CHAPTER 2

Interactions of *Listeria monocytogenes* with the Autophagy System of Host Cells

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Contents

Abstract

Macrophages are immune cells that participate in the host defense against bacterial pathogens. These cells mediate bacterial clearance by internalizing bacteria into a phagosome, which ultimately fuses with lysosomes to kill bacteria. One bacterial strategy to evade killing in the phagosome is to escape from this compartment prior to lysosomal fusion. *Listeria monocytogenes* is a classic example of a "cytosol-adapted pathogen" in that it can rapidly escape from the phagosome in macrophages (and other cell types) and replicate rapidly in the cytosol. Phagosome escape also enables cell-to-cell spread by the bacteria through a bacterial driven actin-based

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motility mechanism. How the bacteria escape the phagosome and evade host cellular defenses, including autophagy, will be discussed in this review. We also discuss an underappreciated population of *L. monocytogenes* that can replicate in macrophage vacuoles and how these may be important for the establishment of chronic infections.

1. INTRODUCTION

Listeria monocytogenes is the causative agent of listeriosis, a gastroenteritis that is self-limiting in healthy individuals but may become severe and systemic in immunocompromised individuals, the elderly and pregnant women (Rocourt and Bille, 1997). This Gram-positive, rod-shaped bacterium provides an important paradigm for host–pathogen interactions since it can replicate within a variety of host cell types during infection. This includes macrophages, cells of the innate immune system that are normally capable of killing bacteria. To replicate in macrophages, bacterial pathogens have evolved different mechanisms to avoid delivery to the lysosome upon uptake by host cells (reviewed in Flannagan *et al.*, 2009; Kirkegaard *et al.*, 2004; Kumar and Valdivia, 2009). What has intrigued researchers in the *L. monocytogenes* field is the ability of these bacteria to escape from the phagosome and replicate rapidly in the cytosol of host cells. The bacteria can escape from the phagosome via the activity of three virulence factors: listeriolysin O (LLO) and two phospholipase C enzymes, PI-PLC and PC-PLC. Upon phagosome escape, *L. monocytogenes* can then replicate rapidly in the nutrient rich cytosol. Another virulence factor, ActA, then mediates the nucleation of an actin tail on one end of the bacteria. Polymerization of the actin tail allows the bacteria to ''rocket'' into neighboring cells, allowing for cell-to-cell spread of the infection (Tilney and Portnoy, 1989). The ability to escape from the phagosome prior to killing in lysosomes ultimately enables *L. monocytogenes* to replicate rapidly in the cytosol and spread to neighboring cells. Below, we will elaborate on the known host and bacterial factors that facilitate phagosome escape by these bacteria.

In addition to phagosomal defenses, *L. monocytogenes* must counter autophagy, which has recently emerged as a key innate immune defense against intracellular pathogens. Autophagy mediates degradation of cytoplasmic contents within lysosomes and is highly conserved in eukaryotic cells (Levine and Deretic, 2007). This process can be subdivided into three types: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (reviewed in Mizushima *et al.*, 2008). Macroautophagy, hereafter referred to as autophagy, is characterized by the presence of double-membrane vesicles termed autophagosomes, which bear the autophagy marker, microtubule-associated protein light chain 3 (LC3)

(reviewed in Hussey *et al.*, 2009). Autophagy can target specific cargoes, including intracellular pathogens, resulting in their clearance in the lysosome (reviewed in Deretic and Levine, 2009; Hussey *et al.*, 2009). Autophagy has been shown to target bacterial pathogens within intact phagosomes, damaged phagosomes, and in the cytosol (Shahnazari and Brumell, 2011). Therefore, *L. monocytogenes* must successfully evade killing by the autophagy system at all stages of its residence within host cells. Below, we discuss the interactions of *L. monocytogenes* with the autophagy system and their outcome for infection by these bacteria.

