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Innate and adaptive immune responses to *Listeria monocytogenes*: A short overview

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Abstract

The Gram-positive facultative intracellular bacterium *Listeria monocytogenes* is a model pathogen for elucidating important mechanisms of the immune response. Infection of mice with a sub-lethal dose of bacteria generates highly reproducible innate and adaptive immune responses, resulting in clearance of the bacteria and resistance to subsequent *L. monocytogenes* infection. Both the innate and adaptive immune systems are crucial to the recognition and elimination of this pathogen from the host.

Keywords

Listeria monocytogenes; immunity; infection

Introduction

A considerable amount of our knowledge of how the immune system functions has been learned from examining innate and adaptive immune responses to experimental *Listeria monocytogenes* infection in mice. Murine listeriosis involves the complex interplay between host and pathogen and has served as an attractive infection model for several key reasons. Infection is very reproducible and bacterial loads in the host are easily enumerated. At sub-lethal doses *L. monocytogenes* induces a strong immune response that leads to bacterial clearance. At the molecular level, *L. monocytogenes* is genetically tractable allowing for deletion of individual virulence factors or insertion of genes expressing different antigens. In this review we will provide an update of recent findings in the field of infection and immunity in which *L. monocytogenes* has served as a model pathogen.

1. Murine listeriosis: An experimental model to study the immune response

L. monocytogenes has been used as a model to study innate and adaptive immunity since the early 1960s when Mackaness demonstrated that cellular immunity was critical for control of infection in mice [1]. In this model, bacteria are intravenously injected into the bloodstream of mice. Within minutes, most bacteria can be found in the spleen and liver where they are quickly internalized by resident macrophages [2]. In a sub-lethal infection, bacteria replicate until their numbers are controlled by activated macrophages. The development of a *Listeria*-

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specific T cell response is necessary to eliminate the bacteria and memory T cells provide protection to reinfection [3,4]. The generation of this protective immunity to rechallenge is dependent on immunization with live *L. monocytogenes*, capable of escaping into the cytosol. Immunization with heat-killed bacteria or bacteria deficient in the key virulence factor listeriolysin O (LLO), which mediates bacterial escape from phagosomes before degradation, does not lead to protective immunity.

Although other animals, such as guinea pigs, are sometimes used to study the immune response to *L. monocytogenes*, mice have proven to be the most useful model for immunological studies due to availability of reagents, including knock-out mice deficient in specific genes. Inbred strains of mice, such as C57BL/6 and BALB/c, allow for ease in the study of epitope-specific T cell responses. In BALB/c mice there are several strong known *L. monocytogenes*-MHC class I restricted epitope responses, including LLO₉₁₋₉₉, and p60₂₁₇₋₂₂₅ [5,6]. In addition to CD8 T cell responses, there is a MHC class II restricted epitope, LLO₁₉₀₋₂₀₁, that generates a strong CD4 T cell response in C57BL/6 mice [7]. These endogenous epitopes have been useful, but studies were limited due to the absence of a strong known CD8 T cell epitope in C57BL/6 mice. This situation has been remedied by the construction of recombinant *L. monocytogenes* expressing foreign H2-K^b epitopes, such as those derived from the model antigen ovalbumin (OVA) or from lymphocytic choriomeningitis virus (LCMV) [8,9]. These *L. monocytogenes* engineered to express model antigens take advantage of immunological reagents such as TCR transgenic mice, tetramers for detecting antigen-specific T cells, and other bacterial or viral pathogens sharing these antigens for heterologous infections.

2. Innate immune responses

Innate immune responses are essential for early control of *L. monocytogenes* infection. Upon infection in the murine spleen, *L. monocytogenes* first localizes within macrophages in the marginal zone between the T cell rich white pulp and the B cell rich red pulp [2]. These infected cells then migrate into the white pulp region and form the beginning of a focus of infection that expands as neighboring cells become infected by the intercellular spread of bacteria. The innate immune response plays an important role in controlling bacterial growth and dissemination, preventing the spread into systemic, lethal infection.

