

Research Paper

# *Listeria monocytogenes* Evades Killing by Autophagy During Colonization of Host Cells

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## KEY WORDS

*Listeria monocytogenes*, bacterial invasion, autophagy, innate immunity, lysosome, macrophage.

## ABBREVIATIONS

CFU colony forming unit  
LAMP-1 lysosomal-associated protein-1  
LC3 microtubule-associated protein light chain-3  
LLO Listeriolysin O  
MEF mouse embryonic fibroblast  
p.i. post infection  
PLC phospholipase C  
TEM transmission electron microscopy

## ABSTRACT

*Listeria monocytogenes* is an intracellular pathogen that is able to colonize the cytosol of macrophages. Here we examined the interaction of this pathogen with autophagy, a host cytosolic degradative pathway that constitutes an important component of innate immunity towards microbial invaders. *L. monocytogenes* infection induced activation of the autophagy system in macrophages. At 1 h post infection (p.i.), a population of intracellular bacteria (~37%) colocalized with the autophagy marker LC3. These bacteria were within vacuoles and were targeted by autophagy in an LLO-dependent manner. At later stages in infection (by 4 h p.i.), the majority of *L. monocytogenes* escaped into the cytosol and rapidly replicated. At these times, less than 10% of intracellular bacteria colocalized with LC3. We found that ActA expression was sufficient to prevent autophagy of bacteria in the cytosol of macrophages. Surprisingly, ActA expression was not strictly necessary, indicating that other virulence factors were involved. Accordingly, we also found a role for the bacterial phospholipases, PI-PLC and PC-PLC, in autophagy evasion, as bacteria lacking phospholipase expression were targeted by autophagy at later times in infection. Together, our results demonstrate that *L. monocytogenes* utilizes multiple mechanisms to avoid destruction by the autophagy system during colonization of macrophages.

## INTRODUCTION

*Listeria monocytogenes* is a Gram-positive foodborne pathogen that causes gastroenteritis, encephalitis and can lead to abortion (reviewed in refs. 1 and 2). The intracellular lifestyle of this bacterium allows it to avoid extracellular host defense systems. *L. monocytogenes* is able to mediate its own uptake into phagocytic and nonphagocytic cells via the bacterial internalins InlA and InlB, and enters a phagosome by a 'zippering' mechanism. However, these bacteria have the capacity to escape from this compartment and grow in the cytosol of host cells during infection. Escape from the phagosome is mediated principally by Listeriolysin O (LLO), a pore-forming protein encoded by the *hly* gene. Expression of LLO alone is sufficient to allow phagosome escape by nonpathogenic bacteria.<sup>3,4</sup> Phagosome escape is enhanced by two phospholipase C (PLC) enzymes, with substrate preferences for phosphatidylinositol (PI-PLC, encoded by *plcA*), or phosphatidylcholine and other phosphoinositides (PC-PLC, encoded by *plcB*). Upon entry into the cytosol, the bacterial protein ActA recruits actin regulatory factors of the host cell. This initiates rapid actin polymerization and motility of the bacteria through the cell cytosol. Actin-based motility generates protrusions at the surface of the host cell, and spread of bacteria to neighboring cells. All known virulence factors of *L. monocytogenes*, including ActA, LLO, PI-PLC and PC-PLC, are controlled by the bacterial transcriptional regulator PrfA.<sup>1,2</sup>

Macroautophagy (hereafter referred to as autophagy) plays a central role in eukaryotic cell physiology.<sup>5</sup> Through this system, protein aggregates, cytosol, and even entire organelles are delivered to the lysosome for degradation. Autophagy is prevalent during times of starvation, cellular stress and the developmental cycle of many organisms, including mammals.<sup>6</sup> Recent studies have also demonstrated a role for autophagy in the immune response to microbial invaders. For example, autophagy has been shown to target viruses, bacteria and parasites.<sup>7,8</sup> Restricted growth of these pathogens in the host cell is ensured by their destruction in the lysosome and the presentation of immunogenic peptides on MHC Class I and II molecules, inducing an adaptive immune response (reviewed in ref. 9). To counter this host defense, some intracellular bacterial pathogens have evolved mechanisms

to evade or subvert autophagy during infection. For example, the Gram-negative *Shigella flexneri* escapes the phagosome after invasion and actively avoids autophagy to colonize the cytosol of host cells.<sup>10</sup> These bacteria express the cell-surface protein IcsA, the functional homologue of *L. monocytogenes* ActA, to drive actin polymerization and motility of the bacteria through the cytosol.<sup>11</sup> Surprisingly, IcsA was shown to interact with Atg5, an essential component of the autophagy machinery. This interaction is sufficient for autophagy of *S. flexneri* mutants lacking the type III secreted effector protein IcsB. However, IcsB binds IcsA at the same site as Atg5, allowing wildtype bacteria to avoid autophagy. Thus, *S. flexneri* appears to express an autophagy target that it masks with a 'molecular shield' to prevent restriction of bacterial growth by autophagy.<sup>10</sup>

As *L. monocytogenes* has a similar intracellular lifestyle to *S. flexneri*, it was originally thought that this bacterium would be able to avoid autophagy. However, a recent study has shown that autophagy targets *L. monocytogenes* before bacterial escape into the cytosol in fibroblasts and epithelial-like cell lines. This targeting limits the onset of bacterial replication, but does not affect replication once the bacteria enter the cytosol in these cell types.<sup>12</sup> As well, Rich et al have shown that a nonmotile *actA* mutant of *L. monocytogenes* treated with the bacteriostatic antibiotic chloramphenicol can be targeted by autophagy in the cytosol of macrophages.<sup>13</sup> However, nontreated bacteria were not addressed in their study. Here, we investigated the interaction of *L. monocytogenes* with the macrophage autophagy system in detail. We demonstrate that *L. monocytogenes* infection induces activation of autophagy in this cell type, and that a population of bacteria within vacuoles is targeted by autophagy in an LLO-dependent manner. However, *L. monocytogenes* utilizes multiple PrfA-regulated mechanisms, including ActA-dependent actin polymerization and bacterial PLC expression, to avoid destruction by the autophagy system to colonize the macrophage cytosol.

## MATERIALS AND METHODS

**Bacterial strains, cell culture and transfection.** *L. monocytogenes* strains used in this study are listed in Table 1. To generate DH-L919, a  $\Delta prfA$  mutant<sup>14</sup> was transformed with a plasmid encoding the *hly* gene under a constitutive promoter.<sup>15</sup>

Mouse RAW 264.7 macrophages were maintained in DMEM growth medium (HyClone) supplemented with 10% FBS (Wisent) at 37°C in 5% CO<sub>2</sub> without antibiotics. For immunofluorescence, macrophages were seeded in 24-well tissue culture plates at 1.25 x 10<sup>5</sup> cells/well 48 h before use or 2.5 x 10<sup>5</sup> cells/well 16–24 h before use. For Western blot analysis, macrophages were seeded in 6-well tissue culture plates at 7.5 x 10<sup>5</sup> cells/well 16–24 h before use. For transmission electron microscopy, macrophages were seeded in 6-well tissue culture plates at 2 x 10<sup>6</sup> cells/well 16–24 h before use. Wildtype and autophagy-deficient (*atg5*<sup>-/-</sup>) mouse embryonic fibroblasts (MEFs) have been previously described,<sup>16</sup> and were maintained in DMEM growth medium supplemented with 10% FBS at 37°C in 5% CO<sub>2</sub> without antibiotics. For Western blot analysis, MEFs were seeded in 6-well tissue culture plates at 1.5 x 10<sup>5</sup> cells/well 16–24 h before use. For gentamicin replication assays, MEFs were seeded in 24-well tissue culture plates without coverslips at 5.0 x 10<sup>4</sup> cells/well 16–24 h before use.

