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Journal
Cellular Microbiology, 22(4)

ISSN
1462-5814

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Publication Date
2020-04-01

DOI
10.1111/cmi.13175

Peer reviewed

HHS Public Access

Author manuscript

Cell Microbiol. Author manuscript; available in PMC 2021 April 01.

Published in final edited form as:

Cell Microbiol. 2020 April ; 22(4): e13175. doi:10.1111/cmi.13175.

Why is *Listeria monocytogenes* such a potent inducer of CD8+ T-cells?

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Abstract

Listeria monocytogenes is a rapidly growing, Gram-positive, facultative intracellular pathogen that has been used for over 5 decades as a model to study basic aspects of infection and immunity. In a murine intravenous infection model, immunization with a sublethal infection of *L. monocytogenes* initially leads to rapid intracellular multiplication followed by clearance of the bacteria and ultimately culminates in the development of long-lived cell-mediated immunity (CMI) mediated by antigen-specific CD8+ cytotoxic T cells. Importantly, effective immunization requires live, replicating bacteria. In this review, we summarize the cell and immunobiology of *L. monocytogenes* infection and discuss aspects of its pathogenesis that we suspect lead to robust CMI. We suggest 5 specific features of *L. monocytogenes* infection that positively impact the development of CMI: (1) the bacteria have a predilection for professional antigen-presenting cells; (2) the bacteria escape from phagosomes, grow, and secrete antigens into the host cell cytosol; (3) bacterial secreted proteins enter the MHC class I pathway of antigen processing and presentation (4) the bacteria do not induce rapid host cell death; (5) cytosolic bacteria induce a cytokine response that favors CMI. Collectively, these features make *L. monocytogenes* an attractive vaccine vector for both infectious disease applications and cancer immunotherapy.

Keywords

bacteria; intracellular pathogen; macrophage; inflammasome; cancer; tumor; vaccine; dendritic cell

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Conflicts of Interest

No conflicts of interest to report

A brief primer on *L. monocytogenes*

L. monocytogenes is a rapidly growing, easily manipulated, Gram-positive bacterium that is taxonomically placed in the Firmicute phylum most closely related to bacterial members of the Bacilli, Lactobacilli and Enterococci (Radoshevich & Cossart, 2018). *L. monocytogenes* is a ubiquitous environmental inhabitant that lives a biphasic lifestyle as both a saprophyte and as a pathogen of many warm-blooded animals including livestock and humans (Freitag, Port, & Miner, 2009). *L. monocytogenes* is a common contaminant of a variety of fresh and processed foods. Humans often consume *L. monocytogenes* contaminated food, but the most identified illness occurs in pregnant women, neonates, the elderly, and individuals whose immune system is compromised where it often causes meningitis and CNS infection (Schlech, 2019). Although disease is relatively rare, it is often fatal, and in the developed world, represents a leading cause of death due to food-borne illnesses.

Although natural infection is clearly via the oral route, most basic research has been conducted in mice using either intravenous or intraperitoneal routes of administration. Indeed, beginning with the classic work of George Mackaness in the 1960s, *L. monocytogenes* emerged as a highly quantitative and reproducible murine model system to study basic aspects of innate and adaptive immunity (D'Orazio, 2019; Mackaness, 1962; McGregor, Koster, & Mackaness, 1970). To very briefly summarize decades of research, an effective innate immune response to *L. monocytogenes* is sufficient to contain the infection and relies on the orchestrated influx of neutrophils and macrophages to the sites of infection followed by the activation of macrophage bactericidal activity. Mice that lack B and T-cells do not succumb to infection, but are unable to clear the bacteria (Bancroft, Schreiber, Bosma, Bosma, & Unanue, 1987; Bhardwaj, Kanagawa, Swanson, & Unanue, 1998). In contrast, conventional mice that survive a primary challenge with a sub-lethal dose of *L. monocytogenes* clear the infection within 7–10 days and become highly resistant to a subsequent lethal challenge. Long-lived adaptive immunity is antibody-independent and depends on the expansion of antigen-specific CD8+ T cells and establishment of memory cells (cell-mediated immunity or CMI). Importantly, induction of adaptive immunity requires live, replicating bacteria; i.e., killed vaccines do not induce protective immunity (Berche, Gaillard, & Sansonetti, 1987; Von Koenig, Finger, & Hof, 1982). The observation that *L. monocytogenes* induces T-cell-mediated immunity suggested to numerous investigators that it might represent a highly amenable and potent recombinant vaccine vector for the induction of CMI (Goossens, Milon, Cossart, & Saron, 1995; Ikonomidis, Paterson, Kos, & Portnoy, 1994; Shen et al., 1995). Indeed, *L. monocytogenes*-based vaccines have been developed as therapeutic vaccines for cancer immunotherapy that have shown promising results in clinical trials (Flickinger, Rodeck, & Snook, 2018). Below, we describe the biological features of *L. monocytogenes* that make it such as potent inducer of CMI.

