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***Listeria monocytogenes*: a model pathogen to study antigen-specific memory CD8 T cell responses**

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Abstract

Memory CD8 T cells play a critical role in providing protection to immune hosts by orchestrating rapid elimination of pathogen-infected cells after re-infection. Systemic bacterial infection with *Listeria monocytogenes* has been a favored approach for researchers to characterize pathogen-specific CD8 T cell responses, and in-depth understanding of *L. monocytogenes* biology has provided invaluable experimental tools that have been used to increase our understanding of memory CD8 T cell differentiation. Here, we describe how the tools from this murine model system of infection have been utilized to characterize pathogen-specific CD8 T cells in inbred and genetically diverse outbred hosts as they undergo naïve-to-memory CD8 T cell differentiation *in vivo*. We also discuss how studying *L. monocytogenes*-evoked CD8 T cell responses have provided insight on the degree of diminished T cell immunity in clinically relevant conditions such as sepsis and obesity. Overall, this review will highlight how infection with the intracellular pathogen *L. monocytogenes* has enabled analysis of systemic CD8 T cell responses and greatly contributed to what is known about memory CD8 T cell generation and differentiation.

Keywords

CD8 T cells; Memory; Inflammation; Sepsis; Obesity; CD11a; Interferon- γ

Introduction

Listeria monocytogenes is a gram-positive facultative intracellular bacterial pathogen that is the causative agent of listeriosis (1). This food-borne pathogen is especially dangerous in pregnant women, newborns, and the immunocompromised (2). The unique ability of *L. monocytogenes* to cross several tight barriers within the infected human host contributes to its pathogenesis and wide range of clinical symptoms (3, 4). Invasion of enterocytes after oral ingestion mediates crossing of the intestinal barrier and access to internal organs, resulting in gastroenteritis. In addition, listeriosis can result in mother-to-fetus infection and septicemia after the pathogen crosses the fetoplacental barrier. Finally, the capacity of *L. monocytogenes* to cross the blood-brain barrier can result in infection of the meninges and the brain (5).

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Experimental listeriosis described in a murine model in the early 1960s by George Mackaness laid the foundation for use of *L. monocytogenes* as a model pathogen to characterize mammalian immune responses (6). Specifically, Mackaness and his fellow colleagues demonstrated that a protective cellular immune response is generated after intravenous infection with *L. monocytogenes*. Further studies then showed that the clearance of this pathogen is T cell-mediated (7). Extensive research has been conducted as a result of this pioneering work to fully characterize host T cell responses after *L. monocytogenes* infection. In fact, CD8 T cell-mediated responses to this pathogen are widely studied due to its ability to survive and replicate within infected host cell cytosol and access the endogenous MHC class I pathway, which results in induction of a robust CD8 T cell response. As a favored pathogen for immunological research, laboratories have utilized *L. monocytogenes* to study CD8 T cell responses. In this review, we will describe the tools afforded by the *L. monocytogenes* model system and how they have been utilized to characterize naïve-to-memory CD8 T cell generation and differentiation. Finally, we will discuss how studying *L. monocytogenes*-evoked CD8 T cell responses provide insight on the degree of diminished T cell immunity in clinically relevant conditions such as sepsis and obesity.

Molecular pathogenesis of *L. monocytogenes* and its impact on the generation of a CD8 T cell response

L. monocytogenes invades both phagocytic and non-phagocytic cells (8). Inside the infected host cell, this pathogen evades membrane-bound phagosomes through secretion of the pore-forming toxin listeriolysin O (LLO) and gains access into the cytosol (9). Although a number of gram-positive bacteria secrete pore-forming cytolysins like LLO, *L. monocytogenes* is unique in its ability to specifically secrete this toxin inside the infected host cell and to replicate within the cytosol. Polymerization of host actin enables pathogen motility, propelling *L. monocytogenes* through the host cell and into neighboring cells (10, 11). Notably, direct cell-to-cell spread of this pathogen enables evasion of extracellular milieu and the consequential effects of antibodies and complement. This process of creating actin polymers is mediated by the expression of the bacterial surface protein actin-assembly-inducing protein (ActA). Since intercellular mobility is an important component of *L. monocytogenes* virulence, the deletion of the *actA* gene attenuates pathogenicity *in vivo* (12). Replication of *L. monocytogenes* in the cytosol and secretion of bacterial proteins within this compartment allows for bacterial antigen processing and presentation in the endogenous MHC class I pathway (13). Therefore, presence in the host cell cytosol induces a strong *L. monocytogenes*-driven CD8 T cell response and results in the establishment of protective immunity (14, 15).

