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Addendum

Avoiding death by autophagy

Interactions of *Listeria monocytogenes* with the macrophage autophagy system

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Autophagy restricts the growth of a variety of intracellular pathogens. However, cytosol-adapted pathogens have evolved ways to evade restriction by this innate immune mechanism. *Listeria monocytogenes* is a Gram-positive bacterial pathogen that utilizes a cholesterol-dependent pore-forming toxin, listeriolysin O (LLO), to escape from the phagosome. Autophagy targets *L. monocytogenes* in LLO-damaged phagosomes and also in the cytosol under some experimental conditions. However, this bacterium has evolved multiple mechanisms to evade restriction by autophagy, including actin-based motility in the cytosol and an as yet undefined mechanism mediated by bacterial phospholipases C's (PLCs). A population of *L. monocytogenes* with inefficient LLO activity forms Spacious *Listeria*-containing Phagosomes (SLAPs), which are autophagosome-like compartments that do not mature, allowing slow bacterial growth within enlarged vesicles. SLAPs may represent a stalemate between bacterial LLO action and the host autophagy system, resulting in persistent infection.

Introduction

Autophagy is involved in controlling a variety of intracellular bacterial infections, and has been shown to target bacteria in intact phagosomes,[1] damaged phagosomes[2,3] or the cytosol.[4-6] In some cases, the targeted bacteria are delivered to autolysosomes in order to restrict infection. However, other bacterial species evade or even exploit autophagy during infection.[7] Cytosol-adapted pathogens, such as *Listeria monocytogenes* and *Shigella flexneri*, have evolved mechanisms to evade restriction by the autophagy system.

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and

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L. monocytogenes is an intracellular pathogen that replicates in the cytosol of host cells (reviewed in refs. 8 and 9). After uptake into a phagosome, *L. monocytogenes* escapes into the cytosol using listeriolysin O (LLO) and two bacterial phospholipases C's (PLCs). LLO, an essential virulence factor, is a cholesterol-dependent pore-forming toxin that generates small pores in the phagosomal membrane.[10] These pores uncouple the pH and calcium gradients across the phagosomal membrane to block lysosomal fusion. This allows time for LLO and the PLCs to mediate bacterial escape into the cytosol.[11] Once in the cytosol, *L. monocytogenes* replicates rapidly and recruits the host actin polymerization machinery using the bacterial protein ActA, resulting in actin-based motility and eventual spread to neighboring cells.[8,9]

Autophagy of *L. monocytogenes* in the Cytosol

Rich et al were the first to demonstrate that autophagy can directly target bacteria in the macrophage cytosol.[6] Nonmotile *actA* mutant *L. monocytogenes* treated with the bacteriostatic antibiotic chloramplenicol are targeted by autophagy in the cytosol and delivered to lysosome-associated membrane protein-1 (LAMP-1)$^+$ compartments, presumably for degradation.[6] Therefore, cytosolic *L. monocytogenes* has an intrinsic target for autophagy, though the identity of this target is still unknown. We find that wild-type bacteria avoid targeting by autophagy in the cytosol under normal infection conditions in an ActA-dependent manner.[12] Whether this is due to bacterial motility or the formation of an actin 'shell' around the bacteria remains to be determined.

In the absence of actin-based motility (i.e., *actA* mutant bacteria), bacterial protein synthesis must be inhibited in order to get efficient autophagy of bacteria.[6,12] Therefore, *L. monocytogenes* has at least one other active mechanism for autophagy evasion in the cytosol of macrophages. Further studies revealed that bacterial PLCs also play a role.[12] How these bacterial enzymes are involved is not clear, though they may affect the autophagosomal membrane. *L. monocytogenes* PLCs have been shown to act on the inner membrane of double-membrane phagosomes created during intercellular spread,[13] structures morphologically similar to double-membrane autophagosomes. As well, phosphotidylethanolamine, a phospholipid present on the autophagosomal membrane, is a *L. monocytogenes* PLC substrate.[14] Therefore, bacterial PLCs may mediate escape from autophagosomes or prevent their formation.

