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Yersinia pestis Can Reside in Autophagosomes and Avoid Xenophagy in Murine Macrophages by Preventing Vacuole Acidification

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Yersinia pestis survives and replicates in phagosomes of murine macrophages. Previous studies demonstrated that Y. pestis-containing vacuoles (YCVs) acquire markers of late endosomes or lysosomes in naive macrophages and that this bacterium can survive in macrophages activated with the cytokine gamma interferon. An autophagic process known as xenophagy, which destroys pathogens in acidic autophagolysosomes, can occur in naive macrophages and is upregulated in activated macrophages. Studies were undertaken here to investigate the mechanism of Y. pestis survival in phagosomes of naive and activated macrophages and to determine if the pathogen avoids or co-opts autophagy. Co-localization of the YCV with markers of autophagosomes or acidic lysosomes and the pH of the YCV were determined by microscopic imaging of infected macrophages. Some YCVs contained double membranes characteristic of autophagosomes, as determined by electron microscopy. Fluorescence microscopy showed that ~40% of YCVs colocalized with green fluorescent protein (GFP)-LC3, a marker of autophagic membranes, and that YCVs failed to acidify below pH 7 in naive macrophages. Replication of Y. pestis in naive macrophages caused accumulation of LC3-II, as determined by immunoblotting. While activation of infected macrophages increased LC3-II accumulation, it decreased the percentage of GFP-LC3-positive YCVs (~30%). A viable count assay showed that Y. pestis survived equally well in macrophages proficient for autophagy and macrophages rendered deficient for this process by Cre-mediated deletion of ATG5, revealing that this pathogen does not require autophagy for intracellular replication. We conclude that although YCVs can acquire an autophagic membrane and accumulate LC3-II, the pathogen avoids xenophagy by preventing vacuole acidification.

Yersinia pestis is a gram-negative bacterium and the cause of plague (32, 34). Zoonotic foci of plague exist in many parts of the world, including North America. Y. pestis infections are most commonly transmitted to humans by infected fleas and typically develop into bubonic plague or, less frequently, into septicemic plague (32). Plague infections in humans can also result from contact with body fluids or from inhalation of respiratory droplets from infected animals or humans. Inhalation of Y. pestis into the lungs can initiate primary pneumonic plague.

Y. pestis is able to survive and replicate in murine macrophages in vitro (5, 6, 18, 35, 36, 44) and in vivo (23) and is therefore classified as a facultative intracellular pathogen. Microscopic examination of tissues of animals experimentally infected with Y. pestis has shown the presence of plague bacilli inside macrophages (11, 23, 25, 50). More often, however, Y. pestis is detected as large numbers of extracellular bacteria in tissues (20, 39, 51). Y. pestis produces several antiphagocytic factors that are upregulated during growth at 37°C. These factors include several Yop proteins and the LcrV protein and their designated type III secretion system encoded on pCD1 (48). In addition, a capsule composed of the F1 protein is maximally expressed after extended growth at 37°C and promotes resistance to phagocytosis (8). When grown at ambient temperatures (e.g., 28°C), Y. pestis is efficiently phagocytosed by macrophages (5). It has been hypothesized that when Y. pestis growing at 28°C is introduced into a mammalian host, it initially survives and replicates within macrophages that internalize the bacteria (5). Subsequently, the bacteria escape or are released from dying macrophages and replicate in an extracellular niche (5).

After a macrophage engulfs a bacterium, the phagosome changes rapidly into a less habitable compartment, termed a phagolysosome, through a series of fusion events with endocytic compartments (49). Within 2 to 5 min after their formation, phagosomes transiently acquire characteristics of early endosomes (49). After 10 to 30 min phagosomes begin to fuse with late endosomes and lysosomes. Late endosomes and lysosomes can be characterized by the presence of components such as antimicrobial peptides, lysosome-associated membrane proteins (LAMPs), and lysosomal proteases (cathepsins B, D, and L). In contrast to late endosomes, only lysosomes and phagolysosomes contain significant amounts of mature proteases. In addition, the pH of lysosomes and phagolysosomes is significantly lower (pH 4.5) than the pH in late endosomes (pH 5.5 to 6.0). The decrease in pH is due to the action of the vacuolar proton ATPase (vATPase).
Early work on the trafficking of *Y. pestis*-containing vacuoles (YCVs) in primary murine macrophages yielded evidence that YCVs fuse with lysosomes (6, 45). It was therefore suggested and expansion of its vacuole in macrophages is not understood. In addition, results of ultrastructural analysis by thin-section electron microscopy (EM) showed that the YCV colocalizes with these markers between 1.5 and 8 h postinfection (13). Results of studies that utilized lysosomal tracers or antibodies to the LAMP1 or cathepsin D protein in conjunction with immunofluorescence and thin-sectional tracers or antibodies to the LAMP1 or cathepsin D protein in conjunction with immunofluorescence and thin-section electron microscopy (EM) showed that the YCV colocalizes with these markers between 1.5 and 8 h postinfection (13). In addition, results of ultrastructural analysis by thin-section EM showed that the YCV has a spacious morphology beginning around 8 h postinfection, at which time bacterial replication begins (13). How *Y. pestis* controls phagosome trafficking and expansion of its vacuole in macrophages is not understood.

