (36), does not generate significant ROS production. Similar inhibitory
effects were observed with Type A *F. tularensis* SchuS4 (Fig. 1C, 1D). Formalin-killed *Francisella* subspecies effectively induced a respiratory burst in MDMS and neutrophils, suggesting that ROS suppression was an active process of live bacteria (Fig. 1). Opsonized zymosan and PMA stimulated the production of ROS in neutrophils and MDMS, as expected (Fig. 1). However, the addition of opsonized zymosan after MDM or PMN infection by *F. novicida* produced significantly higher levels of ROS, with an overall 28-fold increase in neutrophils at 30 min postinfection and a 5.5-fold increase in MDMs at 60 min postinfection versus infection with either the WT strain or the ΔacpA mutant complemented and dramatically reduced ROS induction compared with opsonized zymosan alone (Fig. 1), demonstrating that *Francisella* subspecies are able to suppress ROS production from human phagocytes.

Phagocytes infected with the ΔacpA mutant produced a small amount of ROS, which was particularly evident in MDMS. However, neutrophils and MDMS infected with the ΔABCH strain produced significantly higher levels of ROS, with an overall 28-fold increase in neutrophils at 30 min postinfection and a 5.5-fold increase in MDMS at 60 min postinfection versus infection with *F. novicida*. The magnitude of ROS production in MDMS was significantly less than that observed in neutrophils during *F. novicida* strain infections (compare vertical axis of Fig. 1A with Fig. 1B). The ΔABCH strain was complemented with a plasmid expressing acpA (19). Additional complementation was too difficult to pursue because of the multiple antibiotic resistances of the ΔABCH strain. The results demonstrate that expression of the plasmid-borne acpA in the ΔABCH mutant complemented and dramatically reduced the amount of ROS produced by this strain, but not to the level of the WT *F. novicida* strain (Supplemental Fig. 1). Therefore, these data suggest that loss of acid phosphatases in *Francisella* spp. results in a failure to suppress the production of ROS.

The above experiments were repeated with the addition of diphenylene iodonium (DPI; flavoprotein inhibitor) 15 min prior to bacterial infection. Luminescence was completely abrogated in neutrophils and MDMS postinfection with all bacterial strains as well as with PMA and opsonized zymosan (data not shown).

To confirm that differences in cell association or uptake were not responsible for the observed disparity in ROS production between acid phosphatase mutants and WT *F. novicida*, strains were examined by microscopy upon infection of MDMS and neutrophils in the presence of various concentrations of autologous serum. No significant differences in cell association or uptake were observed (data not shown). Additionally, there were no differences in the deposition of the complement component C3, a central complement factor whose cleavage leads to the deposition of C3bi, a major opsonin, on the surface of the *F. novicida* WT strain or the acid phosphatase mutants (data not shown).

Decreased survival of *F. novicida* acid phosphatase mutants in human macrophages and neutrophils is associated with enhanced ROS production

The ΔABCH mutant of *F. novicida* was reported to have an impaired ability to replicate intracellularly in J774.1 murine and THP-1 macrophages (19). In this study, we measured CFU in neutrophils (15 and 30 min) and MDMS (30 and 60 min) infected with the ΔacpA, ΔABCH, and WT strains of *F. novicida* (Fig. 2). At 30 and 60 min postinfection of neutrophils and MDMS, respectively, there was a 19- and 46-fold decrease in survival of the ΔABCH versus the WT strain (Fig. 2A, 2C). In the presence of DPI, the intracellular growth of WT *F. novicida*, ΔacpA, and ΔABCH strains was nearly identical up to 30 min postinfection in neutrophils and 60 min postinfection in MDMS (Fig. 2B, 2D). Although growth at these time points was nearly identical, a nearly 2-log increase in survival was noted in the
presence versus the absence of DPI. This is likely due to functional bacterial killing by residual ROS production upon infection by the *F. novicida* WT strain (incomplete suppression of ROS production). These results suggest that the reduction in intracellular growth in phagocytes seen with the mutant strains was due to the generation of ROS.

To confirm whether ROS production was responsible for the decreased survival of acid phosphatase-deficient strains, we examined the survival of *Francisella* acid phosphatase mutants in BMDMs isolated from *p47*phox knockout mice. These mice were shown to be more susceptible than WT mice to infection by *F. tularensis* LVS (56), demonstrating a role of ROS in controlling bacterial dissemination and growth. WT and *p47*phox−/− BMDMs were incubated with *F. novicida* WT, *ΔacpA*, and *ΔABCH* strains, and the CFU were compared at different time points. At 12 h postinfection, the *ΔABCH* strain showed a slightly less than 3-log decrease in survival compared with the WT strain (Fig. 3A). This decrease was even greater at 24 h. The *ΔacpA* strain demonstrated intermediate survival, with a >1.5-log decrease in survival at 12 h postinfection and ~1-log decrease in survival at 24 h. In contrast, the *F. novicida* WT, *ΔacpA*, and *ΔABCH* strains grew equally well in infected BMDMs isolated from *p47*phox−/− mice (Fig. 3B). Thus, there is a strong correlation of intracellular survival with the induction of ROS by the NADPH oxidase. These data, combined with the results in the presence of DPI, provide strong evidence that the production of ROS in phagocytes by the NADPH oxidase is responsible for the reduced intracellular survival seen with the *F. novicida* acid phosphatase mutants.