Cell biology of infection

Shortly after IV infection with *L. monocytogenes*, most of the bacteria are found within macrophages and dendritic cells (DCs) of the spleen and liver where the majority of bacteria are killed, but some of the bacteria escape from phagocytic vacuoles, replicate rapidly in the

cytosol of infected cells and exploit host actin dynamics to spread to neighboring cells (Portnoy, Auerbuch, & Glomski, 2002; Serbina, Shi, & Pamer, 2012; Waite et al., 2011). This infectious process occurs in most, if not all adherent cells, including primary or immortalized cultures of bone marrow-derived macrophages (BMMs) which are ideal cells for studies on innate immunity (see below) and can be activated with IFN-γ to kill and degrade phagosomal bacteria (Herskovits, Auerbuch, & Portnoy, 2007).

Listeriolysin O (LLO), a cholesterol dependent cytolysin (CDC), is a secreted virulence factor and a primary determinant of *L. monocytogenes* pathogenesis (Nguyen, Peterson, & Portnoy, 2019). LLO is necessary to escape from both a primary phagosome and the secondary vacuole that forms upon cell-to-cell spread. LLO-damaged phagosomes and free bacteria in the cytosol are recognized by the host autophagy machinery, but the bacteria secrete two phospholipases C (PlcA/B), and utilize actin-based motility, that together allow them to bypass autophagy (Cheng, Chen, Engstrom, Portnoy, & Mitchell, 2018; Mitchell et al., 2018). Importantly, mutants lacking LLO are incapable of growth in BMMs and are 5-logs less virulent in mice (Portnoy, Jacks, & Hinrichs, 1988). Although LLO activity is essential, it is potentially cytotoxic and its activity must be compartmentalized to acidic cellular compartments. *L. monocytogenes* mutants that fail to properly restrict LLO activity to vacuoles kill the infected macrophage *in vitro* and are rendered avirulent *in vivo*. There are multiple mechanisms used by LLO to prevent cellular toxicity, which was recently reviewed (Nguyen et al., 2019).

Upon entering the host cell cytosol, *L. monocytogenes* dramatically up-regulates the expression of a cell surface transmembrane protein called ActA, which recruits and activates the host Arp2/3 complex to induce the polymerization of host actin filaments leading to intra- and intercellular spread (Gouin, Welch, & Cossart, 2005; Pillich, Puri, & Chakraborty, 2017). ActA mutants grow normally within the cytosol of infected cells, but are incapable of cell-to-cell spread and are approximately 1000-fold less virulent in mouse models (Brundage, Smith, Camilli, Theriot, & Portnoy, 1993). However, unlike LLO-minus mutants, which are poor inducers of CMI, ActA mutants are extremely potent inducers of CMI and are the primary basis of attenuation used in vaccine strains safely administered to humans (Flickinger et al., 2018).

Innate immune recognition of *L. monocytogenes*

MyD88-dependent responses:

Within minutes of IV infection, the majority of splenic *L. monocytogenes* can be found within several macrophages sub-types of the marginal zone, subsequently transitioning into dendritic cells (DCs) of the white pulp (Aoshi et al., 2009; Perez et al., 2017). Accordingly, most *in vitro* studies of innate immunity to *L. monocytogenes* utilize infection of BMMs from conventional and knockout mice. The initial innate immune response is somewhat generic and involves the detection of bacteria by Toll-like receptors (TLRs) on the cell surface and within phagosomes of macrophages, leading to the expression of chemokines and both inflammatory and regulatory cytokines (Bahjat et al., 2009; Serbina et al., 2003; Witte et al., 2012). This initial response can be studied using LLO-minus bacteria, which are trapped in phagosomes, and is entirely dependent on the MyD88 adapter molecule (Leber et