Because the expression of LLO by *L. monocytogenes* is not only essential for its pathogenesis, but also necessary for the induction of MHC class I-restricted CD8 T cell responses, researchers aimed to identify the specific epitope of LLO recognized by CD8 T cells. An interesting experimental technique in which an allele-specific motif approach was used for epitope prediction led to the identification of LLO₉₁₋₉₉ as the first MHC class-I restricted bacterial pathogen-derived epitope recognized by CD8 T cells in BALB/c mice

(16). Following identification of LLO₉₁₋₉₉, the epitope from the *L. monocytogenes* protein p60 (p60₂₁₇₋₂₅₅) was classified thereafter (17). MHC class I-restricted CD8 T cells specific for these two epitopes were then shown to provide significant protection following *L. monocytogenes* infection in BALB/c mice (18, 19).

Since epitopes from secreted *L. monocytogenes* protein antigens were identified, it was originally thought that only secreted antigens prime CD8 T cell responses that can mediate protective immunity against *L. monocytogenes* infection. Subsequently, researchers utilized the versatility of the *L. monocytogenes* murine model by engineering recombinant strains of *L. monocytogenes* expressing known CD8 T cell epitopes from other highly-characterized pathogens, and addressed how compartmentalization of bacterial antigens impacts CD8 T cell immunity (20). Recombinant strains of *L. monocytogenes* expressing either the secreted or nonsecreted form of the lymphocytic choriomeningitis virus (LCMV)-derived nucleoprotein epitope (NP₁₁₈₋₁₂₆) elicited NP₁₁₈-specific CD8 T cell responses (21). This suggested that even nonsecreted bacterial antigens can prime CD8 T cell responses. However, memory NP₁₁₈-specific CD8 T cells generated after LCMV infection were only able to protect against *L. monocytogenes* challenge if this recombinant strain expressed the secreted viral antigen (22). Thus, genetically manipulating *L. monocytogenes* and analyzing CD8 T cell responses to a single epitope elegantly demonstrated that a dichotomy exists with regards to eliciting an antigen-specific CD8 T cell response and achieving host protection. In summary, these and other data suggest that thorough understanding of *L. monocytogenes* pathogenesis allows for high-resolution analyses of CD8 T cell responses after bacterial infection.

***L. monocytogenes* as a model pathogen to analyze naïve-to-memory CD8 T cell differentiation**

The identification of specific *L. monocytogenes* epitopes and ability to manipulate this model pathogen have been advantageous to understanding CD8 T cell responses after infection. For example, altering the course of *L. monocytogenes* infection with antibiotic treatment has provided insight on how a decreased inflammatory response impacts phases of the CD8 T cell response including: expansion, contraction, and memory generation (4). Furthermore, using genetically attenuated *L. monocytogenes* has led to important discoveries about antigen-specific CD8 T cell effector mechanisms necessary for controlling infection (23, 24). The various factors regulating the formation of memory CD8 T cells have also been defined after infection with *L. monocytogenes*. The studies outlined below highlight how the *L. monocytogenes* model system has served as a tool for characterizing the generation and differentiation of memory CD8 T cells.

CD8 T cell expansion and acquisition of effector functions

The naïve CD8 T cell repertoire is comprised of relatively small numbers of CD8 T cell precursors specific for a single antigen. In an inbred laboratory mouse this number ranges from 10 to 1,000 cells (25), suggesting that once a naïve CD8 T cell becomes activated it must undergo massive proliferative expansion in numbers to defend against the invading pathogen (Figure 1). Efficient activation of CD8 T cells requires the following signals:

stimulation of the T cell receptor with peptide-MHC class I complexes presented by professional antigen-presenting cells (signal 1), engagement of co-stimulatory molecules such as CD80/86:CD28 (signal 2), and inflammatory cytokines such as IL-12 and type I IFNs (signal 3) (26, 27). Once activated, CD8 T cells have the capacity to give rise to as many as 10,000 daughter cells (28). As previously described, in response to *L. monocytogenes*, primary CD8 T cell expansion peaks between days 7 and 10 depending on the strain of bacteria (29, 30).

It was formerly thought that antigen persistence drives antigen-specific CD8 T cell expansion, since the magnitude of antigen-specific CD8 T cell responses is dependent on the type of pathogen and dose of infection used. However, studies in which the course of *L. monocytogenes* infection was manipulated demonstrated that primary CD8 T cell expansion could be independent of antigen persistence. For instance, antibiotics administered to mice 24 hr after infection with *L. monocytogenes* greatly truncated pathogen replication, while the initial phase of CD8 T cell expansion remained largely unaffected. However, antibiotic treatment starting at 12 h after initial infection greatly reduced CD8 T cell expansion (31). These early experiments suggested that CD8 T cells are programmed to undergo continued expansion following a relatively brief period of antigen stimulation after infection *in vivo*. Similarly, the magnitude of CD8 T cell expansion was significantly reduced in mice pre-treated with antibiotics prior to *L. monocytogenes* infection (32). *In vitro* studies have also reported that T-cell-receptor transgenic (TCR-Tg) CD8 T cells undergo multiple rounds of division and acquire cytolytic functions following brief antigen encounter (33). Overall these early experiments suggested that the program of CD8 T cell expansion, once put in motion, can be independent of further antigen stimulation.

