IMMUNITY TO *LISTERIA MONOCYTOGENES*: THE INFLUENCE OF CD40 AND THE $CD4^+$ T-CELL SUBSET ON THE DEVELOPMENT AND MAINTENANCE OF $CD8^+$ T-CELL MEMORY

by
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ABSTRACT

The interplay between antigen-presenting cells (APC), $CD4^+$ T cells and $CD8^+$ T cells leading to the activation and differentiation of naïve $CD8^+$ T cells to form a protective memory population has been extensively studied, yet further understanding of how these cells interact is required. At the cellular level, the initial activation of $CD8^+$ T cells is dependent on antigen presentation by professional antigen presenting cells (APC) bearing costimulatory molecules. This ability to activate cytolytic effector $CD8^+$ T cells is regulated by the maturation state of the APC. One key interaction that triggers the maturation of APC to a state capable of activating naive $CD8^+$ T-cells is the ligation of the CD40 receptor on the immature APC. This is achieved by CD40 ligand expression on activated $CD4^+$ T cells.

CD40-CD40L interactions are not required for the induction of all $CD8^+$ T-cell responses. For some pathogens, the stimuli provided by the infection itself bypasses the need for CD40-mediated $CD4^+$ T-cell help. It has not been established whether the cellular immune response that develops in the absence of CD40-CD40L interactions is functionally equivalent to the response established by APC that have matured through CD40 ligation. Further, there is evidence suggesting that memory $CD8^+$ T-cell populations generated in $CD4^+$ T-cell deficient environments are functionally defective. As a subset of $CD8^+$ T cells transiently express CD40 following activation, direct $CD4^+$-$CD8^+$ T-cell interactions through CD40 may provide a critical signal that directs the functional quality of memory $CD8^+$ T cells.

I have evaluated both primary and memory $CD8^+$ T-cell responses following systemic *L. monocytogenes* infection in wildtype and $CD40^{-/-}$ BALB/c mice. These experiments revealed that, in the context of *Listeria* infection, the absence of CD40 expression had very few consequences on the functional properties of $CD8^+$ T-cell populations, indicating that direct CD40 ligation on $CD8^+$ T cells is not a global requirement for memory $CD8^+$ T-cell development. Further, transient depletion of $CD4^+$ T-cell populations prior to primary *L. monocytogenes* infection indicated no need for $CD4^+$ T cells to be present at the time of $CD8^+$ T-cell priming to "imprint" the development of protective $CD8^+$ T-cell memory.

Additionally, $CD40^{-/-}$ mice showed enhanced $CD8^+$ T-cell expansion to a secondary *Listeria* challenge that correlated with their reduced $CD4^+$ $CD25^+$ regulatory T-cell population. *In vivo* depletion of $CD4^+$ cells prior to secondary *Listeria* infection resulted in enhanced $CD8^+$ T-cell responses in wildtype mice, but did not further enhance responses in $CD40^{-/-}$ mice. Adoptive transfer of $CD8^+$ T cells from *Listeria*-immune donors into various recipients indicated that cells within the $CD4^+$ cell population influence the magnitude of secondary $CD8^+$ T-cell expansion to antigen. Adoptive transfer of increasing numbers of donor cells suggested that regulatory T cells within the $CD4^+$ subset primarily exert their function as high numbers of antigen-specific $CD8^+$ T cells are achieved. Collectively, these results suggest that $CD4^+$ $CD25^+$ regulatory T cells may function to maintain a balance between pathogen clearance and the immunopathologic consequences of large numbers of effector T-cell populations.

CHAPTER 1: INTRODUCTION

Over 200 years ago Edward Jenner discovered that vaccination could successfully reduce the morbidity and mortality caused by infectious disease. Vaccination is an effective strategy because it provides a strong stimulus to the immune system that results in the development of "immunologic memory" to components within the vaccine. Such memory is defined functionally by the ability of an individual to respond more quickly and more effectively upon re-exposure to that initial vaccine stimulus. In the case of infection by pathogens, immunologic memory develops as part of the response to infection and results in either a significant decrease in severity, or the complete prevention of disease following reinfection. Thus, the immune system can learn from its experiences, and recovery from most infections is accompanied by an ability to resist subsequent infections with the same pathogen. This acquired attribute of immunologic memory is an important hallmark of both humoral and cellular adaptive immune responses.

Although many of the mechanisms that contribute to the generation of immunological memory have been elucidated, there remain additional aspects of cellular immune memory that require a better understanding. This is underscored by the demonstration that many pathogens have evolved strategies to evade components of the immune response designed to target pathogens. For viruses and many bacteria, the approach used to evade the humoral components of the cellular immune response is simply to limit their extracellular lifecycle and establish themselves inside host cells. In order to target this intracellular site of infection that is protected from the humoral

immune response, the cellular arm of the adaptive immune response is required for eradication of intracellular pathogens.

One cellular arm of the adaptive immune response responsible for the elimination of intracellular pathogens is associated with a subpopulation of T lymphocytes that express the CD8 cell-surface antigen. This specialized T-cell subset is responsible for host defense against numerous intracellular infectious agents, including the bacterium *Listeria monocytogenes*. $CD8^+$ T cells mediate immunologic protection against intracellular bacteria and viruses through the secretion of effector cytokines including IFNγ and TNFα. In addition, $CD8^+$ T cells can kill infected cells directly through their ability to ligate cell surface death receptors, as well as through the release of perforin and granzyme proteins.

As a consequence of an initial infection with pathogens such as *L. monocytogenes*, the immune system is stimulated, resulting in the activation of antigen-specific $CD8^+$ T-cell populations. Many of the individual events that lead to the development of effector $CD8^+$ T cells have been described. However, our knowledge of the required stimuli is incomplete, and we remain limited in our ability to manipulate the immune response for vaccine development and therapeutic applications. In this regard, some would suggest that even today, as in the time of Jenner, strong cellular immune responses are established only following infection. Nonviable or subunit vaccines remain of limited value for the generation of specific cellular immunity.

The development of a cellular immune response to an intracellular infection involves recognition of and stimulation by components of the pathogen itself. In addition, the innate inflammatory cascade is triggered following infection. Thus both pathogen-

derived stimuli as well as endogenous cytokines released in response to infection can signal and stimulate the resting immune system into action. It is not clear if different activation signals provide distinct messages to direct the type or strength of an immune response. Alternatively, both pathogen-derived and endogenous inflammatory signals may functionally overlap, resulting in the development of similar adaptive immune responses regardless of which stimuli activate the immune system.

At the cellular level, the initial activation of $CD8^+$ T cells is dependent on the presentation of specific antigen by professional antigen presenting cells (APC) bearing costimulatory molecules. The ability of APC to activate cytolytic effector $CD8^+$ T cells is regulated by the maturation state of the APC. One key interaction that triggers the maturation of APC to a state capable of activating naive $CD8^+$ T-cells is the ligation of the CD40 receptor on the immature APC. This is achieved by CD40 ligand expression on activated $CD4^+$ T cells. This three-cell interaction is thought to be sequential, such that "T-cell help" in the form of CD40 ligation is provided through the initial $CD4^+$ T cell-APC interaction, leading to APC maturation. In turn, subsequent interactions between APC and $CD8^+$ T cells are able to support $CD8^+$ T-cell priming.

However, CD40-CD40L interactions are not required for the induction of all $CD8^+$ T-cell responses. For some pathogens, the stimuli provided by the infection itself bypasses the need for CD40-mediated $CD4^+$ T-cell help. It has not been established whether the cellular immune response that develops in the absence of CD40-CD40L interactions is equivalent in function to the response established by dendritic cells that have matured through CD40 ligation.

In this dissertation I will present an analysis of both primary and memory $CD8^+$ T-cell responses following systemic *L. monocytogenes* infection in wildtype and CD40-deficient BALB/c mice. With these experiments I have evaluated whether the absence of CD40 expression influences functional properties of $CD8^+$ T-cell responses generated in response to infection with *L. monocytogenes*. Further, I have investigated the effects of transient depletion of $CD4^+$ T-cell populations prior to either primary or secondary *L. monocytogenes* infection on the antilisterial $CD8^+$ T-cell response.

1.1 The Immunobiology of $CD8^+$ T cells

Following infection, $CD8^+$ T lymphocytes (CTL) are responsible for host defense against numerous viral and intracellular bacterial infections. In this regard, responding $CD8^+$ T cells become the armed sentinels of the immune system. Upon their activation, following recognition of a specific pathogen-derived peptide antigen presented by class I major histocompatability molecules (MHC-I), effector $CD8^+$ T cells are able to enter peripheral tissues and destroy cells displaying cognate antigen. After a period of time, the majority of the effector $CD8^+$ T-cell population dies, and the immune system returns to homeostatic balance. However, a small population of "experienced" cells is maintained to serve as a memory population to be rapidly recruited again in the event that the host is re-exposed to this specific pathogen. In general, the ability of memory T cell populations to provide enhanced protection against reinfection has been attributed to their ability to respond to lower concentrations of antigen (1), lower dependency on costimulatory signals (2), and the ability to proliferate earlier (3), divide faster (3, 4), and acquire effector functions more rapidly than naive T cells (4, 5).

CD8+ T-cell activation

The activation of naïve $CD8^+$ T cells into effector CTL has been characterized as a two-signal process. The first signal requires T-cell receptor (TCR) recognition of a peptide antigen presented in the context of class I major histocompatability complex (MHC-I) on the surface of an antigen-presenting cell (APC). In addition to the TCR-peptide: MHC-I interaction, the priming of naïve $CD8^+$ T cells requires a second signal provided by the interaction of costimulatory molecules; including CD80 or CD86 on the APC with CD28 on the T cell. Providing both signals to naive T cells in a timely manner is the unique job of professional APC. TCR stimulation (signal 1) in the absence of costimulation (signal 2) leads to tolerance or functional anergy in T cells rather than activation (6).

Following their initial stimulation and activation, $CD8^+$ T cells undergo a program of dramatic proliferation. Following 2 to 24 hours of *in vitro* TCR stimulation, naive $CD8^+$ T cells commit to cellular expansion, regardless of the presence or absence of continued TCR stimulation (7-9). This programmed proliferative response of naive $CD8^+$ T cells is also evident following *in vivo* infection. Following acute infection with lymphocytic choriomeningitis virus (LCMV), a single $CD8^+$ T cell can undergo up to 15 cellular divisions within the first 7 days, generating up to 10^4 progeny (10, 11). Remarkably, at the height of this primary proliferative response, activated cells can divide every 6-8 hours (10).

The continued presence of antigen during the primary expansion phase of CD8+ T-cells does not appear to further influence the effector function or the generation of immunologic memory after the initial stimulation. Antibiotic treatment of mice infected

with *L. monocytogenes* at early timepoints after infection has revealed that a prolonged period of active infection is not required for efficient activation of $CD8^+$ T cells. Animals treated with antibiotics at 24 hours post-infection undergo primary $CD8^+$ T-cell expansion with all response parameters comparable to untreated mice (12). These experiments suggest that $CD8^+$ T-cell expansion becomes "programmed" very early following T-cell activation, leading to the generation of effector CTL populations armed to confront and clear the invading pathogen.

Contraction of effector $CD8^+$ T cells

Following T-cell activation and clonal expansion, activated $CD8^+$ effector T cells enter into a contraction phase, which results in a reduction of their number to approximately 5-10% of the peak numerical level achieved at the height of the primary proliferative phase (10, 13, 14). The onset of contraction is independent of pathogen clearance and appears to be a programmed component of the response (13). Molecular profiling analysis of mRNA expression levels in $CD8^+$ T-cell populations following acute LCMV infection suggests that the transition of cells from the effector phase into resting memory populations is a gradual process that occurs over a period of 30-45 days following acute infection (15). The fraction of $CD8^+$ T cells that survive the contraction phase are maintained as a memory population, capable of quickly responding to a secondary exposure to cognate antigen. Once established, the number of resting memory $CD8^+$ T cells is stably maintained for long periods; at least one year in mice (10). This program of expansion and contraction allows the immune system to maintain a

homeostatic balance following each independent infection, while maintaining a subset of protective, antigen-experienced, memory $CD8^+$ T cells.

Cytokines and memory $CD8^+$ T-cell generation and maintenance

Cytokines are crucial for the expansion, contraction and maintenance of $CD8^+$ T-cell populations. Three cytokines in particular, Interleukin (IL)-2, IL-7, and IL-15, signal through a common γ-chain and have profound effects on $CD8^+$ T-cell responses.

IL-2 is a growth factor that enhances the expansion of naive $CD8^+$ T cells following antigen stimulation. Although IL-2 has been detected in activated DC, the majority of IL-2 produced comes from activated $CD4^+$ and $CD8^+$ T cells (16, 17). Early *in vivo* proliferation of $CD8^+$ T cells following activation is not dependent on IL-2, however continued expansion after the first 3-4 days requires IL-2 (18, 19). Exogenous IL-2 administration during the contraction phase results in a prolonged proliferation of $CD8^+$ T cells and increased survival of effector T-cell populations (20). However, following the primary activation phase of $CD8^+$ T-cell responses, IL-2 is no longer required and the maintenance of memory $CD8^+$ T cells is IL-2 independent (21).

IL-7 is produced by both nonhematopoeitic stromal cells and DC, and appears to influence the survival of activated $CD8^+$ T cells to form the $CD8^+$ memory T-cell population (22, 23). Because IL-7 is essential for the development of lymphocytes, evaluations of the specific role of IL-7 in $CD8^+$ T-cell memory maintenance are still uncertain. However, expression of the IL-7 receptor α-chain (IL-7Rα or CD127) on a subset of effector $CD8^+$ T cells entering into the contraction phase has been indicated as an early marker of cells that will go on to form the memory pool (24, 25). Evidence that

IL-7Rα is an early effector-phase marker for future memory populations was demonstrated through adoptive transfer of purified effector $CD8^+$ T cell subsets: only the transfer of activated $CD8^+$ T cells expressing IL-7Rα went on to form stable memory populations that could provide immunological protection (25). In a separate report, it was demonstrated that when IL-$7R^{-/-}$ TCR-transgenic $CD8^+$ T cells are stimulated with specific antigen *in vivo*, they undergo normal cellular division relative to wildtype transgenic $CD8^+$ T cells, yet are poorly maintained after the contraction phase (26). Together, these reports suggest an important role for IL-7 during the transition of $CD8^+$ T cells from the effector to memory phases.

IL-15 is constitutively expressed by several tissues throughout the body, and by numerous cell types, including monocytes/macrophages and DC (*reviewed in* (27)). Mice deficient in IL-15 show profound defects in memory $CD8^+$ T cells, which is due to a defect in their homeostatic proliferation (28, 29). In contrast, overexpression of IL-15 *in vivo* results in the maintenance of increased numbers of antigen-specific memory $CD8^+$ T cells following infection with *L. monocytogenes* (30). These data implicate IL-15 in controlling the maintenance of antigen-specific memory $CD8^+$ T cells following the contraction phase. Thus, throughout their lifespan, from initial activation to resting memory phases, $CD8^+$ T cells are consistently receiving signals through the cytokine environment that surrounds them.

$CD8^+$ memory T-cell subsets

In addition to the size of the memory $CD8^+$ T-cell population, the type and quality of memory $CD8^+$ T cells generated may influence their ability to control a secondary

infection with a specific pathogen. The peptide-specific memory $CD8^+$ T cells that remain after contraction are a heterogeneous population, with subsets of memory cells showing distinct phenotypes, migratory capacity, and effector functions (31-33).

Memory $CD8^+$ T cells are typically placed into two groups. Effector memory $CD8^+$ T cells (T_{EM}) resemble the primary effector cells generated following the initial T-cell response. T_{EM} lack the lymph node homing receptors CD62L and CCR7, limiting their entry into secondary lymphoid tissues. Thus, it is thought that the T_{EM} population is responsible for patrolling peripheral tissues. Upon recognition of cognate antigen, T_{EM} undergo limited proliferation, but are able to express immediate effector function including cytokine production, and direct cytotoxicity (34). In contrast, a population of cells defined as central memory $CD8^+$ T cells (T_{CM}) express both CD62L and CCR7, allowing their migration into lymph nodes. It is generally held that T_{CM} do not express immediate effector function, but rather proliferate in response to antigen, then differentiate into secondary effector T cells, which subsequently leave the lymph nodes to survey peripheral tissues. Therefore, the current paradigm suggests that T_{EM} function to provide immediate protection to control the secondary infection, while T_{CM} proliferate in response to secondary infection to provide a second wave of effector cells for pathogen clearance.

How are memory cell subsets selected following $CD8^+$ T-cell activation? Two main models of memory T-cell generation have been presented over the past 10 years: linear differentiation vs. progressive development. The linear differentiation model proposes that, in response to activation, $CD8^+$ T cells develop into effector CTL. The majority of this population then undergoes apoptosis during the contraction phase, with a

small fraction of effector CTL surviving contraction to become T_{EM}. Over time, a portion of the T_{EM} population continues to differentiate into T_{CM} (33). In contrast, the progressive model of development proposes that the cytokine context in which naive $CD8^+$ T cells are stimulated determines their differentiation into T_{CM} or T_{EM} memory subsets, and that passage through an effector T-cell stage is not a requirement for memory $CD8^+$ T-cell development (35). Using *in vitro* stimulation with specific antigen, Manjunath *et al.* found that TCR transgenic $CD8^+$ T cells primed under high IL-2 concentrations differentiated into effector CTL, which then contracted into T_{EM} populations following adoptive transfer into naive mice. In contrast, *in vitro* antigen stimulation in the presence of IL-15 resulted in the generation of a $CD8^+$ T-cell population with no effector functions, but which were maintained as T_{CM} following adoptive transfer. However it remains to be established whether the generation of memory $CD8^+$ T cells can bypass the effector stage when primed *in vivo.*

The preferential generation or maintenance of T_{CM} or T_{EM} memory subsets may prove to be dependent upon the type of pathogen encountered. It is possible that, for infectious organisms that trigger widespread tissue damage and inflammation within the host, the immune system might respond to this stimulus by maintaining more T_{EM} memory $CD8^+$ T cells. While T_{CM} would need to proliferate upon reinfection before effector CTL could be generated, T_{EM} memory $CD8^+$ T cells would provide immediate protection, and thus limit host damage by the pathogen. However, the maintenance of large populations of armed T_{EM} might increase the chances that a cross-reactive epitope could induce effector activity and contribute to inappropriate responses or autoimmunity. It is likely that the balanced maintenance of both T_{CM} and T_{EM} memory subsets has

evolved as the most protective strategy to provide immune protection while avoiding autoimmune risk.

1.2 Antigen Presentation and CD8+ T-cell Activation

Multiple cell types can function as APC including dendritic cells (DC), macrophages, and B-lymphocytes, although only DC have the ability to activate naïve T cells. This is attributed to the high levels of MHC and costimulatory molecules present on the DC surface (36), however these properties are dependent on the maturation stage of the DC.

Dendritic cell maturation

"Immature" DC reside in peripheral tissues where they phagocytose and process proteins from their local environment for presentation on major histocompatability complex class II (MHC-II) molecules. DC present peptides in association with MHC-I molecules from endogenous sources, such as self-proteins or products derived from intracellular pathogens. Proteins originating from exogenous sources, such as those originating in dying cells taken up by DC as apoptotic or necrotic debris are also presented as MHC-I: peptide complexes. This latter form of antigen processing/presentation utilizes alternative MHC-I pathways and is referred to as "cross-presentation" (*reviewed in* (37)).

In order to stimulate the T-cell arm of the adaptive immune response, a mature DC phenotype is required. The transition of DC from an immature to mature state is accompanied by decreased phagocytic function and upregulation of several costimulatory molecules including CD86, CD80, CD40, and CD54 (36). Changes in chemokine

receptor expression are also evident, and results in DC migration into the T-cell areas of secondary lymphoid organs (31). Maturation may be induced by various stimuli, including inflammatory cytokines, as well as direct binding of pathogen products (commonly referred to as "danger" signals) on Toll-like receptors (TLR) on the cell-surface (38, 39). Direct infection by viable pathogens such as *L. monocytogenes* or influenza can also induce DC maturation, as can the presence of type I interferons produced in response to viral infection (40-44). Work by several groups suggests that the uptake of necrotic cell debris in particular may also provide a maturation signal to DC (45, 46), so that the resulting cross-presentation of processed antigens to T-cell populations occurs in the presence of appropriate costimulation, a phenomenon referred to as "cross-priming". Following maturation, DC are endowed with all the properties necessary to initiate a cellular immune response.

In the absence of an APC-maturing "danger signal", at least one other mechanism exists to induce DC maturation. Experimental data from several groups has led to the generally-accepted "APC licensing" theory that proposes that $CD4^+$ T cells expressing CD40L are able to stimulate the maturation of immature DC through CD40 ligation (42, 47, 48). In general, this theory proposes that $CD4^+$ T_H cells recognize a peptide: MHC-II complex on the surface of an immature DC, and stimulation through the TCR upregulates the expression of CD40L on the $CD4^+$ T_H-cell surface. These activated $CD4^+$ T_H cells can then ligate CD40 on the immature DC, triggering the maturation process and subsequent trafficking to secondary lymphoid organs. As matured DC are potent stimulators of naive $CD8^+$ T cells, this form of $CD4^+$ T_H-cell help is critical for the initiation of a CTL response in the absence of inflammation or infection-associated danger signals. This

model is supported by data showing that treatment of DC with activating anti-CD40 antibodies leads to the maturation of APC to a state that can stimulate $CD8^+$ T cells *in vivo* (42, 47, 48).

It is unknown whether all DC maturation signals are equivalent in terms of the functional properties of the T cells stimulated by them. Does a pathogen-infected DC prime a T-cell response that is as functionally equivalent as one stimulated by a DC matured by CD40 ligation or following TLR recognition of pathogen-derived products? The answers to these questions need to be addressed in order to improve future vaccine and therapeutic immune strategies.

MHC class I antigen presentation pathways

The classical antigen presentation pathway for MHC-I molecules centers around the presentation of peptides derived from proteins located within the cell. These proteins may be endogenous to the cell, or originate from intracellular pathogen-derived proteins. Upon translation within the cytosol, the majority of newly synthesized proteins are quickly targeted for proteosomal degradation, which releases peptides of 4-20 amino acids in length (49). Following cytosolic processing by the proteosome, peptides are transported by the transporter associated with antigen-processing (TAP) complex into the endoplasmic reticulum (ER) (50). Aminopeptidases within the cytosol and ER continue trimming these peptides into 8-11 amino-acid peptide fragments that can fit into the binding pocket of MHC-I molecules (51, 52). Within the ER, empty MHC-I molecules are loaded with these trimmed peptides through association with a "class I-loading complex" containing the chaperones tapasin, calreticulin and ERp60. Successful binding

of peptide into the MHC-I binding pocket releases the MHC-I molecule from the loading complex, allowing transport to the cell surface via the standard secretory pathway (*reviewed in* (53)). Thus, the historical definition of the classical MHC-I processing pathway allows for the presentation of both endogenous proteins and those derived from intracellular pathogens.

However, the classical MHC-I presentation pathway does not include a mechanism to allow for the presentation of antigens from intracellular pathogens that do not specifically infect APC. Thus, additional pathways of MHC-I antigen presentation have been demonstrated, allowing for the cross-presentation of antigens derived from sources outside the APC by MHC-I molecules. Under the cytosolic processing model, exogenous antigen is phagocytosed by the APC, and then released from the phagosome into the cytosol. At this point, MHC-I processing and presentation follows the classical TAP-dependent MHC-I presentation pathway through the ER (54). Under the poorly understood vacuolar model, phagocytosed antigen is processed within the phagosome by proteolytic enzymes and then loaded onto MHC-I within post-Golgi compartments (55, 56). The third proposed cross-presentation model appears to borrow strategies from both the cytosolic and vacuolar pathways, and involves fusion of the phagosome membrane with the ER membrane (57, 58). This fusion event brings to the phagosome the class-I loading complex components associated with the ER membrane that are utilized for classical MHC-I antigen presentation, including TAP, MHC-I, calreticulin and tapasin. Phagocytosed exogenous antigens are transported by the Sec61 channel from the hybrid phagosome-ER vacuole out into the cytosol for proteosomal processing, then transported back into the vacuole for presentation on MHC-I molecules brought into the hybrid

vacuole with the ER membrane. It is unclear which strategy is ultimately employed *in vivo* for the cross-presentation of exogenous antigens by professional APC.

Intracellular pathogens that do not specifically infect APC are targeted by $CD8^+$ T-cells, thus the variety of antigen presentation pathways that have evolved in mammals provide for efficient $CD8^+$ T-cell priming to both peptides from proteins produced within the APC as well as those derived externally. The strict conditions of APC maturation and the subsequent upregulation of costimulatory molecules that are required for naive $CD8^+$ T-cell priming protect against the potentially problematic priming of $CD8^+$ T cells against endogenous host proteins. These requirements, as well as other regulatory mechanisms (*discussed in Section 5*) attempt to keep $CD8^+$ T-cell activation limited to responses against pathogenic rather than self-antigens.

1.3 CD40 and CD40L Expression

Although many costimulatory molecules have been identified as contributing to the priming and development of adaptive immune responses, the interactions between the CD40-CD40L receptor-ligand pair has been characterized as a key component for both B-cell and T-cell responses. CD40 is a tumor necrosis factor (TNF) family member that is expressed primarily on the surface of antigen presenting cells, including B cells, DC, monocytes and macrophages (59). The CD40 ligand (CD40L or CD154) is expressed at low levels on resting $CD4^+$ T cells, and is upregulated following TCR stimulation and T-cell activation (60). Although transient expression of CD40 on activated $CD8^+$ T cells has been reported, the implications of this expression remain unclear (61).

CD40 ligation is required for optimal B-cell responses

The importance of CD40-CD40L interactions was first appreciated in B-cell biology, with the discovery that CD40 ligation is required for the generation of thymus-dependent humoral immune responses (62). Upon antigen recognition by the B-cell receptor (BCR), B cells internalize antigen for processing into peptides, which are subsequently presented in the context of MHC-II molecules on the B-cell surface. Upon recognition of this MHC-II: peptide complex by antigen-specific $CD4^+$ T-cells, a stable cell conjugate is formed, allowing the delivery of both cytokines and membrane-bound signals between the $CD4^+$ T cell and the B cell. One critical signal is delivered by CD40L expressed on the surface of activated $CD4^+$ T cells to the CD40 receptor on the B-cell surface. These signals induce the proliferation and terminal differentiation of B cells into antibody-secreting cells (63). Proliferation alone is not sufficient for the development of B-cell memory. Rather, B cells can divide extensively and secrete IgM without converting into long-lived memory B-cell populations (64). These short-lived, antibody-secreting plasma cells function to provide a temporary source (3-5 days) of lower affinity antibody that presumably aids the immune response to pathogens while the development of high affinity antibody-secreting B cells is underway (65).

Following their interactions with antigen-specific $CD4^+$ T cells, not all B cells become short-lived plasma cells. A subpopulation of activated B cells move into the follicular region of the secondary lymphoid organs to initiate the germinal center (GC) reaction. Similar to the early stages of antibody production, the formation of GC is entirely dependent on CD40-CD40L interactions (66). Upon entry into the GC, proliferating B cells undergo somatic hypermutation, a process of diversifying the BCR

through random genetic mutations. The new variant BCR is expressed on the B-cell surface, which is then tested for antigen-binding affinity by follicular dendritic cells (FDC) presenting antigen. B-cells that display a new, higher affinity BCR receive a positive signal through their interaction with antigen-bearing FDC that allows their survival, while the majority of the cells are not selected and undergo apoptosis. The surviving cells return to the GC for another round of somatic hypermutation, followed by affinity testing on antigen-bearing FDC (67). These events ultimately result in the development of a long-lived memory B-cell population selected for the production of high affinity antibodies.

Experimental evidence in several infection models confirms the requirement of CD40-CD40L interactions for the development of high affinity antibody production in response to pathogens. In $CD40L^{-/-}$ mice infected with LCMV or VSV, decreased production of serum IgM and IgG isotypes is observed, and the concentration of serum antibodies rapidly decays within 2 months of infection, while it is maintained in wildtype mice (68). These data indicate that memory B-cells are not generated following acute viral infection of $CD40L^{-/-}$ mice. In addition, the absence of CD40 or CD40L expression completely abrogates the development of germinal centers following acute infection with adenovirus, LCMV or VSV (68, 69). In humans, mutation of the CD40L gene causes X-linked immunodeficiency hyper-IgM syndrome, a disease characterized by impaired $CD4^{+}$ T-cell responses, as well as the absence of memory B cells and circulating IgG, IgA and IgE antibodies (70). Collectively, these data indicate a critical and non-overlapping role for CD40-CD40L interactions in shaping the humoral immune response to infection.

The role of CD40-CD40L in $CD4^+$ T-cell responses

The importance of CD40-CD40L interactions in the development of $CD4^+$ T-cell responses has also been evaluated in numerous experimental infection models. Following corneal infection with HSV-1, the expansion of $CD4^+$ T cells is reduced in $CD40L^{-/-}$ mice, and the polarization of HSV-1-specific $CD4^+$ T cells toward a T_H1 phenotype is significantly impaired (71). In this model, *in vitro* IL-12 exposure can restore the polarization of HSV-1 primed $CD4^+$ T cells recovered from $CD40L^{-/-}$ mice, suggesting that decreased IL-12 secretion by APCs as a consequence of the absence of CD40-CD40L interactions may impair effector $CD4^+$ T-cell polarization *in vivo*. Peptide-specific $CD4^+$ T-cell priming following acute LCMV infection is reduced nearly 90% in CD40L-deficient mice (72). This defect in $CD4^+$ T-cell priming in $CD40L^{-/-}$ mice correlates with a severely diminished capability to generate humoral immune responses to acute LCMV infection, reflecting the requirement of CD40 ligation by $CD4^+$ T cells for the development of thymus-dependent antibody responses (68). A similar pattern of decreased $CD4^+$ T-cell priming and defective humoral immune responses is seen in $CD40L^{-/-}$ mice following acute infection with either VSV or adenovirus (68, 69). Although there is a significant reduction in effector $CD4^+$ T cells after primary LCMV infection, a subpopulation of these cells can survive as long-lived memory $CD4^+$ T cells, suggesting that a small memory $CD4^+$ T-cell population can be generated in the absence of CD40-CD40L costimulation (73). In regard to infection with *L. monocytogenes*, the route of infection appears to determine whether CD40-CD40L interactions are required for $CD4^+$ T-cell priming. Following systemic *L. monocytogenes* infection in $CD40L^{-/-}$ mice, the upregulation of the activation marker CD44 on bulk $CD4^+$ T-cell populations is

unimpaired relative to wildtype mice, suggesting no inherent defect in $CD4^+$ T-cell activation in the absence of CD40-CD40L signals (40). In contrast, after oral *L. monocytogenes* infection, differential requirements for CD40-CD40L interactions were apparent in peripheral tissues (74). $CD40L^{-/-}$ mice had a reduced frequency of T_H1, IFNγ-producing $CD4^+$ T cells in the spleen and lamina propria, but enhanced populations in the liver and lung. The frequencies of T_H2, IL-4-producing $CD4^+$ T cells followed a similar pattern of tissue distribution in the absence of CD40L.

Collectively, these results imply that the requirement for CD40-CD40L interactions following acute infection with intracellular pathogens may depend on the pathogen evaluated, as well as the infectious route. Further, the apparent need for CD40-CD40L signals during $CD4^+$ T-cell priming may vary depending on the tissues evaluated.

CD40-mediated APC activation: $CD4^+$ T-cell help for $CD8^+$ T-cell responses

There is a clear dichotomy between the requirement for CD40-CD40L interactions during B-cell and $CD8^+$ T-cell activation. While B-cell maturation and the generation of long-lived memory B cells are dependent on direct CD40-CD40L interactions, CD40-mediated "help" to the developing $CD8^+$ T-cell response is both more complex and variable.

The interplay between APC, $CD4^+$ T cells and $CD8^+$ T cells leading to the activation and differentiation of naïve $CD8^+$ T cells into an effector population has been extensively studied and varies widely across experimental models. The primary CTL response to many bacterial and viral infections, including systemic infection with *L.*

monocytogenes, appears to be independent of both $CD4^+$ T_H cells and CD40-CD40L interactions (40, 75, 76). Other model systems in which $CD8^+$ T-cell priming is $CD4^+$ T cell independent include infection with Sendai virus (77), ectromelia virus (78), influenza (79, 80), VSV (81), and LCMV (68, 72, 81, 82). In these settings, CD40 signaling is presumed unnecessary as pathogen-derived products (including CpG-containing DNA, lipopeptides, peptidoglycan and virally induced Type I IFNs) can be directly recognized by TLR on the APC surface, triggering maturation (38, 40, 83). However, several other experimental model systems require $CD4^+$ T_H-cell help, including the priming of $CD8^+$ T cells to adenovirus (84), the class I histocompatability antigen Qa-1 (85), herpes simplex virus (86), antigen-pulsed DC (48, 87), and cross-presented antigens (47, 48, 88).

It is critical to point out that the original experiments that evaluated the requirement for CD40-CD40L interactions in $CD8^+$ T-cell responses were designed to evaluate whether $CD4^+$ T_H cells were required for the priming of naive $CD8^+$ T-cell responses. Only a very limited subset of these investigations also asked whether memory $CD8^+$ T cells could be generated in the absence of $CD4^+$ T_H cell-help. Further, when memory $CD8^+$ T cells have been evaluated previously, only the presence or absence of these populations was reported. Consequently, there has been very limited published information regarding the functional properties of memory $CD8^+$ T-cell generated in the absence of either CD40-CD40L interactions and/or $CD4^+$ T-cell populations. In this regard, many questions remain. Are $CD8^+$ memory T cells generated in the absence of CD40-signals and/or $CD4^+$ T-cell help protective against a secondary infection? Do these cells produce the normal amount of effector cytokines? Are their TCR of high avidity? This dissertation begins to address these questions by evaluating the functional quality of

memory $CD8^+$ T cells generated in CD40-deficient mice and in mice transiently depleted of $CD4^+$ T cells prior to infection with *L. monocytogenes.*

$CD4^+$ T-cell help influences memory $CD8^+$ T-cell populations

In addition to their well-characterized role in APC maturation, $CD4^+$ T_H cells have been proposed to aid the primary $CD8^+$ T-cell response by producing cytokines, notably IL-2 (89, 90). IL-2 signals are required for sustained $CD8^+$ T-cell proliferation, particularly in non-lymphoid tissues (19, 91). However, complete reliance on IL-2 production by $CD4^+$ T_H cells is unlikely as both activated $CD8^+$ T cells and APC matured with pathogen-derived products can also produce IL-2 (90, 92).

A previously unappreciated form of $CD4^+$ T-cell-help has been brought forward in the past few years. Several investigators have demonstrated that $CD4^+$ T_H cells also strongly influence $CD8^+$ memory function, even in infection models not reliant on CD40-mediated APC-maturation signals for CTL priming. In these experiments memory $CD8^+$ T cells generated in the absence of $CD4^+$ T_H cells show qualitative defects, including attrition of memory cell numbers, decreased secondary proliferation to antigen, and impaired effector cytokine production on a per cell basis (93-95). Importantly, these reports evaluated $CD8^+$ T-cell memory in response to such varied antigens as LCMV, VSV, or *L. monocytogenes* infections, as well as to cross-presented tumor and male H-Y antigens. In all of the infectious models evaluated, $CD4^+$ T-cell help was not required for the primary activation or acquisition of effector functions by $CD8^+$ T cells, presumably as APC maturation was sufficiently triggered during infection. However, the absence of CD4-mediated T-cell help during the transition from the effector to the memory phases

significantly impaired the recall function of memory $CD8^+$ T-cell populations. This held true whether the absence of $CD4^+$ T cells was accomplished through the use of MHC-$II^{-/-}$ or $CD4^{-/-}$ mice, or via *in vivo* antibody depletion with anti-CD4 monoclonal antibodies. Further, by evaluating transient depletion of $CD4^+$ T cells only at the time of secondary antigen challenge (compared with depletion at priming), it was determined that once memory $CD8^+$ T cells are generated in the presence of $CD4^+$ T-cell help, further help from $CD4^+$ T-cell populations is not necessary (94).

What is this critical signal that $CD4^+$ T-cells provide to $CD8^+$ T cells to allow the generation of memory $CD8^+$ T-cell populations with full function? It may reflect a direct CD4-CD8 T-cell interaction that must occur during $CD8^+$ T-cell priming. Alternatively, $CD4^+$ T cells may secrete a cytokine required for $CD8^+$ T cells to survive contraction or be maintained as a memory T-cell population. However, regardless of the mechanism involved, this cluster of reports all point to the surprising conclusion that, even in situations where $CD4^+$ T_H cells are not required for CD40-mediated APC activation (such as helper-independent $CD8^+$ T-cell responses to pathogens), the development of functional memory $CD8^+$ T cells is still reliant on the presence of $CD4^+$ T cells.

In 2002, Benedicta Rocha's laboratory reported that CD40 is transiently expressed during T-cell activation on a subset of TCR-transgenic $CD8^+$ T cells directed against the male H-Y antigen. Further, it was proposed that expression of CD40 specifically on $CD8^+$ T cells was required for the generation of memory $CD8^+$ T cells (61). This work led to a more generalized hypothesis that direct ligation of CD40 on the surface of $CD8^+$ T cells by CD40L-bearing $CD4^+$ T_H cells may be a possible mechanism

of direct CD4-CD8 collaboration and a requirement for $CD8^+$ T-cell memory development (*reviewed in* (96-98)).

This theory was subsequently evaluated in several experimental infectious models. Sun and Bevan reported that the generation of SIINFEKL-specific memory $CD8^+$ T cells following infection with recombinant *L. monocytogenes* expressing ovalbumin was equivalent in wildtype, $CD40^{-/-}$ and $CD40L^{-/-}$ C57BL/6 mice ($H\text{-}2^b$ background) (99). Using mixed bone marrow chimeras containing cells from both wildtype and $CD40^{-/-}$ donors within the same recipient, these investigators evaluated the $CD8^+$ T-cell responses to LCMV and *L. monocytogenes* infection. They found that, within the chimeric mice, both wildtype and $CD40^{-/-}$ $CD8^+$ T cells were equally utilized in the primary and memory $CD8^+$ T cell responses, suggesting that $CD40^{-/-}$ $CD8^+$ T cells are able to effectively compete with wildtype $CD8^+$ T cells. Similarly, other investigators have reported that CD40 expression on $CD8^+$ T cells is not required for the generation of memory $CD8^+$ T cells following influenza infection (100).

In the last year, a new piece of evidence in this has puzzle emerged. Using LCMV-specific memory $CD8^+$ T cells from either wildtype or MHC-II-deficient mice, it was demonstrated that $CD8^+$ T cells generated in the absence of $CD4^+$ T-cell help could be rescued from attrition by adoptive transfer (101). Following acute LCMV infection, transfer of effector $CD8^+$ T-cell populations from MHC-II-deficient donor mice into wildtype ($CD4^+$ T-cell competent) recipients allowed for the generation of a stable memory $CD8^+$ T-cell population, while the same cells were gradually lost in $MHC\text{-}II^{-/-}$ recipients. This argues against the hypothesis that the presence of $CD4^+$ T cells "imprints" the $CD8^+$ T-cell population with the ability to be maintained as immunologic

memory. Further, transfer of memory $CD8^+$ T cells from MHC-$II^{+/+}$ mice (primed in the presence of $CD4^+$ T-cell help) into MHC-$II^{-/-}$ recipients results in the loss of these "helped" memory $CD8^+$ T cells over time. Collectively, these data strongly indicate that the loss of memory $CD8^+$ T cells in $CD4^+$ T cell-deficient hosts is not an inherent defect in the $CD8^+$ T-cell populations. Rather, these data indicate that, not the generation, but the maintenance of memory $CD8^+$ T-cell populations is dependent on the presence of $CD4^+$ T cells.

Thus, depending on the pathogen, there are two potential periods in which $CD8^+$ T cells may be dependent of the presence of $CD4^+$ T cells for function. First, the priming of naive $CD8^+$ T-cell populations may be reliant on $CD4^+$ T cells to fully activate APC through CD40-CD40L mediated maturation. Dependency at this stage appears to be variable, and relates to the nature of the initial stimuli associated with pathogen exposure. Second, following $CD8^+$ T-cell priming and contraction, the maintenance of functional, antigen-specific memory $CD8^+$ T cells appears to rely on the presence of $CD4^+$ T cells. As this has only been addressed following LCMV infection to date (101), it is unclear whether this second stage is also variable, or whether all memory $CD8^+$ T cells have this requirement. Further, the mechanism by which $CD4^+$ T cells help to maintain memory $CD8^+$ T-cell function has not been elucidated. It may be through a direct interaction between $CD4^+$ and $CD8^+$ T cells, the secretion of an unknown cytokine by $CD4^+$ T-cell populations, or though interactions with a third cell type. Finally, although transfer into an environment containing $CD4^+$ T cells can prevent the numerical attrition of "unhelped" $CD8^+$ T cells, there has been no demonstration that these "rescued" memory $CD8^+$ T-cell populations have normal effector functions. Thus, it remains unclear

whether the functional properties of memory $CD8^+$ T-cell populations are influenced by the presence of $CD4^+$ T cells at the first or second potential stages of $CD4^+$ T-cell help to $CD8^+$ T cells.

1.4 Adaptive Immunity to Systemic L. monocytogenes Infection: An Experimental Model

Listeria monocytogenes is a ubiquitous gram-positive bacterium with a broad ecological niche and host range. Human infection is most typically associated with gastroenteritis following ingestion of contaminated foods. However, infections in pregnant women, newborns, the elderly, and immunocompromised patients can become serious to life threatening, leading to stillbirth, spontaneous abortion, sepsis, and meningitis (102).

Murine listeriosis has been extensively studied for the past four decades as a paradigm of the immune response to intracellular bacteria. Intravenous injection of *L. monocytogenes* leads to rapid clearance of bacteria from the bloodstream by the spleen and liver. Between 60-90% of the bacteria are removed within 12 hours of infection (102). As the blood-borne bacteria are filtered through the liver, *L. monocytogenes* adheres to specialized macrophages called Kupffer cells lining the liver sinusoid, stimulating the production of IL-8 and upregulation of LFA and ICAM-1 adhesion molecules, which aid the recruitment and retention of neutrophils, respectively (102-106). Upon recruitment to sites of infection, neutrophils are the first line of defense for combating pathogens through phagocytosis, the production of toxic oxygen and nitrogen metabolites, and the release of granules containing proteolytic enzymes (107). In mice depleted of neutrophils or treated with antibodies that impair neutrophil migration,

infection with *L. monocytogenes* results in accelerated and increased entry of bacteria into hepatocytes and 100% mortality within 2-5 days (108-110). As *L. monocytogenes*-infected hepatocytes rapidly undergo apoptosis, the absence of neutrophils and the corresponding acceleration of infection lead to death from acute liver failure.