Activation by the cytokine gamma interferon (IFN-γ) is known to dramatically alter the trafficking and environment of phagosomes in macrophages (37, 41). *Y. pestis* is able to survive in primary murine macrophages that are activated with IFN-γ (36). A chromosomally encoded operon termed ripCBA that promotes survival of *Y. pestis* in activated macrophages has been identified. The ripCBA genes appear to encode novel metabolic enzymes (36). However, it is not known how the Rip proteins promote intracellular survival, and it is unclear if the morpholgy or trafficking of the YCV is modified in IFN-γ-activated macrophages.

Autophagy is a membrane trafficking process in eukaryotic cells that sequesters cytoplasmic material (e.g., defective mitochondria in the case of macroautophagy) in a vacuole (the autophagosome) and routes the cargo for destruction in an autophagolysosome (19, 21, 28). The autophagy pathway encompasses several different membrane compartments, beginning with the phagophore, which functions to sequester cytoplasmic material. Following trapping of cytoplasmic material by the phagophore, the autophagosome is formed, which matures through interactions with late endosomes and lysosomes into an autophagolysosome, where degradation of luminal components takes place. Autophagy is regulated at multiple steps by several signaling molecules, including the mTOR kinase and class I and class III phosphatidylinositol 3-kinases (19, 21, 28). Recent studies have revealed that autophagy can play an important role in the protective innate immune response to cytoplasmic or vacuolar bacterial pathogens (3, 21, 28). The process that results in breakdown of microorganisms within autophagosomes has been referred to as xenophagy (21). IFN-γ can upregulate autophagy in macrophages (21). In macrophages exposed to IFN-γ, upregulation of autophagy appears to override the phagosome maturation block imposed by *Mycobacterium tuberculosis*, allowing routing of the pathogen to a microbicidal autophagolysosome-like compartment (21, 28). Alternatively, some intracellular pathogens appear to co-opt autophagy for survival within host cells (26, 28).

In this work we examined the possibility that the replication of *Y. pestis* in macrophages exposed to IFN-γ resulted from an increase in autophagy and that the YCV might exploit this process for survival within phagosomes. Our results show that, although the YCV can interact with an autophagy pathway in macrophages, this interaction does not appear to require activation by IFN-γ nor is it required for intracellular survival of *Y. pestis*. Instead, we find that *Y. pestis* prevents phagosome acidification, and it is suggested that this process allows *Y. pestis* to avoid destruction in phagolysosomes or autophagolysosomes of either naive or activated macrophages.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *Y. pestis* strains used (Table 1) were derived from *Y. pestis* KIM (molecular group 2 MED) (1). Ampicillin resistance (Ap r), chloramphenicol resistance (Cm r), and kanamycin resistance (aph-3 ′ or Kn r) was introduced into *Y. pestis* strains in compliance with CDC guidelines. *Yersinia pseudotuberculosis* strain 32777 (previously referred to as strain IP2777) is a serogroup I isolate. *Y. pseudotuberculosis* strain 32777 was grown in Luria-Bertani broth or on Luria-Bertani agar plates at 28°C. *Y. pestis* was killed by fixation for 30 min with 2.5% paraformaldehyde, followed by extensive washing with phosphate-buffered saline (PBS).

**Strain construction.** KIM6 + was converted to the fully virulent background by introducing pCD1 marked with Ap r (pCD1Ap) (12) via electroporation as described previously (13). The resulting strain was designated KIM5 + and is handled according to select agent guidelines and using biosafety level 3 conditions. Screening for spontaneous loss of the pgm locus from KIM6 + (13) resulted in KIM6. Introduction of pCD1Ap into KIM6 resulted in KIM5, a strain that is conditionally virulent and exempt from select agent guidelines. Plasmid pMMB207gp3.1 (Cm r), used to express green fluorescent protein (GFP), was introduced into KIM5 or KIM6 + by conjugation (13). A plasmid expressing mCherry under control of the tac promoter was generated using pRSET-mCherry (40). The mCherry gene was amplified from pRSET-mCherry by PCR using oligonucleotides mCherry-EcoRI-F1 (5 ′-CCGGATATCATGGTGAGCAGGGCGGAG-3′) and mCherry-HindIII-R1 (5 ′-CCCAAGCTTTTACTTGTACCACTCTCGCATGAA-3′). The mCherry coding region was inserted into pMMB207 (27a) downstream of the tac promoter and between the EcoRI and HindIII sites. The resulting plasmid, p207mCherry, was introduced into KIM6 + by conjugation. GFP expression in *Y. pestis* was obtained by incubation in the presence of 500 µM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 to 2 h.

