The impact of lifelong viral infection on development and maintenance of memory $CD8^+$ T cells

by

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ABBREVIATIONS

Ag	Antigen
APC	Antigen Presenting Cell
B6	C57BL/6
CMV	Cytomegalovirus
CNS	Central Nervous System
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
FCM	Flow Cytometry
HCV	Hepatitis C Virus
HSV-1	Herpes Simplex Virus type 1
IFN	Interferon
I.P.	Intraperitoneal
LAT	Latency Associated Transcript
LCMV	Lymphocytic Choriomeningitis Virus
LN	Lymph Node
LM	Listeria monocytogenes
MCMV	Murine Cytomegalovirs
MHC	Major Histocompatibility Complex
PFU	Plaque Forming Unit
p:MHC	peptide:MHC complex
RTE	Recent Thymic Emigrant
P.I.	Post Infection

PNS	Peripheral Nervous System
RAG	Recombination Activating Gene
SCID	Severe Combined Immunodeficiency Disorder
SD	Standard Deviation
SPF	Specific Pathogen Free
TCE	T Cell Clonal Expansion
TCR	T Cell Receptor
TG	Trigeminal Ganglion
TK	Thymidine Kinase

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ABSTRACT

Certain viruses are capable of establishing lifelong persistent infections within vertebrate hosts. Such infections can be chronic or latent, and accumulating evidence suggests that they provide ongoing antigenic stimulation to memory T cells throughout lifetime. In addition, infection with some persistent viruses were implicated in driving inflammation-driven expansions of bystander T cells. Both antigen-driven and bystander stimulation of T cells can be expected to affect the composition and diversity of the T cell pool. These processes were suggested to drive the age-associated changes in maintenance of stable T cell pool, contributing to the process of immunosenescence.

In this thesis I examined the impact of both lifelong viral infections as well as aging on the maintenance of systemic $CD8^+$ T cells over lifetime in a mouse model of Herpes Simplex Virus type 1 (HSV-1) and murine cytomegalovirus (MCMV) infection. Both viruses establish latent infections, however recent evidence implies that infection with MCMV is characterized by frequent viral reactivation, as inferred from ongoing expansion of MCMV-specific $CD8^+$ T cells over the timecourse of infection, a process termed memory inflation. Less is known about the effect of HSV-1 infection on maintenance of antiviral memory $CD8^+$ T cell. Until recently, HSV-1 infection in mice was believed to be truly latent and not associated with ongoing antigen presentation. However, evidence gathered over the last decade implies that this may not be the case and that reactivation of HSV-1 from latency in mice is more frequent than previously thought.

I investigated both primary and memory $CD8^+$ T cell response to HSV-1, with special focus on involvement of antigen in maintenance of the memory population. I

found that the frequency and numbers of HSV-specific memory $CD8^+$ T cells depend on infection route, the extent of initial viral spread and the extent of viral reactivation from latency. Systemic HSV-1 infection correlated with greater viral spread and early onset of $CD8^+$ T cell memory inflation, whereas localized infection resulted in limited viral spread and absence of virus-driven memory $CD8^+$ T cell expansion over the course of latent infection. The extent of memory inflation following systemic infection was antigen-driven, as blocking viral replication from latency could prevent it. Therefore, under certain conditions HSV-1 was able to drive $CD8^+$ T cell memory inflation in mice.

Despite the ability to stimulate $CD8^+$ T cell memory inflation, lifelong HSV-1 or MCMV infection alone was not associated with significant constriction of the TCR repertoire diversity or phenotypic changes among $CD8^+$ T cells in old infected mice. However, combination of both virus-related and virus-independent effects influenced the TCR composition and phenotype of the aging $CD8^+$ T cell pool.

In summary, in this thesis I present evidence that both virus-driven and virus-independent aging-associated mechanisms contribute to changes in the T cell pool homeostasis over lifetime contributing to the state of immunosenescence. Onset of immunosenescence correlates with increased morbidity and mortality in the elderly and understanding the mechanisms of its development and maintenance should aid in designing therapeutic strategies to prevent it.

THESIS SUMMARY

$CD8^+$ T cells are crucial in protection against intracellular pathogens, most notably viruses. Their primary function is eliminating the pathogen, either by production of antiviral cytokines which interfere with viral replication and recruit other effector cells that can clear the virus, or by killing the infected cells. Ideally, the $CD8^+$ T cell response is able to completely eliminate the invading pathogen, thus providing protection to the host, both from the primary infection (by preventing uncontrolled viral replication and spread, which would lead to tissue damage) as well as from possible secondary infection with the same pathogen (by persistence of memory cells). That is true for acute pathogens, which can be completely eliminated and are unable to persist inside an organism in the face of antiviral immune response. However, certain pathogens, including herpesviruses, evolved mechanisms that allow them to establish lifelong infections, despite a vigorous innate and adaptive immune response following initial infection, and despite successful establishment and maintenance of antiviral memory.

The purpose of this dissertation is two-fold. The first is to analyze the relationship between Herpes Simplex Virus (HSV-1), a herpesvirus that establishes a lifelong infection in the nervous system, and the lifelong $CD8^+$ T cell response generated against that infection. The second is to investigate the effects of lifelong viral infections on the lifelong homeostasis of the entire $CD8^+$ T cell compartment, using HSV-1 and murine cytomegalovirus (MCMV) murine infection models.

In Chapter 2 of the dissertation, I investigated the progression of primary infection with HSV-1 and the generation of the antiviral $CD8^+$ T cell response. The

kinetics of viral spread from the infection site to the site of lifelong latent infection were correlated with the kinetics of development and tissue distribution of antiviral $CD8^+$ T cells in an established C57BL/6 mouse model. The results described in this dissertation provide numerous novel findings, deepening and extending our knowledge regarding primary anti-HSV-1 $CD8^+$ T cell response to HSV-1. First, there is a lag of 6 days from the time of infection before antiviral $CD8^+$ T cells reach significant enough numbers to be detected by currently available methods. Second, $CD8^+$ T cell priming is restricted to the draining lymph node, and only cells that have undergone significant differentiation and at least 6 divisions leave the lymph node. Once cells are released from the lymph node, they simultaneously reach other secondary lymphoid as well as the infected non-lymphoid organs. Finally, the replicating virus spread very quickly from the site of infection (cornea) to the periphery, reaching the trigeminal ganglia by day 2 p.i. and brain by day 3 p.i. Combined with the kinetics of the $CD8^+$ T cell response, these data conclusively show that the cytotoxic T lymphocyte (CTL) response is mounted too late to prevent the virus from reaching the nervous system, and establishing lifelong latent infection therein. However, the $CD8^+$ T cells are crucial in limiting the extent of viral replication at the peripheral infection sites and the nervous system, and are necessary to prevent lethal viral encephalitis.

In Chapter 3 of the dissertation I analyzed the memory $CD8^+$ T cell response to HSV-1 and its interaction with this persisting pathogen. First I described the antiviral $CD8^+$ T cell memory following localized corneal infection. In that infection model, the antiviral memory is maintained at a stable, low frequency for most of the life of the infected animal, and there doesn't appear to be any interaction of the persisting virus with

the systemic memory $CD8^+$ T cell pool (i.e. those $CD8^+$ T cells present in sites other than the nervous system, the reservoir of the latent virus), which would be expected to result in periodic rounds of T cell activation and expansion. While the persisting virus had no apparent effect on the systemic memory T cell pool, there was a noticeable effect of old age. In 40% of analyzed mice, there was a significant increase, both by percentage and absolute number of HSV-specific memory $CD8^+$ T cells in old mice (18 m.o. or older). This increase was not likely driven by viral reactivation from latency, as the cells did not display effector memory phenotype, and the age-related memory expansions were also documented in mice treated with the antiviral drug famciclovir during latency, which should have prevented full viral reactivation and, therefore, most of the antigen presentation to T cells.

To further investigate the influence of persisting viruses on memory cell maintenance, Chapter 4 will discuss the results pertaining to maintenance of memory $CD8^+$ T cells following systemic infection with HSV-1. Unlike in the case of localized infection, the memory $CD8^+$ T cells induced by systemic infection do not remain at low, stable frequency for the duration of adult life. Rather, these cells undergo an early memory inflation. I analyzed the influence of the total viral load, viral spread and of ongoing viral stimulation on this process. I demonstrate that reducing initial viral systemic spread, as well as preventing viral reactivation by treatment with antiviral drugs, can limit the extent of memory inflation in this model.

Finally, in Chapter 5 of the dissertation I assessed the impact of lifelong infection with HSV-1 and MCMV on the overall diversity and phenotype of the $CD8^+$ T cell pool in old mice. I found that in a mouse model there isn't a dramatic effect of these lifelong

infections on the homeostasis of the aging $CD8^+$ T cells In studying homeostasis, I have focused on the analysis of TCR repertoire diversity and found that it was not significantly affected by the lifelong infection studied here. Rather, the effects of lifelong infection combine with the effects of virus-independent aging changes that lead to the constriction of repertoire diversity.

In summary, in this dissertation I have investigated the relationship between $CD8^+$ T cells and lifelong latent viral infections. I found evidence for ongoing stimulation of virus-specific memory $CD8^+$ T cells during HSV-1 latency, and the impact of that stimulation on the antiviral memory $CD8^+$ T cell pool depended on the extent of initial viral spread and the ability of the virus to reactivate. However, lifelong infection with HSV-1 and MCMV did not have a significant impact on the homeostasis of the total $CD8^+$ T cell pool, and unlike suggested in the human literature, did not appear to be a major factor leading to constriction of TCR repertoire diversity and phenotypic changes of aging T cells in mice. Finally, I have uncovered one potential mechanism by which T cell expansions, previously shown to be associated with old age, might arise from pre-existing memory cell pool, by an antigen-independent mechanism.

CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Over a lifetime, a host encounters many infectious agents and the immune system is crucial in providing protection against them. The initial defense is provided by innate immunity, however, most often more specialized mechanisms of adaptive immunity are necessary to provide full protection. This is especially true in case of intracellular pathogens, including viruses, which have evolved multiple strategies to avoid recognition by the immune system, both innate and adaptive. Often despite the presence of both innate and adaptive immune response, the pathogen manages to withstand the immune attack, and establishes a lifelong infection. Herpes Simplex Virus type 1 (HSV-1) and murine cytomegalovirus (MCMV) are examples of such pathogens.

Despite not being able to completely eliminate HSV-1 during initial infection, the adaptive immune response generated as a result of that infection still plays a crucial part in protecting the infected host. First, during the primary infection, lack of T lymphocytes can compromise the health or even survival of the host. Then, maintenance of memory $CD8^+$ T cells appears to be important in preventing wide-spread viral reactivation. Finally, as persistent pathogens, HSV-1 and MCMV have the potential to continually influence the homeostasis of T lymphocyte pools, as has been reported for humans infected with another persistent herpes virus, human CMV.

In this section of the dissertation, I shall review the literature relating to the generation of effector and memory $CD8^+$ T cell responses, the mechanisms maintaining

$CD8^+$ T cell homeostasis during adulthood and into senescence, and how they are influenced by persistent pathogens.

1.2 $CD8^+$ T CELLS AND ANTIVIRAL IMMUNITY

Generation of $CD8^+$ T cell responses

Following infection, $CD8^+$ T cells become activated through contact with antigen presenting cells (APCs) presenting peptides derived from viral proteins. This initiates a program of clonal expansion and phenotypic and functional maturation into armed effector cytotoxic T lymphocytes (CTLs). Once activated, the CTLs migrate into the infected tissues and eliminate the cells displaying the viral antigen. The cells remain in the activated effector stage for a short period of time (days), and then the majority of them die, while a small fraction (5-10%) survives and develops into long-lived memory population, maintained by homeostatic mechanisms. Thus, the $CD8^+$ T cell response can be divided into four main phases: activation, expansion, contraction, and memory (rev. in (Haring et al., 2006). The memory $CD8^+$ T cells may be periodically reactivated to armed CTL status, for example in an instance of reinfection with the pathogen for which they are specific.

Antigen presentation

The specificity of the $CD8^+$ T cell response is granted by the ability of each T cell receptor (TCR) to recognize a specific pathogen-derived peptide determinant, termed epitope, presented in the context of a major histocompatibility complex class I (MHC I)

molecule on the surface of an APC. The peptide epitopes presented by MHC I are usually octa-, nona-, or decamers, and are derived from cellular or viral proteins that had been digested by proteasome in the cytoplasm (German et al., 1996; Yewdell and Haeryfar, 2005). The proteasome is a large cylindrical protease complex that binds and degrades intracellular proteins that had been tagged for degradation with polyubiquitin. Viral infection can be associated with formation of immunoproteasome, a complex of proteasome and activator complex PA28 which is induced by interferon-γ (IFNγ) signaling and optimized for generation of epitopes for CTL recognition (Kloetzel, 2004). The MHC I molecule is composed of the heavy α chain and a non-covalently associated light chain (β2-microglobulin). The α chain consists of a cytosolic tail, a transmembrane domain, and three extracellular domains, with the peptide binding groove located in the two cell distal domains of the α chain (α2 and α3). The MHC I binds the peptides tightly, using several peptide side chains and the N and C-terminal residues for binding. The peptide binding groove is closed on both ends, thus limiting the size of the peptides which it can bind. Following processing by proteasome, the peptides are transported to the endoplasmic reticulum (ER) with the help of TAP-1 and -2 (transporter associated with antigen presentation 1 and 2) molecules, where they are loaded onto MHC I molecules and further transported out of the ER via trans-Golgi to the cell surface. The MHC I itself is unstable; it is partially stabilized by binding with β2-microblobulin, but only β2-microglubulin:MHC:peptide complexes are stable enough to be expressed with long half-lives on the cell surface and to enable antigen presentation to T cells.

The expression of MHC I molecules is ubiquitous, and most of the time they are occupied by peptides derived from self proteins. Interactions of T cells with self

peptide:MHC I complexes (p:MHC) play a role in maintenance of naïve T cells (Surh and Sprent, 2002), but do not result in priming, since autoreactive T cells were deleted in the thymus (discussed below). In a healthy organism, i.e. excluding instances of autoimmune disorders, only recognition of foreign peptides can result in $CD8^+$ T cell activation.

The T cell receptor

Each TCR, made up of the Vα and Vβ polypeptide chains, recognizes a specific p:MHC complex (cognate recognition). However, a cross-recognition of highly similar p:MHC complexes is possible, and can result in crossreactivity or heterologous immunity (Selin et al., 2006; Welsh and Selin, 2002). Still, under the majority of circumstances, T cells will recognize only their cognate epitope. T cells are bone-marrow derived lymphocytes which acquire their unique TCRs in the thymus by a process of VDJ recombination (rev. in (Starr et al., 2003)). The TCR genes are rearranged randomly, and not all combinations are productive. Acquisition of the β chain (or β-selection) is required prior to acquisition of the α chain. Once the TCR is formed, the T cells undergo a process of maturation in the thymus, involving positive selection, negative selection, and phenotypic changes such as selection of the CD4 or CD8 coreceptor (Starr et al., 2003). The process of positive selection ensures that the T cells that will be able to survive and migrate into the periphery can recognize the particular MHC molecules expressed by the given individual. During negative selection, the TCR is tested for its reactivity against a wide array of self antigens presented by MHC I on the thymic stromal epithelium, and the self-reactive T cells are eliminated. The mature naïve T cells are then released into the circulation, ready to participate in immune responses.

$CD8^+$ T cell activation and expansion

Events crucial for $CD8^+$ T cell activation begin before the T cells themselves come into contact with their cognate antigen (Ag). Naïve $CD8^+$ T cell precursors become activated, or primed, by interaction with a specialized cell subset called professional APCs (Henrickson and von Andrian, 2007). While MHC I is ubiquitously expressed on the cell surface of most cells, only certain cell types possess either the characteristics or the ability to access the specialized environment necessary to properly activate naïve T cells. Most commonly, dendritic cells (DC) in the lymph nodes (LNs) draining the site of infection serve as professional APCs (Steinman, 1991). Viral infection induces production of inflammatory cytokines, primarily type I interferons and IL-12 by the infected cells (DCs, macrophages), which in turn lead to activation and recruitment of more immune cells into the response, most notably the professional APCs (Hochrein et al., 2001; Joshi et al., 2007; Kolumam et al., 2005).

Interaction of naïve T cells with Ag-loaded professional APCs takes place in the LNs draining the site of infection. The Ag can reach the lymph node by two major mechanisms: 1) within an APC which acquired the Ag at the site of infection, either by direct infection or phagocytosis (Cavanagh and Von Andrian, 2002; Manickasingham and Reis e Sousa, 2001; Randolph, 2001) , or 2) arriving in a soluble form via the reticular network (Sixt et al., 2005). The Ag presentation can take place in two main ways: 1) via direct presentation, where the viral proteins are presented to T cells on the surface of infected cells, or by 2) cross-presentation, where the APC acquires the Ag by means other than direct infection, such as phagocytosis of Ag-containing material (Bevan, 2006; Groothuis and Neefjes, 2005; Shen and Rock, 2006). Regardless of how

the Ag is acquired, following Ag uptake the APC undergoes process of maturation, which involves upregulation of MHC I and costimulatory molecule expression. Presence of proinflammatory cytokines is crucial for this process (Heath and Carbone, 2001; Mescher et al., 2006; Moser, 2003).

Naïve T cells regularly patrol the LNs, and their access to the LNs is aided by expression of homing receptors CD62L and CCR7 (Weninger and von Andrian, 2003). Following activation of T cells, CD62L and CCR7 expression is downregulated, thereby excluding the effector cells' entry into the lymph node and promoting their migration into the infected tissues.

The process of T cell activation involves three "signals" (rev. in (Mescher et al., 2006)). Signal 1 is the engagement of the TCR by its cognate p:MHC complex. Signal 2 delivers costimulatory signals to the T cell by interaction of proteins from B7 family, such as CD80 and CD86, expressed by the APC with CD28 expressed by the T cell. The more recently described signal 3 refers to cytokine milieu provided by DCs or macrophages recognizing the pathogens via Toll-like Receptors (TLRs). TLRs belong to a family of pattern recognition receptors (PRRs), which recognize pathogen determinants termed pathogen-associated molecular patterns (PAMPs), such as non-methylated CpG (Akira and Takeda, 2004; Akira et al., 2006). TLR stimulation induces production of inflammatory cytokines IL-12, type I interferons (interferon α and β) and IFNγ , and they interact with cytokine receptors expressed by the T cell (Hochrein et al., 2001; Mescher et al., 2006). The three signals determine the positive outcome of T cell activation and dictate the characteristics of the effector and memory T cells, such as the extent of their proliferation and survival (rev. in (Haring et al., 2006)). Signals 1 and 2 are crucial, since

TCR engagement without costimulation leads to induction of tolerance or anergy, rather than activation (Heath and Carbone, 2001; Moser, 2003). The role of signal 3 is more complex and dependent on the particular type of challenge and may vary for different pathogens or tumors (Curtsinger et al., 2007; Joshi et al., 2007). As signal 3, type I IFN is crucial for generation of large effector CTL population following LCMV (lymphocytic choriomeningitis virus) infection (Kolumam et al., 2005), *Listeria monocytogenes* infection (LM) (Badovinac et al., 2004), and tumor challenge (Curtsinger et al., 2007), and in case of LCMV it is required not for proliferation, but for survival, of the effector cells. However, the low numbers of LCMV-specific cells that are generated in the absence of type I IFN survive well as a memory population (Kolumam et al., 2005). Similarly, the LM-specific $CD8^+$ T cells activated under low-inflammation conditions result in lack of contraction and stable memory maintenance. This is in line with evidence that decreasing the amount of inflammation (in this case IL-12) during LCMV infection correlates with preferential induction of long-lived memory cells, but correlates inversely with induction of short-lived effector CTLs (Joshi et al., 2007). Importantly, the inflammatory cytokine milieu, as well the extent of production of the various cytokines, depends on the pathogen, and possibly on the route of infection (Thompson et al., 2006). This heterogeneity of cytokine environment induced by different infections is only one of many factors, many of them still unknown, contributing to the different characteristics of effector, and then memory $CD8^+$ T cells reported in various infection models.

Following recognition of its specific target, the process of T cell activation begins. Recent data using multiphoton microscopy (MPM) of intact lymph nodes demonstrated

that there are at least 3 distinct phases of T cell priming (Henrickson and von Andrian, 2007; Mempel et al., 2004a; Mempel et al., 2004b). The first phase (lasting up to 8 hours) consists of short T cell:APC interactions, during which the T cells acquire activation markers such as CD44 and CD69. In the second phase the T cells form longer (1 hour or more) contacts with APCs, acquire fully activated phenotype and start cytokine production. The second phase is the longest and lasts up to 12 hours since the initial antigen encounter. During the final stage of priming the T cells detach from the APCs, extensively proliferate (completing replication cycle every 6-8 hours) and eventually leave the LN to travel to the infected organs. Some reports indicate that the extent of T cell priming does not depend on the length of pathogen persistence. For example, administration of antibiotics 24 hours after infection with LM did not affect the generation of $CD8^+$ T cell response, implicating that once initiated, the program of T cell differentiation from naïve to effector cell is set and does not depend on continued supply of antigen (Mercado et al., 2000). However, other studies provide evidence contrary to that. Experiments in flank scarification model of HSV-1 demonstrated that removal of the Ag within the first 48 hours of infection decreased the size of effector CTL pool, and removing Ag within the first 8 hours post-infection (p.i.) completely prevented generation of an effector response (Stock et al., 2004). This happened despite the fact that Ag presentation was demonstrated to start within 4-6 hours p.i. (Mueller et al., 2002b). Studies of effector CTLs during localized HSV-1 (Stock et al., 2004)and influenza infection (Yoon et al., 2007) demonstrated that Ag presentation and recruitment of new naïve $CD8^+$ T cells into the response can last as long as 7 days p.i., and shortening

that period affected the size of $CD8^+$ T cell response. Therefore, it remains unclear how the length of antigenic stimulation affects the programming of CTL response.

Effector functions of CTLs

The antiviral activity of $CD8^+$ T cells is accomplished by two main mechanisms: cytotoxic killing of infected cells and cytokine secretion. T cell activation in the lymph node results in production of effector cells capable of cytokine secretion and cytotoxic function upon recognition of its cognate MHC:peptide complex on any cell in the periphery. Unlike naïve T cells which need a series of signals in order to achieve activation, effector $CD8^+$ T cells require TCR stimulation alone for effector function (Kaech et al., 2002).

Interferon gamma (IFNγ) is the dominant cytokine by which effector cells perform their antiviral functions. Signaling through the interferon receptor on the infected cells initiates several signaling cascades, leading to interference with viral replication by a number of mechanisms (Ahmed et al., 2007). Another key effector cytokine secreted by activated CTLs is tumor necrosis factor alpha (TNFα), which has direct antiviral effects on the infected cells, in addition to its key role in recruiting inflammatory cells to the site of infection (Hehlgans and Pfeffer, 2005). Effector cytokines are not stored within effector $CD8^+$ T cells, but rather are synthesized *de novo* following TCR stimulation.

Cytotoxic killing by CTLs is accomplished via two types of molecules: perforin and granzymes A and B (Pipkin and Lieberman, 2007). Unlike cytokines, which need to be synthesized once the infected target is recognized, the synthesis of perforin and

granzymes is upregulated upon priming, and they are afterwards stored within lysosome-like membrane-enclosed cytotoxic granules in the cell's cytosol. Once the TCR is stimulated, downstream signals lead to cytoskeleton rearrangements, transporting the cytotoxic granules to the cell surface in the vicinity of the engaged TCR, where they undergo exocytosis. Perforin, a pore-forming protein, creates a junction with the target cells, through which the contents of the cytotoxic granule such as granzymes A and B are delivered (Catalfamo and Henkart, 2003). The granzymes are pro-apoptotic serine proteases, which, once inside the target cell, will initiate a caspase signaling cascade, leading to induction of apoptosis in the target cell (Lieberman, 2003; Russell and Ley, 2002). It is still unclear how the CTL itself is protected from release of granzymes into its own cytosol, but it is known that the CTL survives its attack of the target cell and is capable of launching another (Pipkin and Lieberman, 2007).

Contraction of the effector CD8$^+$ T cells and development of memory

The priming of CD8$^+$ T cell initiates a program of differentiation, which includes clonal expansion followed by a contraction phase. Depending on the pathogen and the infection route, the expansion and effector phase of the response lasts 7-10 days, after which time the majority of the effector CTLs (90-95%) die, and the remaining Ag-specific cells form the memory population (Haring et al., 2006; Sprent and Tough, 2001). A lot still remains unknown about the selection of cells that die or survive, and even about at which point in the immune response the Ag-specific cells choose their effector or memory fate. One factor that correlates with effector cell survival and differentiation into memory is expression of IL-7 receptor alpha (IL-7Rα, or CD127)

during the effector phase of the response. In LCMV infection model, on average only 5% of effector CTLs express IL-7Rα, and only these cells survive the contraction phase and become memory cells (Kaech et al., 2003). However, the IL-7Rα expression on effector cells and the survival of IL-7Rα^{+} memory precursors is not dependent on IL-7 signaling (Klonowski et al., 2006). In fact, IL-7 downregulates its own receptor.

There are two major models of how memory T cell populations develop: the linear differentiation model and the non-linear model (Kalia et al., 2006). The more conventional linear differentiation model suggests that memory cells develop from effector cells (Wherry et al., 2003b). The alternative model proposes that the fate of the cell (short-lived effector or long-lived memory which can be activated to become an effector CTL) is determined at the time of priming, and that a cell can progress directly to the memory cell status and bypass the effector stage. While phenotypic and functional progression from naïve to effector to memory cell can be observed on a population level, each individual cell would progress directly towards its predetermined fate, rather than itself undergo phenotypic and functional transformation.

Recent data show that early events during priming affect the activated cell's fate. A very recent data supporting the divergent differentiation model demonstrates that a choice between becoming an effector and memory cell may be made as early as during the initial division of the progenitor Ag-specific T cell (Chang et al., 2007). The authors demonstrate that via a process of asymmetrical division, the two daughter cells acquire, and then maintain, the phenotype and function of either effector or memory cells, and that upon adoptive transfer only the memory-phenotype cells are able to provide long-lived protection against pathogen challenge.

Aside from cell-intrinsic factors, other factors also may skew the fate of the activated T cells. The importance of cytokine milieu during primary response, particularly the inflammatory cytokines, influences the extent of contraction of effector CTLs (rev. in (Haring et al., 2006)). In one study the extent of early inflammation and IFNγ production following *Listeria monocytogenes* infection was controlled by antibiotic treatment. The treatment did not significantly affect the size of protective effector response, but resulted in less contraction (Badovinac et al., 2004).

A recent study demonstrated another way in which the inflammatory cytokines present during activation of the immune response determine the $CD8^+$ T cell fate very early on. In this study (Joshi et al., 2007), high level of IL-12, but not IFNγ, during primary infection led to elevated expression of transcription factor Tbet in greater proportion of responding $CD8^+$ T cells. The modulation of Tbet expression by IL-12 was dose dependent. Cells expressing more Tbet had a $KLRG1^{hi}$ $IL7^{lo}$ phenotype, and were destined to become short-lived effector cells, unlike the $KLRG1^{lo}$ $IL7R^{hi}$ cells which became long-lived memory cells. KLRG1 (inhibitory killer cell lectin-like receptor G-1) expression by $CD8^+$ T cells has been previously suggested to be a marker of terminally differentiated or senescent cells (Hamann et al., 1997; Voehringer et al., 2001), and this seemed consistent with KLRG1 expression by short-lived effector cells in this study. Therefore, the level of inflammation during initial infection can influence the fate (effector or memory) of a $CD8^+$ T cells very early during viral infection. Similar results were obtained in LM infection model (Badovinac and Harty, 2007).

Another way to control survival of memory $CD8^+$ T cell is by their interactions with other cell types, including helper T cells and regulatory T cells (T_{regs}). The role of

helper T cells in development and maintenance of memory $CD8^+$ T cells depends on the pathogen (rev. in (Bevan, 2004)). Viruses can be classified as CD4-help-dependent or independent. Depending on the infection model, the CD4 T cell help has been shown to be required for efficient activation and memory maintenance (Smith et al., 2004), not needed for priming but required for memory maintenance (Janssen et al., 2003; Sun and Bevan, 2003), or not crucial at any phase of $CD8^+$ T cell response (Shedlock and Shen, 2003). Similarly, the involvement of $CD4^+$ $CD25^+$ $FoxP3^+$ T_{regs} in shaping the effector and memory $CD8^+$ T cell response depends on the type of infection (Rouse et al., 2006; Rouse and Suvas, 2004; von Boehmer, 2003). Since the T_{reg} biology has only recently been under intense scrutiny, their impact on the generation and maintenance of $CD8^+$ T cell response can only be discussed per individual infection model. Regardless, the recent data makes it important to consider the potential impact of these two CD4 T cell subsets while studying any $CD8^+$ T cell response.

The final factor to be considered in the process of contraction and memory development is persistence of Ag. All of the mechanisms described above relate to acute infection models, where the pathogen is completely eliminated within 1-2 weeks post-infection. During persistent infections the ongoing antigenic stimulation contributes to all of the above described factors in determining the size, phenotype and functional characteristics of the memory response. These will be discussed in the next sections.

Phenotypic and functional characteristics of memory $CD8^+$ T cells

On a T cell population level, transformation from effector to memory phase is a gradual process and can take up to 45 days following infection, as was demonstrated in

the LCMV experimental model (Kaech et al., 2003). During that time, the phenotype and the functional characteristics of Ag-specific $CD8^+$ T cells change. While there isn't a single phenotypic marker to distinguish an effector and memory $CD8^+$ T cell, a combination of markers can give a good idea of the activation status of a given cell. Naïve $CD8^+$ T cells would generally have $CD44^{lo}$ $CD62L^{hi}$ $CCR7^+$ $CD43^{int}$ $Ly6C^{int}$ $CD127^+$ $CD69^-$ $CD25^-$ $GzmB^-$ phenotype. Effector CTL are $CD44^+$ $CD62L^{lo}$ $CCR7^-$ $CD43^{hi}$ $Ly6C^{hi}$ $CD127^-$ $CD69^+$ $CD25^+$ $GzmB^+$, although it should be noted that even during the short effector phase the above proteins can be expressed differentially, i.e. CD69, a very early activation antigen, is expressed only for a very short time (hours) following activation, and similarly, expression of CD25 is also transient.

Memory $CD8^+$ T cells are characterized by the greatest phenotypic heterogeneity of all $CD8^+$ T cell subsets, although they all retain high expression of CD44 (Kalia et al., 2006; Masopust et al., 2004a). The reasons for this phenotypic heterogeneity are not completely understood, but appear to be a result of conditions present during priming and then during the memory phase. Some of these factors are the anatomical location and cytokine environment at the time of priming, which in turn is affected by the biology of the pathogen and the infection route used (rev. in (Lefrancois, 2006)). Similarly, the anatomical location, the history of contact with antigen, and division history during the memory phase may be additional factors contributing to the phenotypic diversity of memory $CD8^+$ T cells. For example, memory cells from non-lymphoid organs display different phenotype than those found in secondary lymphoid organs or circulation (Masopust and Lefrancois, 2003; Masopust et al., 2001). $CD8^+$ T cells specific for chronic pathogens display phenotype indicative of ongoing stimulation, i.e. $CD62L^{lo}$

$CD127^{lo}$ $CD27^{lo}$ (Baars et al., 2005; Sierro et al., 2005). Another factor influencing the phenotype of a memory $CD8^+$ T cell pool is the precursor frequency at the time of priming, where high precursor frequency leads to $CD62L^-$ phenotype that can be reverted to $CD62L^+$ phenotype upon adoptive transfer to naïve host. By contrast, the same infection under low-precursor frequency conditions leads to irreversible $CD62L^-$ phenotype of memory cells (Marzo et al., 2005). Finally, naïve $CD8^+$ T cells may acquire memory-like phenotype simply as a result of extensive homeostatic proliferation (Murali-Krishna and Ahmed, 2000).

Another criterion that distinguishes naïve, effector and memory $CD8^+$ T cells is their size. Naïve T cells are small resting cells, whereas effectors are large cells, often termed blasts. Finally, memory cells generally return to small resting cell phenotype, although they tend to be somewhat larger than naïve cells. (rev. in (Lefrancois, 2006)).

The functional features distinguishing naïve and memory $CD8^+$ T cells are the speed and efficacy of their responses. Memory $CD8^+$ T cells respond more rapidly, due to their increased precursor frequency, and heightened signaling capacity (Curtsinger et al., 1998; Farber, 1998; Hussain et al., 2002; Lefrancois, 2006).

Two main categories of memory $CD8^+$ T cells are central memory (T_{CM}, $CD62L^+$ $CCR7^+$) and effector memory (T_{EM}, $CD62L^-$ $CCR7^-$). The central memory cells reside in the lymphoid tissues, have no immediate effector functions, and respond vigorously to contact with antigen by extensive proliferation and differentiation into effector CTLs. By contrast, the effector memory cells can migrate to non-lymphoid tissues, are capable of immediate cytotoxic activity, and do not proliferate as extensively as T_{CM} upon contact

with Ag (Masopust and Lefrancois, 2003; Sallusto et al., 2004; Sallusto et al., 1999; Wherry et al., 2003b).

Effect of persisting infections on phenotype and function of memory CD8$^+$ T cells

Chronic infection and ongoing antigenic stimulation associated with it can compromise the functional responses of memory CD8$^+$ T cells by driving them to functional exhaustion. This has been described for a number of human (including HIV, HCMV, Hepatitis C) and murine (including chronic LCMV, Polyoma virus) pathogens (rev. in (Shin and Wherry, 2007)). Several functions can be affected, as is the case with chronic LCMV infection, where the loss of various functions occurs sequentially (Wherry et al., 2003a). First IL-2 production, cytolysis and robust proliferation are lost, followed by loss of ability to make TNFα and finally IFNγ. The dysfunctional memory CD8$^+$ T cells may continue to persist in the host, or can be deleted, as was documented for chronic LCMV (Wherry et al., 2003a; Zajac et al., 1998).

The functional exhaustion has been linked with two signaling pathways: PD-1:PD-1L and IL-10:IL-10R. PD-1 is an inhibitory receptor belonging to the CD28 superfamily (Sharpe et al., 2007). Exhausted CD8$^+$ T cells in LCMV (Barber et al., 2006), HIV (Day et al., 2006; Trautmann et al., 2006)and Hepatitis C (HCV) chronically infected hosts had elevated expression of PD-1 (Urbani et al., 2006). In HIV-infected humans, elevated expression of PD-1 by memory CD8$^+$ T cells correlated with high viral load and progression of disease. Blocking the PD-1:PD-1L interactions *in vivo* in mice lead to reduction of viral titers and improved function by LCMV-specific memory CD8$^+$ T cells (Barber et al., 2006). Similarly, the loss of function of HIV and HCV specific

memory $CD8^+$ T cells was reversed *in vitro* by blocking the PD-1:PD-1L signaling pathway (Day et al., 2006; Trautmann et al., 2006; Zhang et al., 2007).

IL-10 signaling has been proposed to limit T cell responses during chronic infections. In chronic LCMV model (Brooks et al., 2006; Ejrnaes et al., 2006), treatment with anti-IL-10 mAb early in the infection allowed for clearance of the virus, preventing establishment of chronic infection. The role of IL-10 in curtailing early T cell responses in other chronic infections still needs to be addressed.

Another phenotypic marker expressed on memory $CD8^+$ T cells during some chronic infections is KLRG1 (discussed above as an early marker of short-lived effector CTLs). KLRG1 is an inhibitory NK-cell receptor, and was proposed to serve the same inhibitory function on memory $CD8^+$ T cells. It was described as a marker of senescent cells (Hamann et al., 1997; Voehringer et al., 2001), and its expression correlated with decreased proliferative capacity of memory $CD8^+$ T cells, however this has not been demonstrated directly.

Finally, memory $CD8^+$ T cells specific for certain MCMV epitopes display effector memory phenotype ($CD62L^{lo}$ $CD27^-$ $CD28^-$) suggestive of ongoing Ag stimulation (Baars et al., 2005; Sierro et al., 2005). In murine MCMV infection, there is continuous accumulation of memory $CD8^+$ T cells termed "memory inflation" (Karrer et al., 2003). $CD8^+$ T cell response to MCMV is very broad (Munks et al., 2006b), and not all specificities of memory $CD8^+$ T cells undergo memory inflation (Sierro et al., 2005). Interestingly, the memory T cells that do not undergo inflation display a resting, central memory phenotype, whereas the inflating memory T cells have an activated, effector memory phenotype. The presumed reason for memory inflation and the phenotype of

memory T cells in that model is ongoing stimulation by the persisting virus, although a formal proof of that is still missing.

Migratory properties of $CD8^+$ T cell subsets

As described above, the phenotypic characteristics of memory $CD8^+$ T cells are dependent upon a number of events during priming and memory phase. The same is true for expression of homing receptors by effector and, more importantly, memory $CD8^+$ T cells, which in turn determines their ability to access different tissues (Campbell et al., 2003). In recent years, significant progress was made in understanding the migratory behavior of effector and memory cells (rev. in (Lefrancois, 2006)). The main techniques used for these studies were adoptive transfers of purified T cell subsets, and a parabiosis system (Klonowski et al., 2004), where two congenic mice were joined surgically, allowing exchange of circulating lymphocytes. In general, activated $CD8^+$ T cells are able to access almost any non-lymphoid tissue, regardless of the anatomical site where they were originally activated (Masopust et al., 2004b). Even access to sites with limited vascularization, such as cornea, or sites protected by blood-brain-barrier (brain and spinal cord) is open to activated $CD8^+$ and $CD4^+$ T cells during active infection (Chen et al., 2005; Deshpande et al., 2001a; Deshpande et al., 2001b; Lang and Nikolich-Zugich, 2005; van Lint et al., 2005). Even though virus-specific as well as bystander activated T cells could access the eye (Deshpande et al., 2001a) and brain (Chen et al., 2005), only virus-specific T cells were retained in those tissues, implying involvement of Ag in their retention (van Lint et al., 2005).