**Colocalization of Francisella with the NADPH oxidase complex component p47phox in human neutrophils and macrophages**

Assembly of a functional NADPH oxidase requires the translocation of cytosolic NADPH oxidase components p67phox, p47phox, and p40phox to the membrane component cytochrome b559 (57). However, intracellular *F. tularensis* LVS did not induce ROS production or colocalize with NADPH complex subunits (36). We examined colocalization of p47phox with bacteria in human neutrophils and MDMs to determine whether the *F. novicida*, *ΔacpA*, *ΔABCH*, and Type A *F. tularensis* subspecies *tularensis* associate with NADPH oxidase complex components.

Neutrophils adhered to serum-coated glass coverslips were incubated with *F. novicida*, *ΔacpA*, or *ΔABCH* strains. The colocalization of *F. novicida* with p47phox was detected using confocal microscopy. Representative confocal micrographs of neutrophils infected with the *F. novicida* and *ΔABCH* strains at 30 min postinfection are shown in Fig. 4A. Neutrophils infected with the *ΔABCH* strain showed a maximum colocalization at 30 min postinfection; ~90% of the bacteria were colocalized with p47phox (Fig. 4B). The *ΔacpA* mutant strain colocalized less extensively with p47phox (Fig. 4B). The *F. novicida* WT strain colocalized with p47phox poorly, reaching a maximum ~12% at 30 min postinfection (Fig. 4B). Similarly, the virulent Type A and Type B strains colocalized poorly with p47phox in neutrophils (7% and 8.5%, respectively) and MDMs (5.5% and 6.5%, respectively) at 30 min postinfection (data not shown).

Similar to what was observed in neutrophils, the *ΔABCH* strain demonstrated marked colocalization with p47phox in MDMs (representative confocal micrographs are shown at 60 min postinfection in Fig. 5A), with a maximum of nearly 48% colocalization at 60 min postinfection (Fig. 5B). The *ΔacpA* mutant strain showed an intermediate level of colocalization (Fig. 5B), whereas the WT *F. novicida* strain showed the least association with p47phox over the time period studied, with a maximum of only ~8% colocalization observed at 60 min postinfection.

**NADPH oxidase components show increased phosphorylation following infection with the *ΔABCH* mutant strain relative to *F. novicida* WT in neutrophils and macrophages**

To determine whether the limited generation of NADPH oxidase-mediated ROS in response to WT *F. novicida* involved the phosphorylation state of NADPH complex subunits by the acid phosphatases, we first examined the phosphorylation of p47phox and p40phox during the course of infection. Neutrophils were infected with *F. novicida* strains; at different time intervals, cells were lysed, and the phosphorylation of p47phox or p40phox was detected.
In this study, we used active PKC and Akt to phosphorylate substrates for phosphorylation by PKCs, Akt, and p38 MAPK (46, 59–63). In neutrophils, infection by the WT, but not the ΔABC strain versus infection with the WT strain suggests that these strain differences result in a marked increase in phosphorylation of p47phox within 15 min of infection compared with the WT strain (Fig. 6). Similarly, phosphorylation of p40phox in neutrophils infected with the ΔABC strain showed a dramatic increase within 15 min of infection compared with the WT strain. In addition, MDMs infected with the ΔABC strain showed a steady increase in phosphorylation of p47phox and p40phox over the time course of the experiment, which was not observed with the WT strain (data not shown). The relative increase in p47phox and p40phox phosphorylation during infection of neutrophils and MDMs with the ΔABC strain versus infection with the WT strain suggests that these NADPH complex components do not become phosphorylated by upstream kinases or are dephosphorylated in human phagocytes when infected with the WT, but not the ΔABC strain.