al., 2008). As a Gram-positive bacterium, *L. monocytogenes* lacks LPS and consequently does not stimulate TLR4, but in non-activated BMMs is mostly detected by TLR2. In IFN-γ activated macrophages, the MyD88 response is mostly dependent on Unc93b1, which is a chaperone necessary for the trafficking of nucleic acid sensing TLRs 3, 7, and 9 to the phagosome (Tabeta et al., 2006). Since these TLRs detect nucleic acids, these results imply that lysis of the bacteria exposes their nucleic acids which are detected by Unc93b1-dependent TLRs. In addition to MyD88, there are numerous other innate immune factors that are essential for resistance to primary infection, notably IFN-γ, TNF, and CCR2, much of which is related to macrophage migration and activation (Pamer, 2004). Importantly, as few as 10 bacteria are lethal to MyD88 and IFN-γ KO mice, yet these same mice are not more sensitive to ActA-minus mutants which exhibit an abortive infection due to the defect in cell-to-cell spread and are likely killed by neutrophils subsequent to bacteriolysis (Harty & White, 1999).

STING-dependent responses in the cytosol.

Most of the BMM transcriptional response to *L. monocytogenes* is MyD88-dependent as discussed above. However, upon entering the cytosol, *L. monocytogenes* induces a unique set of IRF3-dependent genes, that leads to the expression of IFN-β and co-regulated genes (Leber et al., 2008). A hallmark of *L. monocytogenes* infection is the induction if type I interferons, that are detected by type I interferon receptor, IFNAR1. Clearly, monocyte recruitment is critical for innate resistance to *L. monocytogenes* (Shi & Pamer, 2011), and while cytosolic bacteria induce chemokine MCP-1 in a MyD88-independent manner, IFNAR MyD88 double mutant mice are defective for monocyte recruitment compared to single IFNAR mutant mice, and are more susceptible to infection than MyD88 KO mice alone (Jia, Leiner, Dorothee, Brandl, & Pamer, 2009), demonstrating that both phagosome and cytosolic pathways are redundant for innate resistance to infection.

The cytosol-specific response to *L. monocytogenes* is induced primarily by the molecule cyclic-di-AMP (c-di-AMP) (Woodward, Iavarone, & Portnoy, 2010). c-di-AMP is a small signaling molecule widespread among bacteria, especially Firmicutes, that controls aspects of bacterial metabolism and osmoregulation (Commichau, Heidemann, Ficner, & Stulke, 2019). *L. monocytogenes* secretes c-di-AMP through multidrug resistance transporters, although the role of its secretion, other than to stimulate the IFN-β response is not yet clarified (Crimmins et al., 2008; Huynh & Woodward, 2016). Bacterial cyclic-di nucleotides, c-di-AMP, c-di-GMP, and 3'−5', 3'−5'cGAMP act by binding to and activating a host protein called STING (Stimulator of Interferon Genes) (Burdette et al., 2011). Importantly, the host response to DNA is also STING-dependent, but requires cGAS, which is activated by cytosolic dsDNA to synthesize a chemically distinct form of cGAMP 2'−5', 3'−5'cGAMP (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013). Thus, STING appears to be a central signaling hub for the recognition of many viral and bacterial pathogens by the recognition of either dsDNA and/or cyclic-di-nucleotides (Danilchanka & Mekalanos, 2013; Konno & Barber, 2014).

Inflammasome activation.

Inflammasomes are multiprotein complexes that assemble in response to microbial ligands, including bacterial flagellin and DNA, resulting in the activation of Caspase-1, and secretion of the inflammatory cytokines IL-1β and IL-18 and culminates in a form of inflammatory programed cell death called pyroptosis (Bergsbaken, Fink, & Cookson, 2009; Fink & Cookson, 2006; von Moltke, Ayres, Kofoed, Chavarria-Smith, & Vance, 2013). Many bacterial pathogens induce pyroptosis, although it typically plays only modest roles in host resistance to infection, probably because *bona fide* pathogens evolve to avoid it (Jorgensen & Miao, 2015). *L. monocytogenes* infection of BMMs results in relatively low levels of pyroptosis, but that which occurs is mostly due to the infrequent lysis of cytosolic bacteria and the activation of the DNA-dependent AIM2 inflammasome (McDougal & Sauer, 2018). In addition, LLO, like other pore-forming toxins, can activate the NLRP3 inflammasome, but this occurs from the outside of cells and probably is not directly relevant to *L. monocytogenes* pathogenesis (Sauer et al., 2010). Strains of *L. monocytogenes* engineered to secrete a fusion of ActA and the *Legionella pneumophila* flagellin activate the NLRC4-dependent inflammasome and are attenuated in WT mice but retain full virulence in NLRC4-minus mice (Sauer, Pereyre, et al., 2011; Warren et al., 2011). Therefore, it appears that avoidance of pyroptosis, and cell death in general, is an essential feature of *L. monocytogenes* pathogenesis. *L. monocytogenes* does not appear to actively inhibit cell death, but rather fails to stimulate it by growing without appreciable lysis, using multiple mechanisms to avoid LLO-mediated cytotoxicity, and not expressing its flagellum at 37°C (Shen & Higgins, 2006).

