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Francisella Acid Phosphatases Inactivate the NADPH Oxidase in Human Phagocytes

Nrusingh P. Mohapatra,*,† Shilpa Soni,*,† Murugesan V. S. Rajaram,*,† Pham My-Chan Dang,§ Tom J. Reilly,§ Jamel El-Benna,§ Corey D. Clay,*† Larry S. Schlesinger,*†,1 and John S. Gunn,*†,1

Francisella tularensis contains four putative acid phosphatases that are conserved in Francisella novicida. An F. novicida quadruple mutant (AcpA, AcpB, AcpC, and Hap [ΔABCH]) is unable to escape the phagosome or survive in macrophages and is attenuated in the mouse model. We explored whether reduced survival of the ΔABCH 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 ΔABCH strain stimulated a significant level of reactive oxygen species. The ΔABCH 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 macrophage (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, PmrA, 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 [ΔABCH]) 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...
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, fMLP, or zymosan. NADPH oxidase induction in neutrophils or macrophages requires the translocation of phosphorylated p47$_{phox}$ and 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 fMLP-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 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 monocytic-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 $g$ 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 $2 \times 10^6$ PBMCs/ml in RPMI 1640 containing 20% autologous 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 $2 \times 10^5$ cells/well in 0.5 ml culture media, resulting in $2 \times 10^5$ 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 replenished with fresh culture medium with 10% autologous serum. For intramacrophage survival assays, MDMs were incubated for an additional 7 days in culture with 20% autologous serum to stabilize the MDM monolayer prior to infection (37). Macrophages were incubated with Francisella at a multiplicity of infection (MOI) $\sim$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 bacterium (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 $\times$ 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 $\times$ 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, Deltaacp, 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 at photometry by $600 \mathrm{nm}$; 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. Opsonized 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 ($2 \times 10^6$ cells/well) were seeded onto human serum-coated 24-well plates in RPMI 1640, and Francisella were added at an MOI $\sim$50:1. At time points $\geq$ 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 ($2 \times 10^6$ cells/well) were seeded onto human serum-coated 24-well plates in RPMI 1640, and opsonized Francisella was added at an MOI $\sim$50:1 and incubated for 10 min at 37°C in 5% CO$_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. In this assay, monocytes/macrophages and phagocytes were 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 (diluted 1:100 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 using an $\times 100$ oil-immersion objective with a wide-bandwidth 570-nm dichroic mirror on a BX51 Olympus fluorescence microscope. Pictures were taken with a CoolSnap digital camera (Roper Scientific, Sunnyvale, CA) at a magnification of $\times 400$. Triplicate coverslips were used for each test group. Attached bacteria were assessed by scoring nonpermeabilized 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 10% human serum albumin, and 50 µm luminol (Invitrogen, Carlsbad, CA) and left on ice for 15 min. Subsequently, serum-opsonized Francisella spp. were added at an MOI $\sim$50:1. The microtiter plate was centrifuged at 400 $\times$ 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 (Bio-Tek Instruments, Winooski, VT). Luminol was diluted 1:5 in PBS and added to the plates at a final concentration of 0.125 µM luminol (Invitrogen, Carlsbad, CA). ROS production in MDMs in response to serum-opsonized Francisella (MOI $\sim$50:1) was determined using the Diogenes enhanced luminescence system for superoxide 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 oposizenzymatic. 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 (ComplementTech, Tyler, TX) was used to evaluate complement component C3 deposition on Francisella strains, as described (39). Briefly, after predigesting microcarrier 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 and once in PBS. 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-human 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 Ab (1:1000 dilution; American Diagnostica, Stamford, CT) was added for 1 h at room temperature. The secondary Ab was added for 1 h at room temperature. Substrate was added for 10 min at room temperature (Bio-Rad), and the reaction was stopped with 2% oxalic acid. Absorbance at 415 nm was measured on a 96-well plate reader (Molecular Devices). Values obtained from reactions with heat-inactivated control serum were subtracted in each case.

Confocal microscopy

Neutrophils (10^6/well) were plated onto human serum-coated glass coverslips in HBSS in a 24-well plate. Serum-opsonized Francisella spp. were added at an MOI ∼50:1, and the infection was synchronized at 12°C by centrifugation at 400 × g for 2 min. At different time intervals, coverslips were washed with HBSS to remove extracellular bacteria, and the cells were fixed with 3.5% paraformaldehyde for 30 min (40). After fixation, cells were washed three times with HBSS and permeabilized with chilled methanol for 15 s (41). Cells were washed again in HBSS and blocked in HBSS containing 20% normal human serum (Cambrex) and 5% BSA (blocking solution) for 2 h. Infected cells were treated with primary Ab consisting of mouse monoclonal anti-F. novicida (1:2000 dilution; Immunoprecise, Victoria, British Columbia, Canada) or mouse monoclonal anti-F. tularensis LPS (1:2000 dilution; Abcam, Cambridge, MA) and/or rabbit anti-p47^phox Abs (1:1000 dilution; Molecular Probes) for 2 h in blocking solution. Coverslips were washed three times in blocking solution and secondary Ab (goat anti-mouse Alexa Fluor 488 or donkey anti-rabbit Alexa Fluor 546, 1:1000 dilution) was added for 1 h. Coverslips were washed and mounted with Prolong anti-fade reagent (Invitrogen).