The migration of central memory $CD8^+$ T cells is more restricted. Study of migration of VSV (vesicular stomatitis virus) and LM-specific memory $CD8^+$ T cells using the parabiosis system (Klonowski et al., 2004) demonstrated that they can freely access the lung and the liver, but their migration into intestinal lamina propria (LP) and brain was restricted. Additional adoptive transfer studies showed that access of memory cells to the intestinal LP, brain and the peritoneal cavity, but not to the lung and liver, depends on G-protein-coupled-receptors, since it was prevented by treatment of cells with pertussis toxin prior to transfer.

1.3 HOMEOSTASIS OF $CD8^+$ T CELLS

Memory $CD8^+$ T cells provide long-term protection against their cognate pathogen. The longevity of this responses is granted by the memory $CD8^+$ T cells' ability to survive for a long time as a population. This process relies on their enhanced survival and constant slow proliferation, termed homeostatic cycling. The mechanisms regulating T cell homeostasis are reviewed in this section.

Homeostatic maintenance of T cell subsets

T cell homeostasis is a process of maintenance of naïve and memory T cell pool numbers and diversity and the ability to restore these numbers and diversity following antigenic challenge. T cell homeostasis is regulated by the response of T cells to environmental trophic and survival signals and by the presence and availability of such signals. The most important and best understood of these signals are the common γ-chain

cytokines (most notably IL-7, IL-15 and IL-2) and self p:MHC complexes (Fry and Mackall, 2001; Surh et al., 2006; Surh and Sprent, 2002). The contribution of each of these signals to homeostatic maintenance varies depending on the T cell subset. Briefly, survival of naïve T cells depends on IL-7 and self p:MHC interactions, and maintenance of memory T cells relies on IL-15-driven proliferation. IL-2 was shown to oppose some of the actions of IL-7 and IL-15(Ku et al., 2000) and its primary role appears to be in immune regulation and production and expansion of regulatory T cells (Malek and Bayer, 2004). Somewhat less-well understood is the role of metabolic signals (Frauwirth and Thompson, 2004) and it is possible that there are other, presently unknown pathways regulating T cell homeostasis.

T cells are bone marrow-derived lymphocytes which undergo maturation in the thymus before their release into the periphery as recent thymic emigrants (RTEs, rev. in section 1.2). Release of RTEs bearing a variety of randomly rearranged TCRs ensures the diversity of the peripheral T cell pool. The rate of RTE production is proportional to the total thymic mass (Hale et al., 2006; Scollay et al., 1980), may depend on the rate of influx of new precursors from the bone marrow (Donskoy et al., 2003), but is independent from signals from peripheral T cell pool. One study (Berzins et al., 1998) demonstrated that in mice with neonatal thymic graft the rate of RTE export from both the donor and host thymus remained unchanged (1% of total thymocytes every day), which resulted in release of overall greater numbers of RTE into the periphery. However, despite this increased RTE output (owing to the presence of additional thymic lobes) the size of the peripheral T cell pool remained unchanged. Therefore, the production and release of RTE is not regulated by signaling by the peripheral T cells. However,

homeostatic mechanisms are able to maintain stable peripheral T cell numbers in the event of altered thymic output.

Once released from the thymus, the RTE join the naïve T cell pool. Naïve T cells have no pre-set lifespans (Sprent et al., 1991; Tough and Sprent, 1995) and are maintained by IL-7 and trophic signals from interaction of their TCR with self p:MHC complexes. When these two signals are present, naïve T cells can survive indefinitely in serial transplantation experiments (rev. in (Surh et al., 2006; Surh and Sprent, 2002)). Murine RTE proliferate faster than naïve peripheral T cells in the first three weeks after export (Berzins et al., 1998) perhaps in order to maximize naïve T cell diversity, before they equilibrate with other naïve T cells. Naïve T cells display very low levels of spontaneous (or homeostatic) cycling *in vivo*. Homeostatic cycling is greatly increased in lymphopenia, where T cells sense the excess of "space", most likely by sensing an excess of unused IL-7 and IL-15. Under lymphopenic conditions T cells undergo Ag-independent homeostatic proliferative expansion (HPE), in a seeming attempt to fill the empty compartment (Fry and Mackall, 2001; Surh and Sprent, 2002)

Unlike naïve T cells, memory T cells do not require specific p:MHC contact for survival. Instead, their survival is dependent on continued homeostatic proliferation, driven mainly by IL-15, or by IL-7 in the absence of IL-15 (Tan et al., 2002). Memory cells cycle and self-renew *in vivo* three to four times faster than naïve T cells and are capable of vigorous proliferation during lymphopenia (Surh and Sprent, 2002).

The above described homeostatic mechanisms function to maintain a balanced and diverse T cell pool. Over lifetime this means regulating the process of Ag-driven expansion of naïve T cells, their contraction, and selection and maintenance of memory T

cells. The role of the homeostatic mechanisms is to balance the composition of the T cell pool so that it contains both naïve precursors with diverse TCRs, as well as Ag-experienced memory $CD8^+$ T cells, as both of these subsets are crucial for the health of the host. The homeostatic forces work very efficiently in adult mice housed under SPF conditions, as evidenced by remarkably similar size and diversity of the T cell pool among individual mice of the same strain (Callahan et al., 1993; Surh and Sprent, 2002). However, maintenance of homeostasis becomes more complicated in the face of repeated antigenic challenges, and also due to poorly understood aging-associated defects. These issues will be discussed below (effect of persisting pathogens on T cell homeostasis) and in section 1.8 (effect of aging on T cell homeostasis).

Effect of persisting viruses on $CD8^+$ T cell homeostasis

Memory $CD8^+$ T cells generated as a result of chronic infections often do not respond to the normal homeostatic signals, and use alternative mechanisms for their long-term maintenance (rev. in(Shin and Wherry, 2007)). Memory $CD8^+$ T cells from mice chronically infected with LCMV do not survive following transfer into naïve hosts, unlike memory cells generated by acute infections (Wherry et al., 2004). Their inability to survive may be related to loss of responsiveness to homeostatic cytokines IL-7 and IL-15, as the cells that do not survive the adoptive transfer have low expression of receptors for these cytokines and exhibit poor responsiveness to cytokine treatment *in vitro (Lang et al., 2005; Shin et al., 2007; Wherry et al., 2004).* This correlates with inability of these cells to undergo homeostatic proliferation upon adoptive transfer into naïve hosts. Defects in perceiving homeostatic signals were also described for memory $CD8^+$ T cells specific for another persistent virus, gamma herpesvirus-68 (MHV-68). These cells were

able to survive well in infected IL-15$^{-/-}$ host, implying that Ag but not IL-15 are important for their maintenance, but they failed to undergo homeostatic proliferation when transferred into wt naïve hosts (Obar et al., 2004).

Two recent studies uncovered new mechanisms utilized by memory CD8$^+$ T cells in chronically infected hosts which allow their survival despite defects in perceiving standard homeostatic signals (Shin et al., 2007; Vezys et al., 2006). As outlined in a recent review (Shin and Wherry, 2007), the possible alternative mechanisms of maintaining memory CD8$^+$ T cell populations during chronic infections are their enhanced longevity, periodic generation of new memory cells by activation of naïve cells, maintenance driven by ongoing inflammatory environment, and ongoing stimulation with Ag. The enhanced longevity hypothesis is unlikely, as adoptively transferred cells do not survive long-term. Study by Vezys et al. (Vezys et al., 2006) used a chronic Polyoma virus and LCMV infection of mice to analyze the involvement of RTE in memory CD8$^+$ T cell maintenance in face of chronic infection. The authors constructed bone marrow chimeras from mice already chronically infected, and showed that within months, donor-derived memory CD8$^+$ T cells could be found in the peripheral blood. Maintenance of memory pool by recruitment of new memory cells from RTEs is most likely only one of mechanisms of memory cell maintenance in chronically infected hosts, as an earlier study focused on maintenance of memory CD8$^+$ T cells following chronic LCMV infection of thymectomized mice did not notice a difference in the size of the memory population (Miller et al., 2005).

Study by Shin et al. (Shin et al., 2007) demonstrated that presence of Ag is required for maintenance of memory CD8$^+$ T cells during chronic LCMV infection, and

in the absence of Ag the inflammatory environment itself was not enough to sustain the memory population. The authors used adoptive transfer of memory cells into congenic mice infected with chronic strain of LCMV that either did or did not express the dominant LCMV epitope. The transferred cells survived only in the presence of their cognate Ag, and their survival in the presence of Ag was not dependent on recruiting RTEs into the memory response. By labeling the memory cells with CFSE prior to transfer the authors demonstrated that the likely mechanism of maintenance of these memory cells is their extensive Ag-driven proliferation.

More work is needed to extend the findings of these experiments to other persistent infection models, however it is clear that the homeostasis of memory $CD8^+$ T cells during persistent infections often follows different rules than the homeostasis of memory cells generated by acute infections.

1.4 THE HERPES SIMPLEX VIRUS

The Herpesvirus family

The herpesvirus family (Herpesviridae) is an ancient, large and diverse family of viruses infecting vertebrates. The members of the family share a high level of homology both in their genes and in the organization of their genome. In addition to genomic similarities, the herpesviruses also share a conserved virion structure with the following common features: an outer lipid membrane surrounding a proteinuos structure called

tegument, which in turn surrounds an icosahedral nucleocapsid, containing linear double-stranded DNA genome (Fields, 2001).

The herpesvirus family consists of three subfamilies: α, β and γ herpesviruses, based on their sequence and biology. The α-herpesviruses include HSV-1 and -2, varicella-zoster virus (VZV), equine herpesvirus type I (EHV-1), pseudorabies virus (PRV), and bovine herpes virus (BHV-1), and they are characterized by a variable host range, relatively short reproductive cycle, rapid spread in culture, lytic replication cycle (the infection destroys the cell), and the ability to establish latency in neurons. The β-herpesviruses include human and murine cytomegalovirus (CMV), and human herpesvirus (HHV) 6 and 7, and are characterized by having a restricted host range, longer reproductive cycle, slow spread in culture and induction of cytomegalia (enlargement of infected cells). Finally, the γ-herpesviruses, which include Epstein-Barr Virus (EBV), herpesvirus saimiri (HVS), equine herpes virus type 2 (EHV-2), Kaposi sarcoma-associated herpesvirus (KSVH) and mouse γMHV68 are defined by their extreme host-specificity, and the ability to establish latency in host T or B cells (Fields, 2001).

As an ancient virus family, herpesviruses evolved many immune evasion mechanisms, which allow them to coexist with their vertebrate hosts, often for the host's lifetime (Fields, 2001). For the purpose of this dissertation, we define any virus capable of establishing a lifelong infection as "persistent". Defined as such, persistent viruses would include those that establish latent infection (for example HSV-1- and -2, EBV, MCMV), as well as chronic viruses (for example HIV or Hepatitis C virus, HCV). Latent infections differ from chronic infections by lack of production of infectious virus, but are

different from acute, or abortive infections, because the latent genome retains the capacity to replicate and cause disease upon reactivation.

HSV-1 cell tropism and life cycle

HSV-1, an α-herpesvirus, is a natural human pathogen, however the virus is capable of infecting mice. HSV-1 can infect a wide range of cell types, possibly due to the fact that it does not require a unique receptor for entry. The receptor most commonly used for attachment, heparan sulfate (HS), as well coreceptors such as nectins, exhibit ubiquitous expression across cell types and their homologs are common in different vertebrate species (Spear, 2004). For example, a murine homolog of human nectin mediates entry of HSV-1 into murine cells (Shukla et al., 2000). However, despite the wide-spread expression of the viral receptors and coreceptors, the viral lifecycle is predominantly restricted to two cell types: epithelial cells and neurons. The virus typically infects the host via epithelial cells of a mucosal surface. Following productive replication in the epithelium at the site of entry, the virus enters the sensory neurons innervating the tissue, from where it may further spread to the central nervous system to cause encephalitis. After a brief period of productive replication in neurons the virus establishes lifelong latency. The latency, defined as lack of productive viral gene expression and replication, can be periodically broken by an event of reactivation, commonly associated with stress. The only viral transcripts produced with relative abundance during latency are the LATs (the latency associated transcripts), whose role in maintaining the latent state is still debated and will be discussed in more detail below. The viral spread, both in the epithelium and in the nervous system, is accomplished by

cell-to-cell transport, which requires viral dimeric glycoprotein complex gE:gI to form a junction between cells (Farnsworth and Johnson, 2006). While infection of lymphocytes is possible, transport of the virus via the lymphatic or circulatory system does not play a part in viral spread (Fields, 2001).

The viral lifecycle is completed in 18-20 hours (Cunningham et al., 2006; Fields, 2001). Viral entry is a three-step process, consisting of receptor binding, coreceptor binding and membrane fusion. First, HS on the cell surface is bound by viral glycoprotein gB or gC, which then allows binding of cellular coreceptors, such as nectins, by viral gD or gH-L complex. Binding induces fusion of viral outer lipid membrane with the cell's plasma membrane, allowing entry. While binding of HS and coreceptor on the host cell allows for the most efficient entry, infection is possible in the absence of HS, suggesting that alternative receptors for entry do exist. Similarly, viruses with non-functional surface gB, gC, or both gB and gC can attach to the cell, but binding is severely impaired. Finally, gB is essential for infectivity, as it participates in the fusion process that takes place after binding (Fields, 2001; Reske et al., 2007; Spear, 2004).

Following attachment and entry, the viral nucleocapsid is transported to the nuclear membrane, and the DNA is further transported to the nucleus. The viral genes are expressed in the cytoplasm, whereas the DNA synthesis and assembly of new viral capsids takes place in the nucleus (Fields, 2001). The viral genome transcription requires both host and viral factors. The newly formed capsids mature and acquire infectivity during transport from the nucleus via Golgi to the plasma membrane, from where they can spread to adjacent cells. Viral gene expression, DNA synthesis and viral assembly are

tightly regulated and occur in a series of sequential steps, as described in the next segment.

Gene expression

HSV-1 contains three classes of genes, called α, β and γ, or, alternatively, immediate early (IE), early (E) and late (L) (Fields, 2001). The IE genes, termed infected cell polypeptides (IPC), include ICP 0, 4, 22, 27 and 47. These proteins are expressed *de novo* within 2 to 5 hours post infection, and their expression is required for viral replication to proceed. ICP4 is particularly important; in fact, it is an essential transactivator of HSV genes. Of interest to this thesis, ICP47 has been reported to inhibit antigen presentation, both in humans (Fruh et al., 1995; Hill et al., 1995) and to a lesser extent in mice *in vivo* (Goldsmith et al., 1998), however despite this infection with HSV-1 results in activation of adaptive immunity.

The next phase of gene expression, the E gene expression, occurs between 5 to 7 hours post-infection (Fields, 2001). The E gene products are predominantly involved in DNA synthesis and nucleotide metabolism, and include viral DNA polymerase, thymidine kinase (TK) and ribonuclease reductase.

Following E gene expression, viral DNA synthesis begins. The extent of the L gene expression, and subsequently the extent of new virion assembly, depends on the DNA synthesis and will not proceed without it (Kosz-Vnenchak et al., 1993; Nichol et al., 1996; Summers and Leib, 2002).

While the gene expression pattern during productive replication has been studied extensively and is well understood and universally accepted, it is still unclear whether the

same program of gene expression is followed in the event of reactivation from latency (Kent and Fraser, 2005).

Latency and reactivation

Following productive viral replication in the epithelial cells at the site of entry, the virus travels via axons of sensory nerves to the nerve cell bodies in the sensory ganglia (retrograde transport). There, it will continue productive replication for a short time (up to 2 weeks, but usually less than that), and then the viral gene expression will cease. However, the viral genome will persist indefinitely in the infected cell's nucleus as a non-integrated circular molecule, and may commence productive gene expression and replication under favorable conditions (Fields, 2001; Mitchell et al., 2003). Stress has long been known as the main factor aiding viral reactivation from latency. Constraint stress, hyperthermic stress, hormonal changes, psychological stress, and immunesuppression all had been demonstrated to serve as triggers of viral reactivation (Anglen et al., 2003; Freeman et al., 2007; Sainz et al., 2001; Sawtell and Thompson, 1992; Sawtell and Thompson, 2004; Wagner and Bloom, 1997; Wang et al., 2001).

The molecular factors determining whether the virus enters, maintains and exits the latent state remain far from being understood. The main players believed to influence the viral replicative status (productive or latent) are intrinsic host cell factors, viral factors, and the host immune system, particularly $CD8^+$ T cells and inflammatory cells.

It is still unclear what triggers the initial switch from productive to latent transcriptional state. It has been suggested that cell factors specific to neurons create an environment permissive for establishment of latency, for example by limited presence of

cellular proteins that aid viral transcription, or by presence of transcriptional repressors (Akhova et al., 2005; Chen et al., 2007; Gordon et al., 1990; Halford et al., 2006; Pinnoji et al., 2007). Once active replication ceases, the viral transcription is restricted to LATs, and their potential role in maintaining the latent state had been under intense scrutiny (rev. in (Bloom, 2004; Fields, 2001; Kent et al., 2003; Millhouse and Wigdahl, 2000)). While some reports indicate that LATs actively participate in maintaining the latent state, others suggest that LAT expression may be more of a phenotypic hallmark rather than functional requirement for maintenance of latency. Despite intense research, the role of LATs in HSV biology is far from resolved at present.

The majority of work on HSV-1 latency has been done using mouse infection models, particularly the ocular infection model that leads to establishment of latency in the sensory trigeminal ganglia (TG). The appeal of the model was the apparent lack of viral reactivation (Margolis et al., 2007). Unlike in humans, where reactivation and virus shedding are known to occur periodically, attempts to detect a similar phenomenon in mice have failed in many studies (Deatly et al., 1988; Devi-Rao et al., 1994; Laycock et al., 1991; Mitchell et al., 1994; Speck and Simmons, 1991; Trousdale et al., 1984; Tullo et al., 1982; Willey et al., 1984). However, there were indications that viral reactivation may indeed be taking place in the latent TG. These included continued presence of inflammatory cell infiltrates and cytokines at the latent sites (Cantin et al., 1995; Halford et al., 1996; Liu et al., 1996), and their decrease following treatment with antiviral drug acyclovir (Halford et al., 1997). In one study, the cell infiltrates surrounding latently infected neurons were shown to be $CD8^+$ T cells specific for a viral epitope derived from gB, a L gene (Halford et al., 1997). Still, the inability to detect viral gene expression,

DNA replication and infectious virus after resolution of primary infection lead to the wide-spread belief that spontaneous HSV-1 does not happen in mice. However, evidence accumulated in the recent years calls for revision of that concept.

Several reports indicate that unlike previously thought, HSV-1 latency in mice is not complete at all times. Rather, there is continued low-level of viral gene expression in addition to LATs, including IE, E and even some L genes (Feldman et al., 2002). In addition, rare cells in the infected TG sections contain high level of viral DNA, and viral TK and ICP0 proteins, similar to the level observed in productively infected cells, which was interpreted as potential event of DNA replication. The expression of viral genes coincided with presence of inflammatory cell infiltrates around neurons undergoing this "molecular reactivation". These findings were extended in another study (Margolis et al., 2007), where spontaneous molecular reactivation was shown to coincide with presence of infectious virus particles in 6% of latently infected TGs at any given time. The authors interpreted the results as evidence that viral reactivation is a relatively common event in mice, and that the mouse model mimics the human infection more closely than previously thought.

However, this viral reactivation, even though more common than initial studies indicated, is still not associated with detectable infectious virus presence in the periphery (outside of the latently infected tissue). One potential reason limiting full reactivation and virus spread from the neurons is the extent of viral DNA synthesis in cells undergoing spontaneous reactivation. The extent of DNA synthesis has been shown to influence the degree of de novo synthesis of IE and E genes in reactivating cells (Kosz-Vnenchak et al., 1993). Using inhibitors of viral replication and viral mutants (TK-null

virus), the authors demonstrated by in situ hybridization (ISH) that unlike during productive infection, a critical threshold of DNA replication must be reached in order to proceed with gene expression in reactivating neurons in explant cultures of sensory ganglia from latently infected mice. While this finding created a long-standing paradigm, recent reports using more refined viral inhibitors indicate that DNA synthesis may not be crucial in allowing IE and E gene expression. One study (Pesola et al., 2005) used two different inhibitors of viral replication: acyclovir, which blocks viral DNA synthesis, and WAY-150138, which permits DNA synthesis but blocks viral encapsidation and therefore release and spread of new virions. The extent of IE and E gene expression was surprisingly similar in acyclovir and WAY-150138-treated cultures, however both inhibitors of viral replication contributed to blocking viral protein synthesis as compared to untreated cultures. The authors proceeded to demonstrate that the difference was not due to the extent of gene synthesis at the individual cell level, but rather the increased gene expression in untreated cultures could be attributed to spread of new viral particles throughout the explanted tissue. These results suggests that neuron-to-neuron spread is more common than previously thought, and that it significantly influences the latent viral burden and the number of latently infected cells. It also indirectly supports the notion that viral reactivation in mouse neurons is more common than previously thought.

In addition to the molecular and cellular factors contributing to the reactivation rates, the frequency of viral reactivation has also been positively correlated with the latent viral load (Hoshino et al., 2007) and the number of latently infected neurons (Sawtell, 1998; Sawtell et al., 1998). The final factor in control of viral reactivation is the host

immune response, particularly IFNγ-producing $CD8^+$ T cells, and which will be discussed in section 1.6.

In the event of reactivation, viral particles are synthesized in the so-far latently infected neurons. From there they can spread to other neurons, or travel to the periphery, once again moving inside the axons on the sensory neurons (anterograde transport). The productive viral replication following reactivation is the main factor contributing to HSV-1 associated pathologies.

Pathology associated with HSV-1 infection

Infection with HSV-1 can lead to several pathological outcomes, many of them severe. The pathology is caused either by the virus or by immune cells activated as a result of infection. The most common symptoms associated with HSV-1 infection are cold sores, extensive lesions at the site of initial infection, herpetic stromal keratitis (HSK) in the event of infection of the eye, blindness as a result of HSK, and neurological complications and death from encephalitis (Jensen et al., 2004; Miserocchi et al., 2007; Raguin and Malkin, 1997; Rezende et al., 2007; Simpson and Lyseng-Williamson, 2006). The most severe pathologies are not extremely common, but given the high prevalence of HSV-1 in the population (up to 80-90% in the US, rev. in (Mitchell et al., 2003; Simpson and Lyseng-Williamson, 2006)) and the high frequency of reactivation events, the possibility of their onset is a constant concern. For example, HSV-1 is the main cause of corneal opacity and infection-related blindness in the industrial world, and the recurrence rate for that condition ranges from 20-67% and increases over time (Rezende et al., 2006). The incidence of reactivation depending on the patient and on the type of

infection they suffer from. In patients with genital herpes, 87% of patients experienced reactivation demonstrated by viral shedding, with individual incidence of reactivation ranging from 3 to 8 times in a year (Gilbert, 2006).

The most severe outcome of HSV-1 infection is the herpes simplex encephalitis (HSE). It has a high mortality rate (44-62%), and the survivors are likely to suffer a relapse of HSE associated with neurological consequences (Skoldenberg et al., 2006). One of such neurological complications is Alzheimer's disease, and while the exact cause-and-effect relationship between HSV-1 and that disease needs to be elucidated, it is known that presence of HSV-1 in the brains of patients with a known Alzheimer's disease susceptibility genetic factor (type 4 allele of apolipoprotein E gene, or APOE-ε4) confers additional risk for development of Alzheimer's (Lin et al., 2001; Wozniak et al., 2005).

Prevention and treatment of HSV-1

Currently there is no vaccine against HSV-1. Inhibitors of viral replication are used with limited success (Simpson and Lyseng-Williamson, 2006). No drug can eliminate the latent virus, however the extent of viral replication during primary infection or reactivation can be curtailed. The most commonly used viral inhibitors are acyclovir and various permutations of this molecule (valacyclovir, pencyclovir, famciclovir). All of these drugs operate on the same principle (reviewed below) but vary in their bioavailability (how well they are absorbed) and route of administration. The drugs have a similar efficacy, however famciclovir is the most commonly used due to fact that it can be taken orally and has high (77%) bioavailability (Simpson and Lyseng-Williamson, 2006).

Famciclovir, a nucleoside analog, blocks HSV-1 replication without affecting cellular DNA synthesis (Darby et al., 1981; Datta et al., 1980; LeBlanc et al., 1999; Simpson and Lyseng-Williamson, 2006). Famciclovir is administered and absorbed by the cells in its inactive form. The molecule undergoes conformational transformation into its active form following phosphorylation by viral TK, which is only synthesized in actively infected or reactivating cells. Famciclovir is then incorporated into viral DNA, leading to production of defective genomes which in turn prevent further viral gene expression and production of infectious virions. Cellular DNA synthesis is not affected, because only viral, but not cellular DNA polymerase has an affinity for famciclovir (Darby et al., 1981).

1.5 OVERVIEW OF THE IMMUNE RESPONSE TO HSV-1

The majority of work done to understand HSV-1 immunity comes from studies done in mouse models. Both innate and adaptive responses were identified to play a role in controlling the severity and outcome of initial infection, while the adaptive immune responses, primarily $CD8^+$ T cells, appear to play a dominant role in responding to viral reactivation. The findings from mouse studies are often difficult to reconcile due to the fact that they use different mouse strains, inoculation routes, and viral strains, all of which can influence the outcome of infection (rev. (Brandt, 2005)).

The resistance loci

Various infection models demonstrated that mouse strains differ in their resistance to infections with a number of pathogens. The resistance in these studies is most often defined as survival rate, the ability of the pathogen to replicate and spread, and by severity of disease induced by infection. For example, C57BL/6 (B6) mice are more resistant than other inbred strains to infection with MCMV, *Trypanosoma congolense*, *Leishmania major*, and other infections (rev. in (Brandt, 2005)). Similarly, B6 mice were also reported to be more resistant to HSV-1 than the susceptible strain BALB/c. The initial study describing this increased resistance (Lopez, 1975)demonstrated better (>1000 times) survival of B6 than of BALB/c mice following intraperitoneal (i.p.) challenge with HSV-1. Whereas B6 mice survived 10^6 PFU infection dose, BALB/c mice had 100% mortality following challenge with 10^4 PFUs and 50% mortality after infection with 200 PFUs. This and subsequent studies using survival rates following i.p. infections with several pathogens classified strains as highly susceptible (A/J, PL), moderately susceptible (Balb/C, AKR/J, DBA/2J) and resistant (C57BL/6, DBA/1. CBA) (rev. in (Lundberg et al., 2003)).

Later studies were refined to identify the mechanism behind the differences in resistance. The resistance was not H-2 linked, as congenic strains expressing MHC alleles of susceptible mice on the resistant strain background did no have increased susceptibility (Lopez, 1980). Several resistance loci were identified: *Hrl* (herpes resistance locus, closely linked to the TNF p55 receptor) on mouse chromosome 6 (Lundberg et al., 2003), *Rhs1* (NK complex-linked) also on mouse chromosome 6

(Pereira et al., 2001), and *igh* locus on mouse chromosome 12 (Norose et al., 2002; Opremcak et al., 1988).

The importance of *igh* locus to conferring HSV-1 resistance was specific to the ocular infection model, in which the resistant mouse strains were protected from development of keratitis. This protection was speculated to result from induction of tolerance via molecular mimicry, since the *igh* locus contains a sequence matching a peptide sequence from viral gene UL6 (Zhao et al., 1998). However, later studies failed to formally prove that hypothesis (Deshpande et al., 2001a; Deshpande et al., 2001b; Ellison et al., 2003). Therefore, the mechanism behind *igh* locus mediated resistance remains unresolved.

Hrl locus was shown to confer resistance as defined by survival rate following lethal challenge, as well as contribute to the control of HSV-1 replication in the eye and the TG (Lundberg et al., 2003). In addition, this study pointed to a potential sex-linked resistance to HSV-1, where *Hrl* in combination with another locus, *Smn* (sex modifier locus), functions to increase resistance of female mice. The mechanism by which *Hrl* locus confers resistance was not identified in this study, but it was suggested to involve one or more TNF receptor family members encoded within the locus.

More clear were the results of the analysis of the type of resistance conferred by the *Rhs* locus, which point towards the importance of early innate responses as the resistance-mediating factors (Pereira et al., 2001). The *Rhs* locus contributed to efficient control of primary viral replication, and this was implied to be mediated by $NK1.1^+$ cells by treating the resistant mice with mAb against NK1.1. While suggestive, this experiment does not exclude the possibility that the cells eliminated by mAb treatment

were not only NK cells, but also activated $CD8^+$ T cells, known to upregulate NK1.1. This was one of many studies underlying the importance of innate responses to control of HSV-1 primary replication, spread, and disease severity.

Overview of innate immune response

The innate immune response is induced very early after infection, before activation of the adaptive immunity, and involves production of type I and type II interferons and other inflammatory cytokines by infected cells, and functions of NK cells, γδ T cells and complement.

Early interferon α/β production was shown to contribute to the increased HSV-1 resistance of B6 mice (Halford et al., 2004). The IFN limited viral replication and spread. Later studies demonstrated that plasmacytoid dendritic cells (Bancroft and Kelly, 1994) and infected macrophages (Malmgaard et al., 2004) are the main cell types making type I IFN early following HSV-1 infection. Production of type I interferons by dendritic cells and macrophages early in the infection is stimulated by signaling through pattern recognition receptors (PRRs), which recognize pathogen determinants termed pathogen-associated molecular patterns (PAMPs). For example, mannose receptor on DCs binds gD of HSV-1, stimulating IFNα production (Milone and Fitzgerald-Bocarsly, 1998). Another example of PRRs is the TLR (toll-like receptor) family, which uses the receptor (TLR) and adaptor molecules (MyD88 or TIR, depending on the TLR), to recognize a wide variety of pathogens. TLR2 and TLR9 are involved in HSV recognition and induction of IFNα/β, as well as proinflammatory RANTES and TNFα production (Malmgaard et al., 2004; Morrison, 2004).

Another important cytokine contributing to control of the virus by the innate immune system is IFNγ. During HSV-1 infection IFNγ enhances the effects of type I IFN, as demonstrated by experiments in mice with targeted mutations abrogating the function of IFNαβR, IFNγR, or both receptors (Vollstedt et al., 2004). IFNαβR$^-$ mice were more susceptible to death from localized ocular or footpad HSV-1 infection than C57BL/6 mice (30% versus 100% survival), which correlated with greater viral titers and systemic spread. The survival of these mice was further compromised by treatment with anti-IL-12 while abrogation of IL-12 activity in wild type mice had no effect on survival, implying that IL-12 contributes to, but is not an essential component, of innate immune response to HSV-1. The survival and viral titers of IFNγR$^-$ mice were the same as in wild type mice, implying that IFNγ production by the innate or the adaptive immune cells is not essential for control of initial viral infection (Ghiasi et al., 2000b). Finally, lack of both type I and type II IFN receptors was fatal (100% mortality), demonstrating the importance of type I interferons as well as their coordinated action with IFNγ to confer antiviral control (Vollstedt et al., 2004). These results were corroborated in another independent study (Luker et al., 2003).

Prior to activation of CD8$^+$ T cells, IFNγ is secreted by macrophages and can also be made by activated NK cells. The literature is divided on the issue of NK cell involvement in anti-HSV protection. Many studies indicate that NK cell responses do not play an important part in mediating antiviral protection, even in C57BL/6 mice, despite the fact that one of the resistance loci, *Rhs*, is NK complex-linked. Halford et al. (Halford et al., 2004) compared viral titers and viral spread in SCID mice that were either untreated or depleted of NK cells. Since SCID mice are deficient in T lymphocytes, the

mAb treatment only have affected NK cells. The experiments showed no differences in viral titers or survival between control and depleted animals, making the contribution of NK cells to the anti-HSV innate response unlikely. Of note, all mice in the experiment died within 14 days post-infection, providing evidence for the importance of the adaptive immunity in controlling primary HSV-1 infection.

To counter that finding, other groups demonstrate that NK cells may play a role in viral control, however not an essential one. In an intranasal HSV-1 infection model, Reading and colleagues (Reading et al., 2006) show that NK cells get activated and recruited to the lung, and in their absence the viral titers in the lungs increase, however the time to clear the virus nor the survival of the mice is affected. In another study (Barr et al., 2007), intravenous (i.v.) injection of HSV-1 resulted in activation of NK cells to produce IFNγ in spleen, peaking at 6 hours p.i. This activation was dependent on IL-18 but not IL-12. The authors went on to identify plasmacytoid DCs as the source of IL-18 required to activate NK cells. However, the activation of NK cells was not correlated with its effect on control of viral replication. Therefore, the exact role of NK cells in anti-HSV response remains unresolved.

Unlike the role of NK cells in controlling HSV-1 infection, the role of other innate IFNγ producers, the macrophages and γδ-T cells, is clear. They infiltrate the infected sensory ganglia and are crucial in controlling the extent of viral replication in the peripheral nervous system early in the infection, prior to arrival of αβ-T cells which are also involved in controlling viral replication (section 1.6) (Kodukula et al., 1999; Liu et al., 1996; Sciammas et al., 1997). In addition to IFNγ, the activated macrophages produce TNFα and nitric oxide (NO), both of which have antiviral activity.

Finally, complement also can play a role in innate immunity against HSV-1, however HSV-1 developed a strategy to evade it. Glycoprotein gC of HSV-1 binds the C3b complement component, blocking activation of the complement cascade (Hook et al., 2006; Judson et al., 2003; Lin et al., 2004; Lubinski et al., 2002). This prevents complement-mediated lysis of infected cells, and complement-mediated neutralization of cell-free virus. Even though HSV-1 is not commonly cell-free during its life cycle (reviewed in section 1.4), the inactivation of complement cascade by gC contributes to its virulence; infection with mutant viruses unable to bind C3b is more attenuated (Lubinski et al., 1999; Nagashunmugam et al., 1998), as is infection of gC-immunized mice, in which the antibodies block viral gC and prevent complement binding (Judson et al., 2003) . Interfering with complement cascade is one of the strategies developed by the virus to evade the immune system.

In summary, the innate response provides significant protection against HSV-1 infection. While some components of the innate immune system, including NK cells and γδ T cells have more mild effects and contribute to alleviation of symptoms or reduction of viral titers, other components, primarily type I IFN production by DCs and macrophages, are critical to viral control and host survival. The role of innate responses is either directly antiviral or indirect, leading to activation of the adaptive immune response, which in turn is critical for a positive outcome following infection.

Overview of adaptive immune response to HSV-1

The adaptive immune response plays a key role in limiting the initial infection and responding to reactivation. Infection with HSV-1 leads to development of antibody,

$CD4^+$ and $CD8^+$ T cell responses (Koelle and Corey, 2003). However, the control of viral replication both during primary and recurrent infection is mediated primarily by the T cells, and not by the antibody. The induction of antibody response extends beyond the phase of infection where cell-free virus can be neutralized, which is most likely why antibody does not play a crucial part mediating protection against infection. Adoptive transfer of sera from immune mice containing HSV-specific IgG and IgA antibodies did not decrease the extent of viral replication in the genital tract (McDermott et al., 1990; Parr and Parr, 1997), although some studies demonstrated that the onset of disease was delayed (Eis-Hubinger et al., 1993; Parr and Parr, 2000). Importantly, both corneal and footpad infection models demonstrated that transfer of immune sera prior to infection does not limit the extent of viral replication and establishment of latency in the sensory ganglia (rev. in (Morrison et al., 2001; Openshaw et al., 1979).

Morrison et al. (Morrison et al., 2001) compared the viral titers, disease severity and survival of genitally infected SCID mice (lacking T and B lymphocytes) and μMT mice (lacking B lymphocytes) with and without transfer of titrated amounts of immune sera prior to infection. Infection of SCID mice was lethal and produced the same viral titers on day 6 p.i. regardless of presence of immune sera. However, serum-treated mice survived longer (13-15 days, whereas the untreated controls died by day 8 p.i.) and had lower disease scores. Both effects were serum dose-dependent. Transfer of immune sera into B-cell deficient mice also did not lower viral titers, but similarly to SCID mice alleviated the disease severity. Interestingly, whereas serum-treated B cell deficient mice survived the challenge, only 50% of control mice survived. This study outlines the importance of T cell responses to protection against HSV-1, and suggests that B cell

immunity, while not crucial to virus control contributes to protective effects of antiviral T cells.

Multiple studies in mice lacking both $CD4^+$ and $CD8^+$ T lymphocytes (SCID mice, $Rag^{-/-}$ mice, antibody depletion studies) demonstrated that the T cell response is crucial in viral control during primary infection (rev. in (Koelle and Corey, 2003)). However, the field still remains divided on the exact contribution of each T cell subset in mediating this effect. While some found that lack of either $CD8^+$ or $CD4^+$ T cells has deleterious consequences (Ghiasi et al., 1999b), others demonstrated that only $CD4^+$ T cells (Manickan and Rouse, 1995; Morrison and Knipe, 1997) play a critical role controlling viral replication and host survival. Contrary to that, multiple studies demonstrated the role for $CD8^+$ T cells in viral clearance and host survival (Banerjee et al., 2004; Ellison et al., 2000a; Ghiasi et al., 2000a; Ghiasi et al., 1999b; Lang and Nikolich-Zugich, 2005; Simmons and Tscharke, 1992; van Lint et al., 2004). The role of $CD8^+$ T cells in controlling primary HSV-1 infection was further strengthened by studies performed in mice lacking molecules involved in cytotoxicity, namely perforin (Chang et al., 2000; Ghiasi et al., 1999a), granzyme A (Pereira et al., 2000), and lymphotoxin α (Kumaraguru et al., 2001).

