11 Adaptive Immunity to *Listeria monocytogenes*

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11.1. Murine Infection with *Listeria monocytogenes* as a Model Intracellular Pathogen

Serious complications resulting from human infection with *Listeria monocytogenes* are usually limited to pregnant women, the very young or very old, or otherwise immunocompromised individuals (Schlech 2000). However, infection of experimental animals with *L. monocytogenes* serves as an extremely useful immunological tool because the bacteria are well characterized, easily manipulated, and infect virtually all mammals (Sixl et al. 1978). In particular, murine listeriosis has been used for many decades to dissect the fundamental components of innate and adaptive immunity to intracellular pathogens (Mackaness 1962; North et al. 1997; Unanue 1997a,b,c; Finelli et al. 1999; Harty et al. 2000).

In a naturally occurring infection, *L. monocytogenes* is introduced into the gastrointestinal tract after consumption of contaminated food products where it binds to, and is taken up by, epithelial cells via interaction of bacterial internalin A and E-cadherin on the host cells (Gaillard et al. 1991). In comparison to humans, mice exhibit markedly reduced susceptibility to intestinal infection with *L. monocytogenes* due to a single amino-acid difference in mouse E-cadherin (Lecuit et al. 2001); therefore, intravenous (i.v.) or intraperitoneal (i.p.) infection of mice is used in most experimental systems. Regardless of the route of infection (i.p or i.v.), administration of one of the many laboratory *L. monocytogenes* strains available results in a highly reproducible infection that can be easily quantitated by assaying bacterial load (colony forming units; CFUs) in the spleen and liver at various days postinfection (White et al. 1999; Messingham et al. 2003). Mortality is dependent on the strain of bacteria used, with LD_{50} ranging from $\sim 1 \times 10^4$ virulent bacteria to 1×10^9 for some attenuated strains (Bouwer et al. 1999; Messingham et al. 2003; Badovinac et al. 2005). Upon infection, the bacteria are taken up by splenic and hepatic (primarily) phagocytes where the majority are killed within the phagosomes; however, a small percentage of bacteria are able to escape destruction and invade the cytosol where the race between bacterial replication and priming of the immune response begins.

Early after infection (hours to days), a cascade of innate immune events ensues that are critical for host survival; either the infection is limited or death results from an inability to control bacterial spread. Early reduction of bacterial numbers is mediated by a cytokine-dependent (primarily IFN-γ and TNF) inflammatory response that results in recruitment of additional activated macrophages and neutrophils primed for bacterial destruction (Nickol and Bonventre 1977; Bancroft et al. 1991). The presence of viable bacteria within a cell results in release of bacterial products into surrounding tissues and production of chemokines that facilitate recruitment of activated phagocytes and their subsequent release of bacteriocidal reactive oxygen species (North 1970; Rogers and Unanue 1993; Conlan and North 1994; Shiloh et al. 1999; Serbina et al. 2003) (see Chap. 12).

If innate immunity can adequately control the level of infection, the elaboration of the slower adaptive immune response results in *L. monocytogenes*-specific $CD8^+$ T-cell-dependent clearance of remaining infected cells (Mackaness 1962; McGregor et al. 1970; Kaufmann 1988; Kaufmann and Ladel 1994a,b; Ladel et al. 1994). Although the adaptive response to *L. monocytogenes* is comprised of a several of cell types (MHC Class I and II restricted $CD8^+$ and $CD4^+$ T cells, respectively), responding to a variety of bacterial antigens, bacterial clearance in an infected mouse is dependent on MHC Class Ia-restricted $CD8^+$ T cells; capable of antigen (Ag)-specific recognition of infected cells. It is the presence of these *L. monocytogenes* Ag-specific memory $CD8^+$ T cells that confer lifelong resistance to subsequent high dose rechallenge (Kaufmann 1988).

11.2. Adaptive Immunity to *L. monocytogenes*

Innate immunity to *L. monocytogenes* serves an essential role in the early control of bacterial numbers, thereby allowing time for the antigen (Ag)-specific adaptive immune response to achieve sterilizing immunity. In the absence of the adaptive response, innate immune mechanisms are unable to effect complete bacterial clearance. This was most clearly demonstrated by the inability of mouse strains that possess innate defenses but lack both T cell and humoral immunity (severe combined immunodeficient (SCID) mice, nude mice) to clear infection, which invariably results in death (Bancroft et al. 1991; Nickol and Bonventre 1977). Additionally, humoral immunity does not appear to play a significant role in the clearance of *L. monocytogenes*; antibody responses are very weak and serum transfer from immune mice does not improve outcome of infected naïve mice (Mackaness 1962; Miki and Mackaness 1964; Edelson and Unanue 2000). Thus, primary sterilizing immunity and long-term protective immunity to *L. monocytogenes* are entirely mediated by listerial-specific T cells. In this chapter, we will discuss the major elements involved in the initiation, execution, and regulation of the T-cell-mediated response to *L. monocytogenes* in the laboratory mouse.

11.3. Initiation of T-Cell-Mediated Immunity to *L. monocytogenes*

The Ag-specific immune response to *L. monocytogenes* is comprised of distinct populations of T cells responding to bacterial antigens presented in the context of MHC Class Ia, MHC Class Ib, or MHC Class II molecules. Regardless of MHC restriction, the Ag-specific response must be initiated through encounter of naïve T-cell clones bearing a TCR specific for bacterial peptide/MHC complexes on the surface of an antigen-presenting cell (APC). By virtue of their high level of expression of a variety of co-stimulatory molecules, dendritic cells (DCs) are the most potent activators of naïve $CD4^+$ and $CD8^+$ T cells (Heath and Carbone 2001; Muraille et al. 2005). In elegant studies using transgenic mice expressing the diphtheria toxin receptor (DTR) primarily on DCs, temporary depletion at the time of infection demonstrated that the listeria-specific $CD8^+$ T-cell response in vivo is dependent on antigen presentation by DCs (Jung et al. 2002).