2. PHAGOSOME ESCAPE

In order for *L. monocytogenes* to replicate in the cytosol, the bacterium must first escape from the phagosome. Numerous studies indicate that the initial phagosome escape requires both bacterial and host factors (Fig. 2.1). Escape to the cytosol can occur as rapidly as 30 min after bacterial entry (Beauregard *et al.*, 1997; Henry *et al.*, 2006). In murine macrophages, *L. monocytogenes* escape from $Rab7^+$, phosphatidylinositol 3-phosphate $(PI(3)P)^+$, $LAMP\text{-}1^-$ phagosomes (Henry *et al.*, 2006). Despite the fact that some bacterial factors have been identified that facilitate phagosome escape, the precise mechanism and the role of host factors is still undefined. It is likely that there is a dynamic interplay between bacterial factors perforating or altering the phagosomal membrane, and host factors being recruited to repair the phagosome which possibly inadvertently aid in bacterial escape.

2.1. Bacterial factors

The primary bacterial factor that mediates phagosome escape in macrophages is the cholesterol-dependent, pore-forming cytolysin LLO (Cossart *et al.*, 1989; Portnoy *et al.*, 1988, 1992a). Studies have shown that mutants lacking LLO cannot escape macrophage phagosomes (Birmingham *et al.*, 2008; Portnoy *et al.*, 1988). Within minutes of phagosome uptake, LLO can make pores in the phagosomal membrane (Beauregard *et al.*, 1997). These pores grow in size in a time-dependent manner which is evidenced by the exchange of fluorescent molecules of increasing sizes (Shaughnessy *et al.*, 2006). Therefore, LLO creates pores that gradually increase in size and become large enough to allow the exchange of proteins with the cytosol (Higgins *et al.*, 1999). Phagosome perforation also allows the exchange of protons and calcium ions with the cytosol, causing an increased pH and decreased Ca^{2+} concentration within the phagosomal compartment (Shaughnessy *et al.*, 2006). However, it has been shown that LLO requires an acidic pH for optimal activity

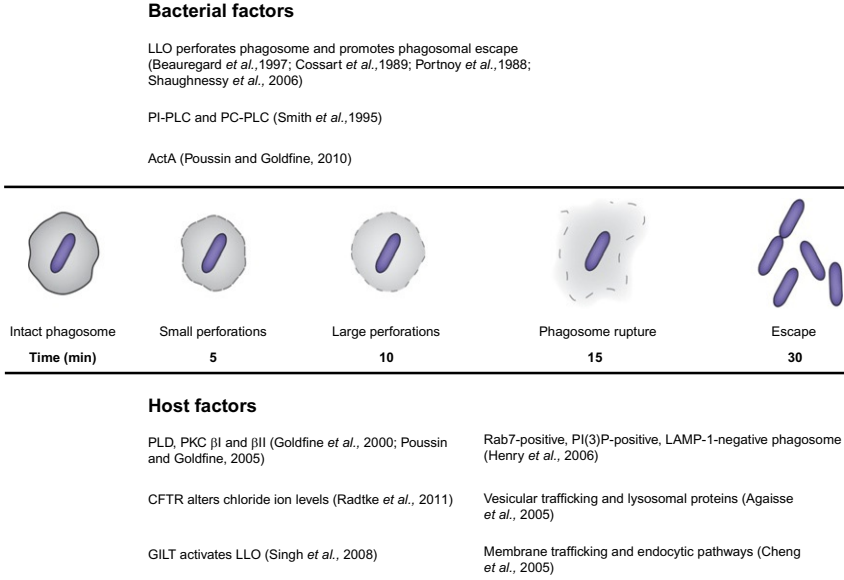

FIGURE 2.1 Kinetics of phagosome escape by *L. monocytogenes* during infection of macrophages. Perforations in the phagosome increase in size until the rupture of the phagosome, facilitating *L. monocytogenes* escape to the cytosol. Escape of *L. monocytogenes* from a macrophage phagosome is dependent on LLO, yet other bacterial and host factors can contribute to phagosome escape.

(Glomski *et al.*, 2002; Portnoy *et al.*, 1992b) and that inhibition of acidification of the phagosome by treatment with bafilomycin A1 decreases *L. monocytogenes* escape from the phagosome (Beauregard *et al.*, 1997). Perhaps initial pore formation requires an acidic pH for LLO to permeabilize the membrane, after which the pH is neutralized by the exchange of ions through pores in the phagosomal membrane.

