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ADVANCES IN  
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VOLUME 66



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# *Advances in* **APPLIED MICROBIOLOGY**

VOLUME **66**

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## Multiple Effector Mechanisms Induced by Recombinant *Listeria monocytogenes* Anticancer Immunotherapeutics

Anu Wallecha,\* Kyla Driscoll Carroll,\* Paulo Cesar Maciag,\* Sandra Rivera,\* Vafa Shahabi,\* and Yvonne Paterson<sup>†</sup>

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## Abstract

*Listeria monocytogenes* is a facultative intracellular gram-positive bacterium that naturally infects professional antigen presenting cells (APC) to target antigens to both class I and class II antigen processing pathways. This infection process results in the stimulation of strong innate and adaptive immune responses, which make it an ideal candidate for a vaccine vector to deliver heterologous antigens. This ability of *L. monocytogenes* has been exploited by several researchers over the past decade to specifically deliver tumor-associated antigens that are poorly immunogenic such as self-antigens. This review describes the preclinical studies that have elucidated the multiple immune responses elicited by this bacterium that direct its ability to influence tumor growth.

## I. INTRODUCTION

*Listeria monocytogenes* is a gram-positive facultative intracellular bacterium responsible for causing listeriosis in humans and animals (Lecuit, 2007; Lorber, 1997; Vazquez-Boland *et al.*, 2001). *L. monocytogenes* is able to infect both phagocytic and nonphagocytic cells (Camilli *et al.*, 1993; Gaillard *et al.*, 1987; Tilney and Portnoy, 1989). Due to its intracellular growth behavior, *L. monocytogenes* triggers potent innate and adaptive immune responses in an infected host that results in the clearance of the organism (Paterson and Maciag, 2005). This unique ability to induce efficient immune responses using multiple simultaneous and integrated mechanisms of action has encouraged efforts to develop this bacterium as an antigen delivery vector to induce protective cellular immunity against cancer or infection. This review describes the multiple effector responses induced by this multifaceted organism, *L. monocytogenes*.

## II. MOLECULAR DETERMINANTS OF *L. monocytogenes* VIRULENCE

To survive within the host and cause the severe pathologies associated with infection such as crossing the intestinal, blood-brain, and fetoplacental barriers, *L. monocytogenes* activates a set of virulence genes. The virulence genes of *L. monocytogenes* have been identified mainly through biochemical and molecular genetic approaches. The majority of the genes that are responsible for the internalization and intracellular growth of *L. monocytogenes* such as *actA*, *hly*, *inlA*, *inlB*, *inlC*, *mpl*, *plcA*, and *plcB* are regulated by a pluripotential transcriptional activator, PrfA (Chakraborty *et al.*, 1992; Freitag *et al.*, 1993; Renzoni *et al.*, 1999; Scortti *et al.*, 2007). Thus, *prfA* defective *L. monocytogenes* are completely avirulent as they lack the ability to survive within the infected host's phagocytic cells such as dendritic cells (DC), macrophages, and neutrophils (Leimeister-Wachter *et al.*, 1990; Szalay *et al.*, 1994).

### A. Virulence factors associated with *L. monocytogenes* invasion

A set of *L. monocytogenes* surface proteins known as invasins interact with the receptors present on host cell plasma membranes to subvert signaling cascades leading to bacterial internalization. The internalins (InlA and InlB) were the first surface proteins that were identified to promote host cell invasion (Braun *et al.*, 1998; Cossart and Lecuit, 1998; Lecuit *et al.*, 1997). InternalinA is a key invasion factor that interacts with the epithelial cadherin (E-cadherin), which is expressed on the surface of epithelial cells and thus promotes epithelial cell invasion and crossing of the gastrointestinal barrier. The efficiency of the interaction between InlA with its receptor E-cadherin is variable in different mammalian hosts. For example, mice are resistant to intestinal infection with *L. monocytogenes* because of a single amino acid difference between mouse and human E-cadherin (Lecuit *et al.*, 1999). InlA is also suggested to be important for crossing the maternofetal barrier since E-cadherin is expressed by the basal and apical plasma membranes of syncytiotrophoblasts and villous cytotrophoblasts of the placenta (Lecuit *et al.*, 1997, 2001). However, the precise role of InlA in crossing the fetoplacental barrier remains to be demonstrated since, fetoplacental transmission occurs in mice that lack the *inlA* receptor and also occurs in guinea pigs that are infected with an *inlA* deletion mutant *L. monocytogenes* (Lecuit *et al.*, 2001, 2004).