To construct a Kn r plasmid expressing inducible GFP, pMMB207gp3.1 was modified to inactivate the Cm r gene and to introduce a Kn r cassette (aph-3 ′). To this end, the Kn r cassette was isolated from pBSL86 (2) by restriction digestion introducing pCD1 marked with Kn r cassette (aph-3′, Ap r, or Kn r) cassettes were introduced into *Y. pestis* strains in compliance with CDC guidelines. *Yersinia pseudotuberculosis* strain 32777 (previously referred to as strain IP2777) is a serogroup I isolate. *Yersinia pseudotuberculosis* strain 32777 was grown in Luria-Bertani broth or on Luria-Bertani agar plates at 28°C. *Y. pestis* was killed by fixation for 30 min with 2.5% paraformaldehyde, followed by extensive washing with phosphate-buffered saline (PBS).

**TABLE 1. Strains used in this study**

<table>
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<th>Strain</th>
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<th>Reference</th>
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<td>13</td>
</tr>
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<td>pCD1 ′, rppC ′, pgm ′, ΔripCBA</td>
<td>This study</td>
</tr>
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<td>22</td>
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<td>KIM10 32777</td>
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</table>

Deletion of the ripCBA operon in KIM6 + was performed by allelic recombination. To this end, the ripCBA operon and flanking sequence were obtained by PCR amplification using primers KIM6 + chromosomal DNA as the template and primers Y2385-R5/SwaI (5 ′-CAGCGTGTGACCAATCATGCAGAAATGCTAAATTT-3′) and Y2385-R5/XmaI (5 ′-TCCCCCAGGTCGAGTATGCAATTCTTTTTTGCCC-3′). The amplified DNA fragment was cloned into the pGEM-T Easy vector (Promega). The resulting plasmid was AfeI, which recognizes and cleaves sites within rppC and rppA. Upon ligation of the ends of the
plasmid, a deletion was obtained that removed the 3′ end of ripC, all of the ripP gene, and the 5′ end of ripA. A DNA fragment with the deletion (ΔripCBA) was liberated from pGEM-T Easy and introduced into the suicide vector pSB890 using NotI restriction sites (30). The resulting plasmid, pSB890ΔripCBA, was conjugated into KIM6+, and allelic exchange was performed as described previously (14), resulting in KIM6+ripCBA.

**Cell culture.** Bone marrow-derived macrophages (BMDMs) were isolated and cultured as described previously (35, 36). BMDMs were prepared from femurs of C57BL/6 mice (Jackson Laboratory), ATG5flx/flx-Lye-Cre mice, or control ATG5flx/flx mice (52). ATG5flx/flx mice were backcrossed five generations to a C57BL/6 line prior to use. 29T3 cells were cultured at 37°C in the presence of 5% CO2 in Dulbecco modified Eagle medium (DMEM) with Glutamax-I (11001) supplemented with 1 mM sodium pyruvate (Invitrogen) and 10% fetal bovine serum (FBS) (HyClone). J774A.1 murine macrophage-like cells were cultured as described previously (13).

**Assays for determination of bacterial survival in macrophages.** The procedures used for infecting macrophages with *Y. pestis* or *Y. pseudotuberculosis* and for measuring intracellular survival by a CFU or GFP induction assay have been described previously (13, 35, 36).

**EM.** BMDMs (1.5 × 105 cells) seeded on plastic coverslips (diameter, 12 mm; Thermacon) were infected with KIM6+ at a multiplicity of infection (MOI) of 10 for 30 min in 200 μl of infection medium. Activation of BMDMs with INF-γ was performed as described previously (30). At the appropriate time, the infected cells were fixed for 1 h with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.5) supplemented with 2.5 mM CaCl2. Fixative buffer baths were renewed after 30 min of incubation. Fixed samples were washed three times for 5 min with 100 mM cacodylate buffer (pH 7.5) supplemented with 0.1% sodium azide and 0.1% Triton X-100 (Bio-Rad) and postfixed with 1% osmium tetroxide (Polysciences) in cacodylate buffer (pH 7.5) for 1 h. Sections were dehydrated in increasing concentrations of ethanol and embedded in Spurr’s resin. Ultrathin sections were contrasted in uranyl acetate and lead citrate and examined in a Zeiss EM 912 electron microscope.