Cells were transfected with FuGene 6 transfection reagent (Roche Diagnostics) 16–24 h before infection according to the manufacturer's instructions. GFP-LC3 was generated as previously described.<sup>17</sup>

Table 1 ***L. monocytogenes* strains used in this study**

Strain	Genotype	Reference
10403S	Wildtype 10403S	Bishop and Hinrichs (1987)
DP-L4137	10403S <i>prfA</i>	Cheng and Portnoy (2003)
DH-L919	10403S <i>prfA</i> + <i>hly</i>	This study
DP-L3078	10403S <i>actA</i>	Skoble et al. (2000)
DP-L2161	10403S <i>hly</i>	Jones and Portnoy (1994)
DP-L4818	10403S <i>hly</i> + <i>hly</i>	Lauer et al. (2002)
DP-L1552	10403S <i>plcA</i>	Camilli et al. (1993)
DP-L1935	10403S <i>plcB</i>	Smith et al. (1995)
DP-L1936	10403S <i>plcA plcB</i>	Smith et al. (1995)
DP-L3177	10403S <i>plcB</i> (H69G)	Zuckert et al. (1998)
DP-L3178	10403S <i>plcB</i> (H118G)	Zuckert et al. (1998)
DP-L2167	10403S <i>plcB</i> + <i>plcB</i>	Smith et al. (1995)

**Bacterial infections, drug treatments and replication assays.** For most experiments, *L. monocytogenes* were grown for ~16 h in BHI at 37°C with shaking, subcultured 1:10 in BHI without antibiotics and grown to an OD<sub>600</sub> of 0.8 at 37°C. Bacterial inocula were prepared by pelleting at 10,000 x g for 1–2 min, washing once and resuspending in PBS. Bacterial inocula were then diluted in DMEM without FBS. Cells were washed twice in DMEM without FBS and infected at an MOI of 10, unless otherwise indicated. Bacteria were centrifuged onto cells at 1,000 rpm for 1 min at room temperature, and infected cells incubated at 37°C with 5% CO<sub>2</sub>. At 30 min p.i., extracellular bacteria were removed by extensive washing with PBS. Gentamicin was added to the media to a final concentration of 10 µg/mL and maintained throughout the duration of the experiment. Where indicated, chloramphenicol was added to the media at a final concentration of 200 µg/mL at 3 h p.i. and maintained throughout the duration of the experiment. The autophagy inhibitors were added to the media at 10 min p.i. and used at the following concentrations: wortmannin (Sigma), 100 nM; 3-methyladenine (Sigma), 10 mM; LY294002 (Sigma), 100 µM. The autophagy inducer rapamycin (Sigma) was used at 25 µg/mL, and was added to the media at 30 min p.i. To induce autophagy using starvation, MEFs were washed and switched from DMEM supplemented with 10% FBS to Hank's Balance Salt Solution (HBS; Cellgro) 4 h before harvesting. Folimycin was used at 200nM and was added to the media for 4 h.

Gentamicin-protected intracellular replication assays were performed by growing *L. monocytogenes* for ~16 h in BHI at room temperature without shaking to an OD<sub>600</sub> of 2. Bacterial inocula were prepared as above and diluted in DMEM supplemented with 10% FBS. Cells were infected at an MOI of 50 with no centrifugation and incubated at 37°C with 5% CO<sub>2</sub>. At 1 h p.i., extracellular bacteria were removed by extensive washing with PBS. Gentamicin was added to the media to a final concentration of 5 µg/mL gentamicin and maintained throughout the duration of the experiment. At 2 h and the indicated times p.i., cells were washed twice in PBS and lysed in 0.2% Triton X-100 by pipetting. The suspensions were then serially diluted 10<sup>-2</sup> to 10<sup>-5</sup> and plated in replicates of 3 on BHI plates. Plates were incubated overnight at 37°C and colony forming units (CFUs; viable intracellular bacteria) were counted. At least three independent experiments were performed. Fold replication was determined by dividing the CFUs at the desired time p.i. by the CFUs at 2 h p.i.