L. monocytogenes is able to enter various cell types, albeit at widely different efficiencies. Nonprofessional phagocytic cells are induced to take up the bacteria following the binding of the bacterial cell surface proteins Internalin A and Internalin B, to host cell E-cadherin and the Met receptor tyrosine kinase, respectively (111). Interestingly, a 15% sequence divergence between murine and human E-cadherin prevents Internalin A from binding to mouse E-cadherin, an explanation for the reduced uptake of bacteria following oral administration in mice (112). Consequently, most murine experimental models utilize an intravenous route of *L. monocytogenes* infection. Following intravenous injection, professional phagocytic cells, such as splenic macrophages and liver Kupffer cells, internalize bacteria that have avoided neutrophil-mediated clearance.

Regardless of the mechanism employed, once entry is gained into host cells the intracellular lifecycle of *L. monocytogenes* begins in earnest. A hallmark of this lifecycle is the ability of the bacteria to escape from the phagosome and enter the cytosol by secretion of the pore-forming toxin listeriolysin O (LLO; Figure 1) (113). Following escape into the cytosol, the production of the bacterial membrane-bound ActA protein allows the nucleation and polymerization of host actin, enabling *L. monocytogenes* to move both within the cell, and to spread to adjacent cells (114). Cell-to-cell spread is accomplished through the polar polymerization of actin filaments that propel the bacteria

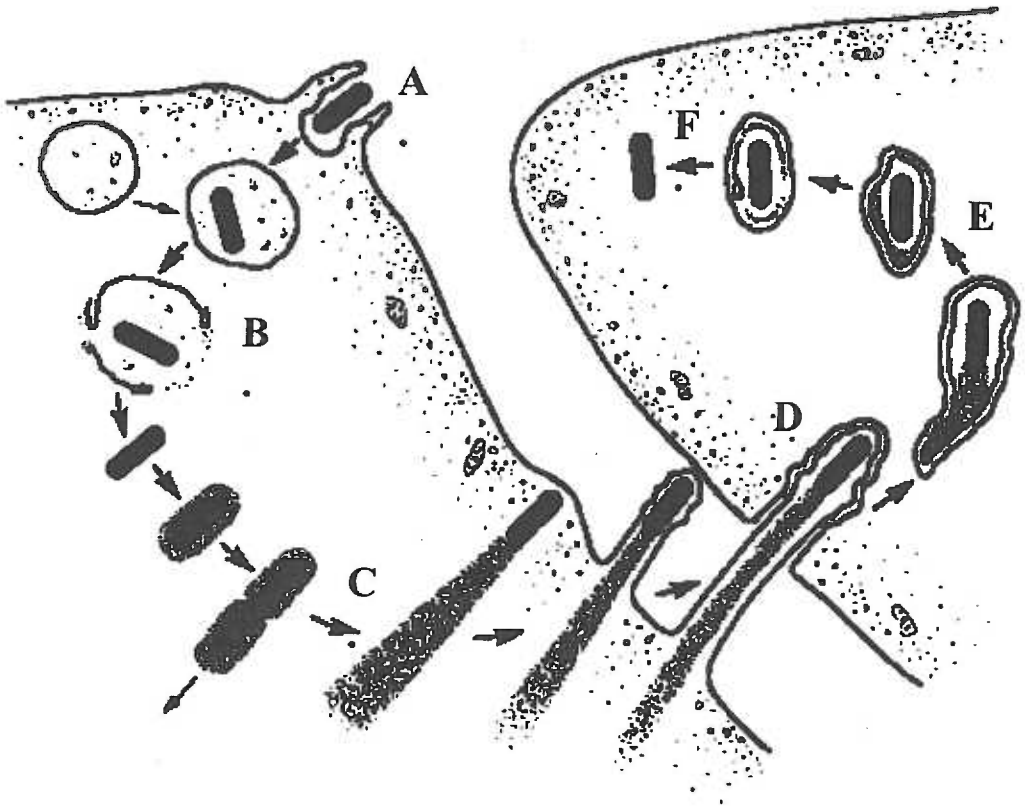


Figure 1. The intracellular lifecycle of *L. monocytogenes*. Following host cell phagocytosis (A), secretion of LLO mediates bacterial escape from the immature vesicle (B). Once in the cytosol, secretion of ActA (C) allows the bacterium to polymerize host actin, creating an actin tail that propels the bacterium through the host cell membrane (D) and into the cytosol of neighboring cells, creating a double walled vacuole (E). Further secretion of LLO again leads to bacterial escape from the vesicle into the host cell cytosol (F). Image © Dan Portnoy, UC Berkeley, CA.

through the cytosol. Upon reaching the cellular membrane, continued actin polymerization pushes the bacteria through the cell membranes of both the original host cell and the neighboring cell, forming fingerlike protrusions into the adjacent cell. The pseudopods are engulfed by phagocytosis and lead to the formation of a double walled vacuole within the new host cell. Again, bacterial secretion of LLO mediates bacterial escape into the cytosol, and the cycle repeats. This results in dissemination of the infection without the extracellular exposure of the bacteria to humoral components of the immune system.

Several other bacterial proteins are essential for full virulence. Two phospholipases (PI-PLC and PC-PLC), and a metalloprotease (mpl) are secreted in a tightly regulated manner, and aid in bacterial escape from the phagosome (115, 116). The constitutively expressed murine hydrolase protein, p60, is involved in bacterial septation following replication. In addition, p60 has a role in the intracellular movement of *Listeria*, as the absence of p60 results in a non-polarized ActA distribution within the bacterium, compromising the formation of actin tails and significantly reducing cell-cell spread and virulence (115).

The initial control of a primary systemic *L. monocytogenes* infection is accomplished through innate resistance mechanisms involving macrophage activation, natural killer (NK) cells and neutrophil recruitment (110, 117). Yet, studies in SCID mice (deficient in both T and B cells) indicate that the innate response can control but not eliminate *L. monocytogenes*, resulting in a chronic infection (118). Transfer of serum from *Listeria*-immune mice does not protect recipients against a challenge with *L. monocytogenes* (119). As *L. monocytogenes* spreads from cell to cell without

extracellular exposure, the lack of antibody-mediated protection is not surprising. The complete elimination of *L. monocytogenes* is dependent on the development of antilisterial T-cell populations. Although $CD4^+$ T-cells can contribute to bacterial clearance following adoptive transfer, sterilizing immunity is though to primarily be the responsibility of cytolytic $CD8^+$ T-cell populations in intact mice (118, 120) Following a primary *L. monocytogenes* infection, the generation of long-lived memory $CD8^+$ T cells confers protection against subsequent, otherwise lethal challenge infections (121).

Classical MHC-Ia-restricted $CD8^+$ T-cell responses to L. monocytogenes infection

Bacteria can encode thousands of distinct proteins that are both distributed within the organism, as well as secreted by the pathogen. During infection with intracellular pathogens, proteins secreted into the cytosol during host-cell infection enter into the classical MHC-I antigen-presentation pathway, and are displayed on the cell surface of infected cells. As infection also results in the killing of many bacteria within the phagosome, non-secreted proteins contained within the bacteria are also be accessible for presentation by the classical MHC-I antigen-processing pathway.

The adaptive immune response of BALB/c mice ($H\text{-}2^d$ background) to systemic infection with *L. monocytogenes* has been extensively characterized, demonstrating that the $CD8^+$ T-cell response is directed to at least four epitopes presented by the classical MHC-Ia $H\text{-}2K^d$ molecule. The four major *L. monocytogenes* $CD8^+$ T-cell epitopes currently known for BALB/c mice all derive from secreted virulence proteins, with the dominant response directed against the $LLO_{91\text{-}99}$ epitope. The $p60_{217\text{-}225}$ epitope is also

recognized by a significant number of $CD8^+$ T cells (122). Additional, subdominant $CD8^+$ T-cell responses are detectable against the $p60_{449-457}$ and mpl_{84-92} epitopes (123).

Two elegant papers have shown that bone marrow-derived APC are required for priming $CD8^+$ T-cell responses to *L. monocytogenes* (124, 125). Lenz et al. generated chimeric mice by the transfer of $H\text{-}2^b$ bone marrow into lethally irradiated ($H\text{-}2^b$x$H\text{-}2^d$) F1 recipient mice (124). In this system, parenchymal cells express both $H\text{-}2^b$ and $H\text{-}2^d$ MHC-I haplotypes, while bone marrow-derived DC only express $H\text{-}2^b$ MHC-I molecules. Following intravenous infection with recombinant *L. monocytogenes* that express $CD8^+$ T-cell peptides presented by either $H\text{-}2^b$ or $H\text{-}2^d$ molecules to peptide-specific $CD8^+$ T-cell populations, these authors demonstrated that priming of $CD8^+$ T cells is specifically dependent upon antigen presentation by bone marrow-derived cells.

Utilizing genetically engineered mice which undergo induced ablation of $CD11c^+$ DC following *in vivo* administration of diphtheria toxin, Jung *et al.* demonstrated that the absence of this APC subset completely abrogates the priming of $CD8^+$ T-cells specific for cell-associated antigens (125). These findings were extended to show that targeted depletion of $CD11c^+$ DC prior to *L. monocytogenes* infection results in a similar lack of CTL priming to *L. monocytogenes* antigens. In contrast, such DC depletion had a minimal effect on the *in vivo* restimulation of adoptively transferred antilisterial $CD8^+$ T-cells. These data indicate that, following *Listeria* infection, the presentation of antigen on APC other than DC occurs in a context that is sufficient to restimulate memory $CD8^+$ T-cells, but that DC are uniquely capable of priming antilisterial $CD8^+$ T-cell populations. In these studies, whether the source of antigen within the DC was a result of direct infection of DC by *Listeria*, or the cross-presentation of antigen from other infected host

cells was not formally demonstrated. However, as infection with *L. monocytogenes* has been reported to induce apoptosis of numerous cell types including DC, the cross-presentation of *Listeria* antigens by DC is likely the significant mechanism for $CD8^+$ T cell priming following *L. monocytogenes* infection (126-128).

As all four major $CD8^+$ T-cell epitopes currently known for BALB/c mice are derived from secreted proteins, the question has been raised of whether secretion of bacterial proteins is a requirement for the priming of protective $CD8^+$ T cells. To evaluate whether non-secreted bacterial proteins can generate protective $CD8^+$ T cells, two recombinant *L. monocytogenes* strains have been developed which express the LCMV $NP_{118-126}$ epitope. One recombinant strain secretes the model LCMV antigen under the control of the *L. monocytogenes* native *hly* promoter, which controls transcription of the LLO protein. The other recombinant strain does not contain the secretion signal sequence, resulting in the sequestration of the LCMV model antigen inside the bacteria. Following systemic infection with these recombinant *L. monocytogenes* strains, bacteria expressing either the secreted or non-secreted LCMV antigens were equally capable of generating memory $CD8^+$ T-cell populations that could provide protection against a challenge infection with LCMV (129). Presumably, priming of $CD8^+$ T cells specific for non-secreted antigens results from bacterial lysis in the phagosome, and the subsequent cross-presentation of the non-secreted antigens by professional APC. These data indicate that secretion of bacterial proteins is not a requirement for the priming of specific $CD8^+$ T-cell populations.

However, when mice were infected with LCMV (which efficiently primes $NP_{118-126}$-specific memory $CD8^+$ T cells), rested 5 weeks to allow viral clearance, and then

challenged with either the secreting or non-secreting *Listeria* strains, protection was only evident against bacteria secreting the target peptide (129). Therefore, cytolytic $CD8^+$ T cells can only provide protection against *L. monocytogenes* strains that secrete their target peptide. This indicates that host cells infected with *Listeria* strains that express the non-secreted form of the LCMV target antigen cannot be recognized by $NP_{118-126}$-specific $CD8^+$ T cells in a timely fashion. This interpretation has been confirmed with a model system using $CD8^+$ T cells specific for the endogenous *Listeria* ActA protein. In this system, following adoptive transfer into naive mice, ActA-specific $CD8^+$ T-cells were unable to confer protection against challenge with *L. monocytogenes*. Using *in vitro* cytotoxicity assays, these investigators demonstrated that the bacterial membrane-bound ActA protein was not presented on the surface of *Listeria*-infected target cells (130). It has been demonstrated that the LLO-mediated escape of the bacteria from the phagosome into the cytosol is required for the infected cell to be recognized by antilisterial effector $CD8^+$ T-cells, suggesting that bacteria trapped within the phagosome are not effectively processed and presented on cell-surface MHC-I molecules (131). Therefore there is a differential effect of antigen compartmentalization within bacteria: $CD8^+$ T cells can be primed to both secreted and non-secreted proteins, but escape from the phagosome and the bacterial secretion of antigenic proteins are necessary in order for *Listeria*-infected cells to become visible targets for cytolytic $CD8^+$ T cell effectors.

MHC-Ia-restricted $CD8^+$ T-cell responses to heat-killed L. monocytogenes

Immunization with heat-killed pathogens, including *L. monocytogenes* (HKLM) has been pursued as a potential vaccination strategy. In theory, the use of heat-killed

pathogens for vaccination is sound, as pathogen cell wall components present in the heat-killed inoculum should provide inflammatory and DC maturation signals to the immune system through TLR triggering. Additionally, the proposal that cross-priming of antigens to $CD8^+$ T cells is a mechanism for priming naive $CD8^+$ T cells following *L. monocytogenes* infection supports this strategy, as presumably, inoculation with HKLM would also require presentation to T cells through cross-priming pathways (125).

The results from attempts to use HKLM to prime naive $CD8^+$ T-cell populations have been disappointing. HKLM immunization stimulates the division of naive TCR-transgenic $CD8^+$ T cells *in vivo*, however the cells appear to undergo an abortive proliferative process resulting in very little accumulation of peptide-specific cells (132, 133). Although the $CD8^+$ T cells recovered following HKLM immunization can bind MHC-peptide tetramers (indicating TCR specificity), these populations show a severely reduced ability to lyse target cells or secrete effector cytokines following peptide stimulation (132, 133). This suggests that $CD8^+$ T-cells activated in response to HKLM may receive an insufficient activation signal, resulting in the acquisition of proliferative potential without effector functions.

This defect in $CD8^+$ T-cell function following vaccination with HKLM may reflect the decreased ability of HKLM preparations to fully upregulate costimulatory molecules on DC *in vitro*, when compared to infection of the DC with viable *L. monocytogenes* (40). When HKLM immunization is given with an agonist anti-CD40 antibody, both $CD4^+$ and $CD8^+$ peptide-specific T cells are primed and capable of secreting IFNγ (134). Importantly, in these experiments, *in vivo* treatment with anti-CD40 antibody during HKLM vaccination allows for the generation of memory $CD8^+$ T-cell

populations that are protective against a secondary *L. monocytogenes* challenge infection. These results suggest that the bacterial components present in the HKLM preparation, and their ability to mediate TLR stimulation are insufficient signals for the priming of protective $CD8^+$ T-cell populations. In contrast, either infection with viable *L. monocytogenes*, or *in vivo* administration of agonist anti-CD40 antibodies at the time of HKLM vaccination can overcome this abortive priming process and allow efficient $CD8^+$ T-cell priming to antigens uniquely contained within the HKLM inoculum.

Nonclassical MHC-Ib-restricted $CD8^+$ T-cell responses to Listeria infection

In addition to the classical MHC-Ia restricted $CD8^+$ T cells, $CD8^+$ T-cell responses directed against peptides presented by the nonclassical H2-M3 and Qa-1 MHC-Ib molecules are also evident following infection with *L. monocytogenes* (135-137). Three peptides presented by H2-M3 have been reported: f-MIVIL (138), f-MIGWII(A) (139), and f-MIVTLF (140). While a Qa-1^b associated *Listeria*-derived peptide has yet to be identified, *Listeria*-infected fibroblasts expressing only Qa-1^b MHC-I molecules stimulate IFNγ production by splenocytes from *L. monocytogenes*-immune BALB/c mice, indicating the priming of a Qa-1^b-restricted $CD8^+$ T-cell population following *Listeria* infection (137). Similar to classically-restricted memory $CD8^+$ T-cells, a subset of peptide-specific antilisterial H2-M3-restricted $CD8^+$ T cells are maintained following *L. monocytogenes* infection as a memory population with similar expression of expression of cell-surface phenotypic markers (141). In genetic knockout mice devoid of

classically restricted $CD8^+$ T-cells, these MHC-Ib-restricted memory $CD8^+$ T-cell populations provide protection against lethal *Listeria* challenge (142).

The kinetics of the primary MHC-Ia-restricted and MHC-Ib-restricted $CD8^+$ T-cell responses differ markedly following infection with *L. monocytogenes.* During the primary response to *Listeria*, the peak magnitude of the H2-M3-restricted $CD8^+$ T-cell response occurs at day 7, nearly two days earlier than the peak of the H2-K^d-restricted classical $CD8^+$ T-cell population (136). In contrast to classical antilisterial $CD8^+$ T-cells, which undergo significant secondary *in vivo* expansion upon reinfection with *Listeria*, neither the H2-M3-restricted or Qa-1-restricted nonclassical $CD8^+$ T-cell populations expand in response to secondary challenge infection (136, 137, 141).

To date, two groups have evaluated the possible reasons for the differential ability of classical vs. nonclassical $CD8^+$ T cell populations to proliferate in response to secondary challenge with *L. monocytogenes.* Kerksiek, *et al.* suggest that the reduced ability of H2-M3-restricted $CD8^+$ T-cells to proliferate upon secondary antigen exposure is programmed based on the priming environment, and is not an inherent property of MHC-Ib-restricted $CD8^+$ T cells. Rather, mice <u>can</u> generate memory H2-M3-restricted $CD8^+$ T cells specific for the f-MIGWIIA peptide that have proliferative potential. In these experiments, *in vivo* priming with the combination of peptide-loaded splenocytes co-injected with anti-CD40 agonist antibody resulted in the generation of f-MIGWIIA-specific $CD8^+$ T-cells that undergo a recall proliferative response in response to *L. monocytogenes* infection (141). These data argue against an inherent difference in the programming of MHC-Ia and MHC-Ib-restricted $CD8^+$ T cells following *Listeria* infection, but rather suggest that the context in which H2-M3-restricted $CD8^+$ T cells are

primed during infection dictates their downstream proliferative potential. In a different approach, Hamilton *et al.* evaluated whether the presence of classical MHC-Ia-restricted $CD8^+$ T-cell populations influences the secondary expansion of memory $CD8^+$ T cells restricted by H2-M3. Utilizing various adoptive transfer approaches with antigen-specific memory $CD8^+$ T-cell populations, they report an inverse correlation between increasing numbers of MHC-Ia-restricted responding $CD8^+$ T cells and the expansion of f-MIGWIIA-specific $CD8^+$ T cells (143). They suggest that competition for APCs may be a mechanism by which classical $CD8^+$ T cells impair the secondary expansion of H2-M3-restricted memory $CD8^+$ T-cell populations.

At this time, many of the requirements for MHC-Ib-restricted $CD8^+$ T-cell priming and memory generation remain poorly understood. It is unknown whether there are differences in either the antigen-presentation kinetics or density of MHC-Ia and MHC-Ib restricted peptides following *Listeria* infection. The type(s) of APC that can efficiently activate antilisterial H2-M3-restricted $CD8^+$ T cells has not been evaluated. Although treatment with anti-CD40 agonist antibody during primary infection with *L. monocytogenes* does not enhance the priming of f-MIGWIIA-specific $CD8^+$ T cells, whether the absence of CD40-CD40L signals influences this response is unknown (141). Further, whether $CD4^+$ T-cell help influences the generation of nonclassically restricted $CD8^+$ T cell responses to *L. monocytogenes* infection has not been addressed.

$CD4^+$ T-cell responses to L. monocytogenes

$CD4^+$ T cells contribute to cell-mediated immunity through several mechanisms including the activation of APC through CD40-CD40L interactions, as well as the

secretion of cytokines including IL-2, IL-4, and IL-12. These cytokines, in turn, aid in the direction of class switching in responding B-cell populations, direct the polarization of $CD4^+$ T cells, and influence activation of $CD8^+$ T cells. In addition to aiding B cells and $CD8^+$ T cells, $CD4^+$ T cells become effector cells themselves, secreting IFNγ and TNFα to activate the antimicrobial properties of macrophages.

Although $CD4^+$ T cells are not required for the clearance of systemic infection with *L. monocytogenes*, they do contribute to pathogen clearance in wildtype mice. The rate of clearance of bacteria following both primary and secondary challenge infection with *L. monocytogenes* is delayed in mice MHC-II-deficient mice (144). Although a *Listeria*-derived $CD4^+$ T-cell epitope was elusive for years, the recent demonstration that a small population of $CD4^+$ T cells specific for the $LLO_{189-200}$ peptide develops following *L. monocytogenes* infection in both BALB/c and C57BL/6 mice has allowed the tracking of peptide-specific $CD4^+$ T-cell populations (145).

On day 7 following systemic *Listeria* infection, $LLO_{180-200}$-specific $CD4^+$ T cells are evident in the spleen and liver by evaluation for both IFNγ and TNFα production in response to specific peptide (40, 146, 147). Following oral infection, the peptide-specific $CD4^+$ T-cell response is more pronounced in the mesenteric lymph nodes and mucosal tissues than in the spleen and liver (146). Thus, the location of the majority of the peptide-specific $CD4^+$ T-cells correlates with the dissemination pattern of the bacteria following intravenous (systemic) vs. oral *Listeria* infection (146). Whether this altered distribution of $LLO_{180-200}$-specific $CD4^+$ T cells reflects differences in the site of $CD4^+$ T-cell priming, or rather is due to subsequent trafficking of $CD4^+$ T cells to the site of infection is unknown. However, both systemic and oral infection with *L. monocytogenes*

results in the activation of significant numbers of $CD4^+$ T cells specific for *Listeria*-derived antigens.

The $LLO_{180\text{-}200}$-specific $CD4^+$ T-cell population undergoes a contraction phase similar to $CD8^+$ T cells and is maintained as a memory population (148). However, the loss of *Listeria*-specific $CD4^+$ T cells over time is much greater than the attrition evident in the antilisterial $CD8^+$ T-cell population (147). This appears to reflect an inherent difference in the maintenance of memory $CD4^+$ and $CD8^+$ T cells, with prolonged survival of $CD8^+$ T cells the norm (149). Further, in contrast to the robust secondary expansion of antilisterial $CD8^+$ T cells, the peak of the secondary $LLO_{180\text{-}200}$-specific $CD4^+$ T-cell response is similar in magnitude to the primary response. Although the magnitude of secondary $LLO_{180\text{-}200}$-specific $CD4^+$ T-cell expansion can be marginally improved by increasing the *L. monocytogenes* challenge dose, the effect is modest compared to the significant increases seen in secondary $CD8^+$ T-cell expansion in response to increased challenge doses (147).

Ampicillin treatment of *Listeria*-infected mice 24 hours post-infection shortens the duration of infection and results in decreased presentation of the $LLO_{189\text{-}200}$ peptide in the context of MHC-II (148). These changes in antigen-presentation correlate with a 2-fold decrease in the peak number of $LLO_{180\text{-}200}$-specific $CD4^+$ T cells at day 8 of the primary infection. However, by day 28, the number of peptide-specific memory $CD4^+$ T cells is equivalent in ampicillin-treated and untreated mice (148). Further, these memory $CD4^+$ T-cells expand normally following secondary *Listeria* challenge, indicating that the duration of *L. monocytogenes* infection does not influence the development of memory $CD4^+$ T-cell populations.

The role of CD40 in the classical CD8+ T-cell response to Listeria infection

As CD40-mediated $CD4^+$ T-cell help has profound effects on the $CD8^+$ T-cell responses to many pathogens, several evaluations on the impact of CD40-CD40L interactions in the development antilisterial immunity have been performed. Previous studies have characterized splenic $CD8^+$ T cell responses following intravenous *Listeria* infection in the absence of CD40-CD40L signals by blocking such interactions through administration of anti-CD40L antibodies *in vivo* (87) or by infection of $CD40L^{-/-}$ mice (40, 150). Experiments performed in $CD40L^{-/-}$ mice following primary infection with *L. monocytogenes* confirmed that functional $CD8^+$ T cells are primed and subsequently clear the primary infection (40, 150). Following *in vivo* CD40L antibody blockade, the number of resting peptide-specific $CD8^+$ memory T cells recovered 4 weeks later was not influenced by the absence of CD40-CD40L interactions (87). Although these data suggest that CD40-CD40L interactions are not required for the generation of memory $CD8^+$ T cells, the functional properties of the recovered memory $CD8^+$ T cells, such as target cell killing or effector cytokine production, have not been evaluated. It remains possible that the recall effector functions of memory $CD8^+$ T-cell populations may be compromised when these cells develop in the absence of CD40-CD40L signaling.

When wildtype, $CD40^{-/-}$ and MHC-$II^{-/-}$ mice (all H-2^b haplotype) were infected orally with recombinant OVA-expressing *L. monocytogenes* (rLM-OVA) tetramer analysis of peptide-specific $CD8^+$ T-cell responses recovered from both peripheral, lymphoid, and mucosal tissues revealed a tissue-specific dependency on CD40-CD40L interactions for $CD8^+$ T-cell priming (75). While the absence of CD40 did not decrease the frequency of peptide-specific $CD8^+$ T cells recovered from the spleen 9 days post-

infection, the liver response was reduced by ~50%. $CD8^+$ T-cell responses in mucosal tissues were even further decreased, with the frequency of peptide-specific cells in the lamina propria (LP) and intraepithelial lymphocyte (IEL) population reduced by 75-90%. The responses in MHC-$II^{-/-}$ ($CD4^+$ T cell-deficient) mice were also differentially influenced depending on the organ evaluated, although the overall reductions in $CD8^+$ T-cell priming were less dramatic than those seen in $CD40^{-/-}$ mice. Again, these experiments evaluated the magnitude of primary $CD8^+$ T-cell responses, while an evaluation of memory $CD8^+$ T-cell function was not performed. Nonetheless, these results suggest that $CD4^+$ T cells (possibly through CD40 ligation) may act in a tissue-specific manner to aid the generation of $CD8^+$ T-cell responses following oral infection with *L. monocytogenes*.

Support for the suggestion that priming of $CD8^+$ T cells in mucosal tissues is more dependent on CD40-CD40L interactions is found in the immune response to vesicular stomatitis virus (VSV). In this system, priming of mucosal $CD8^+$ T cells following intravenous VSV infection is significantly reduced in $CD40^{-/-}$ mice, while there is no impairment of splenic $CD8^+$ T-cell responses (151). Collectively, these reports suggest that the requirement for CD40 expression in priming of mucosal $CD8^+$ T cells is (a) not dependent on the route of infection, and (b) not limited to *Listeria* infection. Thus, it is possible that the costimulation requirements for $CD8^+$ T-cell priming in the mucosa are inherently different than found in the spleen and other secondary lymphoid compartments. Importantly, when $CD40^{-/-}$ mice primed with VSV received a secondary challenge infection with a heterologous VSV strain, the mucosal memory $CD8^+$ T-cell population showed an equal or greater fold-expansion when compared with wildtype

mice (151). This suggests that, although the priming of mucosal memory $CD8^+$ T-cell populations may be numerically decreased in the absence of CD40-CD40L interactions, the memory $CD8^+$ T-cell populations generated in this environment have strong proliferative responses to secondary antigen exposure.

There are still several questions to be addressed regarding how CD40-CD40L interactions might influence $CD8^+$ T-cell memory. It is unknown whether ligation of CD40 on the DC surface results in a unique APC that endows specific functional properties on the T cells it interacts with. Perhaps CD40-mediated APC activation is required for the generation of T_{EM} or T_{CM} memory $CD8^+$ T cell phenotypes. As CD40 ligation has been associated with the upregulation of survival proteins, a direct signal between $CD40L^+$ $CD4^+$ T-cells and $CD40^+$ $CD8^+$ T cells might allow the $CD8^+$ T cell to survive apoptosis during the contraction phase and be maintained as memory (61, 98). It is unknown whether the absence of CD40-CD40L interactions influences the avidity of the $CD8^+$ T cells, or results in a $CD8^+$ T-cell population that secretes lower amounts of cytokines or is slower to kill target cells.

1.5 Regulation of T-cell Responses

Following infection, pathogen-specific $CD4^+$ and $CD8^+$ T cells undergo extensive proliferation upon recognition of antigen in the presence of appropriate costimulatory signals. These effector T-cell populations eventually reach a numeric plateau, and then undergo a contraction phase resulting in the generation of a small population of memory T cells that provide protection against subsequent infection with the same pathogen (13, 148).

Numerous events contribute to the magnitude of $CD8^+$ T-cell expansion, including the number of CTL precursors recruited, antigen concentration, and cytokine availability (8, 90, 152). It is becoming clear that a regulatory force may also act upon the expanding T-cell populations to prevent the accumulation of "too many" cells of any single specificity. Recent evidence indicates that there is a numeric upper limit to the expansion of $CD8^+$ T cells of the same specificity, and that increased antigen density or CTL precursor frequency cannot bypass this upper limit (152, 153).

A number of recent publications have demonstrated that antibody-mediated *in vivo* depletion of either $CD4^+$ or $CD25^+$ cells prior to infection has a profound impact on T-cell responses to pathogens. These studies have revealed a regulatory effect by cells expressing the CD4 and CD25 cell-surface antigens on both primary and secondary T-cell expansion. For example, in mice depleted of $CD25^+$ cells prior to primary infection with herpes simplex virus (HSV), both $CD4^+$ and $CD8^+$ T-cell priming are increased (154, 155). The influence of $CD4^+$ T cells on $CD8^+$ T-cell responses to *L. monocytogenes* is less clear. When mice are rested for 2 months between primary and challenge *L. monocytogenes* infection, both CD4 and CD25-depletion prior to challenge results in a marked enhancement in secondary $CD8^+$ T-cell responses (156). In contrast, other investigators have reported that the secondary $CD8^+$ T-cell response to *L. monocytogenes* is reduced when animals are rested seven months between primary *L. monocytogenes* infection and CD4-depletion and challenge (157).

Regulatory T-cell populations

Several mechanisms have evolved to prevent or minimize immune responses to self-antigens as well as over-exuberant responses to pathogens, both of which can

damage the host. To date, at least three subsets of regulatory T cells have been described that have been shown to limit antigen-specific T-cell responses. These regulatory T-cell populations differ from each other based on cell surface phenotype, cytokine profile, and suppressive mechanism. T_R1 regulatory T cells produce IL-10 and TGF-β, and require IL-10 for both their differentiation and function. T_H3 regulatory T cells also produce IL-10 and TGF-β, but additionally make IL-4. Both T_R1 and T_H3 regulatory T cell subsets suppress immune responses through the secretion of anti-inflammatory cytokines, and thus influence T-cell responses in a cell-contact and antigen-independent manner (158, 159).

$CD4^+$ $CD25^+$ regulatory T cells, (T_{REG}), constitutively express the IL-2Rα chain (CD25), inhibit IL-2 production, and promote cell cycle arrest in activated $CD4^+$ and $CD8^+$ T-cell populations through a contact-dependent mechanism (160-162). These cells constitute ~5-10% of the resting $CD4^+$ T cell population in healthy mice and humans, and appear to suppress other T-cell populations in a non-specific manner (161, 163, 164).

An important distinction between the various regulatory T-cell subsets is their derivation. While T_R1 and T_H3 cells appear to be conventional T cells stimulated under specific conditions (i.e. by immature DC, following antigen feeding, or in the presence of IL-10), $CD4^+$ $CD25^+$ T_{REG} appear to be a specific developmental lineage, generated during thymic development (158, 159, 165). Neonatal thymectomy of mice within three days of birth prevents the emigration of $CD4^+$ $CD25^+$ T_{REG} cells from the thymus, and results in the spontaneous development of autoimmunity, including the development of circulating autoantibodies, gastritis and oophoritis (166). Thus, the $CD4^+$ $CD25^+$ T_{REG} population can be considered to be a "naturally occurring" regulatory T-cell pool that is

endogenous to the host, and not dependent on specific stimulation conditions for their development.

The $CD4^+$ $CD25^+$ phenotypically defined T_{REG} population can suppress T-cell responses both *in vitro* and *in vivo* (*reviewed in* (167)). In addition, regulatory function has been attributed to $CD4^+$ T cells bearing CD62L, CD103, CTLA-4 (CD152), and the glucocorticoid-induced tumor necrosis factor receptor (GITR) (167-169). However, the use of these markers to define the T_{REG} subset is problematic, as activation of conventional T cells induces the expression or upregulation of many of these cell-surface markers as well. Recently it was reported that the *foxP3* gene, which encodes the transcriptional repressor protein Scurfin, is exclusively transcribed in the subset of $CD4^+$ $CD25^+$ T cells with potent regulatory function (170, 171). In contrast to cell-surface markers, the expression of the *foxP3* gene in mice has not been observed in activated T cells that lack regulatory function.

In mice, targeted deletion of *foxP3* results in an animal devoid of T_{REG} cells. These animals exhibit lymphoproliferative disorders similar to those seen in neonatal mice thymectomized on day 3 after birth, including circulating autoantibodies, gastritis and oophoritis (166, 171, 172). This defect appears to involve the development of T_{REG} cells, as transfer of $CD4^+$ $CD25^+$ T_{REG} cells from *foxP3*$^+$ mice into *foxP3*-deficient animals prevents autoimmune disease development (171). In agreement with the theory that *foxP3* is required for T_{REG} cell development, transgenic overexpression of *foxP3* leads to the generation of increased numbers of T_{REG} cells *in vivo* (170). Additionally, retroviral transduction of $CD4^+$ $CD25^-$ non-regulatory T cells with *foxP3* induces the conversion of these conventional T cells into fully functional T_{REG} cells, with the

upregulation of both CD25 and GITR expression on the transduced $CD4^+$ T-cell surface and the ability to suppress effector T cells both *in vitro* and *in vivo* (171, 172).

Signaling requirements for $CD4^+$ $CD25^+$ regulatory T-cells

The mechanisms that facilitate the development and maintenance of $CD4^+$ $CD25^+$ T_{REG} cells remain unclear, although the requirement for several signaling events has been demonstrated. It is generally held that $CD4^+$ $CD25^+$ T_{REG} cells express high affinity TCR sensitive for detecting low concentrations of self-antigen presented by MHC-II molecules (167, 173). The TCR repertoire of the $CD4^+$ $CD25^+$ T_{REG} populations appears to be as diverse as found in the $CD4^+$ $CD25^-$ T-cell population (173). Stimulation of the TCR is required for $CD4^+$ $CD25^+$ T_{REG} cells to exert regulatory function (160, 161). IL-2 appears to be required for their generation and maintenance *in vivo* (165). The requirement for B7-1, B7-2 and CD28 in $CD4^+$ $CD25^+$ T_{REG} development has been demonstrated in the non-obese diabetic (NOD) mouse model of spontaneously-developed type-I diabetes (174), as well as following adoptive transfer of TCR transgenic $CD4^+$ T cells (175). In these experiments, marked reductions in the $CD4^+$ $CD25^+$ T_{REG} population were observed in the absence of B7-CD28 interactions.

In two different mouse genetic backgrounds, $CD40^{-/-}$ mice also show a clear decrease in the number of $CD4^+$ $CD25^+$ regulatory T cells (176, 177). The transfer of T cells from $CD40^{-/-}$ BALB/c mice ($H\text{-}2^d$) into nude recipients (T cell-deficient) leads to the development of autoimmunity that closely resembles the disease seen in neonatal thymectomized mice and *foxP3*$^{-/-}$ mice, including autoantibody production, thyroiditis, sialoadenitis, oophritis, and insulitis (176). This has been attributed to the reduced $CD4^+$

$CD25^+$ T_{REG} subset in the donor cell pool. Additionally, defects in the $CD4^+$ $CD25^+$ T_{REG} population have also been observed in $CD40^{-/-}$ mice on the $H\text{-}2^b$ genetic background (177), suggesting that the expression of CD40 may be important for either the development or maintenance of $CD4^+$ $CD25^+$ T_{REG} cells.

$CD4^+$ $CD25^+$ Regulatory T-cell function in autoimmunity

The prevention of autoimmunity is initially prevented through developmental mechanisms of T-cell tolerance and anergy, antigenic ignorance, and thymic deletion. However, it is very likely that during infection, self-antigens not normally presented to the immune system (and perhaps not available during thymic selection) may be released following cell damage, increasing the possibility that autoreactive T cells may encounter their cognate antigen in peripheral lymphoid compartments. Alternatively, pathogen-derived peptides that mimic self-antigens may be presented to T cells by APC that are fully matured by the infectious process. However, autoimmune responses rarely develop during or following recovery from an infectious disease, suggesting that, in addition to passive, ontogenic regulatory mechanisms, complete protection against self-directed T-cell responses may require specialized regulatory cell populations to actively suppress erroneous responses of self-reactive T-cell populations.

Historically, $CD4^+$ $CD25^+$ regulatory T cells (T_{REG}) have been assigned a role in maintaining self-tolerance by preventing the activation and expansion of self-reactive effector T cells that have escaped thymic deletion (163, 178). Targeted removal of the $CD4^+$ $CD25^+$ T_{REG} population leads to the spontaneous development of a variety of autoimmune diseases in otherwise normal mice, including autoantibodies, gastritis,

oophoritis, thyroiditis and insulitis (163). Targeted depletion of the $CD4^+$ $CD25^+$ T_{REG} population also triggers the activation of T cells specific for antigens derived from commensal intestinal bacteria, leading to the development of inflammatory bowel disease (*reviewed in* (179)).

Investigations of various mouse models of autoimmunity have revealed that $CD4^+$ $CD25^+$ T_{REG} cells exert profound effects on the induction of T-cell mediated autoimmune diseases. $CD4^+$ $CD25^+$ T_{REG} populations influence the spontaneous development of diabetes in the NOD mouse, as adoptive transfer of purified $CD4^+$ $CD25^+$ T_{REG} cells confers significant protection against the onset of diabetes (174). Experimental autoimmune encephalomyelitis is an experimental model of multiple sclerosis that can be induced by immunization of animals with neuroantigens and adjuvant (active induction), or through the adoptive transfer of neuroantigen-specific $CD4^+$ T cell lines (passive induction). Adoptive transfer of naive $CD4^+$ $CD25^+$ T_{REG} prior to active induction of EAE resulted in a marked decrease of disease severity, and the skewing of responding $CD4^+$ T cells toward a nonpathogenic T_H2 phenotype (180). In addition, co-transfer of $CD4^+$ $CD25^+$ T_{REG} cells with neuroantigen-specific effector $CD4^+$ T–cell lines also results in markedly reduced clinical symptoms, indicating that $CD4^+$ $CD25^+$ T_{REG} populations can influence $CD4^+$ effector T-cell function past the priming stage (180). Interestingly, the suppressor function of peripheral blood $CD4^+$ $CD25^+$ T_{REG} populations in human MS patients is severely reduced compared with healthy patients, lending credit to the interpretation of the data from experimental mouse models (181).

$CD4^+$ $CD25^+$ Regulatory T-cells influence $CD8^+$ T-cell activation and function

In vitro analyses have demonstrated that $CD4^+$ $CD25^+$ T_{REG} cells can reduce the activation and effector function of both human and murine $CD8^+$ T cells. Human $CD4^+$ $CD25^+$ T_{REG} cells inhibit the proliferation and IL-2 production of $CD8^+$ T cells following *in vitro* stimulation with mitogen (182). In addition, murine TCR transgenic $CD8^+$ T cells show decreased *in vitro* proliferation and secretion of IFNγ following polyclonal or antigen-specific stimulation in the presence of purified $CD4^+$ $CD25^+$ T_{REG} populations (162). In both studies, the suppressor function was found to require direct T_{REG}-$CD8^+$ T cell contact. Interestingly, both investigations revealed that co-culture of $CD8^+$ T cells with $CD4^+$ $CD25^+$ T_{REG} cells leads to down-regulation of CD25 expression on the $CD8^+$ T-cell population, suggesting that $CD4^+$ $CD25^+$ T_{REG} cells may alter the responsiveness of $CD8^+$ T cells to IL-2. However, at this point, a conclusive understanding of the mechanism(s) by which $CD4^+$ $CD25^+$ T_{REG} cells inhibit $CD8^+$ T cells remains to be established.

$CD4^+$ $CD25^+$ Regulatory T-cells influence effector T-cell responses to infection

An additional role for T_{REG} cells is suggested from reports that T_{REG} populations also limit T-cell responses to pathogens. Several groups have investigated T-cell responses to infection following depletion of either $CD4^+$ or $CD25^+$ cells by *in vivo* antibody administration. $CD25^+$ depletion prior to infection of BALB/c mice with the lethal *Plasmodium yoelli* malaria parasite leads to enhanced $CD4^+$ T-cell priming and

expansion, in addition to host survival following pathogen clearance (183). $CD8^+$ T-cell expansion is enhanced following *L. monocytogenes* challenge when immune BALB/c mice are treated with depleting antibodies specific for either CD4 or CD25 prior to secondary *Listeria* challenge (156). These experiments suggest that the presence of $CD4^+$ $CD25^+$ T_{REG} during both priming and recall phases regulates the magnitude of $CD4^+$ and $CD8^+$ T-cell populations in response to infection.

The ability of $CD4^+$ $CD25^+$ T_{REG} cells to decrease the magnitude of effector T-cell expansion may ultimately be beneficial in some circumstances. For example, following adoptive transfer of $CD4^+$ $CD25^-$ T cells into $RAG^{-/-}$ recipients, the effector T-cell response to infection with the opportunistic pathogen *Pneumocystis carinii* results in pathogen clearance, but also leads to fatal pulmonary hyper-inflammation in these lymphopenic mice (184). In contrast, immunologically intact mice can clear the infection with minimal pulmonary inflammation. This difference has been attributed to the activity of $CD4^+$ $CD25^+$ T_{REG} cells in the normal host that limit the expansion of effector $CD4^+$ T-cell populations, preventing the accumulation of high numbers of effector cells that cause host pathology. In this model, the regulatory function that modulates the adaptive immune response benefits the host, limiting immunopathologic tissue destruction and the release of high levels of proinflammatory cytokines by effector T cells.

Support for the premise that regulation of the effector T-cell population is beneficial to the host is found in mouse models of HSV infection. Depletion of $CD25^+$ cells prior to footpad infection with HSV results in enhanced antiviral $CD8^+$ T-cell responses and improved viral clearance (155). Yet, when ocular HSV infection models are evaluated, the severity of corneal lesions that develop is significantly increased in

CD25-depleted mice compared with normal animals (154). Therefore, the immune response to the same pathogen appears to be delicately regulated by the $CD25^+$ cell population to allow the generation of a sufficient number of effector T cells to combat the pathogen while also attempting to minimize tissue damage by effector T cells that is deleterious to the host.

The influence of $CD4^+$ $CD25^+$ T_{REG} populations on T-cell responses is not limited to infectious settings, but also to T-cell priming following vaccination. Following primary and boost vaccination with a DNA construct, depletion of $CD4^+$ T cells between the two vaccinations leads to enhanced expansion of peptide-specific $CD8^+$ T cells able to secrete effector cytokines and display cytotoxic activity (156). The priming of transgenic $CD4^+$ and $CD8^+$ T cells following *in vivo* transfer of antigen-pulsed mature DC was similarly increased in mice depleted of $CD25^+$ cells (185). HKLM prime-boost vaccination protocols in normal mice result in a memory $CD8^+$ T-cell population defective in effector cytokine secretion and cytotoxic function (132). In contrast, *in vivo* depletion of $CD4^+$ T cells between the HKLM prime and boost injections results in the development of memory $CD8^+$ T cells capable of secreting IFNγ following peptide stimulation, and which confer protection against viable *L. monocytogenes* challenge (186). Collectively, these experiments suggest that $CD4^+$ $CD25^+$ T_{REG} can influence the magnitude of both primary and memory T-cell responses to specific antigen.