**Detection of LC3 by immunoblotting.** BMDMs (6.5 × 105 cells) seeded in six-well plates were infected with *Y. pestis* strains as previously described (35), with the following modifications. An MOI of 10 to 15 was used, and BMDMs were infected for 30 min in a small volume (1 ml) of infection medium. BMDMs were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.5) supplemented with 2.5 mM CaCl2 and postfixed with 1% osmium tetroxide (Polysciences) in cacodylate buffer (pH 7.5) for 1 h. Sections were washed three times with PBS and lysed with 100 μl RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mM Tris [pH 8.0], 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitors (Complete Mini, EDTA-free; Roche). Cell lysate from each well was collected, passed through a 22-gauge needle, and centrifuged for 10 min at 16,000 × g at 4°C. Supernatants were collected, protein concentrations were determined (bicinchoninic acid or Bradford protein assay), and 25 μg of protein from each sample was separated by MiniBiorad electrophoresis apparatus. The blots were probed overnight using a polyclonal anti-actin antibody (catalog no. A1303; Sigma) diluted 1:5,000. Immunoblots were developed using an enhanced chemiluminescent reagent (Perkin-Elmer Life Science) and Kodak X-OMAT film. Band intensities for LC3-II/LC3-I or LC3-II/LC3-I were determined in three different fields per experiment.

**Phagosomal pH assay.** Determination of Lysotracker Red DND-99 localization with YCVs containing *Y. pestis* was performed using J774A.1 macrophage-like cells as described previously for Texas Red ovalbumin (13), except that Lysotracker was added at a concentration of 50 nM h prior to fixation and analysis of samples. In some experiments macrophages were incubated in medium containing 30 μM chloromaphenicol to inhibit bacterial protein synthesis during infection. Coinfection experiments were performed as follows. KIM5/GFP bacteria were induced with IPTG (500 μM) to express GFP and then fixed. Macrophages were infected with 5 × 104 bacteria in 500 μl of medium containing 50 μM chloromaphenicol and 500 μM IPTG and unlabeled live KIM5 (total MOI 10). At 1.25 h postinfection cells were fixed, permeabilized with saponin, and stained with anti-*Yersinia* primary antibody (1:1,000) and Alexa Fluor 350 (Molecular Probes) secondary antibody (1:500). Macrophages were labeled with Lysotracker as described above.

**RESULTS**

**Fully virulent *Y. pestis* replicates in activated macrophages.** We previously demonstrated that *Y. pestis* cured of pCD1 (KIM6+) (Table 1) replicates in murine BMDMs activated with IFN-γ (36). To determine if fully virulent, pCD1-containing *Y. pestis* can survive in activated BMDMs, a GFP induction assay was performed (35). KIM5+/GFP (Table 1) pregrown at 28°C was used to infect BMDMs, and survival of extracellular bacteria was prevented by use of gentamicin (see Materials and Methods). At different time points postinfection de novo synthesis of GFP was induced by addition of IPTG, and live cells were examined using phase-contrast and fluorescence microscopy. As shown in Fig. 1, KIM5+/GFP replicated in activated BMDMs between 6 and 24 h postinfection. At a higher magnification, GFP-positive bacteria were clearly visible within spacious YCVs of activated BMDMs at 24 h postinfection (Fig. 1).
2), with elongated bacteria typically apposed to the phagosomal membrane.

**Ultrastructural analysis of YCV morphology in activated macrophages.** We next looked for evidence that YCVs interact with an autophagy pathway in activated macrophages. Evidence for the interaction of intracellular bacteria with an autophagy pathway can be obtained by analysis of phagosome morphology at the ultrastructural level (19). Nascent autophagosomes typically have a double membrane. Fusion of lysosomes with an autophagosome results in maturation of the vacuole to an autophagolysosome, which has a single delimiting membrane. Therefore, the presence of a double membrane surrounding an intracellular bacterium can be taken as evidence that the pathogen was residing in a nascent autophagosome-like vacuole at the time of cell fixation. Thin-section EM was used to examine the possibility that *Y. pestis* resides in autophagosome-like vacuoles in activated macrophages. BMDMs were infected with KIM5+ (Table 1) pregrown at 28°C and then exposed to IFN-γ. Analysis of the infected macrophages at 4 h postinfection by thin-section EM (see Materials and Methods) showed that some intracellular *Y. pestis* bacteria were present within a tight-fitting double membrane (Fig. 3A and B). However, more commonly, a tight-fitting single membrane surrounded intracellular *Y. pestis* and other vesicular material (Fig. 3C). At a later time point (22 h postinfection) *Y. pestis* bacteria were found to be present in spacious phagosomes with partial double membranes (Fig. 3D and E and Fig. 3F, inset). These results indicated that, in activated macrophages, *Y. pestis* resided in autophagosome-like vacuoles, as well as in vacuoles with morphologies similar to those of standard phagosomes or multivesicular bodies.