Two C-type phospholipases, phosphatidylinositol-specific (PI-PLC) and a broad-range phosphatidylcholine (PC-PLC), also help to mediate *L. monocytogenes* escape from the phagosome, possibly by digesting the phagosomal membrane. While LLO is necessary and sufficient for escape, the PLCs play a supporting role to allow for efficient bacterial escape (Shaughnessy *et al.*, 2006; Smith *et al.*, 1995). In addition to its direct role in permeabilizing the phagosomal membrane, PI-PLC can also mediate the translocation of host protein kinase C (PKC) βI and βII (Poussin and Goldfine, 2005). The consequence of PKC βI and βII downstream signaling can promote *L. monocytogenes* escape as inhibition of host PKCs can limit bacterial escape (Poussin *et al.*, 2009; Wadsworth and Goldfine, 1999, 2002). Therefore, LLO and PI-PLC activity, as well as host PKC pathways

can act in concert to promote phagosome permeabilization and subsequent *L. monocytogenes* phagosome escape. It must be noted that the reverse is true for *L. monocytogenes* escape from the phagosome in human epithelial cells as the PLCs are sufficient for phagosome escape while LLO may be dispensable (Burrack *et al.*, 2009; Gründling *et al.*, 2003). It is currently unclear why different bacterial virulence factors are required for escape from the phagosome in different cell types. However, it is worth noting that LLO is sufficient to activate host phospholipases C and D during infection of macrophages (discussed below). Therefore, LLO may activate a host signaling pathway in macrophages that is sufficient to complement loss of bacterial PLCs in this cell type to promote phagosome escape.

Recently, the bacterial factor ActA has been implicated in phagosome escape (Poussin and Goldfine, 2010). The study of phagosome escape was enabled by a probe encoding the cell wall-binding domain of the Listeriophage endolysin Ply118 fused to yellow fluorescent protein (CBD-YFP; see Henry *et al.*, 2006). This probe allows detection of bacteria as soon as they have ruptured the phagosome sufficiently to allow access of the cytosolic probe to bacteria (Henry *et al.*, 2006). Using the CBD-YFP probe, ActA, a bacterial protein previously thought to be involved in actin-based motility and spreading exclusively in the cytosol, was shown to contribute to phagosome escape (Poussin and Goldfine, 2010). The observation that ActA has a role in phagosome escape leads to new hypotheses: *L. monocytogenes* could conceivably be within a phagosome and use ActA to recruit actin through large LLO and PLC derived pores, which could influence escape (Poussin and Goldfine, 2010). Alternatively, escape may be mediated by unknown protein–protein interactions of ActA (Poussin and Goldfine, 2010). It is worth noting that expression of ActA is thought to occur exclusively in the cytosol (Freitag and Jacobs, 1999). Therefore, the question of why Δ*actA* mutants are impaired in phagosome escape requires further study.

2.2. Host factors facilitating escape from the phagosome

In addition to its bacterial virulence factors, *L. monocytogenes* also requires host factors to assist in bacterial escape from the phagosome. PI-PLC can produce the signaling molecule diacylglycerol (DAG) and inositol phosphate upon cleavage of phosphatidylinositol (Griffith and Ryan, 1999). Despite using a *L. monocytogenes* strain lacking both bacterial PLCs, infection of macrophages resulted in an increased level of intracellular DAG over that of the uninfected cell. This observation indicated that *L. monocytogenes* could modulate DAG levels by activating host PLCs as well as utilizing bacterial PLCs (Smith *et al.*, 1995). Consistent with this idea, host phospholipase C (PLC) and phospholipase D (PLD) were found

to be recruited to *L. monocytogenes* containing phagosomes in a manner that is dependent on LLO expression (Goldfine *et al.*, 2000). Further, treatment of cells with a PLD inhibitor reduced phagosome escape, suggesting a link between PLD activity and LLO-mediated escape from the phagosome (Goldfine *et al.*, 2000).