1.6 Summary and Relevance to Dissertation

A better understanding of the mechanisms that provide for the generation of highly functional immunological memory populations following infection is required for

the development of immune-based therapies for the treatment of infectious disease and tumors. Although CD40-CD40L interactions are not required for the induction of all $CD8^+$ T-cell responses, whether this interaction provides a unique signal to the developing immune response remains unclear. For some pathogens, the stimuli provided by the infection itself appears to bypass the need for CD40-mediated $CD4^+$ T-cell help for APC maturation and the activation of naive $CD8^+$ T-cell populations. However, it has not been determined whether the cellular immune response that develops in the absence of CD40-CD40L interactions is equivalent in function to the response developed in the presence of dendritic cells that have received a signal through CD40 ligation.

With this dissertation I have evaluated both primary and memory $CD8^+$ T-cell responses following systemic *L. monocytogenes* infection in wildtype and CD40-deficient BALB/c mice. With these experiments I have determined whether the absence of CD40 expression influences the expansion and functional properties of $CD4^+$ T cells, as well as both classically and nonclassically restricted $CD8^+$ T-cell responses generated in response to infection with *L. monocytogenes*. Further, I have investigated the effects of transient depletion of $CD4^+$ T-cell populations prior to either primary or secondary *L. monocytogenes* infection on the antilisterial $CD8^+$ T-cell populations. Finally, through *in vivo* depletion of $CD4^+$ T cells, infection of $CD40^{-/-}$ mice (containing a naturally reduced $CD4^+$ $CD25^+$ T-cell population), and adoptive transfer studies, I have evaluated the impact of regulatory T-cell populations on the secondary expansion of memory $CD8^+$ T cells following *L. monocytogenes* challenge.

CHAPTER 2: MATERIALS AND METHODS

Mice and cell lines

BALB/cJ, C57BL/6 and CD40$^{-/-}$ (CNCr.129P2-*Tnfrsf5*^{tm1Kik}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c Thy1.1 mice were kindly provided by Dr. Richard Dutton (Trudeau Institute, Saranac Lake, NY). BALB/c Thy1.1 and CD40$^{-/-}$ mice were maintained by in-house breeding at the VA Medical Center. The absence of CD40 expression in CD40$^{-/-}$ mice was routinely confirmed by flow cytometry. All experiments with animals were conducted under approval of the IACUC at the Veterans Affairs Medical Center in Portland, OR.

RMAS-K^{d} cells (provided by Dr. Michael Bevan, University of Washington, Seattle, WA) were maintained in RPMI 1640 (BD Biosciences, San Jose, CA) containing 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA) and 200 μg/ml G418 (Sigma, St. Louis, MO).

L. monocytogenes culture and infection

L. monocytogenes was inoculated into 2 ml of brain-heart infusion (BHI) broth (BD Biosciences, San Diego, CA) and grown overnight. Prior to use, cultures typically contained ~2x10^{9} colony-forming units (CFU)/ml, as determined by plating serial dilutions onto BHI-agar plates. Plates were incubated at 37° C until CFU could be visualized and counted.

Mice were given a primary systemic infection by intravenous injection of 1-3x10^3 CFU (0.1 LD_{50}) *L. monocytogenes* into the lateral tail vein in a volume of 100 µl. The number of CFU injected was calculated retrospectively by plating serial dilutions of the injected bacterial suspension onto BHI agar and allowing CFU outgrowth by incubation at 37° C.

L. monocytogenes strain #43251 (ATCC, Manassas, VA) was the principal bacterium used for experiments unless otherwise noted. The *L. monocytogenes* strain DP-L2528 (referred to as LLO92F) was provided by Dr. Dan Portnoy, UC Berkeley, Berkeley, CA. The recombinant JJL-OVA strain, expressing ovalbumin protein was provided by Dr. Hao Shen, University of Pennsylvania, Philadelphia, PA.

Unless otherwise noted, the term "*Listeria*-immune mice" is applied to animals that received a primary *L. monocytogenes* infection and then were rested for four weeks prior to subsequent use.

Heat-killed L. monocytogenes (HKLM) production

L. monocytogenes strain #43251 was inoculated into 20 ml of BHI broth and grown overnight at 37° C in a shaking incubator. Cells were pelleted, washed twice with sterile PBS, then resuspended at ~1x10^{10} CFU/ml in sterile PBS. Bacteria were incubated for 3 hours at 70° C, then stored at –20° C. To confirm that no viable bacteria remained in each HKLM stock, the absence of CFU growth on BHI agar was confirmed.

Peptides

Synthetic peptides for LLO_{91-99} (GYKDGNEYI), $p60_{217-225}$ (KYGVSVQDI), $OVA_{261-268}$ (SIINFEKL), and $LLO_{189-200}$ (WNEKYAQAYPNV) were produced at the VA Medical Center using an Applied Biosystems Synergy apparatus (Foster City, CA) and standard

Fmoc chemistry. The f-MIGWII peptide was provided by Dr. Eric Pamer (Sloan-Kettering Institute, New York, NY).

Isolation of lymphocyte populations

Spleens were removed and single cell suspensions were prepared by passage over a cell strainer. For the evaluation of lymphocytes from lung and liver tissues, anesthetized mice were perfused with 10 ml PBS, 75 u/ml heparin (Sigma) at the time of tissue harvest. Lung tissue was minced in HBSS, 1.3 mM EDTA and incubated at 37° C for 30 minutes. Cells were washed twice with PBS, 5% FCS, then digested at 37° C for 30 minutes in RPMI, 5% FCS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 U/ml Collagenase 1A (Sigma). Digested lung tissue was passed over a cell strainer prior to analysis (74). Lymphocytes were collected from liver tissue by passage over a cell strainer, then centrifugation of the cells through 35% Percoll (Sigma) containing 100 U/ml of heparin (Sigma) (75).

ELISPOT assay

96-well nitrocellulose plates (Millipore, Bedford, MA) were coated overnight at 4° C with capture anti-IFNγ antibody (Mouse IFNγ ELISPOT pair, BD Biosciences), then blocked for 2 hours with RPMI, 10% FCS. RMAS-K^d cells were held overnight at room temperature, pulsed with 10^{-6} M LLO_{91-99} or $p60_{217-225}$ peptide for 2 hours, and then washed thoroughly. 10^5 peptide-pulsed RMAS cells and 1-50x10^5 splenocytes from *L. monocytogenes* -infected mice were added to wells in a total volume of 200 µL RPMI 1640 supplemented with 10 mM HEPES, 2mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin (all Sigma) (hereafter referred to as RPMI-GPPS). ELISPOT plates were incubated for 20 hours at 37° C. All wells were set up in duplicate. Plates were developed according to the manufacturer's protocol, and spots

were visualized using BCIP/NBT substrate (KPL, Inc., Gaithersburg, MD). The number of spots per well was assessed with a Zeiss Axioplan-2 microscope and Zeiss KS ELISPOT software (Göttingen, Germany).

L. monocytogenes CFU clearance assay

To evaluate bacterial clearance following primary and challenge *L. monocytogenes* infections the bacterial burdens in the spleens and/or livers was determined by *ex vivo* CFU clearance assays (187). Spleens and livers from infected mice were harvested into sterile PBS, and weighed. Tissues were homogenized mechanically using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland). Serial dilutions were made in sterile PBS and plated onto BHI agar. Plates were incubated overnight at 37° C. The $\log_{10}$ CFU burden/g of tissue was calculated as:

$$\log_{10}[(\text{CFU}/\text{DILUTION FACTOR}) \times ((\text{ORGAN WEIGHT} + \text{HOMOGENATE VOLUME})/\text{ORGAN WEIGHT})]$$

Cell surface staining, intracellular cytokine staining, and flow cytometry

Intracellular cytokine staining of $CD8^+$ T-cell populations was performed by culturing splenocytes or lymphocytes recovered from liver and lung tissues from individual mice for 5-6 hours with 10^{-6} M LLO_{91-99} or $p60_{217-225}$ peptide in the presence of 0.2 μg Brefeldin A (GolgiBlock, BD Biosciences). Cells were set up in 96-well plates in a volume of 100 μl RPMI-GPPS, 5% FCS.

Intracellular cytokine staining of $CD4^+$ T-cell populations was performed by culturing splenocytes from individual mice in the presence of 10^{-6} M $LLO_{189-200}$ or $p60_{367-378}$ peptide, or 10^7 heat-killed *L. monocytogenes* (HKLM) for 6 hours in a volume of 100 µl RPMI-GPPS, 5% FCS. For the last 4 hours of the culture period 0.2 µg of Brefeldin A was added to each well.

At the end of the culture period, cells were washed with PBS, 5% FCS, then incubated overnight at 4° C with antibodies for cell surface antigens. Cells were stained with 0.5 µg of each antibody in 50 µl of PBS, 5% FCS. The next morning, cells were fixed, permeabilized, and stained for intracellular IFNγ and/or TNFα using the Cytofix/Cytoperm kit (BD Biosciences). Following intracellular staining, cells were resuspended in PBS, 5% FCS and analyzed by flow cytometry. Controls for each individual cell population stimulated with peptide or HKLM were also analyzed following culture in the absence of antigen stimulation to determine non-specific levels of cytokines accumulated, as well as non-specific antibody staining.

Various antibodies were used in different combinations depending on the aim of each experiment. Cells were stained with FITC, PE, PE-Cy7 and/or APC-conjugated antibodies to CD8α (clone 53-8.7, BioLegend, San Diego, CA), CD8β.2 (clone 53-5.8, BioLegend), CD4 (clones RM4-5 and GK1.5, BD Biosciences), CD25 (clones PC61 and 3C7, BD Biosciences), CD62L (clone MEL-14, eBioscience, San Diego, CA), GITR (clone 108619, R&D Systems, Minneapolis, MN), THY1.1/CD90.1 (clone HIS51, eBioscience), IFNγ (clone XMG1.2, BioLegend) and TNFα (clone MP6-XT22, eBioscience). Data acquisition was performed on a BD FACSCalibur flow cytometer,

and was analyzed using CELLQuest software (BD Biosciences). A minimum of 10,000 $CD4^+$ or $CD8^+$ events was collected for each stimulation condition.

TCR avidity analysis via intracellular cytokine staining

The TCR avidity of LLO_{91-99} and $p60_{217-225}$ peptide-specific $CD8^+$ T-cell populations was determined by performing intracellular cytokine staining on splenocytes cultured *ex vivo* with a gradient of 100-0.001 ng/ml of LLO_{91-99} or $p60_{217-225}$ peptides. For individual animals, the frequency of IFNγ-producing $CD8^+$ T cells in response to each peptide concentration was determined, then standardized, setting the largest response in each animal to 100%. The avidity of each peptide-specific response was determined to be the concentration of peptide required to stimulate 50% of the potentially responsive $CD8^+$ T cells from each individual animal (188).

In vivo cytotoxicity assay

To evaluate the *in vivo* cytotoxic function of $CD8^+$ T-cell populations, clearance of peptide-loaded target cells was evaluated (189, 190). Naïve BALB/c splenocyte target cells were suspended at 10^7/ml in RPMI, and then labelled for 10 minutes with Vibrant DiI solution (Molecular Probes). Target cells were washed and equally divided into three tubes. Target cells were then labelled with 1 μM, 100 nM or 1 nM CFSE (described below). Finally, the dual-labelled cells were pulsed with 1 μM LLO_{91-99} peptide, $p60_{217-225}$ peptide, or no peptide, for 1 hour at room temperature. $5x10^6$ of each target cell population was injected intravenously into recipient mice. Animals were rested 6 hours before recipient spleens were analyzed by flow cytometry for target cell clearance. Gating on Vibrant DiI^+ cells, the percent killing was calculated as:

100% - ([(% PEPTIDE PULSED IN IMMUNE/% UNPULSED IN IMMUNE) / (% PEPTIDE PULSED IN NAÏVE/% UNPULSED IN NAÏVE)] X 100)

Hybridoma culture and antibody purification

Hybridoma clone GK1.5 (anti-CD4, rat IgG2b, ATCC), was grown in DMEM (BD Biosciences) supplimented with 2mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Cells were grown in 75 mm^2 tissue culture flasks until no longer viable, then the culture supernatants were collected by centrifugation and filtration.

Supernatants were dialyzed overnight into 20 mM sodium phosphate, pH 7.2 (Sigma). Antibodies were purified from the culture supernatants by passage over a Protein G resin (ImmunoPure Plus, Pierce Biotechnology, Rockford, IL), followed by elution with 0.1 mM Glycine-HCl, pH 2.7 (Sigma). Eluted fractions containing antibodies were identified by Bradford Assay (Bio-Rad Protein Assay, Hercules, CA) and pooled. Antibodies were then passed over a desalting column equilibrated with PBS (10DG desalting columns, Bio-Rad), and concentrated by centrifugation. Antibody concentrations were determined by OD_{280} evaluation and adjusted to 1 mg/ml in PBS. Aliquots of concentrated antibodies were filter sterilized, then stored at –20° C.

To ensure the specificity of the purified antibody, *in vitro* blocking experiments were performed. Splenocytes or thymocytes were first incubated with a gradient of concentrations of lab-purified antibody. Cells were washed, and then stained with a fluorescent-tagged, commercially available antibody of the same clone (both from BD

Biosciences). In addition, cells were stained with antibodies from a different hybridoma clone to ensure that blocking was specific. The ability of the lab-purified, unlabelled antibodies to block specific binding of fluorescent-tagged antibodies from the same clone was evaluated by flow cytometry.

In vivo depletion of cell subsets

For depletion of $CD4^+$ cells, 100 μg of purified GK1.5 hybridoma supernatants (anti-CD4) was given by intravenous injection into the lateral tail vein on days –3 and 0 relative to infection with *L. monocytogenes*. The efficiency of antibody treatment was determined by flow cytometry, and routinely resulted in >98% depletion of $CD4^+$ cells.

Positive selection of cell subsets and adoptive transfer

$CD8^+$ cells were purified by positive selection using the EasySep magnetic system according to the manufacturers protocol (StemCell Technologies, Vancouver, BC, Canada). Briefly, splenocytes were first stained with FITC-conjugated anti-CD8α antibodies (clone 53-6.7, BioLegend). Next, cells were incubated with the EasySep FITC-selection cocktail, followed by EasySep magnetic nanoparticles. The tube was placed within a magnet apparatus and incubated, allowing labelled cells coated with magnetic particles to become attracted to the magnet through the wall of the tube. The entire apparatus was lifted to pour off the supernatant, leaving the positively selected cells within the tube/magnet. Finally the tube was removed from the magnet and the positively selected cells collected from the tube. Positively selected cells were incubated for at least 30 minutes in a 37° C water bath prior to injection. The purity of enriched $CD8^+$ cells was evaluated by flow cytometry, and typically was between 90-98% $CD8^+$ cells.

Enriched $CD8^+$ cells were resuspended in RPMI 1640 (Difco), and then injected into the lateral tail vein of recipient mice in a volume of 100 μL. Recipient mice received $2.5x10^6$ – $1x10^7$ cells, depending on the experiment.

Data analysis

Data are expressed as the mean ± standard deviation, and a representative experiment is shown for each figure. Statistical probabilities were evaluated by 'Student's' t Test, with probability values of $p<0.05$ considered to be significant.

CHAPTER 3: RESULTS

Systemic infection with *L. monocytogenes* results in the activation of cellular immune responses evident within both $CD4^+$ and $CD8^+$ T-cell populations. Both classical and nonclassical MHC-I molecules restrict $CD8^+$ T-cell recognition of cognate antigen. Over the last 20 years the ability to specifically monitor peptide-specific T-cell populations during the course of infection has advanced, allowing the detailed characterization of T-cell responses to *L. monocytogenes* in murine models of infection. In mice, both $CD4^+$ and $CD8^+$ T-cell populations contribute to the control and clearance of this intracellular bacterium, yet the classically restricted $CD8^+$ T-cell population is considered to be responsible for complete pathogen clearance (118, 120). Following a primary *L. monocytogenes* infection, the generation and maintenance of long-lived memory $CD8^+$ T cells provides protection against subsequent infections with *L. monocytogenes* (121). After decades of research on *L. monocytogenes*, a large array of information has been collected from many investigators, making infection with *L. monocytogenes* a valuable model for discerning the elements that contribute to the development and expression of cellular immune responses.

The initiation of cellular immune responses has been determined to depend on T-cell stimulation by activated dendritic cells (DC). One accepted mechanism that provides such activation includes the ligation of the CD40 receptor on DC with CD40L as expressed on activated $CD4^+$ T cells. This CD40-CD40L interaction results in the maturation of DC to an activated state capable of priming naive $CD8^+$ T-cell populations, and is considered to be a significant mechanism by which $CD4^+$ T-cells provide help to

$CD8^+$ T-cell responses (42, 47, 48). Dendritic cells are required for the initial priming of antilisterial $CD8^+$ T cells, however the development of a protective, memory $CD8^+$ T-cell population following systemic *L. monocytogenes* infection is not dependent on CD40-CD40L interactions (99, 124, 125). Thus, investigators have rationalized that the inflammatory response and/or direct TLR ligation from bacterial components during *Listeria* infection bypasses this CD40-mediated mechanism of APC maturation. This premise is based on the assumption that all potential maturation signals that might be experienced by a DC feed into a common DC maturation program, which in turn, leads to identical activation of cellular immune responses. These presumptions have not been validated experimentally.

Since stimulation of the immune response to *L. monocytogenes* can occur in the absence of direct CD40 ligation, the question of additional roles for the CD4+ T-cell subset have been considered by a number of investigators. For example, several groups have reported that the magnitude of $CD8^+$ T-cell priming following systemic infection with the intracellular bacterium *L. monocytogenes* is either unchanged or slightly decreased in the absence of $CD4^+$ T cells, however, the primary effector functions of these "unhelped" $CD8^+$ T cells are not compromised (40, 156, 157). Yet, although $CD4^+$ T cells are not required for the primary $CD8^+$ T-cell response to *L. monocytogenes*, it is unclear whether their presence at the time of priming may have a downstream influence on the functional properties of memory $CD8^+$ T cells. It is possible that memory $CD8^+$ T-cell populations generated in the absence of CD40-CD40L signals and/or $CD4^+$ T cells may be functionally compromised.

Recent studies have revealed that memory $CD8^+$ T-cell populations generated in either $CD4^{-/-}$ or MHC-$II^{-/-}$ mice (devoid of $CD4^+$ T cells) are lost over time and become functionally impaired (93-95). In these experiments, the $CD4^+$ T-cell population was continually absent, thus it is difficult to interpret whether the deficiency in $CD8^+$ memory T-cell function is due to a missing $CD4^+$ T-cell dependent signal during $CD8^+$ T-cell priming. Alternatively, the maintenance of highly functional memory $CD8^+$ T cells may be dependent on signals from $CD4^+$ T-cell populations at later stages of the lifespan of the memory $CD8^+$ T-cell population.

Many questions remain as to the role of CD40-CD40L interactions specifically, as well as the influence of $CD4^+$ T cells in general, on long-term $CD8^+$ T-cell memory maintenance and function. For expample, are $CD8^+$ T cells functionally "imprinted" during their primary response to antigen to allow their subsequent conversion into long-lived, memory $CD8^+$ T-cell pools? Is this "imprinting" achieved specifically by the presence of CD40-CD40L signals during $CD8^+$ T-cell priming? Alternatively, does the absence of $CD4^+$ T-cell help influence not the generation, but the <u>maintenance</u> of memory $CD8^+$ T-cell populations?

The extensive knowledge of the antilisterial T-cell response that occurs in wildtype mice provides an ideal environment for the evaluation of these questions regarding the specific role of CD40-CD40L interactions, as well the general need for $CD4^+$ T cell help, on the generation and maintenance of antilisterial memory $CD8^+$ T-cell populations. As memory $CD8^+$ T cells can provide protection against secondary *L. monocytogenes* challenge infection in the absence of $CD4^+$ T_H cells and CD40-CD40L signals (40, 87, 144, 191), the *Listeria* infection model is particularly suited for assessing

the specific contributions of CD40 expression in combination with transient $CD4^+$ T-cell depletions at different stages of the cellular immune response.

Utilizing the *Listeria* infection model in wildtype and $CD40^{-/-}$ BALB/c mice I have evaluated some of the parameters that define functional memory $CD8^+$ T cells: secondary expansion to cognate antigen, TCR avidity, effector cytokine production, protection against challenge infection, and *in vivo* killing of antigen-bearing target cells. In addition, I have determined the impact of transient CD4-depletion administered prior to either the primary or secondary *Listeria* infection in wildtype and $CD40^{-/-}$ BALB/c mice. This approach has allowed the differential evaluation of a role for $CD4^+$ T-cells and CD40-mediated signals during priming of naive $CD8^+$ T cells compared with their influence during recall responses by antigen-experienced memory $CD8^+$ T-cell populations. These studies provide further evidence that neither CD40-CD40L interactions nor the presence of $CD4^+$ T cell populations during priming is required for the generation of highly functional memory $CD8^+$ T-cells in response to systemic infection with *L. monocytogenes.* These studies also suggest that the presence of $CD4^+$ T-cells, particularly the $CD4^+$ $CD25^+$ regulatory T-cell population regulates the magnitude of secondary $CD8^+$ T-cell expansion in response to *Listeria* challenge.

3.1 PRIMARY T-CELL RESPONSES TO SYSTEMIC L. MONOCYTOGENES INFECTION: THE INFLUENCE OF CD40 EXPRESSION AND $CD4^+$ T CELLS

CD40-deficiency influences the kinetics but not the magnitude of primary CD8+ T-cell responses to L. monocytogenes

The development of $CD8^+$ T-cell responses to infection follows a conserved kinetic pattern of three phases. Following *L. monocytogenes* infection, this process appears as an initial expansion of pathogen-specific $CD8^+$ effector T cells which reach a peak number in the spleen by days 7-8 post-infection. These cells then undergo a contraction phase with ~75% of antigen-specific $CD8^+$ T cells being eliminated from the spleen by day 10. By day 30 following infection, $CD8^+$ T cells have completed the contraction phase, resulting in the generation of a stably maintained pool of resting memory $CD8^+$ T cells that constitute 5-10% of the number of $CD8^+$ T cells present during the peak of the primary response (10, 11, 13).

In BALB/c mice ($H\text{-}2^d$), the $CD8^+$ T-cell response to systemic infection with *L. monocytogenes* is dominated by cells specific for the $H\text{-}2K^d$-presented $LLO_{91\text{-}99}$ epitope, a peptide derived from the secreted virulence factor listeriolysin O (122, 123). An additional $CD8^+$ T-cell response specific for the $p60_{217\text{-}225}$ epitope, derived from the mureine hydrolase protein, is also readily detected (123). To evaluate the contribution of CD40 to the generation of functional $CD8^+$ effector T-cell populations, the primary $CD8^+$ T-cell responses to the $LLO_{91\text{-}99}$ and $p60_{217\text{-}225}$ epitopes were evaluated in wildtype and $CD40^{-/-}$ BALB/c mice following systemic infection with *L. monocytogenes*. Throughout the expansion, peak, and contraction phases of the primary T-cell response, the number of $LLO_{91\text{-}99}$ and $p60_{217\text{-}225}$-specific $CD8^+$ T cells were enumerated by ELISPOT analysis to detect IFNγ production in response to peptide stimulation. Although the total number of peptide-specific $CD8^+$ T cells generated in wildtype and $CD40^{-/-}$ BALB/c mice was

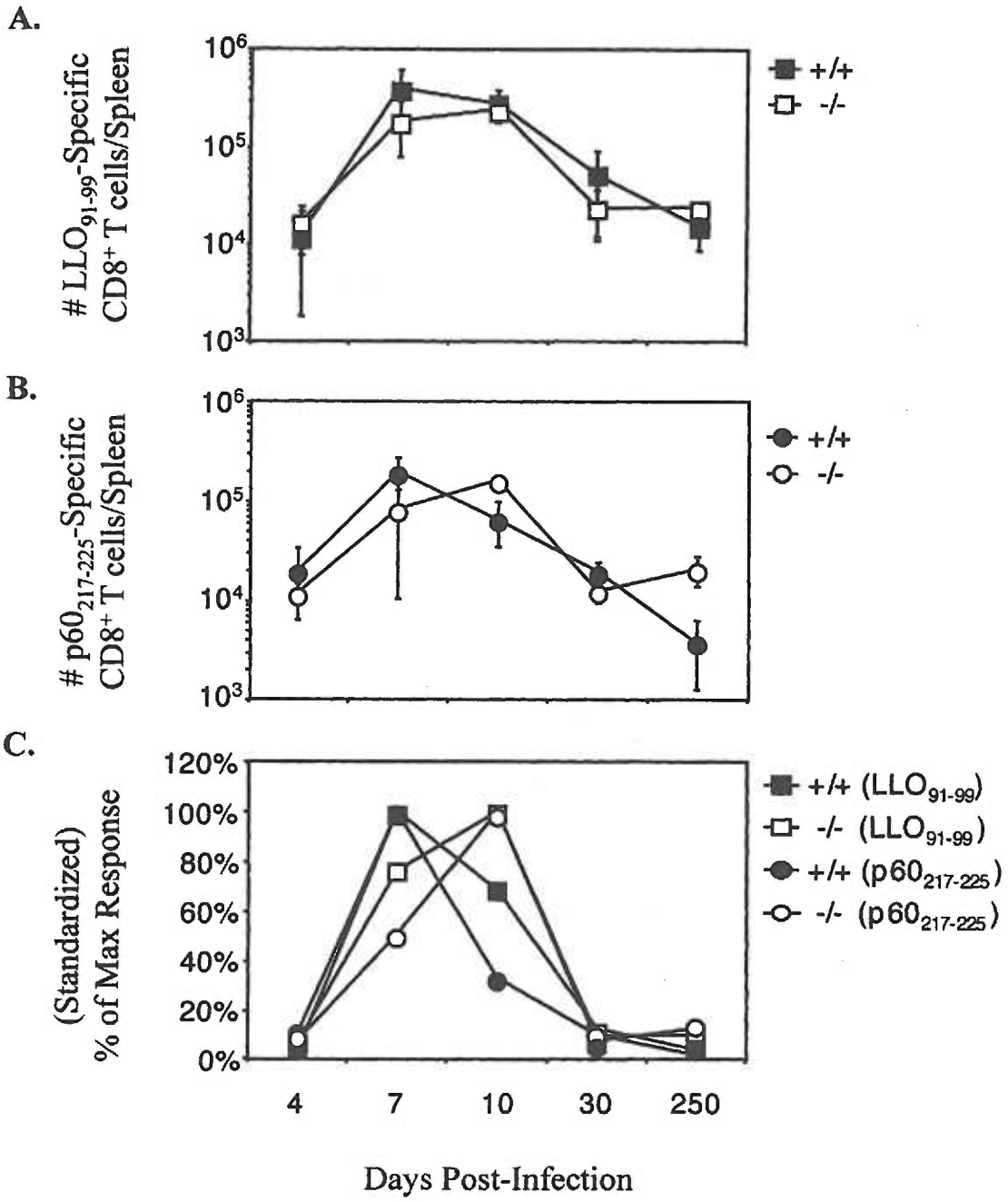


Figure 2. CD40-deficiency influences the kinetics, but not the magnitude of CD8$^+$ T-cell priming to *L. monocytogenes*. Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *Listeria* and the number of (A) LLO_{91-99}-specific and (B) $p60_{217-225}$-specific CD8$^+$ T cells per spleen was evaluated by ELISPOT on days 4, 7, 10, 30 and 250 for IFNγ production. (C) To evaluate the kinetics of the response, the number of peptide-specific CD8$^+$ T cells in each mouse strain was standardized, normalizing the numbers at each time point against the peak (100%). Data represents 1 of 2 experiments with 2-3 mice/group/timepoint.

similar following infection with *L. monocytogenes,* $CD40^{-/-}$ mice showed a delay in the primary expansion of antilisterial $CD8^+$ T-cell populations. At day 7 post-infection wildtype BALB/c mice contained an average of $4.05x10^5$ LLO_{91-99}-specific $CD8^+$ T cells per spleen, compared with $2.44x10^5$ LLO_{91-99}-specific $CD8^+$ T cells at day 10 in $CD40^{-/-}$ mice (Fig. 2A). Although the peak numbers of $p60_{217-225}$-specific $CD8^+$ T cells were comparable between wildtype and $CD40^{-/-}$ mice ($1.98x10^5$ vs. $1.63x10^5$, respectively), the response was similarly delayed in $CD40^{-/-}$ mice, such that the peak occurred three days later than in wildtype BALB/c mice (Fig. 2B).

To compare the kinetics of the $CD8^+$ T-cell responses, the number of LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T cells was determined for each mouse strain along all time points, then each value was standardized with the peak magnitude in each mouse set at 100% (Fig. 2C). This analysis showed a marked difference between the two groups. While wildtype animals reached the peak of both LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cell responses at day 7 following primary *L. monocytogenes* infection, this was delayed in $CD40^{-/-}$ mice until day 10. However, by day 30 following infection, both populations had contracted to a similar frequency in both wildtype and $CD40^{-/-}$ mice (~8-12% of the peak). When the maintenance of the memory $CD8^+$ T-cell populations was evaluated 250 days after primary *L. monocytogenes* infection, no differences were seen in cells specific for LLO_{91-99}, while $p60_{217-225}$-specific $CD8^+$ T-cells appeared to be better maintained in $CD40^{-/-}$ mice. These results suggest that, although a delay in the expansion and contraction kinetics of the $CD8^+$ T-cell response is evident in $CD40^{-/-}$ mice compared with wildtype animals, the number of antigen-specific memory $CD8^+$ T cells generated appears to be normal. Additionally, both animals maintained approximately 10% of the

peak magnitude, a frequency consistent with what is typically maintained as long-lived memory $CD8^+$ T cells (10, 11, 13). Collectively, these data confirm that both the magnitude of the primary $CD8^+$ T-cell response to *L. monocytogenes*, and the maintenance of long-lived memory $CD8^+$ T-cell populations are not impaired in $CD40^{-/-}$ mice.

Primary antilisterial effector $CD8^+$ T-cells display normal function in $CD40^{-/-}$ mice

To determine if the absence of CD40 influences the clearance of *L. monocytogenes* following primary acute infection, the bacterial load in individual mice was assessed throughout the primary *Listeria* infection by CFU clearance assays. As shown in Figure 3A, the number of bacteria recovered from infected mice was comparable between wildtype and $CD40^{-/-}$ BALB/c hosts at all timepoints. By day 10 following infection, no bacteria could be detected in either mouse strain (data not shown). These results are in agreement with previously published work, indicating that CD40-CD40L interactions are not required for the development of the immune response able to resolve a primary systemic *L. monocytogenes* infection (40, 76, 150).

To evaluate the cytolytic activity of effector cells generated in the absence of CD40 following primary infection with *L. monocytogenes, in vivo* cytotoxicity assays were performed in wildtype and $CD40^{-/-}$ BALB/c mice (189, 190). On day 7 after primary *Listeria* infection, mice were infused with target cells coated with either LLO_{91-99} or $p60_{217-225}$ peptides, and the clearance of these target cells from the spleen was evaluated 6 hours later by flow cytometry. As shown in Figure 3B, no significant

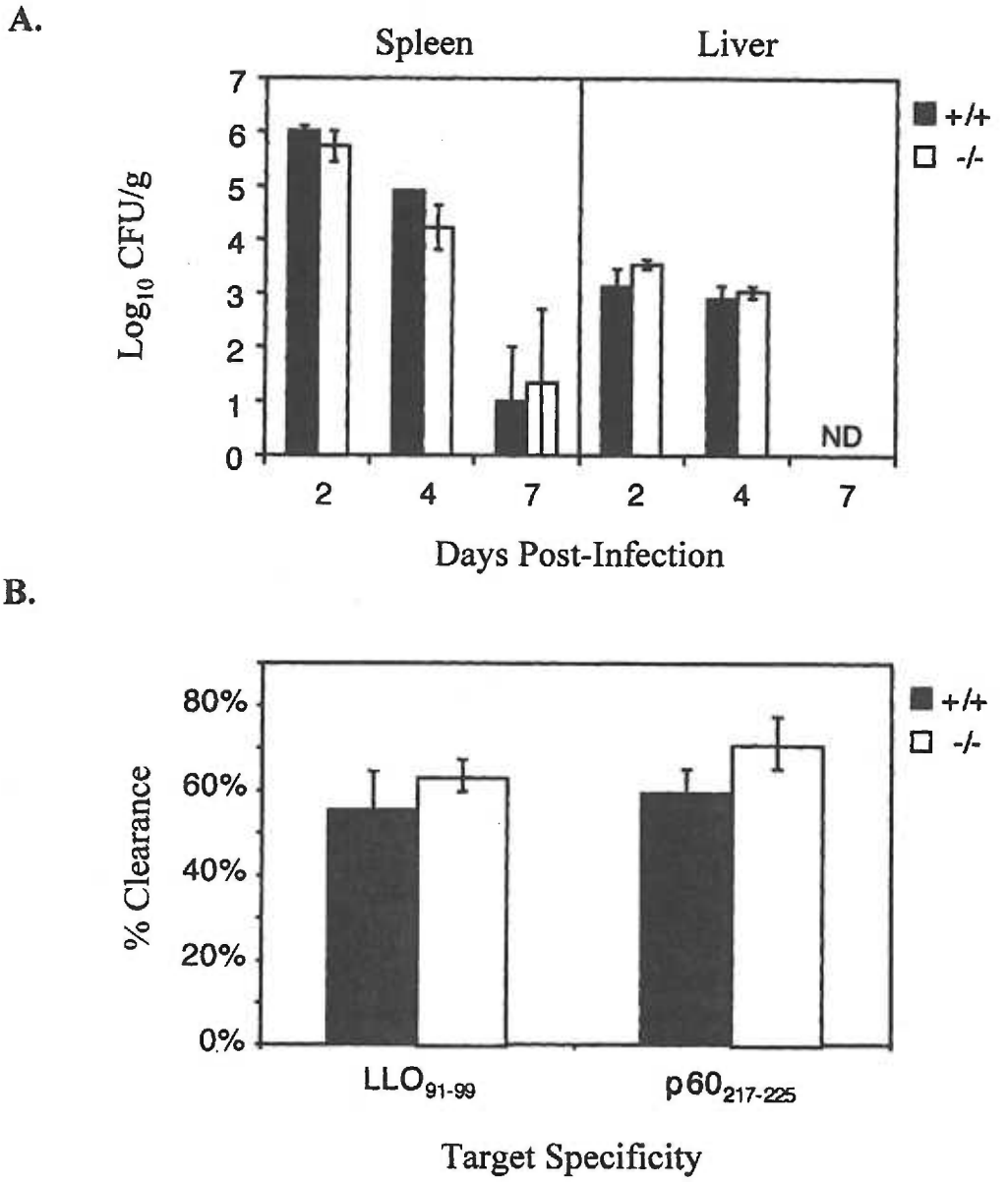


Figure 3. CD40$^{-/-}$ mice show normal cytotoxic function following primary *L. monocytogenes* infection. Wildtype (+/+) and CD40-deficient mice (-/-) were infected with *Listeria*. (A) Bacterial loads in the spleen and liver were determined by CFU clearance assays on days 2, 4, and 7 following infection, and are reported as the $\log_{10}$ number of CFU per gram of tissue. Data represents 1 of 2 experiments with 2 mice/group/timepoint. (ND = none detected). (B) *In vivo* killing assays were performed to determine the ability of CD8$^+$ primary effector T cells to clear $LLO_{91\text{-}99}$ or $p60_{217\text{-}225}$-coated target cells on day 7 after primary *Listeria* infection. Data represents 1 of 3 experiments with 2-3 mice/group.

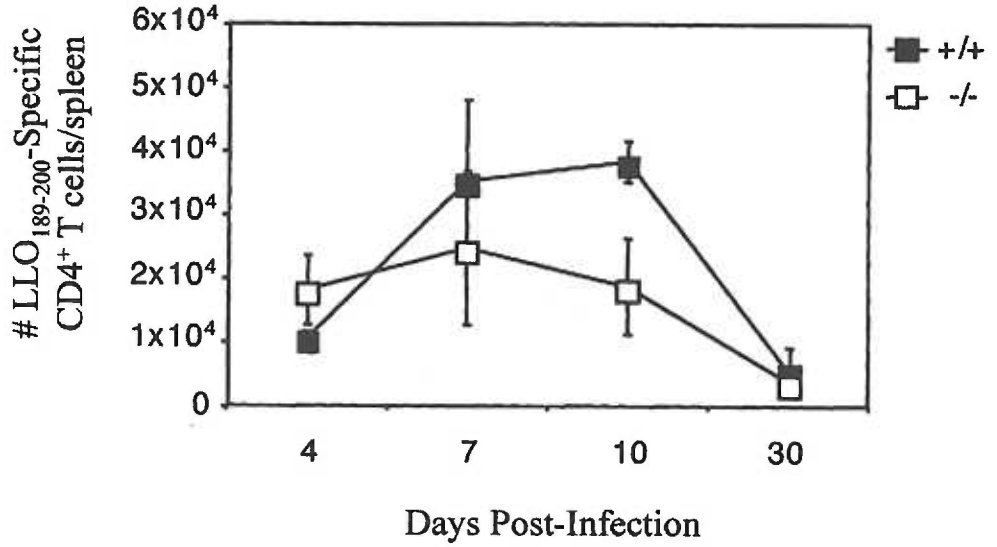
differences were found in the ability of wildtype or $CD40^{-/-}$ mice to clear either target populations. For the LLO_{91-99} coated target cell population, 55.2% clearance was evident in wildtype BALB/c mice compared with 63.2% clearance in $CD40^{-/-}$ mice at 6 hours following target cell transfer. The clearance of $p60_{217-225}$ coated target cells was also similar between mouse strains, with 59.2% vs. 71.1% clearance seen in wildtype and $CD40^{-/-}$ BALB/c mice, respectively. Taken together, these experiments indicate that $CD40^{-/-}$ mice are capable of generating functional effector $CD8^+$ T cells functionally equivalent to those generated in wildtype BALB/c mice following primary *L. monocytogenes* infection.

CD40-deficiency impairs the priming of $CD4^+$ T cells following primary L. monocytogenes infection

The absence of CD40-CD40L interactions impairs $CD4^+$ T-cell responses to LCMV and adenovirus infections (69, 72). In contrast, systemic infection of $CD40L^{-/-}$ C57BL/6 mice with *L. monocytogenes* leads to the normal upregulation of the CD44 activation marker on total $CD4^+$ T-cell populations (40). However, the ability to fully activate naive antigen-specific $CD4^+$ T cells to the effector state was not evaluated in these experiments. Thus, the influence of CD40-CD40L interactions on the priming of antigen-specific $CD4^+$ T cells into functional effector populations following systemic *L. monocytogenes* infection remains unclear.

To address this question, the expansion and contraction of $LLO_{189-200}$ specific $CD4^+$ T_H1 cells in the spleen was evaluated following primary *L. monocytogenes* infection in wildtype and $CD40^{-/-}$ BALB/c mice. ELISPOT analysis was used to detect

A.



B.

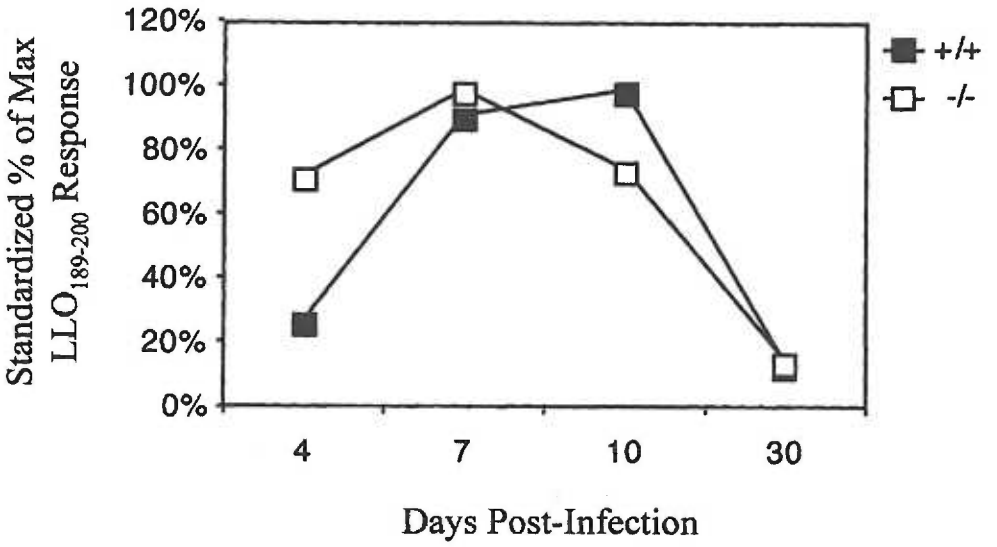


Figure 4. CD40-deficiency impairs CD4⁺ T-cell priming to *L. monocytogenes*. Wildtype (+/+) and CD40-deficient mice (-/-) were infected with *Listeria* and (A) the number of $LLO_{189-200}$-specific $CD4^+$ T cells per spleen was determined by ELISPOT analysis for IFNγ production on days 4, 7, 10, and 30 following infection. (B) To evaluate the kinetics of the response, the number of $LLO_{189-200}$-specific $CD4^+$ T cells in each mouse strain was standardized, normalizing the numbers at each time-point against the peak (100%). Data represents 1 of 2 experiments with 2-3 mice/group/timepoint.

IFNγ production in response to peptide stimulation directly *ex vivo*. As indicated in Figure 4A, $CD40^{-/-}$ mice were capable of priming peptide-specific $CD4^+$ T cells, albeit at lower numbers than observed in wildtype mice. At day 7 after primary infection, $CD40^{-/-}$ mice contained an average of $2.5x10^4$ $LLO_{189-200}$-specific $CD4^+$ T cells per spleen, compared with $3.5x10^4$ specific $CD4^+$ T cells in wildtype BALB/c mice. At day 10, this difference was even more pronounced, with $CD40^{-/-}$ mice averaging $1.9x10^4$ $LLO_{189-200}$-specific $CD4^+$ T cells per spleen, compared with $3.8x10^4$ specific $CD4^+$ T cells in wildtype BALB/c mice. However, by day 30 following infection, differences in the number of $LLO_{189-200}$-specific $CD4^+$ T cells were no longer apparent between wildtype and $CD40^{-/-}$ BALB/c mice.