In various studies it has been demonstrated that ‘signal 3’ cytokines play a critical role in regulating optimal levels of CD8 T cell expansion (34). For example, CD8 T cells lacking certain cytokine receptors undergo reduced levels of expansion after multiple infections. TCR-Tg CD8 T cells lacking the type I IFN receptor (IFNAR) had a dramatic reduction in expansion after LCMV infection, whereas IFNAR deficiency on CD8 T cells did not significantly alter expansion after infection with *L. monocytogenes*. However, the absence of IL-12 signaling greatly diminished CD8 T cell expansion after *L. monocytogenes* infection (35, 36). These data suggested that individual cytokines regulating CD8 T cell responses are greatly pathogen-dependent (37). Interestingly, it has recently been demonstrated that in response to either of the signal 3 cytokines, IL-12 or type I IFNs, activated CD8 T cells maintain high-affinity IL-2 signaling *in vivo* which results in continued expression of cell-cycle associated genes and extended cellular division (38). Thus, inflammatory signals received by activated CD8 T cells shape the magnitude of proliferative expansion.

Proliferating antigen-specific CD8 T cells gain the capacity to elaborate effector mechanisms such as the production of IFN γ and TNF α to alert other cells of the immune system of pathogen invasion and the secretion of perforin to mediate cytolysis of the infected cell (Figure 1). Early studies demonstrated that gene knockout mice either lacking the TNF α type 1 receptor (39) or the IFN γ receptor (40) were more susceptible to *L. monocytogenes* infection compared to wild-type mice. Given that IFN γ and TNF α are important cytokines for innate resistance to *L. monocytogenes* (20), it was thought that CD8

T cell-mediated resistance to *L. monocytogenes* infection primarily depended on the effector molecules IFN γ and TNF α . Investigators utilized the reduced pathogenicity of the attenuated *L. monocytogenes* strain to immunize gene knockout mice and defined the precise mechanisms by which *L. monocytogenes*-specific CD8 T cells mediate protection (24).

Susceptibility to the virulent *L. monocytogenes* strain was significantly increased in gene knockout mice compared to wild-type mice. For instance, the 50% lethal dose (LD₅₀) of virulent *L. monocytogenes* infection in IFN γ ^{-/-} mice has been documented to be 10 CFU, compared to the LD₅₀ of greater than 10⁴ CFU in wild-type mice. (41). Interestingly, after high-dose infection with the attenuated *L. monocytogenes* strain lacking ActA, IFN γ ^{-/-} mice not only elicited an effective LLO- and p60-specific CD8 T cells response, but also survived after clearing the infection (41). This suggested that CD8 T cell-mediated resistance to *L. monocytogenes* can occur in the absence of IFN γ . Adoptive transfer experiments in which antigen-specific CD8 T cells either sufficient or deficient in IFN γ were transferred to new naïve hosts prior to *L. monocytogenes* challenge effectively transferred *L. monocytogenes*-specific immunity, once again demonstrating that CD8 T cells can protect against this intracellular pathogen in an IFN γ -independent fashion. To delineate whether TNF α , like IFN γ , served as a CD8 T cell-derived effector molecule that may be expendable for immunity against *L. monocytogenes*, adoptive transfer experiments were performed using TNF α deficient LLO-specific CD8 T cells. Bacterial burden was significantly reduced in wild-type mice that had received LLO-specific CD8 T cells lacking TNF α prior to virulent *L. monocytogenes* challenge (42). However, TNF α ^{-/-} recipient mice, similar to IFN γ deficient hosts, succumbed to virulent bacterial challenge (42). This suggests that although TNF α derived from antigen-specific CD8 T cells might not be required for antilisterial immunity, host TNF α is important for resistance to *L. monocytogenes* infection.