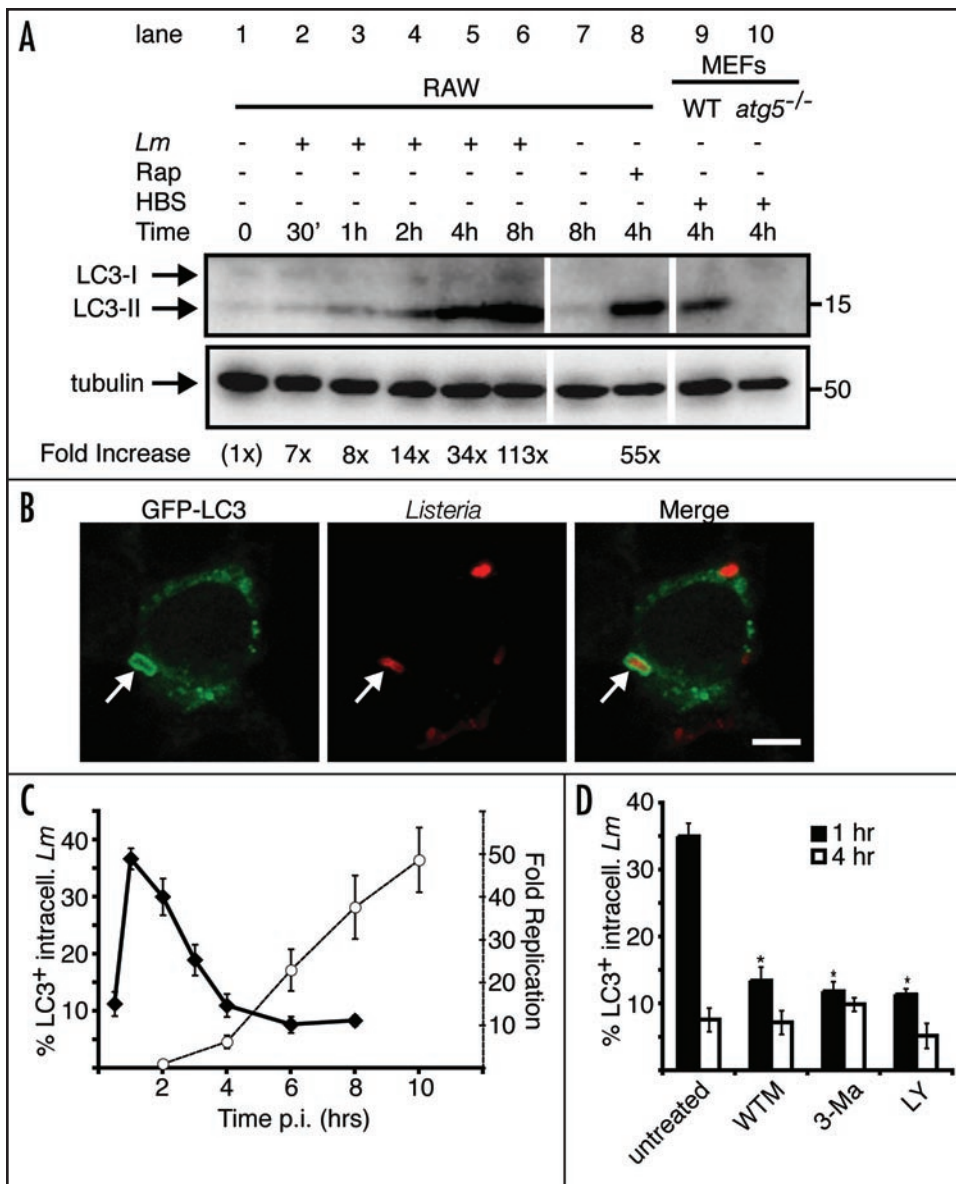


Figure 1. Activation of the autophagy system in macrophages does not restrict intracellular growth of wildtype *L. monocytogenes*. (A) Top panel: Western blot of endogenous LC3 protein levels. Shown are the LC3-I (cytosolic) and LC3-II (lipid conjugated) forms as detected with polyclonal antibodies to LC3.<sup>17</sup> 264.7 RAW macrophages were infected with wildtype *L. monocytogenes* for the indicated times or treated with 25  $\mu$ M rapamycin for 4 h to induce autophagy. As controls, wildtype (WT) or autophagy-deficient (*atg5<sup>-/-</sup>*) mouse embryonic fibroblasts (MEFs) were grown in starvation media (Hank's Balanced Salt Solution; HBS) for 4 h to induce autophagy. Cells were harvested at the indicated times. Numbers on the right indicate protein sizes (kDa). Bottom panel: Loading control Western blot for tubulin for the same membrane as the top panel. The LC3-II fold increase as compared to the uninfected control (lane 1) is shown for the indicated conditions. (B) Confocal images of RAW macrophages transfected with GFP-LC3 (green) and infected with wildtype *L. monocytogenes*. Cells were fixed at 1 h p.i. and stained for *Listeria* (red). The arrow indicates bacteria recognized by the autophagy marker LC3. Size bar = 5  $\mu$ m. (C) Left axis: RAW macrophages were transfected and infected as in (B), fixed at the indicated times p.i. and stained to distinguish between total and extracellular bacteria (see Materials and Methods). The percentage of intracellular *L. monocytogenes* that colocalized with GFP-LC3 was quantified ( $\blacklozenge$ ). Right axis: RAW macrophages were infected with wildtype *L. monocytogenes* in an intracellular replication assay. Cells were lysed at the indicated times and the intracellular bacteria plated to determine colony forming units (CFUs). Shown is the fold replication compared to 2 h p.i. ( $\circ$ ). Error bars indicate  $\pm$  standard error. (D) RAW macrophages were transfected with GFP-LC3 and infected with a high MOI of wildtype *L. monocytogenes* (MOI = 100). Where indicated, cells were treated with the autophagy inhibitors wortmannin (WTM; 100 nM), 3-methyladenine (3-Ma; 10 mM) or LY294002 (LY; 100  $\mu$ M) at 10 min p.i. Cells were fixed at 1 and 4 h p.i., and stained as in (C). The percentage of intracellular *L. monocytogenes* that colocalized with GFP-LC3 was quantified. Asterisks indicate a significant difference from untreated control levels,  $p < 0.05$ .

**Western Blots.** Infected and uninfected cells were lysed in RIPA lysis buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 50 mM Tris pH 7.5, 1% deoxycholic acid sodium salt, 0.1% SDS) in the presence of protease inhibitors (aprotinin, 10  $\mu$ g/mL; leupeptin, 10  $\mu$ g/mL; pepstatin A, 1  $\mu$ M; PMSE, 1mM) and phosphatase inhibitors (sodium fluoride, 5 mM; sodium orthovanadate, 5 mM). Sample buffer (60 mM Tris pH 6.8, 5% glycerol, 1% SDS, 2%  $\beta$ -mercaptoethanol, 0.02% bromophenol blue) was added to the suspensions, and samples boiled for 5 min. Protein quantification was performed using the RC DC Protein Assay Kit (BioRad) according to the manufacturer's instructions. Samples were run on 13% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 1% skim milk at room temperature for 1 h. Rabbit anti-LC3 was generated as previously described,<sup>17</sup> and was incubated with the membrane overnight at 4°C (1:2000 dilution). The monoclonal antibody to tubulin was from Sigma (clone B-5-1-2). Secondary antibodies used were conjugated to horse radish peroxidase (HRP). Densitometry was performed with FluorChem™ Version 3.04A software (Alpha Innotech Corp.). To control for loading levels, the densitometry measurements of the LC3-II bands were divided by those of the corresponding tubulin bands. The resulting ratios were divided by the LC3-II/tubulin ratio of the control to calculate LC3-II fold increase.

**Immunofluorescence and transmission electron microscopy.** For immunofluorescence, cells were fixed with 2.5% paraformaldehyde (PFA) in PBS for 10 min at 37°C. To distinguish between intracellular and extracellular *L. monocytogenes*, extracellular and total bacteria were differentially stained as follows. Fixed cells were blocked overnight with 10% normal goat serum (NGS; Wisent) in PBS at 4°C. Cells were then stained with polyclonal antibodies to *L. monocytogenes* and an AlexaFluor 350 secondary antibody as previously described<sup>18</sup> to label only extracellular bacteria. Cells were permeabilized and blocked in 0.2% saponin (Calbiochem) and 10% NGS for 30 min, and then stained again with polyclonal antibodies to *L. monocytogenes* and an AlexaFluor 568 secondary antibody to label total bacteria. In this way, intracellular *L. monocytogenes* were only visible in the red channel, while extracellular bacteria were visible in both the red and blue channels. For all other staining

procedures, permeabilization and blocking were performed with 0.2% saponin and 10% NGS overnight at 4°C, and stained as previously described.<sup>18</sup> Fixed immunofluorescence samples were mounted on slides using fluorescent mounting medium (Dako Cytomation) and quantifications were performed using a Leica DMIRE2 epifluorescence microscope. Confocal Z-slices were taken using a Zeiss Axiovert confocal microscope and LSM 510 software.

The following antibodies and dyes were used: Rabbit polyclonal antibodies to *L. monocytogenes* (used at 1:500) were generated as previously described.<sup>19</sup> Rat anti-mouse LAMP-1 antibody (clone ID4B) (used at 1:50) was developed by J. Thomas August and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa. Phalloidin conjugated to AlexaFluor 568 (used at 1:100) was from Molecular Probes. Rabbit polyclonal antibodies to ActA (used at 1:500) were generated as previously described.<sup>20</sup> All secondary antibodies used were AlexaFluor conjugates (used at 1:200) from Molecular Probes. DAPI (Molecular Probes) was used according to the manufacturer's instructions.

For transmission electron microscopy, cells were fixed in 2% glutaraldehyde overnight at room temperature and processed as previously described.<sup>21</sup>

**Statistics.** Colocalization quantifications were performed by direct visualization on a Leica DMIRE2 epifluorescence microscope, unless otherwise specified. At least 100 bacteria were counted for each condition in each experiment. For all gentamicin replication assays and colocalization quantifications, at least 3 independent experiments were performed on separate days. The mean  $\pm$  standard error is shown in figures, and p values were calculated using the two-tailed student's t-test.