Detection of phosphorylated p40^phox in cell lysates of neutrophils and MDMs

Neutrophils (10^6/well) or MDMs (10^6/well) were added to a 12-well plate in RPMI 1640. Serum-opsonized F. 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 TN buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM Na_2HPO_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 Signaling Technology, Beverly, MA; 1:500 dilution in Tris-buffer saline with 5% milk) or phospho-p47^phox (Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of 1 μg/ml [32P]-y-ATP for 30 min using published protocols (46). The reaction was terminated by adding the same volume of 2× Laemml sample buffer, boiled, and resolved by SDS-PAGE using 10% polyacrylamide gels. The separated proteins were transferred to a nitrocellulose membrane. Proteins were stained by ponceau red (data not shown), and [32P]-labeled p47^phox, p67^phox, and p40^phox were detected by autoradiography. To further confirm that the signal was not due to the release of the protein from the nitrocellulose, the supernatant was subjected to SDS-PAGE using 15% polyacrylamide gels and analyzed by autoradiography. Experiments were performed in duplicate and repeated twice.

Results

Production of ROS in human neutrophils and macrophages by F. novicida acid phosphatase 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 phosphatase mutants are no longer able to suppress the respiratory burst in infected 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
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 WT F. novicida. The magnitude of ROS production in MDMs was significantly less than that observed in neutrophils during 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 F. novicida 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
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 b558 (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...
by Western blotting using phospho-p40\textsuperscript{phox} and phospho-p47\textsuperscript{phox} Abs (Fig. 6). In neutrophils, infection by the ΔABC\textsuperscript{H} strain resulted in a marked increase in phosphorylation of p47\textsuperscript{phox} within 15 min of infection compared with the WT strain (Fig. 6). Similarly, phosphorylation of p40\textsuperscript{phox} in neutrophils infected with the ΔABC\textsuperscript{H} strain showed a dramatic increase within 15 min of infection compared with the WT strain. In addition, MDMs infected with the ΔABC\textsuperscript{H} strain showed a steady increase in phosphorylation of p47\textsuperscript{phox} and p40\textsuperscript{phox} over the time course of the experiment, which was not observed with the WT strain (data not shown). The relative increase in p47\textsuperscript{phox} and p40\textsuperscript{phox} phosphorylation during infection of neutrophils and MDMs with the ΔABC\textsuperscript{H} 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\textsuperscript{H} strain.

Direct dephosphorylation of p47\textsuperscript{phox} and p40\textsuperscript{phox} by AcpA

Activation of the NADPH oxidase complex depends upon the phosphorylation and subsequent translocation of the cytosolic components p40\textsuperscript{phox}, p47\textsuperscript{phox}, and p67\textsuperscript{phox} 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 p47\textsuperscript{phox}, p40\textsuperscript{phox}, and p67\textsuperscript{phox} proteins in the presence of [\textsuperscript{32}P]-γ-ATP (60). The phosphorylated p47\textsuperscript{phox}, p40\textsuperscript{phox}, and p67\textsuperscript{phox} proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The [\textsuperscript{32}P]-labeled proteins were detected by a phosphoimager and autoradiography (Fig. 7A). These data demonstrated that [\textsuperscript{32}P] was released from the proteins (Fig. 7B). To confirm that [\textsuperscript{32}P] release was not due to the complete release of the nitrocellulose membrane-bound proteins, the supernatants containing the released [\textsuperscript{32}P] were spotted on thin layer cellulose plates and detected by autoradiography (Fig. 7B). Purified AcpA strongly dephosphorylated p47\textsuperscript{phox} and p40\textsuperscript{phox} but only weakly dephosphorylated p67\textsuperscript{phox}. In the presence of AcpC and Hap, p47\textsuperscript{phox} was dephosphorylated to a small extent, whereas none of the other phosphorylated phox proteins tested were dephosphorylated by these two acid phosphatases (Fig. 7B). To confirm that [\textsuperscript{32}P] release was not due to the complete release of the nitrocellulose membrane-bound proteins, the supernatant was subjected to SDS-PAGE and analyzed by autoradiography (Fig. 7B). The data demonstrated that [\textsuperscript{32}P] was released from the proteins (Fig. 7C, 7D). Thus, the results provide evidence that p47\textsuperscript{phox} and p40\textsuperscript{phox} can be directly dephosphorylated by the Francisella 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
Subspecies holarctica phages. Interestingly, although the live organisms induced similar 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 ∆ABCH 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 ∆ABCH 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 ∆ABCH 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 ∆ABCH 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 colocalize with the p47phox component of the NADPH oxidase complex in human neutrophils and macrophages. However, the ∆ABCH strain, and to a lesser extent, the acpA 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...
osomes 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, *Coxella burnetii* produces an acid phosphatase that, similar to *Francisella*, inhibits ROS production from activated human neutrophils by an unknown mechanism (55).