Generation of adaptive immunity

The hallmark of adaptive immunity to *L. monocytogenes* is that naïve mice that recover from a primary challenge develop long-term immunity to reinfection (Condotta, Richer, Badovinac, & Harty, 2012; D'Orazio, 2019). The most common assay used to measure adaptive immunity is to infect immunized mice with a dose of *L. monocytogenes* lethal to non-immunized mice, and determine the number of CFUs in the liver and spleen after 48 or 72h compared to the numbers observed in non-immunized mice. Typically, immune mice have approximately 5-logs fewer bacteria in their organs as compared to naïve mice. Although CD4+ T-cells play a role in the development of immunity, CD8+ T cells are the dominant effector T-cell, which is largely dependent on antigen-specific cytotoxicity (Harty, Schreiber, & Bevan, 1992; Sun, Williams, & Bevan, 2004). T-cell epitopes are derived largely from proteins secreted by *L. monocytogenes* into the host cell cytosol which are processed and presented in the MHC class I pathway of antigen presentation (Pamer, 2004; Pamer, Harty, & Bevan, 1991). Fusion proteins consisting of the N-terminus of ActA or LLO and a foreign protein or epitope lead to the generation of CD8+ T cells to the foreign epitope. Immunization with *L. monocytogenes* ActA mutants expressing an ActA-OVA fusion results in more than 10% of the primary CD8+ T cells being specific for OVA, and up to 20% upon secondary expansion (Bahjat et al., 2006).

What makes *L. monocytogenes* such a potent inducer of CD8+ T cells?

We hypothesize that there are five biological factors that contribute to *L. monocytogenes* as an effective inducer of CMI. Briefly, it requires the correct host cells, bacterial growth and secretion of antigens, the ideal cellular compartment for antigen presentation, lack of host cell death, and induction of a favorable cytokine milieu (Figure 1).

1 The bacteria have a predilection for professional antigen-presenting cells.

Within minutes of intravenous vaccination, the majority of splenic *L. monocytogenes* are found predominantly within macrophages of the marginal zone, where bacterial growth is restricted (Aoshi et al., 2009). As early as 1-hour post infection the majority of splenic *L. monocytogenes* can be found within CD169+ macrophages, such that depletion of CD169+ macrophages dramatically increases susceptibility to disease (Perez et al., 2017). In coordination with DCs, CD169+ macrophages support CMI by providing antigen and producing type I IFN. However, CD169+ macrophages can suppress CMI to self-antigens and phagosome-restricted content including apoptotic cells and exosomes (Grabowska, Lopez-Venegas, Affandi, & den Haan, 2018; McGaha, Chen, Ravishankar, van Rooijen, & Karlsson, 2011; Miyake et al., 2007; Saunderson, Dunn, Crocker, & McLellan, 2014). Indeed, several hours after infection, phagosome confined LLO-minus bacteria are still localized to CD169+ macrophages, whereas bacteria growing in the host cytosol are transferred to CD8-α^+ DCs that serve as a protective niche for bacterial survival (Edelson et al., 2011; Neuenhahn et al., 2006; Perez et al., 2017). Batf3 knockout mice that specifically lack CD8-α^+ DCs are resistant to infection, suggesting that this population of DCs represent a critical host cell for the pathogenesis of *L. monocytogenes* (Edelson et al., 2011). Clearly, professional antigen-presenting cells (APCs) like DCs, have a predominant role in presenting foreign antigens and orchestrating the development of antigen-specific cytotoxic T cells (Merad, Sathe, Helft, Miller, & Mortha, 2013). However, Batf3 knockout mice can still be immunized with high doses of *L. monocytogenes*, suggesting that APCs other than CD8-α^+ DCs are sufficient for induction of CMI (Edelson et al., 2011).