## RESULTS

**Activation of the autophagy system in macrophages by *L. monocytogenes* infection.** Autophagy is upregulated in response to microbial products such as lipopolysaccharide<sup>22</sup> and inflammatory cytokines.<sup>23</sup> *L. monocytogenes* infection is known to induce the expression of type I interferons upon colonization of the cytosol,<sup>24</sup> and these cytokines can upregulate autophagy.<sup>25</sup> As well, *L. monocytogenes* infection of mouse embryonic fibroblasts (MEFs) has been shown to induce autophagy.<sup>12</sup> To test if *L. monocytogenes* infection induces autophagy in macrophages, we examined microtubule-associated protein light-chain 3 (LC3), an essential component and well-characterized marker of the autophagy system.<sup>17</sup> There are two forms of LC3, which can be differentiated by their mobility on SDS-PAGE gels. LC3-II is directly conjugated to phosphatidylethanolamine on the membrane of forming autophagosomes and has a higher electrophoretic mobility than unconjugated LC3-I, which is localized to the cytosol.<sup>17</sup> To test our assay, we verified that LC3-II formation corresponded with an increase in autophagy. We detected low signals for LC3-I and II in uninfected RAW 264.7 macrophages of approximately equal intensities (Fig. 1A, lane 1). In response to rapamycin treatment, which induces autophagy,<sup>8</sup> we

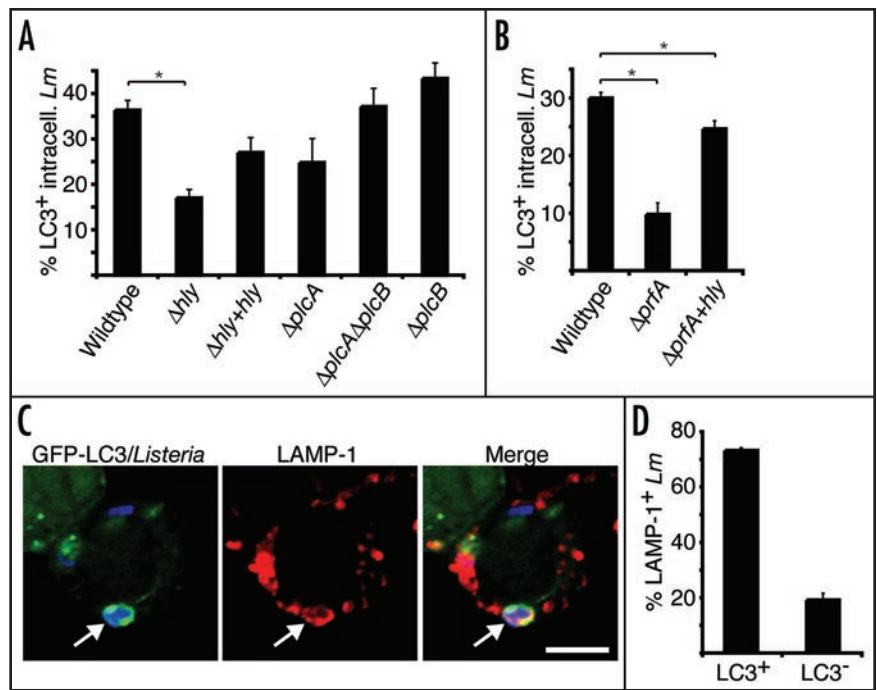


Figure 2. Autophagy targets a population of *L. monocytogenes* in damaged phagosomes. (A) RAW macrophages were transfected with GFP-LC3 and infected with the indicated strains of *L. monocytogenes*. The cells were fixed at 1 h p.i. and stained to distinguish between total and extracellular bacteria. The percentage of intracellular *L. monocytogenes* that colocalized with GFP-LC3 was quantified. Error bars indicate  $\pm$  standard error. Asterisk indicates a significant difference,  $p < 0.05$ . (B) RAW macrophages were transfected with GFP-LC3 and infected with the indicated strains. Cells were fixed, stained and analyzed as in (A). (C) Confocal images of RAW macrophages transfected with GFP-LC3 (green) and infected with wildtype *L. monocytogenes*. Cells were fixed at 1 h p.i. and stained for *L. monocytogenes* (blue) and LAMP-1 (red). Arrow indicates bacteria recognized by autophagy that are also within LAMP-1<sup>+</sup> vacuoles. Size bar = 5  $\mu$ m. (D) RAW macrophages were transfected, infected, fixed and stained as in (C). The percentage of LC3<sup>+</sup> (recognized by autophagy) or LC3<sup>-</sup> (not recognized by autophagy) bacteria that colocalized with LAMP-1 was quantified.

observed a strong signal for LC3-II indicative of autophagy<sup>8,26</sup> (Fig. 1A, lane 8). Similar results were observed with MEFs, as starvation conditions induced the formation of LC3-II in wildtype but not autophagy-deficient (*atg5<sup>-/-</sup>*) cells<sup>16</sup> (Fig. 1A, lanes 9 and 10). Treatment of cells with the vacuolar ATPase inhibitor Folinmycin, which blocks autophagosome maturation and loss of membrane-conjugated LC3, also caused an increase in detectable LC3-II (data not shown). Therefore, our immunoblotting conditions were sufficient to detect autophagy induction.

RAW 264.7 macrophages infected with *L. monocytogenes* and harvested at different times post infection (p.i.) revealed formation of the conjugated LC3-II form following infection (Fig. 1A, lanes 2–6). Autophagy induction was observed as early as 30 min p.i. and increased to a maximum at 8 h p.i., the latest time examined. Therefore, *L. monocytogenes* infection induced activation of the autophagy system in macrophages, consistent with the role of autophagy in immune responses to microbial infection.

**A population of *L. monocytogenes* is targeted by autophagy during early stages of infection in macrophages.** To determine if *L. monocytogenes* is targeted by autophagy during infection of macrophages, we examined the localization of LC3 in infected cells using an N-terminal GFP fusion protein<sup>17</sup> (GFP-LC3). As shown in (Fig. 1B), GFP-LC3 colocalized with a population of intracellular *L. monocytogenes*. Maximum colocalization was observed at 1 h

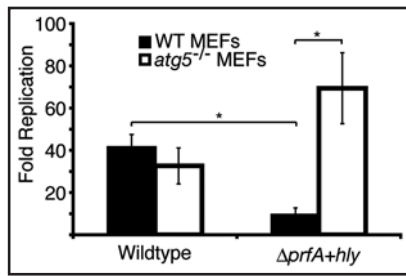


Figure 3. Autophagy restricts intracellular growth of a *L. monocytogenes* *prfA* mutant expressing Listeriolysin O (LLO). Wildtype (WT) or autophagy-deficient (*atg5*<sup>-/-</sup>) mouse embryonic fibroblasts (MEFs) were infected with wildtype or *prfA* mutant bacteria expressing LLO in an intracellular replication assay. Cells were lysed at 8 h p.i. and the intracellular bacteria plated to determine CFUs. Shown is the fold replication compared to 2 h p.i. Error bars indicate  $\pm$  standard error. Asterisks indicate a significant difference,  $p < 0.05$ .

p.i., with ~37% of total intracellular bacteria colocalizing with GFP-LC3 at this time (Fig. 1C, left axis). Treatment of cells with the autophagy inhibitors wortmannin, 3-methyladenine, and LY294002<sup>8</sup> confirmed that LC3 recruitment to intracellular *L. monocytogenes* was via autophagy and not some other event (Fig. 1D). These findings demonstrate that a population of *L. monocytogenes* is targeted by autophagy during the early stages of infection in macrophages. However, the fraction of intracellular *L. monocytogenes* colocalizing with LC3 declined after 1 h p.i., and was as low as ~10% of the total intracellular bacterial population by 4 h p.i. (Fig. 1C, left axis). This result was surprising as autophagy was highly upregulated at this time (Fig. 1A, lane 5), and suggests that intracellular *L. monocytogenes* employ mechanisms to evade targeting/killing by the autophagy system during infection. In accordance with this hypothesis, we observed rapid intracellular growth of these bacteria during the first 8 h p.i., consistent with earlier findings<sup>27-29</sup> (Fig. 1C, right axis).