Dendritic cells (or other APCs) could acquire *L. monocytogenes* antigens by being directly infected with the bacteria or by phagocytosing other infected (live or dead) cells and presenting the processed antigen, a phenomenon termed "cross presentation" or "cross priming" (Heath and Carbone 2001). The unique ability of DCs to present exogenous antigens on either MHC Class I or Class II molecules bypasses the requirement for the DCs themselves to be infected by *L. monocytogenes*. It has not been established how frequently DCs are actually infected by *L. monocytogenes*, although it is likely to occur in vivo. Thus, it is probable that both cross presentation of exogenous listerial antigens and direct presentation of intracellular bacterial antigens contribute to T-cell priming during a primary response to *L. monocytogenes*.

11.3.1. Specificity of MHC Class Ia-Restricted Responses

Early after infection,*L. monocytogenes* is taken up by activated phagocytes and is able to gain access to the cytoplasm through listeriolysin O (LLO)-mediated escape from the phagosome. $CD8^+$ T cells recognize listerial peptides, of typically 8–10 amino acids in length, presented by MHC Class I molecules on the surface of APCs or infected cells (Busch and Pamer 1998). Infection with *L. monocytogenes* results in efficient priming of MHC Class I restricted $CD8^+$ T cells due to the presence of bacterial antigens within the cytosol of the APC, where efficient processing by the endogenous MHC Class I presentation pathway can produce antigen peptides from binding to MHC Class I (Pamer and Cresswell 1998). Due to the intracytoplasmic location of this pathogen, it is not surprising that MHC Class I restricted $CD8^+$ T cells comprise the majority of T cells responding to *L. monocytogenes* infection and, therefore, have been studied extensively in this model.

To be accessible for MHC Class I presentation in an infected cell, a bacterial protein must be secreted into the cytoplasm for degradation by

the proteasome, and resultant peptides must be transported to the golgi via the TAP transporter and loaded onto nascent MHC Class I molecules for expression on the cell surface (Germain 1994; Rock et al. 1994; Pamer and Cresswell 1998). In the case of *L. monocytogenes*, these proteins are virulence factors associated with phagosomal escape (LLO) (Kathariou et al. 1987; Portnoy et al. 1988; Pamer et al. 1991; Lety et al. 2001) or viability factors (p60) (Bubert et al. 1992; Wuenscher et al. 1993; Pamer 1994) that are essential for completion of the bacterial life cycle. Four major *L. monocytogenes* epitopes, presented by $H2-K^d$ MHC Class Ia molecules, have been identified in infected BALB/c ($H\text{-}2^d$) mice (see Table 11.1. for a list of the major *L. monocytogenes* epitopes). Simultaneous recognition of these bacterial proteins results in a reproducible hierarchy of immunodominant and subdominant $CD8^+$ T-cell

TABLE 11.1. *Listeria monocytogenes* T cell epitopes.

Epitope	Antigen	MHC restriction	AA sequence	Reference
Class Ia				
LLO_{91-99}	LLO	K^d	GYKDGNEYI	Pamer et al. (1991) and Pamer (1994)
LLO_{88-99}	LLO	$H2\text{-}K^d$	PRKGYKDGNEY	Geginat et al. (2001)
$p60_{217-225}$	p60	$H2\text{-}K^d$	KYGVSVQDI	Pamer (1994)
$P60_{449-457}$	p60	$H2\text{-}K^d$	IYVGNGQMI	Sijts et al. (1996)
$P60_{476-484}$	p60	$H2\text{-}K^d$	KYLVGFGRV	Geginat et al. (2001) and Skoberne et al. (2001)
Mpl_{84-92}	Mpl	$H2\text{-}K^d$	GYLTDNDQI	Busch et al. (1997)
Class Ib				
f-MIGWII(A)	LemA	H2-M3	f-MIGWII(A)	Lenz et al. (1996) and Princiotta et al. (1998)
f-MIVTLF	AttM	H2-M3	f-MIVTLF	Princiotta et al. (1998)
f-MIVIL	unknown	H2-M3	f-MIVIL	Gulden et al. (1996), Pamer et al. (1992), and Princiotta et al. (1998)
Class II				
$LLO_{215-234}$	LLO	$I\text{-}E^K$ ($I\text{-}A^K$)	SQLIAKFGTAF KAVNNSLNV	Safley et al. (1991)
$LLO_{190-201}$	LLO	$I\text{-}A^b$	NEKYAQ AYPNVS	Geginat et al. (2001)
$LLO_{354-371}$	LLO	$I\text{-}E^K$ ($I\text{-}A^K$)	DEVQIIDGLNG DLRDILK	Safley et al. (1991)
$P60_{301-312}$	p60	$I\text{-}A^d$	EAAKPAPAPSTN	Geginat et al. (1998, 1999)
$3A1.1_{132-148}$	Bacterial surface proteins	$I\text{-}A^K$	IVDDTIDDRDNV VSIGF	Sanderson et al. (1995) and Campbell and Shastri (1998)
12A4.G7	Bacterial surface proteins	$I\text{-}A^K$	DDAVIYPISYDN AVLALDSR	Campbell and Shastri (1998)

For additional Class Ia and Class II epitopes (Geginat et al. 2001).

responses (Sercarz et al. 1993; Vijh and Pamer 1997; Busch et al. 1998a,b), represented as a greater or lesser frequency of responding cells that can be identified by peptide-stimulated intracellular cytokine production (IFN-γ, TNF-α) (Badovinac and Harty 2000), MHC Class I tetramer reagents (Altman et al. 1996; Busch et al. 1998a; Busch and Pamer 1999), or ELISPOT (Vijh and Pamer 1997; Skoberne et al. 2001).

The $CD8^+$ T-cell response to *L. monocytogenes* is multiclonal, comprised of T cells specific for multiple peptide epitopes, and the frequency of cells responding to each epitope can differ dramatically. At the peak (~day 7–9 postinfection; p.i.) of the primary response to sub-lethal *L. monocytogenes* infection, roughly 2–3% of the total $CD8^+$ T-cell population is specific for known listerial antigens (Vijh and Pamer 1997; Busch et al. 1998a,b; Mercado et al. 2000; Badovinac and Harty 2002; Messingham et al. 2003). In BALB/c mice the epitope stimulating the highest frequency of responding cells derives from the LLO protein (residues 91–99), accounting for approximately 1.5–2% of all $CD8^+$ cells in the spleen at the peak of the primary response. The response to p60 (residues 217–225) represents ~0.5% of responding $CD8^+$ T cells. The LLO_{91-99} and $p60_{217-225}$ epitopes comprise the immunondominant responses to *L. monocytogenes* in BALB/c mice. Other subdominant epitopes include $p60_{449-457}$ (Vijh and Pamer 1997) and the metalloprotease peptide, mpl_{84-92} (Busch et al. 1997). These subdominant epitopes account for very few (0.05%) responding $CD8^+$ T cells. The evolution of the Ag-specific $CD8^+$ T-cell response is dependent on a variety of factors that influence bacterial peptide recognition, T-cell activation, and the magnitude of the response.