When the $CD4^+$ T-cell responses were standardized relative to the peak within each animal, it was evident that the kinetics of the $LLO_{189-200}$-specific $CD4^+$ T-cell population – as observed for the $CD8^+$ T-cell response – were influenced by the absence of CD40-expression. By day 10, $CD40^{-/-}$ BALB/c mice showed signs of contraction within the $LLO_{189-200}$-specific $CD4^+$ T-cell population, with only 75% of the peak number of cells remaining (Fig. 4B). In wildtype mice, no significant change in the number of $LLO_{189-200}$-specific $CD4^+$ T cells was evident between days 7 and 10, suggesting a later onset of contraction in wildtype BALB/c mice expressing CD40. These results indicate that, both the magnitude and the kinetics of primary $CD4^+$ T-cell expansion following systemic *L. monocytogenes* infection are altered in $CD40^{-/-}$ mice. However, the reduced primary $CD4^+$ T-cell response in $CD40^{-/-}$ mice results in the generation of $LLO_{189-200}$-specific memory $CD4^+$ T-cell populations at day 30 after *Listeria* infection at numbers equivalent to those observed in wildtype BALB/c mice. Thus, during *L. monocytogenes*

infection, CD40-CD40L interactions are not a requirement for $CD4^+$ T-cell activation or the generation of memory $CD4^+$ T cells.

CD4-depletion prior to Listeria infection does not impair the primary $CD8^+$ T-cell response

The primary CTL response to many bacterial and viral infections, including systemic infection with *L. monocytogenes*, appears to be independent of both $CD4^+$ T_H cells and CD40-CD40L interactions (40, 75, 76). To confirm these results in our model system, I first evaluated whether transient depletion of $CD4^+$ T cells influences the priming of peptide-specific $CD8^+$ T cells in response to *Listeria* infection. Wildtype and $CD40^{-/-}$ BALB/c mice were left untreated, or treated with CD4-depleting monoclonal antibodies on days –3 and 0 relative to primary infection with *L. monocytogenes*. On days 5 and 8 following infection, the number of splenic $p60_{217-225}$-specific $CD8^+$ T-cells was determined by intracellular cytokine staining for IFNγ (Fig. 5). At both time points no significant differences were detectable between any of the animal groups, indicating that the absence of $CD4^+$ T-cells during primary *Listeria* infection does not impair the priming of classically restricted antilisterial $CD8^+$ T-cell populations.

As $CD4^+$ T cells have been shown to contribute to the clearance of *L. monocytogenes* in MHC-$I^{-/-}$ mice devoid of $CD8^+$ T-cell populations (144), it was possible that CD4-depletion during the primary infection might alter the rate of pathogen clearance in these mice. To address this possibility wildtype and $CD40^{-/-}$ BALB/c mice were left untreated, or treated with CD4-depleting antibodies on days –3 and 0 relative to primary infection with *L. monocytogenes*. On days 4 and 7 after infection, the bacterial

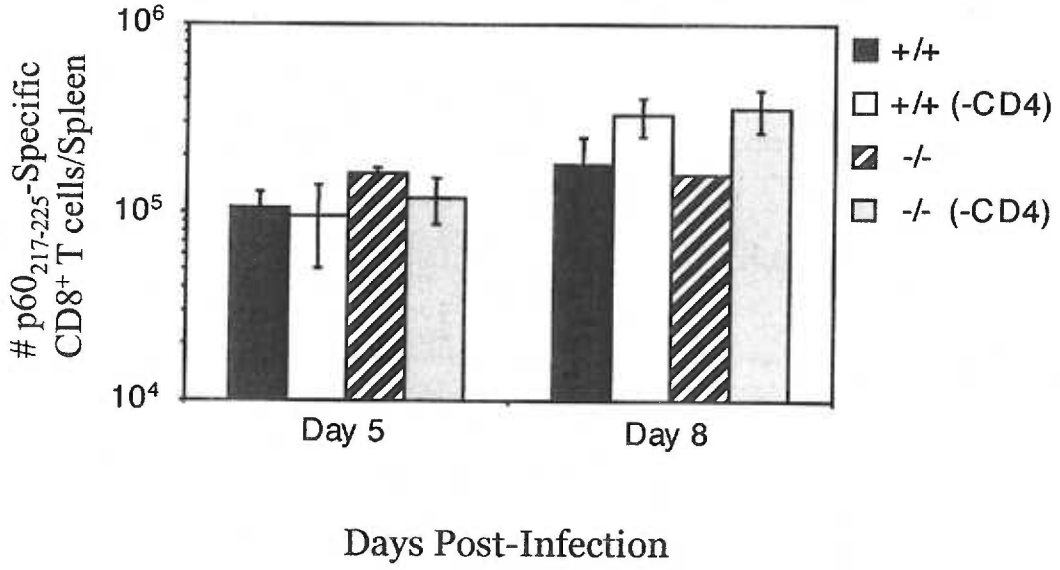


Figure 5. CD4-depletion during primary *Listeria* infection does not impair the primary CD8$^+$ T-cell response. CD4-depleted and untreated wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *Listeria* and (A) the number of $p60_{217-225}$-specific CD8$^+$ T cells per spleen was determined by intracellular cytokine staining for IFNγ production on days 5 and 8 following infection. Data represents 1 of 2 experiments with 2 mice/group/timepoint.

burdens in the spleen and liver were evaluated by CFU clearance assays (Fig. 6). In neither wildtype nor $CD40^{-/-}$ mice did the depletion of $CD4^+$ cells influence the number of bacteria present in either the spleen or liver. By day 10 following infection, bacteria could no longer be detected in any experimental group (data not shown). Collectively, these data confirm that the absence of $CD4^+$ T cells during the primary immune response to *L. monocytogenes* does not impair the priming of pathogen-specific $CD8^+$ T-cell populations or the clearance of bacteria.

Nonclassical H2-M3-restricted $CD8^+$ T-cell priming in response to Listeria infection in not dependent on CD40-expression or $CD4^+$ T cells

Very little data are available regarding the specific requirements for MHC-Ib-restricted $CD8^+$ T-cell priming and memory generation. Similar to classical MHC-Ia-restricted $CD8^+$ T-cells, a subset of peptide-specific MHC-Ib-restricted $CD8^+$ T cells are activated in response to systemic infection with *L. monocytogenes* (135, 136). Following the resolution of *Listeria* infection, H2-M3-restricted $CD8^+$ T cells specific for the f-MIGWII(A) peptide are evident in the memory population. In contrast to classically restricted memory $CD8^+$ T cells, the H2-M3-restricted memory $CD8^+$ T-cell populations that develop following *L. monocytogenes* infection do not show significant *in vivo* expansion upon secondary antigen exposure, a hallmark of classical MHC-Ia-restricted memory $CD8^+$ T cells (136, 137, 141). The signals required for the activation of MHC-Ib-restricted $CD8^+$ T cells have not been extensively characterized, and it is unknown whether CD40-CD40L interactions are involved with the activation of nonclassical $CD8^+$

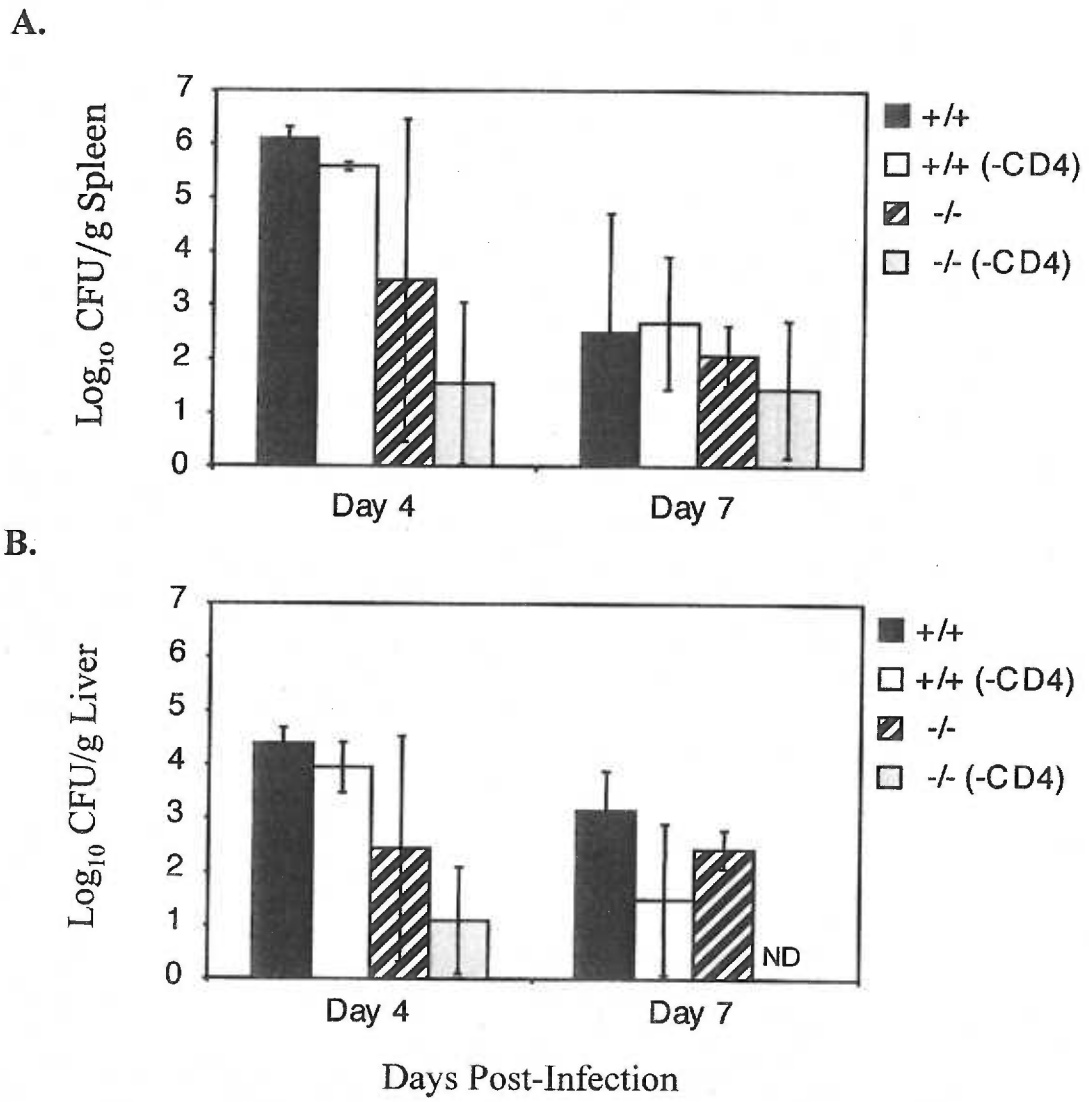


Figure 6. CD4-depletion during primary *Listeria* infection does not influence bacterial clearance. CD4-depleted and untreated wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *Listeria* and the number of bacteria in the (A) spleen and (B) liver was evaluated by CFU clearance assays on days 4 and 7 following infection. (ND = none detected). Data represents 1 of 2 experiments with 2 mice/group/timepoint.

T cells. Further, whether $CD4^+$ T-cell help influences the generation of MHC-Ib restricted $CD8^+$ T cell responses to *L. monocytogenes* infection has not been determined.

To determine the specific influences of both CD40-expression and $CD4^+$ T-cell help on the priming on nonclassical $CD8^+$ T cells following *Listeria* infection the priming of H2-M3-restricted f-MIGWII-specific $CD8^+$ T cells was evaluated. Wildtype and $CD40^{-/-}$ BALB/c mice were either left untreated, or depleted of $CD4^+$ cells prior to infection with *L. monocytogenes*. On days 5 and 8 after infection, the number of f-MIGWII-specific $CD8^+$ T cells in the spleen was determined by intracellular cytokine staining for IFNγ (Fig. 7). Although variation between the groups was evident, at neither time point was the total number of f-MIGWII-specific $CD8^+$ T cells in the spleen significantly influenced by CD40-deficiency and/or CD4-depletion relative to wildtype BALB/c mice (Fig. 7A). These results suggest that, similar to MHC-Ia-restricted $CD8^+$ T cells, neither the presence of CD40-CD40L interactions specifically, nor the presence of $CD4^+$ T-cell populations in general is required for the priming of MHC-Ib-restricted $CD8^+$ T cells.

To evaluate the kinetics of the f-MIGWII-specific $CD8^+$ T-cell response in untreated and CD4-depleted wildtype and CD40-deficient BALB/c mice, the number of peptide-specific $CD8^+$ T cells was determined for each mouse strain at days 5 and 8 following infection, and then all values were standardized relative to the peak value occurring at day 5 in each mouse (Fig. 7B). By day 8 in untreated wildtype BALB/c mice, the f-MIGWII-specific $CD8^+$ T-cell population has contracted to 6.4% of the peak response observed at day 5. In contrast, BALB/c mice depleted of $CD4^+$ cells prior to *Listeria* infection showed very little contraction of the f-MIGWII-specific $CD8^+$ T-cell

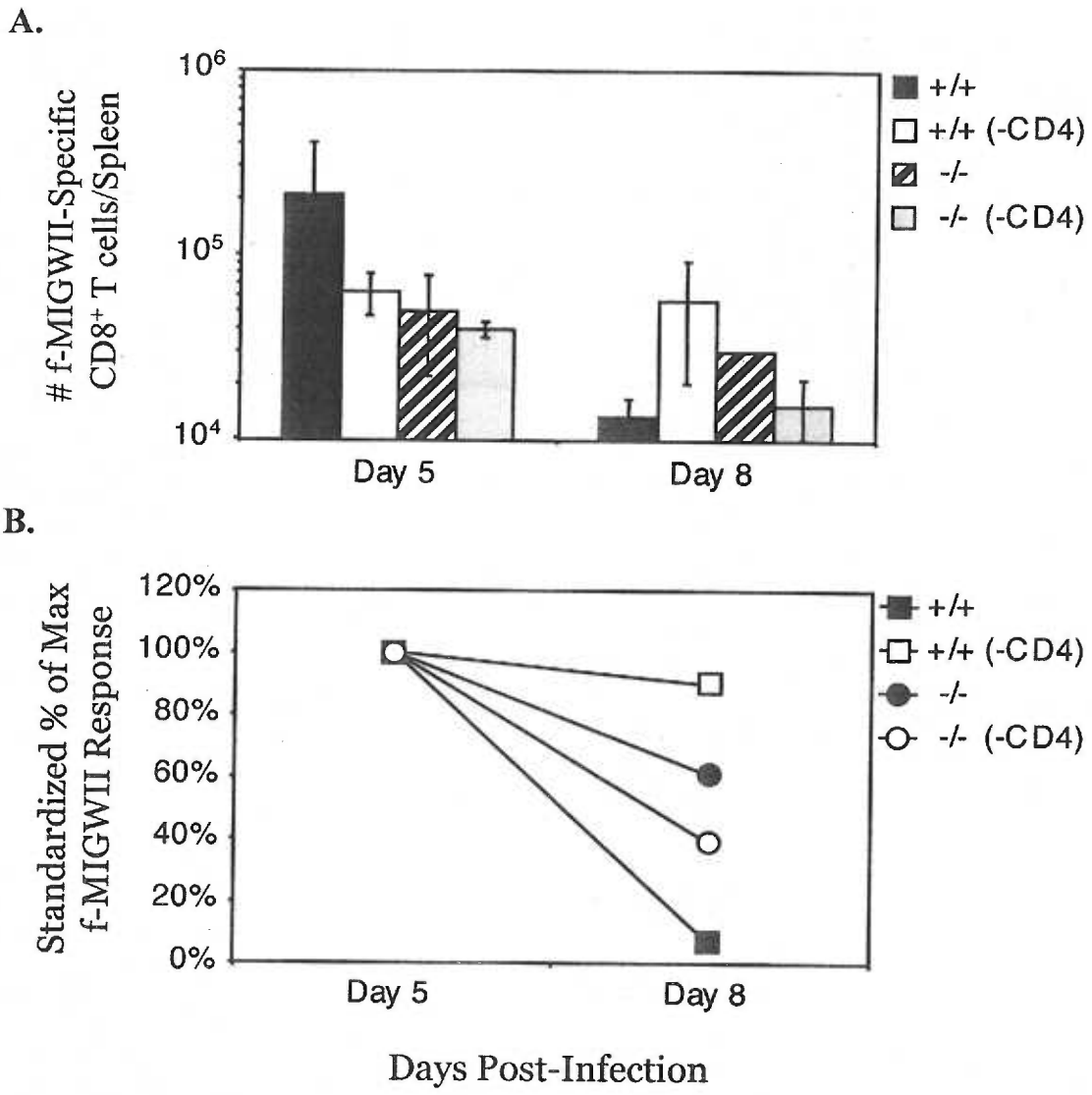


Figure 7. CD4-depletion and CD40-deficiency influence the kinetics but not the magnitude of primary H2-M3-restricted CD8$^+$ T-cell responses to *Listeria*. CD4-depleted and untreated wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *Listeria* and (A) the number of f-MIGWII-specific CD8$^+$ T cells per spleen was determined by intracellular cytokine staining for IFNγ production on days 5 and 8 following infection. Data represents 1 of 3 experiments with 2-3 mice/group/timepoint. (B) To evaluate the contraction kinetics, the number of f-MIGWII-specific CD8$^+$ T cells at day 8 was standardized against the peak number at day 5 post-infection (100%).

population, with 89.8% of the peak value still evident in the spleen by day 8 post-infection. Both untreated and CD4-depleted $CD40^{-/-}$ mice showed intermediate levels of contraction relative to the wildtype BALB/c mice. $CD40^{-/-}$ mice contained 60.5% of their peak numbers at day 8, while CD4-depleted $CD40^{-/-}$ mice contained 38.5%. These results suggest that both costimulation through CD40-CD40L and other interactions with the $CD4^+$ T-cell subset may influence the kinetics of nonclassically restricted $CD8^+$ T-cell responses to infection with *L. monocytogenes*. However, f-MIGWII specific $CD8^+$ T cells are activated and able to secrete IFNγ in the absence of both CD40 expression and $CD4^+$ T-cell populations, demonstrating that these events are also not required for the priming of MHC-Ib restricted $CD8^+$ T cells to *Listeria* antigens.

Summary to Results Section 3.1

In these studies I have evaluated the influence of both $CD4^+$ T cells and CD40 expression on the primary activation and response kinetics of $CD4^+$ T-cell populations, as well as $CD8^+$ T-cell populations restricted by both classical ($H\text{-}2K^d$) and nonclassical (H2-M3) MHC-I molecules following systemic infection with *L. monocytogenes*. These data demonstrate that both $CD4^+$ and $CD8^+$ T-cell responses are generated in the absence of CD40-CD40L interactions, indicating that CD40 expression is not required for the initiation of cellular immunity to *Listeria*. Further, these data indicate that both classical and nonclassical primary $CD8^+$ T-cell responses can also occur in the absence of $CD4^+$ T-cell help.

Classically restricted $CD8^+$ T-cell responses are mostly unaffected by the absence of both CD40 expression and $CD4^+$ T cells during *Listeria* infection. $CD40^{-/-}$ BALB/c

mice show equivalent peak numbers of both LLO_{91-99} and $p60_{217-225}$-restricted primary $CD8^+$ T-cell populations relative to wildtype BALB/c mice. These effector CTL generated in the absence of $CD4^+$ T-cell help or CD40-CD40L interactions display normal cytolytic function as evident by equivalent clearance of both viable bacteria and peptide-coated target cells in CD40-deficient and CD4-depleted BALB/c mice. However, the kinetics of the expansion of MHC-Ia-restricted $CD8^+$ T cells are delayed in $CD40^{-/-}$ mice, with peak numbers of peptide-specific $CD8^+$ T cells taking longer to develop (day 10, compared with day 7 in wildtype BALB/c mice). This may be due to a slower rate of $CD8^+$ T-cell division in the absence of CD40-CD40L interactions, or perhaps reflects a delay in the onset on the contraction phase. How cells transition from the proliferative response into the contraction phase is unknown. Thus, although CD40-CD40L interactions specifically, or $CD4^+$ T-cell help in general are <u>not required</u> for the primary activation of antilisterial $CD8^+$ T cells, the kinetics of the $CD8^+$ T-cell responses are influenced by the absence of CD40 expression.

Similar to the classical $CD8^+$ T-cell response, nonclassical f-MIGWII-specific $CD8^+$ T-cell expansion is not impaired by the absence of CD40 expression or $CD4^+$ T-cells during the primary immune response to *L. monocytogenes*. H2-M3-restricted f-MIGWII specific $CD8^+$ T cells are activated and able to secrete IFNγ in the absence of both CD40 expression and $CD4^+$ T-cell populations, demonstrating that these events are not required for the priming of MHC-Ib restricted $CD8^+$ T cells to *Listeria* antigens. However, both costimulation through CD40-CD40L and other mechanisms of $CD4^+$ T cell-mediated help influence the kinetics of nonclassically restricted $CD8^+$ T-cell responses to infection with *L. monocytogenes*.

In contrast to both classical and nonclassical $CD8^+$ T-cell populations, the magnitude of the $LLO_{189-200}$-specific $CD4^+$ T cell response is dramatically reduced in the absence of CD40. Further, following systemic *L. monocytogenes* infection, the kinetics of the primary $LLO_{189-200}$-specific $CD4^+$ T-cell response is altered in $CD40^{-/-}$ BALB/c mice, with the onset of contraction significantly delayed relative to wildtype BALB/c mice. These data suggest that the absence of CD40 expression may have a greater impact on $CD4^+$ T-cell responses than on $CD8^+$ T-cell responses to *Listeria*.

For all T-cell populations evaluated, neither CD40-deficiency nor the absence of $CD4^+$ T cells impairs effector function. In $CD40^{-/-}$ BALB/c mice, both $CD4^+$ and $CD8^+$ T cells produce IFNγ, and classically restricted $CD8^+$ T-cell populations efficiently clear target cells. Further, the absence of $CD4^+$ T cells and/or CD40-expression has no effect on the clearance of *Listeria* from infected mice. By day 30 following *L. monocytogenes* infection the number of peptide-specific cells is equivalent between wildtype and $CD40^{-/-}$ BALB/c mice, for both $CD4^+$ and classically restricted $CD8^+$ T-cell subsets. Collectively, these data indicate that there is no requirement for the presence of either $CD4^+$ T-cell help in general, or CD40-expression specifically, for the activation of primary cellular immune responses following systemic infection with *L. monocytogenes*.

3.2 CD8+ T-CELL MEMORY FOLLOWING L. MONOCYTOGENES INFECTION: THE INFLUENCE OF CD40 EXPRESSION AND CD4+ T-CELLS

CD40-deficiency does not influence the phenotype of resting memory CD8$^+$ T cells in Listeria-immune mice

Antigen-experienced memory CD8$^+$ T cells can be organized phenotypically into two groups by their surface expression of CD44, CD62L and the IL-7Rα chain (CD127). "Effector memory" CD8$^+$ T cells (T_{EM}, defined as CD44$^+$ CD62L$^-$ CD127$^+$) resemble the primary effector cells generated following the initial T-cell response, and lack the lymph node homing receptors CD62L and CCR7, which restricts their migration primarily to peripheral tissues. In contrast "central memory" CD8$^+$ T cells (T_{CM}; defined as CD44$^+$ CD62L$^+$ CD127$^+$) express both CD62L and CCR7, allowing their migration into secondary lymphoid organs. Although there remains much controversy over whether there are functional differences between T_{CM} and T_{EM} populations, they both differ from primary effector (T_E) CD8$^+$ T cells in that they survive the contraction phase following primary T-cell activation and are maintained together as the long-lived memory population. As shown in Figure 2, the absence of CD40 does not impair the generation of long-lived memory CD8$^+$ T-cell populations following systemic infection with *L. monocytogenes.* Relative to wildtype animals, CD40$^{-/-}$ BALB/c mice possess equal or greater numbers of antigen-specific memory CD8$^+$ T cells at days 30 and 250 following primary *Listeria* infection. However, it is possible that the absence of CD40-CD40L interactions might influence the phenotype of CD8$^+$ T cells maintained in the memory population.

To investigate whether CD40-CD40L interactions influence (1) the development of, or (2) the ratio of the T_{CM} and T_{EM} phenotypically defined memory subsets, wildtype and CD40$^{-/-}$ BALB/c mice were infected systemically with *L. monocytogenes.* Four

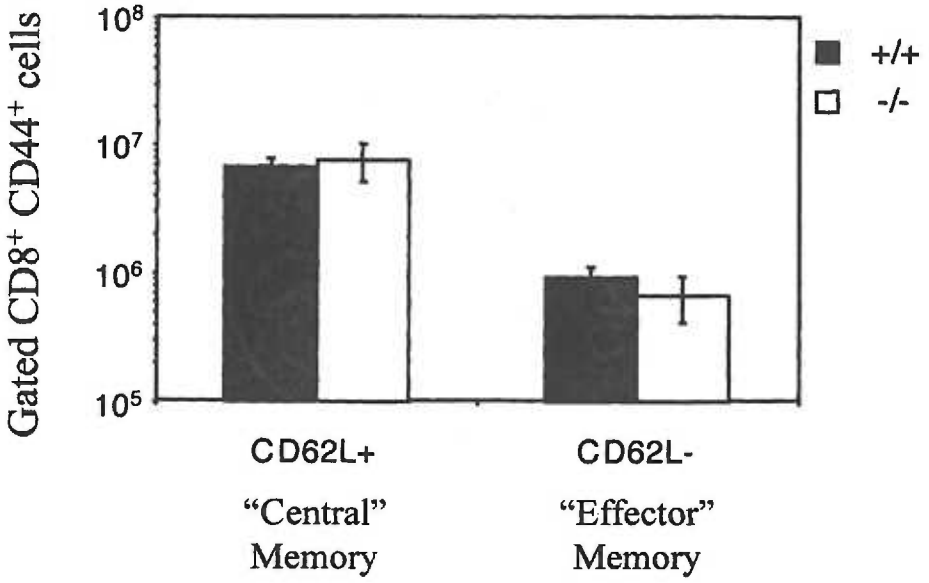


Figure 8. CD40-deficiency does not alter the ratio of resting memory populations following infection with *L. monocytogenes*. Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *Listeria* and rested for 25 days before splenocytes were evaluated by flow cytometry for the expression of CD62L on gated $CD8^+$ T-cell populations co-expressing the CD44 memory marker on their cell surface. Data represents 1 experiment with 4 mice/group.

weeks later, splenocytes were evaluated for the expression of memory phenotypic markers on the surface of $CD8^+$ T cells. Specifically, FACS analysis was used to determine the differential expression of CD62L on cells within the memory $CD8^+$ $CD44^+$ T-cell gate (Fig 8). There was no discernable difference between the number of $CD8^+$ T-cells expressing the T_{CM} and T_{EM} phenotypes on their cell-surface. In both mouse strains, the majority of cells could be classified as T_{CM}, expressing both CD44 and CD62L (averaging $6.7x10^6$ in wildtype and $7.5x10^6$ in $CD40^{-/-}$ spleens). The number of $CD8^+$ T cells expressing the T_{EM} phenotype of $CD44^+$ $CD62L^-$ was also similar, with an average of $9.2x10^5$ $CD8^+$ T cells in wildtype compared with $6.7x10^5$ in $CD40^{-/-}$ mice. These data indicate that the phenotypic ratio of T_{CM}: T_{EM} memory $CD8^+$ T cells maintained 30 days following acute *L. monocytogenes* infection is not influenced by the absence of CD40 expression. Further, these data suggest that the absence of CD40 does not prevent the development of memory $CD8^+$ T cells of either phenotype. Thus, CD40-CD40L signaling does not contribute to the development of a specific memory T-cell subset.

CD40-deficiency does not impair the cytolytic function of antilisterial resting memory $CD8^+$ T cells

The ability to rapidly gain effector function upon recognition of specific antigen is a primary function of resting memory $CD8^+$ T-cell populations. One measure of cytolytic function is the killing and subsequent clearance of target cells presenting peptide antigen in the context of the appropriate MHC-I molecule. Recently, an *in vivo* clearance assay has been developed that allows the evaluation of the cytolytic activity of resting memory $CD8^+$ T-cell populations in the absence of proinflammatory signals that accompany a

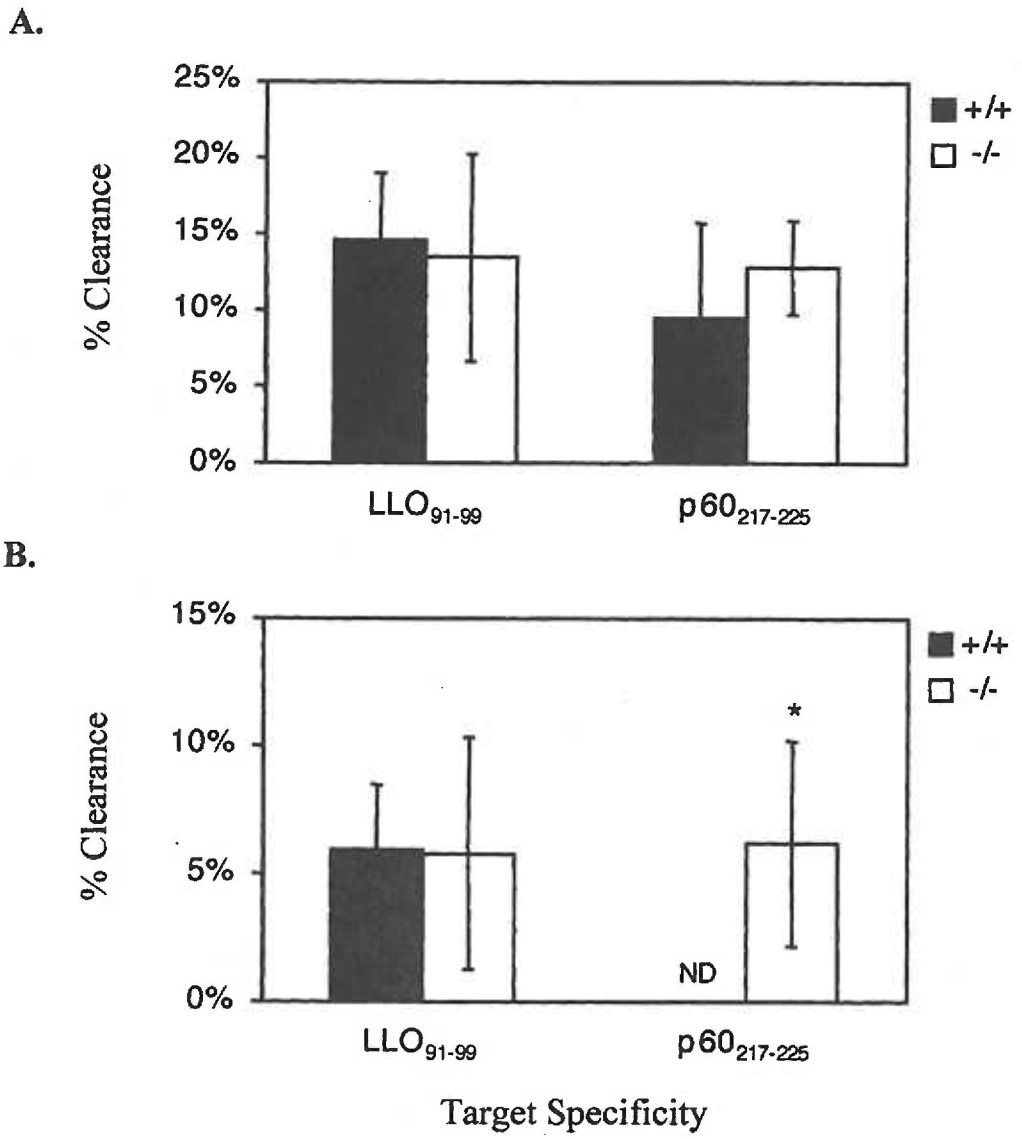


Figure 9. Resting antilisterial CD40$^{-/-}$ memory CD8^{+} T cells display normal cytolytic function. Wildtype (+/+) and CD40 deficient (-/-) BALB/c mice were analyzed for the cytolytic function of resting memory CD8^{+} T cells. Animals were rested either (A) 4 weeks or (B) 8 weeks after primary *L. monocytogenes* infection prior to the evaluation of the *in vivo* clearance of fluorescently labeled, peptide-pulsed target cells. (ND= none detected, * $p<0.0187$ relative to wildtype). Data represents 1 of 2 experiments with 3-5 mice/group/timepoint.

secondary infection (189, 190). To evaluate the cytolytic function of resting memory $CD8^+$ T cells generated in the absence of CD40, I performed *in vivo* cytotoxicity assays in wildtype and $CD40^{-/-}$ *Listeria*-immune BALB/c mice that had been rested for either 4 or 8 weeks following primary *L. monocytogenes* infection (Fig. 9).

Animals were infused with fluorescently labeled, peptide-pulsed target cells, and the *in vivo* clearance of the target populations was evaluated by flow cytometry 6 hours later. In mice rested for 4 weeks after *L. monocytogenes* infection, wildtype and $CD40^{-/-}$ BALB/c mice showed equivalent clearance of LLO_{91-99}-loaded target cells (14.6% WT vs. 13.4% CD40), as well as $p60_{217-225}$-bearing targets (9.5% vs. 12.8%, Fig. 9A). In *Listeria*-immune mice rested 8 weeks, clearance of LLO_{91-99}-pulsed target cells was also equivalent between wildtype (6.0%) and CD40-deficient (5.8%) mice. However, after resting 8 weeks, $p60_{217-225}$-bearing target cell clearance was only evident in $CD40^{-/-}$ hosts (6.2%, Fig. 9B). The ability to clear peptide-coated target cells within a 6-hour assay continued to decrease in both wildtype and $CD40^{-/-}$ BALB/c mice between weeks 4 and 8 post-infection, such that greater clearance was always observed in animals rested 4 weeks compared with those rested 8 weeks. However, there was no setting in which the absence of CD40 expression negatively affected cytolytic function of memory $CD8^+$ T cells compared with that observed in wildtype BALB/c mice. Rather, CD40-deficiency resulted in longer maintenance of $p60_{217-225}$-specific $CD8^+$ cytolytic function.

CD40-deficiency does not impair the cytolytic function of memory $CD8^+$ T cells following secondary L. monocytogenes infection

One hallmark of effective antilisterial cellular immune responses is an ability to provide protection against an otherwise lethal challenge infection. To determine the

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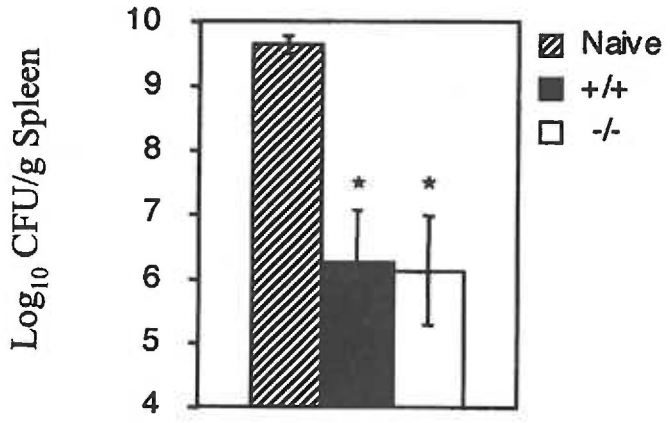

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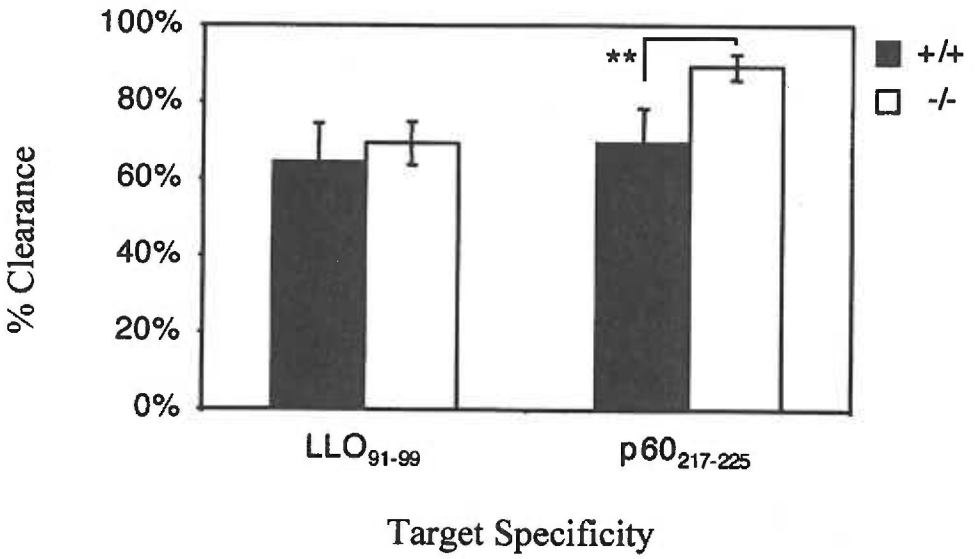

Figure 10. CD40-deficiency does not impair the cytolytic function of $CD8^+$ T cells following secondary *L. monocytogenes* infection. Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were rested for 3 weeks following primary *Listeria* infection, then challenged with a secondary infection. (A) The clearance of bacteria from the spleen was evaluated 2 days after 100 LD_{50} challenge. Naïve mice (hatched bar) did not receive primary infection prior to challenge. (* $p<0.0002$ relative to naïve). Data represents 1 of 3 experiments with 4-5 mice/group. (B) The *in vivo* clearance of using LLO_{91-99} and $p60_{217-225}$-coated target cells was performed 5 days after secondary challenge with 10 LD_{50} *Listeria*. (** $p<0.0024$). Data represents 1 experiment with 4-5 mice/group.

protective capacity of memory $CD8^+$ T cells generated in the absence of CD40, wildtype and $CD40^{-/-}$ BALB/c mice were infected with *L. monocytogenes*, and then rested for 4 weeks. *Listeria*-immune mice were then challenged with a secondary infection of 100 LD_{50}, and two days later, the splenic bacterial burdens were determined by CFU clearance assay (187). As shown in Figure 10A, both wildtype and $CD40^{-/-}$ *Listeria*-immune BALB/c mice showed significant protection, as evident by their reduced bacterial loads relative to naïve mice. Within four days of challenge, both mouse strains had completely cleared *L. monocytogenes* from the spleen, while naïve mice would succumb to this challenge dose within this time frame. This confirms that memory $CD8^+$ T cells generated in the absence of CD40 signals are capable of providing protective immunity at levels comparable to wildtype BALB/c mice.

To specifically evaluate the cytolytic function of the LLO_{91-99} and $p60_{217-227}$-specific $CD8^+$ T-cell populations in wildtype and $CD40^{-/-}$ BALB/c mice, *in vivo* cytotoxicity assays were performed. Wildtype and $CD40^{-/-}$ BALB/c mice were infected with *Listeria,* then rested for 3 weeks before receiving a secondary *L. monocytogenes* challenge. Five days following the secondary *Listeria* infection, animals were infused with fluorescently labeled target cells that had been coated with either LLO_{91-99} or $p60_{217-225}$ peptides. The clearance of target cells from the spleen was evaluated 6 hours later by flow cytometry (Fig. 10B). Although the clearance of target cells coated with the LLO_{91-99} peptide was equivalent between wildtype and $CD40^{-/-}$ mice (64.5% vs. 69.3%), the clearance of $p60_{217-225}$-coated target cells was significantly enhanced in $CD40^{-/-}$ mice relative to wildtype BALB/c (89.5% compared with 69.7%, respectively). These results demonstrate clearly that the peptide-specific $CD8^+$ T-cell populations present in $CD40^{-/-}$

mice following secondary *L. monocytogenes* infection are not functionally compromised in their ability to clear cells bearing cognate antigen. Rather, these data reveal that $CD40^{-/-}$ mice appear to have enhanced cytolytic activity following secondary *Listeria* challenge in response to the subdominant $p60_{217-225}$ epitope compared to that observed in wildtype BALB/c mice.

CD40-deficiency reduces the avidity of antilisterial memory $CD8^+$ T cells

Blockade of CD40-CD40L signaling during *L. monocytogenes* infection has been reported to cause a slight reduction in the number of memory $CD8^+$ T cells generated, however a detailed assessment of the functional capabilities of these cells has not been performed (87). The results from our evaluations of the cytolytic function of memory $CD8^+$ T cells generated in $CD40^{-/-}$ mice suggests no functional defects relative to $CD8^+$ T cells generated in wildtype BALB/c mice. However, these evaluations of target clearance were performed under experimental conditions of either high bacterial challenge dose or *in vitro*-pulsed, peptide-pulsed target cells. Under both conditions, potential differences in the sensitivity of the $CD8^+$ T-cell populations may escape detection, due to the large quantities of antigen available in the system.

To evaluate whether CD40-deficiency influences the sensitivity of memory $CD8^+$ T-cells, two parameters of peptide-specific memory $CD8^+$ T cells were measured: TCR avidity and the amount of IFNγ produced on a per-cell basis. Mice were infected with *L. monocytogenes,* and then rested for 4 weeks before receiving a secondary challenge infection. On day 5 after the secondary *L. monocytogenes* challenge, splenocytes were

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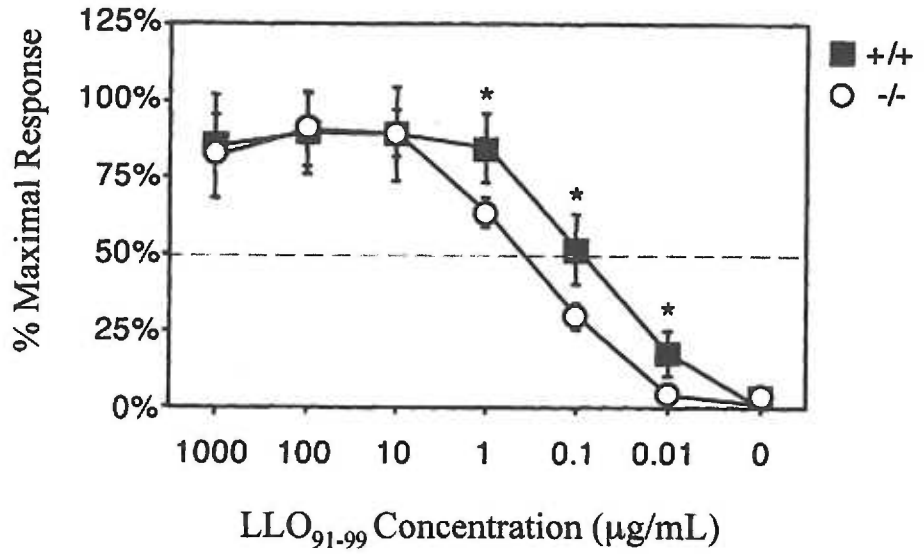

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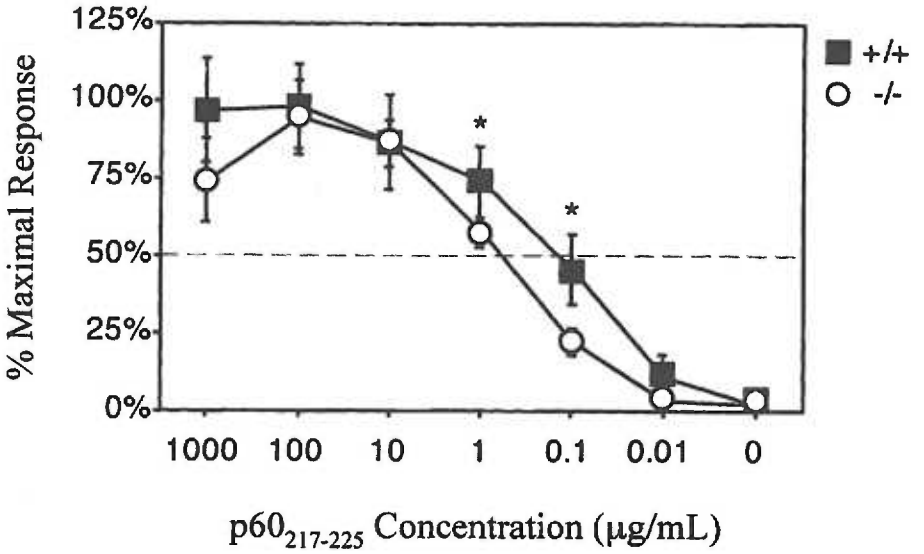


Figure 11. CD40-deficiency influences the avidity of memory CD8$^+$ T cells generated in response to *Listeria* infection. Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice, infected four weeks prior with *L. monocytogenes* were assessed 5 days after secondary *Listeria* challenge for the avidity of (A) LLO_{91-99} and (B) $p60_{217-225}$- specific CD8$^+$ T cells by intracellular cytokine staining for IFNγ. Data represents 1 of 3 experiments with 3-4 mice/group. (* $p<0.02$ relative to wildtype).

cultured *ex vivo* with a gradient of LLO_{91-99} or $p60_{217-225}$ peptide concentrations, then stained for intracellular accumulation of IFNγ or TNFα. The avidity of each peptide-specific $CD8^+$ T-cell response was determined to be the concentration of peptide required to stimulate IFNγ production by 50% of the potentially responsive $CD8^+$ T cells from individual animals (188). As shown in Figure 11, the avidity of both the LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cell populations from $CD40^{-/-}$ mice were reduced in comparison with wildtype BALB/c mice. However, evaluation of the quantity of intracellular IFNγ or TNFα accumulated on a per-cell basis by these same $CD8^+$ T-cell populations revealed no differences between memory $CD8^+$ T cells generated in CD40-deficient or wildtype BALB/c mice (Fig. 12). Together, these results indicate that the absence of CD40 negatively influences the sensitivity of the TCR to antigen. However, once stimulated, $CD40^{-/-}$ $CD8^+$ memory T cells produce equivalent effector cytokines as their wildtype counterparts.