Balance between immune protection and immune pathology

In addition to providing antiviral protection, both $CD8^+$ and $CD4^+$ T cells have been demonstrated to cause pathology in some HSV-1 infection models. This included both HSV-specific and bystander activated T cells.

HSV-1 infection of cornea leads to development of herpetic stromal keratitis (HSK, rev. in (Streilein et al., 1997)). This disease develops only in immunocompetent mice despite clearance of replicating virus from the eye and is primarily driven by $CD4^+$ T cells migrating into the cornea, however contribution of $CD8^+$ T cells to the disease has also been demonstrated (Banerjee et al., 2004; Lepisto et al., 2006). Interestingly, both $CD4^+$ and $CD8^+$ T cells found in the lesions were primarily non-HSV specific. This was demonstrated in a series of elegant experiments using infection of TCR transgenic (Tg) mice on $Rag^{-/-}$ or SCID background, ensuring that they only expressed T cells of desired specificity. In one set of experiments (Banerjee et al., 2005) gBT-I TCR Tg mice expressing $CD8^+$ T cells specific for the dominant viral epitope were shown to develop HSK despite their ability to clear the virus from the eye. The lesions in these mice contained mostly $CD4^+$ T cells and few if any virus-specific $CD8^+$ T cells, demonstrating that virus-responding CTLs are not required for HSK development. Similarly, OT-I TCR Tg mice expressing $CD8^+$ T cells specific for non-HSV epitope, also developed HSK (Banerjee et al., 2004), supporting the finding of the above mentioned study. Infection of these mice was lethal, but death was prevented by transfer of HSV-immune $CD8^+$ T cells. While the transferred cells provided protection, they were not evident in the lesions. In another study, DO11.10 TCR Tg mice specific for non-HSV CD4 epitope (OVA) were demonstrated to develop HSK, and the OVA-specific $CD4^+$ T cells were present in the lesions (Deshpande et al., 2001a). However, this infection was also lethal. Therefore, following ocular infection virus-specific $CD8^+$ and $CD4^+$ T cells play a protective role while bystander $CD8^+$ and $CD4^+$ T cells cause pathology.

Another site where the balance between mediating protection and disease by activated T cells following HSV-1 infection can be difficult to accomplish is brain. Multiple studies demonstrated the requirement for adaptive T cell response to prevent uncontrolled viral replication in the brain, leading to encephalitis (Deshpande et al., 2001a; Ghiasi et al., 2000a; Ghiasi et al., 1999b; Lang and Nikolich-Zugich, 2005; Nash et al., 1987; van Lint et al., 2004). However, excessive infiltration of inflammatory lymphocytes into the brain can exacerbate the disease (Anglen et al., 2003).

1.6 $CD8^+$ T CELL RESPONSE TO HSV-1 IN C57BL/6 MICE

The above summary of literature demonstrates that, depending on the model used, both innate and adaptive immune responses contribute protection against HSV-1 infection. However, regardless of these defenses, the virus is capable of establishing a lifelong infection in neurons. Since the entire work presented in this thesis has been done in C57BL/6 (B6) mice, the $CD8^+$ T cell response to HSV-1 in this mouse strain is reviewed below in greater detail.

The response to the immunodominant epitope

In B6 mice, almost entire $CD8^+$ T cell response is directed towards a single epitope $gB_{498\text{-}505}$ (SSIEFARL) derived from viral surface glycoprotein B (gB-8p in the text) presented in the context of MHC I molecule $H\text{-}2K^b$ (Hanke et al., 1991; Wallace et al., 1999). During primary infection, 70-90% of activated HSV-1 specific $CD8^+$ T cells recognize this peptide, as demonstrated by comparison of IFNγ responses of HSV-

immune CTLs stimulated by APCs infected with wt virus of with gB mutant lacking the epitope (Wallace et al., 1999). The remaining CTLs react with the subdominant K^b-restricted peptide $RR_{822-829}$ derived from viral ribonuclease reductase. The RR –specific CTLs are present in very low frequencies, constituting less than 10% of the total antiviral response and less that 0.5% of total $CD8^+$ T cells. Therefore the response to gB-8p can be used to study the entire anti-HSV response in B6 mice, as has been the case in the majority of studies done since the identification of the gB-8p epitope.

The initial response to gB-8p is dominated by two TCR Vβ families, Vβ8 and Vβ10, which make up 20% and 60% of the gB-8p specific CTLs, respectively. Despite the strong bias in TCR Vβ family usage, the overall response is diverse, owing to great heterogeneity of TCR Vβ sequences (Cose et al., 1995). The diversity of the memory $CD8^+$ T cell response has not been analyzed with respect to its Vβ usage and clonotypic diversity.

Rapid activation of gB-specific CTLs in the cutanous infection model

Activation of gB-specific CTLs has been analyzed in the footpad and flank scarification models of infection (Mueller et al., 2002b; Stock et al., 2004). In those models, activation was demonstrated to take place in the popliteal LNs, which serve as the draining LN in that infection. gB-8p-reactive CTLs were found in the LNs as early as 4-6 hours post-infection (Mueller et al., 2002b; Stock et al., 2004). This was surprising, as gB is a late gene and as such its production would not be anticipated to take place this early in the infection. *In vitro* experiments analyzing the kinetics of activation of gB-specific CTLs by infected APCs demonstrated that presentation of gB takes place as early

as 2 hours post-infection, depends on *de novo* gB synthesis since it was not observed following infection with UV-inactivated virus, and can be blocked by brefeldin A treatment, demonstrating that CTL activation requires the MHC presentation pathway (Mueller et al., 2003). In addition, the kinetics of priming of gB-specific $CD8^+$ T cells *in vitro* were the same as those of CTLs specific for the RR epitope, derived from an early viral gene. The study suggested the still controversial possibility that the expression of late genes, or at least gB, may take place sooner and out of the hierarchical gene expression sequence, creating a new category of "leaky late" genes. While the data on CTL activation are convincing, the expression of gB was not directly demonstrated in these experiments, and the classification of gB as an early, late, or "leaky-late" gene remains controversial.

Activation of gB-specific $CD8^+$ T cells was not correlated with presence of infectious virus in the LNs (Mueller et al., 2002b), implying that priming required recruitment of Ag-loaded APCs into the LN. Further studies shed some light on the way in which Ag reaches naïve $CD8^+$ T cells. First, different DC populations from the draining LNs of infected mice were isolated following footpad infection and tested for their ability to activate gB-specific hybridoma or gB-specific transgenic $CD8^+$ T cells. Only one DC subset, $CD11c^+$ $CD8\alpha^+$ $CD45RA^-$ DCs, was capable of stimulating T cell activation (Smith et al., 2003). Another study demonstrated that Langerhans cells, a specialized skin-resident DC type, do not participate in Ag presentation (Allan et al., 2003). The authors constructed bone marrow chimeras in which the radiation-resistant Langerhans cells were of recipient's origin, whereas all other bone-marrow derived DC populations were of donor origin. Bone marrow from bm1 mice, which have a mutation

in K^b preventing presentation of the gB-8p epitope, was used to reconstitute the recipients. By using these chimeric mice, the authors demonstrated that CTL priming did not take place when the only cells capable of presenting the Ag were Langerhans cells, despite their ability to migrate to the draining LN. It is still unclear how the Ag reaches the site of CTL priming, however it is known that 1) actively replicating virus is absent from the site of priming, 2) Langerhans cells migrating from the periphery do not present the antigen to $CD8^+$ T cells, and 3) Ag presentation is performed by $CD8\alpha^+$ DCs in the LN. Since Langerhans cells do migrate into the LN from the site of infection, it is possible that they aid in transport of Ag to the $CD8\alpha^+$ DCs for CTL priming.

While the events of anti-HSV response generation have been described in detail for the footpad and flank scarification model, it is possible that the kinetics of CTL priming are different depending on the infection route. Since priming of CTLs is not dependent on presence of infectious virus in the draining LNs, but on the arrival of Ag, the mode of Ag arrival in the draining LN may be dependent on accessibility of the cells migrating from the site of active infection. For example, it remains unknown how the Ag reaches the draining LN following infection of the cornea, although it can be expected that drainage of Ag or Ag-loaded APCs is restricted, considering the reduced vascularity of that site as compared to skin.

Role of CD4 help in generation and maintenance of gB-8p specific CD8 response

Jennings et al. (Jennings et al., 1991) used chronic CD4 depletion approach to demonstrate that generation of primary $CD8^+$ T cell response, but not maintenance of memory response following HSV-1 infection is dependent on CD4 help. The

mechanisms of the help provided by $CD4^+$ T cells was revisited by Smith et al. (Smith et al., 2004). The data reveal that DCs must be "licensed" by HSV-specific $CD4^+$ T cells before they can act as APCs for $CD8^+$ T cell priming. The licensing involves p:MHC recognition by $CD4^+$ and $CD8^+$ T cells on the same APC (termed cognate recognition). This was demonstrated *in vivo* by infection of mixed bone marrow chimeras in which the donor bone marrow came either from MHCII-deficient animals (unable to present Ag to $CD4^+$ T cells), or from bm1 animals (unable to present the gB-8p to $CD8^+$ T cells). Generation of $CD8^+$ T cell response in mice where cognate recognition was not possible was severely impaired. DC licensing was also shown to be important for generation (but not maintenance) of long-lived memory. The same number of gB-8p-specific CTLs generated *in vitro* with or without cognate CD4 help were transferred into naïve congenic recipients, which were then challenged. Only CTLs generated in presence of CD4 help generated a detectable memory response.

Role of T_{regs}

Work from the Rouse laboratory examined contribution of naturally occurring $CD4^+$ $CD25^+$ regulatory T cells (T_{regs}) to the control of $CD8^+$ T cell response to HSV-1 in B6 mice (Suvas et al., 2004; Suvas et al., 2003). Mice were depleted of $CD25^+$ cells prior to footpad infection with HSV-1. The depleted animals had and enhanced $CD8^+$ T cell response compared to control mice, as measured by their ex-vivo Ag-driven proliferation, and the size of the *in vivo* response measured by tetramer staining and ex-vivo Ag-specific IFNγ production. The enhanced CTL response correlated with earlier clearance of replicating virus from the site of infection. To control for the possibility that depletion

of $CD25^+$ cells eliminated other cells important to generation of CTL responses, such as activated T cells, the authors compared CTL responses in B6 mice that received an adoptive transfer of sorted $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ T cells. The results confirmed the earlier finding, demonstrating that the size of $CD8^+$ T cell response was decreased in presence of additional T_{regs}. Both the size of primary and the memory response were decreased compared to control mice (Suvas et al., 2003).

Using the same methods (depletion or experiments with sorted $CD4^+$ $CD25^+$ T cells), the same group later demonstrated that T_{regs} control the severity of viral immunoinflammatory lesions following ocular infection (Suvas et al., 2004). T_{regs} were present in the lesions, and their depletion prior to infection correlated with increased influx of activated $CD4^+$ T cells, known mediators of HSK, into the cornea. T_{regs} isolated from HSK^+ corneas produced IL10 upon TCR stimulation with anti-CD3, and were able to suppress in vitro proliferation of $CD4^+$ $CD25^-$ T cells via a mechanisms involving IL-10, as demonstrated by Ab blocking experiments. Involvement of T_{reg}-produced IL-10 in curtailing $CD8^+$ T cell responses following HSV-1 infection in B6 mice has not been addressed so far. However, another secreted factor that may be involved in this process is TGFβ, demonstrated to mediate repressive effects of T_{regs} in other infection models and suggested to modulate the lytic activity of $CD8^+$ T cells during HSV-1 infection (Suvas et al., 2006).

Control of lytic viral replication and spread

Despite the still on-going debate on the involvement of $CD8^+$ T cells in restricting viral replication and spread during primary infection (rev. in section 1.5), their role in

doing so in B6 mice is quite clear (Banerjee et al., 2004; Lang and Nikolich-Zugich, 2005; van Lint et al., 2004). Recent experiments, including work presented in this thesis, addressed the exact mechanisms by which this control is accomplished. It appears that $CD8^+$ T cell responses do not restrict viral spread from the infection site, however they are involved in clearing viral replication at the infected sites, particularly the nervous system ((Lang and Nikolich-Zugich, 2005; van Lint et al., 2004)and Chapter 2 of this dissertation).

Ongoing viral surveillance during latency

Despite activation of innate and adaptive immune response, infection with HSV-1 results in establishment of latency in the neurons of peripheral and central nervous system. This latent infection is associated with ongoing inflammation and corresponding continued presence of inflammatory cell infiltrates, including $CD8^+$ T cells, at the site of latency (Liu et al., 1996). The involvement of these $CD8^+$ T cells in controlling the viral reactivation has been under intense scrutiny over the last several years.

The majority of studies assessing involvement of $CD8^+$ T cells in controlling viral reactivation were done in the ocular infection model where the latency is established in the sensory trigeminal ganglia (TG) (Carr et al., 2001). Early studies demonstrated infiltration of $CD8^+$ T cells into the TG within 5 days p.i. and their continued presence thereafter, albeit in decreased frequency (Liu et al., 1996). These observations were refined following the development of peptide:MHC tetramers and after generation of CD8 TCR transgenic mice specific for the gB-8p epitope (Mueller et al., 2002a). These new tools allowed the discovery that the majority of $CD8^+$ T cells found in the acutely or

latently infected TG are gB-8p specific ((Khanna et al., 2003; Lang and Nikolich-Zugich, 2005; van Lint et al., 2004) and Chapter 2 of this dissertation).

Using TG explant reactivation model, the Hendricks group demonstrated in a series of studies that the gB-8p-specific $CD8^+$ T cells present in the TG are capable of blocking viral reactivation via production of IFNγ (Decman et al., 2005; Liu et al., 2001; Liu et al., 2000). The function of $CD8^+$ T cells in controlling viral reactivation from latency *in vivo* is more difficult to assess, but they are expected to play the same role. The specificity and activated phenotype of $CD8^+$ T cells present in latently infected ganglia is suggestive of their ongoing stimulation. *In situ* staining of latently infected TG sections revealed that gB-8p specific $CD8^+$ T cells surround neurons, with their TCRs polarized to the area in direct contact with the neurons, implying that the TCRs are engaged by their cognate p:MHC (Khanna et al., 2003). However, the neurons in these experiments were not co-stained for HSV antigens, making the results suggestive but not definitive. Another study demonstrated that the parenchymal cells surrounding the latently infected neurons, rather than the neurons themselves, engage the ganglion-resident $CD8^+$ T cells and are required for their retention in the sensory ganglia and maintenance of activated phenotype (van Lint et al., 2005).

An interesting feature of HSV-1 infection of the neurons is that it is not associated with cell death, neither during productive infection or the latent phase. Similarly, the $CD8^+$ T cell control of viral reactivation in TG explants was not associated with cell death *in vitro*, implying that it operates by a non-lytic mechanism (Liu et al., 2001; Liu et al., 2000). Secretion of IFNγ and inhibition of viral replication was one plausible mechanism of non-lytic viral control. $CD8^+$ T cells isolated from latently infected TGs

were capable IFNγ production upon Ag stimulation (Khanna et al., 2003), providing additional implication that the TG-resident memory $CD8^+$ T cells could control viral replication in the TG as they do in explant TG cultures. However, the $CD8^+$ T cells present in the latently infected neurons were shown to contain high level of pre-formed granzyme B (Lang and Nikolich-Zugich, 2005; van Lint et al., 2005), implying their activated phenotype and capability to be lytic.

Suvas et al. investigated this issue further and demonstrated a mechanism by which cytolysis of infected neurons is prevented in the latent TG (Suvas et al., 2006). The gB-8p-specific $CD8^+$ T cells expressed NK-inhibitory receptor CD94-NKG2a complex, and their cytolytic activity was blocked by the receptor's ligand Qa1. Qa1 expression was demonstrated on neurons in the TG by flow cytometry. The authors further demonstrated that blocking the Qa1:CD94-NKG2a interactions during ex-vivo TG explant cultures resulted in neuronal lysis. The high expression of CD94-NKG2a by the $CD8^+$ T cells was dependent on presence of TGF-β, and expression of TGFβ in the TG was confirmed by an ELISA. TGFβ is likely provided by regulatory $FoxP3^+$ $CD4^+$ $CD25^+$ T cells, which were also present in the latent TG.

1.7 IMMUNOSENESCENCE

Immune aging

Aging of the immune system involves a complex set of changes that are collectively termed immune senescence (rev. by (Cambier, 2005; Linton and Dorshkind,

2004; Miller, 1996)). These changes affect both the innate and adaptive immune system, resulting in still incompletely understood state of immune deficiency. Key manifestations of immune senescence include poor responsiveness to vaccination and increased susceptibility to infection (rev. in (Miller, 1996; Nikolich-Zugich, 2005; Wick et al., 2000). This results in part from defects in generating B and T cell responses (Ershler et al., 1994; Ginaldi et al., 1999a; Ginaldi et al., 1999b; Haynes et al., 2000), which in turn can be attributed to age-associated defects in signaling, proliferation and cytokine secretion upon antigenic challenge (Garcia and Miller, 2001; Haynes et al., 1999). Additional age-associated defects in generating immune response relate to impaired Ag processing and presentation (Pamer, 1999) and changes in the cytokine environment (Haynes et al., 1999). The latter can influence the maintenance of the T cell compartment in addition to affecting primary responses to pathogens (Ku et al., 2000).

Overall, the aging immune system exhibits two different types of defects, relating to 1) cell-intrinsic changes, and 2) change in cell population dynamics. The cell-intrinsic defects are a result of accumulation of mutations, and changes in gene expression and signaling. These can be caused by DNA damage, oxidative stress, reduced repair and/or removal of damaged molecules and reduced de novo biosynthesis (Campisi, 2001; Campisi et al., 2001; Hekimi and Guarente, 2003; Masoro, 2001). As a result of these defects, cells can have altered responses to various stimuli, such as antigenic stimulation or homeostatic cytokines in case of T cells. However, cellular aging affects not only T cells, but also all other cells in an organism. In contrast, the age-related changes in maintaining cell population dynamics affect predominantly lymphocytes. This is due to the fact that lymphocytes have relatively high homeostatic turnover (Surh et al., 2006)

and in addition to that they frequently undergo rounds of antigen-driven expansion, followed by contraction and addition of new cells to the memory pool.

T cell senescence and its manifestations

While immune senescence affects many aspects of innate and adaptive immunity, defects in T cell immunity are the best documented and appear to be the most profound (rev. in (Linton and Dorshkind, 2004; Miller, 1996; Nikolich-Zugich, 2005)). What follows is that restoration of T cell population balance and numbers often correlates with improved response to antigenic challenge (Effros et al., 1991; Haynes et al., 2003; Haynes et al., 2005). As described above, age-related changes in T cell immunity fall in two categories. One set of changes occurs on a cellular level, with the cell-autonomous defects in T cell responses to signaling via the T cell receptor (TCR) and costimulatory receptor pathways (rev. in (Miller et al., 2005; Miller et al., 1997)). The second set of changes involves disruption in T cell population balance, which is only beginning to be understood and is one of the topics of this thesis. That disruption is marked by 1) involution of the thymus; 2) decline of naïve T cell numbers; 3) reduction in T cell repertoire diversity; and 4) memory T cell domination (Cambier, 2005; Linton and Dorshkind, 2004). In humans memory domination is associated with accumulation of T cells specific for persisting pathogens (memory inflation), often in the form of expanded clones of pathogen-specific $CD8^+$ T cells (Pawelec et al., 2005). The above phenomena result in decrease in the diversity of the T cell pool and availability of naïve T cells, which is crucial for protection from novel pathogens.

Disruption of T cell homeostasis in aging

Thymic involution begins almost immediately after birth in humans and soon after puberty in mice (Linton and Dorshkind, 2004), and results in decreased RTE output. For example, 22-mo-old mice receive less than 10% of RTE compared to young adult mice (Hale et al., 2006; Heng et al., 2005). Even in old age the thymus continues to produce RTE proportionally to its overall cellularity, but as the cellularity itself decreases, so does the output. The cause of thymic involution is not known at present. Thymic involution presents a challenge for the homeostatic mechanisms, which strive to maintain the size and diversity of the peripheral T cell pool in the face of decreased influx of diverse new T cells. Despite the fact that thymus involution begins early in life, it is only in old age that homeostatic mechanisms begin to malfunction.

A marked difference between the adult and old lymphocyte compartment is an age-related decrease in representation of naïve phenotype T cells and concomitant increase in frequency and numbers of memory phenotype T cells (Linton and Dorshkind, 2004; Pawelec et al., 2004). The exact mechanisms leading to this population shift are not understood, but likely involve a combination of 1) attrition of naïve T cells, 2) their conversion into effector or memory cells as a result of encounters with pathogens, and 3) changes in the environment, such as availability of homeostatic cytokines (IL-7, IL-15, IL-2). For example IL-2 production by $CD4^+$ T cells is decreased in old mice (Haynes et al., 2005; Haynes et al., 2004; Linton and Dorshkind, 2004). Less is known about age-related changes in IL-7 or IL-15 levels (Andrew and Aspinall, 2002; El Kassar et al., 2004; Fry and Mackall, 2005; Fry et al., 2003; Kim et al., 2006) or the expression and function of their receptors on different T cell subsets (Kang et al., 2004; Messaoudi et al.,

2006c). In addition, the naïve T cell pool could be indirectly affected by a growing pool of memory T cells that may compete with naïve T cells. Considering that there is some overlap in the use of survival and maintenance cytokines by these two pools, particularly in case of IL-7 (Fry and Mackall, 2001; Picker et al., 2006; Surh et al., 2006), it is possible that the two are not always independently regulated. Conversely, if naïve T cells continue to decrease in number, this may lead to an excess of survival and maintenance cytokines which normally would have been consumed by naïve T cells. This could trigger homeostatic proliferative expansion (HPE) of the remaining naïve T cells and drive their conversion to memory-phenotype. This was demonstrated in mice under lymphopenic conditions (Tanchot et al., 2001).

Overall, in an aging T cell compartment there is a dramatic increase in representation of memory cells, accompanied by a concomitant and equivalent decrease in representation of circulating naïve T cells. The exact mechanisms leading to this population shift are unknown, but are in part a result of thymic involution, lifelong encounters with pathogens, and less-well defined age-associated changes in cytokine environment.

Impact of persistent infections on T cell senescence

While they are not the only factor leading to loss of naïve T cells over lifetime, pathogens are the obvious culprits behind this process. Infection with an acute pathogen results in generation of effector T cell response, which contracts following resolution of infection. As a result, a small number of pathogen-specific cells enters the memory pool following most acute infections. Multiple lifelong encounters with acute pathogens result

in generation of diverse memory T cell pool, which is then regulated by homeostatic mechanisms which ensure that memory cells of any given specificity do not usurp too much space in the compartment. However, maintenance of memory T cells can be influenced by persistent pathogens which are capable of providing ongoing stimulation to their specific memory T cells. Under such conditions, the homeostatic mechanisms often are not able to control ongoing expansion of memory T cells specific for the persistent pathogen (memory inflation), resulting in their disproportionately large frequency within the T cell pool.

Herpesviruses, particularly CMV, are well known for causing memory inflation (rev. in (Pawelec et al., 2004; Pawelec et al., 2005). $CD8^+$ T cells are the main subset affected, however accumulation of expansions of virus-specific $CD4^+$ T cells was also reported (Fletcher et al., 2005). Large expansions of CMV-specific $CD8^+$ T cells were found both in mice (following MCMV infection, see (Holtappels et al., 2000; Holtappels et al., 2002; Karrer et al., 2003; Karrer et al., 2004)) and humans (Gillespie et al., 2000; Khan et al., 2002a; Pawelec et al., 2005; Weekes et al., 1999; Wikby et al., 2002; Wills et al., 1999). Importantly, CMV seropositivity has been correlated with a number of immunosenescence-associated phenomena. These included restriction of the TCR repertoire diversity due to accumulation of large virus-specific CD8+ T cell expansions and development of an immune risk phenotype (IRP), which predicted mortality within 2 years (Ferguson et al., 1995). The IRP described a number of characteristics of T lymphocyte pool and was reminiscent of old age-associated characteristics of that compartment. Specifically, both in aged and in IRP^+ individuals there was accumulation of effector memory phenotype $CD28^- CD8^+$ T cells, decline in $CD4^+$ T cells, and overall

decline in number of naïve T cells (Ferguson et al., 1995; Weekes et al., 1999). These findings in combination with the known high prevalence of CMV in the population, reported to be 60-90% worldwide (Akbar and Fletcher, 2005)), led to the hypothesis that CMV drives the age-associated changes in the T cell pool composition and phenotype (Pawelec et al., 2005).

In addition to driving virus-specific expansion of $CD8^+$ and $CD4^+$ T cells, persistent HCMV has also been suggested to drive bystander proliferation of CMV-non-specific T cells (Akbar and Fletcher, 2005). Viral infections are associated with production of inflammatory cytokines by infected cells, and it is expected that persistent infections lead to continued presence of these cytokines. In partial support of that hypothesis, Type I IFN was demonstrated to drive both $CD8^+$ (Borthwick et al., 2000) and $CD4^+$ (Fletcher et al., 2005) human T cell conversion to $CD28^-$ phenotype, and stimulation of plasmacytoid dendritic cells (pDCs) with CMV lysates induced IFNα production by these cells (Fletcher et al., 2005). However, the exact role of inflammatory cytokines in driving both CMV-specific and bystander $CD8^+$ and $CD4^+$ T cell proliferation in vivo is not known.

While the data from CMV-seropositive humans is suggestive of the role of CMV in driving the old-age associated changes in T cell composition, phenotype and diversity, this prediction remains to be experimentally tested. Studies in humans are restricted, due to ethical constrictions as well as difficulty in obtaining a true "negative control", given prevalence of CMV and other persistent herpesviruses (i.e. HSV-1, Epstein Barr Virus (EBV), and others). Only comparison of truly naïve and infected subjects will allow to distinguish the relative contribution of aging and persistent infections to the process of

immune aging. Mouse models of persistent infections, such MCMV, would be suitable to test the predictions of the role of persistent viruses on aging of the T cell compartment, since MCMV infection and the MCMV-specific $CD8^+$ T cell response closely mimics that seen in humans(Karrer et al., 2003). Specifically, MCMV appears to provide ongoing stimulation to murine memory $CD8^+$ T cells over the course of lifelong infection, resulting in memory inflation akin to that described in CMV-seropositive humans. Additional advantage of using the mouse MCMV model is that the viral determinants recognized by $CD8^+$ T cells in B6 mice have been mapped (Munks et al., 2006b), allowing to clearly distinguish between virus-specific and bystander T cell proliferation.

1.6 GOALS OF THIS THESIS

Our knowledge of $CD8^+$ T cell biology is constantly increasing, but despite this many unanswered questions remain. In this thesis, two main aspects of $CD8^+$ T cell responses were analyzed. First, their classical role in antiviral protection was tested in the HSV-1 infection model, investigating how if at all these cells control viral replication, where do they migrate and whether or not they establish long-lived memory. The second aspect of the $CD8^+$ T cell response to HSV-1 investigated here was their lifelong maintenance as a population and how it is affected by the persisting virus and aging. Finally, the role of persisting pathogens on driving immunosenescence by affecting the diversity of $CD8^+$ T cell pool was analyzed in a mouse model of HSV-1 and MCMV infection.

CHAPTER 2

Development and migration of protective CD8$^+$ T cells into the nervous system following ocular HSV-1 infection

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ABSTRACT

After infection of epithelial surfaces, Herpes simplex virus-1 (HSV-1) elicits a multifaceted antiviral response that controls the virus and limits it to latency in sensory ganglia. That response encompasses the $CD8^+$ T cells, whose precise role(s) is still being defined; immune surveillance in the ganglia and the control of viral spread to the brain were proposed as the key roles. We tracked the kinetics of the $CD8^+$ T cell response across lymphoid and extralymphoid tissues after ocular infection. HSV-1-specific $CD8^+$ T cells first appeared in the draining (submandibular) lymph node on day 5 and were detectable in both nondraining lymphoid and extralymphoid tissues starting on day 6. However, although lymphoid organs contained both resting ($CD43^{lo}$ $CFSE^{hi}$) and virus-specific cells at different stages of proliferation and activation, extralymphoid sites (eye, trigeminal ganglion and brain) contained only activated cells that underwent more than eight proliferations ($CD43^{hi}$ $CFSE^{neg}$) and promptly secreted IFN-γ upon contact with viral antigens. Regardless of the state of activation, these cells appeared too late to prevent HSV-1 spread, which was seen in the eye (from day 1), trigeminal ganglia (from day 2) and the brain (from day 3) well before the onset of a detectable $CD8^+$ T cell response. However, $CD8^+$ T cells were critical in reducing viral replication starting on day 6 and for its abrogation between days 8 and 10; CD8-deficient animals failed to control the virus, exhibited persisting high viral titers in the brain after day 6 and died of viral encephalitis between days 7-12. Thus, $CD8^+$ T cells do not control HSV-1 spread from primary to tertiary tissues, but rather attack the virus in infected organs and control its replication *in situ*.

INTRODUCTION

Herpes Simplex Virus Type 1 (HSV-1) infection of scarified murine cornea results in retrograde transport of the virus via sensory neurons to the trigeminal ganglion (TG). Following multiplication in the TG, HSV-1 migrates back to the area of sensory innervation covered by the ganglion, and causes typical vesicular rash. HSV-1 often spreads from TG into the brain, also along the sensory nerves (Esiri, 1982; Esiri and Tomlinson, 1984; Lewandowski, 1997; Lewandowski et al., 2002; Liu et al., 2001; Matsubara and Atherton, 1997; Schmutzhard, 2001). The incidence of viral spread to the brain is believed to be enhanced by immunosuppression or stress (Anglen et al., 2003; Kastrukoff et al., 1981), and HSV-1 encephalitis has emerged as one of the serious complications in immunosuppressed individuals (Kleinschmidt-DeMasters and Gilden, 2001; Schiff and Rosenblum, 1998) In the course of this infection, HSV-1 is detected by the immune system, resulting in a multifaceted antiviral response that controls the acute infection and limits the virus to latency in the ganglia of the peripheral nervous system (PNS). In fact, following the initial viral replication and the outbreak of the vesicular rash, it is the antiviral T cells that usually limit and control viral replication (Ghiasi et al., 2000a; Ghiasi et al., 2000b; Lewandowski, 1997; Morrison and Knipe, 1997; Nash et al., 1987; Zhao et al., 1995). By contrast, in the absence of T cell immunity, uncontrolled viral replication typically leads to lethal encephalitis (Deshpande et al., 2002; Ghiasi et al., 2000a; Ghiasi et al., 1999b; Nash et al., 1987). Nevertheless, the exact role, basic biology, and mechanisms of antiviral action of various T cell subsets remain

incompletely understood, including the kinetics of priming, kinetics and parameters of migration into infected organs and interplay with the replicating virus.

$CD8^+$ T cells were implicated as potential mediators of acute viral control in the nervous system and of neuropathogenesis (Banerjee et al., 2002; Deshpande et al., 2001b; Deshpande et al., 2002; Ghiasi et al., 2000a; Ghiasi et al., 2000b; Ghiasi et al., 1999b; Nash et al., 1987), and also as sentinels that potentially control viral reactivation from latency in the TG *in situ* (Khanna et al., 2003; Liu et al., 2000; Liu et al., 1996; Sciammas et al., 1997). Such evidence was obtained using infection of animals treated with depleting antibodies (Ghiasi et al., 1999a; Ghiasi et al., 2000a; Nash et al., 1987) or studies in knockout mice lacking CD8α (Ellison et al., 2000a; Simmons and Tscharke, 1992), IFNγ (Bouley et al., 1995; Cantin et al., 1999; Ellison et al., 2000b; Geiger et al., 1997), or molecules that mediate cytotoxicity (Chang et al., 2000; Ghiasi et al., 1999a; Kumaraguru et al., 2001; Pereira et al., 2000; Simmons and Tscharke, 1992). Direct proof that the missing effector molecules act via CD8 T cells, however, was often not obtained in these studies, and results from some studies questioned an obligatory role of $CD8^+$ T cells in fighting HSV (Manickan and Rouse, 1995; Sciammas et al., 1997).

Perhaps more importantly, the kinetics of priming, proliferation and release of activated $CD8^+$ T cells into lymphoid and non-lymphoid organs in relation to the progression of viral replication following ocular infection has not been defined in this model, and such knowledge is critically important in order to dissect the precise role(s) of $CD8^+$ T cells in controlling the virus during primary infection, as well as in inducing potential immunopathogenesis in the CNS (Anglen et al., 2003). To address the above questions, we studied the kinetics and biology of priming and dissemination of antiviral

CTL, linking it to the kinetic distribution and the level of viral replication in different sites. We exploited the advantage of techniques allowing precise and definitive assessment of the specificity of antiviral $CD8^+$ T cells.

MATERIALS AND METHODS

Mice. Female C57BL/6-NCr (B6) and B6-Ly5.2/Cr (Ly-5.2) mice were purchased from the National Cancer Institute colony (NCI, Frederick, MD). Female $CD8\alpha^{-/-}$ mice were purchased from The Jackson Laboratory and propagated in the VGTI vivarium. Female B6.gBT-I (gBT-I in the text) TCR transgenic mice (Mueller et al., 2002a), carrying rearranged *tcr* genes encoding TCR that recognizes the immunodominant HSV-1 epitope $gB_{498\text{-}505}$ + H-$2K^b$, were generously provided by Dr. F. R. Carbone (University of Melbourne, Melbourne, Australia) and were propagated in the VGTI vivarium by backcrossing to B6 mice. All animals were housed under specific-pathogen free conditions and were used at 6-12 weeks of age.

Reagents, antibodies and flow cytofluorometric (FCM) analysis. The $gB_{498\text{-}505}$ peptide (SSIEFARL) was purchased from Research Genetics. $gB_{498\text{-}505}$:K^b tetramers were prepared in our laboratory according to standard published protocol (Altman et al., 1996).

The following mAb were used: anti-CD8α (clone 53-6.7), anti-CD45.2 (clone 104), anti-CD43 (clone 1B11), anti-CD62L (clone MEL-14), anti-IFNγ (all from BD Pharmingen); and anti-human granzyme B (clone GB12, Caltag Laboratories, Burlingame, CA), the last two for intracellular staining.

For FCM analysis, cells isolated from various organs were dispersed into single cell suspensions, stained on ice for 20' with predetermined optimal concentration of various fluorophore-conjugated mAb, washed and fixed in 2% paraformaldehyde. For IFNγ production assay, $4x10^6$ splenocytes were stimulated with 10^{-6} M $gB_{498-505}$ peptide for 6 hours in RPMI media supplemented with 10% FBS and 1.5 μg/ml brefeldin A. After surface staining the cells were fixed in 2% paraformaldehyde and permeabilized with PermWash (1mg/ml saponin, 1% FBS, 0.1% NaN_3 in PBS). Intracellular staining for IFNγ and granzyme B was then performed in PermWash on ice, exactly as described above. Analysis was performed on FACSCalibur instrument using CellQuest 3.3 software (Becton Dickinson, Mountain View, CA). At least 10^4 cells were analyzed per sample, with dead cells excluded by selective gating based on orthogonal and side light scatter characteristics.

Virus and ocular infection. HSV-1 strain 17 (passage 2, uncloned swarm) was obtained from Dr. D.J. McGeoch (University of Glasgow, Scotland, UK), was grown and titered on Vero cells, and was used in all experiments. Mice were anesthetized by i.p. injection of ketamine cocktail (100mg/ml ketamine, 20mg/ml xylazine, 10mg/ml acepromazine). The corneas were scarified in a crisscross fashion with a sterile 26-gauge needle, and 3μl of HBSS (Cellgro, Mediatech) containing $5x10^5$ PFU of HSV-1 was applied to the surface of each eye.

Adoptive transfer of transgenic T cells. Spleens of 6-12-week old female gBT-I transgenic mice were enriched for $CD8^+$ T cells by depletion of $CD4^+$ and $B220^+$ cells by immunomagnetic sorting (MACS, Miltenyi Biotech, per manufacturer's instructions). Magnetic depletion yielded a cell population that was >80% $CD8^+$, and 80-90% of $CD8^+$

cells stained with $gB_{498-505}:K^b$ tetramer. 1 $x10^6$ $tetramer^+$ cells were adoptively transferred into congenic B6-Ly5.2/Cr female recipients by i.v. injection 24 hours prior to infection. In some experiments, the cells were labeled with 2μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) prior to adoptive transfer (10' incubation at 37°C). The transferred cells were distinguished from the recipient cells by FCM detection of CD45.2 (mAb clone 104, BD Pharmingen); Vα2 (BD-Pharmingen) and/or fluorescein introduced by CFSE labeling.

Isolation of replicating virus and titer determination. At various times post-infection the eyeballs, TG, brains, spleens, lungs, livers and the draining lymph nodes were collected and placed in DMEM media (GibcoBRL) supplemented with antibiotics on ice. The organs were then homogenized using sterile glass homogenizer. The homogenate was centrifuged (800g, 4°C, 10'), the supernatant was frozen at -80°C and subsequently used to determine the titer of replicating virus in each organ in a standard plaque assay on confluent Vero cell monolayers. Briefly, serial 10-fold dilutions of the virus-containing supernatant were made in serum-free DMEM and added to confluent layers of Vero cells in 6-well plates (1 ml per well). After 1 hour incubation at 37°C ("infection") the wells were overlaid with 2 ml of 1.5% methylcellulose in DMEM supplemented with 1% FBS and incubated for 2 days at 37°C. The methylcellulose was then washed off with PBS, and the cell monolayers were fixed with 2% formaldehyde and stained with 0.1% crystal violet/PBS solution. The plaques were counted and the results were expressed as total plaque forming units (PFU) per organ. The limit of detection was 10 PFU/organ.