In addition to the production of cytokines, lysis of pathogen-infected cells is another CD8 T cell-mediated effector mechanism important for the control of intracellular infections. Moreover, early *in vitro* studies demonstrated two major mechanisms by which CD8 T cells lyse target cells, including one that is a perforin-dependent pathway and another that is dependent upon the interaction between Fas (CD95) and Fas ligand (CD95 ligand) (43). Initial experiments assessing the role of perforin in the resistance to *L. monocytogenes* utilized perforin gene knock out mice. Here, it was found that CD8 T cell immunity against *L. monocytogenes* was reduced 10–100 fold in perforin-deficient mice (44). Interestingly in other studies, perforin-deficient CD8 T cells specific for various epitopes of *L. monocytogenes* mediated protection from infection in new naïve hosts (45). Antigen-specific CD8 T cells lacking perforin also mediated protection in CD95 gene knock out mice, suggesting that a pathway of *L. monocytogenes* resistance exists independent of perforin- and Fas-mediated cytolysis (46). Interestingly, TNF α -deficient recipient hosts infected with *L. monocytogenes* were not protected after receiving a transfer of perforin-deficient CD8 T cells. These data further demonstrate that while host CD8 T cell-derived TNF α is not a critical effector mechanism, host TNF α is necessary for controlling *L. monocytogenes* infection.

While gene knock out mice and attenuated strains of *L. monocytogenes* have served to broaden the understanding of specific mechanisms employed by antigen-specific CD8 T

cells in response to infection, this model system has also been used to demonstrate the contribution of effector molecules in CD8 T cell homeostasis. For example, wild-type and perforin-deficient mice cleared the attenuated *L. monocytogenes* strain with similar kinetics, however the magnitude of proliferative expansion of LLO and p60-specific CD8 T cells was greatly increased in the absence of perforin. These data suggested that perforin has a role in regulating CD8 T cell expansion. Moreover, a dual deficiency in both perforin and IFN γ also yielded increased numbers of *L. monocytogenes*-specific CD8 T cells (47). Together these data suggest that independent from their role as effector molecules, perforin and IFN γ are important in the regulation of CD8 T cell expansion after *L. monocytogenes* primary infection.

CD8 T cell contraction

CD8 T cell proliferative expansion is necessary for activated cells to differentiate into effector cells and acquire effector functions. However, to maintain memory of previously encountered pathogens and to preserve the ability to respond to a range of new infections, antigen-specific effector CD8 T cells must undergo a contraction (death) phase (Figure 1). During the contraction phase, 90–95% of effector CD8 T cells undergo apoptosis, while the surviving cells form a stable memory pool (27). The observation that the onset of CD8 T cell contraction correlated with the clearance of various pathogens gave rise to the paradigm that responding CD8 T cells sense pathogen clearance. Although IFN γ ^{-/-} and wild-type mice cleared the attenuated *L. monocytogenes* infection with similar kinetics, it was found that antigen-specific CD8 T cells underwent significantly reduced levels of contraction in these gene knockout mice compared to wild-type mice (47, 48). Thus, these data questioned the notion of pathogen sensing and the onset of contraction. Once again, the ability to manipulate the course of *L. monocytogenes* infection served as an experimental tool to address whether the duration of infection is a factor regulating CD8 T cell contraction. Eradication of *L. monocytogenes* with antibiotic treatment 24 h after infection did not change the onset or kinetics of CD8 T cell contraction (48), suggesting that the contraction phase of CD8 T cell homeostasis, in addition to proliferative expansion, is programmed early after infection.

While antibiotic post-treatment (24 h after infection) did not alter CD8 T cell contraction in mice infected with *L. monocytogenes* (32), levels of CD8 T cell contraction were abrogated in mice pre-treated with antibiotics (48). This was likely due to a dramatic reduction in the levels of IFN γ in pre-treated mice. CD8 T cell contraction was also greatly reduced in IFN γ -deficient mice after *L. monocytogenes* infection (47). Interestingly, CD8 T cell contraction was not reduced in antibiotic pre-treated mice that also received an injection with the TLR9 agonist CpG, a potent inducer of inflammation (48). Together these data suggest that inflammatory signals are important for regulating CD8 T cell contraction and downsizing the pathogen-specific effector CD8 T cell pool.

While these data demonstrated that inflammation is necessary for CD8 T cell contraction to occur, it still remained to be determined how the timing of CD8 T cell contraction was regulated. By comparing attenuated and virulent strains of *L. monocytogenes* infections in mice, it was first found that the peak of infection differs between these two strains (49). CD8

T cell responses in mice infected with attenuated *L. monocytogenes* peaked at 7 days after infection, while this peak was delayed to about 8–10 days after infection with virulent *L. monocytogenes*. It was found that 24 h after initial infection (0.1 CFU LD₅₀ for both strains), bacterial loads differ between attenuated (approximately 1,000,000 bacteria/mouse) and virulent (approximately 1,000 bacteria/mouse) strains of *L. monocytogenes* (30). These data suggested that differences in peaks of infection correlated with differences in the timing of antigen presentation. Moreover, the peak of functional antigen display was 1–2 days after attenuated *L. monocytogenes* infection and favored increased recruitment of naïve CD8 T cells because of higher initial bacterial loads. In contrast, bacterial loads and functional antigen display were delayed to 2–3 days after virulent *L. monocytogenes* infection, resulting in a prolonged transition from peak CD8 T cell expansion to the onset of contraction (30). Overall these data suggested that while inflammatory signals are necessary for CD8 T cell contraction to occur, the timing of CD8 T cell contraction is initiated approximately 5 days after the peak of functional antigen display.