Another host factor, γ-interferon-inducible lysosomal thiol reductase (GILT) is necessary for activation of LLO leading to phagosome escape (Singh *et al.*, 2008). LLO requires activation by GILT via a thiol reductase mechanism in order to form pores and mediate *L. monocytogenes* phagosome escape (Singh *et al.*, 2008). GILT-deficient mice are protected from *L. monocytogenes* infection (Singh *et al.*, 2008). Given that LLO activity is tightly regulated (Schnupf, 2006, 2007), the requirement for a phagosomal protein, GILT, to activate LLO may be an additional bacterial fail-safe mechanism to limit LLO activity to that of the phagosome (Lam and Brumell, 2008).

The cystic fibrosis transmembrane conductance regulator (CFTR) is also reported to be required for phagosome escape by *L. monocytogenes* (Radtke *et al.*, 2011). CFTR is thought to increase the chloride ion concentration in the phagosome, and this may act in concert with LLO to facilitate escape possibly through changes in ion homeostasis (Radtke *et al.*, 2011). How chloride ions facilitate LLO activity in the phagosome remains unclear.

Finally, high-throughput RNA interference (RNAi) screens using macrophage-like *Drosophila* SL2 or S2 cells uncovered a number of additional host factors involved in mediating *L. monocytogenes* phagosome escape. In SL2 cells, host genes involved in vesicular trafficking and lysosomal transport were shown to aid in *L. monocytogenes* escape (Agaisse *et al.*, 2005). Cheng *et al.* (2005) performed an RNAi screen in S2 cells and looked for host genes that affected vacuolar escape in both an LLO-dependent and an LLO-independent manner. Host factors affecting phagosome escape in an LLO-dependent manner included those involved in membrane trafficking and endocytotic pathways (Cheng *et al.*, 2005). A second screen was performed to identify host targets of LLO that can be modulated to allow bacterial escape even in absence of LLO expression (Cheng *et al.*, 2005). When infected with an LLO-deficient mutant, knockdown of host genes involved in late stages of vesicular trafficking allowed vacuolar escape (Cheng *et al.*, 2005). Similar findings were reported in HEK293 cells (Burrack *et al.*, 2009). Therefore, these screens shed light on host factors that aid in *L. monocytogenes* phagosomal escape, both in the presence or absence of LLO expression.

3. AUTOPHAGY AND *L. MONOCYTOGENES*

The importance of autophagy in limiting *L. monocytogenes* replication has been demonstrated *in vivo*. Mice with Atg5-deficient macrophages ($Atg5^{flox/flox}$-Lyz-Cre) exhibit a 50% drop in survival 21 days p.i. with

L. monocytogenes when compared to wild-type mice (Zhao *et al.*, 2008). In particular, significantly greater bacterial load was observed in the livers at day 3 p.i. of the $Atg5^{flox/flox}$-Lyz-Cre mice when compared to control. Further, *Drosophila* mutants deficient in Atg5 or the pattern-recognition receptor, peptidoglycan recognition protein (PGRP)-LE, fail to induce autophagy in response to *L. monocytogenes* infection (Yano *et al.*, 2008). PGRP-LE recognition of the *L. monocytogenes* cell wall component, diaminopimelic acid-type peptidoglycan, results in autophagy targeting of *L. monocytogenes*, as assessed by increased $LC3^+$ double-membrane *L. monocytogenes* containing compartments (Yano *et al.*, 2008). *Drosophila* lacking in PGRP-LE or expressing a mutant PGRP-LE was unable to induce autophagy, resulting in increased susceptibility to *L. monocytogenes* infection. This results in a four-fold decrease in the number of surviving *Drosophila* mutants 8 d p.i. over the wild type (Yano *et al.*, 2008). Thus, these *in vivo* studies provide strong evidence that autophagic induction during *L. monocytogenes* infection is a critical host defense against the bacteria.