Isolation of lymphocytes from non-lymphoid organs. Mice were sacrificed, perfused with PBS (Cellgro, Mediatech) and their eyeballs, the TG and the brains excised. The eyeballs and the TG were incubated in RPMI (GibcoBRL) containing 3mg/ml collagenase type 1 (Sigma) for 1-2 hours, dispersed into single cell suspension by pipetting, and filtered through 40μm filter prior to use in assays. The brains were first dispersed into single cell suspensions by grinding over a metal screen. The cell suspensions were then layered over a 40%/60% Percoll gradient (Amersham Biosciences), centrifuged at 1200g for 30 minutes at room temperature, and the lymphocytes were isolated from the 40%/60% interphase.

RESULTS

Initiation of the HSV-1-specific $CD8^+$ T cell response

To follow the early events in the development of the HSV-1-specific $CD8^+$ T cell response, including proliferation and expression of activation-associated antigens, which are difficult to measure in the endogenous population due to low precursor frequency, we used an adoptive transfer approach. gBT-I TCR transgenic $CD8^+$ T cells (Mueller et al., 2002a) specific for the immunodominant HSV-1 glycoprotein B epitope, $gB_{498\text{-}505}$ (Bonneau et al., 1993; Hanke et al., 1991; Vasilakos and Michael, 1993; Wallace et al., 1999) , overwhelmingly of the naïve phenotype (>92% $CD62L^{hi}$, >95% $CD44^{lo}$, and >98% $CD43^{lo}$) were labeled with CFSE, transferred into syngeneic C57BL/6 (B6) mice. After ocular HSV-1 infection, their proliferation, frequency and surface phenotype were analyzed in lymphoid (spleen, submandibular lymph node, mediastinal lymph node) and

relevant nonlymphoid organs (including the eye, TG and brain) on days 1-12 post-infection.

Clearly detectable proliferation of virus-specific T cells was seen in all animals on day 5 in submandibular lymph node (Figure 2.1 A), albeit with some individual variation (15-40% of proliferating gBT-I $CD8^+$ T cells - Figure 2.1 B). On that day, no other organs contained proliferating gBT-I $CD8^+$ T cells, indicating that this lymph node likely served as the draining lymph node for the infected cornea.

Dissemination of activated HSV-1-specific $CD8^+$ T cells into lymphoid and non-lymphoid tissue

The frequency of gBT-I $CD8^+$ T cells increased from the background level on day 6 in both lymphoid and nonlymphoid organs, one day after proliferation was first detected in the draining lymph node (Figure 2.2). Within the limits of our sampling schedule (daily intervals up to day 6, every other day thereafter) the increase in frequency appeared to occur simultaneously in secondary lymphoid organs (spleen and non-draining mediastinal lymph node) and the extralymphoid organs (eye, TG, and brain). Overall, the activation, expansion and dissemination of virus-specific $CD8^+$ T cells took place in three stages: 1) up to day 5 post-infection, initial proliferation of small number of precursors in the draining lymph node; 2) on day 6, initial dissemination of antiviral $CD8^+$ T cells into both lymphoid and nonlymphoid organs, correlating with an increase in frequency of virus-specific $CD8^+$ T cells in those organs; and 3) days 8-12, further increase in frequency of virus-specific CD8 T cells in lymphoid organs and infected peripheral organs, and maintenance of steady, low frequency of these cells in the lymph nodes.

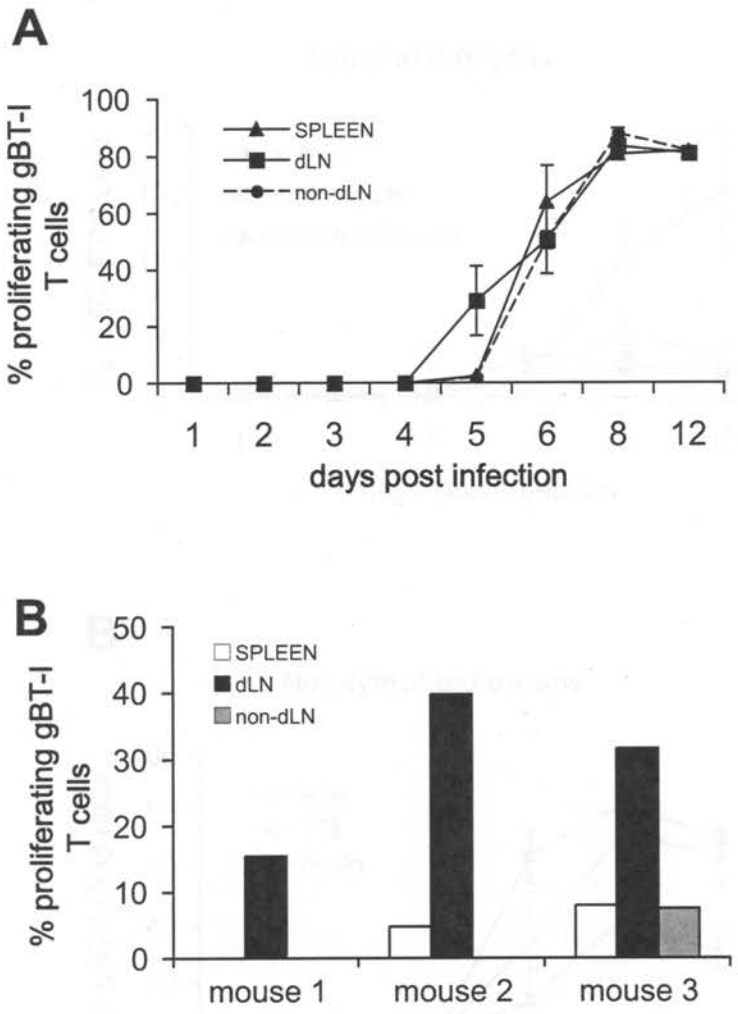


Figure 2.1 Activation of $gB_{498-505}$-specific $CD8^+$ T-cells in the submandibular (draining) lymph node. A. CD8-enriched splenocytes from gBT-I TCR transgenic mice specific for $gB_{498-505}$ were labeled with CFSE and adoptively transferred into congenic B6-Ly5.2/Cr mice 24 hours prior to ocular HSV-1 infection. Lymphocytes were isolated from spleen, the draining (submandibular) and non-draining (mediastinal) lymph nodes on days 1-6, 8, and 12 post-infection. The activation status of the transferred gBT-I $CD8^+$ T-cells was determined based on proliferation (CFSE dilution) of $CD8^+$ $CD45.2^+$ (transferred $CD8^+$ gBT-I) cells. Data are shown as the percantage of donor-derived $CD8^+$ T cells that have undergone one or more divisions (mean ± SD; n = 3) and are representative of three independent experiments. **B.** Data from individual animals providing an example of activation of gBT-I $CD8^+$ T-cells in the submandibular, but not mediastinal LN nor spleen, on day 5 post-infection in individual mice.

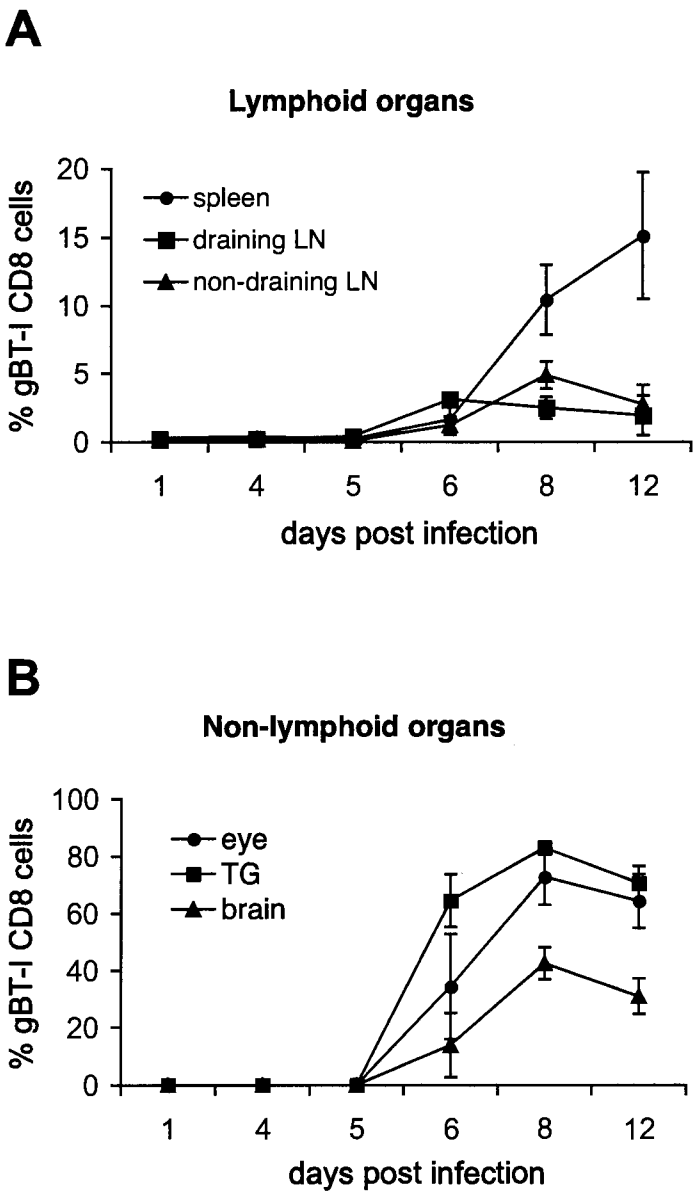


Figure 2.2 Kinetics of infiltration of activated $gB_{498-505}$-specific CD8+ T-cells into lymphoid (spleen) and selected extra-lymphoid organs (brain, eyes, TG). gBT-I $CD8^+$ T-cells were adoptively transferred into congenic recipients and animals infected as in Figure 2.1. The frequency of gBT-I $CD8^+$ T-cells among total $CD8^+$ T cells (host and donor) in lymphoid (left panel) and non-lymphoid (right panel) organs was determined on days 1-6, 8, and 12 post-infection by FCM, based on staining with anti-CD45.2 (marker for donor-derived cells) and anti-CD8. The values are shown as the mean ± SD (n=3) and are representative of two independent experiments.

Consistent with that pattern, on day 6, the relative frequency of $gB_{498-505}$-specific $CD8^+$ T cells was highest in the draining lymph node, but at subsequent time points this was no longer the case, as frequencies rose rapidly in other lymphoid and nonlymphoid organs.

To test whether the above kinetics mirror the kinetics of activation of endogenous $CD8^+$ T cells , a parallel experiment was done in which the kinetics of the endogenous anti-HSV-1 $CD8^+$ T cells response were determined in B6 mice by $gB_{498-505}$-K^b tetramer staining (not shown). The endogenous $CD8^+$ T cells followed the same kinetic pattern of dissemination into peripheral lymphoid and nonlymphoid organs as the transgenic cells, but the detection of these cells was delayed by 24-36h, as expected, due to lower precursor numbers of the latter. As in the transfer experiment, the frequency of virus-specific $CD8^+$ T cells continued to increase in the peripheral organs before reaching the peak on day 8-12, while the frequency remained stable and low in the draining lymph nodes.

Incompletely differentiated CTL are excluded from non-lymphoid organs

We next determined the extent of proliferation (CFSE dilution), activation and functional status of the gBT-I $CD8^+$ T cells across the tested organs. Differences were observed in the proliferation status and phenotype of gBT-I cells isolated from lymphoid and nonlymphoid organs on days 6-12 post-infection. In the lymphoid organs, the gBT-I $CD8^+$ T cells were either $CFSE^{hi}$ (have not started to proliferate), $CFSE^{int}$ (undergoing their first through seventh division) or $CFSE^{neg}$ (have divided more than 7-8 times) (Figure 2.3 A). Of interest, resting cells and cells in the early rounds of proliferation could be detected in the draining lymph node as well as in the spleen and the non-

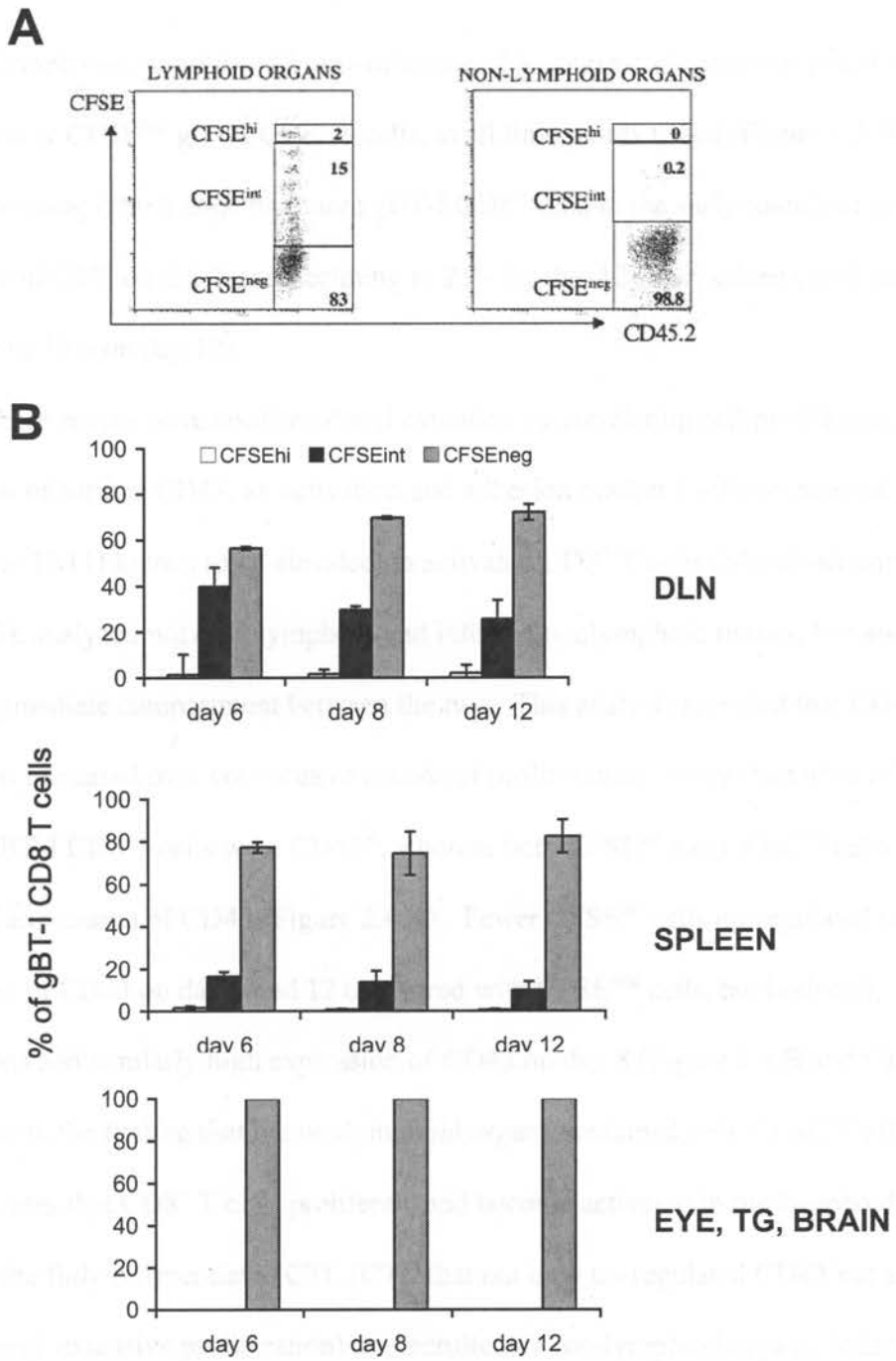
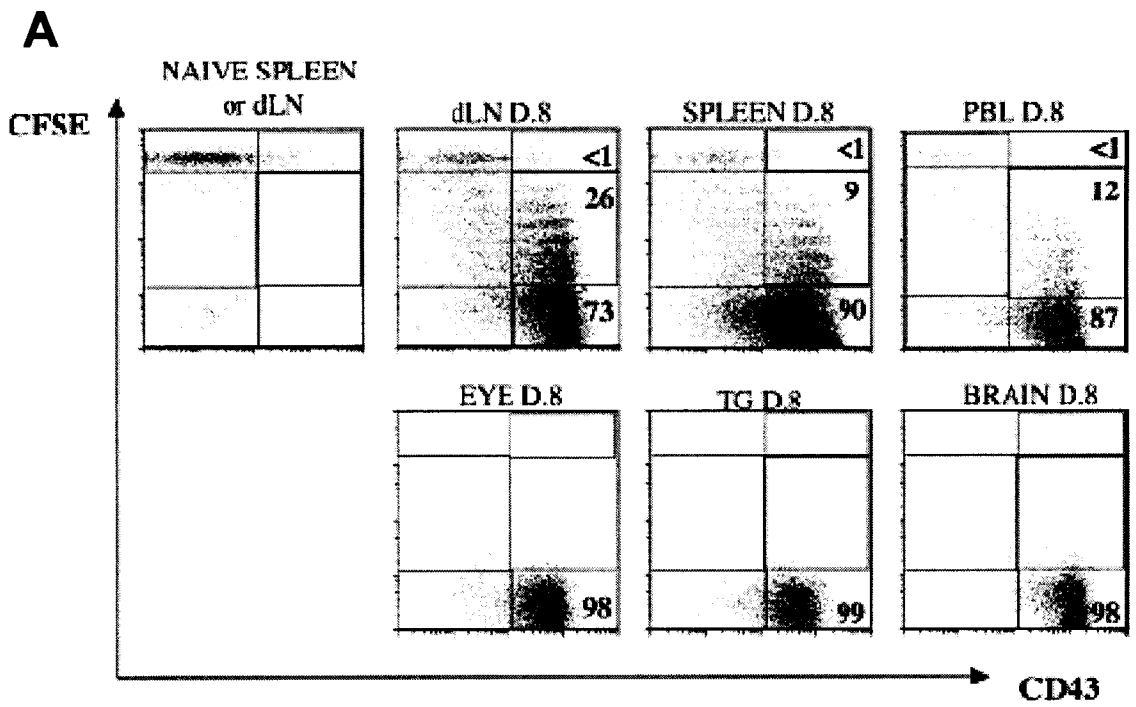


Figure 2.3 CD8$^+$ T-cells at initials stages of proliferation can be found in secondary lymphoid, but are excluded from non-lymphoid organs. A. Representative example of CFSE profiles of gB-specific CD8$^+$ T cells on day 6 post infection in the spleen (left panel) and the brain (right panel). The experiment was performed as in Fig. 2.1, and the analysis conducted as described by selective gating. Profiles of cells from lymph nodes and TG were superimposable on those shown above for spleen and brain, respectively. **B.** The experiment was performed as in A, with 3 animals per organ per time point shown. Results for eye/TG/brain are derived from TG, and are representative of all three tissues. Results are shown as average ± SD and are representative of two experiments.

draining lymph node until day 12 post-infection. By contrast, the non-lymphoid organs harbored only $CFSE^{neg}$ gBT-I $CD8^+$ T cells, at all time points tested (Figure 2.3 B). Note that the draining lymph node had more gBT-I $CD8^+$ cells in the early rounds of division (starting with 39% on day 6 and declining to 25% by day 12) than spleen (16% on day 6 declining to 10% on day 12).

These results were confirmed and extended by correlating cell proliferation to the expression of surface CD43, an activation and adhesion marker (isoform detected by mAb clone 1B11) known to be elevated on activated $CD8^+$ T cells (Murali-Krishna et al., 1998). We analyzed not only lymphoid and infected nonlymphoid tissues, but also blood as an intermediate compartment between the two. This analysis revealed that CD43 expression increased over consecutive rounds of proliferation. More than 95% of all $CFSE^{hi}$ gBT-I CD8 T cells were $CD43^{lo}$, whereas both $CFSE^{int}$ and $CFSE^{neg}$ cells up-regulated expression of CD43 (Figure 2.4 A). Fewer $CFSE^{int}$ cells up-regulated the expression of CD43 on day 6 and 12 compared with $CFSE^{neg}$ cells, but both cell populations had similarly high expression of CD43 on day 8 (Figure 2.4 B and C). This together with the finding that the nonlymphoid organs contained only $CFSE^{neg}$ gBT-I cells indicates that $CD8^+$ T cells proliferate and become activated in the lymphoid organs, and only the fully differentiated CTL (CTL that not only up-regulated CD43 but also went through extensive proliferation) are recruited to non-lymphoid organs. Indeed, the phenotype of gBT-I $CD8^+$ T cells isolated from TG and brain was consistent with this idea (Figure 2.5): these cells were $CD43^{hi}$ and largely capable of IFNγ production upon short stimulation. Of interest, the percentages of $IFN\gamma^+$ gBT-I cells were similar in the spleen, suggesting that peripheral localization was not essential for the acquisition of this



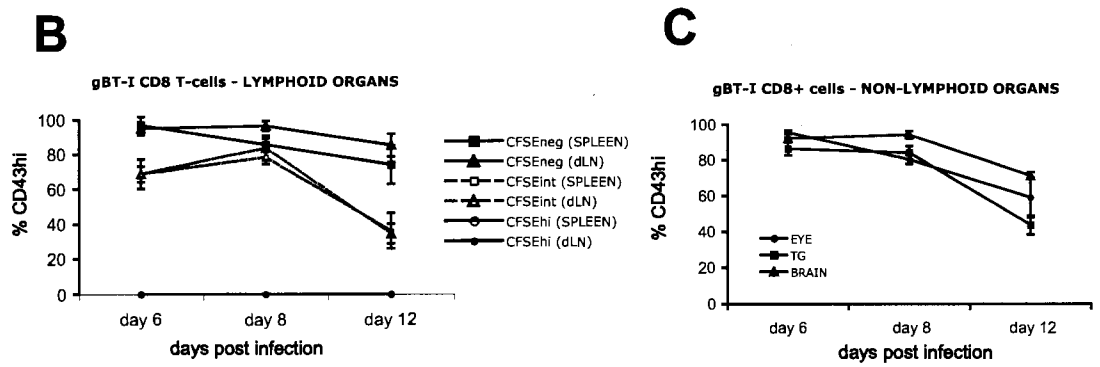


Figure 2.4 CD43 is up-regulated with cell division, recedes after viral clearance, and is uniformly high on cells gaining access to nonlymphoid organs. The experiment was performed essentially identically to that in Figure 2.3, except that CD43 expression was monitored as a function of cell division (CFSE dilution). **A.** Representative graphs of CFSE and CD43 staining on day 8 p.i. The graphs are gated on $CD45.2^{+}$ $CD8^{+}$ T cell. **B.** and **C.** CD43 expression on gBT-I cells from lymphoid (spleen and dLN) and nonlymphoid (eye, TG and brain) sites. Results are shown as a time course, and denote percentage of cells of a given CFSE phenotype (high, intermediate or low) expressing CD43. Note that the nonlymphoid tissues contained only $CFSE^{neg}CD43^{hi}$ cells. Results are shown as mean ± SD (n = 4) and are representative of two experiments.

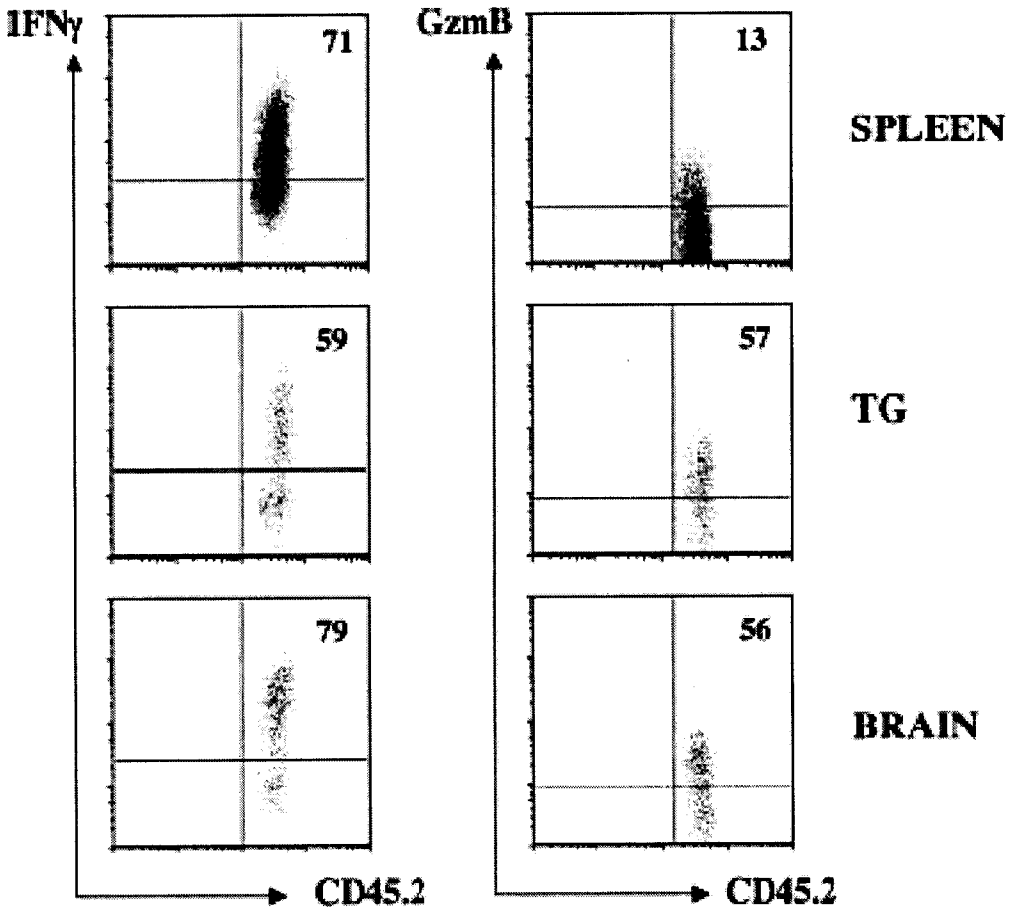


Figure 2.5 The gBT-I CD8 T cells in peripheral sites exhibit elevated granzyme B expression and make IFNg upon ex-vivo $gB_{498-505}$ peptide stimulation. The FACS plots shown are gated on gBT-I CD8 T-cells ($CD45.2^+$ $CD8^+$) on day 8 post-infection. The values are representative of percent of IFN- $^+$ (left panel) or granzyme B^+ (right panel) gBT-I CD8 T-cells within each indicated organ. Indistinguishable results were obtained in two experiments with a total of 6 individual animals analyzed.

capacity. The situation is less clear with regard to the expression of GzmB; there, many fewer splenic gBT-I cells expressed this molecule compared with those in TG and brain. At present we are investigating whether this reflects selective recruitment of GzmB+ cells to the neural tissues or the existence of an in situ maturation step. Regardless of this issue, the finding that the nonlymphoid organs contained mostly, if not exclusively $CFSE^{neg}$ gBT-I cells indicates that $CD8^+$ T cells are activated and become functional in the lymphoid organs, but that only cells that underwent extensive proliferation (and, at least in part, arming) in the lymphoid organs are allowed access to peripheral non-lymphoid tissues. This is consistent with findings of Masopust et al. on the expression of CD43 in lung-residing CTL (Masopust and Lefrancois, 2003).

Presence of antiviral $CD8^+$ T cells in the infected peripheral organs correlates with increased survival and lower viral titers in the nervous tissues

We next examined how the kinetics of the antiviral response correlated with the kinetics of viral spread and replication. First, we determined the kinetics of viral spread by measuring titers of replicating virus in the eye, TG and brain on days 1-12 post-infection (Figure 2.6 A). Replicating virus was initially detected only in the eye (day 1), however it spread rapidly to the nervous tissues, reaching the TG on day 2, and brain on day 3. We reproducibly detected the virus in the brains of all mice, albeit the titers on the average were lower compared to TG in this particular experiment. Between experiments and animals, the variation in viral titers was within 1-2 orders of magnitude (see Fig. 2.6 C for an example). Viral titers begun to decline on day 5 (the eye) and day 6 (TG, brain), and viral replication ceased between days 8 and10 in all organs tested. In parallel, non-

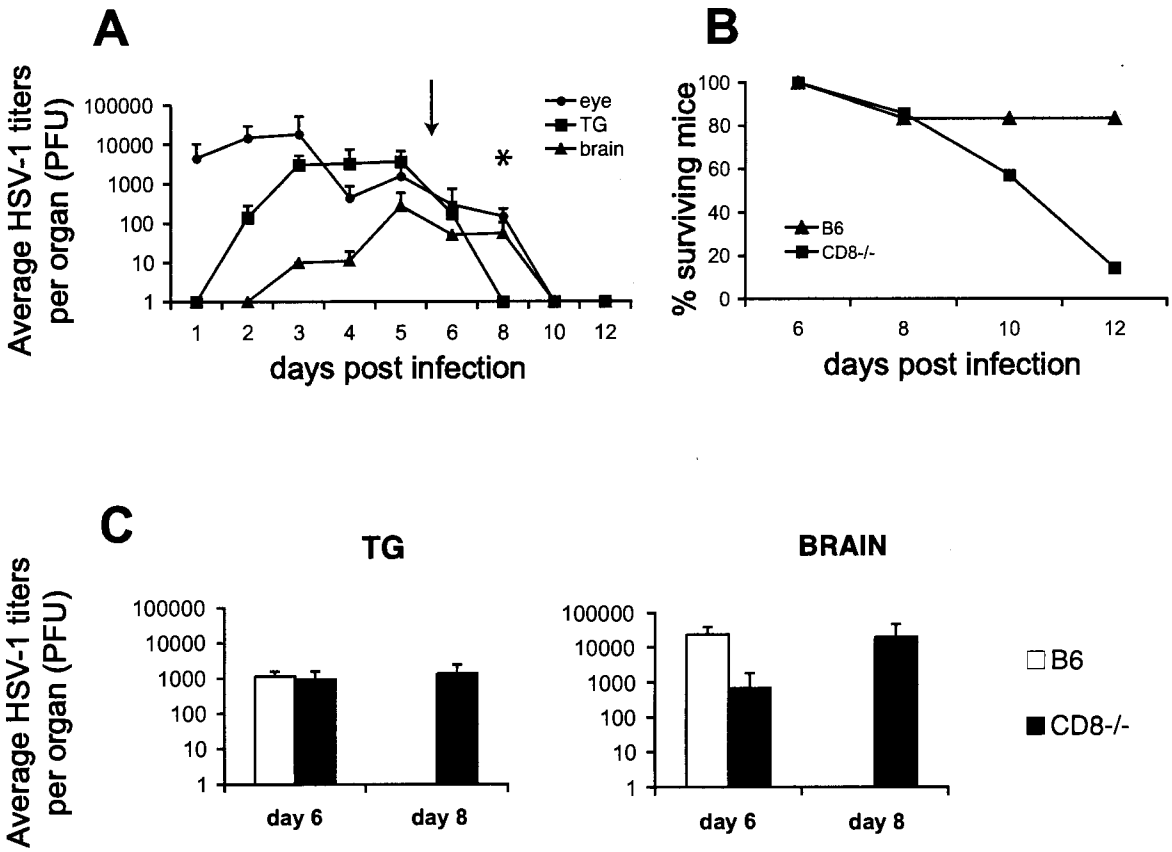


Figure 2.6 Kinetics of viral progression from the eye to the TG and brain and the role of $CD8^+$ T-cells in preventing lethal outcome after ocular infection. A. HSV-1 titers in the eyes, TG, and brains of ocularly infected B6 mice were evaluated on days 1-6, 8, 10, and 12 post-infection. The organs were homogenized and viral titers in the homogenates were determined by a standard plaque assay on Vero cells. Each data point shows an average of six animals and the corresponding SD, and is representative of results from two independent experiments. Note that viral titers begin to decrease from the point of infiltration of HSV-1-specific $CD8^+$ T-cells into the infected organs on day 6 (indicated by an arrow; see also Fig. 2.2). Asterix denotes the earliest timepoint at which some animals cleared the virus (in this experiment, only 2/6 animals had detectable viral loads). **B, C.** Critical role of $CD8^+$ T-cells in preventing lethal HSV-1. **B.** Groups of $CD8^{-/-}$ mice (n = 14) and B6 mice (n = 12) were infected with $1x10^6$ PFU HSV-1 and observed daily for clinical signs of HSV-1 infection. Mice with pronounced leg paralysis and inability to walk and take food/water were considered moribund and were euthanized. **C.** Viral titers in the TG, brain and the eyes of $CD8^{-/-}$ and B6 mice were determined on day 6 and 8 post-infection. The $CD8^{-/-}$ mice examined on day 8 were moribund, while the B6 mice were asymptomatic. The values represent the mean ± SD (n = 4), representative of mice surviving at this point.

lymphoid organs (spleen, submandibular lymph node, mediastinal lymph node) were also tested for presence of replicating virus; no viral replication was detected in these sites at any time. Of note, we also did not detect either the virus or the virus-specific CD8+ T cells in the other nonlymphoid organs tested (lung, liver, fat pad; not shown). Therefore, the progress of infection from the eye was restricted to the nervous tissue and did not cause systemic infection.

When superimposed upon the kinetics of $CD8^+$ T cell response, it is clear that the early spread of the virus occurs much before $CD8^+$ T cells are available to contain it in nonlymphoid organs. If so, one could argue that either $CD8^+$ T cells are not necessary for viral control or that they exercise their effect rather late after dissemination. We thus tested whether $CD8^+$ T cells are responsible for control of viral replication in situ after viral dissemination from the eye into PNS and CNS. If that were true, one would expect that starting on and after day 6 post-infection, CD8α-deficient mice would maintain high virus titers, perhaps for longer periods of time, than the wild type controls. To test this hypothesis, we infected CD8α knockout mice ($CD8\alpha^{-/-}$), and control B6 mice with the same dose of HSV-1, and compared their survival (Figure 2.6 B). We detected a significant difference in survival between the two groups of mice: 83% survival in B6 mice (n=12) and only 14% survival of $CD8\alpha^{-/-}$ animals (n = 14). The difference was statistically significant (p-value =0.0011, two-sided Fisher's exact test). Mortality of CD8-deficient animals occurred after day 8, at the time when wild-type animals exhibited peak frequencies of activated virus-specific $CD8^+$ T cells in the infected organs (Figures 2.2 & 2.4). This correlated with persistence of high viral titers in the TG and in the CNS of the $CD8\alpha^{-/-}$ mice at these late time points, in contrast to wild-type mice in which HSV

was no longer detectable (Figure 2.6 C). These finding indicated that the virus, rather than the $CD8^+$ T cells, is responsible for brain pathology. Moreover, this finding strongly suggests that antiviral $CD8^+$ T cells are critical in controlling the outcome of the ocular HSV-1 infection and survival of the host.

DISCUSSION

The notion that in the course of HSV infection, $CD4^+$ T cells operate to clear local skin/mucosal infection, and that $CD8^+$ T cells chiefly operate in containing the virus in neural tissues was introduced a long time ago (Nash et al., 1987; Simmons and Tscharke, 1992). Yet in some studies, the role of $CD8^+$ T cells in containing the primary, acute infection was deduced to be either not essential or not obligatory (Manickan and Rouse, 1995; Sciammas et al., 1997), and was proposed to be mostly at the level of limiting the viral spread into the CNS. The results reported herein strongly affirm that naïve, resting $CD8^+$ T cells cannot prevent viral spread to different organs if activated at the time of viral infection. These cells get activated and proliferate with kinetics that is incompatible with the idea that they can contain early viral spread into the brain. Of interest, activated HSV-1-specific $CD8^+$ T cells, injected within the first 24 hours of infection in a cutaneous, zosteriform model of HSV, reduced or abrogated viral infection, suggesting that these cells could limit viral spread if present at the right time and in sufficient numbers (van Lint et al., 2004). However, in that model (van Lint et al., 2004) in the course of natural progression of primary infection freshly primed $CD8^+$ T cells were also

reported to arise too late to limit initial viral spread. Our results are entirely consistent with that study.

Our study systematically analyzed the development of the anti-HSV $CD8^+$ T cell response following corneal infection using definitive and conclusive tools: adoptive transfer of naïve, HSV-specific $CD8^+$ T cells. Proliferation of antiviral $CD8^+$ T cells was initiated in the submandibular lymph node, which was previously shown to be the draining lymph node in corneal transplantation experiments (Yamagami et al., 2002). This result confirms and extends earlier results obtained in the flank scarification and footpad infection models (Coles et al., 2002; Mueller et al., 2002b), and formally demonstrates the kinetics of CTL priming in the ocular model. The novel aspects of these studies are that: 1) activated cells were released shortly after initial activation in the draining lymph node; 2) both the draining lymph node and the spleen contained a mixture of resting, Ag-specific cells and cells undergoing a range of cycles of proliferation, as late as day 12 post-infection; and 3) that the cells which infiltrated extralymphoid organs have invariably undergone extensive proliferation, full CD43 up-regulation and arming for IFN-γ secretion, and CD43 expression on these cells begun to recede shortly after termination of viral replication. We also found that in addition to the TG and eye, the brain is a site of a vigorous antiviral CTL response, exhibiting the same infiltration kinetics and functional characteristics as the response in TG.