Memory CD8 T cell generation and maintenance

The 5–10% of activated CD8 T cells that survive contraction establish a stable population of memory CD8 T cells (Figure 1). Enhanced protection after re-infection provided by memory CD8 T cells is due to stable persistence at higher numbers and rapid initiation of effector functions after antigen re-encounter (Figure 1). While it has been shown that memory CD8 T cells persist for great lengths of time after infection or vaccination (50), the maintenance of memory CD8 T cells is a dynamic process. Memory CD8 T cells undergo time-dependent changes in phenotype and function, thus even within a population of memory CD8 T cells specific for a single antigen there is substantial phenotypic and functional heterogeneity. The *L. monocytogenes* model has been used in numerous studies to explore and define mechanism(s) controlling changes in pathogen-specific CD8 T cells as they undergo memory differentiation.

The concept of dividing circulating memory CD8 T cells into subsets based on functionality, migration, and proliferative potential gave rise to the classical paradigm of effector memory (T_{em}) and central memory (T_{cm}) (51). The expression of the lymph node homing molecules, CD62L and CCR7, permits T_{cm} access to lymphoid organs. In contrast, T_{em} predominantly localize within peripheral tissue, as the expression of these homing markers is substantially low. While both subsets of memory CD8 T cells rapidly produce the cytokines IFN γ and TNF α after antigen re-encounter, T_{cm} are potent producers of IL-2. Additionally, T_{cm} undergo a higher magnitude of proliferative expansion after antigen re-stimulation compared to T_{em} (52). This suggests that the T_{cm} population may be better suited to control systemic infections.

Interestingly, the rate at which differentiating CD8 T cells acquire a T_{cm} phenotype with time after infection is pathogen-dependent. For example, CD8 T cells acquire a T_{cm} phenotype faster after infection with *L. monocytogenes* compared with LCMV (52, 53). The rate of memory CD8 T cell differentiation has been shown to be dependent on the inflammatory environment in which naïve CD8 T cells are initially activated, implying that this process can be manipulated. Antibiotic treatment, which resulted in reduced levels of

inflammation following infection with *L. monocytogenes*, led to both decreased magnitudes of expansion and contraction following systemic infection (48). Interestingly, it was also found in this study that these activated CD8 T cells acquired long-term T_{cm} characteristics at an accelerated rate, suggesting that lower levels of inflammatory cytokines at the time of initial activation greatly accelerated memory CD8 T cell differentiation. In another study, mice treated with antibiotics after infection led to the generation of memory CD8 T cells that underwent robust secondary expansion and provided increased protection from re-infection as early as 2 weeks after *L. monocytogenes* infection (54). However, accelerated memory CD8 T cell differentiation was reversed in these mice when they also received an injection of CpG to restore high levels of inflammation (54). Finally, mice that received LPS-matured dendritic-cells (DCs) coated with *L. monocytogenes*-derived peptides generated antigen-specific CD8 T cells that acquired memory characteristics at an accelerated rate (54). Yet, DC-immunization coupled with co-injection with CpG prevented accelerated memory formation. Together, these data support the notion that inflammation present early after initial antigen recognition regulates the rate of memory generation in circulating memory CD8 T cells.

Over time after infection circulating memory CD8 T cells re-express high levels of CD62L and predominantly convert to a T_{cm} phenotype (Figure 1). Since T_{cm} undergo extensive proliferation compared to T_{em}, this suggests that subsets of memory CD8 T cells will be better at secondary expansion upon cognate antigen re-stimulation. Using an experimental system in which the proliferative ability of circulating memory CD8 T cells generated in mice either early (1 month) or late (8 months) after infection with *L. monocytogenes* was evaluated on a per-cell basis, Martin et al. demonstrated that the ability of memory CD8 T cells to undergo proliferation after secondary antigen encounter increases with time after infection (55). Future studies will determine the full extent of changes in the memory CD8 T cell pool at various times after primary antigen-encounter and will help define the mechanisms that control the differentiation and maintenance of memory CD8 T cell responses (56).