2.1 Phagocytes

Neutrophils are important for the initial control of bacterial growth through their antimicrobial activities [10,11]. Neutrophils can kill bacteria through engulfment of the bacteria followed by generation of reactive nitrogen and oxygen intermediates [12] and can kill extracellular bacteria through release of extracellular traps (called NETs) consisting of granule-derived proteins and chromatin that ensnare and kill bacteria [13]. Neutrophils are rapidly recruited into infectious foci by the cytokine IL-6 and other factors [14] and they in turn secrete chemokines such as CSF-1 and MCP-1, that signal to macrophages to traffic to the site of infection [15].

Macrophages have been the focus of innate immunity during *L. monocytogenes* infection since replication occurs primarily within them and they are also an essential cell subset in mediating clearance of bacteria. Resident macrophages, especially Kupffer cells in the liver, are responsible for the initial killing of the majority of the injected bacteria. In response to infection, macrophages secrete TNF α and IL-12 [16–18]. These two cytokines drive natural killer (NK) cells to produce IFN γ , which in turn leads to activation of the macrophages and increases their bactericidal activity. As with neutrophils, generation of reactive oxygen and nitrogen intermediates is important for macrophage-mediated killing of *L. monocytogenes*. Mice deficient in phagocyte oxidase have slightly greater bacterial burden than wild-type controls, and mice deficient in nitric oxide synthase have even greater bacterial burdens [19,20]. Other

interferon-inducible genes, such as the p47 GTPases, are also involved in macrophage killing of bacteria through yet to be determined mechanisms [21].

2.2 Inflammatory cytokines

IFN γ may be the most important cytokine for controlling a primary *L. monocytogenes* infection. NK cells and $\gamma\delta$ T cells are important early sources of IFN γ [17,22]. Mice deficient in IFN γ are highly susceptible to *L. monocytogenes* infection [23]. However, IFN γ plays a less important role for protective immunity against re-infection. If IFN γ deficient mice are immunized with an attenuated strain of *L. monocytogenes*, and then challenged with virulent *L. monocytogenes*, these mice are protected against the challenge infection [24].

In addition to its well-described induction of IFN γ , *L. monocytogenes* has been long known to also induce type I interferons [25,26]. Interferon- α and - β , which are usually associated with anti-viral immune responses, are potent stimulators of anti-viral genes, including pro-apoptotic and antigen presentation genes, as well as down-regulating the cell machinery that viruses hijack in order to produce new viruses [27]. Induction of type I interferons is essential for the immune system to clear many viral pathogens. On the contrary, induction of type I interferons by *L. monocytogenes* is beneficial to the bacteria. In the absence of type I interferon signaling, *L. monocytogenes* cannot reach as high titers in mice and mice with elevated levels of type I interferons have greater bacterial loads [28–30]. This suggests that *L. monocytogenes* induces type I interferon to its benefit to either directly enhance its growth, or more likely, downmodulate a part of the immune response that plays an important role in controlling bacterial growth. A recent study by Carrero *et al.* shows that type I interferons induce T cell apoptosis early during *L. monocytogenes* infection, resulting in greater IL-10 secretion by phagocytic cells in turn dampening the innate immune response [31].

Induction of type I interferon by *L. monocytogenes* requires escape of intracellular bacteria into the cytosol [32,33]. In vitro experiments with cDNA microarrays have shown that *L. monocytogenes* infection of macrophages triggers two distinct, temporally separate waves of gene induction [33]. The earlier wave induces genes dependent on NF- κ B, which likely occurs through TLRs, and is not dependent on the invasion of cells by live bacteria. However, the second wave of induced genes is dependent on *L. monocytogenes* escaping from the phagolysosome. These genes include type I interferons, as well as genes associated with interferon-signaling, including Stats and Jaks, and other interferon-dependent genes. It remains to be determined what known or unknown intracellular recognition pathway is responsible for inducing this interferon response. However, it is independent of Toll-like receptor (TLR) 2, TLR4, receptor interacting protein 2 (RIP-2) or the mitochondria-associated adaptor protein (MAVS) signaling, but dependent on the transcription factor IFN regulatory factor 3 (IRF3) and the serine-threonine kinase TNFR-associated NF- κ B kinase (TANK)-binding kinase 1 (TBK1) [29,34–36]. Recent data also indicate that members of the NOD and NALP family play a role in intracellular recognition of *L. monocytogenes* [37,38].