**IFN-γ and replication of *Y. pestis* increase LC3-II levels in macrophages.** The progress of autophagy is executed by ATG proteins, which comprise two protein conjugation systems. The ATG8 protein (more commonly known as LC3-I in its unconjugated form) becomes conjugated to phosphatidylethanolamine during autophagy, resulting in the formation of LC3-II, which specifically associates with autophagic membranes (19).
The conversion of LC3-I to LC3-II and the turnover or degradation of LC3-II within autophagolysosomes can be followed by immunoblotting, and this procedure has become a useful tool for measuring rates of autophagy within eukaryotic cells (19, 27). The steady-state level of LC3-II, as detected by immunoblotting of cell lysates, was used to determine if autophagy was altered in macrophages after 24 h of treatment with IFN-γ and/or infection by *Y. pestis* KIM6+. The LC3-II signal obtained by immunoblotting was normalized by using two different approaches (19), calculating the ratio of LC3-II to LC3-I and calculating the ratio of LC3-II to a loading control (actin). Treatment of uninfected BMDMs with IFN-γ resulted in an increased ratio of LC3-II to LC3-I compared to that for untreated macrophages (Fig. 4A, compare lanes 3 and 4; Fig. 4B). Interestingly, after infection of BMDMs with *Y. pestis* KIM6+ in the absence of IFN-γ, there were large increases in the steady-state levels of both LC3-I and LC3-II (Fig. 4A, compare lanes 1 and 3) and there was a slight increase in the LC3-II/LC3-I ratio compared to the data for uninfected macrophages (Fig. 4B). Infection of BMDMs with KIM6+ in the presence of IFN-γ resulted in a further increase in the LC3-II/LC3-I ratio (Fig. 4A, lane 2; Fig. 4B). A time course analysis indicated that the increased LC3-II level was observed beginning at 3 h postinfection (data not shown). To determine if the increase in the LC3-II level required survival of *Y. pestis* in macrophages, BMDMs were infected with a KIM6+/phoP mutant (Table 1), which is defective for intracellular survival, or with killed formaldehyde-fixed KIM6+. Compared to the data for macrophages infected with live KIM6+, the LC3-II/actin ratios were lower after challenge with the phoP mutant or fixed KIM6+ (Fig. 4C and D). Moreover, the LC3-II/actin ratio was higher in BMDMs infected with a *Y. pestis* strain containing pCD1 but lacking the pgm locus (KIM5) (Table 1) than in uninfected BMDMs (Fig. 4E and F), although in this case the ratios were higher in nonactivated macrophages than in IFN-γ-treated BMDMs (Fig. 4E and F). We have observed that KIM5 does not replicate in activated BMDMs as well as KIM6+ replicates, in part because it lacks the ripCBA operon in the pgm locus (36). A KIM6+ ripCBA4 mutant (Table 1) had a phenotype similar to that of KIM5 with respect to LC3-II levels (Fig. 4E and F). These results indicated that the levels of LC3-II in macrophages could be increased by treatment with IFN-γ or by infection with replicating *Y. pestis*.

Analysis of GFP-LC3 colocalization with YCVs in macrophages. A GFP-LC3 fusion protein can be used as a specific cytological marker for autophagic membranes in eukaryotic cells (19). Fluorescence microscopy was used to quantify the GFP-LC3 in YCVs in macrophages exposed or not exposed to IFN-γ. For this purpose, BMDMs were transduced with a retrovirus producing GFP-LC3 (see Materials and Methods). One day after transduction, BMDMs were infected with KIM6+/mCherry (Table 1) in the presence or absence of IFN-γ. IPTG was added to the infected wells for 2 h to induce de novo mCherry expression in viable intracellular *Y. pestis*. Eight hours postinfection, the infected wells were fixed and the samples were processed for immunofluorescence microscopy (see Materials and Methods). The colocalization of GFP-LC3 and bacterial signals was determined by confocal fluorescence microscopy. As shown in Fig. 5B, GFP-LC3 could be detected on YCVs in macrophages that were treated with IFN-γ, con-
firming that an autophagic membrane can be recruited to phagosomes containing viable Y. pestis, as suggested by transmission EM results (Fig. 3B). However, quantification by epifluorescence microscopy showed that only 30.4% of YCVs in activated macrophages were positive for GFP-LC3 (Fig. 5B). In addition, a slightly higher percentage of YCVs colocalized with GFP-LC3 in naïve macrophages (42.5%) (Fig. 5A). Thus, although Y. pestis infection modulated autophagy, based on increased steady-state levels of LC3-II (Fig. 4), the majority of YCVs did not colocalize with the GFP-LC3 marker of autophagic membranes in BMDMs. It was anticipated that the percentage of GFP-LC3-positive YCVs would be highest in activated macrophages, conditions under which maximal LC3-II levels were seen (Fig. 4), but this was not the case (see Discussion).

Y. pestis inhibits phagosome acidification. The results described above suggested that the localization of an autophagic membrane to YCVs and the increase in LC3-II levels during infection may not be critical for intracellular survival, but rather a side effect or downstream consequence of the strategy that Y. pestis uses to survive in macrophages. Turnover of LC3-II trapped in autophagosomes is mediated by lysosomal

FIG. 4. Immunoblot analysis of LC3 levels in BMDMs exposed to IFN-γ and infected with Y. pestis. BMDMs were not infected or were infected with KIM6+ in the presence or absence of IFN-γ. At 24 h postinfection cell lysates were prepared and subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-LC3 antibody (A, C, and E) and anti-actin antibody (C and E). For panels C and E, macrophages were infected with the KIM6+ phoP mutant, with KIM6+ fixed with paraformaldehyde, with KIM5, or with the KIM6+ ripCBA mutant. Densitometry was used to quantify band signals for LC3-I and LC3-II (A) or for LC3-II and actin (C and E), and calculated ratios are shown in panels B, D, and F, respectively. For panel B the ratio of LC3-II to LC3-I in uninfected and untreated cells was defined as 1, and other ratios were normalized to this value.