In vitro studies have revealed a complex picture of how LC3 targets *L. monocytogenes*. Depending on the stage of infection or where the bacteria are located in the host cell, LC3 targeting of *L. monocytogenes* may be mediated via different mechanisms. Approximately 35% of intracellular *L. monocytogenes* in RAW264.7 macrophages are targeted by LC3, with the peak of LC3 colocalizing with bacteria in the early stages of infection 1 h post infection (p.i.) (Birmingham *et al.*, 2007; Meyer-Morse *et al.*, 2010; Py *et al.*, 2007). LC3 targeting at 1 h p.i. in murine macrophages was found to be dependent on LLO as LLO-deficient *L. monocytogenes* does not become significantly $LC3^+$ at any point during infection (Birmingham *et al.*, 2007; Meyer-Morse *et al.*, 2010; Py *et al.*, 2007).

During later stages of infection of macrophages at 8 h p.i., when most bacteria are present in the cytosol, only 10% of wild type and ActA-deficient *L. monocytogenes* are $LC3^+$. Interestingly, while Δ*actA* mutants treated with the bacteriostatic agent, chloramphenicol, become 30% $LC3^+$, wild-type *L. monocytogenes* treated with chloramphenicol remain 10% $LC3^+$ (Birmingham *et al.*, 2007). This data suggests that cytosolic autophagy targeting of *L. monocytogenes* may be evaded by expression of ActA (Birmingham *et al.*, 2007; Rich *et al.*, 2003). It must be noted that LC3 targeting of Δ*actA* mutants have been reported to occur even in absence of chloramphenicol treatment (Yoshikawa *et al.*, 2009). This difference may be explained by the fact that different genetic backgrounds were used in these studies. Thus, it appears that different strains of wild-type *L. monocytogenes* may have different kinetics of LC3 targeting.

Despite the complexity of different bacterial backgrounds, it is clear that ActA plays a role in *L. monocytogenes* avoidance of autophagy targeting. It is known that the Δ*actA* mutant colocalizes with ubiquitinated

proteins (Ub) at 8 h p.i. (Perrin *et al.*, 2004). Recent work by Sasakawa and colleagues suggests that this protein ubiquitination event mediates recruitment of adaptor proteins such as p62/SQSTM1, which leads to autophagic targeting of the Δ*actA* mutant (Yoshikawa *et al.*, 2009). Another adaptor, NDP52, has also been implicated in the autophagy targeting of cytosolic *L. monocytogenes* (Mostowy *et al.*, 2011). Thus, these combined observations suggest that ActA may play a critical role in evading autophagy targeting by preventing ubiquitination of *L. monocytogenes* in the cytosol. Further, recent findings suggest that in conjunction with ActA, another bacterial effector, InlK, is also involved in the avoidance of autophagy targeting in the cytosol (Dortet *et al.*, 2011). InlK is thought to mediate recruitment of major vault protein to the bacterial surface, serving as a molecular ''shield'' to prevent targeting by autophagy.

L. monocytogenes mutants deficient in PI-PLC exhibit markedly greater bacterial replication in Atg5-deficient MEFs when compared to wild-type MEFs (Birmingham *et al.*, 2007). This observation suggests that PI-PLC, or perhaps both bacterial PLCs, may also play a role in the evasion of autophagy that is currently unappreciated. Thus, there may be multiple mechanisms employed by *L. monocytogenes* to evade LC3 targeting within host cells.

Current data suggests a possible scenario whereby *L. monocytogenes* is subject to two independent LC3-targeting events at different stages of infection. At 1 h p.i. of murine macrophages, where the majority of *L. monocytogenes* are still inside phagosomes, one-third of the population is targeted by LC3 (Fig. 2.2A). It is unclear how this targeting occurs and if protein ubiquitination plays a role in early LC3 targeting. Early LC3 targeting of *L. monocytogenes* may result in bacterial clearance. Data indicates that it can also lead to the formation of SLAPs (*S*pacious *L*isteri*a*-containing *P*hagosomes). SLAPs are large, non-degradative LAMP-1$^+$, and LC3$^+$ vacuoles that contain *L. monocytogenes* (Birmingham *et al.*, 2008). Since the formation of SLAPs was found to require autophagy in the host cell and expression of LLO by bacteria, it has been proposed that SLAPs represent a ''stalemate'' between the host and bacteria, allowing slow bacterial replication in SLAPs that may allow chronic *L. monocytogenes* infection in a host (Birmingham *et al.*, 2008). Indeed, compartments resembling SLAPs have been observed in a severe combined immunodeficiency (SCID) mouse model of *L. monocytogenes* chronic infection (Bhardwaj *et al.*, 1998). Thus, early LC3 targeting of *L. monocytogenes* results either in bacterial clearance or in SLAP formation which may promote chronic infection. After entry into the cytosol, wild-type bacteria utilize ActA to inhibit their ubiquitination by host E3 ligases and thereby are not targeted by autophagy (Fig. 2.2B). ActA also mediates actin-based motility and spread to neighboring cells. Expression of ActA on the