Autophagy has been shown to target bacteria present in intact phagosomes,<sup>23</sup> damaged phagosomes<sup>30</sup> and the cytosol.<sup>13</sup> Conceivably, the autophagy of *L. monocytogenes* witnessed at 1 h p.i. could have occurred at any of these stages. To address this question, we infected cells with a variety of *L. monocytogenes* mutants lacking membrane-disrupting virulence factors involved in phagosome escape (reviewed in refs. 1 and 2), and determined LC3 colocalization at 1 h p.i. As shown in Figure 2A, *L. monocytogenes* lacking LLO (*hly* mutant) showed significantly less LC3 colocalization than wildtype bacteria. This defect could be partially complemented by expression of an integrated wildtype copy of *hly*. Deletion of either *plcA* or *plcB* (encoding PI-PLC or PC-PLC, respectively) or the double mutant, did not have a significant effect on LC3 colocalization with intracellular *L. monocytogenes* (Fig. 2A). A notable reduction of LC3 colocalization was observed with the *plcA* mutant, though this was not statistically significant. A full kinetic analysis of LC3 colocalization with the *plcA* mutant is discussed below.

Expression of LLO, and all other known virulence factors in *L. monocytogenes*, is regulated by the bacterial transcription factor PrfA.<sup>1</sup> A *prfA* mutant did not colocalize with LC3 during infection (Fig. 2B). However, constitutive expression of LLO in this *prfA* mutant led to almost wildtype levels of LC3 colocalization, indicating that LLO expression is sufficient to induce autophagy of *L. monocytogenes*. Together, these results suggest that either damage to or complete escape from the phagosome is required for autophagy of

*L. monocytogenes* during the early stages of infection, and that LLO is the principal factor that initiates this event.

The majority (~74%) of intracellular *L. monocytogenes* targeted by autophagy at 1 h p.i. colocalized with lysosome-associated membrane protein-1 (LAMP-1) (Fig. 2C and D). These results suggest that *L. monocytogenes* targeted by autophagy at this time are within vacuoles. These vacuoles could be primary phagosomes that have acquired LAMP-1 before the bacteria could escape,<sup>31</sup> or could be bacteria-containing autophagosomes that have matured into autolysosomes. Although both possibilities are valid, acquisition of LAMP-1 by bacteria-containing autophagosomes has been reported to be slow, occurring over several hours.<sup>13,32</sup> Therefore, our data suggest that autophagy targets *L. monocytogenes* within LAMP-1<sup>+</sup> phagosomes in a manner dependent on LLO expression.

***L. monocytogenes* utilizes PrfA-regulated factors to evade growth restriction by autophagy.** Next, we looked at the effect of autophagy on bacterial replication using autophagy-deficient cells. As RAW 264.7 macrophages were difficult to transfect for reliable siRNA knockdown of autophagy genes (data not shown) and standard autophagy inhibitors had nonspecific effects on long-term *L. monocytogenes* growth (data not shown), we examined the growth of these bacteria in autophagy-deficient (*atg5*<sup>-/-</sup>) MEFs.<sup>16</sup> In agreement with previously published results,<sup>12</sup> growth of wildtype *L. monocytogenes* in *atg5*<sup>-/-</sup> cells was comparable to that in normal fibroblasts by 8 h p.i., indicating that autophagy has little effect on the growth of these bacteria (Fig. 3). In contrast to wildtype bacteria, a *prfA* mutant expressing LLO (to allow phagosome escape) grew poorly in control fibroblasts (Fig. 3). Hence, *L. monocytogenes* employs PrfA-regulated factors to promote intracellular growth in normal fibroblasts. Remarkably, the *prfA* mutant grew rapidly in autophagy-deficient cells (Fig. 3). Together, these findings suggest that *L. monocytogenes* utilizes PrfA-regulated virulence factors to evade growth restriction by the autophagy system.

**ActA is sufficient but not necessary for evasion of autophagy by *L. monocytogenes* in the cytosol.** PrfA regulates expression of all known virulence factors of *L. monocytogenes*, including ActA, the bacterial protein responsible for actin-based motility in the cytosol.<sup>1</sup> The polymerization of host cell actin on the surface of bacteria is a well-characterized marker of cytosolic localization.<sup>33</sup> Rich et al. have previously demonstrated that antibiotic-treated *L. monocytogenes* can be recognized by autophagy in the cytosol of macrophages.<sup>13</sup> To test if nontreated *L. monocytogenes* can be targeted by autophagy in the cytosol, we infected cells with *L. monocytogenes* for 4 h and stained for F-actin with phalloidin conjugated to Alexa 568. Those bacteria that exhibited actin 'comet tails' (indicative of actin-based motility) did not colocalize with LC3 (Fig. 4A and B). Similarly, those bacteria associated with diffuse actin 'clouds', precursors to actin-based motility, were almost exclusively LC3<sup>-</sup> (Fig. 4B). In agreement with Figure 1C, only a small amount of bacteria colocalized with LC3 at 4 h p.i., but these bacteria showed negligible association with actin structures (Fig. 4B). Therefore, these observations suggest that bacteria localized to the cytosol are capable of evading autophagy.

We have previously shown that ActA expression is sufficient for evasion of the ubiquitin system in the cytosol of macrophages.<sup>21</sup> To test if ActA expression also plays a role in autophagy evasion, we infected cells for 8 h with either wildtype or *actA* mutant bacteria. As shown in Figure 4C, wildtype bacteria displayed a low level of LC3 colocalization at 8 h p.i. Surprisingly, the *actA* mutant was also competent to evade autophagy. Consistent with this, *actA* mutant *L. monocytogenes* grew similarly to wildtype bacteria for the first 8 h of

infection in macrophages (data not shown and ref. 34). However, ~28% of intracellular *actA* mutant bacteria colocalized with LC3 when chloramphenicol was added to the infected cells at 3 h p.i. and maintained throughout the duration of the experiment (Fig. 4C), as reported previously<sup>13</sup> and confirming that autophagy targeting occurs normally in these cells. Immunoblotting for LC3-II revealed that chloramphenicol alone does not induce autophagy (data not shown). Therefore, our observation that an *actA* mutant of *L. monocytogenes* was capable of evading autophagy in the absence of chloramphenicol suggests that these bacteria can evade autophagy through the expression of other virulence factors.

Surprisingly, in contrast to the *actA* mutant, wildtype bacteria were not subject to autophagy when treated with chloramphenicol (Fig. 4C). Despite chloramphenicol treatment at 3 h p.i., wildtype bacteria were found to undergo actin-based motility at 8 h p.i. (Fig. 4D, top panels). As well, we also detected ActA on the surface of these bacteria (Fig. 4D, bottom panels). Therefore, expression of *actA* during the first 3 h of infection is sufficient to mediate actin-based motility up to 8 h p.i. These results imply that actin-based motility initiated during the first 3 h of infection is sufficient to mediate autophagy evasion, even in the absence of protein synthesis thereafter. Altogether, our findings suggest that *L. monocytogenes* can evade autophagy in the cytosol by at least two mechanisms, one involving actin-based motility and another involving the expression of other PrfA-regulated virulence factors.

***L. monocytogenes* utilizes bacterial phospholipases to evade growth restriction by autophagy.** As PI-PLC and PC-PLC are also PrfA-regulated factors, we next examined their role in autophagy evasion. *L. monocytogenes* mutants lacking each phospholipase were used to infect RAW 264.7 macrophages

and their colocalization with LC3 determined. The *plcA* mutant displayed lower levels of LC3 colocalization than wildtype bacteria at 1 h p.i. (Fig. 5A, also seen previously in Fig. 2A), consistent with the observation that PI-PLC promotes phagosome escape.<sup>2</sup> However, the *plcA* mutant displayed higher levels of LC3 colocalization by 3 h p.i., and these levels were maintained up to 8 h p.i. (Fig. 5A). Similarly, a *plcB* mutant displayed higher levels of LC3 colocalization than wildtype bacteria during the course of infection (Fig. 5A). A double *plcA,plcB* mutant, as well as bacteria expressing catalytic mutations of the *plcB* gene,<sup>35</sup> also showed significantly higher LC3 colocalization than wildtype bacteria at 4 h p.i. (data not shown). LC3 colocalization could be complemented back to wildtype levels with expression of a wildtype copy of *plcB* in the *plcB* mutant background (data not shown). Accordingly, the double *plcA,plcB* mutant grew less well than

wildtype *L. monocytogenes* in macrophages (data not shown), as has been previously shown.<sup>29</sup> Altogether, our results suggest that bacterial phospholipases play a role in autophagy evasion.