FIG. 5. Analysis of GFP-LC3 colocalization with YCVs. BMDMs were transduced with retrovirus producing GFP-LC3. Twenty-four hours posttransduction, the macrophages were infected with KIM6+/mCherry. De novo expression of mCherry was induced in viable intracellular bacteria by addition of IPTG. After 8 h of infection with Y. pestis in the absence (A) or presence (B) of IFN-γ, cells were fixed and processed for examination by fluorescence microscopy. Images were obtained by using confocal fluorescence microscopy. The panels show overlays of GFP-LC3 (green) and mCherry (red) signals from representative images. The percentages of colocalization of signals as determined by epifluorescence microscopy are shown for each condition, and the numbers of phagosomes scored are indicated in parentheses.

42.5% (331) 30.4% (298)
proteases which are activated in autophagolysosomes. Preventing lysosomal degradation by use of protease inhibitors or drugs (such as bafilomycin A1) that inhibit vacuole acidification leads to an increase in the steady-state level of LC3-II (19). *Y. pseudotuberculosis*, which is closely related to *Y. pestis* (1), has been shown to inhibit acidification of phagosomes in primary murine macrophages (46). To determine if *Y. pestis* prevents acidification of phagosomes, colocalization of YCVs with a probe for acidic lysosomes (Lysotracker DND-99) in macrophages was assayed by fluorescence microscopy. When naïve J774A.1 murine macrophage-like cells were infected with KIM5/GFP (Table 1) in the presence of Lysotracker, very few GFP-positive YCVs colocalized with Lysotracker up to 5 h postinfection (Fig. 6A to C and G). In contrast, phagosomes containing killed fixed KIM5/GFP showed increased colocalization with Lysotracker with time, and the value reached 90% by 2.5 h postinfection (Fig. 6D to F and G). This result suggested that live *Y. pestis* could prevent acidification of the YCVs to values below the value required for detection of Lysotracker (pH ≈ 5.5).

We next used live cell ratiometric imaging and *Y. pestis* labeled with the pH-sensitive dye FITC to measure the pH of the YCV over a time course (43). As shown in Fig. 7, the pH...
of phagosomes containing live KIM5 was ~6.5 at 30 min postinfection. Over the next several hours the pH of the YCV was determined to be ~8, confirming the finding of the LysoTracker assay that *Y. pestis* inhibits phagosome acidification. Similar results were obtained for *Y. pseudotuberculosis* 32777 (Table 1) (Fig. 7), confirming that this property is shared by the two *Yersinia* species. In contrast, the pH of YCVs containing fixed KIM5 decreased to ~4.5 within 2.5 h (Fig. 7). These results showed that *Y. pestis* inhibits acidification of its phagosomes, regardless of whether it acquires an autophagic membrane, and prevents maturation of these vacuoles. Thus, in the YCVs that incorporate an autophagic membrane, the normal turnover of LC3-II may be prevented, resulting in accumulation of this autophagy marker.

**Autophagy is dispensable for survival of *Y. pestis* in macrophages.** Based on the results described above (Fig. 5 to 7), we predicted that autophagy is dispensable for survival of *Y. pestis* in macrophages, and to test this hypothesis, we utilized BMDMs deficient for this process. In many studies, 3-methyladenine, an inhibitor of class I and class III phosphatidylinositol 3-kinases, is used to pharmacologically block autophagy in cells. In preliminary control experiments we found that growth of *Y. pestis* in bacterial culture media was inhibited in the presence of 5 mM 3-methyladenine, a concentration that is typically used to block autophagy in cells. We therefore sought an alternative approach to block autophagy that did not involve the potential nonspecific effects of chemical inhibitors. The ATG5 protein is essential for autophagy, as it is required for the formation of the phagophore (19, 21). Mice lacking ATG5 in myelomonocytic cells have been generated by breeding ATG5<sup>flox/flox</sup> mice with mice in which Cre recombinase is expressed from the lysozyme M locus (52). BMDMs derived from the ATG5<sup>flox/flox</sup>-Lyz-Cre mice are defective for autophagy and have been used previously to determine the role of autophagy in coronavirus replication in host cells (52).