InternalinB promotes *L. monocytogenes* entry into a variety of mammalian cell types including epithelial cells, endothelial cells, hepatocytes, and fibroblasts. The hepatocyte growth factor receptor (Met/HGF-R) has been identified as the major ligand for InlB and is responsible for causing the

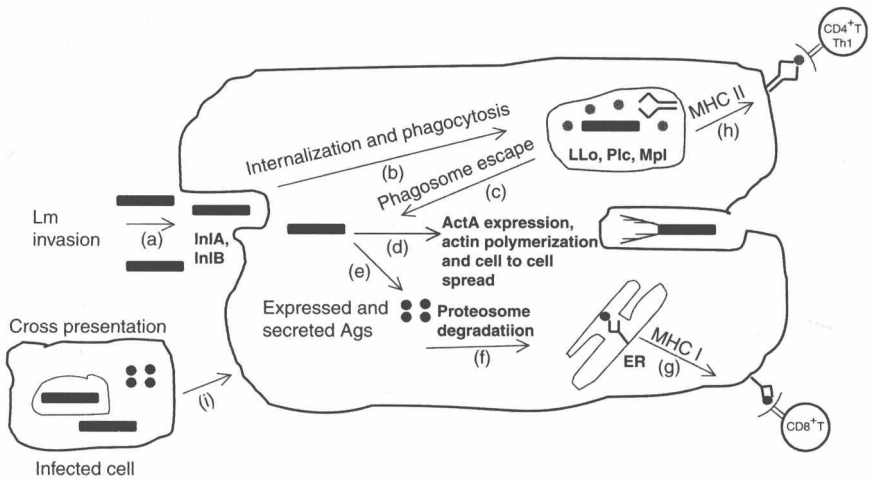
entry of *L. monocytogenes* into nonphagocytic cells (Bierne and Cossart, 2002). Met belongs to the family of receptor tyrosine kinases, one of the most important families of transmembrane signaling receptors expressed by a variety of cells. The activation of Met by InlB is also species specific; indeed InlB fails to activate rabbit and guinea pig Met, but activates human and murine Met (Khelef *et al.*, 2006). *In vivo* virulence studies in mice have shown that InlB plays an important role in mediating the colonization of *L. monocytogenes* in the spleen and liver (Gaillard *et al.*, 1996). InlB is also considered important for crossing the fetoplacental barrier due to the observation that in the absence of InlB, InlA expressing *L. monocytogenes* invaded placental tissue inefficiently (Lecuit *et al.*, 2004). It has also been suggested that InlB is involved in crossing the blood-brain barrier as InlB is necessary for *in vitro* infection of human brain microvascular endothelial cells (Greiffenberg *et al.*, 1998).

Twenty four additional internalins are present in the *L. monocytogenes* genome and could potentially contribute to host cell invasion (Drams *et al.*, 1997). It is plausible that these internalins might cooperate with each other in order to facilitate entry into host cells, for example, InlA mediated entry is enhanced in the presence of InlB and InlC. However, additional studies are required to understand the contributions of each internalin and how these proteins participate in the bacterial entry to establish the successful infection of various cell types.

In addition to the internalins, several other proteins such as Ami, Auto, and Vip are also implicated in the ability of *L. monocytogenes* to enter host cells. In the absence of InlA and InlB, it has been shown that Ami digests the *L. monocytogenes* cell wall and mediates the adherence of a  $\Delta$ *inlAB* bacterial strain to mammalian cells (Milohanic *et al.*, 2001). Auto is another autolysin that regulates the bacterial surface architecture required for adherence (Cabanes *et al.*, 2004). Vip is a cell wall anchored protein that is involved in the invasion of various cell lines. The endoplasmic reticulum resident chaperone gp96 has been identified as a cellular ligand for this protein (Cabanes *et al.*, 2005). Thus, these *L. monocytogenes* cell surface proteins contribute to the ability of *L. monocytogenes* to infect multiple cell types.