Analysis of gB-specific $CD8^+$ T cell proliferation revealed that proliferating cells in early rounds of proliferation, as well as undivided, resting gB-specific CTL, could be found in the draining lymph node as late as day 12 post-infection. Thus, the lymph node remains the site of continuous recruitment and proliferation of naïve $CD8^+$ T cells well

after the initial wave of priming and expansion. Except for day 5, when proliferation was detectable only in the draining lymph node, proliferating cells were also detected in the spleen, indicating that the spleen may also be a site of $CD8^+$ T cell proliferation in this model. Whether the spleen contains any viral antigens at this point (disseminated by local APCs from the site of infection, as we could not detect replicating virus in the spleen at any time point), or whether $CD8^+$ T cells merely proliferate as a consequence of the initial activation program (Kaech and Ahmed, 2001; Wong and Pamer, 2001)is currently under investigation.

Coordinated analysis of proliferation and activation marker acquisition demonstrated that antiviral CD8 T cells up-regulated expression of CD43 over several rounds of proliferation. Undivided $CFSE^{hi}$ gBT-I cells were uniformly $CD43^{lo}$. Cells in the early rounds of proliferation ($CFSE^{int}$) begun to up-regulate CD43, but the frequency of $CD43^{hi}$ cells was the greatest in the $CFSE^{neg}$ cells, which have undergone extensive proliferation. This is consistent with the idea of a $CD8^+$ T cell differentiation program in which proliferation, gradual changes in activation phenotype and differentiation into functional CTL occur across a coordinated continuum. Since only $CFSE^{neg}$ CTL were detected outside of the lymphoid organs, this implies that only differentiated $CD8^+$ T cell can be recruited to the sites of infection. Indeed, we show that the extralymphoid $CD8^+$ T cell from both brain and TG were functional, as shown by IFNγ production after 6-hour ex-vivo peptide stimulation. This is consistent with the results of Khanna et al. (Khanna et al., 2003). In addition, the CTL in the infected organs had elevated granzyme B expression (as compared to naïve controls). Granzyme B is a component of the lytic granule; its expression is elevated in effector CD8 T cells and correlates with the $CD43^{hi}$

and $CD62L^{lo}$ phenotype, as well as with ex-vivo CTL cytotoxic function. Whether the CTL located in the nervous tissues are capable of target cell lysis is currently under investigation.

Finally, our current results suggest that lethal outcome of the HSV-1 infection can be, and often is, prevented by $CD8^+$ T cells. This result is in agreement with the results of a recent study by Anglen et al. (Anglen et al., 2003) in HSV-1-mediated stress-induced encephalitis following intranasal infection, showing that a timely presence of $gB_{498-505}$-specific $CD8^+$ T cells protect against encephalitis. In our experiments using $CD8\alpha^{-/-}$ animals, it is possible that the lack of CD8α affected the otherwise CD8α antigen-presenting dendritic cells (DCs) which in turn may have affected development of the arms of the immune response other than $CD8^+$ T cells. Indeed, in the HSV-1 epidermal infection model (Allan et al., 2003; Belz et al., 2004),the $CD8\alpha^+$ DC subset is critical to priming CTL responses. However, it is unlikely that this defect abolished priming of $CD4^+$ T cells that seem to rely upon $CD11b^+$ DCs but not upon $CD8\alpha^+$ DCs (Iwasaki, 2003; Zhao et al., 2003). Therefore our experiments affirm that, in addition to the proposed role in controlling and surveying viral latency in sensory ganglia (Khanna et al., 2003), the $CD8^+$ T cell response to HSV in the brain is one of the mainstays of antiviral defense during acute infection.

Summary

In this study we have examined the protective role of $CD8^+$ T cells during primary HSV-1 infection. Our data add on to the literature that demonstrates the critical role for this lymphocyte subset in mediating antiviral protection. In the next chapters of this dissertation, the maintenance of HSV-1 specific memory CD8+ T cells will be investigated. While a lot of attention has been paid to the ongoing surveillance of viral latency in the nervous system by the locally present $CD8^+$ T cells, much less is known about the maintenance and function of HSV-specific $CD8^+$ T cells present in the periphery. Given the importance of CD8 memory in case of systemic re-infection or viral reactivation and spread away from the site of latency, we wanted to know 1) how stably is the peripheral memory $CD8^+$ T cell response maintained and 2) whether the presence of the latent infection and periodic viral reactivation is reflected in the size, phenotype and functional responses of the peripheral HSV-specific memory $CD8^+$ T cells. This will be discussed in Chapter 3.

CHAPTER 3

Age-related dysregulation of CD8$^+$ T cell memory specific for a persistent virus is independent of viral replication

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ABSTRACT

The immune system devotes substantial resources to the life-long control of persistent pathogens, which were hypothesized to play an important role in immune aging. Of note, the presence of persistent herpesviruses has been correlated with immune exhaustion and shorter lifespan in octogenarian humans. But neither the causality nor the mechanistic link were established, and the relative roles of persistent antigenic stimulation and of virus-independent homeostatic disturbances in T cell aging remain unresolved. We longitudinally analyzed expansion, contraction and long-term maintenance of murine $CD8^+$ T cells responding to a localized, corneal infection with a typical latent virus, HSV-1. Young mice exhibited the expected expansion of HSV-1 gB-specific cells, followed by their contraction, and the stable maintenance of low numbers of memory T cells into advanced adulthood. However, upon entry into senescence, many (>40%) animals exhibited an increase of Ag-specific cells in blood and spleen, and in some animals this increase (memory inflation) reached the levels comparable to those observed in acute infection. This inflation occurred to the same extent in control mice and the mice continuously treated with the anti-HSV drug famciclovir. Furthermore, the inflating cells largely maintained Ag-specific function, and exhibited typical central memory phenotype, with no signs of Ag-specific activation. They exhibited increased expression of CD122 and CD127, akin to the antigen-independent T cell clonal expansions (TCE) found in old specific pathogen free (SPF) laboratory mice. This collectively suggests that in this model the inflating cells may be selected for high responsiveness to environmental cytokines largely in an antigen-independent manner.

INTRODUCTION

Aging of the immune system is marked by a complex set of changes that are collectively termed immune senescence (Cambier, 2005; Linton and Dorshkind, 2004; Miller, 1996). Senescence of T cells is associated with the involution of the thymus, which drastically curtails production of naïve T cells and reduces the renewal of the naïve T cell compartment. Lifelong encounters with pathogens further deplete the naïve compartment, and precipitate the conversion of many naïve T cells into memory T cells. Therefore, there is a dramatic increase in representation of memory cells, with a concomitant decrease in representation of circulating naïve T cells. This shift is further potentially compounded by homeostatic mechanisms. Low rate of homeostatic cycling of naïve cells ensures their long-term survival as well as the maintenance of T cell repertoire diversity (Fry and Mackall, 2005; Surh et al., 2006). However, in more extreme depletion, this cycling is known to become more pronounced [homeostatic proliferative expansion (HPE)]. This mechanism is likely to maintain repertoire diversity for a while, however, HPE is known to eventually result in phenotypic and functional conversion of naïve cells into memory-phenotype cells (rev. in (Fry and Mackall, 2005; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000; Surh et al., 2006)). Data from humans (Naylor et al., 2005), and non-human primates (L. Cicin-Sain et al., submitted) are consistent with the idea that this may further pronounce the naïve-to-memory shift with age.

While the above forces appear to be the main ones influencing T cell aging at the population level in specific pathogen-free (SPF) laboratory mice, T cell aging in humans is believed to be critically affected by ubiquitous persistent infections, including those by

viruses from the Herpesvirus family. CMV seropositivity in humans has been correlated to an age-related increase in the fraction of memory T cells specific for CMV (rev. in (Pawelec et al., 2004)), a response that is unusual in its strength, breadth and complexity (Munks et al., 2006b; Sylwester et al., 2005). Amongst the octo- and nona-generians, CMV seropositivity has further been correlated to shorter lifespan (Wikby et al., 2005). This provided basis for suggesting a link between immune aging and persistent infections (Pawelec et al., 2004). However, direct causality is difficult to establish in the human model due to ethical constraints and because it is difficult to demonstrate subclinical reactivation of infectious CMV in blood of asymptomatic subjects (Fields, 2001; Jarvis and Nelson, 2002) and mice (Pollock and Virgin, 1995; Reddehase, 2000; Reddehase et al., 2002). Therefore, mechanistic details of the interaction between a persistent virus and T cell memory homeostasis over the lifespan of a mammal remain largely unexplored.

We were spurred by these findings to investigate the role of life-long viral infection in the age-related memory dysregulation in a defined and experimentally versatile murine model. Our findings show that despite viral persistence, many $CD8^+$ memory T cells specific for the virus expand in an old organism independently from antigenic stimulation, strongly suggesting that the primary and decisive role in these age-related disturbances belongs to homeostatic dysregulation.

MATERIALS AND METHODS

Mice. Female C57BL/6-NCr (B6) mice were purchased from the National Cancer Institute colony (Frederick, MD), and used young (8-12 weeks), or at adult (4-12 month -

mo) or old (>20 mo) age. All animals were housed under the SPF conditions, and experiments conducted under IACUC approval and in accordance with the applicable federal, state and local regulations. Animals were inspected at necropsy and those with signs of possible tumors and gross abnormalities excluded from the study.

Viruses and viral infections. HSV-1 strain 17 obtained from Dr. D.J. McGeoch (University of Glasgow, Scottland, UK), cloned as a syn+ variant and titered on Vero cells in our laboratory, was used in all experiments. Localized (intracorneal, i.c.) infections with 10^6 PFU HSV-1 per mouse were performed as described (Lang and Nikolich-Zugich, 2005).

Determination of titers of replicating and reactivated virus. The amount of replicating virus in the trigeminal ganglia (TG) of acutely (day 3-5 p.i.) infected mice was determined by plaque assay of TG homogenates as previously described (Lang and Nikolich-Zugich, 2005). To determine the presence of latent virus in the TG of latently infected mice (>30 days p.i.), the TG were subjected to a standard reactivation assay and the titer of reactivated virus was determined by a plaque assay. Briefly, both TGs/mouse were isolated, cut into 4-6 pieces and cultured on top of Vero cell monolayer in media containing 2% FBS for 5 days in order to induce viral reactivation. At the end of the culture, the TG and the Vero cells were homogenized in the original culture media using sterile glass homogenizer, and the viral titer of the homogenate was determined by a plaque assay. The limit of detection was 10 PFU. Alternatively, the TG from latently infected or control naïve mice were incubated in RPMI (GibcoBRL) containing 3mg/ml collagenase type 1 (Sigma) for 1.5 hours, and dispersed into single cell suspension by pipetting. Total DNA was isolated using DNeasy columns (Qiagen) and was diluted to

10ng/ml. The 25ml PCR reaction contained 8.5ml DNA, 12.5ml TaqMan Universal PCR Master Mix (Applied Biosystems), glycoprotein B-specific primers (Invitrogen) , and probe (Applied Biosystems) . HSV-1 DNA was isolated from a virus stock of known titer and used to generate the standard curve. All samples were analyzed in triplicate. The PCR reaction was carried out using MyIQ PCR cycler (Bio-Rad). The PCR conditions, primer and probe sequences were as described (Corey et al., 2005).

Famciclovir treatment. Where indicated, Famciclovir (Famvir, Novartis) was administered to mice in their drinking water at a concentration of 2mg/ml. The famvir water was changed twice a week.

Reagents, antibodies and flow cytofluorometric (FCM) analysis. The gB-8p peptide (SSIEFARL) was purchased from SynPep Corporation (Dublin, CA), and the gB-8p :K^b tetramer was obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Monoclonal antibodies anti-CD8α (clone 53-6.7), anti-CD27 (clone LG3a10), anti-IFNγ (all from BD Pharmingen, San Diego, CA); anti-CD127 (clone A7R34), anti-CD62L (clone MEL-14), anti-CD44 (Pgp-1, Ly-24), anti-CD122 (clone 5H4) (all from EBioscience, San Diego, CA); and CD43 (clone 1B11; Biolegend, San Diego, CA) were purchased from commercial sources.

FCM analysis with or without intracellular staining to detect IFNγ was performed as previously described (Lang and Nikolich-Zugich, 2005); in the latter case, stimulation was performed with 10^{-6} M gB-8p peptide for 6 h in the presence of 1.5 μg/ml brefeldin A. FCM data was acquired on FACSCalibur instrument using CellQuest 3.3 software or on the FACS LSRII instrument using the Diva software (Becton Dickinson, Mountain View, CA), and analysis performed using FlowJo software (Tree Star). At least 10^4 cells

were analyzed per sample, with dead cells excluded by selective gating based on orthogonal and side light scatter characteristics.

CTL cultures and ^{51}Cr-release assay. On day 8 p.i. splenocytes from infected mice were co-cultured with irradiated, gB-8p-coated syngeneic splenocytes and peptide-specific CTL activity was determined in a standard ^{51}Cr-release assay 5 days later as described previously (Messaoudi et al., 2001).

CDR3 length analysis (spectratyping). Spleens of mice selected for CDR3 length analysis were enriched for $CD8^+$ T cells (>80% $CD8^+$ and <2% $CD4^+$) by depletion of $CD4^+$ and $B220^+$ cells by immunomagnetic sorting (MACS, Miltenyi Biotech). CDR3 length analysis was performed as described (Messaoudi et al., 2001) on Vβ8 and 10 families, which in B6 mice account for >80% of the response to gB-8p (Hanke et al., 1991; Wallace et al., 1999).

RESULTS

Maintenance of stable anti-HSV memory becomes disrupted with age

The key feature of the HSV-1 infection in B6 mice is an unusual degree of immunodominance, whereby >95% of the entire CD8 response is directed against the glycoprotein B (gB) octapeptide 498-505, SSIEFARL, (gB-8p in the text), bound to H-$2K^b$ (Hanke et al., 1991; Wallace et al., 1999) and that response is critical for antiviral protection (Blaney et al., 1998; Lang and Nikolich-Zugich, 2005; Messaoudi et al., 2002; van Lint et al., 2004). To determine whether lifelong infection with a latent virus is

accompanied by stable maintenance of systemic anti-HSV $CD8^+$ T cell memory, we have analyzed the frequency of gB-8p-specific $CD8^+$ T cells at various times post infection (p.i.) in a longitudinal study. A cohort of young mice was infected by corneal scarification, and the frequency of HSV-specific memory $CD8^+$ T cells was periodically assessed by FCM in circulating blood lymphocytes using the gB-8p:K^b tetramer. The infection resulted in expansion of gB-8p-specific $CD8^+$ T cells, which reached the peak on days 7-8 p.i. and then contracted to its memory set point by 4 months p.i. (m.p.i., Figure 3.1 A). Thereafter, the frequency of HSV-specific $CD8^+$ T cells remained stable up to 7-12 m.p.i. (Figure 3.1 A and not shown). The average frequency at set-point in this experiment was 1.6% ± 0.4% of all $CD8^+$ T cells, ranging between 1-2.1%.

To explore memory maintenance at the later time points and into senescence, we followed several mouse cohorts from the time of infection early in life (2-3 mo) until they reached old age (>18 m. p.i., corresponding to >20 months of age). We found that compared to the memory set point observed in adulthood (0.9% ± 0.6%, n =50), there was a significant increase in the frequency of memory $CD8^+$ T cells in the old animals (3.6% ± 4.0%, n =50, Fig. 3.1 B). In some old mice the frequency of memory $CD8^+$ T cells increased to the same level or even above the extent of expansion at the peak of the primary response. Moreover, while not all of the mice exhibited dramatic increases in frequency of memory $CD8^+$ T cells, an increase was seen in most animals, and it was significant at a cohort level (Fig.3.1 B, p-value < 0.001, paired Student's t-test) Similar increase of memory cell frequencies was observed in spleens, where the average frequency of memory $CD8^+$ T cells increased from 0.7% (± 0.2%, n = 12) in adults to 2.2% (± 2.8%, n = 14) in the old (Fig. 3.1 C, p-value 0.036).

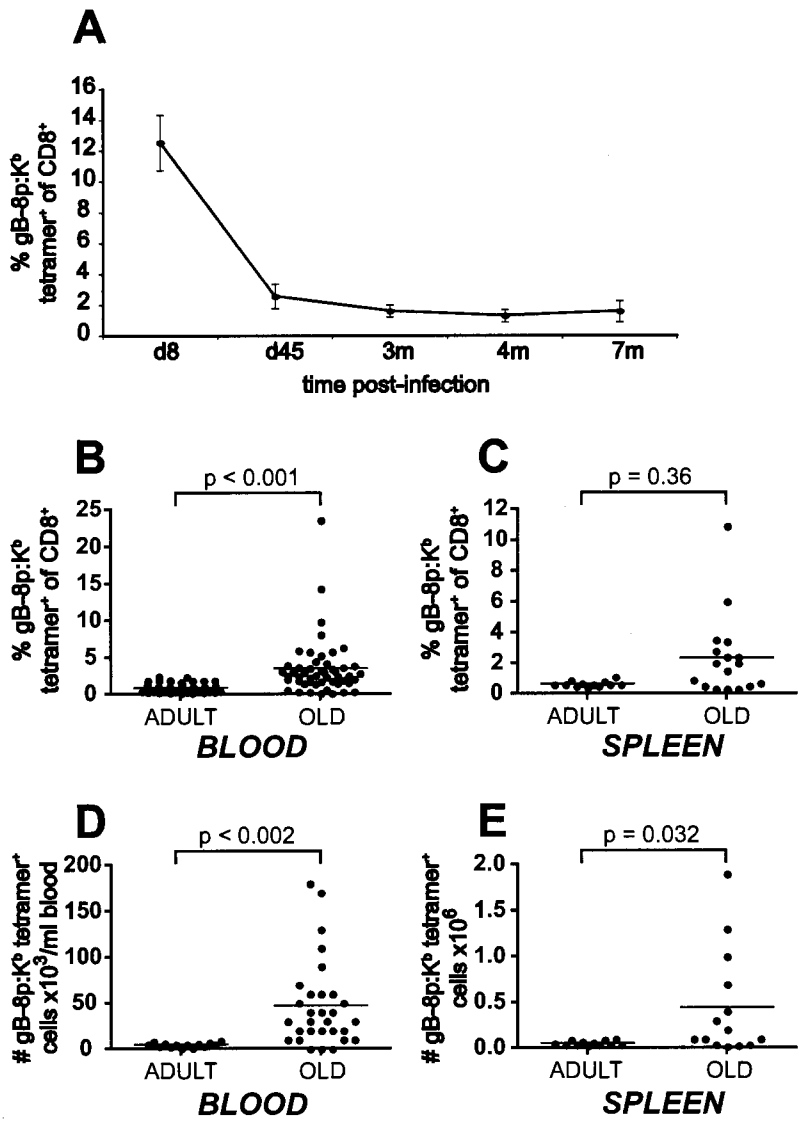

Figure 3.1 CD8$^+$ T cell memory is stable in adult but dysregulated in old animals following ocular HSV-1 infection. A. Stable maintenance of gB-8p specific memory CD8$^+$ T cells in adult animals. A cohort of young B6 mice (n = 9) was infected with 10^6 PFU HSV-1 (i.c. infection). Blood samples were taken at indicated time p.i. and stained with anti-CD8$^+$ and gB-8p:K^b tetramer. The values show the average percent of tetramer$^+$ cells within CD8$^+$ T cells ± SEM. The data is representative of three independent experiments. **B-E.** Memory inflation occurs with advanced age. **B.** A cohort of young B6 mice (n = 50) was infected as in A and followed longitudinally. Blood samples were taken from adult (4 m.p.i.) and old mice (18-24 m.p.i.), and stained as in A. The values show the percent of tetramer$^+$ cells within CD8$^+$ T cells (each individual animal is represented by a dot, the dash reflects the average). **C.** Mice were infected as in A and select animals were sacrificed as adults (n = 12) or old (n = 17) and the frequency of gB-specific memory CD8$^+$ T cells was determined in their spleens as above. **D and E.** Absolute numbers of tetramer$^+$ CD8$^+$ T cells in blood (D) and spleens (E) of mice from part C and D, respectively. Numbers were obtained from absolute counts and percentages of tetramer$^+$ CD8$^+$ T cells.

Most importantly, the memory $CD8^+$ T cells increased in absolute number in blood (from 4.9×10^3/ml ± 2.5×10^3 in adults to 4.7×10^4/ml ± 46.8×10^4 in the old, reaching up to 1.8×10^5 cells/ml in some mice, Fig. 1D), the spleens (from 6×10^4 ± 2×10^4 in adults to 4.5×10^5 ± 5.8×10^5 in the old, reaching up to 1.9×10^5 cells/spleen, Fig. 3.1 E), and the lymph nodes of individual old mice from the above study (not shown). Again, while not all animals showed pronounced absolute accumulation, more than half exhibited an absolute increase over the levels seen in adult animals.

The above results show that the $CD8^+$ memory to localized HSV-1 infection is maintained at a stable, low frequency throughout adulthood in individual mice, but exhibits variable and in many mice significant increase from the set point level in old age, implying a loss of control in memory T cell maintenance. This age-related memory accumulation was observed in 42% of analyzed old mice (n=50), as measured by the fraction of animals whose memory cell frequency was more than two standard deviations above the memory set point.

Memory $CD8^+$ T cells specific for a persisting pathogen retain functionality but show pronounced age-related variability

The uneven memory maintenance seen in the above experiments could be due to homeostatic dysregulation, to repeated viral activation, or both. We set out to distinguish between these two possibilities. In humans, many cells responding to persisting pathogens were described to have dysregulated or diminished functional capabilities (Almanzar et al., 2005; Ouyang et al., 2002; Ouyang et al., 2003), postulated to be due to

exhaustion by repeated Ag stimulation. We therefore asked whether, in addition to loss of stringent control of memory $CD8^+$ T cell numbers, aged HSV-specific T cells may exhibit signs of functional exhaustion. We initially compared the frequency of $tetramer^+$ cells to frequency of cells capable of IFNγ production after short re-stimulation with the gB-8p peptide. In both adult and old mice, the frequencies of $tetramer^+$ and IFNγ-producing cells were comparable, as shown by an excellent correlation of tetramer and IFNγ staining for each individual mouse (Figure 3.2 A, $R^2 = 0.982$). Thus, whereas the old mice exhibited variability in frequency of virus-specific $CD8^+$ T cells by tetramer staining (Figure 3.1), IFNγ secretion closely mirrored this variability and the two traits correlated to each other in individual animals (Figure 3.2 A). Therefore, cells undergoing memory inflation were capable of secreting IFNγ.

We also examined the lytic capacity of these cells after 5-day in-vitro restimulation. Again, tightly clustered lytic activity of adult animals was replaced by high variability in the CTL responses of old animals (Figure 3.2 B), with some exhibiting strong and others poor CTL reactivity. Correlation between lytic activity and the frequency of memory cells at the start of the 5-day culture was less tight in old animals, and several different phenotypes were observed (Figure 3.2 A and B): low accumulation and brisk CTL responses (mouse #6227); low inflation, but poor CTL responsiveness; some or massive accumulation, with good IFNγ response, but poor CTL responses (mouse #3755, and, in particular, 4737), and finally, accumulation with good CTL activity (mouse # 4554 and 7130). These data suggest that CTL activity correlates poorly to memory accumulation during lifelong HSV-1 infection, and fail to provide decisive evidence for antigen-driven exhaustion in this model.

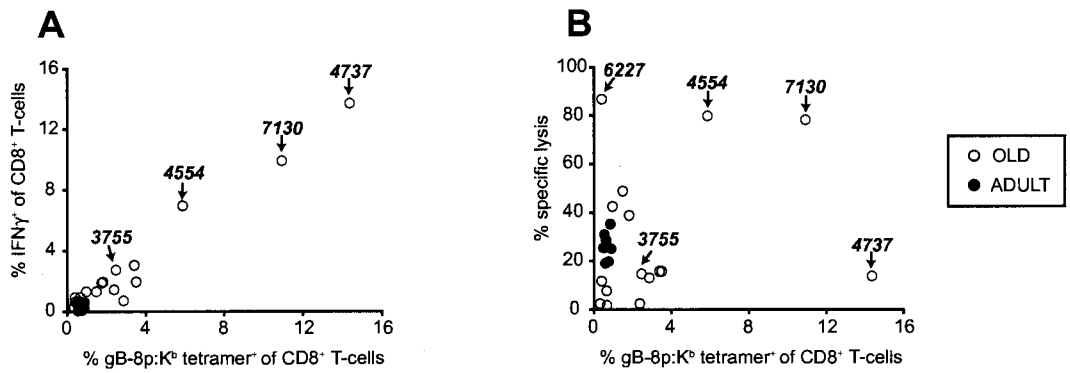

Figure 3.2 Functional responses of HSV-specific memory CD8$^+$ T cells in adult and old mice A. Most gB-8p-specific memory CD8$^+$ T cells produce IFN . Splenocytes from latently infected adult (n = 7, 10 m.p.i., 12 mo) and old (n = 16, 18-24 m.p.i., 20-26 mo) mice were stained with gB-8p:K^b tetramer ex-vivo, or stimulated for 6 hours with 10^{-6} M gB-8p peptide in presence of brefeldin A, and then stained for intracellular IFN . The X values show the percent of tetramer$^+$ cells, and the Y values shows the percent of IFN $^+$ cells within total CD8$^+$ T cell pool of individual mice. The values for IFN show the net IFN (background IFN staining from unstimulated wells was subtracted; background was < 0.2%). Excellent correlation of the tetramer and IFN staining was observed (R^2 = 0.982). **B.** Cytotoxic activity of memory CD8$^+$ T cells from mice at various times post HSV-1 infection. Splenocytes harvested from mice in A were cultured for 5 days in presence of gB-8p:K^b – pulsed stimulators. On day 5 of culture the cytotoxic function of HSV-specific CTLs was tested in a standard ^{51}Cr release assay. The CTLs (effectors) were mixed with gB-8p:K^b – pulsed EL-4 cells that were intracellularly labeled with ^{51}Cr (targets). Background lysis values obtained with peptide-negative targets (<8%) were subtracted. The X axis values show the percent of tetramer$^+$ cells at day 0 of culture, and the Y axis values show the percent of specific lysis at effector-to- target ratio of 80:1. The numbers on the graph indicate the IDs of specific mice as discussed in the results.

Surface phenotype of CD8$^+$ T cells undergoing late memory inflation suggests no recent nor repeated activation by antigen

The above data are somewhat reminiscent of the phenomenon originally described by Reddehase's (Holtappels et al., 2000; Podlech et al., 2000) and subsequently by Klenerman's groups (Karrer et al., 2003; Sierro et al., 2005) (christened by the latter group "memory inflation") in mice infected with the murine cytomegalovirus (MCMV). However, these groups described a much earlier onset of memory inflation (3-4 months p.i.), whereas our results suggested that the loss of numerical control of the memory CD8$^+$ T cell pool occurs only with advanced age during lifelong HSV-1 infection. In a separate study (A. Lang et al, in preparation), we have shown that this is not a consequence of fundamental differences between HSV-1 and MCMV, but rather a likely difference in the latent viral loads, which can be largely equalized if HSV-1 is administered systemically. Our present study, however, sought to understand the interplay of aging and a latent, lifelong infection in the ocular model, which faithfully mimics the fundamental features of natural human infection. To that effect, we proceeded to test whether the accumulating gB-specific CD8$^+$ T cell population bore signs of recent or prolonged contact with antigen.

Multicolor FCM analysis was performed to assess the expression of acute and chronic activation markers. We found no evidence of Ag-specific acute activation as judged by the lack of expression of CD25 and CD69 (not shown). Moreover, the markers which are downregulated with repeated or prolonged activation (CD62L, CD127 and CD27) were all expressed highly on gB-8:K^b tetramer$^+$ cells (Figure 3.3). In addition,

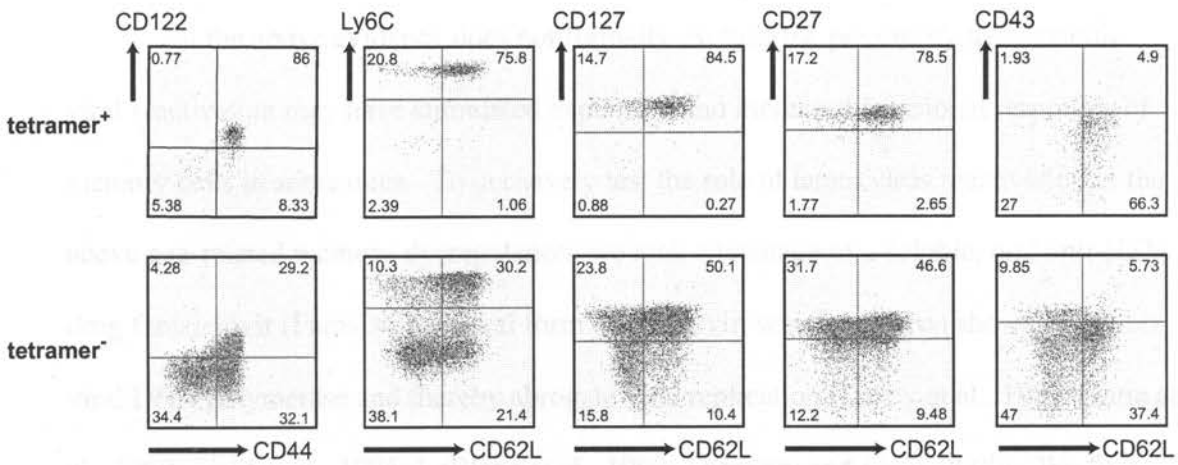

Figure 3.3 Expanded gB-8p-specific memory cells in old HSV-1 infected mice uniformly exhibit central memory phenotype. Splenocytes (shown) or lymphocytes isolated from blood (not shown) from old HSV-1 infected mice with age-related expansions of gB-8p-specific memory $CD8^+$ T cells were stained with CD8, tetramer and a panel of surface markers (CD44, CD62L, Ly6C, CD127, CD122, CD43, CD27). The staining of $tetramer^+$ (top panel) and $tetramer^-$ (bottom panel) $CD8^+$ T cells from a representative mouse is shown. The same phenotype was observed in $CD8^+$ T cells isolated from blood (not shown).

these cells expressed high levels of CD44 and Ly-6C, and intermediate levels of CD43, exhibiting the $CD25^{-}CD69^{-}CD44^{hi}CD62L^{hi}CD27^{hi}CD127^{hi}Ly\text{-}6C^{hi}CD43^{int}$ phenotype, typical of central (resting) memory cells (Fig. 3.3 and not shown). These results provided further evidence against the possibility that memory accumulation in this model may be driven by persistent antigenic stimulation.

Antiviral treatment has no influence upon late-age memory inflation

All the above evidence does not formally exclude the possibility that periodic viral reactivation may have stimulated expansion and increased functional responses of memory cells in some mice. To decisively test the role of latent virus reactivation in the above age-related memory dysregulation, we took advantage of a soluble, oral anti-HSV drug famciclovir (Famvir®), an oral form of acyclovir, which has been shown to inhibit viral DNA polymerase and thereby abrogate viral replication (Darby et al., 1981; Datta et al., 1980; Field et al., 1995; LeBlanc et al., 1999; Thackray and Field, 2000). We first showed that famvir, when administered prior to infection, was able to abrogate acute viral replication (Fig. 3.4 A), prevent establishment of latency (Fig. 3.4 B) in trigeminal ganglia of corneally infected mice, and abrogate generation of antiviral $CD8^{+}$ T cell response (not shown). Having demonstrated the efficacy of famvir in mice, we initiated longitudinal experiments where famvir was administered continuously in drinking water starting on day 14 p.i. In this infection model, productive viral replication ends by day 10 p.i., and from then on the virus remains latent in the trigeminal ganglia (TG) from which it can periodically reactivate. Therefore, initiation of famvir treatment on day 14 p.i. allows for establishment of latency (not shown), but should prevent viral

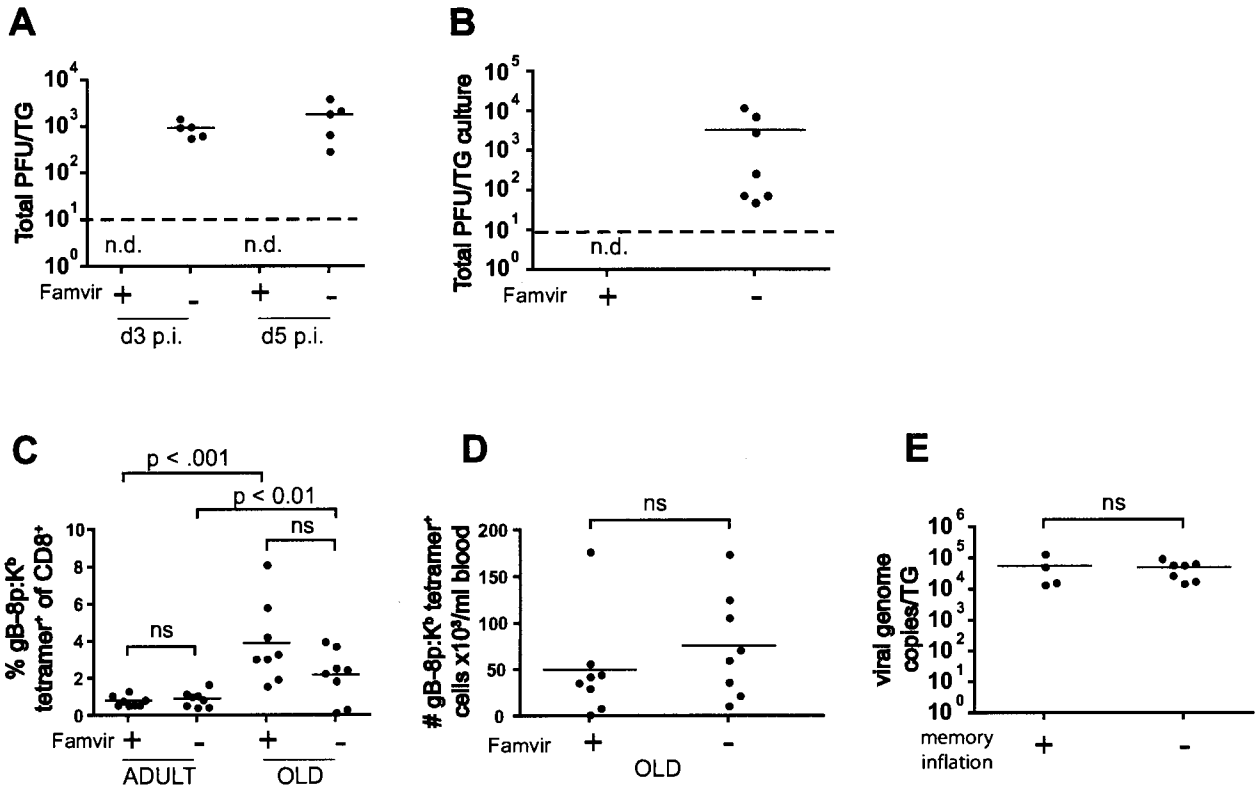

Figure 3.4 Effect of viral reactivation on development of age-related expansions of HSV-specific memory CD8$^+$ T cells A and B. Famvir treatment during acute infection abrogates viral replication and lowers the latent viral load to undetectable level. Young mice were infected i.c. with HSV-1 as described in Materials and Methods. Some mice (n = 5) received famvir continuously in the drinking water starting on day (-7). **A.** On days 3 and 5 p.i. TGs of famvir-treated (n = 5) and untreated (n = 5) mice were isolated and tested for presence of infectious virus by plaque assay. **B.** On day 30 p.i. the TGs from untreated (n = 7) or famvir-treated (n = 10) mice were isolated and subjected to a reactivation assay as described in Materials and Methods. The total titer of the virus reactivated from TG culture from each mouse is shown. The dotted line represents the limit of detection (10 PFUs). No virus was detected in the cultures from famvir-treated mice (n.d., not detected). **C-E.** Continuous famvir treatment of latently infected mice does not prevent the development of age-related memory CD8$^+$ T cell expansions. Young mice (n = 16) were infected i.c. with HSV-1 and their gB-8p-specific CD8$^+$ T cell responses were followed longitudinally. Half of the cohort was continuously treated with famvir starting on day 14 p.i. The same mice were screened for frequency of their HSV-specific memory CD8$^+$ T cells as adults and old. Age-related memory inflation was observed both in control and famvir-treated group, and there were no significant differences in the frequency (C) or absolute numbers (D) of gB-8p-specific memory CD8$^+$T cells in the old mice from either group. Mice undergoing memory inflation had the same latent viral load as the memory inflation-free mice, as determined by real-time PCR at the time of sacrifice at 20 m.p.i. **(E)**. The data is representative of two independent experiments.

reactivation. Figure 3.4 C and D show that famvir treatment did not prevent the age-related memory accumulation. Importantly, this was true both at the level of relative representation (Fig. 3.4 C) and the cell numbers (Fig.3. 4 D). These results further argued against the possibility that viral reactivation and restimulation of the virus-specific $CD8^+$ T cells cause memory accumulation in this model. Finally, if viral factors played a role in memory accumulation, one could expect that animals with pronounced CD8 T cell memory accumulation may have larger viral loads in ganglia. Figure 3.4 E shows that this is not the case, as we found no difference in latent and reactivatable viral loads between animals with large accumulation and those with few, if any, signs of accumulation.