In addition to changes in phenotype and function of memory CD8 T cells over time after infection or vaccination, the number of antigen encounters has also been demonstrated to affect properties of circulating memory CD8 T cells (57). Human hosts are typically infected with pathogens more than once, resulting in populations of memory CD8 T cells that have undergone repeated antigen stimulations. In addition, prime-boost vaccination strategies are often used to generate large numbers of secondary memory CD8 T cells and enhance memory CD8 T cell-mediated protection. Interestingly, longitudinal studies of developing circulating primary and secondary memory CD8 T cells suggest that the rate of acquiring a long-term T_{cm} phenotype and function is distinct between these two populations of memory cells. Utilizing recombinant *L. monocytogenes* expressing OVA to generate primary and secondary circulating memory CD8 T cells, it was shown that primary memory CD8 T cells expressed high levels of CD62L and produced substantial amounts of IL-2 as early as 40 days after infection, while this conversion to a long-term T_{cm} phenotype was markedly delayed in secondary memory CD8 T cells (58). Interestingly, the type of infection and levels of inflammation have been found to modulate both primary and secondary memory

CD8 T cell responses. Infection of mice with a virulent strain of *L. monocytogenes* resulted in higher frequencies of secondary effector and memory CD8 T cells compared to infection with an attenuated strain. As with primary CD8 T cell responses, secondary effector CD8 T cell expansion was also reduced in mice that received antibiotic treatment to curtail *L. monocytogenes* infection (59, 60), suggesting that reducing the duration of infection decreases the overall secondary effector and memory CD8 T cell response to systemic bacterial infection. In addition to antigen, inflammatory signals have also been found to modulate secondary expansion and acquisition of long-term secondary memory characteristics. The magnitude of secondary expansion was increased by fourfold in mice that were immunized with peptide-coated dendritic cells and co-infected with *L. monocytogenes* to induce inflammation compared to mice that received the dendritic cell immunization alone. However, conversion to a long-term T_{cm} phenotype was delayed in secondary memory CD8 T cells that encountered antigen in the presence of *Listeria*-induced inflammation (59, 60). These data suggest that as with primary CD8 T cell responses, systemic inflammation impacts the numbers and phenotype of secondary effector and memory CD8 T cells. It remains to be determined other factors controlling the development of secondary CD8 T cell responses. Additionally, whether the process of primary memory to secondary memory CD8 T cell differentiation can be manipulated, a notion with relevance for the design of prime-boost vaccine strategies.

Our laboratory has previously performed serial adoptive transfers of TCR-Tg CD8 T cells into naïve mice to compare primary and repeatedly stimulated (secondary, tertiary, quaternary) memory CD8 T cells that differed only in the number of times they had encountered antigen (61). Phenotypic analyses of multiply stimulated circulating memory CD8 T cells revealed that reacquisition of CD62L expression and conversion to T_{cm} slowed substantially with each additional antigen encounter. Therefore, the ability of multiply stimulated memory CD8 T cells to localize within the LN and produce IL-2 decreased after each additional antigen encounter (61). It was also found that multiple antigen encounters decreased the capacity of memory CD8 T cells to undergo proliferative expansion. In addition to phenotypic and functional analyses of multiply stimulated memory CD8 T cells, transcriptomic analysis revealed that with each additional antigen encounter a stepwise diversification of genes occurs in populations of repeatedly stimulated memory CD8 T cells (61). Interestingly, despite substantial differences in the expression of genes that regulate effector functions, proliferation, and migration, with each additional antigen encounter in memory CD8 T cells, a core group of genes remains shared between primary memory and memory CD8 T cells responding to repeated antigen stimulations (61). These documented changes in gene expression after multiple antigen encounters demonstrates that diversity in gene regulation also contributes to heterogeneity within the resulting memory CD8 T cell pool.

***L. monocytogenes*-specific CD8 T cell responses in genetically diverse hosts**

Identification of *L. monocytogenes*-derived epitopes recognized by CD8 T cells has allowed extensive research of factors regulating antigen-specific CD8 T cells as they undergo naïve-

to-memory differentiation. In a scenario where antigenic epitopes of relevant pathogens remain undefined or host MHC restriction elements are unknown, a 'surrogate activation marker' approach can be applied to track antigen-specific CD8 T cell responses. It has been previously demonstrated that surface phenotype changes help distinguish antigen-experienced from naïve (antigen-inexperienced) CD8 T cells after infection or vaccination. For example, as previously documented by Rai et al. and depicted in Figure 2, CD8 T cells in cohorts of inbred and genetically diverse outbred mice responding to *L. monocytogenes* infection upregulate the surface expression of the integrin CD11a and downregulate surface expression of CD8α (62). Within the CD11ahi population, pathogen-specific CD8 T cells display additional phenotypic changes including a decrease in CD127 and CD62L and an increase in KLRG1 expression, demonstrating that antigen-experienced CD8 T cells are distinguishable from naïve (antigen-inexperienced) CD8 T cells (Figure 2). These changes are antigen-dependent, as CD11a upregulation and CD8α downregulation did not occur on antigen-specific CD8 T cells in mice that received injections of various TLR agonists to elicit systemic inflammation. Additionally, the surface expression of CD11a and CD8α on OT-I CD8 T cells did not change after infection with a *L. monocytogenes* strain lacking expression of the OVA epitope (62). Therefore, using this 'surrogate activation marker' approach and *L. monocytogenes* as a model of infection has allowed identification of total pathogen-specific CD8 T cells responding to pathogen without prior knowledge of epitope specificity or MHC restriction elements, thus extending analysis of pathogen-specific CD8 T cells to outbred hosts.