S. flexneri is another cytosol-adapted pathogen that evades autophagy in the cytosol. Ogawa et al showed that the essential autophagy protein, Atg5, binds the bacterial cell surface protein IcsA.[5] However, this only occurs in the absence of another bacterial protein, IcsB, which binds to IcsA at the same site as Atg5. In this way, IcsB acts as a 'molecular shield' to mask IcsA from binding to Atg5, thereby preventing autophagy of bacteria.[5] Notably, these *S. flexneri* studies were performed in nonprofessional phagocytic cells.

Autophagy of *L. monocytogenes* During Phagosomal Escape

To reach the cytosol, *L. monocytogenes* escapes from the phagosome using the pore-forming toxin, LLO. Surprisingly, *L. monocytogenes* is also targeted by autophagy during escape in an LLO-dependent manner (~37% of total intracellular *L. monocytogenes* are $LC3^+$ at 1 h post infection).[12] The autophagy target at this time is unknown, though it may be the damaged vacuole itself, as has been previously suggested during infection with *Salmonella typhimurium*[2] and *Toxoplasma gondii*.[3] As *L. monocytogenes* infection progresses, the fraction of bacteria colocalizing with the autophagy marker LC3 drops rapidly (~10% by 4 h post infection).[12] In fibroblasts, bacteria are also targeted by autophagy at 1 h post infection. However, overall bacterial replication is not significantly different in autophagy-deficient versus autophagy-competent fibroblasts.[12] Therefore, even though the bacteria are targeted by autophagy, they do not appear to be killed in the autolysosome in this cell-type.

S. flexneri can also be targeted by the macrophage autophagy system within 30 min post infection (p.i.) in a manner independent of IcsA.[15] Autophagy in this case is dependent on the bacterial type III-secretion system and modulated by the Nod-like receptor protein Ipaf.[15] Whether *S. flexneri* is being targeted by autophagy during escape from the phagosome in a manner similar to *L. monocytogenes* remains to be determined.

Spacious *Listeria*-Containing Phagosomes (SLAPs)

The population of *L. monocytogenes* targeted by autophagy during phagosomal escape has several possible fates, including degradation in the autolysosome (although this is not sufficient to have an impact upon overall replication[12]), escape from the autophagosome[12] or formation of Spacious *Listeria*-containing Phagosomes (SLAPs).[16] SLAPs are spacious vesicular compartments formed by a small population of *L. monocytogenes* (Fig. 1) that do not appear to have escaped from the phagosome.[16] SLAPs require autophagy to form, and label with both endocytic and autophagic markers. *L. monocytogenes* replicate in these compartments, though at a much slower rate compared to cytosolic bacteria. Indeed, maturation of these autophagosome-like compartments is blocked by LLO, which uncouples the pH gradient across the SLAP membrane[16] in a manner similar to LLO-mediated phagosomal escape.[11]

Using *hly* mutant bacteria that express inducible LLO,[13] we found that low levels of LLO expression (~33% of wild-type LLO activity[13]) allow bacterial growth in vesicles. This growth is slower than that of wild-type bacteria in the cytosol, and reaches maximal intracellular bacterial levels after a much longer period of time (3 days versus 12 h, respectively).[16] Therefore, LLO allows replication of *L. monocytogenes* in SLAPs when its activity is not sufficient to drive escape into the cytosol, but is sufficient to block lysosomal fusion. What affects the LLO expression of bacteria in SLAPs is unknown. However,