HSV-specific memory CD8 T cells undergoing age-related accumulation resemble spontaneously arising T cell clonal expansions (TCE)

Another well-described age-related phenomenon bears resemblance to the above situation. Mice older than 18 months were described to develop spontaneous age-related T cell clonal expansions (TCE) in variable percentages (usually 40-50%) (Callahan et al., 1993; LeMaoult et al., 2000), and the same phenomenology has been previously described in humans (Hingorani et al., 1993; Posnett et al., 1994). These $CD8^+$ expansions are monoclonal, can usurp up to 80% of the total $CD8^+$ T cell repertoire (rev. in. (Clambey et al., 2007; Clambey et al., 2005)) and exhibit a uniform central memory phenotype, being $CD8^+CD25^-CD69^-CD44^{hi}CD62L^{hi}CD27^{hi}CD127^{hi}Ly\text{-}6C^{hi}$ $CD43^{int}CD122^{hi}$ (Messaoudi et al., 2004). Their onset can be accelerated and the incidence elevated by lymphopenia (Messaoudi et al., 2006b), and they express higher levels of CD127 and CD122 compared to their normal central memory counterparts that

are not monoclonal nor expanded (Messaoudi et al., 2006c). Given those characteristics and the observation that they do not seem to be selected by antigen (they appear to stochastically express different TCRVαβ protein combinations and were shown to exhibit no particular reactivity pattern), we proposed that these cells are selected by the ability to preferentially respond to homeostatic cytokines and have provisionally classified them as antigen-independent (AI)-TCE (Messaoudi et al., 2006b; Messaoudi et al., 2006c)). We note that the antigen-independent designation refers to their expansion and maintenance, and not necessarily that these cells were never in contact with antigen.

We have already noted marked similarities between the AI-TCE and the expanded memory cells described in this study in the time of onset (both diagnosable after 18 mo of age) and cell surface phenotype. To test whether the expanded memory cells seen in aging mice after life-long ocular HSV-1 infection share other cardinal features of TCE, we first performed TCR Vβ repertoire and CDR3 length analysis on several animals exhibiting pronounced memory inflation, taking advantage of the fact that the anti-gB-8:K^b $CD8^+$ T cells preferentially utilize TCRVβ8 and 10 elements (Cose et al., 1995). Upon TCRVβ staining, we saw the largely expected frequencies of TCR Vβ8 and 10, suggesting that both families are being used in the response, with neither fully dominating, which would be expected if the response was clonal (not shown).
Figure 3.5 A shows the results of CDR3 length analysis. Here too we failed to see the pronounced TCE-like clonality amongst the cells undergoing MI, although several of them exhibited signs of restricted, even oligoclonal, CDR3 profiles (Fig. 3.5 A). Therefore, it appeared that the expanded memory cells, while clearly antigen-selected ($tetramer^+$), were mainly not monoclonal. We next examined in more detail the levels of

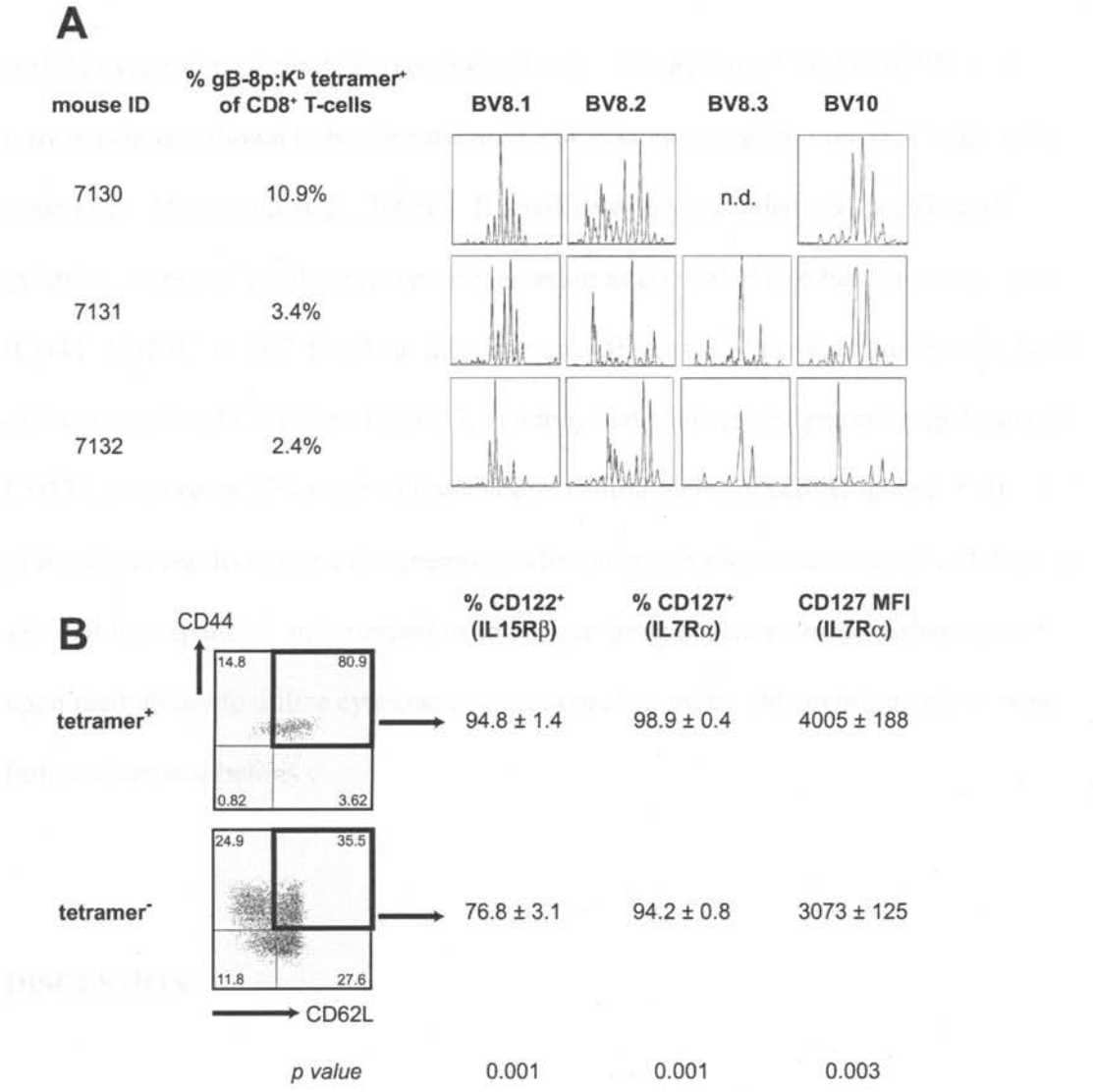

Figure 3.5 Clonal composition and expression of IL7Rα and IL15Rβ by expanded gB-8p-specific memory CD8$^+$ T cells in old mice **A.** CDR3 length analysis of the BV segments involved in gB-8p-specific CD8$^+$ T cell response in mice undergoing late-age memory inflation. CD8$^+$ T cells from mice exhibiting old-age memory inflation were purified using magnetic bead separation, the RNA was extracted and the diversity of CDR3 lengths of BV8 (8.1, 8.2 and 8.3) and BV10 segments was determined as described in Materials and Methods. CDR3 profiles from three representative mice are shown. **B.** Elevated expression of IL7Rα (CD127) and IL15Rβ (CD122) on expanded gB-8p-specific memory cells in old mice. Splenocytes from old latently infected mice (n = 6) undergoing old-age memory inflation were stained with CD8, CD44, CD62L, tetramer, CD127 and CD122. The percentage of CD122$^+$ and CD127$^+$ cells as well as the mean fluorescent intensity (MFI) of CD127 was compared between central memory phenotype (CD44$^+$ CD62L$^+$) tetramer$^+$ (top panels) or tetramer$^-$ (bottom panel) CD8$^+$ T cells. The values represent the average ± SD (first two rows) or the p-value (obtained from paired Student's t-test comparing the values from the tetramer$^+$ and tetramer$^-$ fraction of cells of individual mice), and the data is representative of two independent experiments).

surface expression of cytokine receptors CD122 (IL15Rβ) and CD127 (IL7Rα), which were previously shown to be elevated on AI-TCE as compared to non-TCE cells in the same mice (Messaoudi et al., 2006c). Remarkably, the expanded gB-specific cells exhibited increased cytokine receptor expression as compared to other central memory ($CD44^+$ $CD62L^+$) $CD8^+$ T cells in the same mice (Figure 3.5 B). Specifically, nearly all of them expressed CD122 and CD127, and they had an increased per cell expression of CD127, expressing 25% more of it than other central memory cells (Figure 3.5 B). All of the above results suggest that memory cells undergoing late-age memory inflation are selected for expansion and survival in an antigen-independent manner, perhaps based upon their ability to utilize cytokine resources present in the old environment, an issue further discussed below.

DISCUSSION

Latent viruses – initiators or drivers of age-related memory dysregulation?

Accumulating evidence has linked persistent (viral) infections and T cell senescence (rev. in (Pawelec et al., 2004)). Here, we used the term "persistent" to indicate that the virus remains associated with the host, without any reference to the status of viral replication. Defined as such, persistent viruses can be either chronic (continuously replicating; e.g. HIV, HCV) or latent (HSV, VZV, CMV). Given that chronic viruses often produce disease even in immunocompetent hosts, they are considered not to be associated with normal aging.

Latent viruses, on the other hand, coexist with immunocompetent hosts for life, and are therefore considered as potential partners of the aging process. HSV-1 is a persistent, latent virus, that initially replicates at the site of epithelial/mucosal entry, enters the sensory nerves, and travels to the nearest sensory ganglia and the CNS, where it is ultimately contained by the $CD8^+$ T cells ((Divito et al., 2006; Lang and Nikolich-Zugich, 2005; Pawelec et al., 2004) and references therein). Once controlled, the virus establishes latency for the life of the organism, remaining in the ganglia, and, possibly, the brain (Liu et al., 2000; Thomas et al., 1997). In mice, HSV never shows clinical signs of spontaneous reactivation (Freeman et al., 2007; Thackray and Field, 1996), and this lack of clinical reactivation may be due to $CD8^+$ T cells, which were proposed to control the virus in the ganglia prior to manifest clinical reactivation (Khanna et al., 2003; Liu et al., 2001; Liu et al., 2000; Sheridan et al., 2006).

So is this virus contributing to CD8 T cell aging and how? We show that following local infection in youth, HSV-1 memory is maintained at stable, low levels throughout the adulthood in mice. However, old age leads to improper memory maintenance, marked by an accumulation of memory cells specific for the original immunodominant peptide antigen, gB-8p. One possibility is that memory accumulation arises due to a loss of antiviral $CD8^+$ T cell function (primary or due to exhaustion by stimulation), which results in viral reactivation, feeding into a positive feedback loop, whereby the virus continuously stimulates the immune system and contributes to the accumulation of memory T cells. The $CD8^+$ T cells found at the sites of latency (trigeminal ganglia, brain) bear the effector-memory phenotype ((Khanna et al., 2003; Lang and Nikolich-Zugich, 2005; Liu et al., 2000) and A.L. & J.N-Z., unpublished

observations), whereas, the HSV-specific CD8 T cells which accumulate with aging exhibit uniform central memory phenotype. This suggests that the exchange of cells between the latently infected organs and the systemic virus-specific $CD8^+$ T cell pool, if any, is probably numerically negligible. Neither the levels of latent virus nor the inhibition of viral replication by antiviral drugs had any impact upon the extent of the late-life memory cell accumulation. Moreover, the accumulating cells were highly positive for the receptors for common γ chain cytokines IL-7 and IL-2/15. The sum of our results, therefore, argues against the Ag-driven accumulation.

Homeostatic disturbances are critically involved in T cell memory dysregulation

It is pertinent to compare and contrast the above results with other cases of dysregulation of the $CD8^+$ memory T cell compartment. Systemic infection with MCMV was shown to induce rapid (detectable in 2-4 m.p.i.) and vigorous memory inflation (Karrer et al., 2003; Sierro et al., 2005) . Such cells bear all the characteristics of repeated antigenic stimulation and the phenotype of effector memory T cells ($CD62L^{lo}$, $CD27^{int/lo}$), and can accumulate to impressive levels, up to 20-30% of the total CD8 compartment. Accumulating cells in our current study, in a model that closely mimics natural human infection with HSV-1, share very few common characteristics with these MCMV-specific cells, differing in phenotype (central vs. effector memory), time of onset (>18 mo, and 12-16 m.p.i., vs. 2-4 m.p.i.) and the extent of accumulation (2.4-23% vs 20-30% of $CD8^+$ T cells).

This prompted us to compare and contrast the properties of expanded memory cells from this study to another dysregulated memory T cell type, the spontaneously

arising TCE. Spontaneously arising TCE are clonal in nature (Callahan et al., 1993; Hingorani et al., 1993; LeMaoult et al., 2000; Posnett et al., 1994), they exhibit the central memory phenotype (Ku et al., 2001; Messaoudi et al., 2006b), can make up to 80% of the total $CD8^+$ T cell pool, their onset can be accelerated and incidence increased by lymphopenia and/or increased turnover (Messaoudi et al., 2006b) and they express elevated levels of the receptors for key cytokines that regulate survival, maintenance and homeostatic proliferation of T cells, IL-7 and IL-15 (Messaoudi et al., 2006c). The sum of characteristics of HSV-specific cells undergoing late-onset memory inflation in this study is reminiscent of the spontaneously arising TCE. Two features differed between these cells and TCE: most gB-specific expanded cell populations did not appear to be fully clonal in nature (although evidence for oligoclonality was present), and their upper level of expansion documented so far (23% of total blood $CD8^+$ T cells) fell short of the very large spontaneous TCE (which can make up 80% or more of $CD8^+$ T cells). All other characteristics were either highly similar or superimposable between the two.

Based upon the above discussion, we propose the following scenario for the generation of expanded T cell populations in old age. Memory T cell pool will be formed by antigenic contact, generating larger or smaller Ag-specific subsets. From these subsets, the aging microenviroment will select cells based on their ability to survive and accumulate in response to homeostasis-regulating cytokines. Thus, antigenic stimulation plays a role only inasmuch as it contributes to the generation of the total memory T cell population. Because this process is not antigen-driven, the selected cells will carry random repertoire of T cell receptors. The most successful selectees will accumulate to large numbers, and will become large TCE. This is consistent with the results of Callahan

et al. (Callahan et al., 1993), as well as our own data (LeMaoult et al., 2000), where no enrichment for particular T cell specificity or TCRVβ segment could be linked to the "spontaneously" arising TCE. Accordingly, we believe that the gB-8p:K^b-specific CD8 T cells that accumulate with aging have been selected stochastically from the available pool of memory cells. We would predict that in a model where memory inflation is even more pronounced, such as is the case with systemic MCMV infection (Holtappels et al., 2000; Karrer et al., 2003), the dysregulation of memory homeostasis in old age would include an even higher frequency of MCMV-specific T cell expansions. The mouse model of aging and infection with HSV-1, MCMV and other persistent pathogens should be highly conducive to conclusively test this hypothesis.

Summary

Here we demonstrated that following localized infection with HSV-1, stable antiviral memory $CD8^+$ cell pool is maintained for most of the lifespan, until this maintenance becomes dysregulated late in life. The dysregulation of memory maintenance manifested itself by development of expansions of gB-8p-speicifc memory cell populations in some mice. This age-related dysregulation was independent of viral reactivation. Based on these results, it could be inferred that lifelong infection of mice with HSV-1 is truly latent and not associated with significant viral reactivation. Conversely, if HSV-1 periodically reactivated, one would expect to detect evidence for that in the phenotype and possibly changes in the size of the virus-specific memory $CD8^+$ T cell pool over time. While

latency in mice was previously believed to be absolute (i.e. no viral reactivation), recent literature suggests that viral reactivation from latency is possible in mice, and is a relatively common event (see Chapter 1). Therefore we wanted to further investigate the issue of the relationship of latent and possibly reactivation-prone infection with the dynamics of maintenance of memory $CD8^+$ T cell pool. Localized infection results in containment of the virus predominantly in the TG, however depending on the virus administration route, tissue accessibility of the virus could be modified. We were curious how differences in the initial viral load and viral spread, which we reasoned would affect the latent viral load and number of latently infected cells and tissues, would in turn affect the subsequent maintenance of CD8 memory. These issues will be investigated in the next chapter.

CHAPTER 4

Systemic HSV-1 infection is associated with ongoing antigenic stimulation of peripheral memory CD8$^+$ T cells

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ABSTRACT

Herpes Simplex Virus (HSV-1) establishes a lifelong latent infection in the neurons of the peripheral and central nervous system. While periodic viral reactivation is commonly associated with HSV-1 infection in humans, spontaneous viral reactivation was believed until recently to be extremely rare in mice, leading to the assumption that in the murine model the latent virus is invisible to the immune system. However, recent studies, both virological and immunological, suggest that this may not be true. Rather, evidence is accumulating for a more dynamic interaction of the persisting virus and the immune system during the lifelong infection, particularly involving HSV-1-specific memory $CD8^+$ T cells. We have analyzed the effect of lifelong HSV-1 infection on the maintenance of virus-specific memory $CD8^+$ T cells present in the systemic circulation and away from the latent virus reservoir. In this study, we show that the size and phenotype of the systemic memory $CD8^+$ T cell pool is responsive to treatments that modulate the extent of viral spread and viral reactivation *in vivo*. Under conditions of extensive systemic viral spread and in the absence of control of viral reactivation, HSV-specific memory $CD8^+$ T cells continue to expand over time, and the development and maintenance of these memory $CD8^+$ T cell expansions depends on the presence of antigen and is in part driven by antigen-induced proliferation.

INTRODUCTION

Herpes Simplex Virus type 1 (HSV-1) is an α-herpesvirus that establishes lifelong latent infection in the neurons of the peripheral and central nervous system. In humans, this lifelong infection is associated with periodic viral reactivation and migration of the reactivated virus from the site of latency into the periphery, usually to the site of primary infection. It was thought for a long time that in mice, unlike in humans, HSV-1 reactivation from latency is a very rare event (Shimeld et al., 1990; Willey et al., 1984), and some studies even argued that it does not happen at all (Gebhardt and Halford, 2005). This lead to the belief that during lifelong infection the virus remained invisible to the murine immune system. However, despite the difficulties in demonstrating viral shedding in mice after resolution of primary infection, several observations suggested that latent HSV-1 infection in mice is not as silent as previously thought. First, several studies demonstrated ongoing presence of inflammatory cell infiltrates at the site of latency, associated with an elevated level of proinflammatory cytokines (Cantin et al., 1995; Halford et al., 1996; Liu et al., 1996; Shimeld et al., 1990; Willey et al., 1984). Both presence of the inflammatory cell infiltrates and inflammatory cytokines could be curtailed by administration of acyclovir (Halford et al., 1997). More specifically, HSV-1-specific $CD8^+$ T cells of effector memory phenotype were demonstrated to be continually present in the trigeminal ganglia (TG) of ocularly infected mice (Khanna et al., 2003; Suvas et al., 2006)and to proliferate at a greater rate than memory $CD8^+$ T cells present in an uninfected lung (Sheridan et al., 2006). In addition, only virus-specific cells were retained in the latently infected ganglia and the maintenance of their activated phenotype depended on the ability of the host to present antigen (van Lint et al., 2005).

This suggested ongoing presentation of viral antigen(s) within the tissue serving as the reservoir of the latent virus. Recent data provided some evidence for that. First, low level of expression of lytic viral genes, termed "spontaneous molecular reactivation", was documented in unmanipulated immunocompetent mice (Feldman et al., 2002). This finding was accompanied by demonstration of elevated number of viral genomes in some latently infected neurons *in vivo*. Finally, infectious virus was demonstrated to be present in a small proportion (6%) of latently infected murine TG without any treatment to induce viral reactivation (Margolis et al., 2007). Based on these data, a new view of HSV-1 latency is emerging, in which the consequences of periodic viral reactivation should be considered.

One of such potential consequences is the effect of the periodic viral reactivation on the maintenance of memory $CD8^+$ T cells specific for HSV-1. Much work has been done analyzing the relationship of the latent virus with the memory $CD8^+$ T cells present in infected sensory ganglia (Khanna et al., 2003; Sheridan et al., 2006; Suvas et al., 2006), however less is known about the effect of the lifelong HSV-1 infection on the numbers and phenotype of systemic $CD8^+$ T cell pool. Given that spontaneous viral reactivation may take place in mice, we expected to find some evidence of ongoing or periodic activation of the systemic memory $CD8^+$ T cells of HSV-1 infected mice. However, our analysis in ocularly infected C57BL/6 (B6) mice of systemically circulating memory $CD8^+$ T cells specific for gB-8p, the dominant HSV-1 epitope recognized by $CD8^+$ T cells, demonstrated that these memory cells were maintained at a stable frequency and displayed resting central memory phenotype, arguing against the possibility of ongoing interaction with viral antigens (Chapter 3). In addition, the size

and phenotype of the memory $CD8^+$ T cell pool was the same in mice treated with antiviral drug famciclovir, further arguing against the ongoing viral antigen presentation in this infection model.

In the present study, we wanted to further analyze the impact of lifelong HSV-1 infection on memory $CD8^+$ T cell maintenance in B6 mice. We speculated that one of the reasons for the seeming lack of interaction of the persisting virus with the systemic $CD8^+$ T cell pool was the very localized nature of the ocular infection model used in our earlier experiments. During ocular infection, the virus spread is very restricted, limited strictly to the corneal epithelium, the neurons in the TG and the brain (Lang and Nikolich-Zugich, 2005). Therefore the accessibility of viral antigens for presentation to $CD8^+$ T cells during a reactivation event would be expected to also be very limited outside of these tissues. To test this prediction, we compared the $CD8^+$ T cell response generated by a localized (ocular) infection to that resulting from systemic (intraperitoneal, or i.p.) infection with HSV-1. We speculated that modulating the initial viral titer, or the viral systemic spread, or both, might change the accessibility of viral antigens during reactivation events and possibly result in periodic activation of systemic memory $CD8^+$ T cell pool.

We demonstrate that the size and phenotype of HSV-1-specific $CD8^+$ T cell systemic memory is dependent on presence of antigen, and can be modulated by regulating the extent of viral spread and viral reactivation. Our results show that akin to the murine cytomegalovirus (MCMV), HSV-1 is also capable of driving expansion of antiviral $CD8^+$ T cell pool even after resolution of primary infection, a process termed "memory inflation" (Holtappels et al., 2000; Holtappels et al., 2002; Karrer et al., 2003).

This model may prove useful in studying the effect of persisting infections associated with ongoing antigen presentation on the homeostasis of not only virus-specific $CD8^+$ T cells, but also of the whole $CD8^+$ T cell compartment. Persisting viruses, both chronic and latent, have been implied to affect the homeostasis of the $CD8^+$ T cell compartment, primarily by decreasing its T cell receptor (TCR) diversity due to accumulation of large expansions of memory-phenotype $CD8^+$ T cells (rev. in (Akbar and Fletcher, 2005; Davenport et al., 2007; Pawelec et al., 2005)). Therefore, availability of a suitable mouse model in which the relative contribution of the virus-driven and virus-independent factors in affecting the diversity and composition of the $CD8^+$ T cell compartment could be precisely dissected should be of considerable interest.

MATERIALS AND METHODS

Mice. Male C57BL/6-NCr (B6) mice and B6-Ly5.2/Cr (Ly-5.2) were purchased from the National Cancer Institute colony (Frederick, MD). B6.gBT-I (gBT-I in the text) TCR transgenic(Tg) mice (Mueller et al., 2002a), carrying rearranged *tcr* genes encoding TCR that recognizes the immunodominant HSV-1 epitope gB-8p + $H\text{-}2K^b$, were generously provided by Dr. F. R. Carbone (University of Melbourne, Melbourne, Australia). All animals were housed under the SPF conditions, and experiments conducted under IACUC approval and in accordance with the applicable federal, state and local regulations.

Viruses and viral infections. HSV-1 strain 17 obtained from Dr. D.J. McGeoch (University of Glasgow, Scotland, UK), cloned as a syn+ variant and titered on Vero cells

in our laboratory, was used in all experiments. Localized (intracorneal, i.c.) and intraperitoneal (i.p.) infections with 10^6 PFU HSV-1 per mouse were performed as described (Lang and Nikolich-Zugich, 2005). Recombinant Vaccinia Virus expressing the SSIEFARL minigene (rVV-gB-8p) was a generous gift from Dr. Satvir Tevethia (Pennsylvania State University, Hershey, PA) and was grown on thymidine kinase deficient L-cells and titered on Vero cells. Mice were infected i.p. with $4x10^6$ PFUs of rVV-gB-8p.

Determination of titers of replicating virus. The amount of replicating virus in the indicated organs (Table 4.1) of acutely (day1, 3 or 5 p.i.) infected mice was determined by plaque assay of TG homogenates as previously described (Lang and Nikolich-Zugich, 2005).

Famciclovir treatment. Where indicated, Famciclovir (Famvir, Novartis) was administered to mice in their drinking water at a concentration of 2mg/ml. The famvir water was changed twice a week.

BrdU labeling Mice were given BrdU in drinking water at 0.8 mg/ml as described elsewhere (Messaoudi et al., 2006b; Tough and Sprent, 1994) for 3 weeks. BrdU incorporation was measured by FCM using a kit from BD Pharmingen as per the manufacturer's recommendation.

Adoptive transfers and CFSE labeling. Splenocytes from naïve young female gBT-I transgenic mice or from HSV-immune mice were enriched for CD8 T cells by depletion of CD4 and B220 cells by MACS (Miltenyi Biotec) according to the manufacturer's instructions. Magnetic depletion yielded a cell population that was >80% $CD8^+$. Splenocytes containing $3 x10^6$ gB-8p :K^b $tetramer^+$ naïve $CD8^+$ T cells

(naïve gBT-I cell transfers) were adoptively transferred into each congenic B6-Ly5.2/Cr female recipient by i.v. injection 24 hours prior to infection, exactly as described previously (Lang and Nikolich-Zugich, 2005). The cells were labeled with 2μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) prior to adoptive transfer (10' incubation at 37°C). Alternatively, splenocytes containing $3x10^5$ gB-8p :K^b tetramer$^+$ memory $CD8^+$ T cells (memory cell transfers) were transferred into congenic naïve or HSV-1 infected B6-Ly5.2/Cr mice. The transferred cells were distinguished from the recipient T cells by FCM detection of the CD45.2 molecule (mAb clone 104, BD Pharmingen).

Reagents, antibodies and flow cytofluorometric (FCM) analysis. The gB-8p peptide (SSIEFARL) was purchased from SynPep Corporation (Dublin, CA), and the gB-8p :K^b tetramer was obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Monoclonal antibodies were purchased from commercial sources.

FCM analysis was performed as previously described (Lang and Nikolich-Zugich, 2005). Anti-Ki-67 mAb was purchased from BDPharmingen and the intranuclear staining was performed per manufacturer's instructions. FCM data was acquired on FACSCalibur instrument using CellQuest 3.3 software or on the FACS LSRII instrument using the Diva software (Becton Dickinson, Mountain View, CA), and analysis performed using FlowJo software (Tree Star). At least 10^4 cells were analyzed per sample, with dead cells excluded by selective gating based on orthogonal and side light scatter characteristics.

Statistics. Student's t-tests were performed with Excel (Microsoft), using a 2-tailed analysis with equal variance.

RESULTS

Early onset of CD8$^+$ T cell memory inflation after systemic HSV-1 infection

To test whether the maintenance of gB-8p-specific memory CD8$^+$ T cells is affected by virus administration route, we compared memory responses generated by localized and systemic HSV-1 infection of B6 mice (Figure 4.1). There was no statistically significant difference in the extent of expansion during the effector phase (day 8 p.i., Figure 4.1 A), with the average frequency of HSV-specific CD8$^+$ T cells of 16.7% ± 3.8% (systemic infection) and 12.5% ± 5.8% (localized infection) of total CD8$^+$ T cell pool. By day 45p.i., however, the HSV-specific CD8$^+$ T cells exhibited significant differences between the cohorts. Systemically infected mice showed significantly higher percentages of antigen (Ag) specific cells than the ocularly infected mice (average of 8.6% and 2.6% of total CD8$^+$ T cell pool, respectively, p-value < 0.00001). Thereafter, the difference in the maintenance of memory CD8$^+$ T cells between mice infected via two different routes became even more pronounced. While the frequency of CD8$^+$ memory T cells remained stable in mice following localized infection, the memory CD8$^+$ T cell pool in systemically infected mice continued to expand over time (Figure 4.1 A). At all times during the memory phase, the difference in the percentage of gB-8p-specific CD8$^+$ T cells between locally and systemically infected mice was significant (Figure 4.1 A and B). Similarly, the numbers of memory CD8$^+$ T cells circulating in blood were also significantly greater following systemic infection (Figure 4.1 C). The onset of memory inflation was not likely a result of the particular conditions granted by the systemic infection route, because systemic infection with an acute virus, recombinant Vaccinia

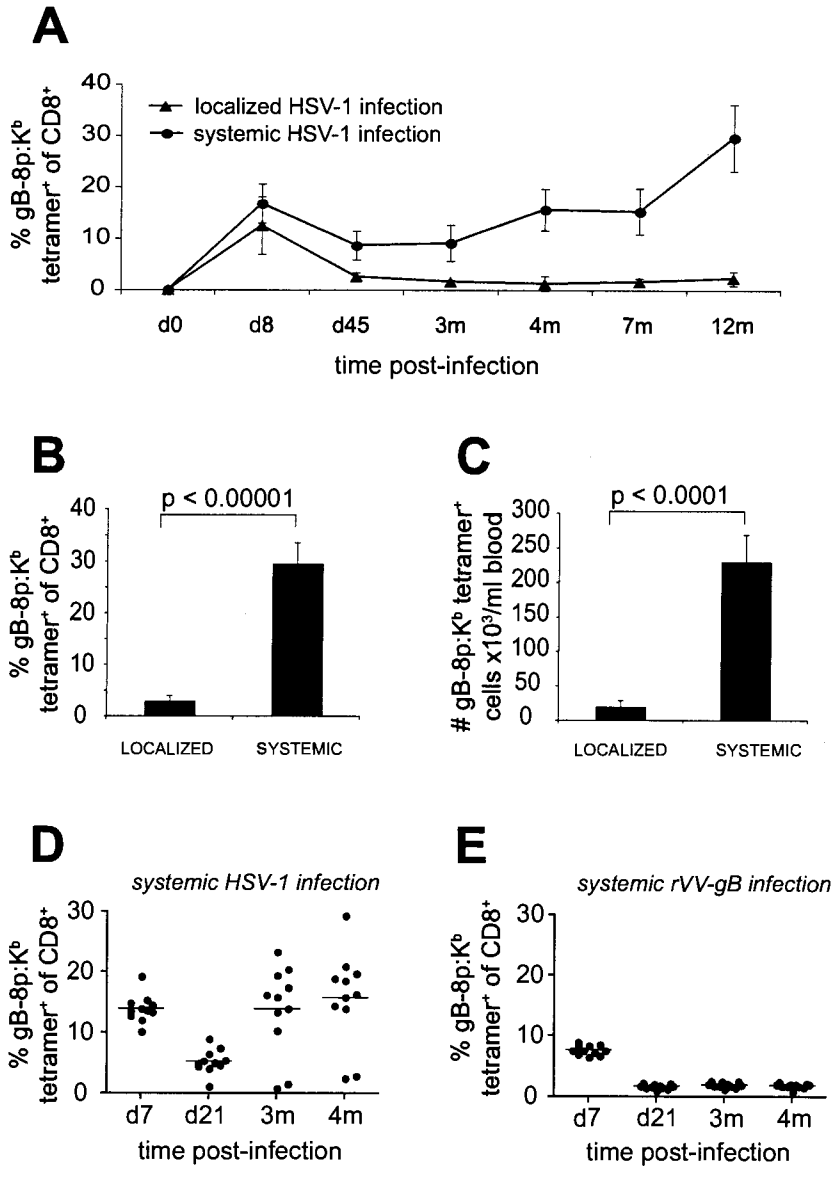

Figure 4.1 Early onset of memory inflation following systemic HSV-1 infection
A-C. Two cohorts of young mice were infected with 10^6 PFU HSV-1 via localized infection (triangles, n = 10) or systemic (circles, n = 8) infection route. Blood samples were taken at day 8, 45, and 3, 4, 7, and 12 m.p.i. and stained with anti-CD8 and gB-8p:K^b tetramer. The values **(A)** represent the average percent of gB-8p:K^b tetramer$^+$ cells within $CD8^+$ T cells over time (± SD). The difference in the percentage **(B)** and numbers **(C)** of tetramer$^+$ cells is statistically significant at 12 m.p.i. time-point. **D - E.** Infection with acute virus, rVV-gB, does not results in memory inflation. Two cohorts of young mice were infected with 10^6 PFU HSV-1 (**D**, n=11) or rVV-gB (**E**, n=11) via systemic infection route. Blood samples were taken at day 7, day 21, 3m.p.i., and 4 m.p.i. and stained with anti-CD8 and gB-8p:K^b tetramer. Each data point represents the percent of gB-8p:K^b tetramer$^+$ cells within $CD8^+$ T cells of individual mice. Memory inflation was observed only in the HSV-1 infected group.

Virus expressing the gB-8p epitope (rVV-gB) was not associated with memory inflation, when compared side-by-side to systemic infection with HSV-1 (Figure 4.1 D and E). Instead, rVV-gB infection resulted in generation of an effector and memory $CD8^+$ T cell response comparable in size to that generated by localized infection (Figure 4.1 E).

Peak viral load and systemic viral spread affect the process of memory inflation

The above data suggested that the difference in memory $CD8^+$ T cell maintenance following infection through localized and systemic route is related to the viral persistence beyond the primary infection, since mice systemically infected with an acute virus did not develop memory inflation. The extent of viral replication and/or spread is one factor that could directly influence memory inflation and explain the differences between the two infection routes. We have previously shown that HSV-1 is contained by $CD8^+$ T cells following ocular infection so that no infectious virus can be detected by day 10 (and often by day 8 in most mice) (Lang and Nikolich-Zugich, 2005), and that kinetics of elimination of replicating virus are similar, if not slightly more rapid, in systemically infected mice (data not shown). We therefore compared the viral titers (Figure 4.2) and systemic virus spread in select organs (Table 4.1) following localized and systemic infection.

Because of the known HSV tropism (Cunningham et al., 2006; Spear, 2004), we focused the analysis of the viral titers in the nervous system. We reasoned that the total amount of virus present in the nervous system would correlate with the viral load during latency, as has been suggested in the literature (Hoshino et al., 2007; Sawtell, 1998; Sawtell et al., 1998). Therefore we determined the total titer of HSV-1 in the nervous

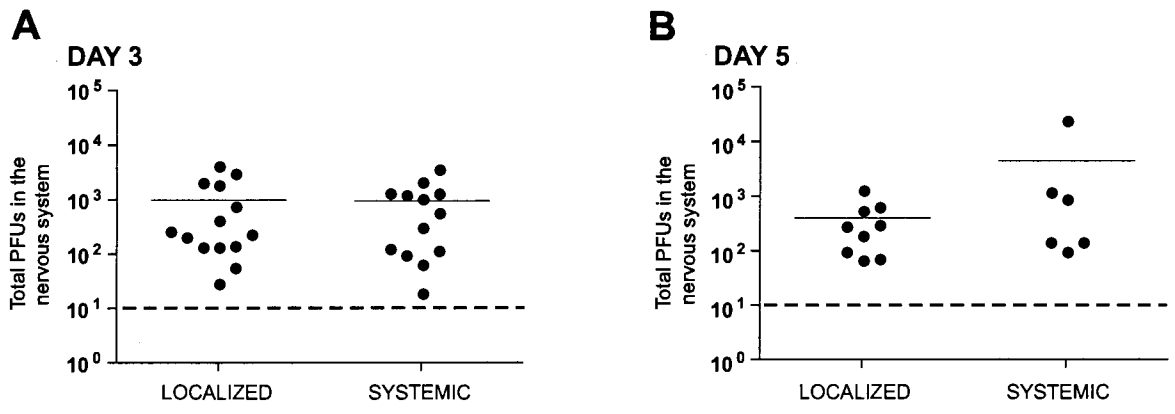

Figure 4.2 Total viral titers in the nervous system following systemic and localized HSV-1 infection Mice were infected with 10^7 PFU HSV-1 via the localized (n = 4) or systemic (n = 4) infection route. Tissues (brain, TG, spinal cord and associated ganglia) were harvested on days 3 and 5 p.i.. The amount of actively replicating virus present in the tissues at the time of harvest was determined by a plaque assay. The values show the combined total viral titer (PFUs) in the nervous system (brain, trigeminal ganglia, spinal cord and associated ganglia) of individual mice on day 3 **(A)** and 5 p.i. **(B)**. The dotted line represents the limit of detection (10 PFUs).

system by adding the viral titers obtained from all individual nervous tissues (TG, brain, spinal cord and associated ganglia) at the peak of viral replication on day 3 (Figure 4.2 A) and day 5 (Figure 4.2 B). Unexpectedly, the total viral titer in the nervous system was comparable between mice infected via the localized and systemic route. However, we found that as anticipated, systemic infection with HSV-1 resulted in much greater systemic viral spread than localized infection (Table 4.1).

Confirming our earlier results, the virus spread following localized infection was restricted to the eye, the TG, and in some mice brain (Table 4.1). In contrast, HSV-1 injected systemically was able to reach more tissues, including the fat pads, spleen, lung, liver, kidney and lymph nodes (Table 4.1). The viral spread differed between individual mice. Some organs, including the fat pads, spinal cord and the peritoneal cavity were uniformly infected at some point of infection in all systemically infected mice tested. The incidence of infection of other organs, including the lymph nodes, spleen, lung, kidney, liver, and brain was more variable, however viral spread to them was documented in significant proportion of mice. For example, about 50% of systemically infected mice had infectious virus present in their spleens on day 5, and in 44% of systemically infected mice the virus has spread to brain by day 5 (Table 4.1).