The factors that contribute to the relative immunodominance of one epitope over another are complex. The role of bacterial Ag secretion, rate of proteasomal degradation, efficiency of peptide loading onto MHC, and peptide/MHC stability have all been investigated (Pamer et al. 1997; Skoberne and Geginat 2002; Pamer 2004). It appears that each epitope, including those derived from the same peptide (p60), has unique properties (rate of initial peptide availability, peptide/MHC stability) that influence its ultimate availability for presentation on the surface of the APC (Sijts et al. 1996; Pamer et al. 1997). Kinetic analysis of Ag presentation by *L. monocytogenes*-infected cell lines or in vivo infected cells suggest that the presentation of each antigen is dynamic and the magnitude of the responding T-cell population is not dictated solely by the relative epitope abundance (Sijts et al. 1996; Pamer et al. 1997; Skoberne et al. 2001; Skoberne and Geginat 2002).

Independent of Ag presentation, the frequency of naive precursors displaying a TCR capable of binding a particular bacterial antigen/MHC Class I complex will influence the magnitude of the $CD8^+$ T-cell response. Although the number of available precursors of any given specificity is below our level of detection, estimates suggest that anywhere from 100's–1000 naïve precursors of each specificity exist in the spleen prior to infection (Bousso et al. 1998; Casrouge et al. 2000; Blattman et al. 2002). The overall magnitude of the *L. monocytogenes*-specific T-cell response is dictated by initial level of

infection and is most likely a reflection of the number of available precursors recruited to undergo division (Shen et al. 1998; Kaech and Ahmed 2001).

It is important to note that nonsecreted listerial antigens are also capable of priming $CD8^{+}$ T-cell responses. This probably occurs via a cross-priming mechanism after DC uptake of digested bacteria within dead or dying neutrophils (Tvinnereim et al. 2004). However, CTL specific for nonsecreted antigens do not confer protective immunity due to the limited presentation of nonsecreted antigens within viable infected cells (Shen et al. 1998; Zenewicz et al. 2002). In this scenario, the majority of infected cells would escape detection during an acute infection because $CD8^{+}$ T cells specific for nonsecreted antigens would only encounter their cognate antigen through cross-presentation by an APC.

11.3.2. Specificity of the MHC Class Ib-Restricted Responses

The MHC Class Ib molecules share many structural similarities with MHC Class Ia but are much more highly conserved resulting in limited diversity even among different mouse strains. The most clearly defined nonclassical MHC molecule in mice, H2-M3, is capable of presenting peptides that contain N-formyl methionine (f-Met) at the amino terminus, a property exclusive to bacterial and mitochondrial proteins (Pamer and Cresswell 1998). Murine infection with *L. monocytogenes* results in the presentation of three known peptides by H2-M3. The presented peptides are relatively short and are referred to by amino acid sequence; f-MVIL, f-MIGWII(A), f-MIVTLF (Gulden et al. 1996; Lenz et al. 1996; Pamer et al. 1992; Princiotta et al. 1998; Tawab et al. 2002). It appears that individual responding clones are cross reactive so that a single bacterially derived N-formylated peptide is capable of activating H2-M3 restricted cells of multiple specificities (Ploss et al. 2003). While promiscuous antigen recognition is common to the innate response, this property is so far exclusive to the H2-M3 restricted adaptive response to bacterial antigens. There is limited additional evidence that presentation of *L. monocytogenes* antigens by Qa-1b MHC molecules also contributes to antilisterial immunity (Bouwer et al. 1997).

11.3.3. Specificity of the MHC Class II-Restricted Responses

In addition to the robust responses of MHC Class I restricted $CD8^{+}$ T cells, infection with *L. monocytogenes* also induces strong activation of MHC Class II restricted $CD4^{+}$ T cells. Several MHC Class II restricted listerial epitopes, derived primarily from LLO (Safley et al. 1991; Sanderson et al. 1995; Campbell and Shastri 1998) and p60 (Geginat et al., 1998, 1999, 2001), have been identified (see Table 11.1.). Typically, MHC Class II restricted antigens are acquired by APCs through phagocytosis of extracellular bacteria; a key component in the control of bacterial spread after intraveinous infection with *L. monocytogenes*.

However, the rapid LLO-mediated escape of bacteria into the cytosol would limit the accessibility of bacterial antigens to the MHC Class II presentation. Rather, it is likely that naïve $CD4^+$ T cells are stimulated by *L. monocytogenes* antigens that are cross-presented on the surface of DCs (Skoberne and Geginat 2002).

11.4. Kinetics of the T-Cell Response to *L. monocytogenes*

11.4.1. MHC Class Ia-Restricted Responses

Upon activation, massive clonal expansion of *L. monocytogenes* epitope-specific $CD8^+$ T cells results in amplification of virtually undetectable levels of naïve cells of a given specificity to levels that are readily detectable (1–2% cells in the spleen) (Busch et al. 1998b). To achieve this expansion, Ag-specific $CD8^+$ T cells exhibit doubling times of 6–8 h/division (Blattman et al. 2002). Although responses to *L. monocytogenes* are multiclonal, $CD8^+$ T-cell populations specific for independent antigens undergo expansion with coordinate kinetics. Within 7–9 days after infection, the rapidly expanding $CD8^+$ T cells of differing specificities (i.e., LLO_{91-99}, $p60_{217-225}$) reach their numerical peak in unison (Busch et al. 1998b). This point marks the onset of the death or contraction phase of the response where $> 90\%$ of cells specific for each epitope die within 3–5 days and the remaining cells comprise the Ag-specific memory cell pool (see Figure 11.1.). This initial memory cell pool is maintained in number and function for the life of the host (Ku et al. 2000); through homeostatic proliferation mechanisms independent of antigen (Lau et al. 1994; Murali-Krishna et al. 1999; Wong and Pamer 2001; Jabbari and Harty 2005), but dependent on the presence of cytokines, such as IL-15 (Ku et al. 2000).