2.3 Toll-like receptor recognition

Many different bacterial-, viral- and protozoan-derived ligands, as well as synthetic ligands, have been identified for the eleven Toll-like receptors (TLRs) expressed by mice. TLRs are expressed by many different immune cell subsets, including macrophages. TLR recognition of pathogen-derived products leads to activation of these cells, resulting in upregulation of expression of different inflammatory cytokines. As a Gram-positive bacterium, *L. monocytogenes* does not express the prototypical TLR ligand lipopolysaccharide (LPS). However, it does express a myriad of other TLR ligands, including peptidoglycan, flagellin, and bacterial DNA which can activate macrophages. Studies of TLR-deficient macrophages have revealed roles for these pattern recognition receptors in *L. monocytogenes* infection. The

most important TLR for *L. monocytogenes* recognition appears to be TLR2. TLR2-deficient macrophages after in vitro infection with *L. monocytogenes* secrete less TNF α , IFN γ , IL-1 β and IL-12 [39–41]. However, mice deficient in TLR2 have little increase in bacterial burden compared to wild-type mice [40]. It remains to be determined if mice deficient in other TLRs that recognize bacterial products, such as TLR9 (recognizes CpG motifs found in bacterial DNA) and TLR5 (flagellin), will also be able to control *L. monocytogenes* infection [42]. Since infection involves many different TLR ligands, a deficiency in recognition of one bacterial-derived ligand appears negligible to the overall immune response. However, TLRs clearly play an important role in immune recognition of *L. monocytogenes* and initiation of a protective response. Binding of the TLR ligand to its appropriate receptor initiates a signaling cascade that results in activation of the transcription factor NF- κ B resulting in expression of different cytokine and antigen presentation related genes. Mice deficient in the key adaptor molecule, MyD88, which is important for signaling from several TLRs, are highly susceptible to *L. monocytogenes* infection [39,40].

2.4 Intracellular immune recognition of *L. monocytogenes*

Recently, it has become apparent that the infected cell itself has mechanisms to detect infection within. Live bacteria that are capable of escaping from the phagosome are better stimulators of the immune response than heat-killed or escape-defective bacteria. Dendritic cells (DCs) infected in vitro with live *L. monocytogenes* express greater levels of surface co-stimulatory molecules for T cell activation and secrete greater amounts of pro-inflammatory cytokines than DCs stimulated with heat-killed bacteria [43]. In macrophages, a similar phenomenon has been observed in that bacteria that can escape the phagosome induce a unique gene expression pattern several hours after infection [33]. In vivo experiments have complemented in vitro findings. Splenic DCs from mice infected with live *L. monocytogenes* express higher surface levels of the co-stimulatory molecules CD40, CD80, and CD86 than mice immunized with heat-killed bacteria [44].

What cellular surveillance system is recognizing cytosolic bacteria and what is initiating these responses? This is an active area of investigation and in the past few years several key molecules have been identified that are involved in this process. The NLR family of cytosolic proteins contains about 20 members and includes both nucleotide-binding oligomerization domain (NOD) proteins and NACHT-, LRR- and pyrin-domain-containing proteins (NALPs) [45]. Some of these proteins have been identified as intracellular recognition molecules that operate independently of TLR signaling. Recognition of cytosolic microbial products initiates signaling cascades distinct from TLR signaling, involving different signaling molecules and results in distinct immune responses. The best studied NLR family members are NOD2 and NALP3, due to their association with the inflammatory disorders Crohn's disease and Muckle-Wells syndrome, respectively.

Both NOD2 and NALP3 play a role in recognition of intracellular *L. monocytogenes*. The ligand for NOD2 is muramyl dipeptide (MDP), a component of both Gram-negative and Gram-positive peptidoglycan [46]. In the absence of NOD2, mice are more susceptible to oral, but not intravenous, *L. monocytogenes* infection [37]. NALP3, also known as cryopyrin, is one component of an intracellular inflammatory complex that activates caspase-1, leading to processing and secretion of mature IL-1 β and IL-18, which are important for the activation of innate and adaptive immune responses. When macrophages deficient in NALP3 are infected with *L. monocytogenes*, they have reduced caspase-1 activation and release significantly less IL-1 β [38].