We conclude that despite having comparable viral titers in the nervous system at the peak of productive replication, the systemically and locally infected mice differ in the extent of systemic viral spread. The virus is able to spread to more tissues following systemic infection, which might result in establishment of more latent virus reservoirs. The exact spread of the virus within the nervous system was not mapped after systemic infection, but the increased spread through the parenchymal may allow the virus to access

infection route	day post-infection	spinal cord	brain	trigeminal ganglia	spleen	lungs	liver	eye	draining lymph nodes	kidneys	peritoneal wash	fat pads
localized	day 1	0/5	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5
	day 3	0/13	4/13	9/9	0/13	0/5	0/5	13/13	0/5	0/5	0/5	0/5
	day 5	0/9	7/9	9/9	0/9	0/5	0/5	9/9	0/5	0/5	0/5	0/5
systemic	day 1	5/5	0/5	0/5	1/5	0/5	1/5	0/5	2/5	4/5	5/5	5/5
	day 3	13/22	1/14	0/14	13/25	1/9	2/9	0/9	0/5	0/5	4/11	9/19
	day 5	3/9	4/9	0/9	0/9	0/9	1/9	0/9	0/5	0/5	0/5	0/5

Table 4.1. Differences in viral spread following systemic and localized HSV-1 infection Mice were infected as in Figure 4.2 and presence of actively replicating virus in the listed tissues at the time of harvest was determined by a plaque assay. The data shows number of mice with actively replicating virus in the given tissue over the total number of mice tested.

neurons innervating these sites, and from there travel to multiple ganglia and different sections of the central nervous system. In contrast, following localized infection the virus reaches only one ganglion (TG) and brain, where it is mostly restricted to the olfactory bulbs, the trigeminal pathway and brainstem (Esiri, 1982; Esiri and Tomlinson, 1984; Valyi-Nagy et al., 2000).

Kinetics of CD8$^+$ T cell activation following systemic and local infection suggest that memory inflation are dictated by events following acute infection

The above data could be interpreted to suggest that the difference in memory CD8$^+$ T cell maintenance is linked to differences in viral spread. However, it was also possible that infection via different routes resulted in generation of different CTLs, with different responsiveness to homeostatic stimuli, or different ability to survive beyond the effector phase. To discriminate between these possibilities, we determined the detailed kinetics of CD8$^+$ T cell expansion and contraction and the expression of molecules that mark activation and memory formation on CD8$^+$ T cells generated by infection via systemic and localized route.

We first examined the propensity of virus introduced by either route to induce proliferation of the indicator population of Ag-specific cells. To that effect, we performed adoptive transfers of fluorescein (CFSE)-labeled gBT-I TCR Tg cells (as previously described, (Lang and Nikolich-Zugich, 2005)) into congenic animals that were then infected by either route, and evaluated the kinetics of their *in vivo* proliferation. The systemic infection triggered proliferation of HSV-specific CTL precursors sooner than ocular infection (Figure 4.3 A, bottom panel). Proliferating HSV-specific CD8$^+$ T cells

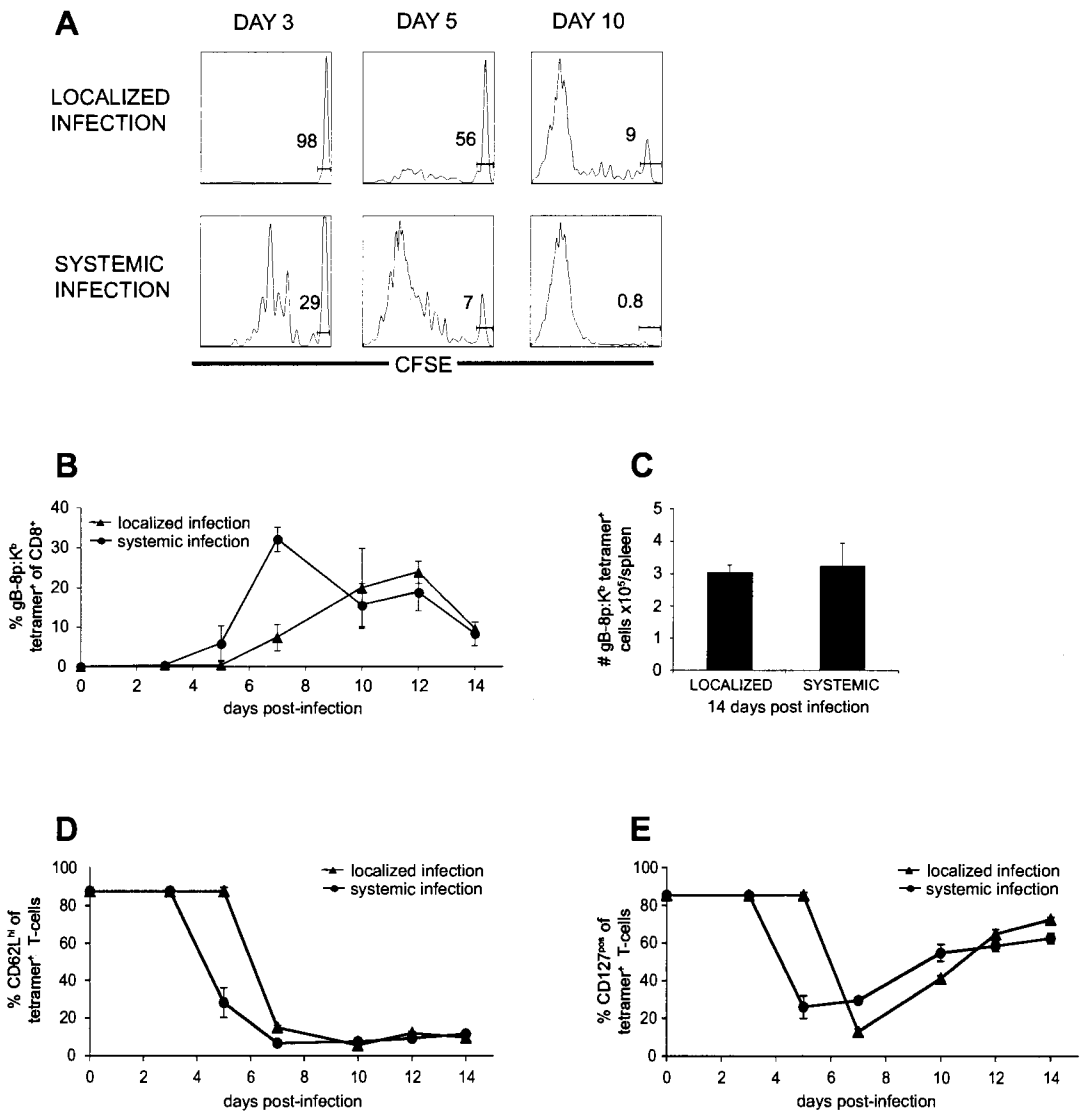

Figure 4.3

Kinetics, extent of expansion and phenotype of HSV-specific CD8$^+$ T cells following systemic and localized HSV-1 infection **A.** Splenocytes from naïve gBT-I TCR transgenic mice were labeled with CFSE and splenocytes containing 2x10^6 TCR transgenic CD8$^+$ T cells were transferred into each congenic Ly5.2$^+$ recipient. 24 hours post-transfer the recipient mice were infected with HSV-1 via the localized (n = 5) or the systemic (n = 5) infection route. Blood samples were taken at day 3, 5 and 10 p.i.. The graphs are gated on HSV-specific CD8$^+$ T cells (CD8$^+$ gB-8p:K^b tetramer$^+$ Ly5.1$^+$) from representative mice and show dilution of CFSE in HSV-specific CD8$^+$ T cells at a given time point. The numbers above each gate marker represent percent of undivided cells within the HSV-specific CD8$^+$ T cells. The data is representative of 2 independent experiments. **B.** Cohorts of B6 mice were infected with 10^6 PFUs HSV-1 via localized (n = 5, triangles) or systemic (n= 5, circles) infection route. Blood samples were taken at indicated time p.i.. and stained with anti-CD8 and gB-8p:K^b tetramer. The values show

the average percent of gB-8p:K^b tetramer$^+$ cells within $CD8^+$ T cells (± SD) The data is representative of two independent experiments. **C.** The mice were infected as in B. On day 14 p.i. the absolute number of HSV-specific $CD8^+$ T cells in spleens of infected mice was determined by staining splenocytes with gB-8p:K^b tetramer and CD8. The values show the average number of tetramer$^+$ $CD8^+$ T cells (± SD). **D -E.** Expression of CD62L (**D**) and CD127 (**E**) by gB-8p:K^b tetramer$^+$ $CD8^+$ T cells from part B was determined by FCM. The values show the average percent of $CD62L^{hi}$ and $CD127^+$ cells within gB-8p:K^b tetramer$^+$ $CD8^+$ T cells (± SD). The data is representative of two independent experiments.

were detected in blood as early as day 3 p.i. following systemic infection, whereas proliferating cells were only beginning to circulate in blood on day 5 post ocular infection (Figure 4.3 A, top panel). However, despite the delay in onset of proliferation, by day 10 the HSV-specific $CD8^+$ T cells have divided to the roughly same extent regardless of infection route, although there were more undivided cells and cells in the intermediate rounds of division following localized infection as compared to the systemic infection (Figure 4.3 A). This likely reflects the differences in the timing of Ag presentation, its dependence on viral replication, or the ease of Ag access to the APCs and subsequently the T cells.

Similarly, the analysis of frequency of endogenous $tetramer^+$ $CD8^+$ T cells in infected B6 mice showed that the early onset of proliferation of HSV-specific $CD8^+$ T cells correlated with different kinetics of $CD8^+$ T cell expansion, with the peak of expansion falling either on day 7 (systemic) or day 8 (ocular) p.i. (Figure 4.3 B). However, by day 12 p.i. and later, (by which time replicating virus can no longer be detected in any of the organs – (Lang and Nikolich-Zugich, 2005)) $CD8^+$ T cells in both groups have begun to contract, and by that time exhibited super-imposable frequencies (Figure 4.3 B), and absolute numbers (Figure 4.3 C). This is consistent with the possibility that the differences seen at later time points (beyond 1.5-2 months, Figure 4.1) are due to the events occurring after resolution of the acute infection.

We next examined the changes in surface expression of intermediate activation markers CD62L and CD127 in both groups. At the peak of the response the majority (>90%) of HSV-specific $CD8^+$ T cells were $CD62L^{lo}$ regardless of the fact that kinetic differences were noted in downregulation of this molecule between the two groups

between days 4 and 7 (Figure 4.3 D and E). The CD62L surface expression was still not regained by day 12 regardless of infection route (Figure 4.3 D). Similarly, the majority of effector CTLs lost surface expression of CD127 at the peak of the response (Figure 4.3 E). The greatest decline in surface CD127 expression was observed on day 5 after systemic infection (74% of tetramer$^+$ CD8$^+$ cells were CD127^{neg}) and on day 7 after localized infection (87% of tetramer$^+$ CD8$^+$ cells were CD127^{neg}). However, at day 12 p.i., when the infectious virus is no longer detectable and the gB-8p-specific CD8$^+$ T cells begin transition from effector to memory CTLs, virus-specific CD8$^+$ T cells generated by the two different routes exhibited the same CD62L/CD127 phenotype. Specifically, frequency of gB-8-p-specific cells within the CD8$^+$ pool was comparable, and their pattern of CD62L and CD127 expression was the same (Figure 4.3 B,C, D and E). Of importance, however, the expression of CD62L and CD127 became again significantly different between the groups during the memory phase. A representative phenotype of memory CD8$^+$ T cells in mice infected for 12 months is shown in Figure 4.4 A and summarized in Figure 4.4 B. CD62L expression begun to recover on Ag-specific CD8$^+$ T cells from mice infected through the localized route, whereas it remained very low on cells from systemically infected mice (Figure 4.4 A). More dramatically, CD127 was expressed on >90% of Ag-specific cells from locally infected mice, but on only less than half of the cells from systemically infected animals (Figure 4.4 A). Since the virus-specific CD8$^+$ T cells regain expression of CD127 early during the effector-to-memory transition phase (day 14 p.i., Figure 4.3 E), lack of CD127 expression by memory CD8$^+$ T cells 12 months p.i. indicates that it was downregulated by an activation event beyond the acute phase of infection. Also, expression of CD27 on

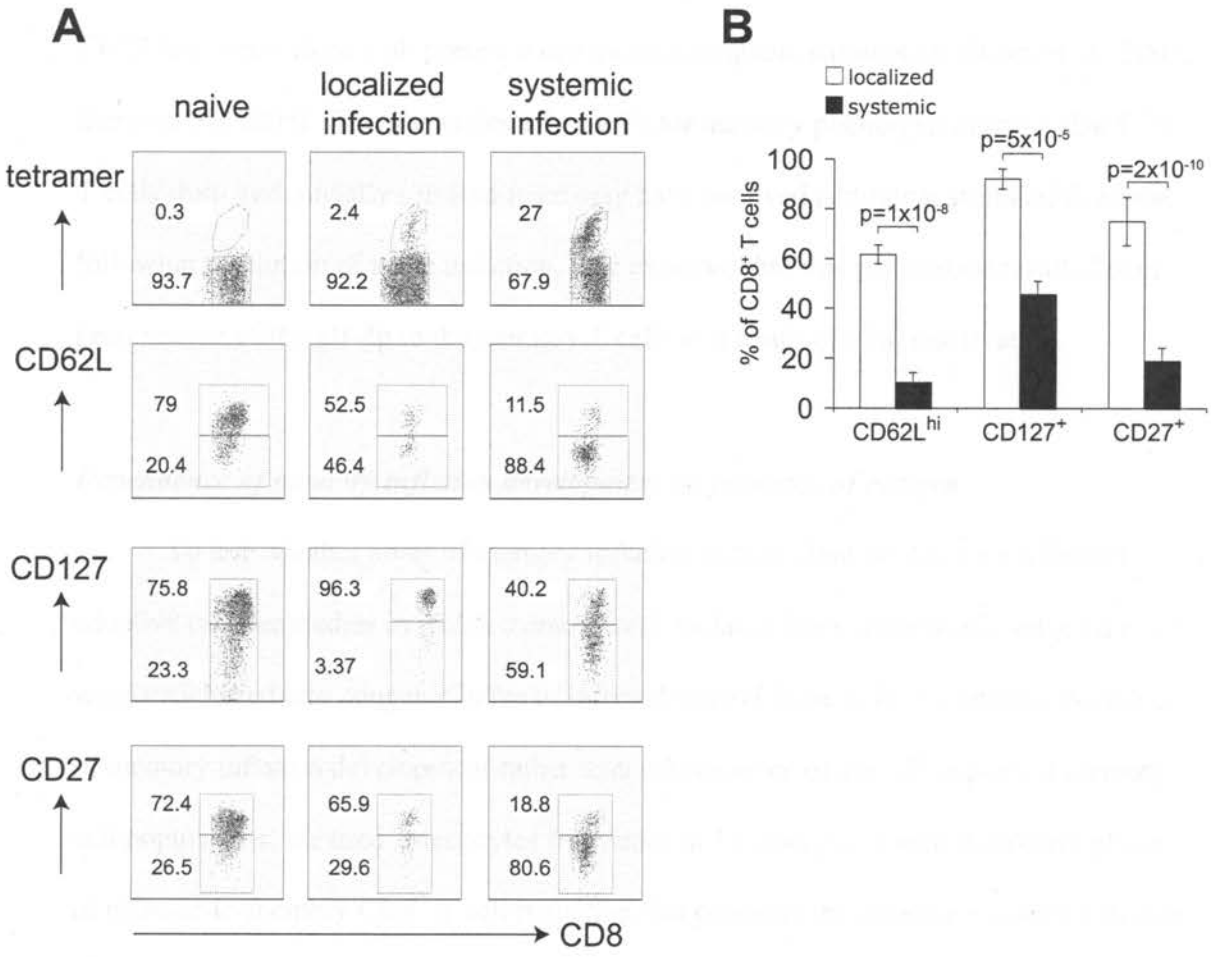

Figure 4.4 Memory cells in systemically infected mice undergoing memory inflation differ in their phenotype from non-inflating memory cells generated by localized HSV-1 infection. A. Examples of phenotypes displayed by inflating and non-inflating memory $CD8^+$ T cells. At 12 m.p.i., blood samples from mice infected with HSV-1 via localized or systemic route were stained with CD8, tetramer, CD62L, CD127, and CD27. The graphs are gated on $tetramer^+$ cells (infected mice) or on total $CD8^+$ T cells from naïve age-matched mouse. The numbers represent the percent of gated cells expressing the given marker, and are representative of values obtained from the entire experimental cohort. **B.** Summary of expression of CD62L, CD127 and CD27 on mice infected with HSV-1 via localized (n = 10) or systemic (n = 8) route. The values show the average (± SD) percentage of $CD62L^{hi}$, $CD127^+$ and $CD27^+$ within the $tetramer^+$ $CD8^+$ T cells at 12 m.p.i. The data is representative of 3 independent experiments.

$CD8^+$ T cells is downregulated in systemically infected mice as opposed to the locally infected mice or to $CD8^+$ T cells from age-matched naïve mice. Downregulation of CD27 has been linked with presence of repeated antigenic stimulation (Baars et al., 2005; Sierro et al., 2005). The above described effector memory phenotype suggests that $CD8^+$ T cells from systemically infected mice may have received additional stimulation *in vivo* following resolution of acute infection. We expected that this stimulation resulted from presentation of the gB-8p to the memory T cells as a result of viral reactivation.

Dependence of memory inflation development on presence of antigen

To test whether onset of memory inflation is dependent on Ag, we performed adoptive transfer studies in which memory cells isolated from systemically infected mice were transferred into congenic naïve or infected mice (Figure 4.5). To address the issue of memory inflation development rather than maintenance of already expanded memory cell populations, we used splenocytes from mice at 14 days p.i., which is an early phase of effector-to-memory $CD8^+$ T cell transition that precedes the onset of memory inflation. The recipients were either naïve, or infected via the localized or systemic route, and they were in the same stage of infection as the donor mice (day 14). The CD8-enriched splenocyte population containing $3x10^5$ gB-8p-specific $CD8^+$ T cells was transferred into each recipient and the frequencies of donor-derived (Figure 4.5 A) and host-derived (Figure 4.5 B) memory $CD8^+$ T cells were monitored in blood.

As demonstrated in Figure 4.5 B, the host-derived gB-8p-specific $CD8^+$ T cells followed the expected pattern of contraction and stable frequency maintenance (localized infection), or contraction followed by onset of memory inflation (systemic infection).

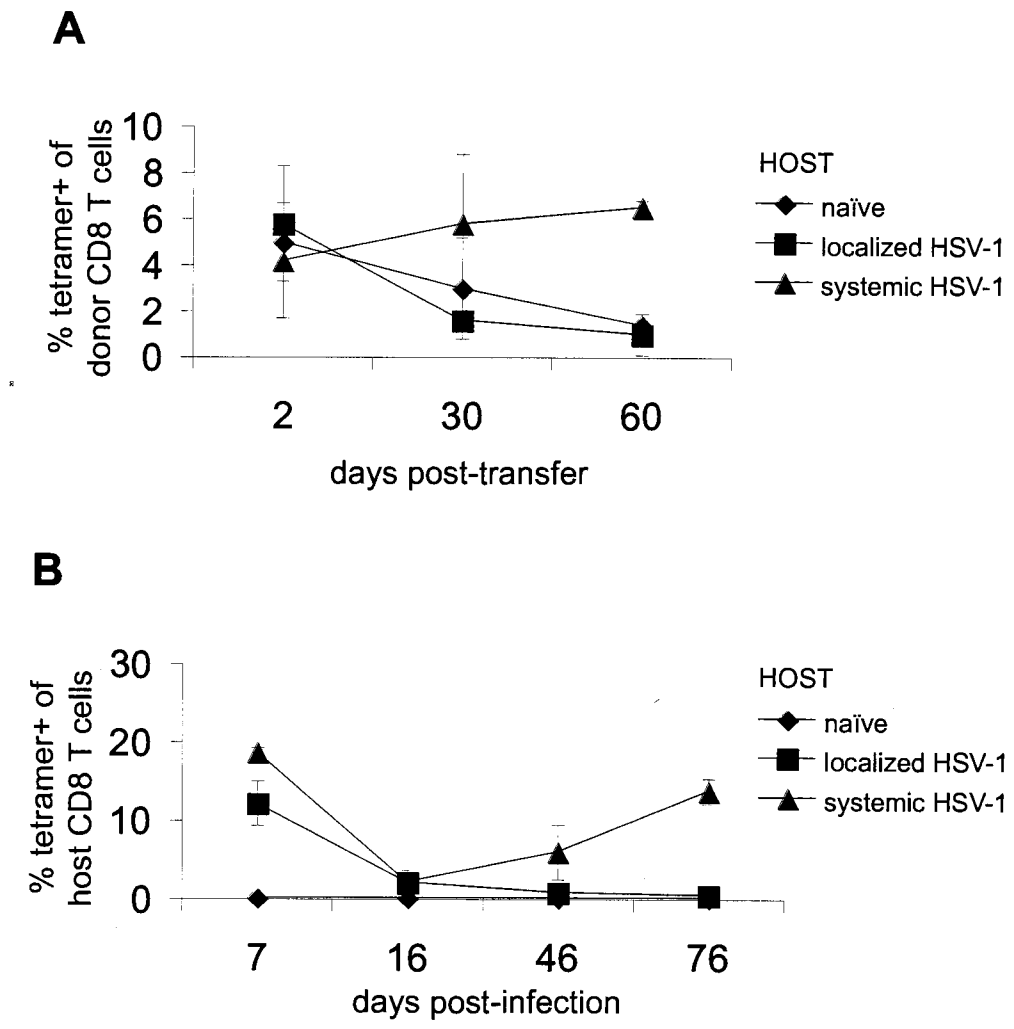

Figure 4.5 Dependence of memory inflation development on presence of antigen. CD8-enriched splenocytes (see Materials and Methods) pooled from mice infected i.p. with 10^6 PFU HSV-1 14 days earlier (n = 12, average % of tetramer$^+$ CD8 T cells in donors was 3.5% at the time of transfer) were transferred into congenic Ly5.2$^+$ recipients that were either naïve (n = 3), or infected with HSV-1 via localized (n = 3) or systemic (n = 3) route. Each recipient received $3x10^5$ gB-8p-tetramer$^+$ CD8$^+$ T cells. **A.** The percentage of tetramer$^+$ cells within the donor CD8$^+$ T cell population was monitored in blood on days 2, 30 and 60 post-transfer. Average values (±SD) are shown. **B.** The percentage of tetramer$^+$ cells within the host CD8$^+$ T cell population is shown as average (± SD).

The frequency of donor-derived memory $CD8^+$ T cells transferred into naïve or locally infected recipients declined over time, mirroring the maintenance pattern of the locally infected host's memory $CD8^+$ T cells, whose frequency also continued to slightly decline throughout the experiment (Figure 4.5 A and B). In the systemically infected host, the frequency of donor-derived memory $CD8^+$ T cells slightly increased (from the average of 4% at day 2 post-transfer to 6% at day 60 post-transfer). Throughout the experiment, donor memory $CD8^+$ T cells were able to maintain and somewhat increase their initial frequency, whereas memory cells transferred into naïve or locally infected mice declined in their frequency. These results suggest that presence of Ag helps maintain an elevated frequency of circulating memory $CD8^+$ T cells.

Dependence of memory inflation maintenance on presence of antigen

We next wanted to test whether maintenance of already expanded memory $CD8^+$ T cell populations requires presence of Ag. We performed an adoptive transfer study similar to the one above, except in this case we used $CD8^+$ T cells isolated from mice that were infected for 7 months (systemic infection) and already developed memory inflation (Figure 4.6). CD8-enriched splenocytes containing $3x10^5$ gB-8p-specific $CD8^+$ T cells were adoptively transferred into naïve congenic recipients, and their frequency was monitored in blood for 120 days. The average frequency of the $tetramer^+$ cells within the donor $CD8^+$ T cells was 18% ex-vivo, and was similar early after transfer (14.8%, day 2). However, this frequency declined over time, down to only 2.8% of donor $CD8^+$ T cells being $tetramer^+$ 120 days post-transfer. This experiment is in agreement with the adoptive transfer experiment discussed above, in that it suggests that maintenance of an

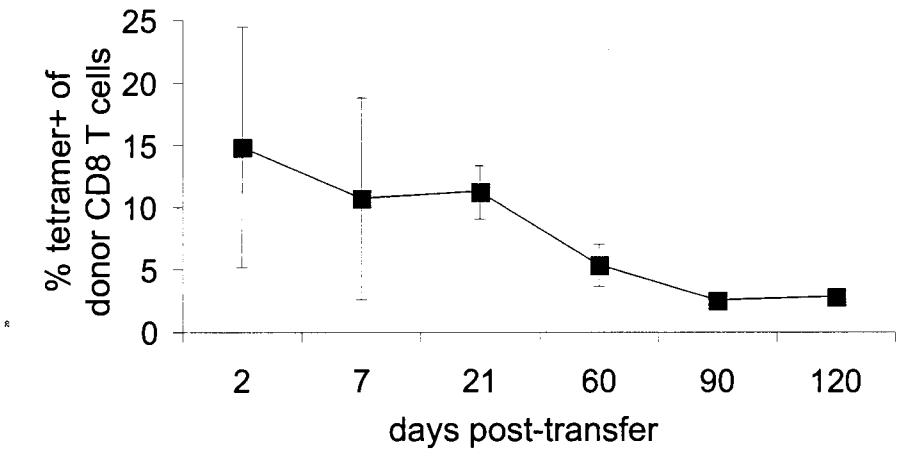

Figure 4.6 Dependence of memory inflation maintenance on presence of antigen. CD8-enriched splenocytes (see Materials and Methods) from mice infected i.p. with 10^6 PFU HSV-1 7 months earlier (n = 3, average % of tetramer$^+$ CD8 T cells in donors was 18% at the time of transfer) were transferred into naïve congenic Ly5.2$^+$ recipients (n = 3). Each recipient received $3x10^5$ gB-8p-tetramer$^+$ CD8$^+$ T cells. The percentage of tetramer$^+$ cells within the donor CD8$^+$ T cell population was monitored in blood on days 2, 7, 21, 60, 90 and 120 post-transfer. Average values (±SD) are shown.

elevated frequency of memory $CD8^+$ T cells is dependent on presence of antigen. However, the survival of some of the memory $CD8^+$ T cell population was not absolutely dependent on the presence of antigen, as the transferred memory cell populations were still detectable 120 days post-infection. However, as the phenotype of the transferred cells was heterogeneous (Figure 4.4), we cannot at the present say whether cells of both central and effector memory phenotype exhibit the same survival. Studies involving more mice and monitoring the survival of sorted transferred cell populations for longer periods of time are needed to definitively address the role of Ag in development of maintenance of expanded memory $CD8^+$ T cell populations specific for HSV-1.

Approach to modulate memory inflation by interfering with viral replication

Another way to test the dependence of Ag on the development and maintenance of HSV-1-specific memory $CD8^+$ T cell expansions is by regulating the viral load and extent of viral reactivation in the infected mice. We attempted to accomplish this in a series of experiments where treatment with antiviral drug famciclovir was used to limit the extent of viral replication and spread during primary infection (Figure 4.7), or to prevent viral reactivation during the latent phase (Figure 4.8).

Famciclovir, also known as famvir, is an inhibitor of HSV-1 replication (Simpson and Lyseng-Williamson, 2006). It is a nucleoside analog with strong affinity for the viral, but not cellular DNA polymerase. Famciclovir is ingested in its inactive form, and becomes activated by viral thymidine kinase (TK), selectively targeting cells with actively replicating virus (Darby et al., 1981; Datta et al., 1980; LeBlanc et al., 1999; Simpson and Lyseng-Williamson, 2006). We and others have previously demonstrated

that famvir treatment during acute ocular HSV-1 infection successfully blocks viral replication *in vivo* (Chapter 3 and (LeBlanc et al., 1999)). Now we decided to use famvir during systemic infection to test the relationship between viral replication and memory inflation.

Decreasing viral load, viral spread and viral reactivation can prevent memory inflation following systemic HSV-1 infection

In a first set of studies we administered famvir to mice during primary systemic infection in order to limit the extent of viral replication and spread. Three cohorts of mice were analyzed: 1) control HSV-1-infected group, 2) HSV-1-infected group treated with famvir starting on day 1 post-infection and continuing thereafter, and 3) HSV-1-infected group that was pre-treated with famvir for seven days prior to infection (Figure 4.7). We reasoned that in famvir pretreated group, viral replication would be abrogated and therefore establishment of latency would be prevented. Mice treated with famvir from day 1 p.i. onwards would have some, but limited level of viral replication and spread as compared to famvir-untreated control HSV-infected group.

Figure 4.7 shows the comparison of the antiviral $CD8^+$ T cell response and viral titers between the control HSV-1-infected mice and the group pretreated with famvir for 7 days prior to infection. Pretreatment with famvir (Figure 4.7 H and I) significantly reduced, and in most cases completely blocked viral replication in the fat pads close to injection site (Figure 4.7 H) and prevented viral spread (no virus present in spleens, Figure 4.7 I). The gB-8p-specific $CD8^+$ T cell response in the control HSV-1-infected group followed the previously described pattern of expansion, contraction and early onset

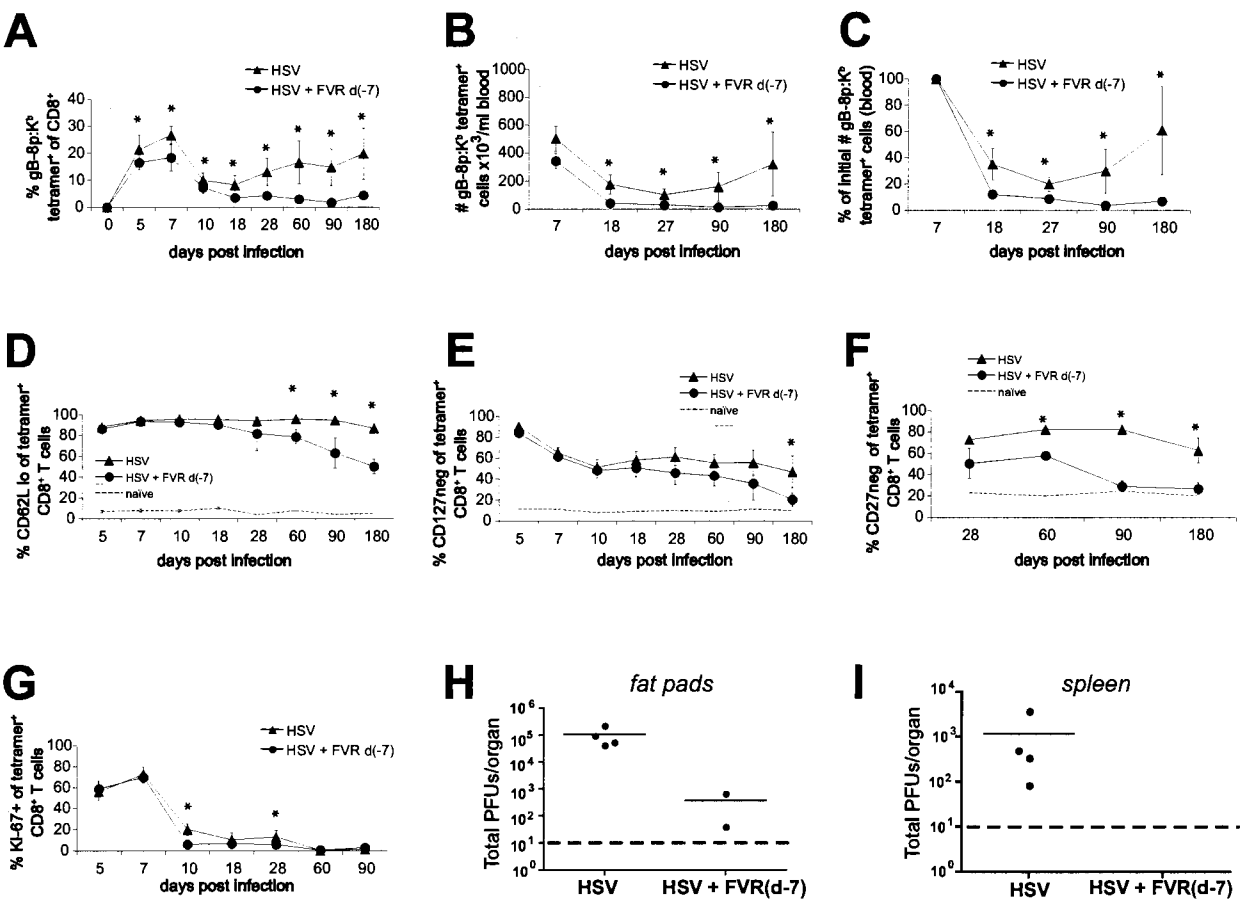

Figure 4.7 Decreasing initial viral load and interfering with viral reactivation can prevent memory inflation following systemic HSV-1 infection. Two groups of mice (n = 4 per group) were infected with HSV-1 via systemic route and their $CD8^+$ T cell responses were followed longitudinally by staining blood samples with CD8 and tetramer. One cohort (labeled HSV+FVR d(-7) in the figure) was given antiviral drug famciclovir (FVR) starting on day (-7), and continuing thereafter. The second cohort (HSV) was left untreated. The average percentage (**A**) and number (**B**) of $tetramer^+$ cells within $CD8^+$ T cells was determined. To better assess the extent of memory inflation, the number of $tetramer^+$ $CD8^+$ T cells over time was expressed as percent of the initial $tetramer^+$ cell number, adjusted to be 100% at day 7 p.i. (**C**). In all the panels, the asterix indicates a statistically significant ($p < 0.05$, Student's t-test) difference between the FVR-treated and control groups. **E-G.** Divergence of phenotypes in inflating (HSV group) and non-inflating (FVR-treated group) memory $CD8^+$ T cells. Expression of CD62L (**D**), CD127 (**E**) and CD27 (**F**) was determined on tetramer+ $CD8^+$ T cells over time. The data shows the average (± standard deviation) percentage of $CD62L^{lo}$, $CD127^{neg}$ and $CD27^{neg}$ cells within the $tetramer^+$ $CD8^+$ T cells per group. **H.** Famciclovir treatment shortens the period of initial $tetramer^+$ $CD8^+$ T cell proliferation. Expression of Ki-67 on $tetramer^+$ $CD8^+$ T cells was determined by FCM as described in Materials and Methods. **I and J.** Famciclovir treatment curtails the extent of viral proliferation and spread. On day 3 p.i., mice from famciclovir-treated and control group (n = 4/group) were sacrificed and the amount of actively replicating virus was determined in fat pads (**I**) and in spleen (**J**). In the famciclovir treated group, only 2 out of 4 mice had any detectable virus in their fat pads, and no virus was detected in their spleens (n.d.).

of memory inflation (first observed on day 28 p.i. in this cohort). Mice pretreated with famvir also developed a robust, but overall smaller $CD8^+$ T cell response, as demonstrated by percentage (Figure 4.7 A) and numbers (Figure 4.7 B) of $tetramer^+$ $CD8^+$ T cells. Interestingly, famvir-pretreated mice did not develop memory inflation. This is best demonstrated in Figure 4.7 C, where the numbers of virus-specific memory cells are represented as the percent of initial number of virus-specific cells at the peak of their expansion (day 7). Therefore, by limiting viral replication and spread we were able to prevent memory inflation.

We next analyzed the phenotype of the effector and memory $CD8^+$ T cells in the different experimental groups (Figure 4.7 D, E, and F). The expression of activation markers CD62L and CD127 was identical during effector phase and early during transition from effector to memory (Figure 4.7 D and E). However, the phenotypes of memory $CD8^+$ T cells from the various mouse cohorts diverged over the course of experiment, with the famvir-treated mice undergoing slow but steady conversion into central memory phenotype (mostly $CD62L^+$ $CD127^+$ $CD27^+$), and the control HSV-1 infected mice acquiring an effector memory phenotype. Whereas the expression of CD127 and CD27 reached the levels comparable to those observed in age-matched naïve mice, in the HSV-control group expression of CD127 and CD27 remained low (Figure 4.7 E and F). These results were very similar to the phenotypic analysis of memory $CD8^+$ T cells resulting from systemic and localized HSV-1 infection (Figure 4.3), with the famvir-pretreated group behaving very much like the ocularly infected group with respect to their $CD8^+$ T cell response. Similarly, the virus-specific $CD8^+$ T cell response in systemically infected cohort that was treated with famvir starting on day 1 p.i. was

identical to that in mice pretreated with famvir for 7 days prior to infection (data not shown). The only difference was that unlike in the famvir-pretreated group, the mice given the drug on day 1 p.i. had the same percentages and numbers of virus-specific $CD8^+$ T cells as the control HSV-group on day 7. However, similarly to famvir-pretreated group they did not develop memory inflation and converted to central memory phenotype within 180 days post-infection.

Famvir pretreatment resulted in early termination of $tetramer^+$ $CD8^+$ T cell proliferation (Figure 4.7 G). This was determined by co-staining cells with CD8, tetramer and Ki-67. Ki-67 is a nuclear proliferation-associated antigen, is expressed during all active stages of the cell's proliferation, and can therefore be used to determine the cell's proliferative status. Equal percentage of $tetramer^+$ T cells was $Ki\text{-}67^+$ on day 5 and 7 p.i. in famvir pretreated and control group, indicating that blocking viral replication and therefore *de novo* viral protein synthesis did not affect the program of cell activation and division. Expression of Ki-67 could be detected on $tetramer^+$ $CD8^+$ T cells in both mouse cohorts as late as day 28 p.i. before returning to background levels by day 60 p.i. However, between days 10-28 p.i. a significantly greater proportion of $tetramer^+$ $CD8^+$ T cells in control HSV-1-infected group was $Ki\text{-}67^+$, as compared to $tetramer^+$ cells in mice treated with famvir (Figure 4.7 G). Active viral replication is no longer detected at this time, and is not the likely cause for this difference. Regardless, from day 60 p.i. onwards Ki-67 expression was at background level in both mouse cohorts.