BMDMs prepared from ATG5<sup>flox/flox</sup>-Lyz-Cre mice or control ATG5<sup>flox/flox</sup> mice were infected with *Y. pestis*. Immunoblotting for LC3-I and LC3-II confirmed that the ATG5<sup>flox/flox</sup>-Lyz-Cre BMDMs were defective for autophagy (data not shown) (52). The BMDMs were infected with KIM5 or the KIM5<sup>phoP</sup> mutant, and a CFU assay (see Materials and Methods) was performed at different time points after infection. As shown in Fig. 8, KIM5 survived equally well in BMDMs from ATG5<sup>flox/flox</sup>-Lyz-Cre mice and BMDMs from control ATG5<sup>flox/flox</sup> mice. The *phoP* mutant showed a similar survival defect in both types of macrophages (Fig. 8). Therefore, although YCVs can interact with an autophagy pathway in macrophages, recruitment of an autophagic membrane does not appear to be required for survival of *Y. pestis* in phagosomes. Instead, a primary mechanism of *Y. pestis* survival in macrophages seems to be inhibition of YCV acidification.

**Inhibition of phagosome acidification by *Y. pestis* does not require de novo bacterial protein synthesis.** To determine if de novo bacterial protein synthesis was required for *Y. pestis* to inhibit phagosome acidification, J774A.1 macrophages were infected in the presence of the bacteriostatic antibiotic chloramphenicol at a final concentration of 30 μg/ml. The results of a Lysotracker assay performed between 1.5 and 5 h postinfection showed that there was no difference in the ability of KIM5/GFP to inhibit phagosome acidification in the presence and in the absence of chloramphenicol (data not shown). To determine if chloramphenicol was effectively inhibiting bacterial protein synthesis under the conditions used for the infection assay, macrophages were infected with KIM5/GFP that had not been preinduced to express GFP. GFP expression was not upregulated in bacteria in chloramphenicol-treated macrophages following addition of IPTG, showing that the antibiotic effectively inhibited de novo protein synthesis. The results of a coinfection experiment with live and fixed KIM5 showed that live bacteria were unable to prevent colocalization of Lysotracker with phagosomes containing fixed bacteria when they were in the same macrophage (data not shown). Taken together, these results suggest that *Y. pestis* is able to inhibit phagosome acidification using a factor(s) that is produced by the pathogen prior to phagocytosis and that this factor does not act globally within the macrophage to inhibit phagosome acidification.

**Inhibition of phagosome acidification by *Y. pestis* does not require known virulence factors.** KIM10, a *Y. pestis* strain lacking pCD1, pPCP1, and the pgm locus (Table 1), was tested for the ability to prevent phagosome acidification in J774A.1 cells. The results of a Lysotracker assay with KIM10 showed that genes in pCD1, pPCP1, or the pgm locus were not required to prevent phagosome acidification (data not shown). In addition, although a *Y. pestis* phoP mutant is defective for intracellular survival, phagosomes containing the KIM6<sup>– phoP</sup> mutant did not colocalize with Lysotracker for the limited period during which the bacteria remained intact within macrophages, suggesting that the factor(s) required to inhibit phagosome acidification is not under control of the PhoP regulon.

**DISCUSSION**

Several species of pathogenic bacteria subvert the function of macrophages in order to survive and replicate within these...
cells. Examples include *Francisella*, *Brucella*, *Legionella*, *Listeria*, *Mycobacterium*, and *Salmonella* species. These bacteria escape from the phagosome and replicate in the macrophage cytosol or modify the phagosome to reach a replicative niche. Pathogenic bacteria that survive within macrophage phagosomes utilize several different strategies to avoid being killed in a vacuole that could potentially mature into a phagolysosome (9, 24). *M. tuberculosis* survives within macrophages by stalling maturation of its vacuole at an early stage. The mycobacterial phagosome is characterized by the absence of a number of markers that are usually associated with late endosomes (37, 47). For example, *Mycobacterium* inhibits acidification of its vacuole by preventing the accumulation of vATPase. Mature lysosomal proteases are also excluded from the mycobacterial phagosome.

We have shown that the YCV has an unusual trafficking pattern as it can acquire markers of late endosomes (LAMP1 and cathepsin D) (13) and autophagosomes (GFP-LC3) (Fig. 5), yet it does not undergo acidification (Fig. 6 and 7). It is thought that acidification is required for efficient phagosome-lysosome or autophagosome-lysosome fusion (17, 28, 49). We hypothesize that the YCV fuses with several vesicular compartments, including late endosomes, multivesicular bodies, and autophagosomes, but fusion with lysosomes and thus full maturation of the YCV are inefficient due to its lack of acidification. The observation that *Y. pestis* infection results in increased steady-state levels of LC3-II (Fig. 4) is consistent with this model, since LC3-II turnover would be prevented in nonacidified autophagosomes. We previously showed that *Y. pestis* could replicate in BMDMs exposed to IFN-γ (36). IFN-γ treatment is known to stimulate autophagy, and some pathogens appear to hijack the autophagy pathway for intracellular replication (26, 28). However, in some cases IFN-γ-induced autophagy is protective, leading to efficient killing (i.e., xenophagy) of intracellular bacteria, such as *M. tuberculosis*. Our results suggest that *Y. pestis* has a strategy to avoid the protective role of xenophagy in either naïve or activated phagocytes and that the increase in the steady-state levels of LC3-II in *Y. pestis*-infected macrophages is an effect, rather than a cause, of YCV formation.