Transient CD4-depletion during primary L. monocytogenes infection does not impair memory CD8+ T-cell functions following secondary Listeria challenge

It has been proposed that the presence of $CD4^+$ T cells during the priming of $CD8^+$ T-cell "imprints" these $CD8^+$ T cells with the potential to be maintained as long-lived, highly functional memory populations. When memory $CD8^+$ T cell maintenance is tracked in MHC-$II^{-/-}$ mice, the number of cells gradually decreases over time, and their proliferative potential is diminished (93, 95). It is important to emphasize that prior experiments that formed the basis for this "imprinting" hypothesis utilized models in

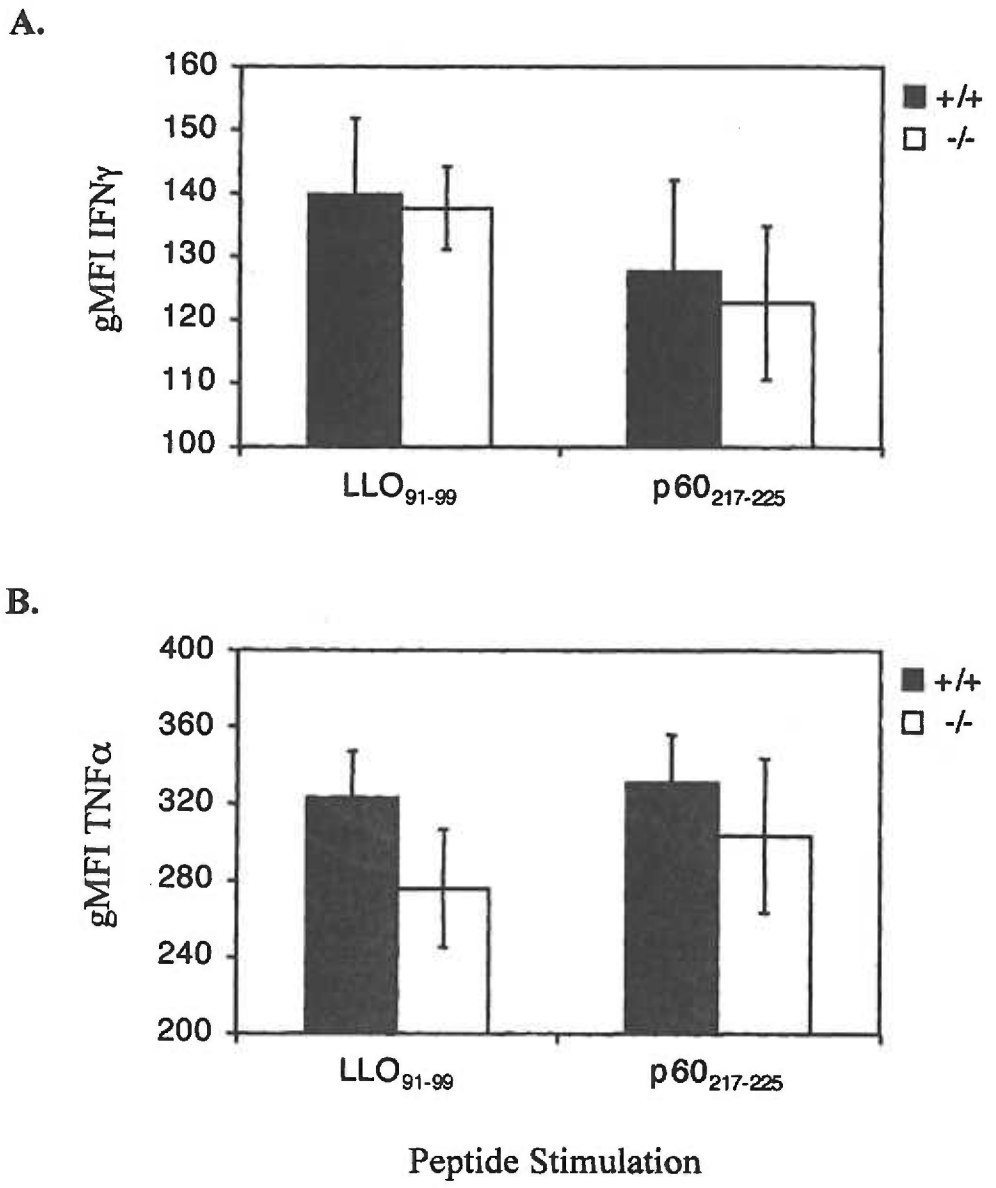


Figure 12. CD40-deficiency does not impair effector cytokine production by antilisterial memory CD8$^+$ T-cell populations. Wildtype (+/+) and CD40 deficient (-/-) *Listeria*-immune BALB/c mice were challenged four weeks after primary *L. monocytogenes* infection with a secondary *Listeria* infection. Five days after secondary challenge the geometric mean fluorescence intensity (gMFI) of (A) IFNγ and (B) TNFα accumulation was evaluated by intracellular cytokine staining of LLO_{91-99} and $p60_{217-225}$-specific CD8$^+$ T cells. Data represents 1 of 4 experiments with 3-5 mice/group.

which the $CD4^+$ T-cell population is continuously absent due to the genetic disruption of CD4 or MHC-II gene expression. Therefore, if the hypothesis that $CD4^+$ T cells provide a specific imprinting signal to $CD8^+$ T cells during priming is sound, then the transient depletion of $CD4^+$ T cells at the time of $CD8^+$ T-cell priming should similarly influence the functional properties of $CD8^+$ T-cells downstream after their conversion to a resting memory state.

I specifically tested this hypothesis in two different mouse strains. To this end BALB/c and C57Bl/6 mice were treated *in vivo* with CD4-depleting monoclonal antibodies on days –3 and 0 relative to primary *L. monocytogenes* infection. Mice were rested for 4 weeks before receiving a secondary challenge with *Listeria.* Within the C57BL/6 mouse model, a native *Listeria*-derived epitope has yet to be discovered, thus a recombinant *L. monocytogenes* strain expressing the ovalbumin protein is commonly used. This recombinant LM-OVA strain efficiently activates $CD8^+$ T cells specific for the $OVA_{261-268}$ epitope in C57BL/6 mice (75, 93).

The TCR avidity of H-$2K^d$-restricted LLO_{91-99} and $p60_{217-225}$-specific memory $CD8^+$ T cells in BALB/c mice and the H-$2K^b$-restricted $CD8^+$ T cells specific to $OVA_{261-268}$ in C57BL/6 mice was assessed. Splenocytes were stimulated 5 days following *Listeria* challenge with a gradient of each peptide, then stained for intracellular accumulation of IFNγ. The avidity of each peptide-specific response was determined to be the concentration of peptide required to stimulate IFNγ production by 50% of the potentially responsive $CD8^+$ T cells from individual animals (188). In BALB/c mice, a significant decrease in the avidity of the $CD8^+$ T-cell population was only evident at one high concentration of LLO_{91-99} peptide (10 μg/ml) (Fig. 13A). No differences in the avidity of

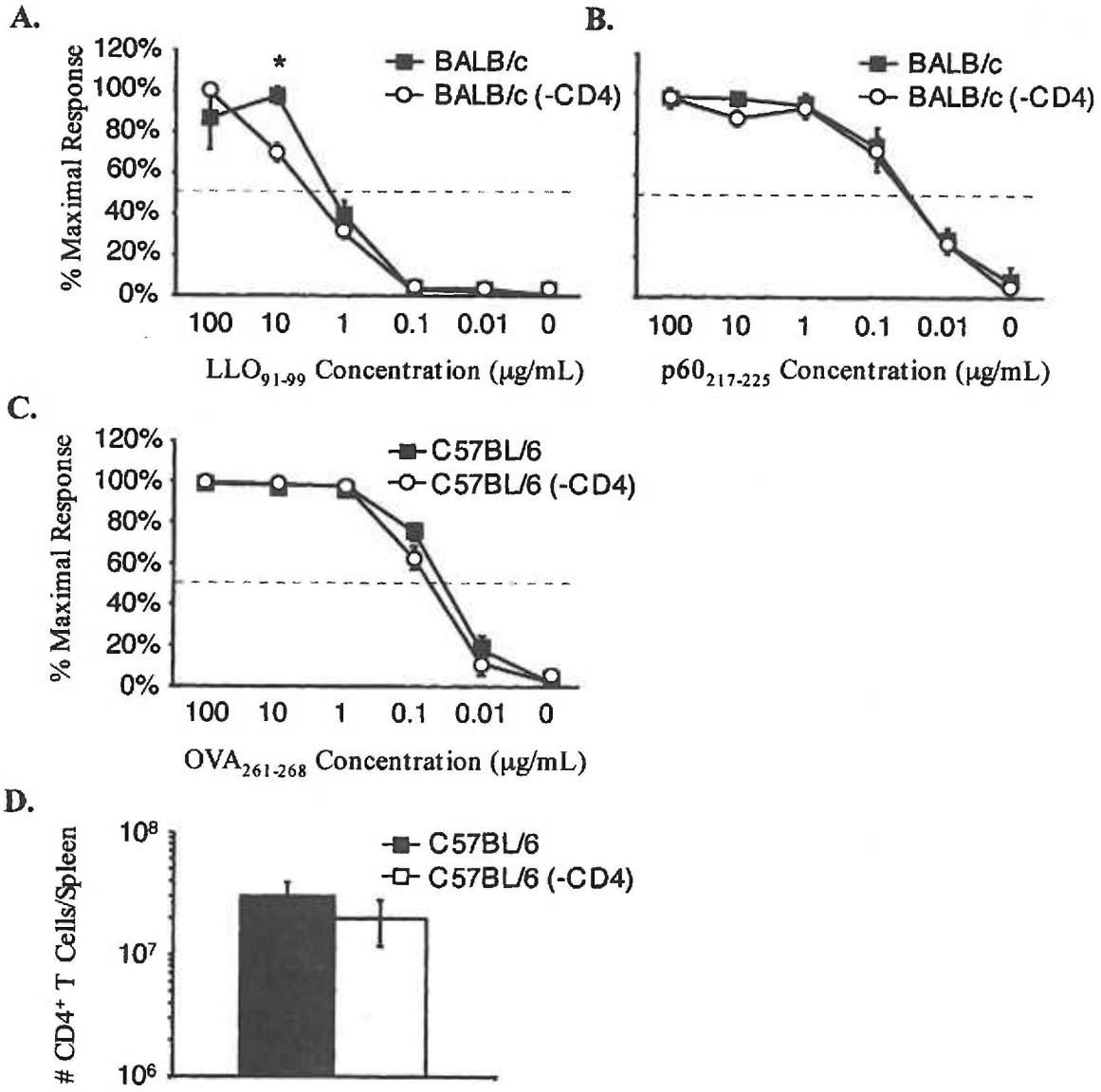


Figure 13. CD4-depletion during primary *L. monocytogenes* infection does not impair the avidity of memory CD8⁺ T-cells. The TCR avidity of antilisterial, peptide-specific CD8⁺ T-cell populations was evaluated 5 days after secondary *Listeria* challenge in (A-B) BALB/c and (C) C57BL/6 mice. (D) The total number of CD4⁺ T cells per spleen in undepleted controls and mice treated 4 weeks previously with CD4-depleting antibodies was evaluated by flow cytometry. Data represents 1 of 4 experiments with 3-5 mice/group.. (* $p<0.0001$).

$p60_{217-225}$-specific $CD8^+$ T cells were seen in CD4-depleted BALB/c mice relative to untreated controls (Fig. 13B). In C57BL/6 mice, CD4-depletion during $CD8^+$ T-cell priming had no significant influence on the avidity of the $OVA_{261-268}$-specific $CD8^+$ T-cell population (Fig. 13C). Importantly, by 4 weeks after CD4-depletion and infection with *L. monocytogenes*, the number of $CD4^+$ T cells present in depleted mice had recovered to numbers comparable to untreated control mice (Fig. 13D).

Additionally, the amount of IFNγ secreted on a per-cell basis was evaluated in peptide-specific memory $CD8^+$ T-cells 5 days following secondary *Listeria* infection. Splenocytes were stimulated with a gradient of LLO_{91-99} and $p60_{217-225}$ peptides (BALB/c), or $OVA_{261-268}$ peptide (C57BL/6) and the fluorescence intensity of accumulated intracellular IFNγ was determined 6 hours later (Fig. 14). In both BALB/c and C57BL/6 mice, peptide-specific $CD8^+$ T-cells primed in the absence of $CD4^+$ T cells were equally abundant in their secretion of IFNγ relative to $CD8^+$ T cells primed in untreated control mice.

Collectively, these data strongly suggest that $CD4^+$ T-cells need not be present during $CD8^+$ T-cell priming to imprint primary effector $CD8^+$ T cells with the potential to become memory $CD8^+$ T cells. Memory $CD8^+$ T cells primed in the absence of $CD4^+$ T-cell help have high avidity TCR and are equally capable of secreting IFNγ following recall recognition of a broad range of antigen concentrations. Thus, the presence of $CD4^+$ T cells at the time of primary $CD8^+$ T-cell activation is not required for the generation of functional $CD8^+$ T-cell memory following *L. monocytogenes* infection.

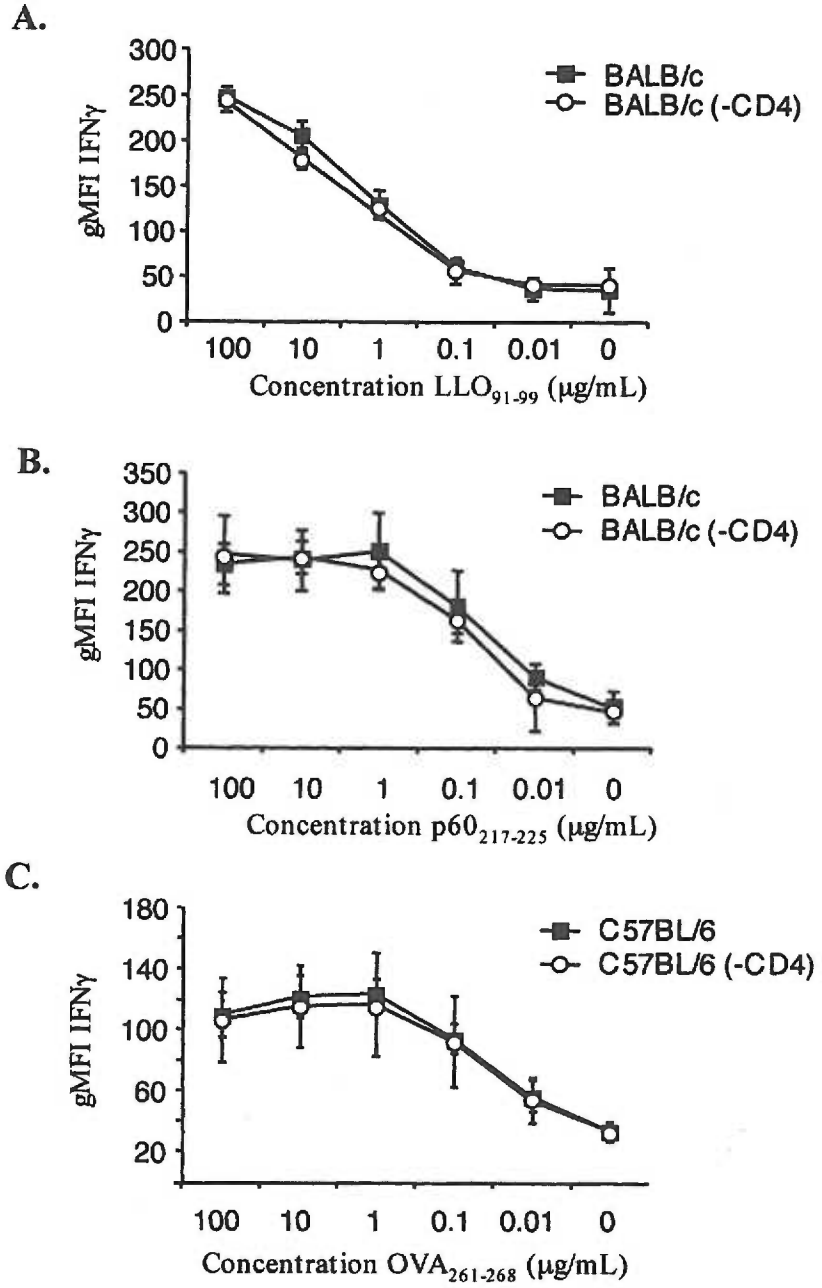


Figure 14. CD4-depletion during primary *Listeria* infection does not impair effector cytokine production by memory CD8⁺ T-cells. (A-B) BALB/c and (C) C57BL/6 mice were left untreated, or underwent CD4-depletion during the primary *L. monocytogenes* infection. Animals were rested 4 weeks, then challenged with a secondary *Listeria* infection. The geometric mean fluorescent intensity (gMFI) of intracellular IFNγ staining was evaluated 5 days later on splenocytes stimulated *in vitro* with a gradient of peptide concentrations. Data represents 1 of 4 experiments with 3-5 mice/group.

CD40$^{-/-}$ mice show enhanced CD8^{+} T-cell expansion to a secondary L. monocytogenes infection

Although there is evidence that memory CD8^{+} T-cell populations in animals devoid of CD4^{+} T cells show a decreased proliferative response following secondary antigen exposure, the mechanisms responsible for this defect are unclear (93-95). To determine if the absence of CD40 similarly impairs the proliferative potential of memory CD8^{+} T cells, I evaluated the *in vivo* expansion of CD8^{+} memory T-cell populations following secondary *L. monocytogenes* challenge. Wildtype and CD40$^{-/-}$ BALB/c mice were rested for four weeks between primary and challenge infections, then evaluated 5 days post-challenge by intracellular cytokine staining for IFNγ to evaluate the magnitude of expansion of LLO_{91-99} and $p60_{217-225}$-specific CD8^{+} T-cell populations.

When the frequencies of peptide-specific CD8^{+} T cells in the spleen were assessed, the LLO_{91-99}-specific populations were found in similar numbers in both wildtype and CD40$^{-/-}$ BALB/c mice. In contrast the frequency of $p60_{217-225}$-specific cells was significantly increased in CD40$^{-/-}$ mice compared with wildtype controls (Fig. 15A, $p<0.004$). When these frequencies were used to calculate the absolute number of peptide-specific cells per spleen in individual mice (Fig. 15B), the number of $p60_{217-225}$-specific CD8^{+} T cells was also significantly greater in the spleens of CD40$^{-/-}$ mice relative to wildtype BALB/c mice. This observation is not due to a defect in T-cell trafficking within the CD40-deficient host, as enhanced numbers of $p60_{217-225}$-specific CD8^{+} T cells were also evident in the liver and lung of CD40$^{-/-}$ mice 5 days after secondary *L. monocytogenes* challenge (Fig. 15C-D). As the number of both LLO_{91-99} and $p60_{217-225}$-specific CD8^{+} T-cell populations are equivalent at day 30 prior to secondary *L.*

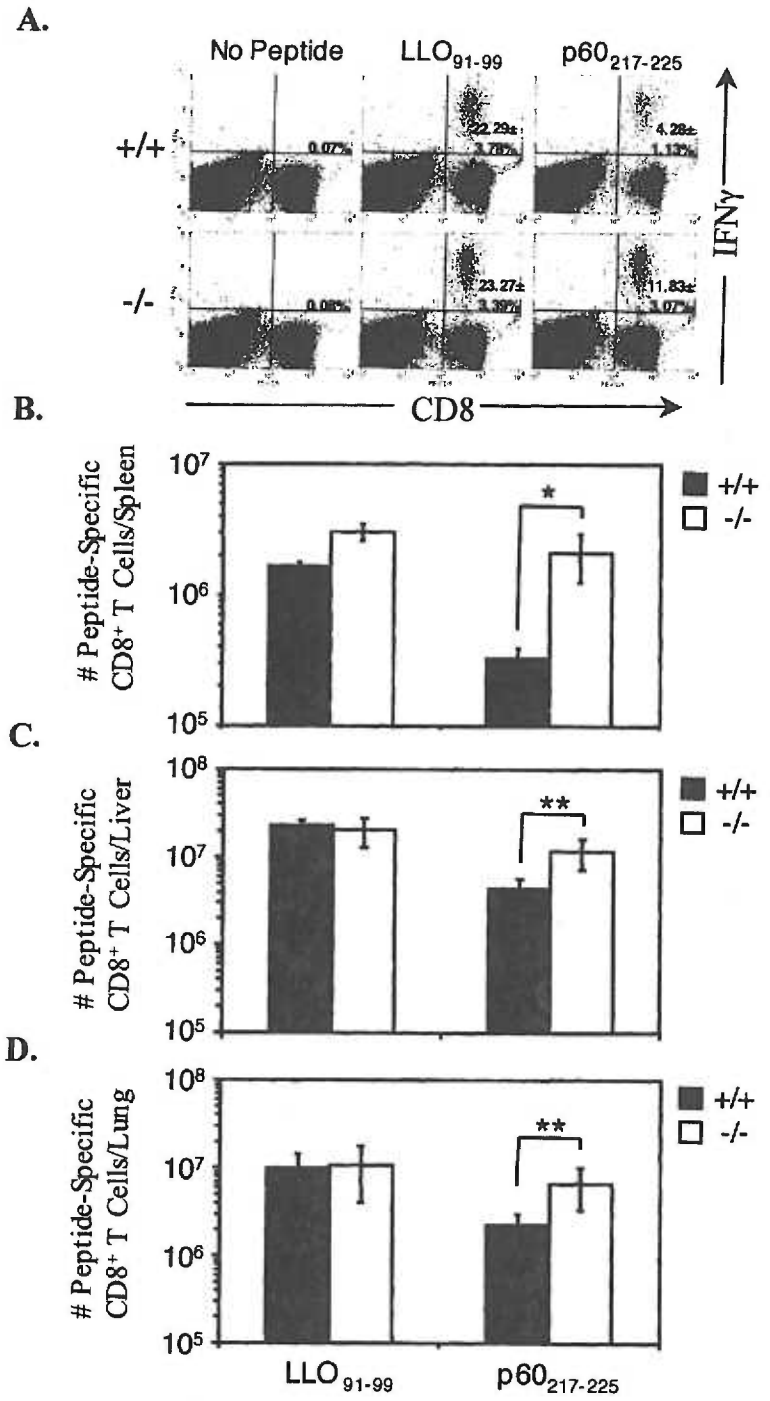


Figure 15. CD40$^{-/-}$ memory CD8$^+$ T cells show enhanced secondary *in vivo* expansion following *Listeria* challenge. Wildtype (+/+) and CD40 deficient (-/-) *Listeria*-immune BALB/c mice were analyzed 5 days after secondary *Listeria* challenge by intracellular cytokine staining for the (A) frequency LLO_{91-99} and $p60_{217-225}$-specific CD8$^+$ T cells in the spleen. Numbers in plots indicate the frequency ± standard deviation. (B) Frequencies of peptide-specific cells were converted to absolute numbers of peptide-specific, IFNγ$^+$ CD8$^+$ T cells in the (B) spleen, (C) liver and (D) lungs for individual mice. Data represents 1 of 8 experiments (A-B) and 1 of 3 experiments (C-D) with 3-5 mice/group. (* $p<0.0046$, ** $p<0.0392$).

monocytogenes challenge (Fig. 2), these results indicate that the absence of CD40 does not impair the proliferative potential of memory $CD8^+$ T cells generated in response to *L. monocytogenes* infection. In contrast, these data suggest that the absence of CD40 contributes to enhanced expansion of peptide-specific $CD8^+$ T cells following a secondary challenge infection with *Listeria*.

$CD40^{-/-}$ mice show enhanced expansion of $CD4^+$ T-cell populations following secondary L. monocytogenes challenge

To evaluate whether enhanced secondary T-cell expansion was limited to the $CD8^+$ T-cell population or was evident in additional responding cells, the secondary expansion of antilisterial $CD4^+$ T-cell populations was determined. Wildtype and $CD40^{-/-}$ BALB/c mice were rested for four weeks between primary and challenge infections, then evaluated 5 days post-challenge by intracellular cytokine staining for IFNγ to evaluate the magnitude of numerical expansion of $LLO_{189\text{-}200}$-specific $CD4^+$ T-cells. As the number of $LLO_{189\text{-}200}$-specific $CD4^+$ T-cells is near the limit of detection by ICS (<1% of total $CD4^+$ T cells), bulk $CD4^+$ T-cell responses to heat-killed *L. monocytogenes* (HKLM) were evaluated as well. Similar to our observations in the $CD8^+$ T-cell compartment (Fig. 15), the magnitude of the $CD4^+$ T-cell responses to both the $LLO_{189\text{-}200}$ peptide, as well as to HKLM were significantly increased in $CD40^{-/-}$ mice (Fig. 16). These data extend our findings to include memory $CD4^+$ T cells as an additional population that demonstrates enhanced expansion in $CD40^{-/-}$ BALB/c mice following secondary antigen exposure. Collectively, these results suggest that a general disregulation of secondary T-cell responses occurs in $CD40^{-/-}$ mice following challenge with *L. monocytogenes*.

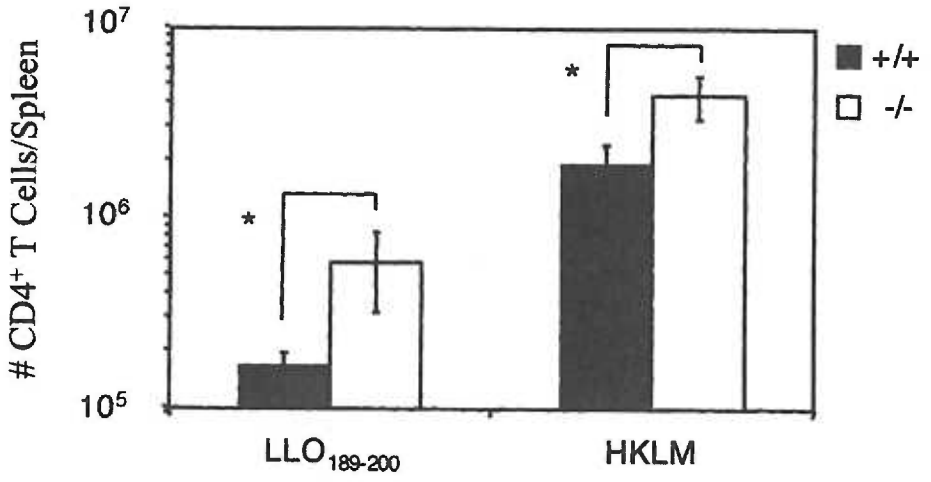


Figure 16. Memory CD4$^+$ T cells in CD40$^{-/-}$ mice undergo enhanced *in vivo* expansion in response to secondary *L. monocytogenes* challenge. *Listeria*-immune wildtype (+/+) and CD40 deficient (-/-) BALB/c mice were analyzed 5 days after secondary *Listeria* challenge. The number of peptide-specific CD4$^+$ T cells in the spleen was evaluated by intracellular cytokine staining for IFNγ-producing cells in response to stimulation with $LLO_{189-200}$ peptide or heat-killed *Listeria* (HKLM). Data represents 1 of 2 experiments with 4-5 mice/group. (* $p<0.005$).

The phenotype of secondary CD8$^+$ T-cell responses to Listeria challenge is unaffected by CD40-deficiency

The phenotype of resting memory CD8$^+$ T-cell populations 4 weeks following primary *L. monocytogenes* infection is similar between wildtype and CD40$^{-/-}$ BALB/c mice (Fig. 8). As there is a marked increase in secondary CD8$^+$ T-cell expansion in CD40$^{-/-}$ mice following *L. monocytogenes* challenge (Fig. 15), I evaluated whether this expansion was restricted to a specific, phenotypically defined memory CD8$^+$ T-cell population.

To this end, wildtype and CD40$^{-/-}$ BALB/c mice received a primary *L. monocytogenes* infection, and then were rested for 4 weeks. Five days following secondary *Listeria* challenge, the expression of CD62L and CD127 on gated CD8$^+$ IFNγ^+ T-cell populations was determined following *in vitro* peptide stimulation. Within the $LLO_{91\text{-}99}$ specific memory CD8$^+$ T-cell population, no differences between the number of T_E, T_{EM} or T_{CM} phenotype cells were evident in the spleens of CD40$^{-/-}$ mice compared with wildtype BALB/c controls (Fig. 17A). In contrast, the number of $p60_{217\text{-}225}$-specific T_E and T_{EM} phenotype cells was increased in the spleens of CD40$^{-/-}$ mice compared with wildtype BALB/c animals (Fig. 17B). Similar results were found when peptide-specific CD8$^+$ T cells were evaluated for the expression of CD44 and CD62L as representative markers of memory phenotypic subsets. These data indicate that the increased secondary expansion observed in the $p60_{217\text{-}225}$-specific CD8$^+$ T-cell population in CD40$^{-/-}$ mice is primarily within the effector cell subsets, and not a generalized expansion within all peptide-specific CD8$^+$ T-cell compartments.

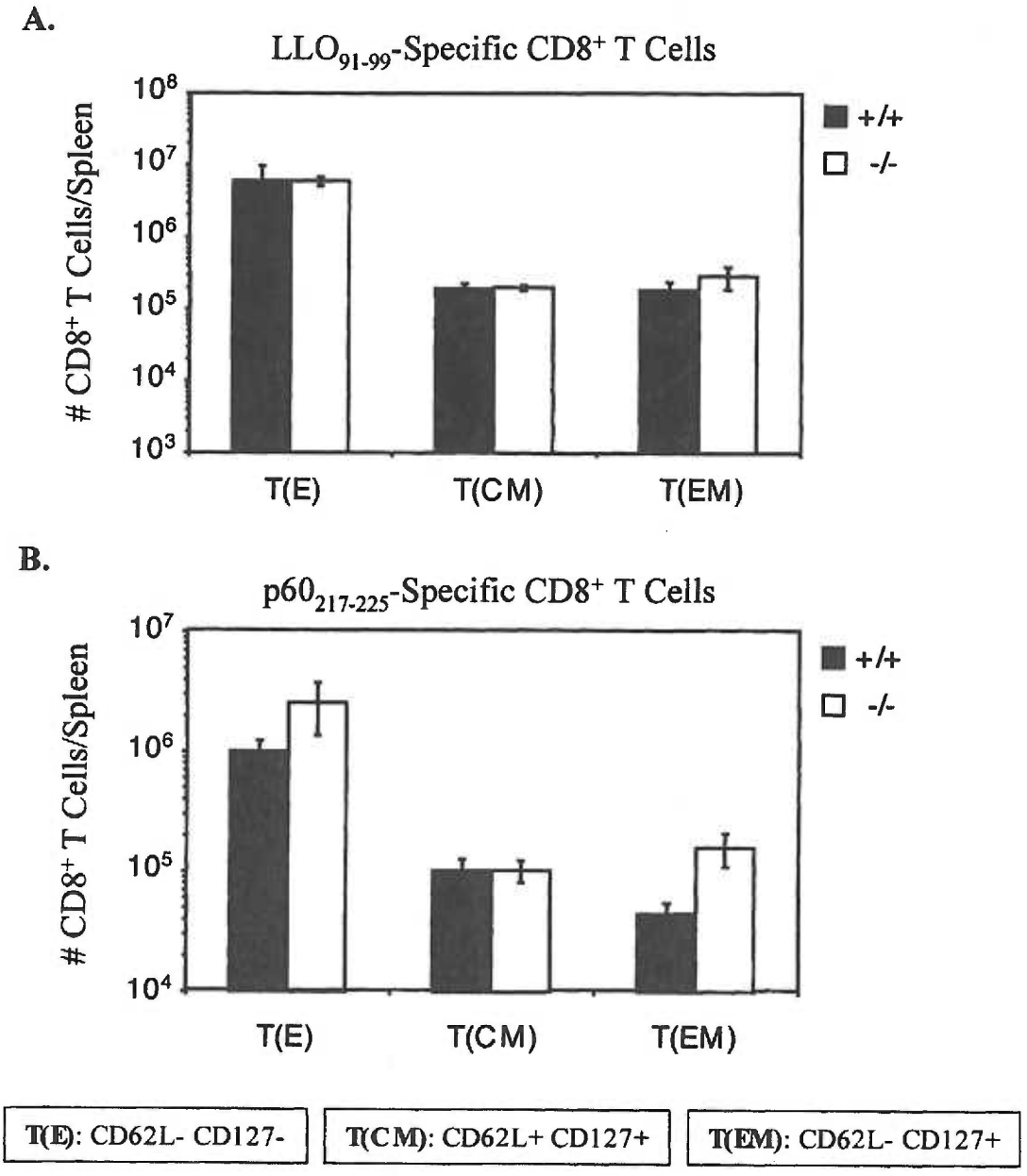


Figure 17. The phenotype of antigen-specific CD8$^+$ T cells following secondary *Listeria* challenge is not altered in CD40$^{-/-}$ mice. Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *L. monocytogenes*, then rested for 4 weeks prior to secondary *Listeria* challenge. Five days after challenge, the (A) LLO_{91-99} and (B) $p60_{217-225}$-specific CD8$^+$ T cells, as determined by intracellular cytokine staining for IFNγ, were further analyzed by flow cytometry for the surface expression of CD62L and CD127 (IL-7Rα). Data represents 1 of 2 experiments with 2-4 mice/group.

CD40$^{-/-}$ mice show similar Vβ TCR usage during the secondary CD8$^+$ T-cell response to L. monocytogenes

One potential mechanism for increasing the number of peptide-specific CD8$^+$ T cells is through the recruitment of additional CD8$^+$ T cell precursors into the response. To determine whether the secondary CD8$^+$ T-cell response in CD40$^{-/-}$ mice was comprised of different or additional CD8$^+$ T-cell populations than observed in wildtype BALB/c mice, the TCR Vβ chains utilized by $LLO_{91\text{-}99}$ and $p60_{217\text{-}225}$-specific CD8$^+$ T-cells was evaluated in wildtype and CD40$^{-/-}$ BALB/c mice 5 days after secondary *Listeria* infection (Fig. 18). Replicate samples of individual splenocyte populations were stimulated with either $LLO_{91\text{-}99}$ or $p60_{217\text{-}225}$ peptide, then stained for intracellular IFNγ as well as cell-surface expression of TCR Vβ chains. By gating on only CD8$^+$ IFNγ$^+$ cells, the distribution of TCR Vβ chains utilized within each peptide-specific CD8$^+$ T-cell population was determined. Although slight variations were seen between individual animals, the frequencies of antigen-specific cells utilizing each Vβ TCR chain were comparable between wildtype and CD40$^{-/-}$ mice for each peptide-specific CD8$^+$ T-cell population. These results suggest that the increased expansion of antigen-specific CD8$^+$ T cells in CD40$^{-/-}$ mice reflects enhanced proliferation, rather than recruitment of additional CTL precursors from distinct TCR families into the antilisterial response.

CD40$^{-/-}$ mice show typical secondary CD8$^+$ T-cell response kinetics following L. monocytogenes challenge

Upon evaluating the kinetics of the primary CD8$^+$ T-cell response to systemic *L. monocytogenes* infection, I observed a marked alteration of the response in CD40$^{-/-}$ mice

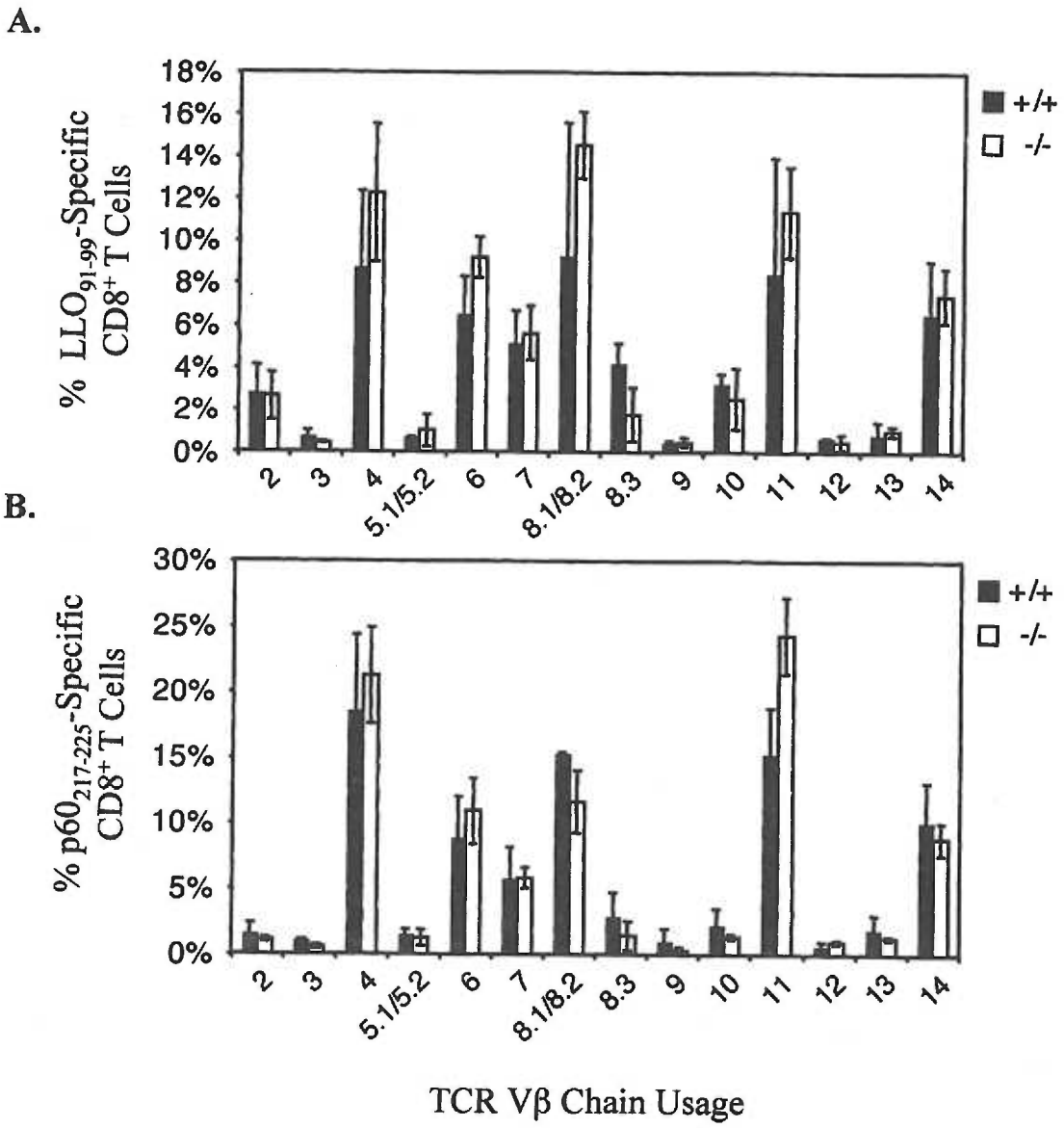


Figure 18. Wildtype and CD40$^{-/-}$BALB/c mice use similar TCR Vβ chains in their secondary CD8$^+$ T-cell response to *Listeria*. Wildtype (+/+) and CD40$^{-/-}$ BALB/c mice were infected with *L. monocytogenes*, then rested for 4 weeks prior to secondary *Listeria* challenge. Five days later, (A) LLO_{91-99} and (B) $p60_{217-225}$-specific CD8$^+$ T cells were gated by flow cytometry using intracellular cytokine staining for IFNγ. The TCR Vβ usage for each peptide-specific CD8$^+$ T-cell population was determined by cell-surface staining of gated IFNγ$^+$ CD8$^+$ T-cell populations with a panel of Vβ antibodies. Data represents 1 of 2 experiments with 2 mice/group.

compared with wildtype BALB/c mice (Fig. 2C). The onset of the contraction phase for both LLO_{91-99} and $p60_{217-225}$-specific responses are delayed in $CD40^{-/-}$ mice: wildtype mice reach the peak of both LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cell responses at day 7 following primary *L. monocytogenes* infection, while the peak is delayed in $CD40^{-/-}$ BALB/c mice until day 10 post-infection.

Is the kinetic delay of primary $CD8^+$ T-cell expansion observed in $CD40^{-/-}$ mice a hallmark of all T-cell responses in these animals, or is this alteration specific to the primary $CD8^+$ T-cell response? To this end, wildtype and $CD40^{-/-}$ BALB/c mice were infected with *L. monocytogenes*, and then rested for 4 weeks. On day 30 following primary infection, animals were challenged with 10 LD_{50} of *Listeria* and the secondary response of LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T cells was determined at various timepoints after challenge by intracellular cytokine staining analysis of splenocytes (Fig. 19). Peptide-specific $CD8^+$ T-cell responses were standardized relative to the peak response in each mouse. In contrast to the altered kinetics evident in $CD40^{-/-}$ mice following primary infection, no differences in the kinetics of either the LLO_{91-99} or $p60_{217-225}$-specific secondary $CD8^+$ T-cell response were seen in $CD40^{-/-}$ mice compared with wildtype BALB/c controls.