After *L. monocytogenes* infection, the exact timing of the transition from the expansion to contraction phase of the $CD8^+$ T-cell response is dependent on the strain of bacteria used; the peak response to virulent *L. monocytogenes* is slightly delayed (8–9 days p.i.) compared to attenuated strains (day 7 p.i.) (Pope et al. 2001; Badovinac and Harty 2002; Wong and Pamer 2003; Porter and Harty 2006). Using highly sensitive methods of Ag detection ("Direct Ex vivo Antigen Display (DEAD)" and "functional Ag display" assays), it was demonstrated that infection with virulent *L. monocytogenes* results in delayed peaks in the bacterial load and resultant Ag presentation compared to infection with attenuated (*actA*-deficient) *L. monocytogenes* (Wong and Pamer 2003; Porter and Harty 2006). In either case, the peak of functional Ag display was followed ~ 5 days later by the transition from expansion to contraction of $CD8^+$ T-cell numbers. Although the reason for the 5-day interval between peak Ag levels and the onset of $CD8^+$ T-cell contraction is unknown, it is possible that the peak of functional Ag display stimulates the highest relative number of precursors programmed to undergo a set number of divisions resulting in a synchronized peak (expansion to contraction transition). Alternatively, it may be that continued

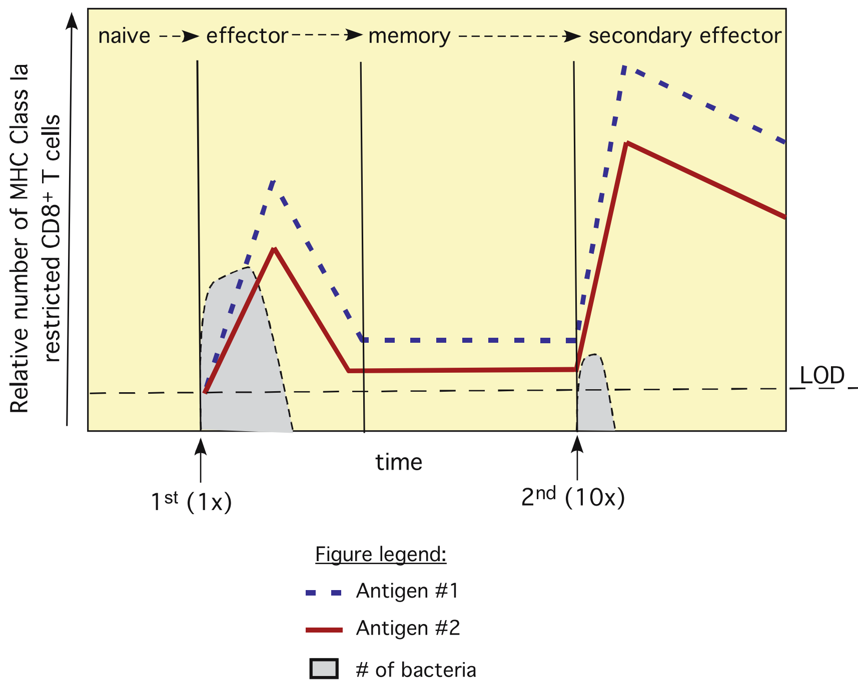

FIGURE 11.1. MHC Class Ia-restricted CD8$^+$ T cell response to *L. monocytogenes*. Total number of T cells specific for *L. monocytogenes*-derived antigens (antigens 1(*dashed*) and 2 (*solid*)). The coordinate expansion of Ag-specific CD8$^+$ T cells after infection (1×) is followed by a rapid and reproducible contraction phase leading to long-lived Ag-specific CD8$^+$ T cell memory. Reexposure to previously lethal doses of *L. monocytogenes* (10×) results in a more rapid and robust expansion resulting in Ag-specific CD8$^+$ T cell numbers in marked excess over the peak of the primary response. The responding Ag-specific CD8$^+$ T cells are capable of dramatically limiting bacterial burden. In comparison to primary CD8$^+$ T cell response, the contraction phase of the secondary response is delayed.

Ag interaction from the onset of infection to the peak of functional antigen display can influence the number of divisions achieved by responding CD8$^+$ T cells (Porter and Harty 2006). In this scenario, it is the loss or absence of Ag interaction shortly after peak Ag display that leads to conclusion of the "program" of expansion ~ 5 days later. Finally, both mechanisms may participate in the shaping of the CD8$^+$ T-cell response.

Depending on the type of infection, a relatively diverse responding TCR repertoire will become focused so that cells of the highest affinity comprise the majority of the Ag-specifc cells (Malherbe et al. 2004). In the case of murine listeriosis, the responding T-cell populations have relatively diverse TCR repertoire utilization throughout the primary response into memory (Busch et al. 1998a; Opferman et al. 1999). As will be discussed later, some focusing of the TCR repertoire does occur during the secondary response to *L. monocytogenes* (Busch et al. 1998a).

Memory T cells are a phenotypically and functionally heterogeneous population and it is unknown when cells with memory characteristics develop during the immune response. In recent years, attempts have been made at identifying memory cell precursors shortly after Ag exposure by virtue of expression of a variety of surface markers found on memory cells. No single marker of "memory cell precursors" has been identified; however, these studies have identified two populations of memory cells defined largely by their tissue distribution (Sallusto et al. 1999; Wherry et al. 2003; Jabbari and Harty 2006). Effector (T_{EM}) and central (T_{CM}) memory populations are defined by their relative expression of low or high levels, respectively, of surface homing molecules, such as CD62L and CCR7, that permit entry into lymph nodes. It has been hypothesized that T_{EM} serve a primary surveillance role based on their increased ability for target-cell killing. T_{CM} may serve the complimentary role as a reservoir of Ag-specific cells poised for expansion after reencountering cognate Ag.