## B. *L. monocytogenes* survival in the macrophage

Upon infection of host cells such as macrophages and DC, a majority of the bacteria are killed in the phagolysosome of the host cell with less than 10% of the *L. monocytogenes* escaping into the host cell cytosol. This escape from the phagolysosome is mediated by the expression of Listeriolysin O (LLO), a pore forming hemolysin, which is the product of the *hly* gene and phospholipases (PlcA and PlcB) (Fig. 1.1). LLO is the first identified major virulence factor of *L. monocytogenes* and is a member of the cholesterol-dependent cytolysin family (CDC) (Portnoy *et al.*, 1992a,b; Tweten, 2005).



**FIGURE 1.1** Intracellular growth of *L. monocytogenes* in an antigen-presenting cell and antigen presentation. Internalization of *L. monocytogenes* on the host cell is mediated by phagocytosis in macrophages but in other host cells such as epithelial and endothelial cells it requires invasins such as InlA and InlB (a). After cellular entry *L. monocytogenes* escape the phagolysosome by secreting Listeriolysin O (LLO), phospholipase (Plc), and metalloprotease (Mpl) resulting in the lysis of the vacuolar membrane, releasing the bacteria in the host cytosol (b and c). Cytosolic bacteria express protein ActA that polymerizes actin filaments and mediates cell to cell spread of *L. monocytogenes* (d). Cytosolic antigens produced after *L. monocytogenes* escape from phagosome are degraded by the proteasome to antigenic epitopes and presented by MHC class I molecules (e, f, and g). Bacterial antigens inside the phagosome are processed as exogenous antigens and epitopes are presented on the membrane surface in the context of MHC class II molecules (h). An alternate route for antigen presentation involves cross presentation with the antigens derived from an *L. monocytogenes* infected cell (i).

LLO binds to the host cell membrane initially as a monomer but then forms oligomers composed of up to 50 subunits, which are inserted into the membrane to form pores of diameter ranging 200–300Å (Walz, 2005). The function of LLO is very crucial for the cellular invasion of *L. monocytogenes* in both phagocytic and nonphagocytic cells.

After entry into the cytosol, another *L. monocytogenes* secreted protein called ActA enables bacterial propulsion in the cytosol leading to the invasion of neighboring uninfected cells by a process called cell to cell spreading (Alvarez-Dominguez *et al.*, 1997; Suarez *et al.*, 2001). In the cytoplasm, *L. monocytogenes* replicates and uses ActA to polymerize host cell actin to become motile enabling spread from cell to cell (Dussurget *et al.*, 2004; Fig. 1.1). As a result, the deletion of *actA* from *L. monocytogenes* results in a highly attenuated bacterium and thus establishes that ActA is a major virulence factor.



### III. IMMUNE RESPONSE TO *L. monocytogenes* INFECTION

#### A. Innate immunity

Innate immunity plays an essential role in the clearance of *L. monocytogenes* and control of the infection at early stages. Mice deficient in T and B cell responses, such as *SCID* and *nude* mice, have normal early resistance to sublethal *L. monocytogenes* infection. However, *SCID* and *nude* mice eventually succumb to infection because complete clearance of *L. monocytogenes* requires T-cell mediated immunity (Pamer, 2004). Upon systemic inoculation of *L. monocytogenes*, circulating bacteria are removed from the blood stream primarily by splenic and hepatic macrophages (Aichele *et al.*, 2003). In the spleen, the bacteria localize within macrophages and DC of the marginal zone, between the white and red pulp (Conlan, 1996). Within the first day of infection, these cells containing live bacteria migrate to the T-cell zones in the white pulp, establishing a secondary focus of infection and attracting neutrophils. Interestingly, this process has been associated with lymphocytopenia in this compartment (Conlan, 1996), as T cells undergo apoptosis induced by the *L. monocytogenes* infection in an antigen-independent manner (Carrero and Unanue, 2007).