2 The bacteria escape from phagosomes and grow in the host cell cytosol.

Once inside APCs, *L. monocytogenes* escapes from phagosomes and grows rapidly in the host cell cytosol. Entry into the host cytosol is necessary for bacterial replication and is a prerequisite for all subsequent steps in pathogenesis. Furthermore, cytosolic access is required for CMI, but in the absence of bacterial replication, cytosolic access is not sufficient to induce robust CMI, since killed but metabolically active *L. monocytogenes* fails to induce optimal CMI due to defective DC maturation (Bahjat et al., 2006). Indeed, cytosolic localization is required for recruitment of CD8-α^+ DCs to the site of infected CD169+ macrophages (Perez et al., 2017). In addition, escape from a phagosome provides a mechanism to avoid the MyD88-dependent responses, such as IL-10 expression, which suppress immunity (Bahjat et al., 2009) as discussed below. Strikingly, whereas as few as 1000 CFUs of a cytosolic ActA-minus mutant can induce long-lived immunity, as many as 10^8 of a phagosome confined LLO-minus mutant fails to induce an appreciable level of immunity (Archer, Durack, & Portnoy, 2014; Bahjat et al., 2006; Berche et al., 1987; Orgun & Way, 2008).

3 Bacterial secreted proteins enter the MHC class I pathway of antigen processing and presentation.

Entry and growth of *L. monocytogenes* in the host cytosol promotes secretion of bacterial proteins directly into the cytosol where they are processed and presented into the MHC class I pathway without a requirement for cross presentation. In BALB/c mice, a dominant MHC I epitope is derived from LLO and inhibition of proteasomes blocks antigen presentation (Villanueva, Sijts, & Pamer, 1995). However, cytosolic access of secreted proteins, is strikingly more efficient at inducing CD8+ T cells compared to the cross priming of antigens derived from the bacterial cytosol (Shen et al., 1998).

4 The bacteria do not induce rapid cell death.

Whereas many pathogens cause cell death, *L. monocytogenes* grows in APCs without causing appreciable cell death until there are over 200 bacteria per cell (Brundage, Smith, Camilli, Theriot, & Portnoy, 1993). Bacteria that are engineered to kill infected cells prematurely by necrosis, apoptosis, or pyroptosis are defective for the induction of adaptive immunity (Theisen & Sauer, 2016). The simplest explanation is that premature killing of the APCs prevents all aspects of immunity including secretion of cytokines and presenting of antigen. However, infected host cell death does not preclude that killed cells are phagocytosed by DCs and processed by cross-presentation (Alloatti, Kotsias, Magalhaes, & Amigorena, 2016; Edelson, 2012), and indeed, although CMI is diminished, there remains substantial levels of immunity in mice immunized with cytotoxic strains (McDougal & Sauer, 2018; Sauer, Pereyre, et al., 2011).

5 Cytosolic bacteria induce a cytokine response that favors CMI.

Surprisingly, when mice are simultaneously co-infected with both phagosome confined LLO-minus bacteria, and ActA-minus mutants, immunity is decreased by over 100-fold (Bahjat et al., 2009). Analysis of serum cytokines revealed that the addition of LLO-minus bacteria suppressed the host inflammatory response, and resulted in acute elevation of IL-10, a MyD88-dependent cytokine known to suppress both innate and adaptive immunity to various microbial pathogens (Couper, Blount, & Riley, 2008; Moore, de Waal Malefyt, Coffman, & O'Garra, 2001; Saraiva & O'Garra, 2010; Slobedman, Barry, Spencer, Avdic, & Abendroth, 2009). Blockade of the IL-10 receptor at the time of immunization eliminated suppression caused by the LLO-minus bacteria and even enhanced immunity in mice immunized with LLO-minus alone (Bahjat et al., 2009). These data suggest that one of the reasons that LLO-minus bacteria fail to induce protective immunity is that they suppress immunity via the MyD88 pathway (Bahjat et al., 2009). Indeed, MyD88 KO mice vaccinated with ActA-minus bacteria show enhanced immunity compared to wild type mice, demonstrating the suppressive role MyD88 signaling can play during vaccination with live bacteria (Archer et al., 2014). *In vitro*, BMMs are potent inducers of MyD88-dependent cytokines in response to LLO-minus bacteria. However, the *in vivo* cellular source of IL-10 during immunosuppression by LLO-minus strains is not known, though it likely involves CD169+ macrophages, as they are the host cells that predominantly capture systemic bacteria and demonstrate prolonged retention of LLO-minus bacteria (Perez et al., 2017).