Expression of the IL-7 receptor (CD127) has been investigated as an early marker of memory. IL-7 is a critical cytokine for the survival of T cells, and it has been suggested that expression of its receptor in a small fraction of Ag-specific $CD8^+$ T cells at the peak of expansion may mark the cells that will survive contraction to become memory (Kaech et al. 2003). In support of this, increased CD127 expression is observed in experimental systems where contraction is limited or absent (Badovinac et al. 2004). On the other hand, recent studies utilizing a variety of vaccination models show that Ag-specific cells expressing high levels of CD127 contract normally (Badovinac et al. 2005; Lacombe et al. 2005). Taken together, these studies suggest that CD127 expression is not the defining factor in the identification of cells that will survive contraction to become memory T cells.

11.4.2. MHC Class Ib-Restricted Responses

During primary infection with *L. monocytogenes*, H2-M3 restricted $CD8^+$ T cells go through expansion and contraction and reach stable memory levels in a manner similar to classically restricted T cells (Kerksiek et al. 1999, 2001). During their expansion phase, H2-M3-restricted $CD8^+$ T cells reach peak frequencies within the spleen at 5–6 days p.i., preceding the peak of classically restricted $CD8^+$ T cells on days 7–9 p.i. Contraction of the H2-M3-restricted response also occurs more rapidly; memory levels are attained within 2–3 days after the peak (Kerksiek et al. 1999, 2001). Due to the specificity of H2-M3-restricted $CD8^+$ T cells for antigens common to all bacterial species, and their early appearance after primary infection, it is thought that the primary contribution is to aid (or prolong) the innate responses' early control of bacterial numbers (Kerksiek et al. 1999, 2001; Hamilton et al. 2004)).

11.4.3. MHC Class II-Restricted Responses

Upon interaction with their cognate antigen in the context of MHC Class II and costimulation, $CD4^+$ T cells progress through similar kinetics of expansion and

contraction to memory levels as observed with MHC Class I-restricted CD8$^+$ T cells (Geginat et al. 2001; Skoberne and Geginat 2002; Corbin and Harty 2004). Likewise, the initiation of the CD4$^+$ T-cell response to *L. monocytogenes* appears to be regulated by initial antigen exposure (discussed in Programming of the T-cell response) (Corbin and Harty 2004). Although the precise role for CD4$^+$ T cells in the control of *L. monocytogenes* infection remains undefined, antigen recognition by these cells stimulates the production of copious quantities of Th1 cytokines that aid in bacterial clearance via activation of other cell types, including DCs and bacteriocidal macrophages (Hsieh et al. 1993; Bouwer et al. 1997).

Although there are many similarities in the CD4$^+$ and CD8$^+$ T-cell responses to *L. monocytogenes*, several differences also exist. In contrast to the stable number of memory CD8$^+$ T cells, *L. monocytogenes*-specific memory CD4$^+$ T-cell numbers decline over time (Schiemann et al. 2003). Additionally, secondary encounter with *L. monocytogenes* may result in selective expansion of high affinity CD4$^+$ T-cell clones resulting in repertoire focusing (Savage et al. 1999). Careful comparison of *L. monocytogenes*-specific CD4$^+$ and CD8$^+$ T-cell responses within the same host suggest that the elaboration of effector molecules is differentially regulated by Ag presence (Corbin and Harty 2005). It appears that while the production of cytokines (IFN-γ and TNF) by *L. monocytogenes*-specific CD8$^+$ T cells is rapidly down regulated in the absence of Ag, CD4$^+$ T cells will continue to proliferate and produce cytokines in response to persistent Ag exposure (Corbin and Harty 2005; Obst et al. 2005). An additional role for CD4$^+$ T cells in supporting the generation and maintenance of functional CD8$^+$ T-cell-mediated protective immunity will be discussed in the next section.

11.5. Programming of the T-Cell Response

Upon infection with *L. monocytogenes*, the rapid response of the innate immune system functions to limit infection until the slower adaptive immune response can develop. This coordinated effort typically results in pathogen clearance within a week of infection (Harty and Bevan 1995; Badovinac and Harty 2000). The peak of the MHC Class Ia-restricted CD8$^+$ T-cell response is coincident with pathogen clearance followed by rapid contraction of Ag-specific cell numbers. The timing of these events led to the hypothesis that the CD8$^+$ T-cell response was dependent on prolonged antigen presentation, and thus the presence of infection. Recently, reports have emerged that support the concept that only a brief exposure to antigen is necessary to initiate all phases of the CD8$^+$ T-cell response (Mercado et al. 2000; Kaech and Ahmed 2001; Badovinac et al. 2002). These studies utilized antibiotic treatment of mice at various times after *L. monocytogenes* infection to kill all viable bacteria by day 3 p.i. and thus limit functional antigen display. Within the spleens of antibiotic-treated mice, Ag-specific CD8$^+$ T cells did not decrease their rate or peak of expansion, or

the timing of contraction, when compared with mice that were not antibiotic treated (Mercado et al. 2000; Badovinac et al. 2002). However, antibiotic administration within 24 h of infection negatively influences the rate and magnitude of the anti-listerial $CD8^+$ T-cell response. Similar studies suggest that the kinetics and development of functional memory $CD4^+$ T cells responding to *L. monocytogenes* infection are also regulated by initial antigen exposure (Corbin and Harty 2004; Williams and Bevan 2004). These data suggest that events during the first few days of infection are critical for establishment of the maximal T-cell response to *L. monocytogenes*; once initiated, the kinetics and magnitude of the T-cell response are antigen independent.

In recent years, intense debate has focused on a role for $CD4^+$ T cells in the development of $CD8^+$ T-cell-mediated protective immunity. Previously, it was thought that because adoptive transfer of *L. monocytogenes*-specific $CD8^+$ T cells could protect naïve mice from high-dose bacterial challenge, $CD4^+$ T cells were dispensable in protective immunity. It was suggested that $CD4^+$ T cell help could contribute to protective immunity by stimulating a more robust expansion of Ag-specific $CD8^+$ T cells than could be realized by "unhelped" $CD8^+$ T cells (Bourgeois et al. 2002; Marzo et al. 2004). More detailed studies utilizing *L. monocytogenes* and other intracellular pathogens identify a possible role for $CD4^+$ T cells in the generation and maintenance of functional Ag-specific memory $CD8^+$ T cells. In these studies, the absence of $CD4^+$ T-cell stimulation, through either systemic depletion or absence of MHC Class II, resulted in a memory $CD8^+$ T-cell population that was not maintained long term (Shedlock et al. 2003; Sun and Bevan 2003). Surprisingly, this defect in the memory phase of the response occurred despite normal magnitude and kinetics of the primary response. Removal of $CD4^+$ T cells after $CD8^+$ T-cell priming did not effect the responding $CD8^+$ T-cell population. These findings suggest that $CD4^+$ T cell help plays a role in the initiation of the $CD8^+$ T-cell program, but are not required thereafter. In contrast, it has also been suggested that $CD4^+$ T cell help maintains functional $CD8^+$ T-cell memory and, therefore, is required during the memory phase of the response (Sun et al. 2004). The exact nature of the signal supplied by the $CD4^+$ T cells in these models is unclear. It may be that direct contact between $CD4^+$ and $CD8^+$ T cells (possibly through CD40/CD40L) (Bourgeois et al. 2002) is necessary for optimal $CD8^+$ T-cell memory; however, the requirement for CD40 ligation may depend on the type of infectious agent utilized (i.e., virus or intracellular bacteria) (Lee et al. 2003; Marzo et al. 2004).