Interestingly, this surrogate activation marker approach was utilized by Rai et al. to analyze pathogen-specific memory CD8 T cells longitudinally in inbred C57BL/6 and outbred Swiss Webster mice. While polyclonal CD8 T cell responses to *L. monocytogenes* in an inbred strain were coordinated and highly reproducible throughout experiments when analyzed in individual mice, substantial variability in the kinetics and magnitude of the response was observed in outbred mice, suggesting that genetic background influences host-pathogen interactions.

In summary, these data demonstrate how the *L. monocytogenes* model of infection can be used to advance our understanding of pathogen-specific CD8 T cell responses even in scenarios where knowledge of antigen specificity of responding CD8 T cells is lacking.

***L. monocytogenes* as a resource to investigate clinically relevant diseases and conditions**

Given that systemic infection with *L. monocytogenes* enables characterization of pathogen-specific CD8 T cell responses, this relevant pathogen can be used to determine the degree of diminished T cell immunity in clinical conditions such as sepsis and obesity.

Our laboratory has recently shown in several studies that a cecal-ligation and puncture (CLP) murine model of polymicrobial sepsis impacts CD8 T cell-mediated immunity to systemic bacterial infection (63). Condotta et al. demonstrated that early after CLP injury, septic hosts had an impaired ability to mount an effective CD8 T cell response after systemic *L. monocytogenes* infection. These sepsis-induced changes were long lasting, since CD8 T

cell responses to bacterial challenge were still impaired months after the initial septic insult. Sepsis-stimulated inflammation induced rapid apoptosis of naïve CD8 T cell precursors leading to holes in the CD8 T cell compartment and rendering septic hosts unable to effectively mount primary CD8 T cell responses to pathogen-derived antigens (64). Our studies also examined the impact of sepsis on pathogen-specific memory CD8 T cell responses. Similar to what was observed in naïve CD8 T cells, Duong et al. found that sepsis induces apoptosis of pre-existing memory CD8 T cells (65). Since the level of CD8 T cell-mediated protection depends on the number of memory CD8 T cells present at the time of re-infection, sepsis-induced decline in memory CD8 T cell numbers leads to the decrease in protection after pathogen re-encounter. In addition to the numerical loss of memory CD8 T cells in septic mice, the functionality of pre-existing memory CD8 T cells was also significantly altered, a notion that helps further explain the extent to which sepsis impairs pre-existing CD8 T cell-mediated immunity.

It has been shown that memory CD8 T cells can be activated in an inflammation-dependent, antigen-independent bystander manner and produce the cytokine IFN γ (66). In some instances, this innate function of memory CD8 T cells can provide a measureable level of protection early after heterologous infection (67). Utilizing a virulent strain of *L. monocytogenes*, Duong et al. evaluated the ability of pre-existing memory CD8 T cells in septic hosts to sense inflammatory changes in the environment in an antigen-nonspecific manner. LCMV-immune mice containing memory GP33-specific TCR-Tg P14 CD8 T cells that experienced sepsis were infected with a high-dose of virulent *L. monocytogenes* that did not express the cognate LCMV-derived GP33 epitope. While memory P14 CD8 T cells in sham-control mice responded to the high-dose challenge and produced IFN γ in an antigen non-specific fashion, bystander responses by pre-existing memory P14 CD8 T cells in septic mice were significantly impaired (65). Overall, these data show that *L. monocytogenes* represents an invaluable tool used to precisely define the role of sepsis on naïve and memory CD8 T cell responses *in vivo*.