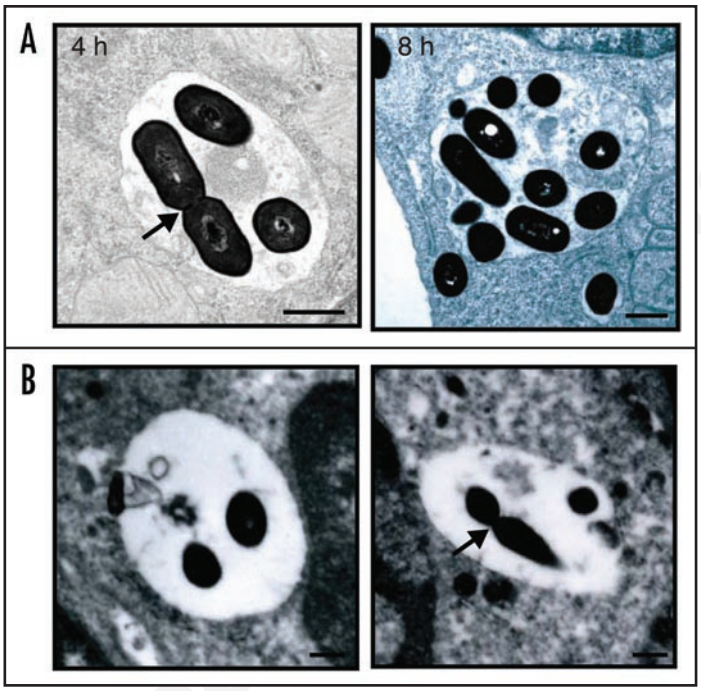

Figure 1. Spacious *Listeria*-containing phagosomes (SLAPs) in vitro and in vivo. TEM images of A) RAW 264.7 macrophages infected for 4 h or 8 h, and B) liver granulomas from SCID mice infected for 21 days with *L. monocytogenes*. Shown are examples of spacious vesicles containing multiple bacteria (SLAPs). Arrows indicate septa of dividing bacteria. Size bars = 0.5 μm. Figure adapted (from Birmingham et al., ref. 16).

LLO activity is inefficient in LAMP-1^+[17] and alkaline[18] compartments, both characteristics of SLAPs. As well, LLO function can be impaired by innate immune defenses, such as reactive oxygen and nitrogen intermediates[19] and cathepsin D.[20] Phagosome maturation is heterogeneous, and therefore bacteria in SLAPs may be targeted by host innate factors differently than those that successfully escape from the phagosome.

SLAPs may occur under specific conditions in vivo. SCID mice lack adaptive immunity, which normally clears *L. monocytogenes* infection, and can develop a persistent infection in which *L. monocytogenes* is present in the liver for up to 21 days p.i.[21] In these animals, bacteria localize to spacious vesicles in macrophages, structures that are morphologically indistinguishable from SLAPs observed in tissue culture cells (Fig. 1).[16,21] Therefore, it is possible that SLAP formation is a mechanism by which persistent *L. monocytogenes* infection is established.

Summary and Future Considerations

In Figure 2, we propose a model summarizing the multiple populations that arise during *L. monocytogenes* infection of macrophages. *L. monocytogenes* can be targeted by autophagy, but has evolved multiple mechanisms to evade restriction by this system, including ActA and PLC-dependent mechanisms, and SLAP formation. SLAPs appear to represent a stalemate between macrophage innate immunity (including autophagy) and bacterial virulence (including LLO). Therefore, LLO plays several important roles during intracellular infection. LLO blocks fusion of the primary phagosome with lysosomes,[11] allows bacterial escape into the cytosol to cause