To address how PI-PLC and PC-PLC may be contributing to autophagy evasion, we used transmission electron microscopy (TEM) to analyze macrophages infected with either wildtype *L. monocytogenes* or mutant bacteria lacking PLC expression. A hallmark of autophagy is the formation of a double-membrane organelle around the target to be degraded.<sup>8</sup> Interestingly, we did not observe wildtype bacteria in double-membrane compartments at 1 h p.i., the time when LC3 colocalization with *L. monocytogenes* is maximal. These bacteria were either in the cytosol (data not shown) or within single membrane compartments that may have been partially disrupted, possibly *L. monocytogenes*-containing phagosomes (Fig. 5B). However, *prfA*

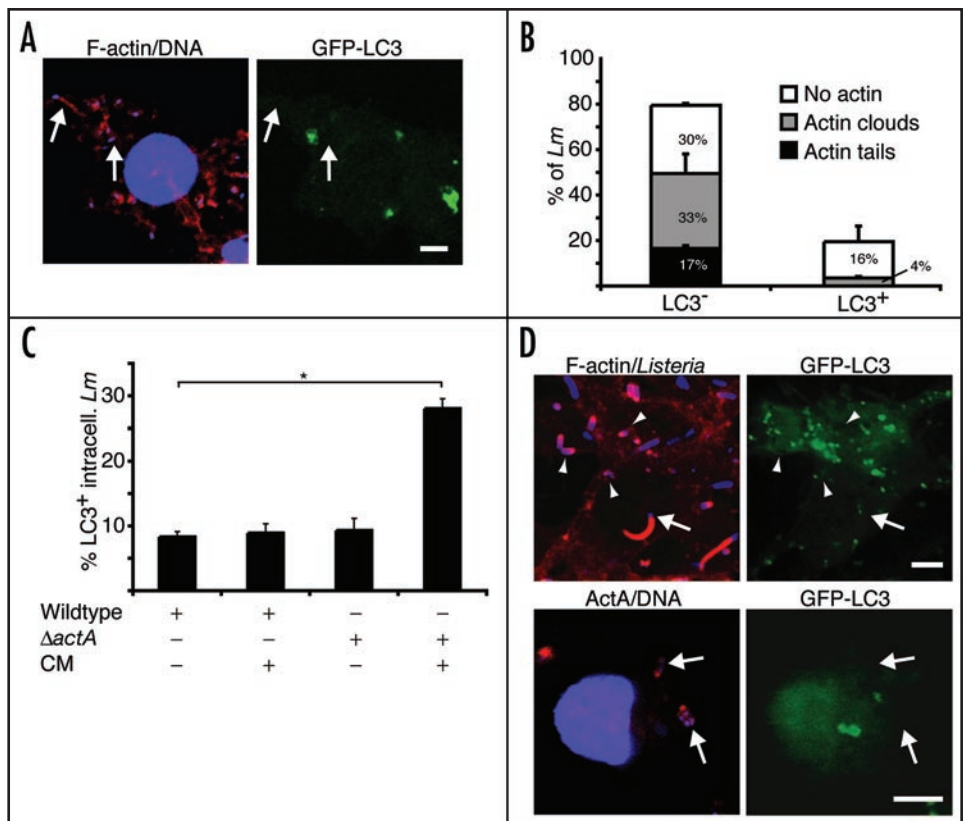


Figure 4. ActA is sufficient but not necessary for evasion of autophagy by *L. monocytogenes* in the cytosol. (A) Confocal images of RAW macrophages transfected with GFP-LC3 (green) and infected with wildtype *L. monocytogenes*. Cells were fixed at 4 h p.i. and stained with phalloidin conjugated to Alexa 568 to visualize F-actin (red) and DAPI to visualize DNA, including bacteria (blue). Arrows indicate bacteria on actin 'comet tails' that are not recognized by autophagy. Size bar = 5  $\mu$ M. (B) RAW macrophages were transfected, infected, fixed and stained as in (A). All bacteria in one confocal plane were counted and grouped according to being LC3<sup>+</sup> or LC3<sup>-</sup>, and then further grouped as having actin 'tails', actin 'clouds' or no actin. Error bars indicate  $\pm$  standard error. (C) RAW macrophages were transfected with GFP-LC3 and infected with either wildtype or *actA* mutant *L. monocytogenes*. Where indicated, chloramphenicol (CM; 200  $\mu$ g/ml) was added to the media to inhibit bacterial protein synthesis at 3 h p.i. Cells were fixed at 8 h p.i. and stained to distinguish between total and extracellular bacteria. The percentage of intracellular *L. monocytogenes* that colocalized with GFP-LC3 was quantified. Asterisk indicates a significant difference,  $p < 0.05$ . (D) Confocal images of RAW macrophages transfected with GFP-LC3 (green) and infected with wildtype *L. monocytogenes*. CM was added to the media at 3 h p.i. and the cells were fixed at 8 h p.i. Top panels: RAW macrophages were stained for *L. monocytogenes* (blue) and with phalloidin conjugated to Alexa 568 to visualize F-actin (red). Bottom panels: Cells were stained for ActA (red) and with DAPI to visualize DNA, including bacteria (blue). Arrows indicate wildtype bacteria treated with CM exhibiting actin 'tails' or ActA staining, and not recognized by autophagy. Arrowheads indicate bacteria exhibiting actin 'clouds' and not recognized by autophagy. Size bars = 5  $\mu$ M.