The specific ligand(s) for activation of the NALP3 pathway by *L. monocytogenes* remains unknown. On the other hand, *L. monocytogenes* DNA in the host cell cytosol is a ligand for a yet to be identified pattern recognition receptor that signals through interferon regulatory factor

(IRF) 3 [47]. IRF3 is an important transcription factor in the induction of IFN β and is downstream of several different cytosolic pattern recognition receptors, including retinoic acid inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) [48]. However, recognition of *L. monocytogenes* DNA is independent of these two receptors since their shared adaptor molecule, MAVS, is not essential for *L. monocytogenes*-induced IRF3 activation [35,36].

2.5 Autophagy

Once intracellular bacteria are recognized by the cell, it can attempt to destroy the pathogens. This can be accomplished by the process of autophagy [49]. First discovered as a way for cells to recycle their own intracellular organelles and cytoplasmic contents, a double membrane vacuole forms in the cytosol around the target. This vacuole can then be shuttled into the lysosome pathway, where with maturation the contents are degraded. Intracellular bacteria can also be sequestered in these vacuoles, leading to their destruction. This phenomenon has been well described for intracellular pathogens including invasive group A *Streptococcus* and *Mycobacterium tuberculosis* [50,51]. Many intracellular bacteria can be found in autophagolysosomes and cells that are deficient in essential components of the autophagolysosomal machinery are unable to destroy these bacteria. Intracellular *L. monocytogenes* can be autophagocytosed, but this has only been observed when the macrophages are first treated with chloramphenicol [52]. Possibly, autophagocytosis of *L. monocytogenes* may be a rare event compared to other bacterial pathogens since its intracellular motility and secretion of the pore-forming LLO may impede membrane formation around the bacterium.

3. Adaptive immune responses

The *L. monocytogenes*-specific adaptive immune response follows the innate immune response. DCs are an important link between innate and adaptive immunity [53]. Specific recognition of pathogen-derived products by TLRs initiates a signaling cascade that leads to activation of the DC. Activation leads to greater expression of co-stimulatory molecules and cytokines, both of which enhance the DC's ability to stimulate T cells. DCs respond to different pathogens and initiate the appropriate type of T cell response needed to control infection. In response to *L. monocytogenes* infection, DCs are critical in priming the T cell response since mice depleted of these cells are unable to generate a CD8 T cell response to infection [54]. Due to the intracellular niche of the bacteria, CD4 and CD8 T cells comprise most of the adaptive immune response. Other cell subsets, including B cells, regulatory T cells and non-classical MHC T cells also contribute to this response by mainly influencing CD4 and CD8 T cell responses.

3.1 CD4 and CD8 T cell responses

Although innate immune cells are important for initial control of *L. monocytogenes* infection, T cells are needed for final clearance of bacteria. CD4 and CD8 T cells are important for conferring sterilizing immunity since SCID mice develop a chronic infection [3,4]. While both CD4 and CD8 T cells contribute to protective immunity, in vivo depletion and adoptive transfer studies have clearly demonstrated that memory CD8 T cells are the most effective T cell subset capable of mediating protection [55,56].

Recognition of infection by the immune system is the first and most important step to eliminating pathogens. For an intracellular bacterial pathogen such as *L. monocytogenes*, recognition of infected cells is critical to controlling infection. *L. monocytogenes* antigen can be presented in several ways depending on the type of cell infected. When *L. monocytogenes* is in the cytosol of almost any cell type, proteins it secretes are subject to

degradation by the host proteasome. These peptides are then transported to the endoplasmic reticulum, loaded onto MHC class I molecules, and presented on the cell surface to CD8 T cells. Furthermore, professional antigen presenting cells are able to present antigen from bacteria destroyed in lysosomes via the MHC class II pathway to CD4 T cells. This antigen is comprised of bacterial secreted, non-secreted and surface proteins. Antigens from the lysosome can also traffic to the endoplasmic reticulum where they are presented by MHC class I molecules via a process called cross-presentation [57–59]. In addition, uninfected professional antigen presenting cells are also able to present bacterial antigen found in debris from dead cells to both CD4 and CD8 T cells. Thus, presentation of *L. monocytogenes* antigens to T cells is dependent on the type of cell infected as well as compartmentalization of the antigen.

