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# The role of the activated macrophage in clearing *Listeria monocytogenes* infection

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## 1. ABSTRACT

Macrophage activation often contributes to the strong immune response elicited upon infection. The ability of macrophages to become activated was discovered when sub-lethal primary infections of mice with the bacterium *Listeria monocytogenes* provided protection against secondary infections through non-humoral immunity. *L. monocytogenes* infect and propagate in macrophages by escaping the phagosome into the cytosol, where they avoid humoral immune mediators. Activated macrophages kill *L. monocytogenes* by blocking phagosomal escape. The timing of the antimicrobial activities within the phagosome is crucial to the outcome. In non-activated macrophages, bacterial factors generally prevail, and *L. monocytogenes* can escape from the vacuoles and grow within cytoplasm. Activated macrophages generate reactive oxygen or nitrogen intermediates early after bacterial uptake, which prevent the bacteria from escaping vacuoles into cytoplasm. The heterogeneity in the interactions between *L. monocytogenes* and the macrophage indicate a complex relationship between the host and the pathogen governed by chemistries that promote and inhibit escape from vacuoles. This review examines the mechanisms used by activated and non-activated macrophages to kill microbes, and how those mechanisms are employed against *L. monocytogenes*.

## 2. INTRODUCTION

### 2.1. Macrophages and phagocytosis

Macrophages are key mediators in eliciting both innate and adaptive immune responses. Monocytes produced in the bone marrow travel via the circulation to surrounding tissues, where they differentiate into macrophages. Macrophages perform multiple functions, including the phagocytosis and digestion of invading pathogens, presentation of antigen to T lymphocytes, and the production of cytokines that activate various other cell types. Activation of macrophages with soluble stimuli enhances all three of these activities.

Phagocytosis is a process by which macrophages, neutrophils and dendritic cells ingest particles, microbes or apoptotic cells (1). Ingestion and degradation of microbes by macrophages provides a first line of defense in the innate immune response to infection (2). Phagocytosis is also important for antigen processing and presentation, which helps to drive the adaptive immune response.

### 2.2. Phagosome maturation

Although the mechanisms of entry are heterogeneous, the signals involved in phagosome maturation are similar for phagosomes containing a wide range of phagocytosed particles. Phagosome maturation in the endocytic pathway involves a series of fusion events

(3), which are regulated in part by acidification of the endosome (4) (Figure 1). As vesicular compartments mature inside macrophages their luminal pH decreases, eventually reaching pH 4.5-5.0 after fusion with lysosomes (Figure 1). The acidic pH and the hydrolytic enzymes associated with the lysosomal compartment are toxic to most microorganisms (5, 6).

A number of markers allow characterization of phagosome maturation inside macrophages. Rab GTPases regulate endocytic trafficking through molecular tethering events that precede fusion between endosomal membranous compartments (7, 8). Rab5a and Rab7 are GTPases that coordinate membrane fusion of early and late endosomes, respectively (7, 8) (Figure 1). During endosome or phagosome maturation, Rab7 is recruited to membranes as Rab5a leaves (9, 10). Early Endosome Antigen 1, EEA1, is another early endosomal protein that co-localizes with Rab5 but not Rab7 and is required for endosomal transport (11, 12). Other phagosomal and endosomal markers include phosphatidylinositol 3-phosphate (PI3P) and lysosome-associating membrane protein-1 (LAMP-1), a trans-membrane glycoprotein of late endosomes, trans-Golgi vesicles and lysosomes (Figure 1). Type III PI 3-kinases generate PI3P on phagosomal membranes (13, 14), which subsequently recruits effector proteins that mediate endocytic trafficking and microbial killing (15) (Figure 1). Phagosomes typically mature in a uniform sequence of marker arrival and departure (10).