Phagosome-confined bacteria activate the MyD88-dependent pathway, which enhances the secretion of the immunosuppressive cytokine IL-10 and thus counteracts the proinflammatory signals needed for CMI (Bahjat et al., 2009). On the other hand, cytosolic *L. monocytogenes* not only induces expression of MyD88-dependent genes like IL-6, IL-12, and TNF, which are beneficial for CMI, but also secretes c-di-AMP which activates STING and leads to a strong type I IFN dependent pathway (Sauer, Sotelo-Troha, et al., 2011; Woodward et al., 2010). While under some experimental conditions, type I IFN promotes CMI (Diamond et al., 2011; Stetson & Medzhitov, 2006), in the case of *L. monocytogenes*, it paradoxically has the opposite effect, as mice lacking the type I IFN receptor or STING induce a stronger CD8+ T cell response (Archer et al., 2014). Interestingly, in sepsis mediated immune suppression, early type I IFN secretion by splenic macrophages decreases DC antigen presentation and inflammatory cytokine secretion (Schwandt et al., 2012) supporting the notion that during systemic bacterial infections overproduction of type I IFN negatively affects CMI. More surprisingly, a MyD88/STING double mutant, which produces almost no detectible cytokine response to *L. monocytogenes* is fully immunized with an ActA-minus mutant (Archer et al., 2014). Indeed, nearly all KO mice tested, other than those lacking T-cells or factors necessary for T-cell-mediated killing are still effectively vaccinated by the ActA-minus strain (Harty & White, 1999). One exception is mice defective for prostaglandin E2, that show a diminished level of immunity which correlates with fewer antigen specific CD8+ T cells (Theisen et al., 2018). Therefore, we suspect that multiple and redundant innate immune pathways lead to CMI.

L. monocytogenes vaccines

The observation that attenuated strains of *L. monocytogenes* induce robust CMI led to studies showing that *L. monocytogenes*-induced CMI can be directed towards elimination of cancer cells *in vivo* (Pan, Ikonomidis, Lazenby, Pardoll, & Paterson, 1995). *L. monocytogenes* engineered to secrete Tumor Associated Antigens (TAAs) can effectively treat established tumors in mice and the therapeutic response depends greatly on CD8+ T cells (Brockstedt et al., 2004). This approach has also proven safe in humans and has led to several clinical trials, some of which are ongoing (Flickinger et al., 2018; Maciag, Radulovic, & Rothman, 2009).

Although the *L. monocytogenes* strain used in all clinical trials, 10403S, is clearly potent at inducing CMI, it is difficult to imagine that the strain cannot be improved. There are several pros and cons to the therapeutic use of strain 10403S. 10403S has been studied extensively over the past several decades, is well characterized and genetically tractable. However, additional issues should be considered. For example, the streptomycin resistance mutation in 10403S reduces virulence by approximately 3-fold (Bishop & Hinrichs, 1987). Furthermore, unlike many *L. monocytogenes* isolates found in nature, which downregulate expression of flagellin at 37°C and during intracellular infection, 10403S contains a point mutation in the ribosome binding site for flagellar repressor gene *mogR*, such that 10403S still expresses some flagella, even at 37°C (Shen & Higgins, 2006). The effect of flagellar expression by 10403S may have unknown consequences such as affecting pyroptosis during therapeutic vaccinations.

On the other hand, some features conserved in 10403S may be well suited for CMI. Several clinical isolates contain mutations in *tetR*, a negative regulator of MDR transporters that efflux cdi-AMP (Schwartz et al., 2012). Indeed, TetR mutants express elevated levels of MdrT and secrete more c-di-AMP, leading to enhanced type I interferon expression (Crimmins et al., 2008). Overstimulation of the STING pathway may diminish CMI, as discussed above, whereas 10403S induces a balanced level of c-di-AMP secretion favoring CMI (Archer et al., 2014). Similarly, a balanced level of STING stimulation is important for tumor immunotherapy (Sivick et al., 2018).