11.6. Secondary Immunity to *L. monocytogenes*

11.6.1. MHC Class Ia-Restricted Responses

Protective immunity to *L. monocytogenes* is solely dependent on MHC Class Ia-restricted $CD8^+$ T cells. Re-exposure to *L. monocytogenes* results in a rapid mobilization of Ag-specific cells from memory $CD8^+$ T-cell pool,

resulting in efficient clearance of challenge doses that are lethal for naïve mice (Lalvani et al. 1997). *L. monocytogenes*-specific memory $CD8^+$ T cells are increased in frequency and number over their naïve precursors, and possess increased capacity for proliferation and elaboration of effector functions in response to antigen levels that are reduced in comparison to a primary infection (Ahmed and Gray 1996). This increased ability to respond to secondary infection is also less dependent on the costimulaory molecules required for priming antigen-specific naïve T cells (Iezzi et al. 1998); however, it is likely that maximal responses are attained in the presence of robust costimulation supplied by DCs (Zammit et al. 2005). Together, unique qualities of memory $CD8^+$ T cells are responsible for very rapid recognition of, and response to, *L. monocytogenes* antigens resulting in the rapid and complete elimination of all infected cells.

After *L. monocytogenes* challenge, Ag-specific memory $CD8^+$ T cells reach their numerical peak approximately 5–6 days p.i. The elevated frequency of Ag-specific precursors in the memory pool, combined with their increased sensitivity for activation, results in secondary expansion to peak numbers in marked excess (10 to 50-fold; Badovinac et al. 2003, 2005) over primary memory numbers. The rapid expansion memory $CD8^+$ T cells exerts a negative influence on the expansion of naïve precursors so that the magnitude of the memory response is inversely proportional to the population of newly recruited cells (Badovinac et al. 2003). This is likely due to more rapid elimination of matured APCs, required for activation of naïve cells, by responding memory $CD8^+$ T cells (Wong and Pamer 2003; Yang et al. 2006). In comparison to the primary response to *L. monocytogenes*, the contraction of the secondary response is markedly protracted (see Figure 11.1.) (Badovinac et al. 2003).

During the secondary $CD8^+$ T-cell response to *L. monocytogenes*, a modest narrowing of the TCR repertoire occurs that is likely due to preferential activation of clones bearing TCRs with higher affinity for antigen (Busch et al. 1998a; Busch and Pamer 1999; Pamer 2004). Although the mechanism responsible for this preferential activation is not entirely known, it could result from a limited availability of antigen due to a very rapid clearance of bacteria after re-exposure.

Bacterial challenge in an *L. monocytogenes* immune mouse ultimately generates a new pool of secondary memory $CD8^+$ T cells. In comparison to primary memory cells, secondary memory $CD8^+$ T cells are more effective at reducing bacterial numbers and exhibit increased capacity for Ag-specific target cell killing and production of associated effector molecules (Jabbari and Harty 2006). Phenotypically, secondary memory $CD8^+$ T cells are slower to re-express hallmarks of memory, such as CD62L expression and IL-2 production, in response to Ag-stimulation. This difference in primary and secondary memory characteristics is associated with decreased expression of the receptor for IL-15, a cytokine known to be critical for the proliferation-driven maintenance of the memory cell pool. In these studies, increased proliferation of memory $CD8^+$ T cells resulted in increased expression of CD62L suggesting that memory cell phenotype and function are dynamic and specific to host environment.

11.6.2. MHC Class Ib-Restricted Responses

In contrast to the explosive expansion of MHC Class Ia-restricted CD8$^+$ T cells upon secondary infection with *L. monocytogenes*, the H2-M3-restricted memory CD8$^+$ T cells fail to undergo significant expansion, despite similar acquisition of the phenotypic markers of activation on both cell populations (Kerksiek et al. 2003). This finding led to the hypothesis that MHC Class Ib-restricted cells were functionally incapable of secondary expansion and, therefore, could not contribute to protective immunity (Urdahl et al. 2002; Kerksiek et al. 2003). In contrast, MHC Class Ib-restricted primary effector cells can contribute to protective immunity to *L. monocytogenes*, but only in the absence of an MHC Class Ia-restricted response (Kaufmann et al. 1988; Lukacs and Kurlander 1989; Seaman et al. 2000; D'Orazio et al. 2003). This is most simply demonstrated by the discovery that adoptive transfer of either effector or memory *L. monocytogenes*-specific CD8$^+$ T cells from MHC Class Ia-deficient hosts protect naïve mice from *L. monocytogenes* infection (Seaman et al. 2000). In WT mice undergoing a secondary response to *L. monocytogenes*, expansion of H2-M3-restricted memory CD8$^+$ T cells is suppressed by the memory Class Ia-restricted memory responses (Hamilton et al. 2004; Ploss et al. 2005). However, if primary responses are elicited using DC-peptide immunization for MHC Class Ib antigens, in the absence of MHC Class Ia responses, the H2-M3-restricted cells are capable of secondary expansion when challenged with LM, but do not contribute to protective immunity (Hamilton et al. 2004). Thus, MHC Class Ia-restricted memory responses limit expansion of an ineffective MHC Class Ib-restricted memory cell population. The precise mechanism of suppression remains unclear, but it appears that factors, possibly linked to the site of infection, such as antigen load, cytokine milieu, etc., may be involved.