However, I did observe a marked change in the onset of contraction between the LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cell responses in both wildtype and $CD40^{-/-}$ BALB/c mice following secondary *L. monocytogenes* challenge. LLO_{91-99}-directed $CD8^+$ T-cells are considered to be the immunodominant response in the BALB/c genetic background, and following secondary *Listeria* challenge, the LLO_{91-99}-specific $CD8^+$ T-cell population was indeed larger than the $p60_{217-225}$-specific $CD8^+$ T-cell population in

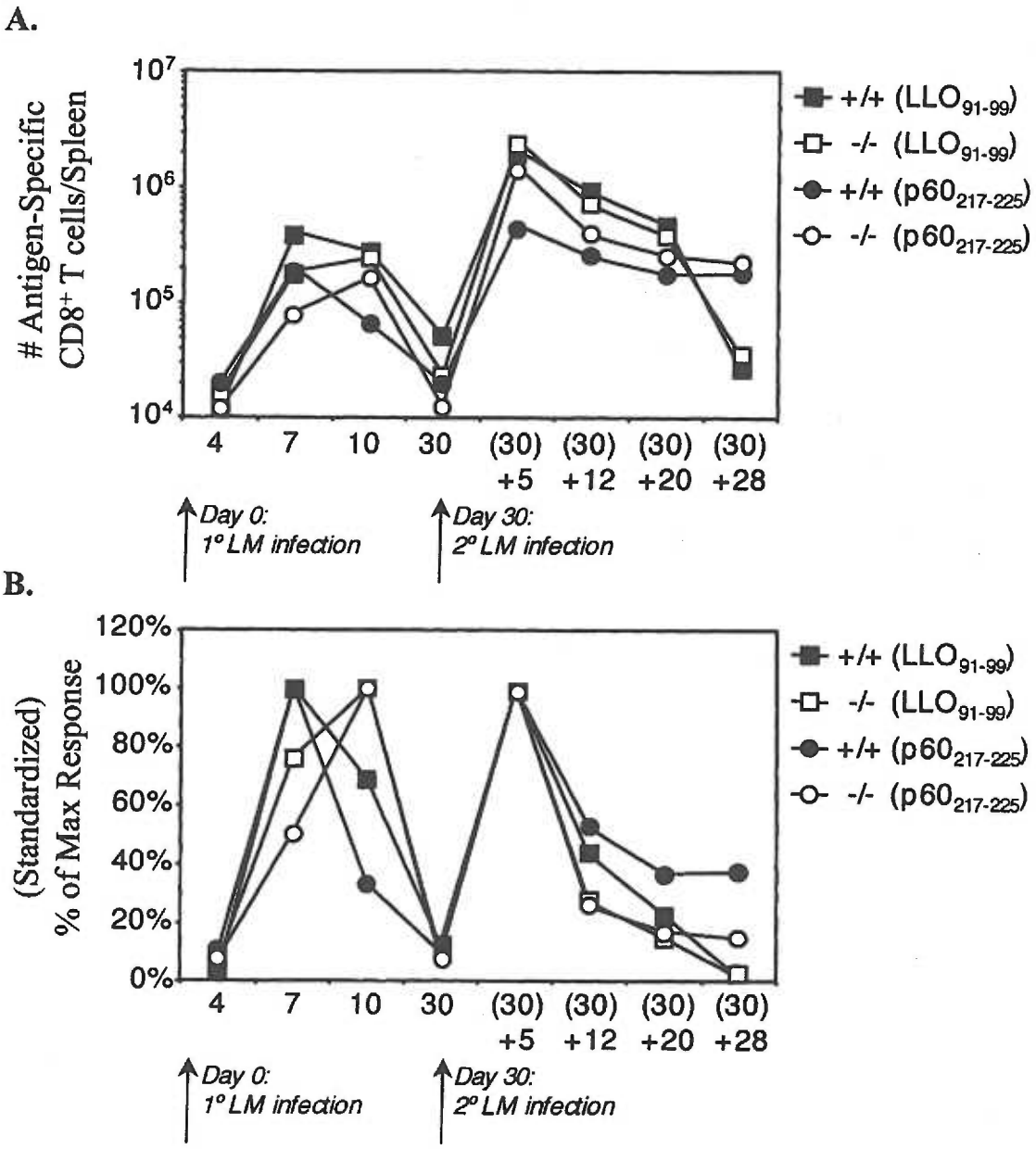


Figure 19. Primary and secondary CD8⁺ T-cell kinetics following *Listeria* infection. (A) Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *L. monocytogenes* and the number of LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cells/spleen was evaluated at days 4, 7, 10 and 30 following primary infection. For secondary responses, Listeria-immune mice were challenged 30 days after primary infection (30), and the number of LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cells/spleen was evaluated at days (30) +5, +12, +20 and +28 following secondary *Listeria* challenge. (B) For kinetic evaluations, the number of $CD8^+$ T cells specific for LLO_{91-99} and $p60_{217-225}$ peptides was normalized against the peak of each response (100%). Data compiled from 4 experiments with 2-3 mice/group/timepoint.

both mouse strains. However, in both wildtype and $CD40^{-/-}$ mice, the LLO_{91-99}-specific $CD8^+$ T-cell population had undergone secondary contraction to a much greater degree than the $p60_{217-225}$-specific $CD8^+$ T-cell population by day 28 after secondary *L. monocytogenes* infection. These results suggest that the hierarchy of peptide-specific responses may not be as rigid as previously thought. To my knowledge, this is the first demonstration that the secondary contraction of peptide-specific $CD8^+$ T-cell populations may not be uniform for all populations.

The $CD8^+$ T-cell response magnitude is enhanced in $CD40^{-/-}$ mice by increasing the Listeria challenge dose

To determine if the enhanced secondary expansion of $CD8^+$ T-cell populations observed in $CD40^{-/-}$ mice was dependent on the bacterial challenge dose, *Listeria*-immune wildtype and $CD40^{-/-}$ BALB/c mice were subsequently challenged with an increasing number of bacteria, and the antigen-specific $CD8^+$ T-cell response was determined 5 days later (Fig. 20). By including the analysis of unchallenged memory *Listeria*-immune mice, the fold-expansion of peptide-specific $CD8^+$ T-cell populations was calculated. Surprisingly, this revealed that increasing the challenge dose only influenced the magnitude of secondary $CD8^+$ T-cell expansion in $CD40^{-/-}$ mice. At a low challenge dose (1 LD_{50}), the fold-expansion of LLO_{91-99}-specific $CD8^+$ T cells was similar in $CD40^{-/-}$ mice compared with wildtype BALB/c controls. However, as the challenge dose was increased, further expansion of antigen-specific $CD8^+$ T cells was only observed in $CD40^{-/-}$ mice; wildtype mice challenged with either 1, 10 or 100 LD_{50} showed no appreciable difference in the fold-expansion of peptide-specific $CD8^+$ T cells.

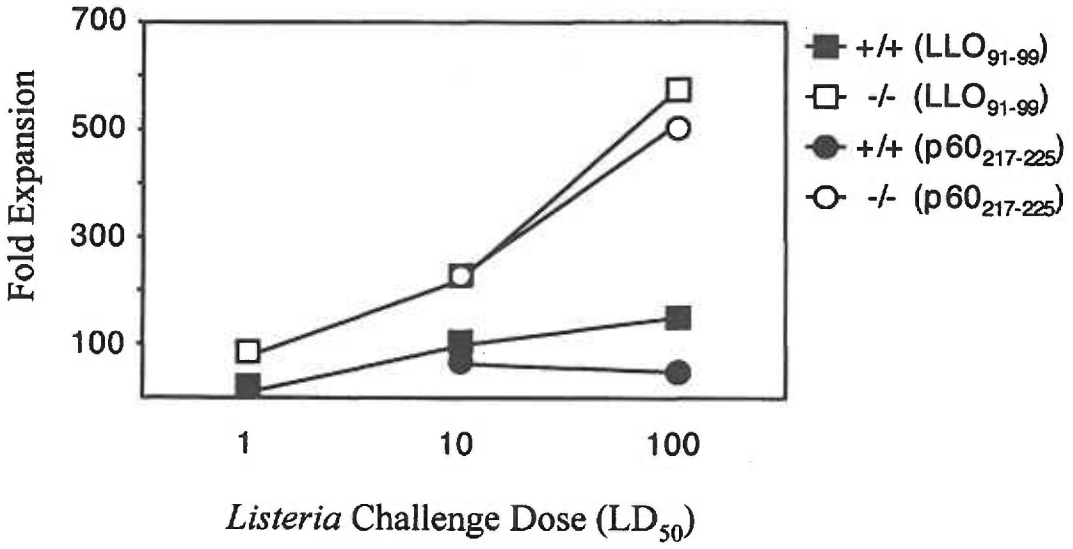


Figure 20. CD40$^{-/-}$ mice show increased secondary CD8^{+} T-cell expansion as the *Listeria* challenge dose is increased. The fold-expansion of peptide-specific CD8 $^{+}$ T cells in *Listeria*-immune wildtype (+/+) and CD40 deficient (-/-) BALB/c mice was evaluated 5 days after secondary *Listeria* challenge with either 1, 10, or 100 LD_{50} bacteria. The number of peptide-specific CD8^{+} T cells was evaluated in the spleen. Data compiled from 10 experiments with 3-5 mice/group.

As wildtype and CD40$^{-/-}$ *Listeria*-immune mice have equivalent numbers of resting memory CD8^{+} T cells prior to secondary *L. monocytogenes* infection (Figs. 2 and 8), this indicates a clear difference in the regulation of secondary CD8^{+} T-cell expansion in wildtype and CD40$^{-/-}$ mice. These data imply that some level of regulation that normally exists to limit the continued expansion of peptide-specific CD8^{+} T-cell populations under increasing infectious dose is impaired in CD40$^{-/-}$ mice compared with wildtype BALB/c animals.

CD40$^{-/-}$ mice show atypical CD8^{+} T-cell responses to HKLM challenge

Immunization with heat-killed pathogens, including *L. monocytogenes* (HKLM) has been extensively investigated as a vaccination strategy. In theory, HKLM should provide a sufficient APC maturation signal through TLR triggering, to activate DC to a maturation state capable of priming naive CD8^{+} T cells. In practice, HKLM vaccination appears to provide an incomplete signal to naive CD8^{+} T cells, triggering an abortive proliferative response and a severely diminished ability to lyse target cells or secrete effector cytokines following peptide stimulation (132, 133). When *Listeria*-immune mice are injected with HKLM, there is no enhancement of protection against viable *L. monocytogenes* challenge, suggesting that HKLM is also incapable of stimulating the expansion of memory CD8^{+} T-cell populations (192). As I have found evidence for differential regulation of secondary T-cell responses in CD40$^{-/-}$ mice, these references led me to ask whether the memory CD8^{+} T-cell population resident in *Listeria*-immune CD40$^{-/-}$ mice might respond to *in vivo* administration of HKLM.

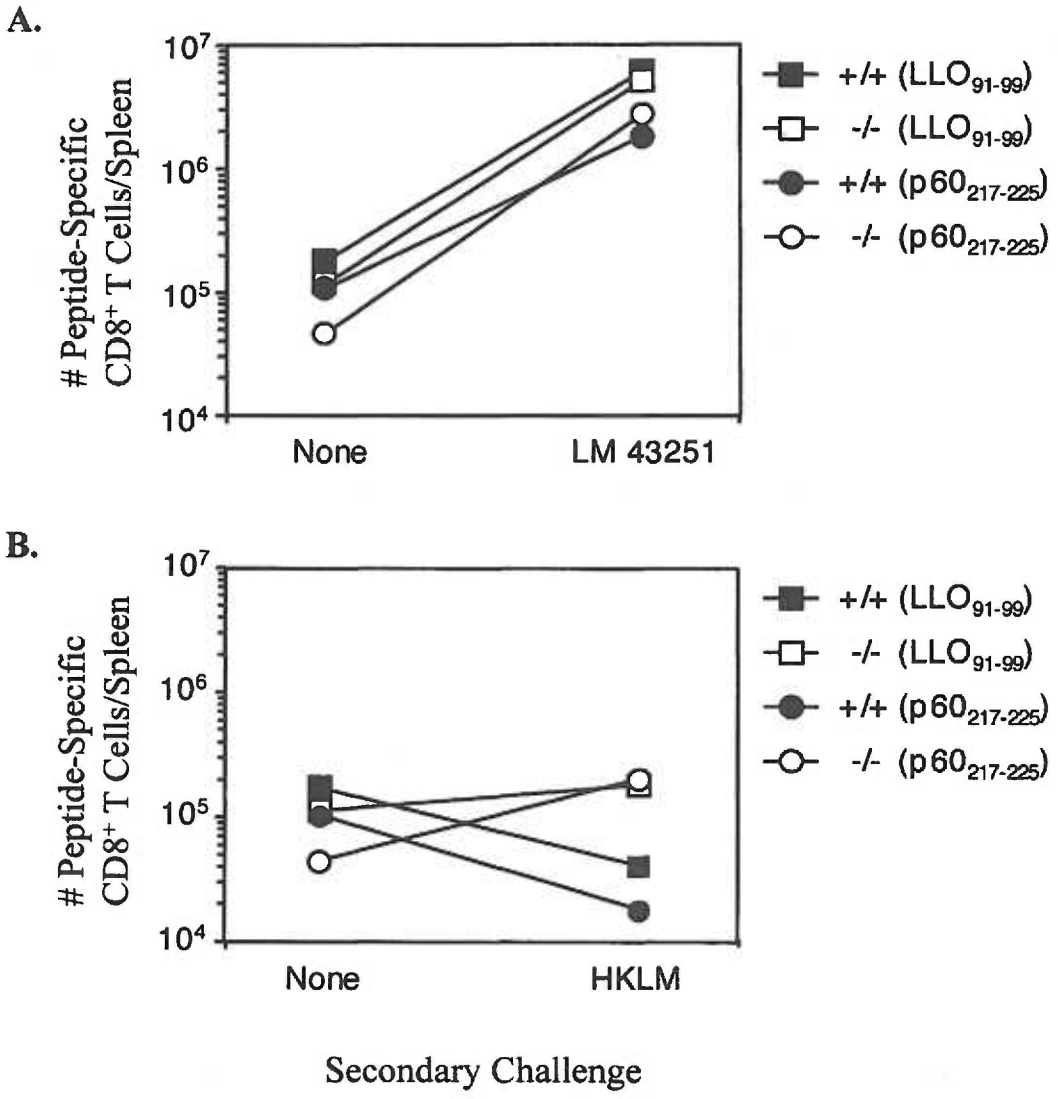


Figure 21. Antilisterial memory CD8$^+$ T-cells in CD40$^{-/-}$ mice expand in response to HKLM challenge. Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *Listeria*, then rested for 4 weeks. Listeria-immune mice were challenged with either (A) viable *Listeria* (strain 43251) or (B) heat-killed *Listeria* (HKLM). After 5 days, the number of peptide-specific CD8$^+$ T cells in the spleen was determined by intracellular cytokine staining for IFNγ. Data represents 1 of 2 experiments with 2-3 mice/group.

Wildtype and $CD40^{-/-}$ BALB/c mice were infected with *L. monocytogenes* then allowed to recover for 4 weeks. *Listeria*-immune animals were challenged with HKLM or viable *Listeria* and the number of peptide-specific LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T cells in the spleen was evaluated 5 days later by intracellular cytokine staining for IFNγ (Fig. 21). Following challenge with viable *Listeria*, the number of $CD8^+$ T-cells specific for $p60_{217-225}$ was significantly enhanced in $CD40^{-/-}$ mice relative to wildtype BALB/c controls. These results confirm that the secondary expansion of antilisterial $CD8^+$ T cells, particularly the $p60_{217-225}$-specific populations, is markedly enhanced in $CD40^{-/-}$ mice. In animals challenged with HKLM, $CD40^{-/-}$ mice showed a small, but significant expansion within the $p60_{217-225}$-specific $CD8^+$ T-cell population relative to wildtype BALB/c mice. The frequency of $p60_{217-225}$-specific cells was increased from 0.14% of the wildtype $CD8^+$ T-cell subset to 1.06% in $CD40^{-/-}$ mice (data not shown). When the total number of $p60_{217-225}$-specific $CD8^+$ T cells per spleen was determined, $CD40^{-/-}$ responses were increased by 6-fold, with a mean of $1.09x10^5$ $p60_{217-225}$-specific $CD8^+$ T cells in $CD40^{-/-}$ mice compared with $1.74x10^4$ in wildtype BALB/c mice. These results suggest that the requirements for triggering the secondary expansion of memory $CD8^+$ T cells in $CD40^{-/-}$ mice is less stringent than initiating such expansion in wildtype BALB/c mice. Thus, memory $CD8^+$ T-cell populations in $CD40^{-/-}$ mice respond to stimuli that are insufficient to trigger the same response in wildtype BALB/c mice. It is unclear why this effect is pronounced in the memory $CD8^+$ T-cell population specific for the $p60_{217-225}$ epitope and not the LLO_{91-99}-specific response.

Independent regulation of peptide-specific CD8+ T-cell responses to secondary Listeria challenge is evident in CD40-/- mice

It has been previously established that the $p60_{217-225}$ epitope is displayed at a much greater density that the LLO_{91-99} epitope on the surface of *L. monocytogenes*-infected cells (193). Further, these two antigen-specific $CD8^+$ T-cell populations do not compete with each other in wildtype BALB/c mice. Infection with mutant *L. monocytogenes* strains that are completely deficient in LLO production or contain a point mutation in the LLO_{91-99} epitope (LLO92F strain, no presentation of LLO_{91-99} epitope), prevents the priming of LLO_{91-99}-specific $CD8^+$ T cells, yet has no effect on the magnitude of the $p60_{217-225}$-specific $CD8^+$ T-cell response (194, 195). Thus, when there is no secondary expansion of the immunodominant LLO_{91-99}-directed $CD8^+$ T-cells, $p60_{217-225}$-specific and other antilisterial $CD8^+$ T-cell populations are not correspondingly increased. This argues against the premise that memory $CD8^+$ T cells specific for different epitopes compete with each other for resources, such as growth cytokines.

The $CD8^+$ T-cell response observed in $CD40^{-/-}$ mice following secondary *L. monocytogenes* challenge is significantly increased relative to that seen in wildtype BALB/c animals (Figs. 15, 20, 21). This observation suggests a level of active regulation in wildtype mice that is less evident in $CD40^{-/-}$ mice. In order to further study this observation, I utilized the LLO92F *Listeria* strain for secondary challenge infection. This approach allows for evaluation of the $p60_{217-225}$-specific $CD8^+$ T-cell population in the absence of secondary expansion within the immunodominant LLO_{91-99}-specific $CD8^+$ T-cell population.

To this end, wildtype and $CD40^{-/-}$ BALB/c mice were infected with *L. monocytogenes* #43251, which primes $CD8^+$ T-cells specific to both LLO_{91-99} and $p60_{217-}$

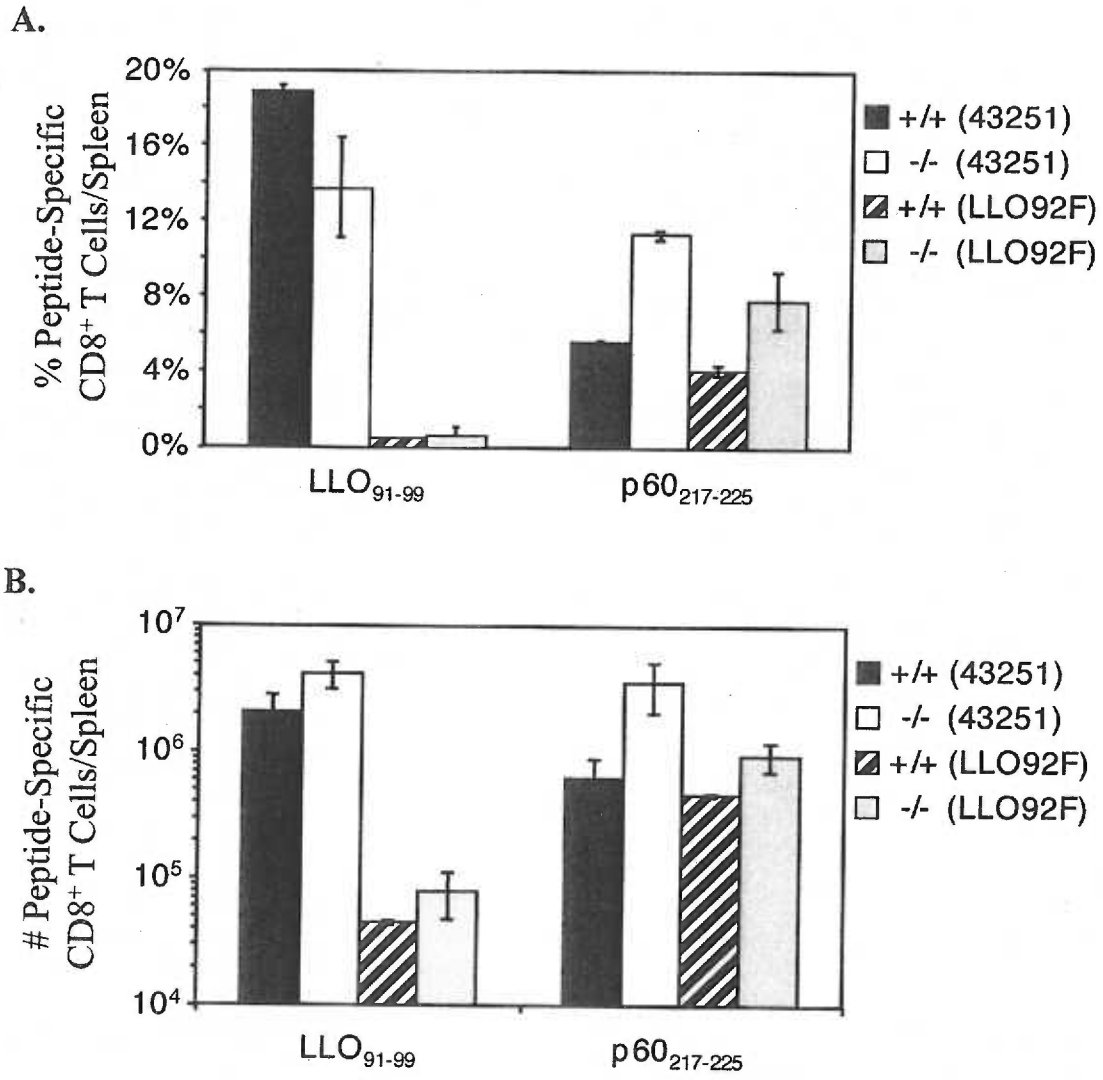


Figure 22. Elimination of the secondary LLO_{91-99}-specific $CD8^+$ T-cell response does not further enhance $p60_{217-225}$-specific $CD8^+$ T-cell expansion in $CD40^{-/-}$ mice. Wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were infected with *Listeria*, then rested for 4 weeks. Listeria-immune mice were then challenged with either *Listeria* 43251 or LLO92F, which cannot present the LLO_{91-99} peptide. After 5 days, (A) the frequency and (B) number of peptide-specific $CD8^+$ T cells in the spleen was determined by intracellular cytokine staining for IFNγ. Data represents 1 of 2 experiments with 2-3 mice/group.

$_{225}$ epitopes. Following 4 weeks of recovery, mice were challenged with either *Listeria* #43251 or the LLO92F mutant strain, which secretes wildtype levels of LLO, but contains a Y→F amino acid substitution at position 92, preventing the presentation of the LLO_{91-99} epitope (194).

Splenocytes were evaluated five days after secondary *L. monocytogenes* challenge for the number of LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T cells (Fig. 22). As seen previously, the secondary expansion of LLO_{91-99}-specific memory $CD8^+$ T cells was only evident in animals challenged with *Listeria* #43251 (expressing LLO_{91-99}). Both wildtype and $CD40^{-/-}$ mice challenged with the LLO92F mutant strain (no expression of LLO_{91-99}) showed no expansion of LLO_{91-99}-specific $CD8^+$ T cells. In agreement with my previous results, the magnitude of the $p60_{217-225}$-directed $CD8^+$ T-cell response was enhanced in $CD40^{-/-}$ mice challenged with either 43251 or LLO92F strains of *L. monocytogenes* when compared to the response observed wildtype BALB/c mice. However, the absence of the secondary expansion of LLO_{91-99}-specific $CD8^+$ T cells in *Listeria*-immune animals challenged with the LLO92F *L. monocytogenes* mutant did not increase the number of $p60_{217-225}$-specific $CD8^+$ T cells in either $CD40^{-/-}$ or wildtype BALB/c mice.

Summary to Results Section 3.2

These experiments provide an evaluation of the functional properties of memory T-cell populations that develop following systemic infection with *L. monocytogenes*. In $CD40^{-/-}$ BALB/c mice infected 4 weeks previously with *L. monocytogenes*, the expression of both central memory and effector memory phenotypic markers on resting $CD8^+$ T cells is comparable to wildtype BALB/c mice, indicating that CD40-CD40L

interactions are not required for the generation of a specific memory $CD8^+$ T-cell subset. Further, resting *Listeria*-immune memory $CD8^+$ T cells in $CD40^{-/-}$ mice are fully functional, as evident by their equal ability to clear peptide-coated target cells *in vivo*, relative to wildtype BALB/c mice.

Evaluation of peptide-specific $CD8^+$ T-cell populations in $CD40^{-/-}$ mice revealed a reduction in the sensitivity of the TCR to antigen relative to wildtype mice. However, once stimulated, the quantity of effector cytokines produced by memory $CD8^+$ T-cells in $CD40^{-/-}$ mice is comparable to that seen in wildtype BALB/c mice. Thus, although antilisterial memory $CD8^+$ T-cells from $CD40^{-/-}$ mice bear TCR that are modestly lower in sensitivity to detecting small antigen concentrations, once activated these cells are fully functional effectors equivalent to those found in wildtype BALB/c mice. These findings are supported by the analysis of bacterial clearance following secondary *L. monocytogenes* challenge in *Listeria*-immune wildtype and $CD40^{-/-}$ mice: both strains show significant protection, as observed by their equal reduction of bacterial loads relative to naïve mice.

By day five following secondary *L. monocytogenes* challenge, the expansion of $p60_{217\text{-}225}$-specific $CD8^+$ T cells is significantly increased in $CD40^{-/-}$ mice compared with wildtype BALB/c controls, resulting in an increased ability to clear $p60_{217\text{-}225}$-pulsed target cells. Although increased expansion in the $LLO_{91\text{-}99}$-specific $CD8^+$ T-cell population is not observed to the same degree in $CD40^{-/-}$ mice, secondary $CD4^+$ T-cell responses to both the $LLO_{189\text{-}200}$ peptide as well as bulk $CD4^+$ T-cell responses to HKLM stimulation are increased. These data suggest a fundamental alteration in the regulation of secondary T-cell responses in $CD40^{-/-}$ mice following challenge with *L. monocytogenes.*

The enhanced expansion of $p60_{217-225}$-specific $CD8^+$ T cells in $CD40^{-/-}$ mice appears to result from enhanced proliferation, rather than recruitment of additional TCR family precursors into the antilisterial response, as the frequencies of antigen-specific cells utilizing each Vβ TCR chain are comparable between wildtype and $CD40^{-/-}$ mice within the $p60_{217-225}$-specific $CD8^+$ T-cell population. The number of $p60_{217-225}$-specific T_E and T_{EM} phenotype cells was increased in the spleens of $CD40^{-/-}$ mice compared with wildtype BALB/c animals, suggesting a preferential expansion of $CD8^+$ T-cell populations bearing effector phenotypic markers.

I have demonstrated that, as the *Listeria* challenge dose is increased, further expansion of antigen-specific $CD8^+$ T cells is only observed in $CD40^{-/-}$ mice; within wildtype BALB/c mice, there appears to be a regulatory process that prevents the continued expansion of antilisterial $CD8^+$ T-cell populations. Further, in *Listeria*-immune mice challenged with HKLM, $CD40^{-/-}$ mice showed a small, but significant expansion within the $p60_{217-225}$-specific $CD8^+$ T-cell population compared with wildtype BALB/c mice. These results suggest that mechanisms to control both the initiation and magnitude of secondary $CD8^+$ T-cell expansion may be less effective in $CD40^{-/-}$ mice than in wildtype BALB/c mice.

Finally, I have presented data that clearly indicates that $CD4^+$ T-cells need not be present during $CD8^+$ T-cell priming to imprint primary effector $CD8^+$ T cells with the potential to become memory $CD8^+$ T cells. Transient *in vivo* depletion of $CD4^+$ T cells at the time of primary *L. monocytogenes* infections results in the generation of memory $CD8^+$ T cells equally capable of secreting IFNγ following recall recognition in response to a broad range of antigen concentrations. These findings were observed in both BALB/c

and C57BL/6 mice. Further, following secondary *Listeria* infection, the TCR avidity of antigen-specific $CD8^+$ T cells primed in the absence of $CD4^+$ T-cell help is generally unaffected, and in both BALB/c and C57BL/6 mice, peptide-specific $CD8^+$ T-cells primed in the absence of $CD4^+$ T cells are equally abundant in their secretion of IFNγ relative to $CD8^+$ T cells primed in untreated control mice.

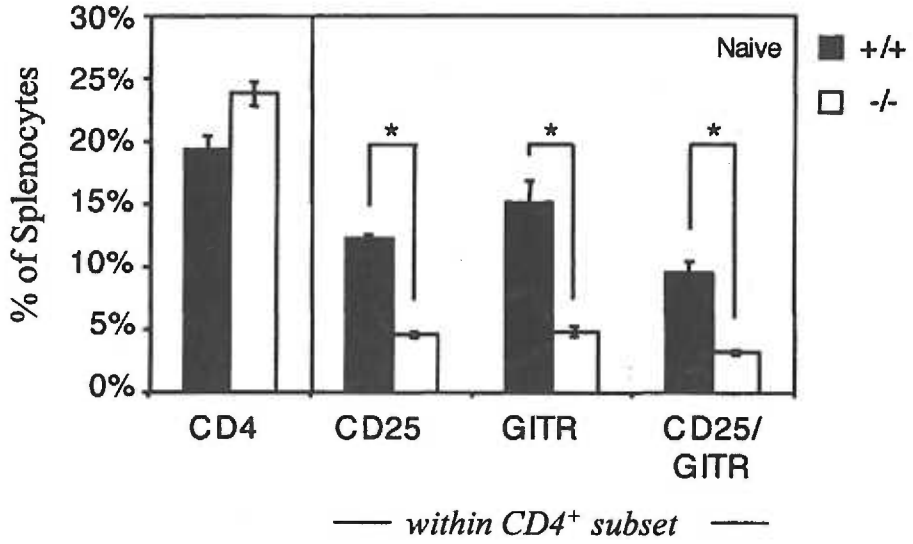
3.3 Regulatory T cells and the secondary $CD8^+$ T-cell response to L. monocytogenes

$CD40^{-/-}$ mice contain a reduced regulatory T-cell population

Previous evaluations of $CD4^+$ $CD25^+$ regulatory T cells (T_{REG}) in $CD40^{-/-}$ mice of two different MHC haplotypes have demonstrated that the absence of CD40 results in a marked reduction within the T_{REG} population (176, 177). To confirm this finding in my experimental system, I evaluated the frequency of T_{REG} cells in both naive and *Listeria*-immune wildtype and $CD40^{-/-}$ BALB/c mice by flow cytometry. *Listeria*-immune mice were analyzed 4 weeks after primary infection to reduce the possibility that activated effector $CD4^+$ T cells expressing the CD25 activation marker might be included in the calculation of T_{REG} populations defined as $CD4^+$ $CD25^+$ $GITR^+$ (163, 168, 169).

In both naive and *Listeria*-immune $CD40^{-/-}$ mice, a marked decrease was observed in the frequency of $CD4^+$ $CD25^+$ $GITR^+$ cells compared with wildtype BALB/c mice (Fig. 23). In naive wildtype mice, $CD25^+$ $GITR^+$ cells constituted 9.5% of the $CD4^+$ T-cell population. In contrast, $CD25^+$ $GITR^+$ cells made up only 3.2% of the $CD4^+$ population in naive $CD40^{-/-}$ mice, a 66% reduction of the T_{REG} population compared with wildtype mice. In animals infected 4 weeks previously with *L. monocytogenes* infection,

A.



B.

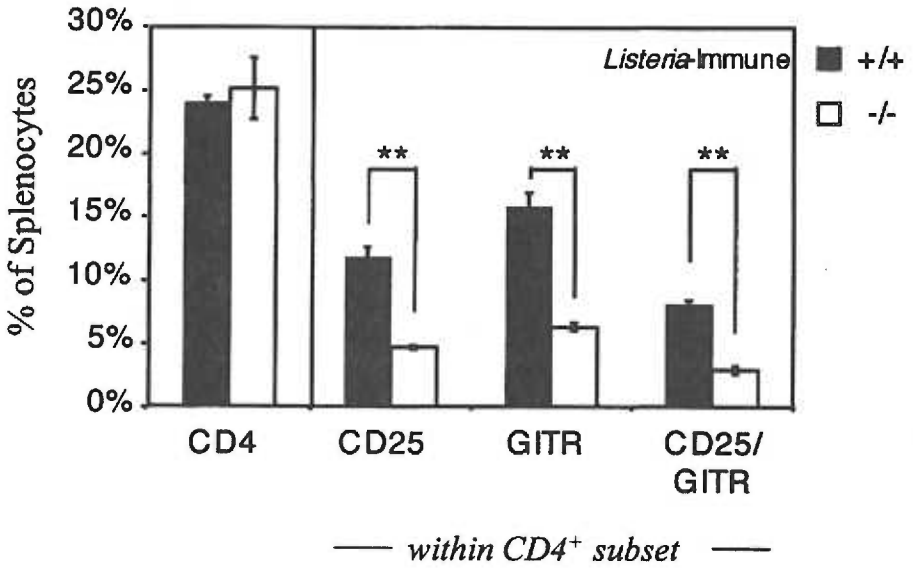


Figure 23. CD40$^{-/-}$ mice have a reduced regulatory T-cell population. (A) Naive or (B) *Listeria*-immune wildtype (+/+) and CD40-deficient (-/-) BALB/c mice were evaluated for regulatory T cells in the spleen. CD4, CD25 and GITR staining was evaluated by flow cytometry. *Listeria*-immune mice were analyzed 4 weeks after primary *L. monocytogenes* infection. Data represents 1 of 3 experiments with 3-5 mice/group. (* $p<0.002$, ** $p<0.007$).

a similar decrease in the T_{REG} population was observed in $CD40^{-/-}$ mice. Within the $CD4^+$ subset, the frequency of $CD25^+$ $GITR^+$ cells averaged 15.8% in wildtype mice, but only 6.4% of the $CD4^+$ population in $CD40^{-/-}$ mice. Importantly, in both naive and *Listeria*-immune animals the frequency of total $CD4^+$ cells was equivalent or slightly greater in $CD40^{-/-}$ than wildtype BALB/c mice, indicating that the reduction observed in the $CD25^+$ $GITR^+$ subset was specific for T_{REG} cells and not a general deficiency in the ability of $CD40^{-/-}$ mice to generate or maintain $CD4^+$ T cells. These data confirm that $CD40^{-/-}$ mice contain a naturally reduced frequency of T_{REG} cells.

To verify that the reduction of T_{REG} observed in $CD40^{-/-}$ mice resulted specifically from CD40-deficiency, and not some other variation unique to our breeding colony, $CD40^{-/-}$ mice were backcrossed to wildtype BALB/c mice. Then ($CD40^{-/-}$ x BALB/c) F1 animals were bred to each other, and $CD40^{-/-}$ mice reselected based on flow cytometry. The frequency of T_{REG} cells in the spleen of these backcrossed $CD40^{-/-}$ mice was evaluated based on cell-surface staining for CD4, CD25 and GITR expression (Fig. 24A). When compared with wildtype BALB/c mice and animals from the original $CD40^{-/-}$ breeding colony, the reselected $CD40^{-/-}$ animals from F1xF1 mating showed the same reduction in T_{REG} cells as previously observed in the original $CD40^{-/-}$ mice. This independent observation supports the premise that CD40 expression is involved with either the generation or maintenance of $CD4^+$ $CD25^+$ regulatory T-cell populations.

To evaluate whether CD40 expression was involved in the maintenance of T_{REG} cells, the frequency of $CD4^+$ $CD25^+$ regulatory T cells in the spleens of naive female $CD40^{-/-}$ mice was evaluated at 6, 11 and 13 weeks of age (Fig. 24B). These experiments revealed a loss of regulatory cells over time, with T_{REG} cells comprising 5.28% of the

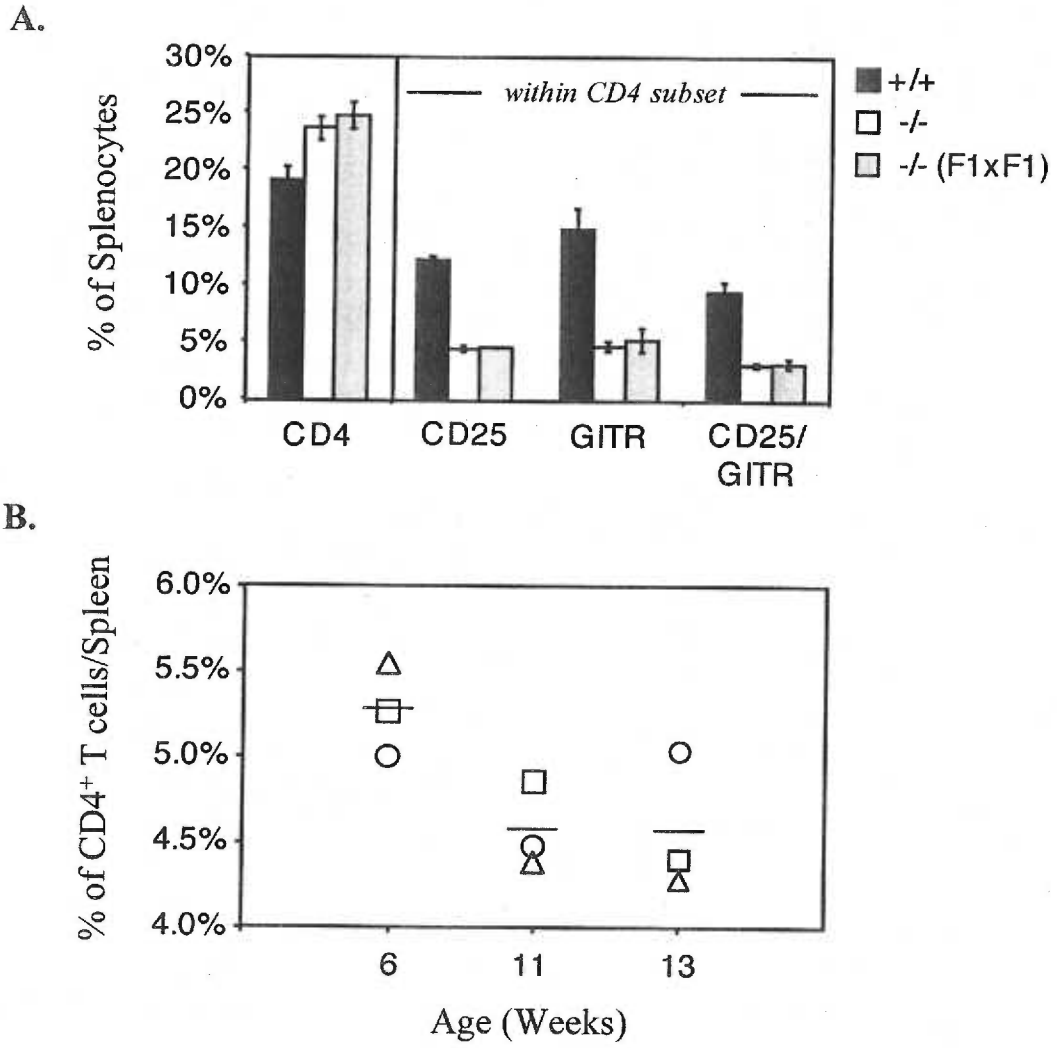


Figure 24. CD40$^{-/-}$ mice have reduced regulatory T-cell populations within the spleen. (A) 12 week-old female BALB/c, CD40-/- mice, and (CD40$^{-/-}$x BALB/c) F1xF1 mice (reselected for the absence of CD40 expression by flow cytometry) were evaluated for regulatory T-cell markers by flow cytometry. CD4, CD25 and GITR staining was performed on splenocyte populations. (B)The frequency of CD4^{+}CD25^{+} cells in individual CD40$^{-/-}$ mice of various ages was evaluated. Data represents 1 of 2 experiments with 3 mice/group.

$CD4^+$ T-cell population at 6 weeks of age, and 4.58% at 11 weeks of age. No further loss was evident between weeks 11 and 13 (4.58% and 4.57%, respectively). These data suggest that CD40 expression is involved in the peripheral maintenance of T_{REG} cells after their emigration from the thymus.

CD4-depletion prior to L. monocytogenes challenge increases the magnitude of secondary $CD8^+$ T-cell responses

In addition to decreased $CD4^+$ $CD25^+$ T_{REG} cells, the absence of CD40 expression may impact other aspects of the immune response to *L. monocytogenes*. To evaluate more closely the influence of T_{REG} cells on the magnitude of secondary $CD8^+$ T-cell expansion following *Listeria* challenge, I performed *in vivo* depletion of all $CD4^+$ cells prior to secondary *L. monocytogenes* challenge in *Listeria*-immune wildtype and $CD40^{-/-}$ BALB/c mice. The use of antibody-mediated in vivo depletion of $CD4^+$ cells as a tool for interpreting the influence of regulatory cell populations on $CD8^+$ T-cell responses is complicated by the fact that classical helper $CD4^+$ T cells are depleted as well. However, as $CD8^+$ T-cell responses to *L. monocytogenes* have been shown to occur in the absence of $CD4^+$ T cells, I felt that the *in vivo* CD4-depletion approach could potentially provide indirect evidence for T_{REG} activity in the *Listeria* infection model.

Four weeks following the primary *L. monocytogenes* infection, wildtype and $CD40^{-/-}$ mice were treated with depleting anti-CD4 monoclonal antibodies, or left untreated, and then all animals were challenged with 10 LD_{50} *L. monocytogenes*. On days 5 and 11 after secondary *Listeria* challenge, the number of peptide-specific $CD8^+$ T cells in the spleen was determined by intracellular cytokine staining (Fig. 25).