Another consideration for vaccine development using live *L. monocytogenes* is providing the vector with optimal access to the cytosol. As mentioned above, the majority of injected bacteria are killed upon phagocytosis and likely induce unfavorable MyD88 signaling. Suppressive signaling may be reduced by using strains that contain a point mutation in the master virulence regulator PrfA that locks it in an active state, termed PrfA*. These strains escape more readily into the cytosol and are enhanced for CMI (Lauer et al., 2008; Qiu et al., 2011). Alternatively, pretreatment of *L. monocytogenes* with reducing agents, which mimic the reducing environment experienced by *L. monocytogenes* in the cytosol, can also pre-activate PrfA (Portman, Dubensky, Peterson, Whiteley, & Portnoy, 2017). Finally, we have recently completed a forward genetic screen for LLO-minus mutants that induce aberrant IL-10 induction in BMMs. We find that mutants that fail to stimulate TLR2, show diminished IL-10 induction, whereas mutants that are prone to lysis, induce enhanced Unc93b1 dependent IL-10 (Unpublished data, Nguyen & Chávez-Arroyo, Cheng, Krasilnikov, Louie, & Portnoy). This suggests that *L. monocytogenes* can be tailored for either enhanced or diminished induction of these pathways to optimize vaccination using live bacteria in cancer therapy.

Future Directions

An exciting future direction in the fields of CMI and microbial pathogenesis is to gain a better understanding of the relationship between virulence and the capacity to induce adaptive immunity. For example, loss of ActA-dependent cell-to-cell spread reduces *L. monocytogenes* virulence by approximately 1000-fold, yet CMI remains remarkably robust. On the other hand, LLO-minus bacteria remain in phagosomes, are up to 100,000-fold less virulent, and are poor inducers of adaptive immunity. Although not fully understood, it appears that immunosuppression occurs during vaccination with LLO-minus bacteria, and IL-10 is involved (Bahjat et al., 2009). Development of a strategy to bypass phagosomal escape while retaining the induction of CMI is an exciting prospect for the field of translational immunology, as it has the greatest potential for increasing the safety of live vaccines. A deeper understanding of the events that occur during the first few hours of infection is still needed. Many questions remain about the early responses that lead to CMI, including the host cell types involved and the optimal cytokines that lead to CMI.

The relevance of chronic versus acute infection is also an important area to consider, as chronic infections tend to induce T cell exhaustion (Wherry & Kurachi, 2015). We suggest that the acute nature of *L. monocytogenes* infection is an additional feature responsible for inducing a favorable environment for the induction of CMI because it does not lead to T cell

exhaustion. A possible explanation for why acute infections with *L. monocytogenes* leads to robust CMI may involve the rapid and acute induction of type I IFN. In contrast, chronic infections with some viruses leads to immunosuppression due to prolonged type I IFN signaling (Dagenais-Lussier et al., 2017). This observation may explain why *L. monocytogenes* mutations that cause enhanced production of type I IFN show diminished CMI in mice, and may even explain why some clinical isolates harbor similar mutations (Archer et al., 2014; Schwartz et al., 2012).

Although many findings observed in mice are conserved in humans, key biological differences between humans and mice influence our ability to extrapolate findings. One example is in species-specific polymorphisms found in innate immune signaling proteins, as is the case with STING. While bacterial c-di-AMP is a potent inducer of mouse STING, it is less potent at activating certain human STING variants (Patel & Jin, 2019).

Finally, our understanding of how *L. monocytogenes* induces CMI, has motivated an exciting frontier for personalized *L. monocytogenes* vaccines for cancer immunotherapy (Flickinger et al., 2018). Advancements in sequencing and computational technology now allow for the identification of patient-specific tumor neoantigens. The identified neoantigen epitopes can be expressed by *L. monocytogenes* to direct CMI to those tumor cells *in vivo* (Figure 1). Indeed, combining the factors that make *L. monocytogenes* such a potent generator of CMI, along with newly developing therapies, like checkpoint blockade, is an exciting prospect for the future of cancer immunotherapy using live *L. monocytogenes* as vaccines (Deng et al., 2018; Rosenberg et al., 2016).

Clearly, decades of research have placed *L. monocytogenes* as an excellent model system for studying innate and adaptive immunobiology, in particular the development of potent CMI. We have reviewed five reasons why *L. monocytogenes* is such a robust inducer of CD8+ T cells. These, and other factors, yet to be discovered, make *L. monocytogenes* a powerful system to study CMI with implications for vaccine development and cancer immunotherapy.