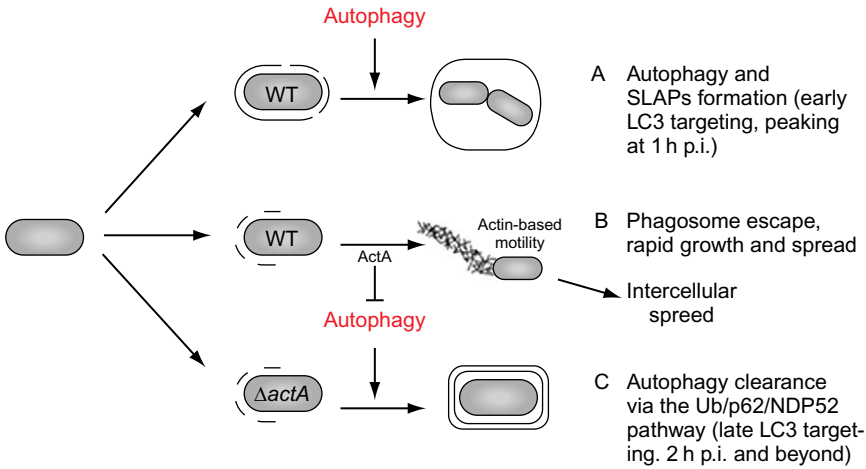

FIGURE 2.2 Pathways of autophagy targeting of intracellular *L. monocytogenes* in murine macrophages. (A) Early LC3 targeting of *L. monocytogenes* which peaks at 1 h p.i. (B) As the infection progresses, wild-type *L. monocytogenes* escapes from the phagosome and, via the activity of ActA, prevents ubiquitination, thus avoiding autophagy targeting in the cytosol. (C) *L. monocytogenes* lacking ActA expression cannot prevent ubiquitination. As such, Δ*actA L. monocytogenes* is ubiquitinated and targeted for autophagy clearance.

bacterial surface may involve a significant delay after escape from the phagosome (Freitag and Jacobs, 1999). During this window of time, bacteria may employ other factors such as InlK and PLCs to evade autophagy. It is also possible that a subset of these bacteria do not express ActA fast enough to prevent bacterial ubiquitination, leading to recruitment of adaptor proteins such as p62 or NDP52, and targeting of bacteria to autophagy (Fig. 2.2C).

4. CONCLUSION

The fate of *L. monocytogenes* inside a macrophage depends on both bacterial factors (LLO, PLCs, and ActA) and host factors (autophagy), giving rise to different populations of *L. monocytogenes* that experience different intracellular fates (Fig. 2.2). While one population of *L. monocytogenes* escape from the phagosome and participate in acute bacterial infection, replication, and cell-to-cell spread, another population of *L. monocytogenes* that is targeted by LC3 gives rise to SLAPs which may be important for chronic infections. The evolution of bacterial strategies for both acute and chronic infection may not be limited to *L. monocytogenes*. SLAP-like structures have also been observed for other intracellular bacteria including

Staphylococcus aureus (Kubica *et al.*, 2008), *Yersinia pestis* (Pujol *et al.*, 2009), *Helicobacter pylori* (Allen *et al.*, 2005), as well as Uropathogenic *Escherichia coli* (UPEC) (Kerrn *et al.*, 2005; Mysorekar and Hultgren, 2006). Thus, the use of *L. monocytogenes* as a model intracellular pathogen may provide insight into how other bacterial pathogens escape from the phagosome or persist in vacuoles inside host cells.

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