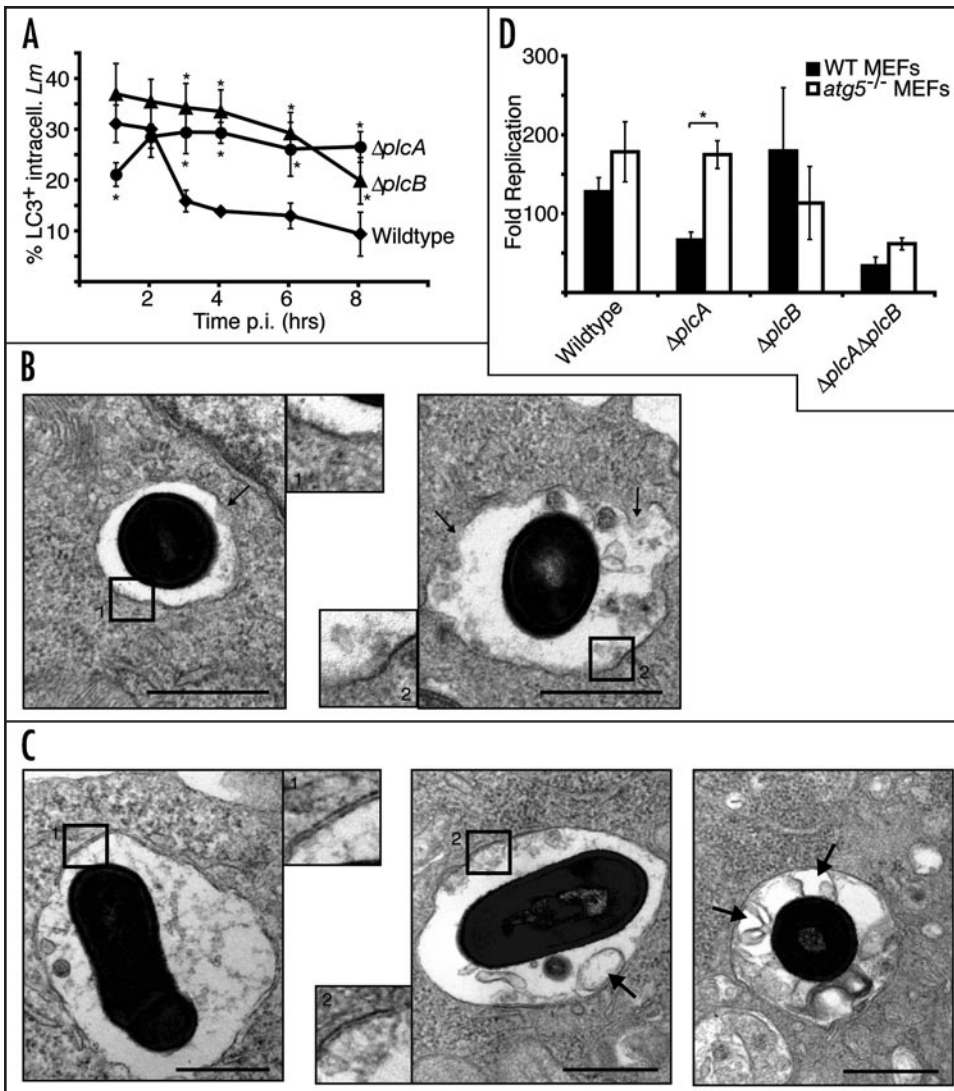


Figure 5. *L. monocytogenes* utilizes bacterial phospholipases to evade growth restriction by autophagy. (A) RAW macrophages were transfected with GFP-LC3 and infected with the indicated strains of *L. monocytogenes*. The cells were fixed at the indicated times p.i. and stained to distinguish between total and extracellular bacteria. The percentage of intracellular *L. monocytogenes* that colocalized with GFP-LC3 was quantified. Error bars indicate  $\pm$  standard error. Asterisks indicate a significant difference from wildtype levels,  $p < 0.05$ . (B) RAW macrophages were infected with wildtype *L. monocytogenes*, fixed at 1 h p.i. and processed for TEM analysis. Boxed areas show magnified images of single-membrane compartments containing bacteria. Small arrows indicate possible signs of vacuole disruption. Size bar = 0.5  $\mu$ m. (C) RAW macrophages were infected with *prfA* mutant *L. monocytogenes* constitutively expressing LLO, fixed at 1 h p.i. and processed as in (B). Boxed areas show magnified images of double-membrane compartments containing bacteria. Arrows indicate vesicles within the bacteria-containing compartments. (D) Wildtype (WT) or autophagydeficient (*atg5*<sup>-/-</sup>) mouse embryonic fibroblasts (MEFs) were infected with the indicated strains of *L. monocytogenes* in an intracellular replication assay. Cells were lysed at 10 h p.i. and the intracellular bacteria plated to determine CFUs. Shown is the fold replication compared to 2 h p.i. Error bars indicate  $\pm$  standard error. Asterisk indicates a significant difference,  $p < 0.05$ .

mutant bacteria constitutively expressing LLO (which do not express PI-PLC or PC-PLC) were often detected within double-membrane structures resembling autophagosomes (Fig. 5C, see magnified images). As these bacteria also do not express ActA, the generation of double-membrane vacuoles by intercellular movement was excluded. We often observed internal vesicles within these bacteria-containing compartments (Fig. 5C, see arrows). Interestingly, Alberti-Segui et al have previously reported that *L. monocytogenes* PLCs may act to degrade the inner membrane of double-membrane vacuoles

during cell-to-cell spread.<sup>36</sup> Our findings suggest that PrfA-dependent factors, such as PI-PLC and PC-PLC, may mediate the disruption of double-membrane autophagosomes during infection of macrophages by wildtype bacteria.

To confirm a functional role for *L. monocytogenes* PLCs in evasion of growth restriction by the autophagy system, we examined the growth of bacteria lacking expression of PI-PLC or PC-PLC in normal and autophagy-deficient MEFs. It has previously been shown that a double *plcA,plcB* mutant of *L. monocytogenes* exhibits a greater degree of growth in *atg5*<sup>-/-</sup> MEFs compared to wildtype fibroblasts.<sup>12</sup> Under our conditions, we observed that a single *plcA* mutant displayed a drop in intracellular growth compared to wildtype bacteria in normal fibroblasts but not in *atg5*<sup>-/-</sup> cells (Fig. 5D). However, deletion of *plcB* alone did not have a significant effect on bacterial growth in either normal or *atg5*<sup>-/-</sup> cells. A double *plcA,plcB* mutant grew less well than the single *plcA* mutant in both normal and *atg5*<sup>-/-</sup> cells, indicating a potentially cooperative function by the two phospholipases. We did observe an increase in replication of the *plcA,plcB* mutant bacteria in *atg5*<sup>-/-</sup> cells compared to wildtype MEFs, though this was not statistically significant (Fig. 5D). Bacteria lacking expression of both phospholipases are trapped in secondary vacuoles following spread, which may account for the lower amount of replication by these bacteria compared to wildtype *L. monocytogenes* in both cell types.<sup>36</sup> Interestingly, these mutant bacteria have been localized to double-membrane structures resembling autophagosomes upon spread to neighboring macrophages,<sup>36</sup> indicating a possible inability to avoid autophagy.

## DISCUSSION

This study is the first in-depth examination of the interaction between *L. monocytogenes* and the autophagy system in macrophages. Infection with *L. monocytogenes* caused a considerable induction of autophagy, and immunofluorescence studies showed targeting of a significant population of intracellular bacteria by autophagy. However, *L. monocytogenes* replicates well in macrophages, which is an important component of in vivo infection.<sup>2</sup> As *L. monocytogenes* is a cytosol-adapted pathogen, it is not surprising that it has developed mechanisms to evade killing by autophagy. However, our studies reveal a surprising complexity to these mechanisms of evasion.

In Figure 6, we propose a model depicting populations of intracellular *L. monocytogenes* that arise during infection of macrophages.