In addition to sepsis, another major public health problem in the United States and worldwide is the obesity epidemic. Recently, studies have demonstrated an association between obesity and increased susceptibility to infection and decreased vaccine efficacy (68). Given that memory CD8 T cells play a vital role in establishing protective immunity, we established a model of diet-induced obesity to determine the extent to which obesity compromises antigen-specific CD8 T cell responses and renders obese hosts susceptible to infection. Interestingly, we found that inbred and genetically diverse outbred hosts receiving a continuous high-fat diet were not impaired in their ability to evoke and maintain a primary *L. monocytogenes*-specific CD8 T cell response. We also found that obesity did not alter naïve-to-memory CD8 T cell differentiation after this systemic bacterial infection, suggesting the obese environment does not influence the development of pathogen-specific CD8 T cells and their ability to acquire long-term memory characteristics. Furthermore, obesity-associated alterations in the inflammatory milieu did not alter primary CD8 T cell expansion or influence the rate memory CD8 T cell differentiation after a peptide-coated dendritic cell immunization (69). Overall, infection with *L. monocytogenes* enabled

thorough analysis of CD8 T cell responses to determine the role of obesity in a host's ability to evoke and maintain a pathogen-specific memory CD8 T cell pool.

In our established models of sepsis and obesity, intravenous infection with *L. monocytogenes* has allowed us to investigate systemic CD8 T cell responses. However, it is difficult to study CD8 T cell responses after natural route of infection because mice are substantially resistant to oral *L. monocytogenes* infection, as the translocation of this pathogen across the intestinal barrier is low. In humans, the interaction between the host receptor E-cadherin on epithelial cells lining the intestinal mucosa and the surface protein internalin A on *L. monocytogenes* mediates bacterial entry. However, murine E-cadherins have a glutamic acid in place of a proline in the sixteenth amino acid, thus preventing recognition by internalin A proteins (70). This suggests that the nature of this amino acid dictates the permissiveness of E-cadherin receptors to internalin proteins. The lack of a small-animal model that mimicks human listeriosis was first addressed by the generation of a transgenic mouse that was genetically humanized. These transgenic mice expressed the human E-cadherin solely in enterocytes and were found to be highly susceptible to oral infection with *L. monocytogenes* (71). In another approach, a mutation in the internalin A protein of a recombinant *L. monocytogenes* bacterium facilitated invasion and entry into murine epithelial cells, thereby broadening host accessibility to mice (72). Recently, investigators utilized this recombinant strain to evaluate the robust generation of intestinal CD8 T cell responses (73). Overall, the existence of murine models in which *L. monocytogenes* infection recapitulates human disease will serve to broaden the analysis of CD8 T cells to include mucosal CD8 T cell responses.

Conclusion

The tools afforded by the *L. monocytogenes* model system have greatly contributed to the vast knowledge of systemic CD8 T cell responses and the factors that shape them. Intravenous infection with *L. monocytogenes* has proven invaluable in understanding phases of the CD8 T cell response, including naïve-to-memory CD8 T cell differentiation. In addition, the study of *L. monocytogenes*-evoked CD8 T cells has served as an effective method to determine the extent to which sepsis and obesity compromise memory CD8 T cell-mediated immunity. Continued research with this model pathogen will further our comprehension of host-pathogen relationships and contribute to our understanding of the biology of CD8 T cells.

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Figure 1. Generation of a memory CD8 T cell response after *L. monocytogenes* infection
After infection, a small number (10–1,000) of naïve antigen-(Ag) specific CD8 T cells are activated upon recognition of cognate Ag presented by mature dendritic cells (DC). Activation is followed by a phase of proliferative expansion, where the number of Ag-specific CD8 T cells increases by more than 10,000-fold, and differentiation into an effector population of cells. Effector CD8 T cells downregulate the expression of CD62L, which is uniformly high on naïve CD8 T cells, and acquire ability to kill pathogen-infected cells secrete cytokines (i.e. IFN γ and TNF α). A majority of the effector CD8 T cells generated during primary expansion are eliminated by apoptosis during the contraction phase, leaving behind surviving cells to initiate the early memory CD8 T cell pool. Memory CD8 T cells persist for long periods of time after infection and undergo time-dependent changes, like slowly reacquiring the expression of CD62L and gaining the ability to produce the cytokine IL-2 after Ag re-encounter. Memory CD8 T cells provide long-lasting protection to immune hosts by rapidly responding to reinfection.

Figure 2. Pathogen-specific CD8 T cells can be identified using a ‘surrogate activation marker’ approach

A) In contrast to naïve (Ag-inexperienced) CD8 T cells, pathogen-specific CD8 T cells upregulate expression of CD11a and downregulate expression of CD8α molecules after infection. B) Pathogen-specific CD11a^{hi} population of CD8 T cells display unique phenotypic changes, including downregulation of CD127 and CD62L and upregulation of KLRG1. Therefore, the CD11a^{hi}CD8α^{low} phenotype distinguishes pathogen-specific from naïve CD8 T cells after infection. This ‘surrogate activation marker’ approach represents an invaluable tool used to study polyclonal CD8 T cell responses to infection in the scenarios where *a priori* knowledge of antigen-specificity and/or MHC restriction is lacking.