### 2.3. Regulation of phagosome superoxide production

Phagosomes also recruit the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (phagocyte oxidase or Nox2), which generates the highly reactive superoxide into the phagosomal lumen (16, 17). Superoxide is produced from the electron donor NADPH and the one-electron reduction of oxygen (18). The inactive phagocyte oxidase complex consists of two membrane components, $gp91^{phox}$ and $p22^{phox}$, and a set of cytosolic components, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac2 (19). Upon phagocytosis, vesicles containing $gp91^{phox}$ and $p22^{phox}$ fuse with the nascent phagosomal membranes and the cytosolic components associate with the $gp91^{phox}$ and $p22^{phox}$ to form active phagocyte oxidase complexes (19, 20). Luminal superoxide dismutates to various additional reactive oxygen intermediates (ROI) which degrade phagosomal contents and facilitate killing. Patients with chronic granulomatous disease exhibit increased susceptibility to bacterial or fungal infections, likely because their phagocytes are unable to produce superoxide due to deficiencies or mutations in components of the phagocyte oxidase. This indicates the importance of the phagocyte oxidase in the initial response to pathogens (20-23).

The macrophage must regulate the quantity and location of superoxide it produces to prevent damage to itself. Cytosolic superoxide dismutase converts superoxide to hydrogen peroxide (18). The macrophage also produces catalase which dismutates hydrogen peroxide into water and oxygen, a mechanism that limits self-inflicted damage (18). Once inside the phagosome, many pathogens subvert degradation by inhibiting lysosome fusion, or by countering the effects of ROI and related molecules such as reactive nitrogen intermediates (RNI) (24-27). Many bacteria express their own superoxide dismutase that can counteract superoxide generated inside phagosomes (28).

## 3. LISTERIOSIS

*L. monocytogenes* is a Gram-positive bacterium found in soil, water, sewage and decaying vegetation that can infect a wide range of animals, including humans (29). Listeriosis in people typically occurs after ingestion of *L. monocytogenes* in contaminated food, such as pre-packaged meat and cheese (30, 31). Because it can grow at food storage temperatures (4$^{o}$C), *L. monocytogenes* is a serious concern in the food processing industry. *L. monocytogenes* affects primarily immuno-compromised individuals, neonates, and pregnant women (29). Infection by foods contaminated with *L. monocytogenes* can cause gastroenteritis, meningitis, meningoencephalitis, and abortions. Ingested *L. monocytogenes* reaches the large intestine where it infects intestinal epithelial cells, M cells, dendritic cells and macrophages (32-34). When systemic infection occurs, *L. monocytogenes* enter the circulation and transit to the liver and spleen, where they infect hepatocytes and macrophages (35, 36). Both innate and acquired immune responses work in concert to control *L. monocytogenes* infections (31).

## 4. CELL BIOLOGY OF *L. MONOCYTOGENES* INFECTION

*L. monocytogenes* infection of mice has provided a good model system to study innate and adaptive immune responses. Bacteria are internalized by the host cell into a phagosome, or vacuole, from which they escape into the cytosol, grow, and spread to neighboring cells without lysing the primary host cells (Figure 2). Upon entering the neighboring cell, the bacterium occupies a double membrane-bounded vacuole, which it lyses to enter the cytosol for continued growth. The distinct intracellular life-cycle of *L. monocytogenes* allows the bacterium both to escape the harsh environment of the phagosome and to evade humoral defenses in the extracellular milieu.

*L. monocytogenes* secretes many virulence factors that contribute to its proliferation in epithelial cells and macrophages. Escape from the vacuole requires a pore-forming cytolysin, listeriolysin O (LLO), produced by the bacterium (37-39). LLO is a major determinant of *L. monocytogenes* pathogenesis, as mutants lacking LLO (*hly*-) are avirulent in mice due to their inability to escape from vacuoles. When *Bacillus subtilis*, a Gram-positive bacterium that normally cannot escape vacuoles, was engineered to secrete LLO; it acquired the capacity to escape (40). LLO belongs to a family of cholesterol-dependent cytolysins (CDCs) that are secreted by other Gram-positive bacteria (41). However, LLO is unique among CDCs because of its preferred activity in the vacuole (42). The pore-forming activity of LLO is optimal at pH 5.0 and the protein is unstable at neutral pH (43), which helps to explain its increased activity in acidic vacuolar compartments and the subsequent low cytotoxicity