Acknowledgments

This work was supported by National Institutes of Health grants 1P01 AI063302 (D.A.P.) and 1R01 AI027655 (D.A.P).

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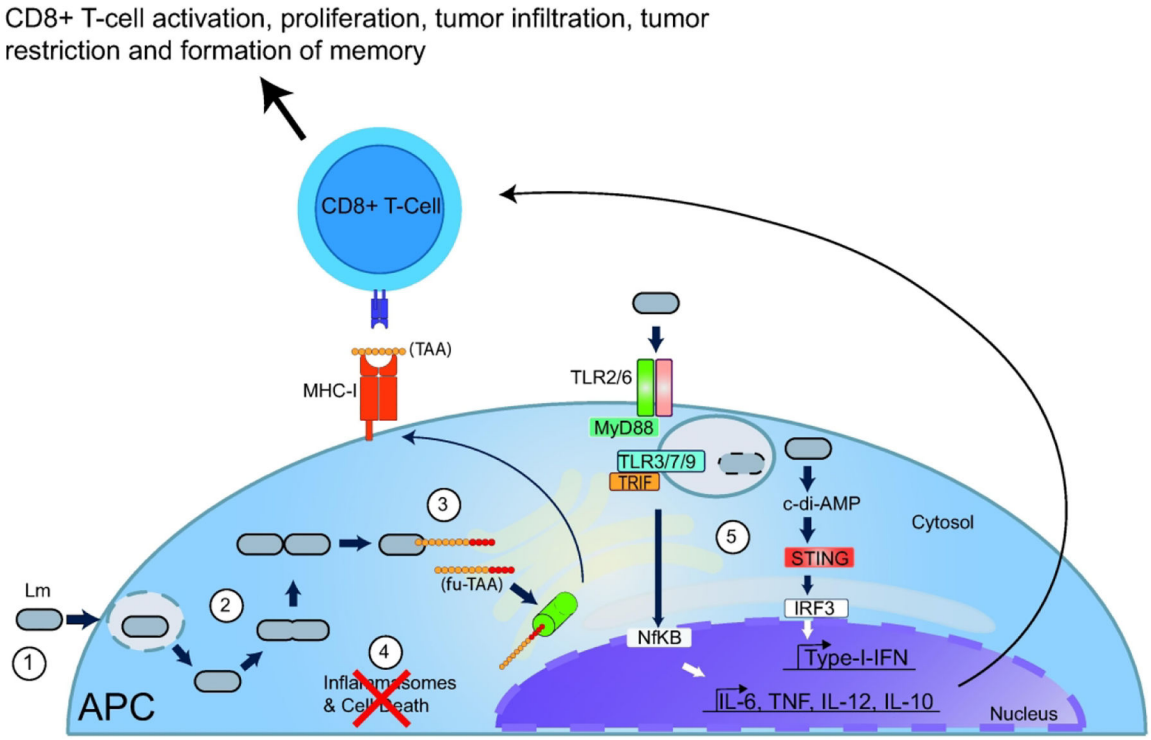

Figure 1: Biological factors that impact the development of cell-mediated-immunity (CMI) during *L. monocytogenes* infection and their role in tumor therapy:

1) Upon intravenous inoculation, *L. monocytogenes* (*Lm*) is internalized by professional antigen presenting cells (APC), such as splenic macrophages or dendritic cells. 2) *L. monocytogenes* escapes the phagosome, enters the cytosol, rapidly replicates, and secretes proteins. 3) *L. monocytogenes* can be engineered to secrete recombinant tumor associated antigens fused to virulence factors, termed here as fusion-tumor-associated antigens (fu-TAA). Secreted proteins are degraded by proteasomes (green cylinder) and enter the MHC class I pathway of antigen presentation. 4) *L. monocytogenes* minimally activates inflammasomes and consequently, infection does not lead to rapid host cell death. 5) Unlike phagosome confined bacteria that can lyse within phagosomes and primarily activate the MyD88-dependent pathway through Toll Like Receptors (TLRs), cytosolic bacteria induce cytosolic innate immune pathways by secreting cyclic di-AMP (c-di-AMP) thereby inducing an overall balanced cytokine milieu that favors CMI and tumor restriction by antigen specific cytotoxic CD8+ T cells.