11.6.3. MHC Class II-Restricted Responses

Because protective immunity to *L. monocytogenes* is dependent on MHC Class Ia-restriced CD 8$^+$ T cells, Ag-specific CD4$^+$ T cells have not been studied as extensively in this infection model. However, *L. monocytogenes*-specific CD4$^+$ T cells are capable of providing protective immunity to naïve mice independent of CD8$^+$ T cells, although to a lesser extent (Bishop and Hinrichs 1987; Czuprynski and Brown 1987; Lukacs and Kurlander 1989; Harty et al. 1992; Harty and Bevan 1996; Geginat et al. 1998). As mentioned previously, CD4$^+$ T-cell-mediated clearance of infected cells is dependent on Ag-specific cytokine-driven phagocyte recruitment and subsequent nonspecific target-cell killing.

11.7. CD8$^+$ T-Cell Effector Mechanisms

During expansion, activated T cells differentiate into effector populations, which leave the secondary lymphoid organs in search of *L. monocytogenes*-infected

cells. Upon recognition of an infected cell, activated effector cells will produce cytokines known to recruit and/or activate microbiocidal effector cells, such as macrophages and neutrophils, and up-regulate molecules necessary for target cell lysis (Harty and Bevan 1999). Both expression of cytokine molecules and the execution of cytolytic effector mechansims by T cells are tightly regulated through TCR-dependent signals. Given that virtually every T-cell effector function is also employed by other cells (NK cells, macrophages, DC, etc.) of the immune system, understanding which mechanisms are important for cell-mediated resistance to infection is a substantial challenge. To this end, gene knockout mice, lacking specific T-cell effector molecules, have become important tools in addressing the biology of the adaptive response to *L. monocytogenes*. Although protective immunity to *L. monocytogenes* in WT mice is dependent on MHC Class Ia-restricted $CD8^+$ T cells, examination of gene knockout mice has not identified a single effector mechanism that is absolutely required for protective immunity.

11.7.1. IFN-γ

IFN-γ plays a central role in innate resistance to *L. monocytogenes* infection through the activation of phagocytic cells and resultant reduction in bacterial cell numbers. The absence of IFN-γ from the host mouse, through either antibody depletion or inactivation of the IFN-γ gene (GKO mice) or its receptor, results in a nearly 1000-fold decrease in the LD_{50} of *L. monocytogenes* in naïve mice (Buchmeier and Schreiber 1985; Huang et al. 1993; Harty and Bevan 1995). These findings clearly demonstrate the requirement for IFN-γ in the innate response to *L. monocytogenes*. However, infection of GKO mice with an attenuated strain of *L. monocytogenes* (*actA*-deficient) that renders the bacteria deficient in cell-to-cell spread (Kocks et al. 1992) is tolerated similar to WT mice and results in efficient $CD8^+$ T-cell priming and long-lasting protective immunity to high-dose challenge. Once vaccinated, GKO demonstrate ~20,000-fold increase in resistance to virulent *L. monocytogenes* (Harty and Bevan 1995) and exhibit increased memory $CD8^+$ T-cell numbers due to protracted contraction (discussed below) in comparison to similarly vaccinated WT mice (Badovinac et al. 2000). These findings indicate that IFN-γ is not required for the development of functional protective immunity to *L. monocytogenes*. Upon infection, both $CD8^+$ and $CD4^+$ T cells produce IFN-γ in an antigen-specific manner (Hamilton et al. 2004; Corbin and Harty 2005). Whether the production of IFN-γ by *L. monocytogenes*-specific $CD8^+$ T cells contributes to, or improves, protective immunity is currently under investigation.

In addition to initial control of bacterial numbers, IFN-γ serves as a multi-potent regulator of Ag-specific $CD8^+$ T-cell homeostasis (Harty and White 1999; Harty and Badovinac 2002). It is well known that IFN-γ can enhance $CD8^+$ T-cell expansion by increasing the ability of APCs to process and present antigens to T cells (Fruh and Yang 1999). However, IFN-γ can

also serve as a negative regulator of MHC Class I-restricted $CD8^+$ T-cell expansion in an epitope-specific fashion due to differential processing and presentation of the antigenic peptides (Skoberne and Geginat 2002). Infection of IFN-γ-deficient mice with attenuated *L, monocytogenes* results in $CD8^+$ T-cell response with an altered immunodominance hierarchy when compared to WT mice (Badovinac et al. 2000). Specifically, increased expansion of the $CD8^+$ T cells specific for the subdominant $p60_{217-225}$ epitope, relative to those specific for the LLO_{91-99}, was observed in IFN-γ-deficient mice. Thus, IFN-γ serves as a key regulator of immunodominance after infection.

The IFN-γ exerts its most potent effect on $CD8^+$ T-cell homeostasis through regulation of the contraction phase of the response to *L. monocytogenes* infection (Badovinac et al. 2000). After infection with attenuated *L. monocytogenes*, Ag-specific $CD8^+$ T cells from both WT and IFN-γ-deficient mice reach peak numbers on approximately day 7 p.i. followed by a rapid contraction, where the majority of responding cells are eliminated from the spleen of WT, but not GKO, mice by day 10–11 p.i. Elevated Ag-specific $CD8^+$ T-cell numbers persist in the spleen of IFN-γ deficient mice indefinitely, resulting in increased levels of *L. monocytogenes*-specific memory in GKO mice. Vaccinated IFN-γ deficient mice display a three- to –six-fold increase in the number of Ag-specific $CD8^+$ T cells in their spleens that is associated with increased protective immunity to high level challenge with virulent *L. monocytogenes* when compared to similarly vaccinated WT mice. A careful comparison of the per-cell protective capacity of Ag-specific memory $CD8^+$ T cells from WT and GKO mice will determine if the increased resistance to virulent *L. monocytogenes* results purely from the elevated memory levels in vaccinated GKO mice or if IFN-γ-deficient memory $CD8^+$ T cells are intrinsically different from WT. In addition, recent experiments also show that Ag-specific $CD4^+$ T cells do not contract in the absence of IFN-γ (Haring and Harty 2006).