The prevailing notion is that CD8 T cells mediate anti-listerial immunity through two synergistic mechanisms: (1) lysing infected target cells via perforin and granzymes to expose intracellular bacteria for killing by activated macrophages, and (2) secreting IFN γ to activate macrophages [60]. Complete protective immunity requires infection with live *L. monocytogenes* capable of escaping from the vacuole [61]. This is in part due to the generation of the CD8 T cell response and a proper memory T cell population [62]. The role of CD4 T cells in controlling *L. monocytogenes* infection is less well understood than that of CD8 T cells. Depletion of CD4 T cells during primary *L. monocytogenes* infection results in diminished granuloma formation [63]. *L. monocytogenes* induces a strong Th1 response and like CD8 T cells, *L. monocytogenes*-specific CD4 T cells also secrete IFN γ which may aid macrophage activation [64].

T cells respond to *L. monocytogenes* infection in a predictable manner. Due to a low precursor frequency, endogenous naïve antigen-specific T cells cannot be detected in mice by current methods. As these populations of cells expand during the immune response, they begin to become detectable four to five days post-*L. monocytogenes* infection. The primary T cell response peaks seven to nine days post-infection [65] and the extent of this proliferation and differentiation is independent of antigen [66–68]. The population of antigen-specific T cells then contracts to a smaller population of stable memory T cells.

Compared to naïve T cells, these memory T cells are greater in number, have a lower stimulation threshold for activation and upon secondary infection can quickly exert effector functions allowing rapid control of infection and elimination of the bacteria [69]. Memory T cells swiftly mobilize to the site of infection, and secrete IFN γ leading to activation of macrophages, and rapidly secrete molecules which are directly toxic to infected cells such as perforin and granzyme. *L. monocytogenes*-specific memory T cells are very specific for the elimination of only bacteria that they can recognize and there is little bystander killing of non-recognizable bacteria [70]. Memory T cell killing is also limited to the ability of the T cells to recognize all infected cells—if some infected cell subsets cannot present the recognized antigen, then memory T cells are not be able to adequately control infection [71,72]. In fact, perforin is perhaps the most important effector protein to provide protective immunity against *L. monocytogenes*. Mice deficient in perforin are able to clear primary infection, albeit slightly slower than wild-type mice, but have a severe defect in clearing a challenge infection after immunization [73]. Interestingly, perforin-dependent killing is only important when *L. monocytogenes* has its natural ability to spread intercellularly. Challenge infection of immunized perforin-deficient mice with *L. monocytogenes* lacking the virulence factor needed for motility (ActA) results in the same rate of clearance as in immunized wild-type mice [74]. Thus, memory T cells are essential for protection from re-infection with *L. monocytogenes* due to their ability to quickly recognize and destroy infected cells.

3.2 Regulatory T cells

Regulatory T cells are a subset of T cells that have suppressive effects on the activities of other T cells by secreting such immunosuppressive cytokines as TGF- β and IL-10, and thus are responsible for limiting the extent of the immune response. Naturally occurring regulatory T cells are characterized as CD4⁺CD25⁺ and express the transcription factor Foxp3, which is essential for their function. Important for preventing autoimmunity, during infection regulatory T cells play a role in down-regulating the T cell responses. No role has been shown for these cells during primary *L. monocytogenes* infection, but during a rechallenge response CD4⁺CD25⁺ cells are important for limiting the expansion of memory CD8 T cells [75].

3.3 Non-classical MHC T cell responses

Besides classical MHCIIa antigen presentation, *L. monocytogenes* antigens can be presented by non-classical MHCIIb molecules. These molecules have similar structure to MHCIIa molecules and also associate with β 2-microglobulin, however MHCIIb molecules lack the polymorphicity of MHCIIa molecules. In murine *L. monocytogenes* infection, the best studied MHCIIb molecule is H2-M3, which binds peptides that contain a formylated amino terminal methionine residue. Since N-formylation of proteins is mainly limited to bacteria (the bacterial evolutionary remnants of mitochondria and chloroplasts also have this activity), the H2-M3 molecule specifically binds bacterial-derived peptides. Several *L. monocytogenes*-derived peptides presented by H2-M3 have been identified and used for studying MHCIIb restricted responses.