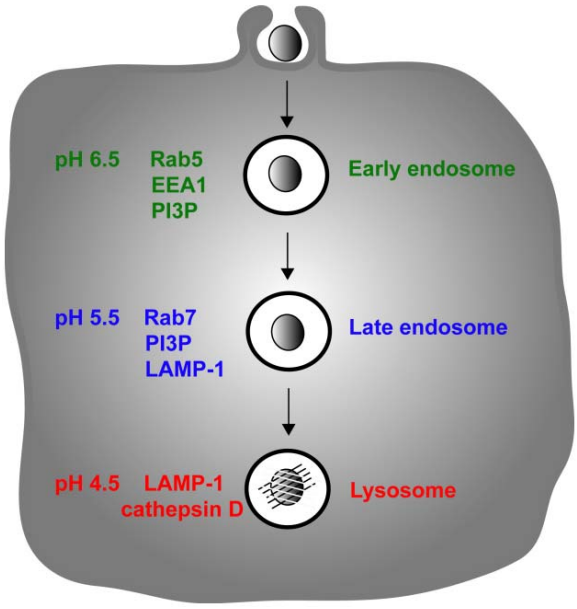


**Figure 1.** Macrophages phagocytose foreign particles into membrane-bounded compartments that undergo fusion events guiding their maturation. After phagosome closure, the phagosome resembles an early endosome that transitions into a late endosome and fuses with the lysosomes.

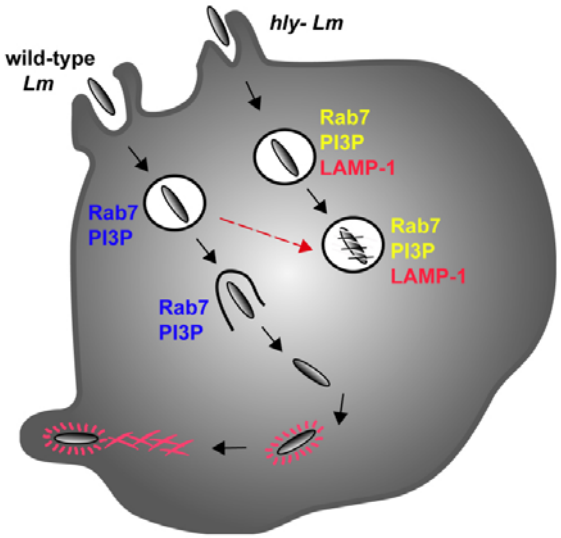


**Figure 2.** In RAW 264.7 macrophages, wild-type *L. monocytogenes* vacuoles recruit and escape from Rab7- and PI3P- positive compartments. Wild-type *L. monocytogenes* also delay lysosome fusion, relative to *hly- L. monocytogenes* vacuoles. *hly- L. monocytogenes* vacuoles also label with Rab7 and PI3P. They eventually also acquire LAMP-1, an indication of their failure to escape.

in the cytosol (44). In addition to LLO, *L. monocytogenes* secrete a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad range phospholipase C, which contribute to vacuolar escape and pathogenicity (45). *L. monocytogenes* also secrete ActA, a protein that mediates actin-based motility of bacteria inside host cells (46, 47).

## 5. MECHANISM OF *L. MONOCYTOGENES* ESCAPE FROM VACUOLES

Before *L. monocytogenes* escape into cytoplasm, they disrupt the maturation process of the phagosomal vacuoles that contain them, thereby inhibiting several macrophage antimicrobial processes (Figure 2). Escape from the phagosome occurs within 30 minutes following phagocytosis (48). Therefore, the chemistries of the anti-microbial attack by the macrophage and the response by *L. monocytogenes* must occur soon after phagocytosis.