**Direct dephosphorylation of p47phox and p40phox by AcpA**

Activation of the NADPH oxidase complex depends upon the phosphorylation and subsequent translocation of the cytosolic components p40phox, p47phox, and p67phox to the phagosomal membrane (58). Several studies reported that phox components are substrates for phosphorylation by PKCs, Akt, and p38 MAPK (46, 59–63). In this study, we used active PKC and Akt to phosphorylate purified p47phox, p40phox, and p67phox proteins in the presence of [32P]-γ-ATP (60). The phosphorylated p47phox, p40phox, and p67phox proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The [32P]-labeled proteins were detected by a phosphoimager and autoradiography (Fig. 7A). The relative increase in p47phox and p40phox phosphorylation within neutrophils and in virulence, because the loss of four acid phosphatases (AcpA, AcpB, AcpC, and Hap) in Francisella acid phosphatases play a major role in survival of *F. novicida* acid phosphatases, particularly the AcpA protein.

**Discussion**

*F. tularensis* LVS is unable to stimulate the production of an oxidative burst in infected human neutrophils and macrophages and survives within these host cells (36, 51). We previously reported that *F. novicida* acid phosphatases play a major role in survival within phagocytic cells and in virulence, because the loss of four acid phosphatases (AcpA, AcpB, AcpC, and Hap) in *F. novicida* acid phosphatases (AcpA, AcpB, AcpC, and Hap) in *F. novicida*...
Subspecies holarctica of ROS in human neutrophils, as well as within human macrophages. Triplicate samples were included in each test group.

The observed decrease in virulence and intraphagocytic cell survival of the WT strain and mutant strains with p47phox, ΔABCH, or ΔacpA was determined at 30 and 60 min postinfection in MDMs. Francisella was detected following staining with goat anti-mouse Alexa Fluor 488 (green color), and p47phox was detected following staining with donkey anti-rabbit Alexa Fluor 546 (red color). Representative confocal microscopy images of F. novicida and ΔABCH colocalized with p47phox within MDMs are shown 60 min postinfection. The images are representative of 1000 infected cells examined from triplicate coverslips in three independent experiments. Original magnification ×63. B, Colocalization of the F. novicida WT, ΔacpA, and ΔABCH mutant strains with p47phox was quantified at 30 and 60 min postinfection. Analyses were based on examination of 1000 infected cells examined from triplicate coverslips in three independent experiments. The results shown are cumulative data of three experiments (mean ± SD of nine samples; triplicate samples were included in each test group in each experiment).

(DΔABCH) rendered it unable to survive within and escape from human and murine macrophage phagosomes (19). The ΔABCH strain was also attenuated in the mouse model of tularemia. In addition, purified AcpA was reported to block the generation of an oxidative burst in neutrophils (33). These data led us to investigate the connection between acid phosphatases and ROS production in human neutrophils and macrophages as a potential mechanism for the observed decrease in virulence and intraphagocytic cell survival of the ΔABCH strain.

In this study, we extend the work with F. tularensis LVS (36, 51) to show that F. tularensis subspecies tularensis SchuS4, F. tularensis subspecies holarctica, and F. novicida also suppress the production of ROS in human neutrophils, as well as within human macrophages. Interestingly, although the live organisms induced similar low levels of ROS, formalin killed F. novicida induced considerably more ROS than did formalin-killed F. tularensis SchuS4, suggesting that there may be something inherently different about the surfaces of these bacteria that affect the respiratory burst. In addition, we provide evidence that infection with the ΔABCH strain results in a significant induction of ROS, suggesting that the acid phosphatases directly or indirectly interfere with the oxidative burst and that increased ROS production is responsible for the killing of the ΔABCH strain within these phagocytes. Indeed, blocking the assembly and/or function of the NADPH oxidase complex with DPI or mutation of a critical cytosolic component (murine p47phox−/− macrophages) allowed for a WT level of survival of the ΔABCH strain in phagocytes. The loss of only AcpA in F. novicida produced an intermediate amount of ROS production in neutrophils and MDMs and resulted in intermediate survival, suggesting that it was a major, but not the only, acid phosphatase contributing to the observed ΔABCH strain phenotypes.

Assembly of the NADPH oxidase requires that cytosolic phosphorylated p47phox and p40phox heterodimers associate to form p47/p67/p40phox heterotrimers prior to their membrane translocation and subsequent association with flavocytochrome b558 (64–67). Similar to what was observed with the F. tularensis LVS strain, the F. novicida WT strain did not significantly co-localize with the p47phox component of the NADPH oxidase complex in human neutrophils and macrophages. However, the ΔABCH strain, and to a lesser extent, the ΔacpA mutant, significantly colocalized with the p47phox in human neutrophils and human MDMs, correlating with their observed levels of ROS induction.

Several other bacteria and parasites were shown to mediate exclusion or disruption of NADPH oxidase assembly or ROS production during phagocytosis (52–55, 68–72). For example, Helicobacter pylori disrupts phagosomal NADPH oxidase targeting, because superoxide anions are released extracellularly.
Confirmation of release of $^{32}$P. To confirm that the above signal was not due to the release of proteins from the nitrocellulose, the supernatant was treated with active PKC or Akt in the presence of $^{32}$P, and phosphorylated by PKC or Akt instead of within the phagosomes, and these phagosomes do not contain p47 or p67 instead of within the phagosomes, and these phagosomes do not contain p47 or p67. Moreover, the supernatant was spotted onto a nitrocellulose membrane and detected by autoradiography. No signal was observed, suggesting that the signal was not due to the release of proteins from the nitrocellulose membrane.