Although results described here indicate that autophagy is not required for survival of *Y. pestis* in macrophage phagosomes (Fig. 8), it is possible that recruitment of an autophagic membrane to the YCV has important consequences for the outcome of the phagosome-host interaction. Autophagosomes may provide a source of membrane, along with late endosomes, for expansion of the YCV into a spacious compartment. Alternatively, recruitment of an autophagic membrane to the YCV may prevent use of the autophagy pathway for its normal functions in macrophages, such as clearing damaged mitochondria or survival during starvation or growth factor withdrawal. There is evidence that death of macrophages infected with *Salmonella enterica* serovar Typhimurium can occur by autophagy (16). We suggest that recruitment of an autophagic membrane to the YCV and prevention of LC3-II turnover allow intracellular *Y. pestis* to slowly compromise the viability of the macrophage. This could provide a mechanism for release of the bacteria from the dying host cell once an intracellular replication cycle has been completed. In this context, we observed that treatment with IFN-γ decreased recruitment of GFP-LC3 to the YCV (Fig. 5), which may represent a protective response on the part of the macrophage to prevent sequestration of autophagic membrane by the pathogen.

The ability to prevent phagosome acidification appears to be an attribute common to several pathogenic bacteria (17). As discussed above for *M. tuberculosis*, the arrest in maturation of its phagosome precludes acquisition of a sufficient density of vATPase, and the failure to acidify is most likely a consequence of the block in membrane trafficking (17). It is currently unclear how *Y. pestis* prevents phagosome acidification. It is possible that, like *M. tuberculosis*, *Y. pestis* prevents association of the vATPase with the YCV. Alternatively, the vATPase may associate with the YCV but is inactivated, as has been suggested for *Y. pseudotuberculosis* (46). The use of anti-vATPase antibodies in conjunction with microscopy techniques should allow us to distinguish between these possibilities.

Recently, *Listeria monocytogenes* has been shown to be capable of replicating slowly in macrophage phagosomes that expand and do not acidify (4). The pore-forming hemolysin listeriolysin O is required for this process, and it was suggested that pore formation by listeriolysin O allows dissipation of the pH gradient (4). *Y. pestis* is not known to secrete a pore-forming hemolysin, and analysis of the sequenced genome does not reveal predicted proteins with significant homology to pore-forming hemolysins (unpublished observations). The putative factor that *Y. pestis* secretes to inhibit phagosome acidification appears to be produced by the bacterium prior to macrophage interaction, and it appears to act on or within the phagosome in a local manner. There is precedence for the production of vATPase-inhibiting molecules by microorganisms (e.g., bafilomycin A1), but to our knowledge there is no known bacterial factor that can mediate this activity directly. It is not inconceivable that a bacterial protein secreted in a phagosome could inhibit the vATPase. The V₀ domain of the vATPase is exposed on the luminal side of the vacuole, and there is previously obtained evidence that a monoclonal antibody that is specific for the a subunit of the V₀ domain can inhibit vATPase activity when this antibody resides in the endosomal compartment (38).

Straley and Harmon showed that genes in pCD1, pCP1, and the pgm locus were not required for replication of *Y. pestis* in murine macrophages (44). Using a Lysotracker assay, we found that these genetic elements are also not required for inhibition of phagosome acidification by *Y. pestis*. The *phoP* gene is important for survival of *Y. pestis* in macrophages (29). PhoP-regulated genes important for survival of *S. enterica* serovar Typhimurium in the phagosomes of macrophages include genes of the *pmr* operon. The products of the *pmr* operon function to modify lipid A with aminoarabinose; this increases bacterial resistance to antimicrobial peptides (10, 15). Genes under control of PhoP in *Y. pestis* that are important for survival in macrophages include *ugd*, *pmrK*, and *mgbC*, which are predicted to promote resistance to antimicrobial peptides (*ugd* and *pmrK*) or low-Mg²⁺ conditions (*mgbC*) of a phagosome (13). Phagosomes containing *Y. pestis* *phoP* mutants do not colocalize with Lysotracker for the period of time that the bacteria remain intact within macrophages. This result indicates that inhibition of phagosome acidification is not sufficient for intracellular survival of *Y. pestis*; resistance to antimicrobial peptides and resistance to environmental stress en-
countered within the YCV are also important for replication of the pathogen in macrophages. Further understanding of the mechanism by which Y. pestis inhibits phagosome acidification requires identification of genes essential for this process.

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REFERENCES


27. Morales, V. M., A. Bäckman, and M. Bugdasarian. 2004. A series of wide-


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