### 5.1. Role of early endosomal GTPase, Rab5a, in *L. monocytogenes* infection

Early studies indicated that Rab5a, the small GTPase that regulates trafficking of early endosomes, contributes to macrophage resistance to *L. monocytogenes*. Alvarez-Dominguez and colleagues demonstrated that increased expression of Rab5a in macrophages increased lysosome fusion and subsequent degradation of intracellular *L. monocytogenes* (49). They also showed that down-modulation of Rab5a blocked lysosome fusion and extended the survival of *L. monocytogenes*, indicating that Rab5a was important in early endosome fusion events governing phagosome maturation. Later, Prada-Delgado *et. al.* demonstrated that Rab5a was important in mediating the interferon (IFN)–gamma-induced listericidal activities of macrophages (50). They identified a role for Rab5a in translocation of Rac2 (a component of the phagocyte oxidase) to the *L. monocytogenes* phagosome, which indicated phagocyte oxidase activation and superoxide production. However, recent studies examining the dynamics of Rab5a-yellow fluorescent protein (YFP) chimeras in RAW 264.7 macrophages found that *L. monocytogenes* vacuoles do not recruit Rab5a, indicating that even early in infection, *L. monocytogenes* may disrupt the maturation of the phagosome by excluding Rab5a (Figure 2) (51). Moreover, over-expression of a dominant-negative Rab5a did not affect *L. monocytogenes* escape from vacuoles (51). Nonetheless, Rab5a has been implicated in macrophage responses to *L. monocytogenes* (49, 50, 52, 53), so it remains likely that Rab5a contributes in some way to *L. monocytogenes* pathogenesis.

### 5.2. Avoidance of late endosomes and lysosomes by *L. monocytogenes*

*L. monocytogenes* vacuoles do contain Rab7 and PI3P in RAW 264.7 macrophages, and Rab 7 in J774 macrophages (50, 54) indicating partial maturation of the vacuole (Figure 2) (51). Compared to Fc-gamma receptor - mediated phagocytosis of IgG-opsonized red blood cells, which exhibit transient localization of endocytic markers, the *L. monocytogenes* vacuoles persist as Rab7- and PI3P-positive compartments, and *L. monocytogenes* escape from such vacuoles (Figure 2) (10, 51). Wild-type *L. monocytogenes*, but not *hly- L. monocytogenes*, delay vacuole fusion with LAMP-1-positive compartments; thus indicating a role for LLO in disrupting vacuole maturation

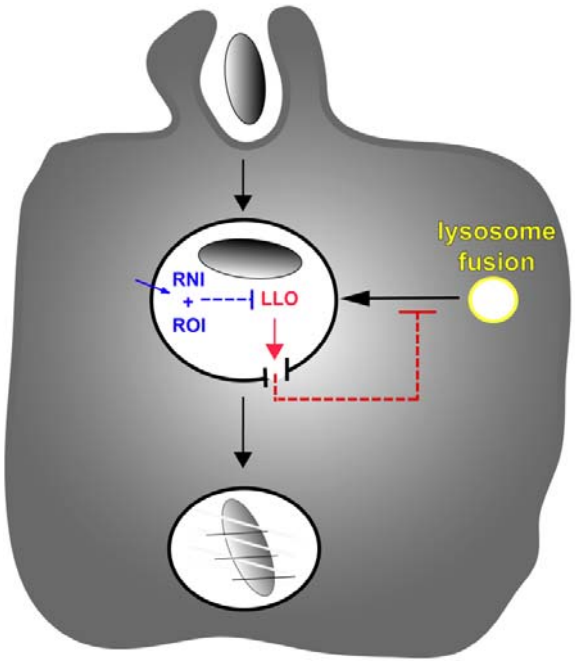


**Figure 3.** Macrophages activated with IFN-gamma and LPS generate reactive oxygen (ROI) and nitrogen (RNI) intermediates within or near the *L. monocytogenes* vacuole, thereby blocking the early perforations by LLO that otherwise allow *L. monocytogenes* to escape the vacuole.