H2-M3 restricted T cells respond differently than classical MHCIIa restricted T cell responses during *L. monocytogenes* infection. Firstly, the kinetics of T cell expansion and contraction are faster for H2-M3 restricted cells. The number of H2-M3 *L. monocytogenes*-specific cells peaks five to six days post-infection, compared to MHCIIa-restricted cells which peak seven to nine days post-infection. Contraction of H2-M3 restricted T cells does result in generation of a pool of memory cells, but they only have some of the hallmarks of traditional memory cells. When rechallenged with a second *L. monocytogenes* infection, although these cells rapidly upregulate surface expression of activation markers, these cells do not proliferate [76,77]. This suppression of proliferation is mediated by the expansion of the MHCIIa response in turn limiting available DCs for antigen presentation [78]. Not surprisingly, without expansion of H2-M3-restricted cells these cells cannot provide protection to secondary *L. monocytogenes* infection. However, these cells do play a role in control of primary infection since H2-M3 knockout mice have a defect in bacterial clearance suggesting that early expansion and IFN γ production by these cells cannot be compensated by other cell subsets [79].

3.4 B cell responses

Since the 1960s when Mackaness showed that transfer of cells from *L. monocytogenes*-immunized mice, but not serum, provided protection against *L. monocytogenes* infection of naïve mice, it has been known that antibodies do not have a major role in *L. monocytogenes* infection. Since the majority of the bacteria remain intracellular during infection and spread intercellularly without encountering the extracellular milieu, *L. monocytogenes*-specific antibodies would be of limited to no use in controlling bacterial spread. However, under certain experimental conditions antibodies can affect the course of infection. Although infection itself does not generate high titers of antibodies, a monoclonal antibody against the pore-forming virulence factor LLO can provide protection by acting intracellularly to neutralize LLO and block bacterial escape from the phagosome [80]. Also, using B cell-deficient mice, natural antibodies in naive animals may play a role in reducing early dissemination of *L. monocytogenes* into vital organs [81]. By trapping bacteria and their antigens in secondary lymphoid organs where specific immune responses are initiated, B cells and antibodies can facilitate the generation of the protective T cell response. Lastly, B cells have been shown to

be important in the maintenance of memory CD8 T cells generated during *L. monocytogenes* infection [82]. Thus, B cells and antibodies do play a minor, yet significant, role during *L. monocytogenes* infection.

4. Conclusions

Infection is a complex interplay between host and pathogen. The model of murine listeriosis allows for careful dissection of both host and bacterial factors that are important for infection and immunity. Infection outcome depends on the effectiveness of the host immune response against a particular pathogen. In the case of *L. monocytogenes*, both innate and adaptive immunity are critical for controlling infection of the intracellular bacterium. Infection is first recognized by the innate immune system, leading to the rapid production of anti-microbial factors, as well as chemokines and cytokines that aid in initiation of the adaptive immune response. *L. monocytogenes* antigen presented by DCs drives strong CD4 and CD8 T cell responses that result in a stable population of *L. monocytogenes*-specific memory T cells. This has made *L. monocytogenes* infection a useful model for recent vaccine-related studies in the generation, maintenance, and challenge responses of memory T cells. In addition, as a cytosolic intracellular pathogen, *L. monocytogenes* is an ideal model for the burgeoning field of intracellular recognition.

Even though *L. monocytogenes* has been used as a model pathogen for over forty years, the recent studies highlighted in this review suggest there are still unidentified immunological aspects of *L. monocytogenes* infection and immunity. Many of the immune system mechanisms elucidated in murine listeriosis serve homologous functions in other hosts, including humans. In the future, *L. monocytogenes* will continue to be a key pathogen in the identification of new molecules in the regulation of the innate and adaptive immune responses.

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Abbreviations

DC	Dendritic cell
LLO	Listeriolysin O
TLR	Toll-like receptor