Recently published work suggested that AcpA, AcpB, and AcpC do not play a role in the pathogenesis of the *F. tularensis* SchuS4 strain (using a triple mutant) (76). This is contrary to our previous finding with *F. novicida*, which demonstrated increased virulence defects upon the accumulation of *acp* deletions, culminating with a strong virulence defect for the ΔABC strain (quadruple mutant) (19). Our ongoing work with the acid phosphatases (Acps) in *F. tularensis* SchuS4 (data not shown), coupled with the data presented in this study, suggests that the acid phosphatases play a role in *F. tularensis* SchuS4 pathogenesis but perhaps not to the same extent as observed in *F. novicida*.

The hypothesis we developed based on the data presented in this work states that the acid phosphatases collectively directly participate in the dephosphorylation of *phox* 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 *phox* components directly, phosphorylation of p47$^{phox}$ was weak and phospho-p40$^{phox}$ was undetectable at early time points post-infection of human neutrophils with the *F. novicida* WT strain. However, ΔABC strain infection resulted in a marked increase in the phosphorylation of these *phox* components. To determine whether the Acps had the capacity to directly dephosphorylate the *phox* components, phosphorylated p40$^{phox}$, p47$^{phox}$, and p67$^{phox}$ proteins were treated individually in vitro with three purified *Francisella* Acps. AcpA strongly dephosphorylated the p40$^{phox}$ and p47$^{phox}$ proteins, but dephosphorylated p67$^{phox}$ weakly, demonstrating specificity in this reaction. As opposed to the actions of AcpA, AcpC and Hap weakly dephosphorylated p47$^{phox}$ but not p40$^{phox}$ or p67$^{phox}$. Although AcpA demonstrated the most activity toward the *phox* components in the in vivo assay, the virulence data (19) and the data presented in this article (including the colocalization data with p47$^{phox}$) 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 *phox* 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 *phox* 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 induced 219- and 10-fold, respectively, at 2 h postinfection with *T. gondii* (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 pneumophilia*, a pathogen that resembles *F. tularensis* in

![FIGURE 7](image)

**FIGURE 7.** The effect of *F. novicida* Acps on phosphorylation of p40$^{phox}$ and p47$^{phox}$ in vitro. A. Phosphorylation of p47$^{phox}$, p67$^{phox}$, and p40$^{phox}$ by AKT and PKC. Recombinant p47$^{phox}$, p67$^{phox}$, and p40$^{phox}$ (5 µg each) were phosphorylated by active PKC or Akt in the presence of 1 µCi of [32P]-[γ-glu-ATP for 30 min. The reaction was terminated by adding the same volume of 2× Laemml sample buffer, and the proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The proteins were stained by ponceau red (data not shown), and [32P]-labeled p47$^{phox}$, p67$^{phox}$, and p40$^{phox}$ were detected by phoshoimaging and autoradiography. B. Dot blot of released [32P]-P. The nitrocellulose area containing [32P]-labeled p47$^{phox}$, p67$^{phox}$, and p40$^{phox}$ was cut and incubated for 30 min at 37°C with polyvinylpyrrolidone, washed, and then incubated with the different purified acid phosphatases. Supernatants containing released [32P] were spotted onto a thin-layer cellulose plate and detected by autoradiography. C and D. Confirmation of release of [32P]. To confirm that the above signal was not due to the release of proteins from the nitrocellulose, the supernatant was subjected to 15% SDS-PAGE and analyzed by autoradiography. C, Phosphorylation by PKC. D, Phosphorylation by Akt.
a number of ways, is also secreted (77). None of the other Francisella Acps were reported to be secreted in vitro, but nothing is known about the potential secretion of these Francisella Acps in vivo. Thus, we hypothesize that Francisella 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 phoX components normally necessary for proper NADPH oxidase assembly and function. A less likely possibility is that the ΔAchBt 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 Francisella WT strains (12). Additional molecular and biochemical characterization, including localization, of the Francisella 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 Francisella infection.

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