(Figure 2) (51). Earlier studies showed that *hly- Lm* delay phagosome-lysosome fusion relative to heat-killed *hly- Lm*, indicating an additional LLO-independent mechanism for delaying lysosome fusion (55).

How does LLO delay vacuole maturation? One possible explanation is that LLO forms small holes in the vacuolar membrane which allow equilibration of vacuolar contents with cytoplasm (56). Vacuoles containing wild-type *L. monocytogenes* and fluorescent dye molecules show a transient size-selective loss of fluorescent molecules. Such vacuoles also frequently have higher pH and lower calcium concentrations than macropinosomes or vacuoles containing *hly- L. monocytogenes*, consistent with the presence of small pores formed by LLO that allow protons and calcium to equilibrate with the cytoplasm. Disruption of pH and calcium gradients in the endocytic pathway can inhibit fusion events and stall the maturation of endocytic compartments (4, 5, 57-59). Accordingly, the small perforations caused by LLO after some acidification of the *L. monocytogenes* vacuole (60) may delay its fusion with the lysosomes, thereby allowing *L. monocytogenes* to evade degradation (Figure 3).

Thus, the escape of *L. monocytogenes* from vacuoles in non-activated macrophages is preceded by two alterations of vacuolar trafficking. First, in some macrophages the newly formed *L. monocytogenes* vacuole lacks Rab5a, and this exclusion is independent of the presence of LLO (51). This may inhibit the macrophage's ability to launch an early attack by ROI or RNI. Second, phagosome maturation to a LAMP-1-positive stage is further delayed by the action of LLO in the phagosome, possibly via perforations that interrupt vacuole maturation. LLO-dependent inhibition of vacuole maturation may allow the bacterium more time to escape.

LLO-dependent delays of vacuole fusion with lysosomes may allow *L. monocytogenes* to avoid the inhibitory cathepsin D, thereby expanding the window of time available for escape. del Cerro-Vadillo *et. al.* demonstrated that the lysosomal aspartyl-protease cathepsin D inhibits *L. monocytogenes* propagation (61). By degrading LLO, cathepsin D inhibits *L. monocytogenes* growth in macrophages and fibroblasts, and escape from vacuoles (61).

The 30-minute window of opportunity to escape into the cytoplasm may reflect the time *L. monocytogenes* takes to reach the late endocytic compartments (i.e. – those containing LAMP-1) (48). LLO works less efficiently from inside LAMP-1-positive compartments (51), and it may be that the bacteria can only escape early compartments. Only 30-80% of the wild-type *L. monocytogenes* internalized by macrophages escape the vacuole (48, 56, 62). The heterogeneity in both the timing and efficiency of escape reflects the balance between the mechanisms *L. monocytogenes* use to escape the vacuole and those that macrophages employ to block escape. The timing of those counteracting activities during the first 30 minutes after *L. monocytogenes* entry determines the fraction of bacteria that escape.

## 6. REGULATION OF ACTIVATED MACROPHAGES

### 6.1. Early evidence of macrophage activation

The concept of the activated macrophage was first described in early work by Mackaness showing that macrophages were capable of acquiring resistance and increased ability to inhibit *L. monocytogenes* infection (63, 64). In mice susceptible to *L. monocytogenes* infection, bacteria multiplied in macrophages. Macrophages isolated from mice after a sub-lethal infection with *L. monocytogenes* were more microbicidal *in vitro*. IFN-gamma was later shown to be the principle cytokine effector of macrophage activation that protected mice from both local and systemic infection with *L. monocytogenes* (65).

Death and degradation of phagocytosed bacteria and the processing of derived antigens are greatly increased following macrophage activation. Historically, IFN-gamma and lipopolysaccharide (LPS), derived from Gram-negative bacterial cell walls, were used as stimulating factors for the classical model of macrophage activation (66, 67). Together, they prime the macrophage for activation by binding to receptors that induce signal transduction cascades, specifically STAT1 and NF-kappaB activation, leading to the activation of pro-inflammatory genes and anti-microbial factors. Infected, non-activated macrophages produce and secrete IL-12, which stimulates T-cells to produce IFN-gamma, creating a feedback loop in which IFN-gamma then activates macrophages and other cells at the site of inflammation (68). Sensing of LPS by

macrophages induces post-translational regulation of NF-kappaB and leads to the production of Tumor Necrosis Factor-alpha (TNF-alpha). TNF-alpha stimulates both pro-inflammatory and apoptotic responses.