At day 5 after *L. monocytogenes* challenge, the depletion of $CD4^+$ cells in either wildtype or $CD40^{-/-}$ mice had no effect on the magnitude of LLO_{91-99}-specific $CD8^+$ T-cell expansion. In contrast, depletion of $CD4^+$ cells had a dramatic effect on the magnitude of the $p60_{217-225}$-specific population in wildtype mice, more than doubling the number of $p60_{217-225}$-specific cells from $1.3x10^6$ to $3.2x10^6$. The influence of CD40-deficiency was similar, with these animals yielding an average of $3.77x10^6$ $p60_{217-225}$-specific $CD8^+$ T cells per spleen. CD4-depletion in $CD40^{-/-}$ mice did not significantly enhance either peptide-specific $CD8^+$ T-cell response. Importantly, CD4-depletion did not influence the total number of $CD8^+$ T cells (Fig. 25C), indicating that the enhanced responses observed in CD4-depleted BALB/c and $CD40^{-/-}$ mice reflect specific expansions within peptide-specific $CD8^+$ T-cell populations.

The influence of CD4-depletion was even more apparent at day 11 after secondary *L. monocytogenes* challenge, after the onset of contraction. Significant differences in the maintenance of LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cell populations were evident in both wildtype and $CD40^{-/-}$ BALB/c mice depleted of $CD4^+$ cells, relative to untreated controls. CD4-depletion in wildtype BALB/c mice increased the number of LLO_{91-99}-specific $CD8^+$ T cells remaining approximately three-fold, from $1.1x10^6$ to $3.6x10^6$ per spleen. A similar effect was seen in $CD40^{-/-}$ mice, with CD4-depletion increasing the number of LLO_{91-99}-specific $CD8^+$ T cells from $1.6x10^6$ to $3.5x10^6$ per spleen. The $p60_{217-225}$-specific $CD8^+$ T-cell population in wildtype mice was increased from $2.5x10^5$ to $8.2x10^5$ cells per spleen in animals depleted of $CD4^+$ cells. In $CD40^{-/-}$ mice, CD4-depletion prior to *L. monocytogenes* challenge increased the $p60_{217-225}$-specific population from $5.5x10^5$ to $2.0x10^6$ cells per spleen.

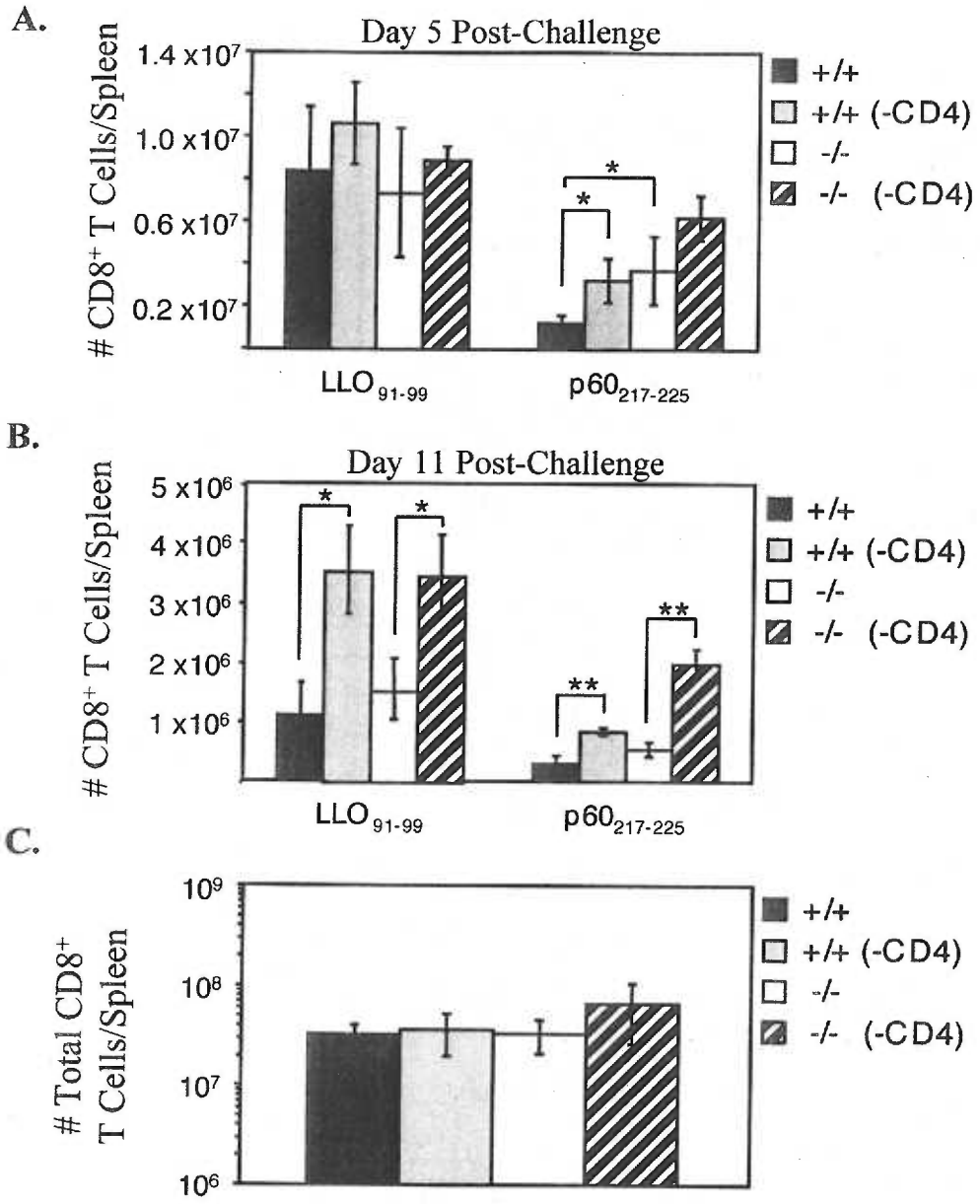


Figure 25. CD4-depletion prior to secondary *Listeria* challenge enhances the expansion of antigen-specific CD8$^+$ T cells. Wildtype (+/+) and CD40-deficient (-/-) *Listeria*-immune BALB/c mice were treated with depleting anti-CD4 antibodies prior to secondary *Listeria* challenge. The expansion of peptide-specific CD8$^+$ T cells in the spleen was analyzed on day 5 (A) and day 11 (B) after secondary *Listeria* challenge by intracellular cytokine staining for IFNγ-producing CD8$^+$ T cells in response to LLO_{91-99} and $p60_{217-225}$ peptides. (C) The total number of CD8$^+$ T-cells in CD4-depleted and untreated mice was evaluated on day 5 after challenge. Data represents 1 of 3 experiments with 2-3 mice/group. (* $p<0.02$, ** $p<0.003$).

Thus, in the absence of $CD4^+$ cells, two events are apparent: enhanced expansion of $CD8^+$ T-cell populations at earlier timepoints following secondary *Listeria* challenge, as well as a slower decline in these $CD8^+$ T-cell populations during contraction.

$CD40^{-/-}$ mice poorly regulate the expansion of adoptively transferred memory $CD8^+$ T cells in response to L. monocytogenes challenge

My data suggest that the decreased T_{REG} population present in $CD40^{-/-}$ BALB/c mice results in poorly regulated secondary expansion of $CD8^+$ T cells following *L. monocytogenes* challenge. However, it is also possible that enhanced $CD8^+$ T-cell expansion in CD40-deficient mice reflects an inherent difference in the "permissiveness" of $CD40^{-/-}$ $CD8^+$ T cells to be regulated, rather than a difference in the number of T_{REG} cells present. To investigate these possibilities I performed a series of adoptive transfer experiments. I hypothesized that, if the reduced T_{REG} population in $CD40^{-/-}$ mice was responsible for the enhanced $CD8^+$ T-cell response, then memory $CD8^+$ cells from *Listeria*-immune wildtype donors adoptively transferred into $CD40^{-/-}$ recipients should also show enhanced secondary expansion following challenge. In contrast, if an inherent difference in the $CD8^+$ T cells' "permissiveness to be regulated" results from their development in wildtype or CD40-deficient environments, then memory $CD8^+$ cells from wildtype donors should show equivalent secondary responses to *Listeria* challenge, regardless of the whether these cells were transferred into wildtype or $CD40^{-/-}$ recipient mice.

To this end, $CD8^+$ T cells from *Listeria*-immune wildtype BALB/c mice ($Thy1.1^+$) were isolated, and 10^7 total $CD8^+$ T cells were transferred into naive wildtype or $CD40^{-/-}$ BALB/c recipients ($Thy1.1^-$). Following transfer, recipient mice were

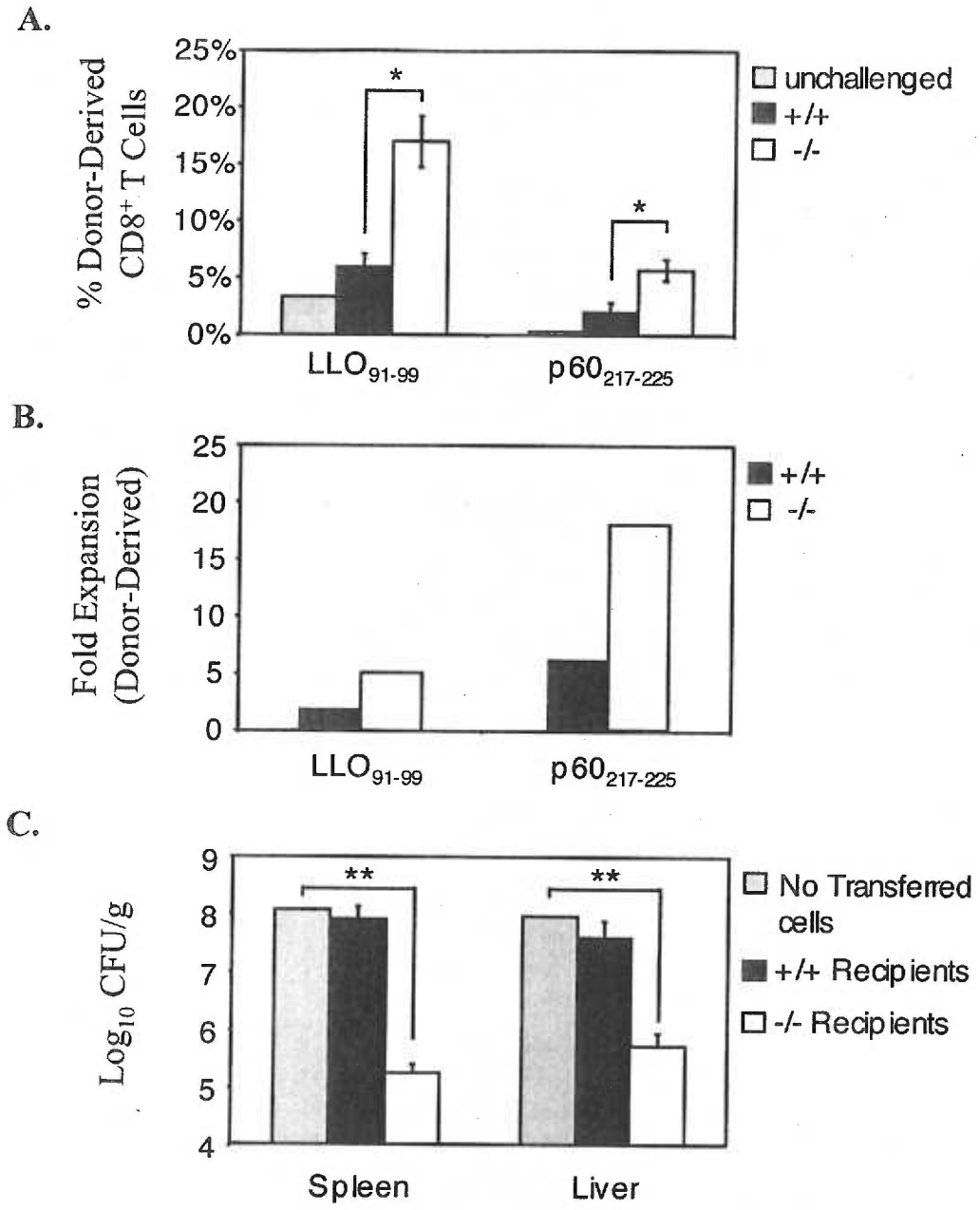


Figure 26. CD40$^{-/-}$ recipients of BALB/c memory cells show enhanced secondary CD8$^+$ T-cell expansion following *Listeria* challenge. Naive wildtype (+/+) and CD40-deficient (-/-) BALB/c mice received 0.6-1x10^7 CD8$^+$ cells from *Listeria*-immune wildtype BALB/c mice. Recipient mice were challenged with *Listeria*, and (A) the frequency of donor-derived, LLO_{91-99} and $p60_{217-225}$-specific CD8$^+$ T cells was determined 5 days after later by intracellular cytokine staining for IFNγ. (B) The fold expansion of peptide-specific populations in each recipient strain was calculated. Data represents 1 of 2 experiments with 4 mice/group. (C) The *in vivo* clearance of *Listeria* from the spleen and liver of recipient mice was determined 4 days after *Listeria* challenge. Data represents 1 of 1 experiment with 3 mice/group. (* $p<0.04$, ** $p<0.0009$).

challenged with *L. monocytogenes*, and the expansion of donor-derived (Thy1.1$^+$) CD8$^+$ T cells was evaluated 5 days later. As indicated in Figure 26, the expansion of peptide-specific CD8$^+$ T cells was markedly different depending upon the recipient mouse strain; wildtype LLO_{91-99} and $p60_{217-225}$-specific memory CD8$^+$ T cells transferred into CD40$^{-/-}$ recipients underwent greater expansion in response to *L. monocytogenes* infection compared to the same cell population following transfer into naive wildtype BALB/c mice. In wildtype recipients, the frequency of LLO_{91-99}-specific CD8$^+$ T cells reached 2.0% of the donor-derived populations, whereas in CD40$^{-/-}$ recipients, this same population expanded to 6.3% of the donor-derived CD8$^+$ T-cell pool. The response of $p60_{217-225}$-specific CD8$^+$ T cells showed a similar pattern of enhanced expansion in CD40$^{-/-}$ recipients, comprising 1.6% of the donor-derived CD8$^+$ T cell population compared with 0.6% in wildtype BALB/c recipients. By comparing the frequency of donor-derived peptide-specific CD8$^+$ T cells recovered from unchallenged recipient mice, the fold-expansion of both peptide-specific populations was calculated (Fig. 26B). Both the LLO_{91-99} and $p60_{217-225}$-specific, donor-derived CD8$^+$ T-cell populations underwent significantly greater expansion in CD40$^{-/-}$ recipients compared with wildtype BALB/c recipients.

I next evaluated whether this enhanced secondary expansion of antigen-specific CD8$^+$ T-cell populations conferred a protective advantage in CD40$^{-/-}$ recipients. Wildtype and CD40$^{-/-}$ BALB/c mice received CD8$^+$ cells from *Listeria*-immune wildtype mice, and were challenged the following day with *L. monocytogenes*. Bacterial clearance from the liver and spleen of recipient mice was determined on day 4 after *L. monocytogenes* challenge. As shown in Figure 26C, a clear difference was noted between wildtype and

$CD40^{-/-}$ recipients, with $CD40^{-/-}$ recipients showing nearly 1000-fold greater protection in both the liver and the spleen; wildtype recipients were unable to reduce the bacterial load in either organ. These results suggest that the enhanced expansion of transferred memory $CD8^+$ T cells observed in $CD40^{-/-}$ recipients 5 days after *Listeria* challenge (Fig. 26B) reflects a more rapid secondary expansion and/or acquisition of effector function by memory $CD8^+$ T-cells in $CD40^{-/-}$ environments, leading to early protection against bacterial challenge in CD40-/- recipients relative to wildtype BALB/c recipient mice.

Collectively, these results indicate that, when the memory $CD8^+$ T-cell pool is transferred into naive $CD40^{-/-}$ recipients, antigen-specific memory $CD8^+$ T cells undergo greater expansion in response to *Listeria* challenge than when the same cells are transferred into naive wildtype BALB/c mice. This supports the hypothesis that a difference exists in the ability of $CD40^{-/-}$ recipient mice to regulate the secondary expansion of memory $CD8^+$ T cells. In addition, in both wildtype and $CD40^{-/-}$ recipients, $p60_{217\text{-}225}$-specific cells showed a greater fold-expansion than the $LLO_{91\text{-}99}$-specific population in each respective host strain. This data reveals that, following adoptive transfer, antigen-specific populations present at lower frequencies ($p60_{217\text{-}225}$) undergo greater fold-expansion than more immunodominant populations ($LLO_{91\text{-}99}$), and suggests that the secondary expansion of $CD8^+$ T-cell populations of different specificities may be regulated independently.

Expansion of adoptively transferred memory $CD8^+$ T cells in response to Listeria is enhanced in CD4-depleted recipient mice

Factors other than the reduction within the T_{REG} population also may influence the secondary expansion of memory $CD8^+$ T cells in CD40-deficient environments. To

evaluate directly the impact of $CD4^+$ T-cell populations on the secondary expansion of memory $CD8^+$ T cells, we performed another series of adoptive transfer experiments. Purified $CD8^+$ T cells from *Listeria*-immune wildtype mice ($Thy1.1^+$) were transferred into naive or CD4-depleted wildtype recipients ($Thy1.1^-$). Recipient mice were challenged the following day with *L. monocytogenes*, and the expansion of donor-derived, peptide-specific $CD8^+$ T cells was evaluated 5 days later (Fig. 27). I found a pattern of enhanced secondary $CD8^+$ T-cell expansion in CD4-depleted wildtype recipients, similar to the response in $CD40^{-/-}$ recipients of wildtype memory $CD8^+$ T cells (Fig. 27). CD4-depletion in recipient wildtype mice resulted in the expansion of LLO_{91-99}-specific $CD8^+$ T cells to an average of 18.2% of the donor-derived $CD8^+$ T-cell population, compared with 3.5% in untreated wildtype recipients. Additionally, the depletion of $CD4^+$ cells within wildtype recipient mice increased the average frequency of $p60_{217-225}$-specific $CD8^+$ T cells from 2.7% to 4.4%. These results suggest that the presence of $CD4^+$ T cells within naive wildtype mice reduces the secondary expansion of antilisterial memory $CD8^+$ T cells stimulated by *L. monocytogenes* challenge.

I also evaluated whether CD4-depletion in $CD40^{-/-}$ recipients would further increase the secondary expansion of adoptively transferred $CD8^+$ memory T cells. Purified $CD8^+$ T cells from *Listeria*-immune wildtype mice ($Thy1.1^+$) were transferred into naive or CD4-depleted $CD40^{-/-}$ recipients ($Thy1.1^-$), followed by challenge with *L. monocytogenes*. The expansion of donor-derived, peptide-specific $CD8^+$ T cells was evaluated 5 days later. In contrast to the impact seen when $CD4^+$ cells were depleted from wildtype recipients, the depletion of $CD4^+$ cells from $CD40^{-/-}$ recipients had a minimal effect on the expansion of adoptively transferred memory $CD8^+$ T cells. This supports the

hypothesis that the residual T_{REG} population within the $CD4^+$ subset in $CD40^{-/-}$ mice has a reduced impact on the secondary expansion of antigen-stimulated memory $CD8^+$ T cells.

The expansion of antilisterial memory $CD8^+$ T cells is primarily regulated at high effector cell numbers

My data suggest that one property of T_{REG} cells is to set an "upper limit" on the number of peptide-specific $CD8^+$ T cells than can be generated in response to cognate stimulation. In Figure 20, I show that increasing the challenge dose in *Listeria*-immune BALB/c mice was not sufficient to increase the fold-expansion of either LLO_{91-99} or $p60_{217-225}$-specific $CD8^+$ T cells. This suggests that the expansion of these populations may have already reached a theoretical "allowable" upper limit in the wildtype BALB/c host. In contrast, when the *L. monocytogenes* challenge dose was increased in $CD40^{-/-}$ BALB/c mice, the fold-expansion of both peptide-specific $CD8^+$ T-cell populations was dramatically increased (up to ~700-fold expansion, compared with ~100-fold at 1 LD_{50}). This data argues against a passive mechanism that may limit the magnitude of secondary $CD8^+$ T-cell expansion in BALB/c mice, such as insufficient levels of cytokines to support proliferation, or access to APCs to receive TCR stimulation. Rather, this result suggests that there is an active regulatory event, potentially mediated by T_{REG} cells, which functions to suppress continued $CD8^+$ T-cell expansion in an environment with sufficient cytokine production and levels of antigen presentation. My data indicates that this regulatory factor is impaired in $CD40^{-/-}$ mice.

To evaluate whether the presence of $CD4^+$ T-cell populations function to set an upper limit on the expansion of peptide-specific $CD8^+$ T cells following antigen exposure, I transferred increasing numbers of $CD8^+$ cells from *Listeria* -immune BALB/c

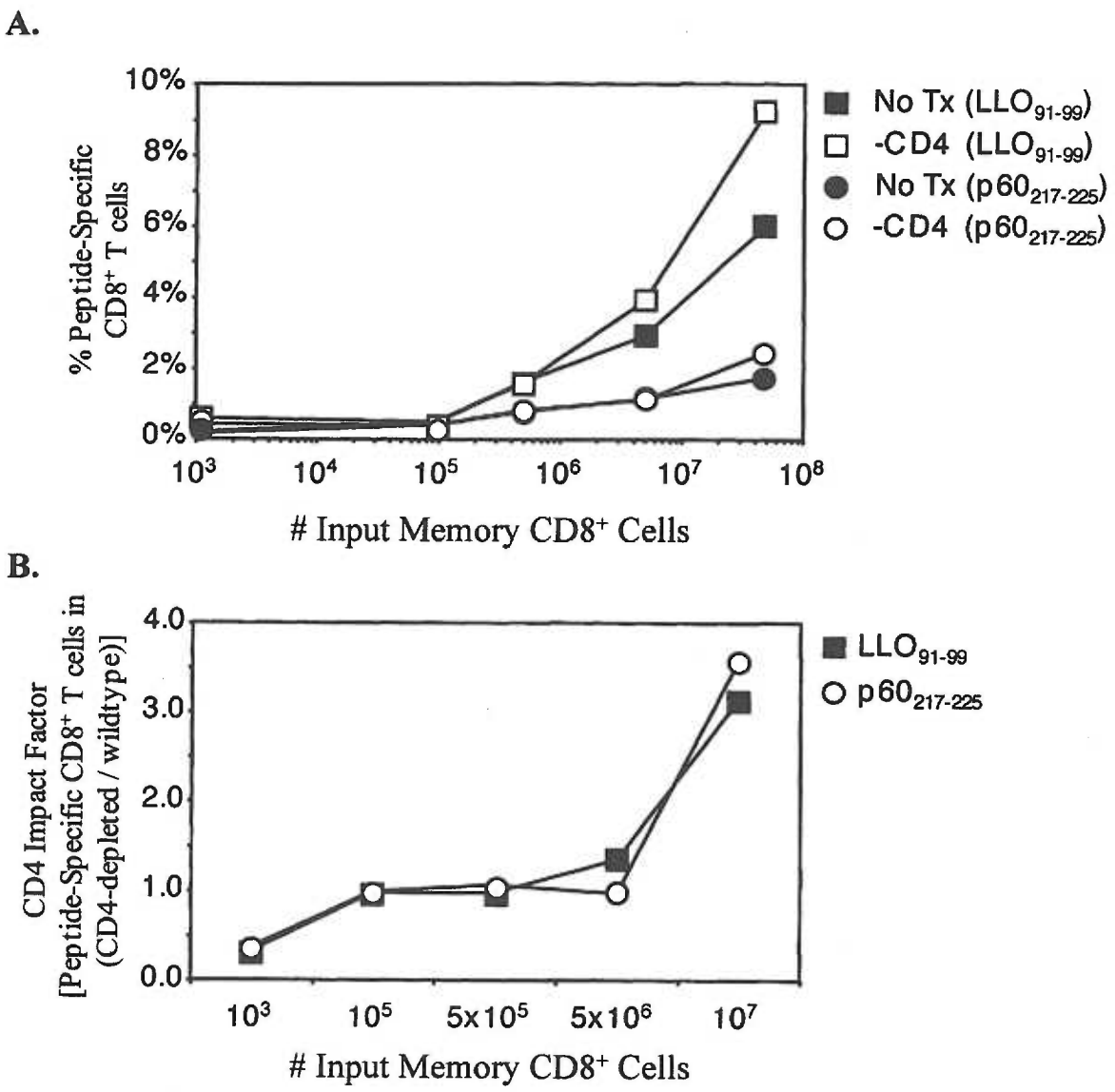


Figure 28. CD4-depletion influences CD8⁺ memory T-cell expansion at high input-cell numbers. Untreated or CD4-depleted BALB/c mice were transferred with a gradient of CD8⁺ cells from *Listeria*-immune BALB/c mice and then challenged with *Listeria*. (A) The frequency of peptide specific CD8⁺ T cells was calculated 5 days later by intracellular cytokine staining for IFNγ. (B) The ratio of cells in CD4-depleted / untreated recipient mice was calculated for each input cell number to determine the impact factor of CD4⁺ cells on the secondary expansion of transferred CD8⁺ T cells. Data represents 2 experiments with 2 mice/group/input cell number.

mice (Thy1.1$^+$) into naive BALB/c and CD4-depleted BALB/c recipients (Thy1.1$^-$). Following challenge with *L. monocytogenes*, the frequency of donor-derived, peptide-specific CD8$^+$ T cells was evaluated (Fig. 28). I discovered a differential influence of CD4$^+$ cells depending on the input cell number. By comparing the ratio of peptide-specific CD8$^+$ T cells within these two recipient environments (CD4-depleted recipients/untreated recipients), we calculated an "impact factor" (IF) that the presence of CD4$^+$ cells had on the expansion of each peptide-specific CD8$^+$ T-cell population. At lower numbers of input cells, CD4-depleted recipients showed decreased CD8$^+$ T-cell expansion relative to wildtype BALB/c recipients, indicating that the presence of CD4$^+$ cells was beneficial for the expansion of the transferred memory CD8$^+$ T-cell population (IF < 1.0). At intermediate numbers of input cells, the presence or absence of CD4$^+$ cells had little influence on the magnitude of CD8$^+$ T-cell expansion (IF ~ 1.0). In contrast, at higher input cell numbers, the presence of CD4$^+$ cells inhibited the expansion of the donor-derived CD8$^+$ T cells compared with the expansion seen in CD4-depleted recipients (IF > 1.0). These results support the hypothesis that a population of cells within the CD4$^+$ subset is responsible for controlling the magnitude of CD8$^+$ T-cell expansion following secondary antigen exposure. Additionally, these data suggest that this regulatory function becomes evident as effector T-cell populations expand numerically.

Summary to Results Section 3.3

The data presented in sections 1 and 2 demonstrate that the absence of CD40 expression had very little effect on the functional properties of memory CD8$^+$ T cells generated in response to systemic infection with *L. monocytogenes.* Rather, following

secondary *Listeria* challenge, $CD40^{-/-}$ mice show enhanced expansion of antigen-specific T-cell populations relative to wildtype BALB/c mice. To begin to evaluate the mechanisms behind the differential T-cell expansion observed in wildtype and BALB/c mice, I evaluated the frequency of phenotypically defined regulatory T-cell populations in both strains by cell surface staining for CD4, CD25 and GITR expression.

$CD40^{-/-}$ mice harbor a markedly reduced frequency of $CD4^+$ $CD25^+$ $GITR^+$ T cells compared with wildtype BALB/c mice. $CD40^{-/-}$ mice were backcrossed with wildtype BALB/c animals, then ($CD40^{-/-}$ x BALB/c) F1 animals were bred to each other, and new breeding pairs of $CD40^{-/-}$ mice were reselected based on flow cytometry. Similar to the original $CD40^{-/-}$ mice, animals from the backcrossed $CD40^{-/-}$ colonies had a significantly reduced number of $CD4^+$ $CD25^+$ $GITR^+$ regulatory T cells. Evaluation of the frequencies of T_{REG} cells in $CD40^{-/-}$ mice of different ages suggests that $CD40^{-/-}$ mice show a loss of regulatory cells over time.

Depletion of $CD4^+$ T cells in both wildtype and $CD40^{-/-}$ *Listeria*-immune BALB/c mice prior to secondary challenge with *L. monocytogenes* resulted in increased numbers of peptide-specific $CD8^+$ T-cells at both 5 and 11 days following challenge. Yet, CD4-depletion did not influence the total number of $CD8^+$ T cells, indicating that this enhancement observed in CD4-depleted mice reflect a specific influence on peptide-specific $CD8^+$ T-cell populations in response to infection. Therefore, although the frequency of T_{REG} cells is reduced in $CD40^{-/-}$ mice, the residual T_{REG} population is capable of exerting a suppressive influence on the secondary expansion of $CD8^+$ T cells. These data further support the hypothesis that CD40 expression influences the

development or maintenance of T_{REG} cells, but that the functional activity of T_{REG} cells is not dependent on CD40 expression.

By adoptively transferring memory $CD8^+$ T cells from *Listeria*-immune wildtype and $CD40^{-/-}$ BALB/c mice, I began to evaluate the parameters that might influence the enhanced secondary expansion of $CD8^+$ T cells observed in $CD40^{-/-}$ mice. Wildtype BALB/c memory $CD8^+$ T cells were transferred into wildtype, CD4-depleted, and $CD40^{-/-}$ recipients, followed by challenge with *L. monocytogenes*. Analyses of the secondary expansion of donor-derived $CD8^+$ T cells in these experiments indicate that the magnitude of the recall proliferative response of the donor-derived $CD8^+$ T cells is dependent on the recipient environment. In both CD4-depleted wildtype recipients and $CD40^{-/-}$ recipients, significantly enhanced donor-derived $CD8^+$ T-cell expansion is seen compared to wildtype recipient mice. In contrast to the impact seen when $CD4^+$ cells are depleted from wildtype recipients, the depletion of $CD4^+$ cells from $CD40^{-/-}$ recipients has a minimal effect on the expansion of adoptively transferred memory $CD8^+$ T cells. This suggests that the residual T_{REG} population within the $CD4^+$ subset in $CD40^{-/-}$ mice has a reduced impact on the secondary expansion of antigen-stimulated memory $CD8^+$ T cells.

I hypothesized that T_{REG} cells function to set an upper limit on the expansion of peptide-specific $CD8^+$ T cells. To address this, increasing numbers of wildtype memory $CD8^+$ T cells were transferred into untreated and CD4-depleted wildtype BALB/c recipients, then recipients were challenged with *Listeria*. These data demonstrate that the presence of $CD4^+$ cells in the recipient has the greatest impact on the expansion of the donor-derived $CD8^+$ T cells at higher input cell numbers. These results support the

hypothesis that a population of cells within the $CD4^+$ subset is responsible for controlling the magnitude of $CD8^+$ T-cell expansion following secondary antigen exposure.

In summary, I find that $CD40^{-/-}$ mice contain a reduction in the $CD4^+$ $CD25^+$ regulatory T-cell population that correlates with enhanced expansion of antigen-specific $CD8^+$ T cells following secondary *L. monocytogenes* challenge. CD4-depleted BALB/c mice also show enhanced $CD8^+$ T-cell expansion to *Listeria*, yet CD4-depletion in $CD40^{-/-}$ mice does not further enhance $CD8^+$ T-cell expansion to *Listeria*. Adoptive transfer of memory $CD8^+$ T cells into naive wildtype, $CD40^{-/-}$, or CD4-depleted BALB/c recipients suggests that cells within the $CD4^+$ cell population limit the magnitude of secondary $CD8^+$ T-cell expansion to antigen, primarily as high numbers of antigen-specific $CD8^+$ T cells are achieved. Collectively, the *in vivo* depletion and adoptive transfer experiments collectively indicate that a regulatory $CD4^+$ cell population, most likely the $CD4^+$ $CD25^+$ subset, is very influential in limiting the magnitude of secondary $CD8^+$ T-cell expansion to *Listeria* infection.

CHAPTER 4: DISCUSSION

The interplay between APC, $CD4^+$ T cells and $CD8^+$ T cells leading to the activation and differentiation of naïve $CD8^+$ T cells to form a protective memory population has been extensively studied, and further understanding of how these cells interact will assist the development of future vaccination strategies. Experimental data from several groups has led to the generally-accepted "APC licensing" theory that proposes $CD4^+$ T cells expressing CD40L are able to stimulate APC maturation through CD40 ligation, resulting in a mature APC capable of priming naïve $CD8^+$ T cells (42, 47, 48). However, CD40-CD40L interactions are not required for the induction of all $CD8^+$ T-cell responses, demonstrating additional pathways of APC licensing. For some pathogens, TLR ligation by pathogen-derived products and/or other stimuli provided by the infection itself are sufficient to produce mature APC capable of inducing peptide-specific $CD8^+$ T-cell responses. However, it remains unclear whether the mechanism of this APC maturation influences the functional properties of the subsequent cellular immune response stimulated by these APC. For example, does a dendritic cell activated by CD40 ligation stimulate an antigen-specific memory $CD8^+$ T-cell population with equivalent function as antigen-specific $CD8^+$ T cells primed by a dendritic cell activated through TLR and/or inflammatory cytokine signals? Does the mechanism of APC activation have downstream effects on the functional properties of the cellular immune response generated?

In addition to facilitating APC maturation through CD40-CD40L interactions, a second mechanism of $CD4^+$ T-cell-help to $CD8^+$ T-cell responses has been brought forward in the past few years. Several groups have demonstrated that memory $CD8^+$ T

cells generated in $CD4^+$ T-cell deficient mice show qualitative defects, including attrition of memory cell numbers, decreased secondary proliferation to antigen, and impaired effector cytokine production on a per cell basis (93-95). In all of these reports, $CD4^+$ T-cell help was not required for the primary activation or acquisition of effector functions by $CD8^+$ T cells. Rather, defects in $CD8^+$ T-cell function in CD4-deficient mice only became apparent after the generation of resting memory $CD8^+$ T-cell populations.

Collectively, these various reports support a model in which there are two distinct periods during which $CD4^+$ T cells influence $CD8^+$ T-cell responses though markedly different mechanisms. First, depending on the antigen source, the priming of naive $CD8^+$ T-cell populations may be reliant on $CD4^+$ T cells to fully activate APC through CD40-CD40L mediated maturation. While $CD8^+$ T cell responses to numerous pathogens can occur without CD40-mediated APC activation, the activation of naive $CD8^+$ T cells in response to other pathogens, as well as cross-presented antigens, requires $CD4^+$ T-cell mediated APC activation through CD40 ligation (47, 48, 77, 79, 82, 84-87). Dependency at this stage appears to be variable, and relates to the nature of the initial stimuli.

Second, the presence of $CD4^+$ T cells influences the function of antigen-specific memory $CD8^+$ T cells, although how this is accomplished is unclear. One possibility is that a specific signal is provided to responding $CD8^+$ T cells by cells within the $CD4^+$ T-cell subset, "imprinting" them with the ability to become protective memory populations. The timing of this potential signal is uncertain, and such imprinting of $CD8^+$ T cells may occur at the time of priming, during the transition from effector to memory phases, or following the development of memory $CD8^+$ T-cell populations. Delivery of this signal might be achieved through a specific CD4-CD8 T-cell interaction. Alternatively, $CD4^+$ T

cells may condition the environment to allow the development or maintenance of functional memory $CD8^+$ T cells through interactions with a third cell population, such as APC or stromal cells. Regardless of the mechanisms involved, several reports support the hypothesis that, even in situations where $CD4^+$ T_H cells are not required for CD40-mediated APC activation (such as helper-independent $CD8^+$ T-cell responses to pathogens), the development of functional memory $CD8^+$ T cells is still considerably influenced by the presence of $CD4^+$ T cells (93-95).

With the data presented in this dissertation I have evaluated both primary and memory $CD8^+$ T-cell responses following systemic *L. monocytogenes* infection in wildtype and CD40-deficient BALB/c mice. These experiments revealed that, in the context of *Listeria* infection, the absence of CD40 expression has very few consequences on the functional properties of $CD8^+$ T-cell populations. Thus, direct CD40 ligation on responding $CD8^+$ T cells is not a global requirement for memory $CD8^+$ T-cell development, nor is it involved with imprinting subsequent memory $CD8^+$ T-cell function. Further, transient depletion of $CD4^+$ T-cell populations prior to primary *L. monocytogenes* infection indicated no need for $CD4^+$ T cells to be present at the time of $CD8^+$ T-cell priming to "imprint" the development of protective $CD8^+$ T-cell memory. Finally, utilizing $CD4^+$ T-cell depletion prior to secondary *Listeria* infection, the experiments described in this dissertation suggest an additional role for regulatory T cells contained within the $CD4^+$ T-cell subset in controlling the magnitude of the memory $CD8^+$ T cell response to cognate antigen.

Primary CD8+ T-cell responses to systemic infection with L. monocytogenes are not dependent on CD40 expression

The primary activation of $CD8^+$ T-cell responses to pathogens can be divided into two categories based on their dependency for $CD4^+$ T cells to provide an APC maturation signal through CD40-CD40L interaction. The $CD8^+$ T-cell response that develops following systemic infection of mice with the intracellular bacterium *L. monocytogenes* has been characterized as being both $CD4^+$ T cell and CD40-CD40L independent, as the priming of protective antilisterial $CD8^+$ T-cell populations occurs readily in the absence of both (40, 75, 76). Previous characterizations of the CD40-CD40L independent $CD8^+$ T-cell response following intravenous *L. monocytogenes* infection have been accomplished by blocking such interactions through administration of anti-CD40L antibodies *in vivo* or by infection of $CD40L^{-/-}$ mice (40, 87, 150). In response to *Listeria* infection, the only notable contribution of CD40-CD40L interactions on $CD8^+$ T-cell responses was observed following *in vivo* CD40L antibody blockade, where the number of resting $CD8^+$ memory T cells generated was slightly reduced (87).

The data presented in this dissertation indicate that neither the absence of CD40-CD40L interactions, nor transient depletion of $CD4^+$ T cells weakens the magnitude or functional properties of the primary $CD8^+$ T-cell response to *L. monocytogenes* infection. CD4-depleted and $CD40^{-/-}$ BALB/c mice showed equivalent peak numbers of both $LLO_{91\text{-}99}$ and $p60_{217\text{-}225}$-restricted primary $CD8^+$ T-cell populations relative to wildtype BALB/c mice (Figs. 2 and 5). The primary effector CTL generated in the absence of $CD4^+$ T-cell help or CD40-CD40L interactions displayed normal cytolytic function as indicated by equivalent clearance of both peptide-coated target cells and viable bacteria *in vivo* (Figs. 3 and 6).

Kinetic differences, however, were observed in the primary expansion of MHC-Ia-restricted $CD8^+$ T cells following *L. monocytogenes* infection in wildtype and CD40-deficient BALB/c mice. In $CD40^{-/-}$ mice, the primary expansion of antigen-specific $CD8^+$ T-cells was slower, taking several more days to reach an equivalent peak magnitude in both the LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cell populations relative to wildtype BALB/c mice (Fig. 2C). It is unknown whether this kinetic difference is caused by a slower cellular division rate in the absence of CD40-CD40L interactions, or alternatively, reflects a decrease in the number of responding $CD8^+$ T cells. If so, this smaller responding population within $CD40^{-/-}$ mice might undergo a prolonged proliferative phase to reach a similar peak number before entering into the (delayed) contraction phase.

Similar to the classical $CD8^+$ T-cell response, the magnitude of nonclassical f-MIGWII-specific $CD8^+$ T-cell expansion was not influenced by $CD4^+$ T-cell mediated help during the primary immune response to *L. monocytogenes*. H2-M3-restricted f-MIGWII specific $CD8^+$ T cells were activated and able to secrete IFNγ in the absence of both CD40 expression and $CD4^+$ T-cell populations, demonstrating that these events are not required for the priming of MHC-Ib restricted $CD8^+$ T cells to *Listeria* antigens (Fig. 7). Together these results suggest that primary $CD8^+$ T-cell responses to systemic infection with *L. monocytogenes* harbor no intrinsic requirement for either the presence of $CD4^+$ T-cell help in general, or CD40-expression specifically.

Primary CD4+ T-cell responses to systemic infection with L. monocytogenes do not require CD40 expression

Following systemic *L. monocytogenes* infection in $CD40L^{-/-}$ mice, the upregulation of the CD44 activation marker on $CD4^+$ T-cells is similar to that observed in wildtype mice, suggesting no inherent defect in $CD4^+$ T-cell activation in the absence of CD40-CD40L signals (94). However, these experiments were conducted on total $CD4^+$ T-cell populations, and may not detect specific differences in the response of *Listeria*-specific $CD4^+$ T cell populations. The primary activation of peptide specific $CD4^+$ T cells following LCMV infection is markedly reduced in $CD40L^{-/-}$ mice, yet a subpopulation of these cells survive as long-lived memory $CD4^+$ T cells (196). These findings suggest that the development of memory $CD4^+$ T-cell populations can occur in the absence of CD40-CD40L costimulation, although perhaps at a reduced number. The data presented in this dissertation support this interpretation.

In contrast to both classical and nonclassical $CD8^+$ T-cell populations, the absence of CD40 expression resulted in a marked reduction in the magnitude of the primary $LLO_{189\text{-}200}$-specific $CD4^+$ T-cell response to *L. monocytogenes* infection (Fig 4A). Further, the kinetics of the primary $LLO_{189\text{-}200}$-specific $CD4^+$ T-cell response was altered in $CD40^{-/-}$ BALB/c mice, with the onset of contraction significantly delayed relative to wildtype BALB/c mice (Fig. 4B). Combined with the evaluation of the primary $CD8^+$ T-cell responses discussed above, these data suggest that the absence of CD40 expression may have a greater impact on the primary $CD4^+$ T-cell responses than on $CD8^+$ T-cell responses to *Listeria*, yet appears to influence the contraction kinetics of both T-cell populations. However, regardless of the numeric reduction in the magnitude of the primary $CD4^+$ T-cell response observed in $CD40^{-/-}$ mice, by day 30 after *L.*

monocytogenes infection these cells were maintained as memory T-cell populations at numbers equivalent to that in wildtype BALB/c mice (Fig. 4). Collectively, these results indicate that CD40 expression is not required for the development of cellular immunity to *L. monocytogenes*.

The functional properties of antilisterial memory CD8+ T-cells are not directed through CD40-CD40L interactions

Many signals that influence primary $CD8^+$ effector T-cell populations to expand, then subsequently contract and be maintained as protective memory populations have been extensively studied, providing pieces of the larger picture of how multiple signals are integrated to direct memory $CD8^+$ T-cell development. However, a clear and complete model of this process has not yet been achieved.

Recent reports have suggested that, although $CD8^+$ T cells can be primed in the absence of $CD4^+$ T cells, after conversion to the memory state, these $CD8^+$ T cells have functional defects that become apparent upon secondary antigen exposure (61, 93-95). Importantly, these findings have been generalized to include memory $CD8^+$ T cells specific for minor transplantation antigens as well as several different infectious agents. In these reports, normal primary activation of $CD8^+$ T-cells was observed in the absence of $CD4^+$ T-cell help relative to wildtype mice; only after contraction into memory $CD8^+$ T-cell populations were the functional defects of "unhelped" $CD8^+$ T cells revealed. Several theories as to how $CD4^+$ T-cells might influence the functional quality of memory $CD8^+$ T-cell populations have been advanced. As CD40 is transiently expressed on a subset of $CD8^+$ T cells during activation, direct ligation of CD40 on the surface of $CD8^+$ T cells by CD40L-bearing $CD4^+$ T cells has been proposed to be a potential

mechanism of direct $CD4^+$-$CD8^+$ collaboration and a contributor to $CD8^+$ T-cell memory development (61, 97, 98).