Both macrophages and neutrophils have essential roles in controlling *L. monocytogenes* infection at early time points. Recruitment of monocytes to the site of infection is an important characteristic of *L. monocytogenes* infection. In the liver, the Kupffer cells clear most of the circulating bacteria. As early as 3 h after systemic injection, *L. monocytogenes* can be found inside the Kupffer cells, followed by granulocyte and mononuclear cell infiltration and formation of foci of infection (Mandel and Cheers, 1980). Neutrophils are rapidly recruited to the site of infection by the cytokine IL-6 and other chemo-attractants, which secrete IL-8 (Arnold and Konig, 1998), CSF-1 and MCP-1. These chemokines are important in the inflammatory response and for attracting macrophages to the infection foci. In the following few days, granulocytes are gradually replaced by large mononuclear cells and within 2 weeks the lesions are completely resolved (Mandel and Cheers, 1980). Further studies have shown that mice depleted of granulocytes are unable to control *L. monocytogenes* infection (Conlan and North, 1994; Conlan *et al.*, 1993; Czuprynski *et al.*, 1994; Rogers and Unanue, 1993). In murine listeriosis, *L. monocytogenes* replicates inside hepatocytes, which are lysed by the granulocytes recruited to the infection foci, releasing the intracellular bacteria to be phagocytosed and killed by neutrophils (Conlan *et al.*, 1993). Although neutrophils are very important in fighting *L. monocytogenes* infection in the liver, depletion of neutrophils does not significantly change the infection course in the spleen (Conlan and North, 1994). Interestingly, mice

depleted of mast cells have significantly higher titers of *L. monocytogenes* in the spleen and liver and are considerably impaired in neutrophil mobilization (Gekara *et al.*, 2008). Although not directly infected by *L. monocytogenes*, mast cells can be activated by the bacteria and rapidly secrete TNF- $\alpha$  and induce neutrophil recruitment (Gekara *et al.*, 2008).

At the cell surface, toll like receptors (TLRs) play a role in the recognition of *L. monocytogenes*. TLRs are important components of innate immunity, recognizing conserved molecular structures on pathogens, and signaling through adaptor molecules, such as MyD88, to induce NF- $\kappa$ B activation and transcription of several proinflammatory genes. NF- $\kappa$ B is a heterodimeric transcription factor composed of p50 and p65 subunits and activates several genes involved in innate immune responses. Mice lacking the p50 subunit of NF- $\kappa$ B are highly susceptible to *L. monocytogenes* infections (Sha *et al.*, 1995).

In particular, TLR2 seems to play a role during *L. monocytogenes* infection because mice deficient in TLR2 are slightly more susceptible to listeriosis (Torres *et al.*, 2004). TLR2 recognizes bacterial peptidoglycan, lipoteichoic acid, and lipoproteins present in the cell wall of gram-positive bacteria, including *L. monocytogenes*. TLR5, which binds bacterial flagellin, however, is unlikely to be involved in *L. monocytogenes* recognition since flagellin expression is downregulated at 37 °C for most *L. monocytogenes* isolates. In addition, TLR5 is not required for innate immune activation against this bacterial infection (Way and Wilson, 2004).

The presence of unmethylated CpG dinucleotides in the bacterial DNA also has stimulatory effects on mammalian immune cells. CpG motifs present in bacterial DNA act as pathogen associated molecular patterns (PAMPs) (Hemmi *et al.*, 2000; Tsujimura *et al.*, 2004) interacting with TLR-9 to trigger an innate immune response in which lymphocytes, DC, and macrophages are stimulated to produce immunoprotective cytokines and chemokines (Ballas *et al.*, 1996; Haddad *et al.*, 1997; Hemmi *et al.*, 2000; Ishii *et al.*, 2002; Tsujimura *et al.*, 2004).

Although TLRs are important in bacterial recognition, a single TLR has not been shown to be essential in innate immune responses to *L. monocytogenes*. On the other hand, the adaptor molecule MyD88, which is used by signal transduction pathways of all TLRs, except TLR-3, is critical to host defense against *L. monocytogenes* and infection with *L. monocytogenes* is lethal in MyD88-deficient mice. Additionally, MyD88<sup>-/-</sup> mice are unable or severely impaired in the production of IL-12, IFN- $\gamma$ , TNF- $\alpha$ , and nitric oxide (NO) following *L. monocytogenes* infection. MyD88 is not required for MCP-1 production and monocyte recruitment following *L. monocytogenes* infection but is essential for IL-12 and TNF- $\alpha$  production and monocyte activation (Serbina *et al.*, 2003). The NOD-LRR receptor interacting protein 2 (RIP2) kinase, identified as