### 6.2. Nitric oxide production in activated macrophages

Activated macrophages produce nitric oxide (NO) (69). Macrophage inducible nitric oxide synthase (iNOS) catalyzes two monooxygenase reactions, hydrolyzing L-arginine and producing NO. Of the three isoforms of nitric oxide synthase, iNOS is the most prevalent isoform expressed in murine macrophages (from the NOS2 gene) (70). Numerous cytokines (IFN-gamma) and bacterial products (LPS) stimulate macrophage expression of iNOS. NO is detrimental to intracellular pathogens, and the combination of NO and superoxide can make the highly reactive product peroxynitrite. Nitric oxide and the reactive nitrogen intermediates (RNI) that derive from it, including nitrite, nitrate and peroxynitrite, are all bactericidal and play a central role in the ability of the activated macrophage to kill ingested pathogens (71).

### 6.3. The role of interferons in macrophage activation

Type I (alpha/beta) and type II (gamma) interferons are important immunomodulatory cytokines during microbial infection (72-74). Interferons mediate a variety of functions through the transcriptional induction of IFN-stimulated genes (75, 76). IFN-gamma stimulates IFN-regulated transcription factors (IRFs) which activate genes for IFN-alpha/beta and inducible nitric oxide synthase (iNOS). Macrophages responding to IFN-gamma are capable of killing microorganisms more readily through increased production of NO. IFN-gamma stimulation also increases expression of major histocompatibility complex class two (MHC II) molecules essential for antigen presentation.

One set of IFN-gamma-stimulated genes up-regulated in response to pathogens includes six different p47 GTP-ases (Igtp, Lrg47, Irg47, Tgtp/Mg21, Iigp, and Gtpi) (77, 78). p47 GTPases are important immune mediators of intracellular pathogens (79). Mice lacking particular p47 GTPases are more susceptible to microbial infection, with each GTP-binding protein having a pathogen-specific response (80-82). LRG-47, which is induced by LPS, IFN-gamma, and IFN-alpha/beta (77), is up-regulated upon infection with *L. monocytogenes* and is important for resistance to *L. monocytogenes* infection (80, 83). These IFN-gamma-induced GTP-binding proteins are located in the ER and recruited to phagosomes, indicating their role in controlling phagosomal bacteria (78, 84-86). Infection of macrophages from LRG-47 -/- mice with *Mycobacterium tuberculosis* determined that phagosomal acidification and lysosome fusion were impaired compared to infection in wild-type macrophages (85).

Microbial products also induce a strong type I interferon response. Although type I interferons have been well studied as anti-viral cytokines, their role in bacterial infections is not yet clear. Interestingly, only *L. monocytogenes* capable of entering the cytosol (LLO+) induce a strong IFN-beta response (87). Mice lacking the type I IFN receptor have increased susceptibility to viral infections, demonstrating the anti-viral immune response elicited by type I interferons. However, analogous studies of *L. monocytogenes* infection showed increased resistance in mice lacking the type I IFN receptor (88-90). This indicates that cytosolic *L. monocytogenes* elicit the normally anti-microbial IFN-beta immune response, but somehow use it to their advantage. Induction of type I interferons also increases apoptosis in *L. monocytogenes*-infected macrophages (91).

### 6.4. Toll-like receptor signaling

Macrophages are also activated through cytokines elicited by microbial molecules, such as LPS (92). Macrophages recognize a number of molecules exhibiting pathogen-associated molecular patterns (PAMPs) which have limited variability and which are not shared by metazoan cells (93). PAMP recognition occurs through pattern recognition receptors (PRRs), primarily Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (92, 94-96). For example, Toll-like receptor 4 (TLR4) and CD14 are PRR co-receptors that recognize the PAMP, LPS (97). LPS signaling through TLR4 in macrophages leads to synthesis of TNF-alpha, IL-1beta, and IL-12. The cytokines modulated by TLR signaling are important for the subsequent innate and adaptive immune response.