The data presented in this dissertation demonstrated that the number of classically restricted memory $CD8^+$ T-cells maintained in $CD40^{-/-}$ mice 30 days following *L. monocytogenes* infection was comparable to that found in wildtype BALB/c mice (Fig. 8). To determine whether these memory $CD8^+$ T-cells generated in the absence of CD40 were functionally compromised, I evaluated some of the parameters that define functional memory $CD8^+$ T cells: *in vivo* killing of antigen-bearing target cells (Figs. 9 and 10), protection against challenge infection (Fig. 10), TCR avidity (Fig. 11), effector cytokine production (Fig. 12), and secondary expansion to cognate antigen (Fig. 15). These experiments indicated that the majority of these hallmarks of $CD8^+$ T-cell memory were expressed fully in CD40-deficient mice following *L. monocytogenes* infection, suggesting that direct $CD4^+$-$CD8^+$ interactions mediated through CD40 are not required for the generation of functional $CD8^+$ memory T cells.

Although a small decrease in the TCR avidity was observed in memory $CD8^+$ T cells generated in $CD40^{-/-}$ mice (Fig. 11), this had no functional consequences in this model system, as $CD40^{-/-}$ mice were fully protected against an otherwise lethal secondary *Listeria* challenge infection (Fig. 10). Therefore, although antilisterial memory $CD8^+$ T-cells from $CD40^{-/-}$ mice may be less sensitive to small antigen concentrations, they are fully functional effectors once activated. In the absence of CD40 expression, similar numbers of antigen-specific $CD8^+$ T cells were recovered expressing markers for both central memory (T_{CM}) and effector memory (T_{EM}) phenotypes (Fig. 17). Collectively, these data indicate that neither the activation of APC via CD40 ligation, nor direct

ligation of CD40 on the surface of $CD8^+$ T cells directs a specific functional program in the developing $CD8^+$ T-cell response.

Recently, Sun and Bevan reported that the generation of SIINFEKL-specific memory $CD8^+$ T cells following infection with recombinant *L. monocytogenes* expressing ovalbumin is equivalent in wildtype, $CD40^{-/-}$ and $CD40L^{-/-}$ mice on the $H\text{-}2K^b$ MHC-I background (99). Utilizing mixed bone marrow chimeric mice containing cells from both wildtype and $CD40^{-/-}$ donors within the same recipient, they evaluated the $CD8^+$ T-cell responses to LCMV and *Listeria* infection. They found that, within these chimeric animals, both wildtype and $CD40^{-/-}$ $CD8^+$ T cells were equally utilized in the primary and memory $CD8^+$ T-cell responses. This is consistent with a separate report suggesting that CD40 expression by $CD8^+$ T cells is not required for the generation of memory $CD8^+$ T cells in response to influenza infection (100). Collectively, these experiments confirm the interpretation that CD40 expression is not a global requirement for generating $CD8^+$ T-cell memory.

$CD4^+$ T-cells are not required to imprint responding $CD8^+$ T-cells with memory potential

The "imprinting" model has been proposed as a potential mechanism by which the presence of $CD4^+$ T cells directs the functional properties of memory $CD8^+$ T-cell populations. Although the data presented in this dissertation demonstrates that CD40-CD40L ligation is not involved in this hypothetic process, numerous other interactions between APC, $CD4^+$ and $CD8^+$ T cells remain possibilities. Although most discussions suggest that the presence of $CD4^+$ T cells is necessary during $CD8^+$ T-cell priming, thus providing the putative imprinting signal at early time points during the developing

immune response, the timing of such a signal is uncertain. The majority of the studies that imply an early requirement for $CD4^+$ T-cell imprinting of responding $CD8^+$ T cells have been evaluated in $CD4^{-/-}$ or MHC-$II^{-/-}$ mice, which are continuously deficient in $CD4^+$ T-cell populations (93-95). In these settings, the influence of early imprinting signals compared with possible later effects of $CD4^+$ T-cell deficiency are difficult to distinguish.

By utilizing the strategy of transient *in vivo* $CD4^+$ T-cell depletion prior to primary infection with *L. monocytogenes*, I evaluated whether $CD4^+$ T cells are required specifically at the time of $CD8^+$ T-cell priming to influence memory $CD8^+$ T-cell function. In these experiments, *in vivo* depletion of $CD4^+$ T cells results in a 99% reduction of this population for a brief time, followed by $CD4^+$ T-cell recovery. Thus using antibody-mediated depletion, the specific influence of $CD4^+$ T cells at priming can be separated from potential influences at later stages in the $CD8^+$ T-cell response to *L. monocytogenes*.

Transient *in vivo* depletion of $CD4^+$ T cells at the time of primary *L. monocytogenes* infections resulted in the generation of functionally normal memory $CD8^+$ T cells. In both BALB/c and C57BL/6 mice, peptide-specific $CD8^+$ T-cells primed in the absence of $CD4^+$ T cells were equally abundant in their recall production of IFNγ relative to $CD8^+$ T cells primed in untreated control mice (Fig. 14). Further, following secondary *L. monocytogenes* infection, the TCR avidity of antigen-specific $CD8^+$ T cells primed in the absence of $CD4^+$ T-cell help was generally unaffected (Fig. 13). As $CD4^+$ T-cell populations recover to wildtype levels during the 4 week resting period between primary and challenge *L. monocytogenes* infection used for these evaluations, these data

support a model in which $CD4^+$ T cells influence the maintenance of memory $CD8^+$ T-cell populations, rather than provide a specific imprinting signal at the time of $CD8^+$ T-cell priming. These data indicate that the recovery of $CD4^+$ T cells following transient depletion occurs at a sufficient rate to generate an environment conductive to $CD8^+$ memory maintenance within four weeks of depletion. Alternatively, $CD4^+$ T cells may play no role in either the development or maintenance of memory $CD8^+$ T-cell populations following *L. monocytogenes* infection.

Using LCMV-specific memory $CD8^+$ T cells from either wildtype or MHC-II-deficient mice, Sun *et al.* recently demonstrated that $CD8^+$ T cells generated in the absence of $CD4^+$ T-cell help could be rescued from attrition by adoptive transfer (101). Following acute LCMV infection, transfer of effector $CD8^+$ T-cell populations from MHC-II-deficient donor mice into wildtype ($CD4^+$ T-cell competent) recipients allowed for the generation of a stable memory $CD8^+$ T-cell population, while the same cells were gradually lost in MHC-$II^{-/-}$ recipients. These findings further challenge the hypothesis that the presence of $CD4^+$ T cells "imprints" the $CD8^+$ T-cell population with the ability to become immunologic memory. Further, transfer of memory $CD8^+$ T cells from MHC-$II^{+/+}$ mice (primed in the presence of $CD4^+$ T-cell help) into MHC-$II^{-/-}$ recipients, results in the loss of these "helped" memory $CD8^+$ T cells over time. Collectively, these data support the premise that the gradual loss of memory $CD8^+$ T cells in $CD4^+$ T cell-deficient hosts is not an inherent defect in the generation of memory $CD8^+$ T-cells. Rather, these data suggest that the maintenance of memory $CD8^+$ T-cell populations is dependent on the presence of $CD4^+$ T cells.

Although transfer into an environment containing $CD4^+$ T cells can prevent the numerical attrition of "unhelped" $CD8^+$ T cells, there has been no demonstration that these "rescued" memory $CD8^+$ T-cell populations have normal effector function transfer (101). Thus, it is uncertain whether the presence of $CD4^+$ T cells at the first or second potential stages of $CD4^+$ T-cell help to $CD8^+$ T cells influences the functional properties of memory $CD8^+$ T-cell populations that develop. The data presented in this dissertation demonstrate that transient depletion of $CD4^+$ T-cells during primary infection with *L. monocytogenes* and their subsequent recovery to wildtype levels within 4 weeks does not impair the functional quality of the antilisterial memory $CD8^+$ T-cell population that develops. Thus, a specific signal from $CD4^+$ T cells to $CD8^+$ T cells during the primary effector phase does not direct the downstream function of memory $CD8^+$ T-cell populations.

$CD40^{-/-}$ mice have a reduced $CD4^+$ $CD25^+$ regulatory T-cell population

The experiments included in this dissertation have additionally evaluated the influence of transient depletion of $CD4^+$ T-cell populations at the time of secondary *L. monocytogenes* infection in both wildtype and $CD40^{-/-}$ BALB/c *Listeria*-immune mice. These experiments revealed that the secondary expansion of memory $CD8^+$ T-cell populations is significantly enhanced in CD4-depleted wildtype mice, suggesting a regulatory component within the $CD4^+$ T-cell population (Fig. 25).

Similar to the enhanced secondary $CD8^+$ T-cell expansion observed in CD4-depleted, *Listeria*-immune mice, $CD40^{-/-}$ *Listeria*-immune mice also exhibited increased numbers of antigen-specific $CD4^+$ and $CD8^+$ T cells following secondary *L.*

monocytogenes challenge infection (Figs. 15, 16, 20). To evaluate potential mechanisms that might account for this differential secondary $CD8^+$ T-cell response, I observed that $CD40^{-/-}$ mice harbor a markedly reduced frequency of $CD4^+$ $CD25^+$ $GITR^+$ T cells compared with wildtype BALB/c mice (Fig. 23). Similar to the original $CD40^{-/-}$ mice, reselected $CD40^{-/-}$ colonies backcrossed to wildtype BALB/c mice maintained this significantly reduced number of $CD4^+$ $CD25^+$ $GITR^+$ regulatory T cells (Fig. 24A), indicating a relationship between CD40 expression and either the development or peripheral maintenance of $CD4^+$ $CD25^+$ regulatory T cells.

To date, the most well-characterized regulatory cell population is contained within the $CD4^+$ $CD25^+$ T-cell subset. The function of these T_{REG} cells has been associated with a role in maintaining self-tolerance by preventing the activation and expansion of self-reactive effector T cells that have escaped thymic deletion. Targeted removal of the $CD4^+$ $CD25^+$ T_{REG} population leads to the spontaneous development of a variety of autoimmune diseases in otherwise normal mice. In these settings, autoimmunity can present as the generation of autoantibodies, gastritis, oophoritis, thyroiditis and insulitis (163). Targeted depletion of the $CD4^+$ $CD25^+$ T_{REG} population also triggers the activation of T cells specific for antigens derived from commensal intestinal bacteria, leading to the development of inflammatory bowel disease (*reviewed in* (179)).

An additional role for T_{REG} cells is suggested from reports that T_{REG} populations limit T-cell responses to pathogens. The ability of $CD4^+$ $CD25^+$ T_{REG} cells to decrease the magnitude of effector T-cell expansion may protect the host against immunopathologic tissue destruction and the release of high levels of proinflammatory cytokines by

pathogen-specific effector T cells. In support of this premise, the $CD4^+$ T-cell response to infection with the opportunistic pathogen *Pneumocystis carinii* is responsible for pathogen clearance, but also causes fatal pulmonary hyper-inflammation in lymphopenic mice (184). In contrast, immunologically intact mice can clear the infection with minimal pulmonary inflammation. This difference has been attributed to the activity of $CD4^+$ $CD25^+$ T_{REG} cells in the normal host that limit the expansion of effector $CD4^+$ T-cell populations, thereby preventing the accumulation of high numbers of effector cells that cause to host pathology. In this model, the regulatory function that modulates the adaptive immune response benefits the host by limiting the size of effector T-cell populations.

Further support for the hypothesis that regulation of the effector T-cell population is beneficial to the host is found in mouse models of HSV infection. Depletion of $CD25^+$ cells prior to footpad infection with HSV results in enhanced antiviral $CD8^+$ T-cell responses and improved viral clearance (155). Yet, when ocular HSV infection models are evaluated, the severity of corneal lesions that develop is significantly increased in CD25-depleted mice compared with normal animals (154). Therefore, the immune response to the same pathogen appears to be delicately regulated by the $CD25^+$ cell population to allow the generation of a sufficient number of effector T cells to combat the pathogen while also attempting to minimize tissue damage by effector T cells that is deleterious to the host.

$CD4^+$ $CD25^+$ regulatory T cells influence memory $CD8^+$ T-cell responses to L. monocytogenes infection

I hypothesized that the decreased $CD4^+$ $CD25^+$ regulatory T–cell population evident in $CD40^{-/-}$ mice results in a numerically enhanced secondary $CD8^+$ T-cell response to antigen. In support of this, the expansion of antigen-specific $CD8^+$ T cells in $CD40^{-/-}$ mice appeared to reflect increased proliferation, rather than recruitment of additional CTL precursors into the antilisterial response, as the frequencies of antigen-specific cells utilizing each Vβ TCR chain were comparable between wildtype and $CD40^{-/-}$ mice within the $p60_{217\text{-}225}$-specific $CD8^+$ T-cell population (Fig. 18). The number of $p60_{217\text{-}225}$-specific T_E and T_{EM} phenotype cells was increased in the spleens of $CD40^{-/-}$ mice compared with wildtype BALB/c animals (Fig. 17), suggesting a preferential expansion of $CD8^+$ T-cell populations bearing phenotypic markers of $CD8^+$ effector T cells.

Immunization with heat-killed *L. monocytogenes* (HKLM) does not provide an adequate stimulation signal to $CD8^+$ T cells, with a resulting abortive proliferative process that leads to the accumulation of very few peptide-specific cells with a severely reduced ability to lyse target cells or secrete effector cytokines (132, 133). Recently, the role of $CD4^+$ $CD25^+$ regulatory T cells in controlling the immune response to HKLM vaccination has been explored (186). Utilizing a HKLM prime/boost vaccination strategy, these investigators report that depletion of $CD4^+$ T cells prior to a secondary boost with HKLM increases the number of peptide-specific antilisterial $CD8^+$ T-cells. This, in turn, results in the generation of a $CD8^+$ T-cell population that can provide modest protection

against a subsequent challenge with viable *L. monocytogenes*. Thus, these data suggest that it is possible to use HKLM to stimulate antilisterial immunity under conditions of decreased immune regulation.

I have found that $CD40^{-/-}$ *Listeria*-immune mice challenged with HKLM show a small, but significant expansion within the $p60_{217-225}$-specific $CD8^+$ T-cell population compared with a decrease in antigen-specific $CD8^+$ T-cell populations in wildtype *Listeria*-immune BALB/c mice challenged with HKLM (Fig. 21). These results suggest that regulatory mechanisms to control both the initiation and magnitude of secondary $CD8^+$ T-cell expansion may be less effective in $CD40^{-/-}$ mice compared with wildtype BALB/c mice.

What role do $CD4^+$ $CD25^+$ regulatory T cells play in a wildtype animal to control the magnitude of secondary $CD8^+$ T-cell expansion? I have found that, as the *Listeria* challenge dose was increased, further expansion of antigen-specific $CD8^+$ T cells was only observed in $CD40^{-/-}$ mice; within wildtype BALB/c mice, there appeared to be an intact regulatory process to prevent the continued expansion of antilisterial $CD8^+$ T-cell populations (Fig. 20). One possibility is that $CD4^+$ $CD25^+$ regulatory T cells may function in a global manner to restrict the general expansion of peptide-specific $CD8^+$ T-cell populations. Alternatively, suppression of effector T-cell expansion by T_{REG} populations may become more evident as the number of peptide-specific T cells responding to cognate antigen increases beyond a certain set point or threshold.

To attempt to evaluate whether the increased $CD8^+$ T-cell expansion observed following *L. monocytogenes* challenge in CD40-deficient mice was a direct consequence of the reduced regulatory T-cell population within these animals, I utilized *in vivo*

depletion of all $CD4^+$ cells prior to secondary *L. monocytogenes* challenge. The use of antibody-mediated *in vivo* depletion of $CD4^+$ cells as a tool for interpreting the influence of regulatory cell populations on $CD8^+$ T-cell responses in some systems is complicated by the fact that classical helper $CD4^+$ T cells are depleted as well.

Previous studies evaluating the influence of $CD4^+$ T cells on the secondary $CD8^+$ T-cell response to *L. monocytogenes* infection have yielded mixed results. When BALB/c mice are rested 60 days following primary systemic infection, then depleted of $CD4^+$ cells prior to secondary *Listeria* challenge, the magnitude of the $LLO_{91\text{-}99}$-specific $CD8^+$ T-cell population is increased 2-fold in the spleen (156). In contrast, Marzo *et al.* report that depletion of $CD4^+$ cells in *Listeria*-immune mice prior to secondary infection results in a severe reduction of the antigen-specific $CD8^+$ memory T-cell response (157). These disparate findings may reflect the experimental approach, as widely different intervals between primary and challenge infections were used in these evaluations (two vs. seven months of rest). In fact, when Marzo *et al.* evaluated different time intervals between the primary and secondary challenge infections, depletion of $CD4^+$ T cells prior to secondary *Listeria* challenge was more detrimental to the expansion of $CD8^+$ T cells as the animals were rested for longer periods of time between infections (157). It is possible that, as more time elapses between primary and secondary *L. monocytogenes* infections, the baseline activation state of the memory $CD8^+$ T-cell population changes. Thus, memory $CD8^+$ T cells could function in a state of CD4-independence shortly after the primary infection. As the time interval between primary and secondary infections increases, these memory $CD8^+$ T cells might transition into a state that is more dependent on classical $CD4^+$ T-cell help in order to undergo a secondary response to antigen. Alternatively, a

short-lived CD4-independent memory population may be present at early timepoints following primary infection, then be lost over time, leaving a CD4-dependent population as long-lived memory $CD8^+$ T cells. My evaluations of memory $CD8^+$ T-cell responses to *Listeria* challenge were performed following a four-week rest between primary and secondary *L. monocytogenes* infections, an interval in which the recall $CD8^+$ T-cell response is not dependent on the presence of classical $CD4^+$ helper T-cell populations (156).

For these experiments, wildtype and CD40-deficient mice were infected with *Listeria*, and then rested for four weeks. Immune animals were treated with depleting anti-CD4 antibodies, and challenged with a secondary *L. monocytogenes* infection. $CD8^+$ T-cell responses were analyzed at days 5 and 11 following *Listeria* challenge by intracellular cytokine staining. Evaluation of the magnitude of peptide-specific $CD8^+$ T-cell responses on day 5 post-challenge revealed that CD4-depletion in either wildtype or $CD40^{-/-}$ mice had no effect on the magnitude of the LLO_{91-99}-specific $CD8^+$ T-cell response. In contrast, CD4-depletion prior to secondary *L. monocytogenes* challenge increased the $p60_{217-225}$-specific response in wildtype mice to a similar magnitude as seen in CD40-deficient mice (Fig 25A). However, CD4-depletion in $CD40^{-/-}$ mice did not lead to further enhancement of the $p60_{217-225}$-specific $CD8^+$ T-cell response (Fig 25A).

The influence of CD4-depletion on secondary $CD8^+$ T-cell responses was more apparent 11 days following *Listeria* challenge, after the onset of contraction. At this time point the maintenance of both LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cells was prolonged in either strain depleted of $CD4^+$ cells (Fig. 25B). Thus, in the absence of the $CD4^+$ population, two separate events were apparent: enhanced $CD8^+$ T-cell expansion at

earlier timepoints, as well as a slower decline in the $CD8^+$ T-cell populations during the contraction phase. Importantly, CD4-depletion did not influence the total number of $CD8^+$ T cells in either strain (Fig. 25C), indicating that the enhanced responses observed reflect specific events within peptide-specific $CD8^+$ T-cell populations. Collectively, these experiments suggest that the decreased regulatory T-cell population present in CD40-deficient mice, or following CD4-depletion in wildtype mice, markedly influences the secondary $CD8^+$ T-cell response to *L. monocytogenes*.

To further evaluate the influence of $CD4^+$ T-cell populations on the magnitude of secondary $CD8^+$ T-cell expansion, I performed a series of adoptive transfer experiments. $CD8^+$ cells were positively selected from *Listeria*-immune wildtype donors marked by Thy1.1 expression. The selected cells were adoptively transferred into wildtype and $CD40^{-/-}$ naive recipient mice, as well as both strains depleted of $CD4^+$ cells prior to transfer. Recipient mice were challenged with *L. monocytogenes*, then evaluated either 4 days later for bacterial clearance, or 5 days later for the expansion of donor-derived, peptide-specific $CD8^+$ T cells. This strategy allowed me to specifically evaluate how the "regulatory environment" within the recipient animal impacted the response of identical donor-cell populations.

Adoptive transfer of wildtype *Listeria*-immune resting memory $CD8^+$ T-cell populations into CD4-depleted or untreated wildtype mice again revealed a pattern of enhanced secondary $CD8^+$ T-cell expansion in CD4-depleted recipients compared with wildtype recipients (Fig. 27A). This was evident in both the LLO_{91-99} and $p60_{217-225}$-specific populations, suggesting that the presence of $CD4^+$ cells impacts the expansion of antigen-specific memory $CD8^+$ T cells stimulated by *Listeria* challenge.

I evaluated whether CD4-depletion in $CD40^{-/-}$ recipient mice would similarly influence the secondary expansion of adoptively transferred memory $CD8^+$ T cells. Similar to the results seen in CD4-depleted wildtype recipients, CD40-deficient recipients showed enhanced donor $CD8^+$ T-cell expansion relative to wildtype animals (Fig. 27B). However, following CD4-depletion in CD40-deficient recipients, no further expansion was seen relative to undepleted CD40-deficient recipients (Fig. 27B).

The enhanced expansion observed in $CD40^{-/-}$ recipients correlated with enhanced protection, as evident by the decreased bacterial burden recovered four days after *L. monocytogenes* challenge of $CD40^{-/-}$ recipients (Fig. 26C). In contrast, naive wildtype recipients of the same memory T-cell pool exhibited insignificant antilisterial protection, with bacterial burdens similar to those seen in naive animals that did not receive *Listeria*-immune $CD8^+$ T cells (Fig. 26C). Typically, *in vitro* culture-activation has been a required step in studies evaluating adoptive transfer of cellular immunity to *L. monocytogenes*. Transfer of splenocytes from mice at the peak of the primary $CD8^+$ T-cell response to *L. monocytogenes* (days 7-9 post-infection) can directly transfer protection, yet once these cells have contracted to form a resting memory population (~4 weeks post-infection) they are no longer protective following direct transfer into naive recipients (197, 198). In contrast, I found that transfer of resting memory $CD8^+$ T cells from *Listeria*-immune wildtype mice into naive $CD40^{-/-}$ recipients provided protection in the absence of prior *in vitro* culture-activation. These results highlight a clear, functional difference in the secondary $CD8^+$ T-cell responses occurring in wildtype and $CD40^{-/-}$ recipient environments, and further support the hypothesis that the reduced $CD4^+$ $CD25^+$

regulatory T-cell population in $CD40^{-/-}$ mice results in enhanced memory T-cell responses to cognate antigen.

To further define the conditions in which cells within the $CD4^+$ population influence the magnitude of $CD8^+$ T-cell expansion, I transferred increasing numbers of $CD8^+$ cells from *Listeria*-immune wildtype donors into naive and CD4-depleted wildtype recipients. Recipients were challenged with *Listeria*, and the frequency of peptide-specific $CD8^+$ T cells was evaluated 5 days later (Fig. 28). This experiment revealed a differential influence of $CD4^+$ cells depending on the input cell number. At lower numbers of input cells, the presence or absence of $CD4^+$ cells within the recipient had little influence on the magnitude of $CD8^+$ T-cell expansion. In contrast, at higher input cell numbers, the presence of $CD4^+$ cells inhibited the CD8 T-cell expansion relative to that seen in CD4-depleted recipients. These results indicate that a population of cells within the $CD4^+$ subset influences the magnitude of $CD8^+$ T-cell expansion following secondary antigen exposure. Additionally, these data suggest that this regulatory function primarily becomes evident as high numbers of effector T-cells are achieved.

Other investigations also suggest an upper limit of $CD8^+$ T-cell expansion, and that increased antigen load or CTL precursor frequency cannot bypass this upper limit (152, 153, 199, 200). Vijh *et al.* evaluated the relationship between the density of antigen display and the magnitude of $CD8^+$ T-cell priming with *L. monocytogenes* strains containing mutations in the flanking regions of the $p60_{217\text{-}225}$ $CD8^+$ T-cell epitope that impaired the efficiency of MHC-I antigen processing and presentation (152). These investigators found that, once a threshold of antigen density was reached to initiate $CD8^+$ T-cell priming, further increases in the amount of antigen presented could not influence

the magnitude of the $CD8^+$ T-cell response. A similar approach using modified vaccinia virus to vary antigen display confirmed that high epitope densities could not improve $CD8^+$ T-cell priming (199). By titrating 10-fold dilutions of TCR-transgenic OT-1 $CD8^+$ T-cells into naive mice followed by immunization with antigen-pulsed DC, Kemp *et al.* found that the absolute number of OT-1 OVA-specific cells recovered was the same regardless of input OT-1 cell number (153). When lower numbers of input cells were transferred, the OT-1 cells underwent greater cellular division than when higher numbers of OT-1 cells were injected, with all conditions reaching the same numeric ceiling of transgenic $CD8^+$ T cells. Further support is found in an evaluation of the simultaneous *in vivo* response of (a) endogenous, naive $CD8^+$ T cells, and (b) adoptively-transferred memory $CD8^+$ T cells specific for the same antigen. Following antigenic challenge, the total number of peptide-specific $CD8^+$ T cells reached the same number, regardless of the naive: transferred memory $CD8^+$ T-cell ratio within the host (200). Collectively, these data support both my findings as well as the hypothesis that a regulatory mechanism exists to prevent the accumulation of potentially deleterious numbers of peptide-specific T cells.

It is interesting that the influence of CD40-deficiency (and presumably decreased T_{REG} frequencies) as well as *in vivo* depletion of $CD4^+$ T cells appeared to have a greater influence on the expansion of the subdominant $p60_{217\text{-}225}$-specific $CD8^+$ T-cell population than on the dominant $LLO_{91\text{-}99}$-specific response. In support of this, I consistently found that the fold-expansion of $p60_{217\text{-}225}$-specific $CD8^+$ T cells was much greater than the fold-expansion of $LLO_{91\text{-}99}$-specific $CD8^+$ T cells when T_{REG} cells were decreased (Fig. 26B). It is unclear why regulation would have a greater impact on one antigen-specific

population than another. It has been previously established that the $p60_{217-225}$ epitope is displayed at a much greater density than the LLO_{91-99} epitope on the surface of L. monocytogenes-infected cells (193). Further, these two antigen-specific $CD8^+$ T-cell populations do not compete with each other. Infection with mutant *L. monocytogenes* strains that are completely deficient in LLO production or contain a point mutation within the LLO_{91-99} epitope (abolishing the LLO_{91-99}-specific $CD8^+$ T-cell response) have no effect on the magnitude of the $p60_{217-225}$-specific $CD8^+$ T-cell response (Fig. 22) (194, 195). Simultaneous evaluations of additional peptide-specific $CD8^+$ T-cell responses in the absence of T_{REG} cells and a more complete understanding of the mechanisms by which T_{REG} interact with specific T-cell populations will be required to address these questions.

In summary, I find that $CD40^{-/-}$ mice contain a reduction in the $CD4^+$ $CD25^+$ regulatory T-cell population that correlates with enhanced expansion of antigen-specific $CD8^+$ T cells following secondary *L. monocytogenes* challenge. Adoptive transfer of memory $CD8^+$ T cells into naive wildtype, $CD40^{-/-}$, or CD4-depleted BALB/c recipients suggests that the presence of T_{REG} cells inhibits the secondary expansion of memory $CD8^+$ T cells, which in turn, impairs pathogen clearance. Further, this regulatory effect primarily influences continued T-cell expansion when high numbers of $CD8^+$ T cells are responding. Collectively, these results suggest that T_{REG} cells may function to maintain a balance between pathogen clearance and the immunopathologic consequences of large numbers of effector T-cell populations.

Chapter 5: Summary and Conclusions

What types of immune cell interactions are necessary for the development of immune memory to provide protection against reinfection with a particular pathogen? Are specific interactions between $CD4^+$ and $CD8^+$ T cells required for the development of memory $CD8^+$ T-cell populations? In this dissertation, I've used a mouse model of *Listeria monocytogenes* infection to evaluate how CD40-CD40L costimulatory interactions specifically, and the $CD4^+$ T-cell subset in general, influence memory $CD8^+$ T-cell development and function.

$CD4^+$ T cells, CD40 expression and memory $CD8^+$ T-cell populations

Over the last few years, several reports in the literature have indicated that memory $CD8^+$ T cells generated in the absence of $CD4^+$ T cells show qualitative defects, including attrition of memory cell numbers, decreased secondary proliferation to antigen, and impaired effector cytokine production on a per cell basis (93-95). Additionally, it was reported that CD40 is transiently expressed of a subset of activated $CD8^+$ T cells, suggesting that direct CD40 ligation on $CD8^+$ T cells might direct either the development or function of memory $CD8^+$ T cell populations (61). As a result of these reports, it was hypothesized that $CD4^+$ T cells are required at the time of $CD8^+$ T-cell priming to "imprint" responding $CD8^+$ T cells with the ability to develop into functional memory populations. However, the experiments that led to this "imprinting hypothesis" were typically performed in genetic knockout animals that were continuously devoid of $CD4^+$ T-cell populations. Therefore, a specific CD4-derived imprinting signal at the time of

$CD8^+$ T-cell priming could not be distinguished from other CD4-mediated signals that might occur at a later time.

Although $CD8^+$ T-cell priming to *Listeria* has been characterized as a "helper independent" response that occurs in the absence of both $CD4^+$ T cells and CD40-CD40L interactions, these early experiments primarily evaluated the presence or absence of antigen-specific $CD8^+$ T cells (40, 75, 87, 150). A detailed analysis of $CD8^+$ memory T-cell function on a per-cell basis had not been performed. I hypothesized that, although memory $CD8^+$ T-cell populations are evident in the absence of both CD40-CD40L interactions specifically, and $CD4^+$ T cells in general, these "unhelped" antilisterial $CD8^+$ T cells may be functionally compromised. To evaluate this premise, I determined whether the cellular immune response that develops following *Listeria monocytogenes* infection is functionally altered when stimulated in the absence of either CD40 expression (using $CD40^{-/-}$ mice) or $CD4^+$ T cells (through transient depletion of $CD4^+$ cells *in vivo*).

In the context of *Listeria* infection I found no requirement for either CD40 expression or $CD4^+$ T-cells in directing the functional properties of either primary effector $CD8^+$ T-cell populations or memory $CD8^+$ T cells. Memory $CD8^+$ T cells generated in $CD40^{-/-}$ mice, or in wildtype mice depleted of $CD4^+$ cells prior to primary *Listeria* infection showed equivalent protection upon secondary *Listeria* challenge. These findings refute the general hypotheses: 1) that $CD4^+$ T cells are required at the time of $CD8^+$ T-cell priming to imprint memory function in responding $CD8^+$ T cells, and 2) that specific CD40-CD40L interactions are required for memory $CD8^+$ T-cell development.

It is important to consider the methodology used when considering my results. For instance, it is possible that $CD40^{-/-}$ mice develop or utilize compensatory signaling

cascades to overcome the absence of CD40-specific signals. This may be particularly true for mechanisms of APC activation, a possibility I did not evaluate in my studies. Even in light of this possibility, I still conclude that a specific CD40-CD40L signal (particularly a direct signals between $CD4^+$ and $CD8^+$ T-cell populations) is not required for the development of memory $CD8^+$ T-cell populations with protective function. Similarly, we must acknowledge that manipulating the *in vivo* environment by antibody-mediated depletion of all $CD4^+$ cells may have effects on cell populations other than $CD4^+$ T cells. For example, this approach would also result in the depletion of $CD4^+$ DC subsets. However, as my depletion studies did not result in a diminishing of T-cell priming or pathogen clearance, I feel it's reasonable to assume that the $CD4^+$ DC populations does not play a unique and irreplaceable role in antilisterial immunity.

My results do not rule out the possibility that the presence of $CD4^+$ T-cell populations after $CD8^+$ T-cell priming influence the maintenance of memory $CD8^+$ T cells, a hypothesis supported by the findings of Sun *et al.* (101). These investigators found that LCMV-specific memory $CD8^+$ T cells generated in MHC-$II^{-/-}$ mice (devoid of $CD4^+$ T cells) could be rescued from attrition by adoptive transfer. Unfortunately, these experiments did not demonstrate that these "rescued" memory $CD8^+$ T-cell populations retained normal effector function. Thus, additional experiments in which $CD4^+$ cell populations are continuously absent are required to evaluate this putative maintenance role in preserving both the number and function of pathogen-specific memory $CD8^+$ T cells. This question might be addressed by repeated *in vivo* anti-CD4 antibody administration following the T-cell priming phase. Alternatively, CD4-depleted animals could be thymectomized following acute infection in CD4-depleted animals to prevent

reconstitution of the $CD4^+$ T-cell subset.

How might $CD4^+$ T cells promote the maintenance of memory $CD8^+$ T-cell populations? My results indicate that a direct CD4-CD8 T-cell interaction at the time of $CD8^+$ T-cell priming is not involved. Perhaps $CD4^+$ T cells secrete a cytokine required for $CD8^+$ T cells to survive contraction or be maintained as a memory T-cell population. It is also possible that the nonspecific $CD4^+$ T-cell interactions with a third cell population, such as stromal cells, are required to somehow "condition" the environment for memory $CD8^+$ T-cell maintenance. Many questions remain in this field.

The nature of the infection is likely an important factor in determining whether CD40 expression and/or $CD4^+$ T-cell help is required for efficient $CD8^+$ T-cell priming and the subsequent development of memory $CD8^+$ T-cell populations. For example, vaccination with heat-killed pathogens (including heat-killed *Listeria*) is generally insufficient for the development of protective $CD8^+$ memory T-cell populations. As these preparations should contain TLR ligands to trigger the maturation process of immature DC, this suggests that the infectious process itself is an important component in creating an efficient priming environment. All of the experiments I performed in $CD40^{-/-}$ mice utilized a highly virulent *L. monocytogenes* strain characterized as a hyper-secretor of LLO. Initially, this approach was selected in an attempt to reduce differences in the quantity of LLO and p60 antigens available to responding $CD8^+$ T-cell populations. However, it must be acknowledged that use of the hyper-LLO *Listeria* may have heightened the inflammatory response during infection, thus enhancing alternative APC maturation signals. Consequently, my use of a highly virulent *Listeria* strain may have masked the potential need for $CD4^+$ T cell-mediated help in the development of $CD8^+$ T-

cell responses following an infection with a less virulent strain. Further experiments to evaluate $CD8^+$ T-cell responses in $CD40^{-/-}$ and CD4-depleted mice following infection with attenuated *Listeria* strains are warranted.

Likewise, it is difficult to say that the inflammatory responses generated following infection with various different pathogens results in similar priming environments. For instance, which parameters should be measured to argue that the priming environment following LCMV infection is the same as that following *Listeria* infection? The fact that CD40-mediated APC maturation is not bypassed by all pathogen infections, as might be expected if TLR ligation by pathogen products were a sufficient signal for APC maturation and $CD8^+$ T-cell priming, argues that the specific influences of CD40 expression and $CD4^+$ T cells on must be considered independently for each pathogen. It is entirely possible that, in addition to APC maturation, other influences of $CD4^+$ T cells, such as "imprinting" the memory potential of $CD8^+$ T-cell populations, might be different for individual pathogens.

It is also questionable to compare the requirements for CD40 expression and $CD4^+$ T cells for $CD8^+$ memory development in the context of infection with the potential need for these signals during $CD8^+$ T-cell priming in the absence of infection-associated inflammation. I would argue that, although my results indicate that these signals can be bypassed during infection with a virulent pathogen such as *Listeria*, this may not be true for $CD8^+$ T-cell priming to antigens presented in the absence of active infection, such as tumor antigens, soluble antigens, heat-killed pathogens, minor histocompatability antigens, or those delivered by peptide-pulsed APC.

Thus, I would not presume that my findings indicating that neither CD40 expression nor $CD4^+$ T cells are required during $CD8^+$ T-cell priming to imprint memory potential are a global truth. Rather, I would argue that the context of the priming environment plays a critical role in determining the need for $CD4^+$ T cell-mediated help. Attenuation of pathogen virulence, or the absence of inflammatory responses are likely to be important factors in whether additional signals provided by $CD4^+$ T-cell populations are necessary for the development and maintenance of protective memory $CD8^+$ T cells.

$CD4^+$ $CD25^+$ regulatory T cells and memory $CD8^+$ T-cell responses

I observed that, in both $CD40^{-/-}$ mice and wildtype mice depleted of $CD4^+$ cells prior to secondary *Listeria* challenge, secondary $CD8^+$ T-cell expansion was enhanced. Upon confirming others' findings that the $CD4^+$ $CD25^+$ regulatory T-cell population is markedly reduced in $CD40^{-/-}$ mice, I hypothesized that $CD4^+$ $CD25^+$ regulatory T-cells function to limit the magnitude of memory $CD8^+$ T-cell expansion following secondary antigen exposure.

To evaluate this hypothesis I performed a series of adoptive transfer experiments in which $CD8^+$ cells were positively selected from *Listeria*-immune wildtype donors marked by Thy1.1 expression. These cells were adoptively transferred into wildtype and CD40-knockout recipient mice (Thy 1.2), as well as both strains depleted of $CD4^+$ cells prior to transfer. Recipient mice were challenged with *Listeria*, and then evaluated for the expansion of donor-derived, antigen-specific $CD8^+$ T cells. This strategy allowed a specific evaluation of how the "regulatory environment" within the recipient animal would influence the expansion of identical donor-cell populations.

Upon transfer of $CD8^+$ cells into either CD4-depleted or untreated wildtype mice I

observed a pattern of enhanced secondary $CD8^+$ T-cell expansion in CD4-depleted recipients compared with wildtype recipients. This was evident in both the LLO_{91-99} and $p60_{217-225}$-specific $CD8^+$ T-cell populations, suggesting that the presence of $CD4^+$ cells impacts the expansion of antigen-specific memory $CD8^+$ T cells stimulated by *Listeria* challenge. Similar to my observation in CD4-depleted wildtype recipients, $CD40^{-/-}$ recipients also showed greater $CD8^+$ T-cell expansion than wildtype recipients. However, CD4-depletion in $CD40^{-/-}$ recipients did not further enhance this expansion relative to undepleted $CD40^{-/-}$ recipients. These data further support my hypothesis that the reduced $CD4^+$ $CD25^+$ regulatory T-cell population in $CD40^{-/-}$ mice results in enhanced memory $CD8^+$ T-cell expansion upon secondary antigen exposure. However, a definitive conclusion that the $CD4^+$ $CD25^+$ regulatory T-cell defect in $CD40^{-/-}$ mice is the sole cause cannot be made. Experiments to reconstitute the $CD4^+$ $CD25^+$ regulatory T-cell population in $CD40^{-/-}$ mice (and presumably control the magnitude of $CD8^+$ T-cell expansion) would strengthen this interpretation.

Upon transfer of increasing numbers of $CD8^+$ cells from *Listeria*-immune wildtype donors into naive and CD4-depleted wildtype recipients, I discovered a differential influence of $CD4^+$ cells depending on the input cell number. At lower numbers of input cells, the presence or absence of $CD4^+$ cells within the recipient had little influence on the magnitude of $CD8^+$ T-cell expansion. In contrast, at higher input cell numbers, the presence of $CD4^+$ cells inhibited the CD8 T-cell expansion relative to that seen in CD4-depleted recipients. These results indicate that a population of cells within the $CD4^+$ subset (most likely the $CD4^+$ $CD25^+$ regulatory population) influences the magnitude of $CD8^+$ T-cell expansion following secondary antigen exposure.

Additionally, these data suggest that this regulation of $CD8^+$ T-cell expansion is activated only when effector populations approach a putative numeric threshold. In this setting, $CD4^+$ $CD25^+$ regulatory T cells would serve to prevent the accumulation of high numbers of effector T cells in order to limit immunopathologic damage to host tissues, in essence setting an upper limit on the number of antigen-specific cells that are allowed to respond to cognate antigen within the animal.

In this model, $CD4^+$ $CD25^+$ regulatory T cells would not influence the $CD8^+$ T-cell population until this numeric threshold of effector $CD8^+$ T cells was broached. Based on the data presented in this dissertation, I propose that, during the primary $CD8^+$ T-cell response, the number of effector $CD8^+$ T cells generated stays below a numeric threshold at which $CD4^+$ $CD25^+$ regulatory T-cell populations become activated to influence the magnitude of the response. However, following secondary *Listeria* infection, when memory $CD8^+$ T-cell populations undergo profound expansion to generate much larger numbers of effector $CD8^+$ T cells than seen during the primary response, the $CD4^+$ $CD25^+$ regulatory T-cell population is activated to limit the continued accumulation of large numbers of $CD8^+$ effector T cells.

It is interesting to speculate on how regulatory $CD4^+$ $CD25^+$ T cells might preferentially be stimulated to regulate $CD8^+$ T-cell responses when high numbers of effector $CD8^+$ T cells are reached. One possibility is that large numbers of effector $CD8^+$ T cells may result in significant increases in the circulating concentration of secreted effector $CD8^+$ T-cells products, such as IFNγ, TNFα, perforin or granzymes. These molecules are typically targeted to infected cells. However, in the context of large effector $CD8^+$ T-cell populations, these molecules may be released in quantities too large

to be completely bound or internalized by their intended target cells. This could signal to the immune system that the effector $CD8^+$ T-cell population has outgrown its necessary size, stimulating regulatory $CD4^+$ $CD25^+$ T cells into action. Alternatively (or additionally) the release of endogenous intracellular host proteins into circulation may signal to the immune system that extensive tissue damage is occurring as a consequence of effectors $CD8^+$ T-cell populations killing large numbers of infected host cells. Under these conditions, the host may be better off limiting the magnitude of the immune response (and the consequential host-cell damage), at the expense of pathogen clearance.

Thus, an additional function of the regulatory $CD4^+$ $CD25^+$ T-cell population is envisioned: to protect the host against immunopathologic damage resulting from uncontrolled expansion of effector $CD8^+$ T-cell populations in response to antigen. Many questions remain as to how this might be accomplished. Do $CD4^+$ $CD25^+$ regulatory T-cells specifically target effector $CD8^+$ T-cell populations and turn off effector functions? Alternatively, do $CD4^+$ $CD25^+$ regulatory T-cell population simply prevent the generation of any more effector $CD8^+$ T-cells through interactions with APC? A better understanding of how regulatory $CD4^+$ $CD25^+$ T cells interact with $CD8^+$ T-cell populations is required. Perhaps manipulation of the regulatory $CD4^+$ $CD25^+$ T-cell subset can be partnered with vaccination to improve weak responses to target antigens. It might also be possible to improve $CD8^+$ T-cell priming toward tumor antigens by coupling these therapies with transient regulatory T-cell depletion. However, the consequences of interfering with immune regulation must also be appreciated and further characterized. Ultimately, a better understanding of the regulatory mechanisms that

influence immune responses may provide surprising insights to aid the development of new therapeutic strategies.

CHAPTER 6: REFERENCES

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