The effect of $^{32}$P in vitro. Phosphorylation of p47 and p47 was detected by phosphorylation and autoradiography. However, phosphorylation of p47 and p47 was weak and p67 was undetectable at early time points post-infection with F. novicida WT strain. Thus, the peak activation may be prior to the 2 h time point. This induction decreased over time, suggesting that the peak activation may be prior to the 2 h time point.

The hypothesis we developed based on the data presented in this work states that the acid phosphatases collectively participate in the dephosphorylation of p47 and p47 components and/or of their kinases, which inhibits NADPH oxidase assembly and ROS production. In support of an effect on the upstream kinases, human MDMs infected with the ΔABC strain have an increased level of p38 MAPK phosphorylation, but not Akt and Erk1/2, compared with the MDMs infected with F. novicida WT strain (data not shown). Further studies are required to fully understand the phosphorylation patterns of the upstream kinases, including p38 MAPK, during ΔABC strain infection. To address the effect of the phosphatases directly, phosphorylation of p47 was weak and phospho-p40 was undetectable at early time points post-infection with human neutrophils with the F. novicida WT strain. However, ΔABC strain infection resulted in a marked increase in the phosphorylation of these p47 components. To determine whether the Acps had the capacity to directly dephosphorylate the p47 components, phosphorylated p40, p47, and p67 were treated individually in vitro with three purified Francisella Acps. AcpA strongly dephosphorylated the p40 and p47 proteins, but dephosphorylated p67 weakly, demonstrating specificity in this reaction. As opposed to the actions of AcpA, AcpC and Hap weakly dephosphorylated p47 but not p40 or p67. Although AcpA demonstrated the most activity toward the p47 components in the in vivo assay, the virulence data (19) and the data presented in this article (including the colocalization data with p47) suggest that a combined effort of the phosphatases is required for a maximal effect toward ROS suppression. Thus, there are likely targets of these phosphatases beyond the p47 components, such as upstream kinases mentioned above, that are necessary for proper NADPH oxidase activation.

The rapid kinetics of NADPH oxidase inactivation by Francisella spp., coupled with the location of the targeted p47 components, provide a conceptual challenge to these findings. Thus, how might AcpA or the other acid phosphatases be able to mediate these effects? Several pieces of data support a role for the Francisella acid phosphatases in this process. We previously demonstrated that two of the acid phosphatases, AcpA and Hap, were activated 219- and 10-fold, respectively, at 2 h postinfection with THP-1 macrophages (19). This induction decreased over time, suggesting that the peak activation may be prior to the 2 h time point. In addition, AcpA is outer membrane-associated in logarithmically growing cells (19), and it has been described as a Francisella-secreted protein, although this has not been observed in all studies that have screened for secreted factors. An acid phosphatase of Legionella pneumophila, a pathogen that resembles F. tularensis in phagosomes in a lipophosphoglycan-dependent fashion (68, 69). In contrast, Salmonella excludes flavocytochrome b$_{558}$ from the phagosomal membrane, thus preventing NADPH oxidase assembly (54, 72). Finally, Coxiella burnetii produces an acid phosphatase that, similar to Francisella, inhibits ROS production from activated human neutrophils by an unknown mechanism (55).
a number of ways, is also secreted (77). None of the other Franciscella Acps were reported to be secreted in vitro, but nothing is known about the potential secretion of these Franciscella Acps in vivo. Thus, we hypothesize that Franciscella affects the phagocyte (e.g., kinase dephosphorylation) very soon after contact, which may be Acp dependent and independent. Early secretion/release of acid phosphatases within the phagosome, or after phagosomal escape, which was described to occur rapidly after phagocytosis (17, 78), would result in a further reduction of the phosphorylation of p horx components normally necessary for proper NADPH oxidase assembly and function. A less likely possibility is that the ΔABCH mutant uses an unknown receptor for phagocyte entry that is associated with a more robust oxidative burst than normally occurs following entry via CR3 and the mannose receptor, known receptors for Franciscella WT strains (12). Additional molecular and biochemical characterization, including localization, of the Franciscella acid phosphatases within host phagocytes will further define the mechanisms by which this bacterial pathogen evades an early innate immune response, allowing for bacterial colonization and disease progression. Targeted inhibition of these phosphatases could be a therapeutic strategy against Franciscella infection.

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Disclosures

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References

NADPH OXIDASE INACTIVATION BY Francisella Acps