TLR-mediated signaling has been implicated in *L. monocytogenes* infections (31). The TLR signal adaptor protein MyD88 is necessary for resistance to *L. monocytogenes* infection and for activation of the innate immune response (98, 99). TLR2, which recognizes lipotechoic acid from Gram-positive bacteria, is not necessary for resistance to *L. monocytogenes* infection (98). Interestingly, neither MyD88 nor TLR2 are necessary for the listericidal activities of activated macrophages (98). LLO and other CDCs also stimulate TLR signaling. CDCs induce TNF-alpha and IL-6 in a TLR4-dependent manner and activate macrophages by inducing iNOS (100).

Another signaling protein that contributes to LPS-induced macrophage activation and defense against bacterial infection is protein kinase C (PKC) epsilon (101). PKC epsilon belongs to the novel subgroup in the PKC family of serine/threonine kinases which are specifically activated by diacylglycerol. Mice deficient in PKC epsilon have a decreased survival rate after bacterial infection (101), apparently as a result of deficient macrophage activation. Macrophages from PKC epsilon -/- mice produce less nitric oxide, TNF alpha, and IL-1 beta in response to LPS and IFN-gamma (101). PKC epsilon is also required for LPS-induced secretion of IL-12 in macrophages (102) and dendritic cells (103). This indicates a link between PKC epsilon and TLR signaling (104, 105). Vacuole perforation by LLO also activates PKC epsilon during *L. monocytogenes* infection (Shaughnessy, L.M., *et. al.*, unpublished data).

## 7. THE ROLE OF THE ACTIVATED MACROPHAGE IN BLOCKING *LISTERIA MONOCYTOGENES* ESCAPE FROM VACUOLES

Peritoneal macrophages activated with IFN-gamma restrict the growth of *L. monocytogenes* by

preventing its escape from the vacuole into the cytosol (62) (Figure 3). Bone marrow-derived macrophages (BMDM) activated with IFN-gamma, LPS, IL-6, and a neutralizing antibody against IL-10 inhibit escape and cytoplasmic growth of *L. monocytogenes* (48).

*L. monocytogenes* escape is inhibited in activated macrophages by both ROI and RNI. Activated BMDM from mice deficient in ROI (gp91$^{phox}$-/-) or RNI (NOS2-/-) production block *L. monocytogenes* escape poorly compared to activated BMDM from wild-type mice (48). NOS2 -/- mice infected with *L. monocytogenes* do not survive as well as wild type mice (106). Additionally, macrophages from gp91$^{phox}$-/-/NOS2-/- mice cannot kill *L. monocytogenes* as readily as wild-type mice (107). ROI are essential for inhibiting escape, and RNI augment those inhibitory effects (48). Reactive oxygen production is localized to *L. monocytogenes* vacuoles, indicating that the macrophage can direct its microbicidal activities into individual phagosomes (48).

It is not yet known how ROI and RNI inhibit escape in activated macrophages. ROI may be generated earlier after phagocytosis or at higher concentrations in vacuoles of activated macrophages (48). Rab5a activities on *L. monocytogenes* vacuoles of activated macrophages may accelerate maturation and allow earlier activation of the phagocyte oxidase or an enhancement of activities provided by localized synthesis of NO near the vacuole (49, 50, 108). LLO contains a single cysteine that must be reduced for hemolytic activity (109); ROI may inhibit LLO by affecting the vacuolar redox potential.

Macrophage activation does not always block *L. monocytogenes* escape (48, 62). Variable amounts of ROI are produced in the vacuoles of activated macrophages, either because of variable activity of the phagocyte oxidase or variable sizes of *L. monocytogenes* vacuoles (48). More spacious vacuoles may reduce the effective concentrations of ROI. Thus, although ROI and RNI are central to the ability of an activated macrophage to control *L. monocytogenes* infection, the mechanisms of their interference with *L. monocytogenes* escape are not known.