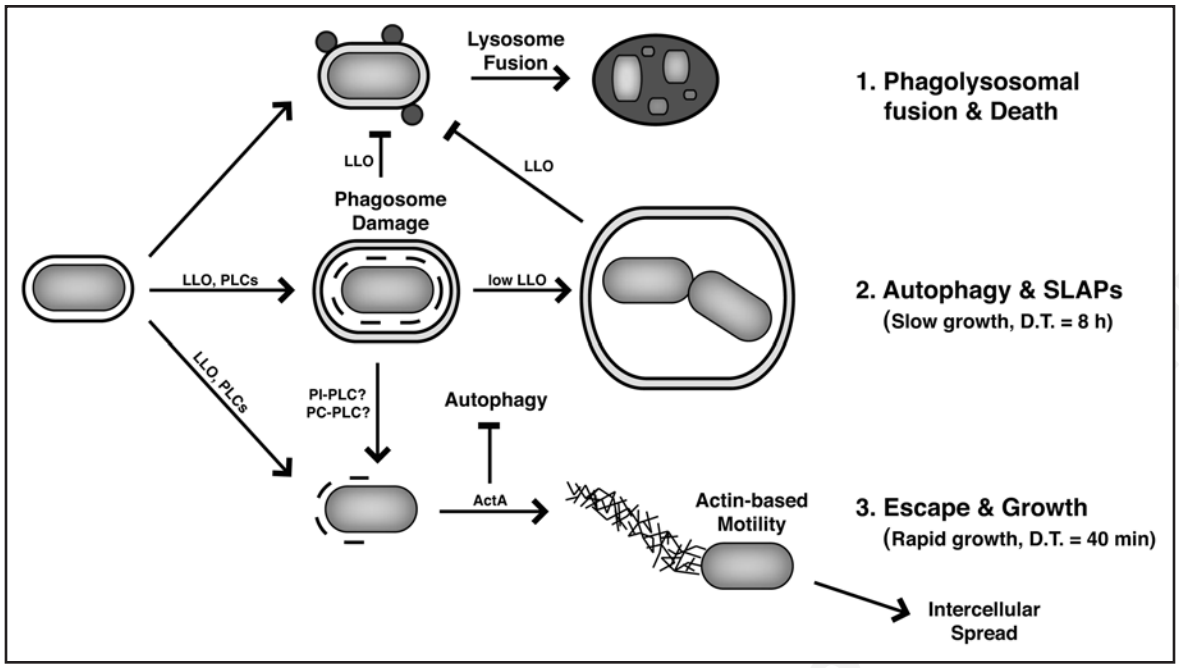

Figure 2. Model of bacterial populations during *L. monocytogenes* infection of macrophages. Bacteria are taken up by macrophages into a phagosome, and a proportion of these bacteria are degraded in a phagolysosome[30] (Population 1). To avoid this fate, *L. monocytogenes* expresses LLO and PLCs to disrupt the phagosomal membrane. Pores created by LLO in the phagosomal membrane block lysosomal fusion and maturation of this compartment.[11] The host autophagy system may target bacteria during phagosomal escape. Bacteria targeted by autophagy may then be degraded in autolysosomes or evade growth restriction by the autophagy system, possibly through the use of bacterial PLCs. A small population of *L. monocytogenes* has low or inefficient LLO activity. This low LLO activity is sufficient to block maturation of the vacuolar compartment, but is not sufficient to mediate escape. Continual damage to the compartment membrane causes more recruitment of autophagy, resulting in the formation of large spacious vesicles or SLAPs (Population 2).[16] The formation of these structures drastically slows bacterial replication (doubling time (D.T.) of approximately 8 h[16]), but does not clear infection. Therefore, SLAP formation may be the result of a stalemate between LLO-mediated bacterial virulence and the macrophage autophagy system, resulting in persistent infection.[16] The proportion of intracellular *L. monocytogenes* believed to cause acute infection escapes from the phagosome using LLO and PLCs, and replicates rapidly in the cytosol (doubling time of approximately 40 min[9,30]). ActA recruits the host actin polymerization machinery, resulting in actin-based motility, autophagy evasion and spread to neighboring cells (Population 3).

acute infection,[8] and damages the phagosomal membrane to trigger autophagy, allowing *L. monocytogenes* growth in SLAPs and possibly persistent infection.[16] As well, LLO has been shown to be involved in signal transduction activation (i.e., NFκB, PKC and MAPK pathways)[10,22] and epigenetic changes in the host cell,[23] indicating that there is a remarkable array of virulence functions associated with LLO.

Pathogen growth in SLAP-like structures may occur during other bacterial infections. Spacious endosome- or autophagosome-like compartments containing multiple bacteria have been observed during infection with *Francisella tularensis*,[24] *Staphylococcus aureus*,[25] *Porphyromonas gingivalis*,[26] *Coxiella burnetti*[27] and *Helicobacter pylori*.[28] Mysorekar and Hultgren showed that uropathogenic *Escherichia coli* can reside in large endocytic vesicles in bladder epithelial cells of mice.[29] These quiescent intracellular bacterial reservoirs can cause recurring bladder infection.[29] It is tempting to speculate that other pathogens known to cause persistent infections may also grow in vesicles formed by the host autophagy system.

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