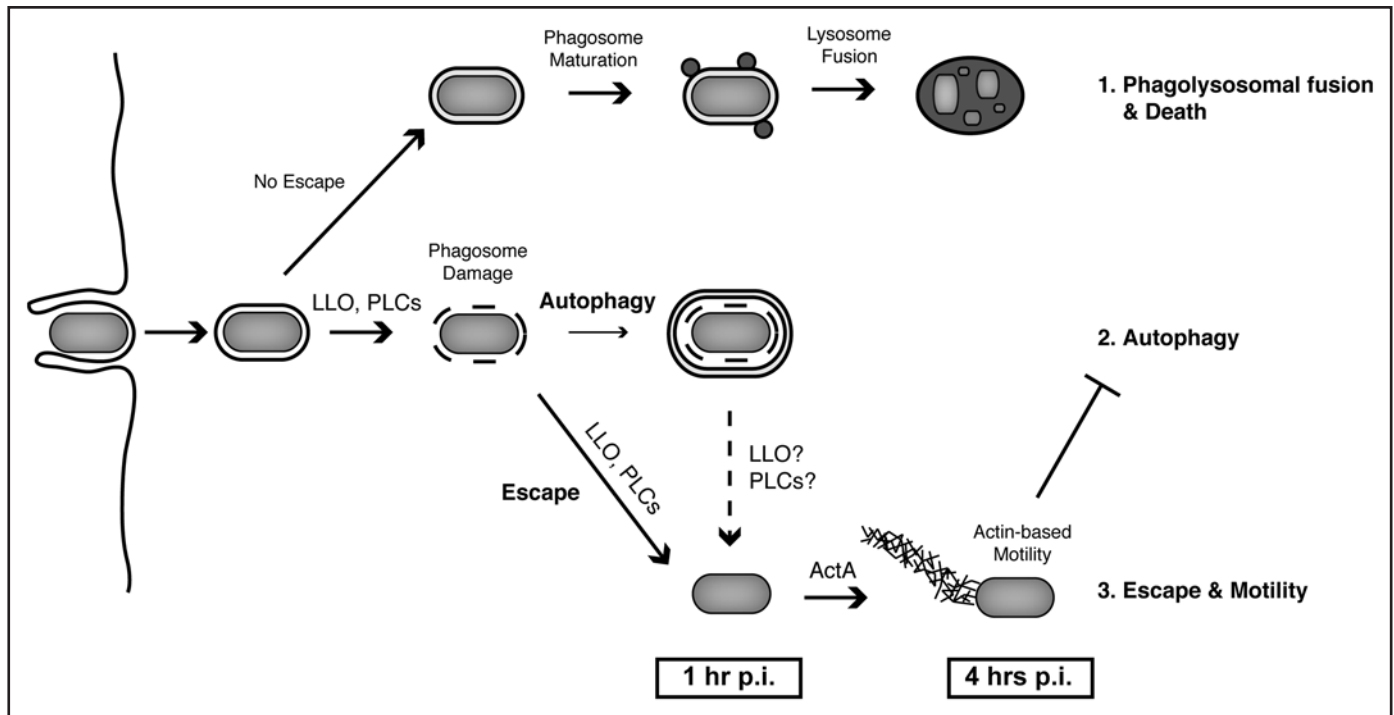


Figure 6. Model for *L. monocytogenes* interaction with the autophagy system during infection of macrophages. *L. monocytogenes* is taken up by host macrophages and enters a phagosome. Upon sensing the environment of the phagosome, the expression of PrfA-dependent virulence factors is upregulated.<sup>1</sup> Population 1: A proportion of bacteria are unable to escape from this compartment, which continues along the phagocytic pathway to fuse with lysosomes. These bacteria are eventually degraded within phagolysosomes.<sup>28</sup> To avoid this fate, the bacteria express membrane-disrupting factors, including LLO, PI-PLC and PC-PLC, to damage the phagosome. Population 2: A proportion of *L. monocytogenes* damages the phagosome, which is then recognized by the host autophagy system. These bacteria may be delivered to degradative autolysosomes or may evade growth restriction by autophagy. Population 3: The best studied population of intracellular *L. monocytogenes* lyses the phagosome using LLO and PLCs, and exists in the cytosol (previously determined to be ~14% of total bacteria at 1 h p.i., see ref. 28). The bacterial protein ActA then induces host cell actin polymerization, allowing actin-based motility and cell-to-cell spread. The bacteria are also able to use this actin polymerization to avoid cytosolic degradative systems, including autophagy (this study) and the ubiquitin system.<sup>21</sup> As well, bacterial phospholipases contribute to autophagy evasion in a manner that remains to be determined.

The phagosomal system of macrophages is very rapid, and a large proportion of *L. monocytogenes* taken up into cells do not succeed in escaping the primary phagosome and are degraded within phagolysosomes (previously estimated to be ~60%, see ref. 28; Fig. 6, Population 1). However, *L. monocytogenes* have developed membrane-disrupting factors and, in essence, there is a race to subvert or escape from the phagosome before it matures into a degradative compartment. These factors include LLO and the complementary phospholipase C's, PI-PLC and PC-PLC, all of which are regulated by the major virulence regulator PrfA.

Here we demonstrate that a population of *L. monocytogenes* is targeted by autophagy during the early stages of infection in macrophages (Fig. 6, Population 2). Autophagy was maximal at 1 h p.i. and dependent on expression of LLO, indicating that intact phagosomes containing the bacteria are not targeted. It is possible the bacteria are targeted by autophagy while within a damaged phagosome, as previously reported for *S. Typhimurium* and *Toxoplasma gondii* infection.<sup>30,37</sup> Live cell imaging techniques, such as those recently developed by Swanson and colleagues,<sup>31,38</sup> will be required to examine this possibility in more detail. The fate of bacteria targeted by autophagy is still unclear and is an active area of investigation. *L. monocytogenes*-containing autophagosomes may mature into degradative autolysosomes as has been suggested to occur during group A *Streptococcus* and *S. Typhimurium* infection.<sup>30,32</sup> However, this would necessarily have to have a minimal impact on overall bacterial

growth as the intracellular growth of wildtype *L. monocytogenes* is not significantly affected in autophagy-deficient cells (Fig. 3 and 5D).

The best characterized population of intracellular *L. monocytogenes* efficiently escapes from the primary phagosome and enters the cytosol. These bacteria polymerize host cell actin to spread from cell-to-cell and perpetuate infection<sup>1,2</sup> (Fig. 6, Population 3). Our data suggests that ActA expression may be sufficient to mediate autophagy evasion in the cytosol at later times in infection. However, the mechanism by which this evasion occurs remains unclear. It is possible that the actin-based movement of the bacteria evades the autophagy system (literally outrunning autophagy), or that the presence of the polymerized actin surrounding the bacteria excludes targeting by autophagy. Actin-based motility is employed by other cytosol-adapted pathogens, including *Rickettsia* species, *Shigella flexneri*, *Burkholderia pseudomallei* and *Mycobacterium marinum*,<sup>39</sup> suggesting that this may be a conserved mechanism to avoid autophagy in the host cell. Interestingly, the *S. flexneri* functional homologue of ActA, IcsA, is directly targeted by the autophagy system.<sup>10</sup> This could represent a host cell adaptation to prevent autophagy evasion. However, this is clearly not the case for *L. monocytogenes* as autophagy targeting can occur in the absence of ActA (refs. 12, 13 and this study). The target for autophagy recognition of cytosolic *L. monocytogenes* still needs to be determined and may be distinct from that which is required for autophagy of bacteria within vacuoles earlier in infection. It is possible that autophagy may target an intrinsic property of



Gram-positive bacteria, or that other host innate immunity factors, such as Nod-like receptors<sup>40-42</sup> or p47 GTPases,<sup>23,43</sup> recognize the invading pathogens and signal for autophagy induction.

Although ActA expression may be sufficient to allow evasion of autophagy by cytosolic *L. monocytogenes*, it is not strictly necessary. Our data suggests that the phospholipases PI-PLC and PC-PLC may also contribute to this function. Deletion of either phospholipase led to an increase in LC3 colocalization with bacteria in macrophages at later times in infection (Fig. 5A). Interestingly, *L. monocytogenes* PLCs have previously been suggested to contribute to autophagy evasion in fibroblasts.<sup>12</sup> The phospholipases may prevent autophagy of bacteria by eliminating the target for autophagy recognition, modulating signaling mechanisms,<sup>44</sup> or perhaps directly mediating escape from autophagosomes. It has been suggested that *L. monocytogenes* PLCs play a role in disrupting the inner membrane of double-membrane compartments.<sup>36</sup> As well, phosphatidylethanolamine, the phospholipid to which LC3 is conjugated on the autophagosome membrane, is a known substrate of PC-PLC.<sup>45</sup>

Altogether, the results of this study demonstrate that *L. monocytogenes* employs multiple PrfA-dependent mechanisms to evade killing by the host autophagy pathway. Importantly, these mechanisms appear to work during multiple stages of the *L. monocytogenes* intracellular lifestyle, and include the avoidance of autophagy in the cytosol.

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