## 8. ADAPTIVE IMMUNITY CONTRIBUTES TO *LISTERIA MONOCYTOGENES* CLEARANCE BY MACROPHAGES

The intracellular life cycle of *L. monocytogenes* allows it to avoid humoral defenses such as antibodies and complement. Hence, innate immune responses are important during the initial stages of infection and for final clearance of *L. monocytogenes* (110). During primary infection, IL-12-producing macrophages elicit IFN-gamma secretion by NK cells. Both TLR-dependent secretion of TNF-alpha and the early IFN-gamma response are important for the activation of macrophages and clearance of bacteria (31, 111) as well as for the priming of the adaptive arm of the immune response.

A strong T-cell-mediated response to *L. monocytogenes* is required for clearance (112). *L. monocytogenes* invasion of the cytosol triggers an inflammatory response and gives rise to a protective immune response. LLO-expressing *L. monocytogenes* elicit CD8+ T cells, whereas CD4+ T cells are elicited by LLO+, LLO-, and heat-killed *L. monocytogenes* (113). Lysis of infected cells by cytotoxic T-cells is necessary for full clearance of *L. monocytogenes* (114). LLO is important for the generation of protective immunity, as LLO-derived peptides are dominant CD8+ T-cell epitopes (115).

## 9. PERSPECTIVES

Macrophages are important for eliciting both innate and adaptive immune responses. The activation of macrophages by IFN-gamma and the cytokines elicited by LPS or other microbial products prime macrophages to clear infections. Importantly, activated macrophages are the principle effectors of a strong immune response against *L. monocytogenes*. *L. monocytogenes* growth inside activated macrophages is restricted and bacteria are actively cleared. ROI and RNI, delivered into the *L. monocytogenes*-containing vacuoles, block both pore-forming activity and escape into the cytosol.

Thus, macrophage activation tips the balance of host-*L. monocytogenes* interactions in favor of the host, most likely by affecting the timing of ROI generation into *L. monocytogenes* vacuoles. In non-activated macrophages, *L. monocytogenes* perforate vacuoles quickly enough to inhibit fusion with lysosomes and the generation of high concentrations of ROI and RNI in the vacuole. This early perforation buys the bacterium time to finish its escape from the vacuole. Activated macrophages generate ROI earlier after *L. monocytogenes* phagocytosis, perhaps in combination with RNI, thereby preventing *L. monocytogenes* from generating the perforations that slow maturation and allow vacuolar escape. Future studies should reveal whether altered timing and magnitude of these chemistries are sufficient to kill *L. monocytogenes* in vacuole or if additional activities are needed beyond those that inhibit escape.

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**Abbreviations:** BMDM: bone marrow-derived macrophage, CDC: cholesterol-dependent cytolysin, EEA1: early endosome antigen 1, IFN: interferon, IFR: interferon-regulated transcription factor, LLO: listeriolysin O, LPS: lipopolysaccharide, LAMP-1: lysosomal-associating membrane protein 1, MHCII: major histocompatibility complex class II, NADPH: reduced nicotinamide adenine dinucleotide phosphate, NLR: Nod-like receptor, NF-kappaB: nuclear factor-kappaB, NO: nitric oxide, NOS: nitric oxide synthase, PAMP: pathogen-associated molecular pattern, PI3P: phosphatidylinositol 3-phosphate, ROI: reactive oxygen intermediate, RNI: reactive nitrogen intermediate, PRR: pattern recognition receptor, PKC: protein kinase C, TNF-alpha: tumor necrosis factor alpha, TLR: toll-like receptor, YFP: yellow fluorescent protein.

**Key Words:** listeriolysin O, ROI, RNI, NOS2, Phagosome Maturation, Cholesterol-Dependent Cytolysin, Interferon-Gamma, Rab5, Lysosome, Review

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