11.7.2. TNF-α

Antigen-stimulated $CD4^+$ and $CD8^+$ T cells produce TNF, a cytokine that also plays an important role in the innate immune response to *L. monocytogenes* infection (Havell 1987; Nakane et al. 1988; Bancroft et al. 1989). Mice lacking the TNF-α gene (Rothe et al. 1993) or receptor (Endres et al. 1997) cannot survive primary infection with virulent *L. monocytogenes*. However, similar to IFN-γ, TNF is not required for the development of a protective $CD8^+$ T-cell response (White et al. 2000).

There are three possible mechanisms by which Ag-specific production of TNF could contribute to bacterial resistance; (1) through stimulation of bacteriocidal macrophages (Endres et al. 1997); (2) induction of apoptosis in target cells through receptor (TNFR1) ligation (Ashkenazi and Dixit 1998); and/or (3) stimulation/recruitment of phagocytes through increased adhesion molecule expression (Lukacs et al. 1994; Henninger et al. 1997; Kondo S and Sauder 1997). Adoptive transfer experiments utilizing *L. monocytogenes* memory $CD8^+$ T cells deficient

in other modes (perforin/FAS) of cytolysis found that Ag-specific production of TNF can contribute to protective immunity (White and Harty 1998); however, the cellular source of TNF, and the nature of its contribution to anti-listerial immunity in a WT mouse, is unclear.

11.7.3. Cytolytic Effector Mechanisms: Granule Exocytosis and Receptor-Mediated Pathways

The presence of *L. monocytogenes* peptide–MHC complexes on the surface of infected cells serves as a beacon identifying targets for destruction by $CD8^+$ T cells. Ag-specific target cell lysis is a function largely limited to $CD8^+$ T cells. TCR recognition of an infected cell through binding cognate peptide–MHC complexes results in the directed exocytosis of cytotoxic granules containing perforin and granzymes that breach the target cell membrane (perforin) and activate the caspase cascade (granzymes) leading to apoptosis (Lieberman 2003). Regarding primary infection with *L. monocytogenes*, the contribution of perforin to control of bacterial numbers is marginal; perforin-deficient mice exhibit resistance (LD_{50}) similar to WT mice despite delayed bacterial clearance in the spleens, but not livers, of infected mice (Kagi et al. 1994; White et al. 1999).

In contrast to primary immunity, perforin-mediated cytotoxicity is critical for optimal protective immunity to virulent *L. monocytogenes* (Kagi et al. 1994; Messingham et al. 2003). Compared with WT mice, *L. monocytogenes*-vaccinated perforin-deficient (PKO) mice have elevated levels (two- to four-fold) of $CD8^+$ T-cell memory but exhibit reduced levels of protection against virulent *L. monocytogenes*. Although the existence of functional protective immunity to *L. monocytogenes* in vaccinated PKO mice underscores the existence of perforin-independent pathways for $CD8^+$ T-cell immunity to *L. monocytogenes*, perforin-deficient memory $CD8^+$ T cells display a fivefold reduction in the per-cell protective capacity when compared to WT cells five-fold (Messingham et al. 2003). Therefore, in some cases, increased levels of $CD8^+$ T-cell memory can compensate for the absence of an important effector molecule.

In recent years, a role for perforin in the regulation of the expansion phase of MHC Class I-restricted $CD8^+$ T-cell homeostasis has emerged (Harty and White 1999; Harty and Badovinac 2002). In an allogenic cell transfer model, where antigen load is identical perforin deficient $CD8^+$, but not $CD4^+$, T cells expand significantly more after antigen stimulation resulting in higher numbers at the peak of the response. This enhanced expansion of perforin-deficient cells appears to be the result of reduced $CD8^+$ T-cell death (AICD), rather than altered persistence of APC (Spaner et al. 1999; Ludewig et al. 2001). After *L. monocytogenes* infection of perforin-deficient mice, both MHC Class I (LLO_{91-99}, $p60_{217-225}$, $p60_{444-459}$) – and H2-M3 (f-MIGWII(A))-restricted $CD8^+$ T cells show three- to fourfold higher expansion despite similar rates of clearance of an attenuated strain of *L. monocytogenes* (Badovinac and Harty 2000; Messingham et al. 2003). Importantly, the contraction phase of the

response after *L. monocytogenes* infection of perforin-deficient mice was normal (Badovinac and Harty 2000).

It also appears that the extent to which perforin influences $CD8^+$ T-cell expansion can be modulated by the host environment. For example, enhanced $CD8^+$ T-cell expansion is not observed in perforin-deficient mice ($H\text{-}2^d$) after primary infection with virulent *L. monocytogenes*, where deficient mice exhibit substantially delayed clearance in the spleen. However, during secondary infection, when clearance kinetics are virtually identical, $CD8^+$ T-cell expansion in perforin-deficient mice is increased (White et al. 1999). Ultimately, the increased expansion paired with a normal contraction phase has the net of effect of increasing the absolute number of functional Ag-specific memory cells and, in turn, increased resistance to *L. monocytogenes* rechallenge.

It should be noted that activated $CD8^+$ T cells also express CD95 ligand which can also activate the caspase cascade through ligation of its receptor (CD95/Fas) on the target cell. Normally, this pathway likely results in the elimination of self-reactive T cells that are repeatedly activated (AICD) (Van Parijs et al. 1998), and does not appear to make a significant contribution to the anti-listerial immunity in the presence or absence of perforin (White and Harty 1998). These findings also demonstrate functional T-cell-mediated immunity in the complete absence of the major pathways of cytolysis.

11.8. Conclusion

For many decades, murine listeriosis has been utilized as a highly reproducible model to safely study both innate and adaptive immune responses. Although T cells are not required for resistance to primary infection with *L. monocytogenes*, the identification of defined peptide epitopes derived from *L. monocytogenes* has allowed immunologists to utilize this infection model to dissect many aspects of the antigen-specific T-cell response to intracellular pathogens. The relative ease by which the bacterium can be manipulated to decrease virulence or express a plethora of exogenous T-cell epitopes has allowed this model to be used as a vehicle for the study of a wide variety of other pathogens. In this way, infection with *L. monocytogenes* also serves as a prototypical candidate for vaccine development for humans. In addition, human studies support the potential utility and relative safety of attenuated *L. monocytogenes* as a vaccine delivery vector (Angelakopoulos et al. 2002). As our knowledge base expands and the tools available improve, experimental infection with *L. monocytogenes* will no doubt remain a valuable system for the investigation of all aspects of immunity.

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