The above data demonstrate that development of HSV-specific CD8 memory inflation and the effector memory phenotype associated with it can be prevented by limiting viral replication and spread during primary infection. This strongly suggests that

memory inflation is driven by viral reactivation, since it was absent in mice in which the viral load and viral spread were restricted, and in which viral reactivation from latency was prevented by continuous treatment with an antiviral drug.

Effects of blocking viral reactivation on memory inflation

In the above experiments we severely decreased the viral load and viral spread during primary infection, in addition to preventing potential viral reactivation from latency by continuing famciclovir treatment throughout the experiment. As a result, we could not tell with certainty whether the effects on memory inflation we observed were due to blocking viral reactivation, or whether they were a direct result of decreased viral titer present in these mice. To clearly distinguish between these two possibilities, we analyzed $CD8^+$ T cell response in a cohort of mice in which the primary systemic infection with HSV-1 and establishment of latency were uninterrupted, but viral reactivation from latency was prevented by commencing famciclovir treatment on day 14 p.i. and continuing it for the duration of the study. By doing so we were able to compare maintenance of memory $CD8^+$ T cells that were generated under the same conditions as in control HSV-infected mice, and in animals that had the same latent viral load. Therefore, any difference seen in the antiviral memory $CD8^+$ T cell response would be due to effects mediated by viral reactivation from latency.

Figure 4.8 A shows the comparison of the effector and memory $CD8^+$ T cell response in control and famvir treated mice. Mice in both groups had the same average frequency of virus-specific $CD8^+$ T cells up to day 45 p.i., however after that memory inflation begun in the control group. Famciclovir treatment of latently infected mice was

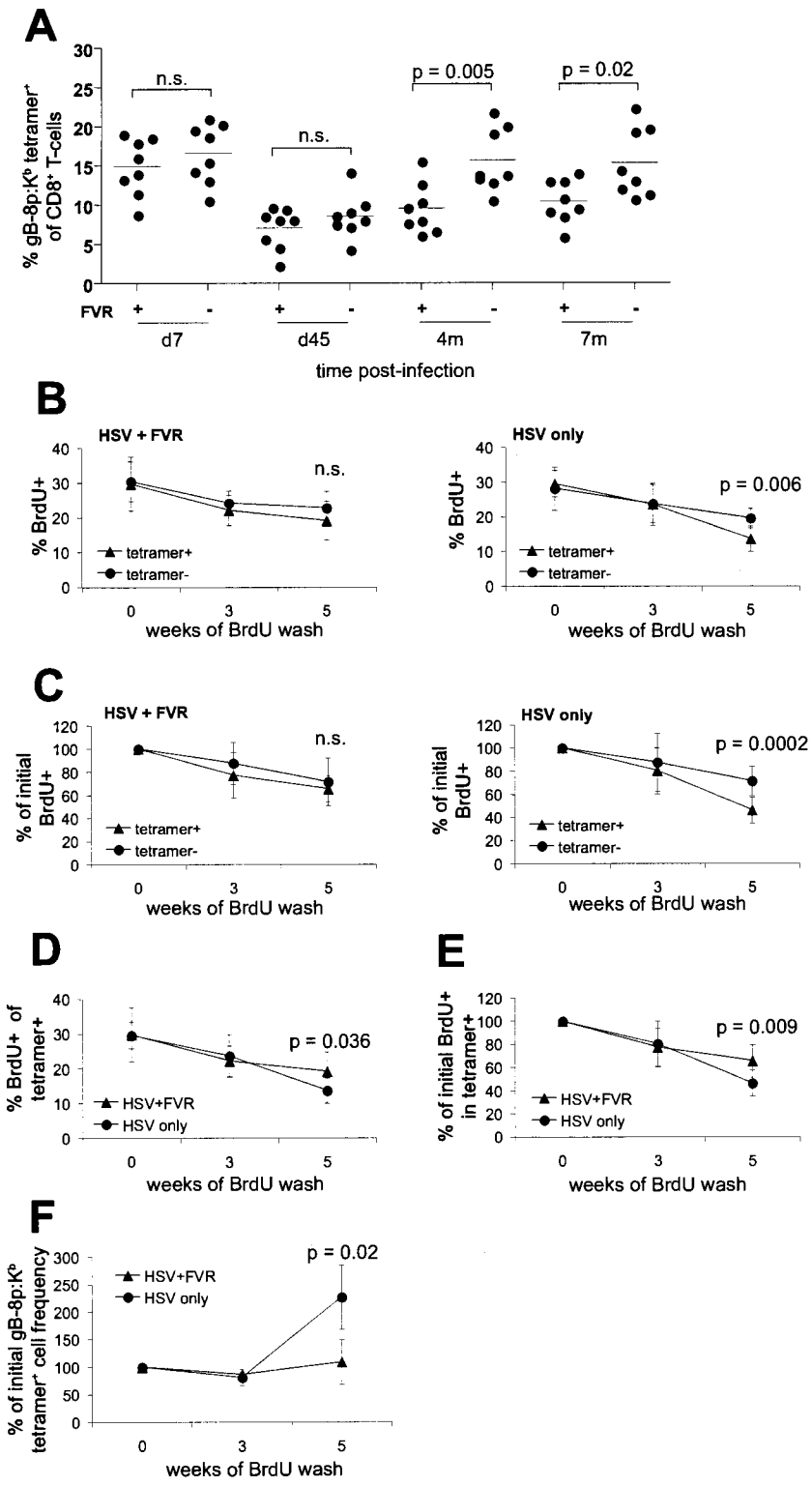

Figure 4.8 Dependence of *in vivo* proliferation of memory CD8$^+$ T cells on presence of antigen. Cohorts of B6 mice were systemically infected with HSV-1 and their gB-8p-specific CD8$^+$ T cell response was monitored in blood over time. One group was left

untreated (HSV only, n = 8), whereas the second group was continuously treated with famciclvovir starting on day 14 p.i. (HSV + FVR, n = 8). At 7 m.p.i. mice from both groups were given BrdU in their drinking water for 3 weeks to monitor *in vivo* T cell proliferation. The loss of BrdU label (BrdU wash) was monitored over the course of 5 weeks following termination of BrdU treatment. Mice in HSV+FVR group continued to receive famciclovir during BrdU treatment. **A.** Frequency of gB-8p-tetramer$^+$ cells in CD8$^+$ T cells in mouse cohorts described above over time. The values for individual mice are shown. + and – indicate presence or absence of famciclovir treatment. **B.** Percent of BrdU$^+$ cells within tetramer$^+$ or tetramer$^-$ CD8$^+$ T cell populations in famciclovir treated (left panel) or untreated control (right panel) group. Average values (±SD) are shown. **C.** Percent of initial BrdU level present in tetramer$^+$ or tetramer$^-$ CD8$^+$ T cell populations in famciclovir treated (left panel) or untreated control (right panel) group. The percent of BrdU$^+$ cells in given population at the termination of BrdU treatment was used as 100%, and the loss of BrdU staining was determined in relation to that value over time. Average values (±SD) are shown. **D.** Percent of BrdU$^+$ cells within tetramer$^+$ CD8$^+$ T cell populations of famciclovir treated or untreated control group. **E.** Percent of initial BrdU level present in tetramer$^+$ CD8$^+$ T cell populations of famciclovir treated or untreated control group (calculated as in panel C). **F.** Percent of initial frequency of gB-8p- tetramer$^+$ CD8$^+$ T cells during BrdU wash period. The frequency of tetramer$^+$ cells at the beginning of the BrdU wash period was used as 100%. Average values (±SD) are shown.

able to prevent memory inflation on a cohort level, with the average frequency of memory $CD8^+$ T cells being significantly smaller in famvir-treated group at 4 and 7 m.p.i. (Figure 4.8 A). However, famvir treatment did not completely prevent memory inflation, as demonstrated by slow but gradual increase in virus-specific $CD8^+$ T cells in some famvir-treated mice.

Dependence of in vivo proliferation of memory $CD8^+$ T cells on presence of antigen

To better understand the mechanism leading to development and maintenance of memory cell expansions, we have analyzed *in vivo* turnover of memory $CD8^+$ T cells in mice undergoing memory inflation and in mice where memory inflation was controlled by blocking viral replication, using mouse cohorts from Figure 4.8 A.

Original experiments using Ki-67 as an indicator of cell proliferation implied that gB-8p-specific memory $CD8^+$ T cells do not cycle above background level beyond day 28 p.i. (Figure 4.7 G). However, Ki-67 only detects cells that are actively proliferating. It was possible that in order to observe the proliferation dynamics of gB-8p-specific $CD8^+$ T cells, we would need to follow their proliferation over a longer period of time. To achieve that, we used BrdU to label actively dividing cells *in vivo*. Mice that were already infected for 7 months, including a cohort continuously treated with famvir starting on day 14 p.i., were given BrdU in their drinking water for 3 weeks. The dynamics of proliferation of $tetramer^+$ and $tetramer^-$ $CD8^+$ T cells in famvir-treated and control infected mice were then determined by measuring the loss of BrdU over the period of 5 weeks after BrdU pulse ended (BrdU wash).

At the peak of BrdU labeling (end of the 3-week-long BrdU pulse, denoted as week 0 of BrdU wash in Figure 4.8), no significant differences were detected between tetramer$^+$ and tetramer$^-$ cells in either mouse cohort, or between tetramer$^+$ cells from mice from the two different cohorts, with an average of 30% of CD8$^+$ T cells being BrdU$^+$ in each analyzed cell subset (Figure 4.8 B). All of the analyzed cell subsets continued to lose their BrdU label slowly but continuously over the next 5 weeks. In latently infected mice treated with famvir, no significant differences were observed between proliferation of tetramer$^+$ and tetramer$^-$ CD8$^+$ T cell populations, as demonstrated by analysis of the percentage of BrdU$^+$ cells (Figure 4.8 B, left panel) or by calculating the percent of initial BrdU label still present 3 and 5 weeks after BrdU pulse ended (Figure 4.8 C, left panel). In contrast, statistically significant difference in proliferation between tetramer$^+$ and tetramer$^-$ CD8$^+$ T cells emerged by 5 weeks after labeling was stopped in control HSV-infected mice (Figure 5.8 B and C, right panels). In these mice, the tetramer$^-$ CD8$^+$ cell population still retained an average of 71% of the initial BrdU label, unlike the tetramer$^+$ cell population which proliferated more extensively and retained only 46% of the peak BrdU level (Figure 4.8 C, right panel).

Unfortunately we did not co-stain the cells in this experiment with markers that would allow us to distinguish between memory and naïve CD8$^+$ T cells. Naïve CD8$^+$ T cells are known to cycle more slowly than memory cells (Surh and Sprent, 2002), and this provided a caveat for the analysis described above. In order to compare proliferation of equivalent CD8$^+$ T cell populations, we compared cycling of tetramer$^+$ CD8$^+$ T cells in mice that either were or were not treated with famvir throughout the experiment (Figure 4.8 D and E). By this comparison we found that the tetramer$^+$ cells in famvir

treated group proliferated more slowly than tetramer$^+$ cells in control group, as demonstrated by the level of the peak BrdU label retained at 5 weeks of the BrdU wash period (Figure 4.8 E, tetramer$^+$ cells in famvir treated mice retained 65% of peak BrdU level as compared to 46% of the initial BrdU retained by control mice). The difference in the proliferation was subtle but significant, and correlated with an increase in the percent of tetramer$^+$ cells within CD8$^+$ T cells of the control, but not the famvir-treated group during the BrdU wash period (Figure 4.8 F). These results suggest that increased proliferation rate of tetramer$^+$ cells is driven by viral reactivation, and this process is likely one of the mechanisms leading to development of memory inflation in mice where viral reactivation is not controlled.

DISCUSSION

In this study we have examined the relationship between lifelong HSV-1 infection and maintenance of CD8$^+$ T cells specific for it. We demonstrate that depending on the administration route, infection with HSV-1 can either result in establishment of stable systemic memory pool with central memory phenotype (localized infection), or may be associated with expansion of effector-memory phenotype virus specific CD8$^+$ T cells over time (systemic infection). We further demonstrated that the extent of virus-specific CD8$^+$ T cell expansion during the memory phase is influenced by the extent of systemic viral spread and the ability of the latent virus to reactivate. By a combination of adoptive transfer experiments and treatments controlling viral reactivation, our data shows that HSV-related memory CD8$^+$ T cell inflation requires presence of antigen, which is provided by the reactivating virus. One of the mechanisms by which expansions of virus-

specific $CD8^+$ T cells may arise is virus-driven proliferation, as demonstrated by decreased *in vivo* turnover of memory $CD8^+$ T cells in famciclovir treated mice as compared to HSV-1-infected controls.

Our results demonstrate that controlling viral replication can affect the magnitude of virus-specific $CD8^+$ T cell expansion during primary and latent infection, and imply that this is accomplished by regulating the extent of gB-8p presentation to T cells. However we still do not know how the Ag reaches the T cells, particularly during latency. The results presented here provide several clues. First, a combination of limiting viral spread and blocking reactivation results in complete abrogation of memory inflation (Figure 4.7 A). Second, blocking viral reactivation alone controls memory inflation, but it is not completely effective, resulting in significantly decreased but still evident memory inflation (Figure 4.8 A). This suggests that famvir is either unable to block reactivation, or that expression of gB takes place even when viral reactivation is blocked. The latter is likely when one considers both the great efficacy with which famvir blocks productive replication *in vivo*, and the mechanism by which famvir affects viral replication. Specifically, the program of viral replication is blocked only after expression of viral TK commences (Darby et al., 1981), and literature suggests that by that time there is some expression of late genes (Mueller et al., 2003; Pesola et al., 2005). In fact, studies in latently infected TGs demonstrated that acyclovir treatment of TG during ex-vivo reactivation assay did not affect the viral gene expression in reactivating neurons, but rather decreased the extent of productive viral replication by preventing cell-to-cell virus spread (Pesola et al., 2005). This is in line with evidence that infection of cells with TK-deficient HSV-1 is associated with gB production even in the absence of

productive viral replication, and immunization with TK-deficient HSV-1 leads to generation of protective immune response (Brehm et al., 1997). The ability to express gB even in the absence of productive viral replication is the likely explanation for the robust $CD8^+$ T cell response generated in mice pretreated with famvir prior to systemic infection (Figure 4.7 A). Therefore, it is likely that in systemically infected mice the extensive systemic spread of the virus during primary infection led to establishment of large number of latent virus reservoirs, and the incidences of spontaneous reactivation even in presence of viral replication inhibitor generated enough gB for presentation to peripheral T cells. One study (Sawtell, 1998) found a positive correlation between viral reactivation rates and the number of infected neurons. We expect that greater virus spread through the parenchymal organs would allow the virus to infect larger number of neurons, resulting in more frequent reactivation. An alternative explanation for the inability of famvir to completely prevent viral reactivation is that by continuous famvir treatment we have selected drug-resistant viral mutants. Such mutants were described and they usually have a defect in viral TK or DNA polymerase (Besecker et al., 2007; Lebel and Boivin, 2006; Strick et al., 2006; Wang et al., 2007). Isolation and sequencing of latent virus from mice experiencing memory inflation despite famvir treatment would be required to test this possibility.

The mechanism of generation and maintenance of expanded gB-8p-specific $CD8^+$ T cell populations needs to be further characterized, but our data implies that it depends on periodic encounters with Ag which induce proliferation of memory $CD8^+$ T cells. However, as a population, the memory cells do not proliferate extensively during latency, as indicated by very low level of Ki-67 staining in gB-8p-specific memory $CD8^+$ T cells

(Figure 4.7 G). This clearly distinguishes them from memory $CD8^+$ T cells during chronic LCMV infection, which are maintained by robust Ag-driven proliferation (Shin et al., 2007). Therefore, while both chronic and latent viruses provide antigenic stimulation during lifelong infections, our data in conjunction with results obtained in chronic infection models (Shin et al., 2007; Vezys et al., 2006) demonstrates that the intensity of that stimulation is much stronger in case of chronic infections.

In this study we focused on the importance of viral Ag in driving expansion of HSV-specific memory $CD8^+$ T cells. We found that as a population, the expanding and non-expanding memory $CD8^+$ T cells can be distinguished phenotypically, with the expanding memory cell populations exhibiting predominantly effector memory phenotype, and the non-expanding populations displaying a uniform central memory phenotype. This was consistent with the evidence that viral Ag drives the memory T cell inflation. However, the memory $CD8^+$ T cell population in mice undergoing memory inflation was not uniform, and while the majority of the cells were of effector memory phenotype, the population also contained a significant fraction of central memory phenotype cells (Figure 4.4). It will be of interest to examine how the different subsets of the gB-8p-specific memory $CD8^+$ T cells are maintained and whether or not they differ in their ability to respond to homeostatic and virus-derived stimuli during latent HSV-1 infection.

Another factor that one should consider when examining the $CD8^+$ T cell response is the cytokine and chemokine environment during the primary infection, which affects the resulting effector and memory response (Haring et al., 2006; Joshi et al., 2007). Although we did not test for it, it is very likely that by decreasing the extent of

viral replication and spread, we have altered, most likely decreased, the inflammation normally associated with systemic HSV-1 infection. The extent of inflammation can affect the initial programming of $CD8^+$ T cells, and decreased inflammation has been associated with smaller effector responses and accelerated development of memory (Badovinac and Harty, 2007; Badovinac et al., 2004; Joshi et al., 2007). However, our data imply that the size and the pattern of maintenance of gB-8p-specific $CD8^+$ T cells is not a result of cell programming during priming, but rather is influenced by stimuli encountered during the latent period. Specifically, the gB-8p-specific effector $CD8^+$ T cells contract to similar extent regardless of infection route or the extent of viral replication during acute phase of infection, and this contraction is associated with conversion indicating transition to memory (upregulation of CD127). Memory inflation and reversion to effector memory phenotype take place after that initial contraction. Furthermore, memory $CD8^+$ T cells resulting from systemic infection, presumably associated with extensive inflammation, were maintained differently following adoptive transfer into naïve or infected host, implying that presence of Ag rather than intrinsic cell factors are involved in regulating the expansion of memory cells during latency. Finally, both localized and systemic infection are associated with induction of inflammatory cytokines, yet memory maintenance differs between these two models. Therefore, the memory inflation documented for systemic HSV-1 infection is most likely a result of direct antigenic stimulation.

In summary, lifelong infection with HSV-1, which for a long time was considered to be truly latent in mice, appears to be more prone to reactivation and periodic stimulation of the immune system than previously believed. However, much remains

unknown about the intensity and nature of the virus:immune system communication during the lifelong infection. This and other recent studies suggest that HSV-1 provides ongoing antigenic stimulation to memory $CD8^+$ T cells, but the characteristics of that stimulation lead to a somewhat different outcome than stimulation provided by chronic viral infections (LCMV, HCV or HIV) or latent infection with MCMV. Unlike chronic viral infections, HSV-1 has a moderate rather than overwhelming influence on memory $CD8^+$ T cell pool. For example, HSV-associated memory inflation does not lead to functional exhaustion (data not shown). While development and maintenance of large expansions of HSV-1-specific memory $CD8^+$ T cells depend on presence of Ag, some of these cells are capable of long-term survival in the absence of Ag, which is in marked contrast to memory $CD8^+$ T cells generated by chronic LCMV (Shin et al., 2007) or polyomavirus infections (Vezys et al., 2006). Finally, while lifelong HSV-1 infection under certain conditions can drive memory inflation of $CD8^+$ T cells, the expansion often reaches a plateau where the expanded memory $CD8^+$ T cell population usually will not exceed 30% of total CD8 T cell pool, and in most cases averages 15-20% (see Figure 5.1 A and D, Figure 5.7 A, Figure 5.8 A). This differs from CD8 memory inflation described for MCMV, where accumulation of virus-specific $CD8^+$ T cells often does not plateau, but rather continues throughout the latency (Karrer et al., 2003; Karrer et al., 2004). The analysis of systemic $CD8^+$ T cell response to HSV-1 presented here is in agreement with the data implying that viral reactivation takes place regularly, but only in small percent of latently infected cells at a time, most likely resulting in periodic, but not chronic stimulation of $CD8^+$ T cells in the periphery.

Summary

Understanding the impact of lifelong infections on maintenance of memory $CD8^+$ T cells is of great interest, as it was suggested to influence the homeostasis of the whole $CD8^+$ T cell compartment. Persistent viral infections may contribute to the process of immunosenscence, in particular by decreasing the diversity of the $CD8^+$ T cell repertoire in the elderly. Both chronic and latent infections can lead to ongoing accumulation of memory $CD8^+$ T cells over time, and in extreme cases this may result in domination of the repertoire by the expanded antiviral $CD8^+$ T cells. This in turn can compromise the host's ability to mount immune responses to new pathogens. Murine models of persistent viral infections in which the contribution of ongoing viral stimulation to the homeostasis of virus-specific memory cells as well the homeostasis of the whole $CD8^+$ lymphocyte compartment can be clearly analyzed is needed to test the above predictions. The data presented in this chapter suggest that systemic HSV-1 infection of B6 mice may be a suitable setting in which to investigate the impact of latent, periodically reactivating virus on the lifelong $CD8^+$ T cell homeostasis. Results from the HSV-1 infection model could be compared to other persistent infection models in which the ongoing viral activation of the $CD8^+$ T cells is more pronounced, such as in case of MCMV. In the next chapter, we will investigate the impact of both of these infections on maintenance of diverse $CD8^+$ T cell repertoire during lifelong murine infection.

CHAPTER 5

Impact of lifelong viral infections on the development of $CD8^+$ T cell expansions in old mice

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Contribution of authors to the presented work:

Drs Michael Munks and Ann Hill designed and performed the experiments with MCMV in conjunction with Anna Lang. Anna Lang's contribution to the MCMV experiments involved participation in organ harvest, design of the antibody staining panels, and participation in the ex-vivo IFNγ assays, surface antibody staining, flow cytometry and data analysis. The MCMV virus strains were generated in the Hill laboratory, and the mice were infected with MCMV by Michael Munks.

All the experiments involving HSV-1 were designed by Dr. Janko Nikolich-Zugich and Anna Lang and performed by Anna Lang. This included preparation of the viral stock, infection of mice , and all the experimental procedures. Bree Fisher assisted in spleen harvests.

ABSTRACT

Aging is associated with a complex set of changes in the immune system, including changes at the T cell population level. One of them is development of large T cell clonal expansions (TCE) within the aging $CD8^+$ T cell pool. TCE arise both in humans and mice, and the mechanism(s) behind their generation remain incompletely understood. Some TCE appear to arise due to changes in how they perceive homeostatic signals promoting their proliferation (IL-15) and survival (IL-7). These TCE appear to be Ag-independent, as they were documented to arise in specific pathogen-free (SPF) mice, to survive long-term upon transfer into naïve hosts in the apparent absence of antigenic stimulation, and to express elevated levels of IL-7R and IL-15R. However, data from humans indicate that development of TCE may also be a result of persistent infection, most notably with Human Cytomegalovirus (HCMV), via direct ongoing antigenic stimulation or via stimulation of bystander T cells by chronic inflammation due to persistent viral infection. In this study we sought to determine whether lifelong persistent infection with Herpes Simplex Virus (HSV-1) and murine cytomegalovirus (MCMV) can drive development of $CD8^+$ T cell expansions in mice. Neither infection had a significant effect on the incidence of expansions, or on the overall size and phenotype of $CD8^+$ T cell pool. However, certain $CD8^+$ T cell expansions detected in infected mice were partially virus specific, and the phenotype of the large MCMV-specific memory $CD8^+$ T cell expansions had a unique $KLRG1^+$ phenotype, which was not seen in virus-nonreactive expansions. We conclude that in the mouse model persistent viral infections are not the likely drivers of the development of large TCE by either direct or indirect mechanisms. However, identification of phenotypic markers that distinguish virus-

specific and virus-independent $CD8^+$ T cell expansions in mouse studies may prove useful to further address the importance of HCMV in driving TCE development in humans, where detailed analysis of viral specificity of expanded cell populations is not always possible.

INTRODUCTION

In young mammals, including humans and mice, the T cell receptor (TCR) repertoire of $CD8^+$ T cells is diverse, owing to ongoing supply of diverse recent thymic emigrants (RTE) and the homeostatic maintenance mechanisms (Callahan et al., 1993; Hingorani et al., 1993; Surh and Sprent, 2002). T cell homeostasis, defined by maintenance of numbers and diversity of naïve and memory T cells, is achieved by regulating their proliferation and survival by cytokines, self-MHC contact and other less defined factors (Fry and Mackall, 2001; Surh et al., 2006; Surh and Sprent, 2002). The mechanism of maintaining the TCR repertoire diversity is very poorly understood (Davenport et al., 2007), however it is evident that there is an age-associated breakdown in the organisms' ability to maintain diverse repertoire (Cambier, 2005; Clambey et al., 2005). This manifests itself most drastically in development of T cell clonal expansions (TCE), which affect predominantly $CD8^+$ T cells (rev. in (Clambey et al., 2005)). TCE can occupy up to 90% of murine and up to 50% of human $CD8^+$ T cell pool, thus severely constricting the repertoire (Callahan et al., 1993; Clambey et al., 2007; Hingorani et al., 1993; LeMaoult et al., 2000; Messaoudi et al., 2006c; Posnett et al., 1994; Schwab et al., 1997). While TCE themselves are not malignant and do not affect the overall size of the $CD8^+$ T cell pool (Clambey et al., 2005), they can severely impair the host's ability to generate an immune response against an invading pathogen in a TCR and Ag-specific manner. Mice which had TCE in Vβ family dominantly involved in anti-HSV-1 response (TCR Vβ8 or 10), but where the TCE itself was not HSV-specific, had an impaired anti-HSV response and compromised survival upon challenge (Messaoudi et al., 2004). However, presence of TCE in a TCR Vβ family not involved in

anti-HSV-1 response did not have suppressive effect. Therefore, depending on the TCR Vβ family affected and on the TCR mobilized to respond against the given pathogen, TCE can affect the immunity of the host by occupying the space that normally would have been available to naïve precursors or memory cells capable of mounting an immune response.

With respect to mechanism of their generation, TCE are thought to arise either due to an age-dependent dysregulation in perceiving homeostatic signals (Ag-independent TCE, or AI-TCE), or by expansion driven by ongoing antigenic stimulation (Ag-responding TCE, or AR-TCE) (rev. in (Clambey et al., 2005)). Evidence for the homeostatic dysregulation-driven development of AI-TCE was obtained in SPF mice (Messaoudi et al., 2006c). Large TCE in these mice had a uniform resting central memory phenotype, indicating lack of recent contact with Ag and characterized by elevated expression of CD122 (IL15Rβ) and CD127 (IL7Rα) as compared to other memory ($CD44^+$) $CD8^+$ T cells. In addition, they did not respond normally to environmental on or off cues such as transfer into lymphopenic or full lymphoid compartment, but rather continued their constant, slow proliferation regardless of their environment. These results suggested that TCE acquired the ability to survive independently of homeostatic signals, and that their dominance in the $CD8^+$ T cell pools may be in part mediated by depriving the remaining $CD8^+$ T cells from homeostatic signals due to the elevated expression of, and suspected usage of, homeostatic cytokines IL-7 and IL-15. Whether AI-TCE act as "cytokine sinks" needs to be further elucidated.

While in some experiments the TCE have a uniform phenotype, others reported greater heterogeneity of surface molecule expression (Clambey et al., 2007). One

possibility for these phenotypic differences may be the origin (AI or AR) of the TCE. The best documented cases of AR-TCE come from humans. HCMV seropositivity in elderly humans has been correlated with occurrence of large clonal expansions of T cells, particularly $CD8^+$ T cells, as well as with phenotypic and functional changes within the $CD8^+$ T cell compartment that collectively are considered to be biomarkers of immunological aging (Pawelec et al., 2005). Most of these changes relate to an accumulation of $CD28^-$ $CD8^+$ cells (Akbar and Fletcher, 2005; Ferguson et al., 1995; Pawelec et al., 2005; Weekes et al., 1999). Since this is the also the phenotype of HCMV-specific memory $CD8^+$ T cells, and since some large HCMV-specific oligoclonal expansions can also be seen in the elderly (Gillespie et al., 2000; Khan et al., 2002a; Khan et al., 2002b; Wikby et al., 2002; Wills et al., 1999), it is commonly assumed that most of the TCR Vβ expansions and the increased $CD28^-$ $CD8^+$ cells in the elderly are specific for CMV. Some have proposed that the HCMV-specific response simply becomes so large that it limits the ability of the immune system to respond to other challenges (Pawelec et al., 2004; Pawelec et al., 2005). However, an alternative viewpoint is that by increasing the level of IFNα, CMV drives cells of various specificities towards expansion and the $CD28^-$ phenotype (Akbar and Fletcher, 2005). In humans it is extremely difficult to define the whole HCMV-specific CD8 T cell population because of lack of identified epitopes, so the antigenic specificity both of the large clonal expansions and the $CD28^-$ $CD8^+$ population as a whole is difficult to address. However, in C57BL/6 (B6) mice, Munks et al. (Munks et al., 2006b) recently mapped the entire $CD8^+$ T cell response to MCMV, allowing to address this questions in a mouse model.

In this study we set out to address the possible role of lifelong persistent infections in driving the development of $CD8^+$ T cell expansions and their overall impact on the aging $CD8^+$ T cell compartment. We wanted to do this in a mouse model where the specificity of $CD8^+$ T cell expansions could be determined To this effect, we analyzed the TCR Vβ in old mice that had been infected for >2 years with a persistent herpesvirus, HSV-1 or MCMV. Systemic infection with HSV-1 and MCMV is associated with $CD8^+$ T cell memory inflation in mice (Chapter 4 and (Karrer et al., 2003; Karrer et al., 2004)), and the effector memory phenotype of the virus-specific $CD8^+$ T cell expansions in both models was consistent with the effector memory phenotype described for HCMV-specific expansions of $CD8^+$ T cell in humans. Therefore we expected that the mouse infection could provide a suitable model in which to test the involvement of persistent pathogens in driving the development of AR-TCE. Mice with TCR Vβ $CD8^+$ T cell expansions were tested for the expansions' reactivity against viral antigen to determine how commonly the expansions result from Ag-specific expansion driven by persistent virus. We found that the overall impact of persistent viral infections on the size and phenotype of $CD8^+$ T cell pool is negligible in our study, however we did notice some unique phenotypic differences between Ag-specific $CD8^+$ T cell expansions and viral Ag-unrelated expansions. This study demonstrates that there may be differences in the murine and human responses to CMV, or these differences may reflect the differences between the murine and human CMV itself. While additional studies involving more mice are needed to definitively address the involvement of persistent infections in driving development of AR-TCE in mice, one important result

that emerged from our initial studies was identification of KLRG1 as a unique marker of CMV-specific but not CMV-unreactive TCR Vβ expansions.

MATERIALS AND METHODS

Mice. The mice listed in Table 5.1 were purchased from Jackson Laboratories and housed at Oregon Health & Science University. Mice listed in Table 5.2 were purchased from the National Cancer Institute colony (NCI, Frederick, MD) and housed under the specific-pathogen free conditions at the Vaccine and Gene Therapy Institute vivarium.

Viruses and infections. MCMV strain MW97.01, a BAC-derived virus from the Smith strain, was used as wt MCMV. The MCMV mutant Δm4+m6+m152 (triple-knockout, or tko in the text), derived from MW97.01, has been previously described (Wagner et al., 2002). Both viruses were grown on C57BL/6 mouse embryonic fibroblasts in Dr. Ann Hill's laboratory. The mice listed in Table 5.1 were infected at 6-12 weeks of age with 2 x 10^6 PFU wt or tko MCMV i.p., or alternatively were left uninfected.

HSV-1 strain 17 obtained from Dr. D.J. McGeoch (University of Glasgow, Scotland, UK), was grown, cloned as a syn+ variant and titered on Vero cells in our laboratory, and was used in all experiments. The mice listed in Table 5.2 were infected at 8-12 weeks of age with 10^6 PFU of HSV-1 either i.p. (systemic infection) or by ocular infection (localized infection), or were left uninfected. For localized infections, mice were anesthetized by i.p. injection of ketamine cocktail (100mg/ml ketamine, 20mg/ml xylazine, 10mg/ml acepromazine). The corneas were lightly scarified in a crisscross

fashion with a sterile 26-gauge needle, and 3µl of HBSS (GibcoBRL) containing $5x10^5$ PFU of HSV-1 was applied to the surface of each eye. For systemic (i.p.) infections, mice were injected i.p. with 200µl HBSS (GibcoBRL) containing indicated dose of the virus.

Reagents, antibodies and flow cytofluorometric (FCM) analysis. The gB-8p peptide was purchased from SynPep Corporation (Dublin, CA), and the gB-8p :K^b tetramer was obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA). The IE3, m38 and m139-specific tetramers were provided by Dr. Ann Hill's laboratory. Monoclonal antibodies were purchased from commercial sources.

FCM analysis with or without intracellular staining to detect IFNγ was performed as previously described (Lang and Nikolich-Zugich, 2005); in the latter case, stimulation was performed with 10^{-6} M gB-8p peptide (HSV-infected mice) or a panel of MCMV-specific peptides (Munks et al., 2006b) for 6 h in the presence of 1.5 µg/ml brefeldin A. FCM data was acquired on FACSCalibur instrument using CellQuest 3.3 software or on the FACS LSRII instrument using the Diva software (Becton Dickinson, Mountain View, CA), and analysis performed using FlowJo software (Tree Star). At least 10^4 cells were analyzed per sample, with dead cells excluded by selective gating based on orthogonal and side light scatter characteristics.

Statistics. Student's t-tests were performed with Excel (Microsoft), using a 2-tailed analysis with equal variance.

RESULTS

Experimental cohorts

To assess the influence of lifelong persistent infections on the development of $CD8^+$ T cell expansions in old mice, we infected cohorts of adult mice (8-12 weeks old) with HSV-1 or MCMV and analyzed them for presence of $CD8^+$ T cell expansions at an old age (26-30 m.o.). The HSV-1 infected mice and their age-matched uninfected controls were housed in a different facility than the MCMV infected mice and their corresponding naïve controls, and since housing conditions may have an effect on the history of antigenic encounters and therefore on the phenotype and composition of T cell pool, the HSV-1 and MCMV cohorts were analyzed independently.

The MCMV-infected mouse cohorts are summarized in Table 5.1. Two different strains of MCMV were used, wild type (wt) and triple knockout virus (tko), which is deficient in three known MCMV immune evasion genes, m04, m06 and m152 (Kavanagh et al., 2001; Pinto et al., 2006; Reusch et al., 1999; Ziegler et al., 1997). The mice used for this study were a part of experiments aimed at determining the impact of these immune evasion genes on acute and memory $CD8^+$ T cell response to MCMV *in vivo*. These results were published (Munks et al., 2007) and are outside of the scope of the analysis presented here. Briefly, the authors found that the size, kinetics and immunodominance hierarchy of $CD8^+$ T cell response elicited by the two viruses *in vivo* was comparable, however the tko-infected animals had an overall larger MCMV-specific response in old mice. Therefore, the wt and tko-infected mice were analyzed separately

infection status	age (months)	mouse id	expanded Vβ family	% expanded Vβ+ (of CD8+)	avg %Vβ+ in control young mice	fold expansion	% mice with VBE per cohort
naïve	30	B2	6	17.0	7.9	2.2	50%
n = 6	30	B6	4	15.6	4.1	3.8	(3/6)
	30	B7	3	8.8	2.7	3.3	
avg						**3.1**	
sd						0.8	
							50%
wt MCMV	30	B20	14	47.3	2.8	16.9	(11/22)
n = 22	30	B8	13	9.2	4.7	2.0	
	30	B22	2	21.0	5.2	4.0	
	30	B22	4	23.8	4.1	5.8	
	26	7529	11	24.1	7.2	3.3	
	26	7503	5	42.1	14.8	2.8	
	26	7505	8	85.2	20.4	4.2	
	26	7508	14	80.0	2.8	28.6	
	26	7530	11	18.5	7.2	2.6	
	26	7532	6	30.7	7.9	3.9	
	26	7533	8	50.2	20.4	2.5	
	26	7533	11	18.7	7.2	2.6	
	26	7534	11	20.3	7.2	2.8	
avg						**6.3**	
sd						7.7	
							45%
tko MCMV	30	B19	14	45.1	2.8	16.1	(9/20)
n = 20	30	B16	3	31.6	2.7	11.7	
	26	7510	14	35.4	2.8	12.6	
	26	7513	7	26.8	6.9	3.9	
	26	7525	11	24.5	7.2	3.4	
	26	7514	11	30.4	7.2	4.2	
	26	7521	2	23.0	5.6	4.1	
	26	7518	11	20.0	7.2	2.8	
	26	7520	11	20.0	7.2	2.8	
avg						**6.8**	
sd						5.1	

* only VBE+ mice are listed individually

Table 5.1. Summary of wt MCMV-infected, tko MCMV-infected and control naïve mouse cohorts. The total mouse number per group (n) is denoted directly underneath the group name in the infection status column. All mice that developed VBE are listed, and the TCR Vβ family, its size (represented as percentage of given $V\beta^+$ cells within total $CD8^+$ T cell pool), the size of the given TCR Vβ family in control young mice, and the fold expansion (determined as fold increase from the percentage of that TCR Vβ family in young mice) are summarized for each VBE. The percentage of mice that developed VBE per each cohort is shown in the last column.

to assess the possible role of the immune evasion genes on development of $CD8^+$ T cell expansions in old mice.

The HSV-1-infected mouse cohorts are summarized in Table 5.2. While in earlier experiments we determined that memory inflation of HSV-1-specific memory $CD8^+$ T cells takes place both following localized (Chapter 3) and systemic (Chapter 4) infection, we suspected that if ongoing viral stimulation indeed contributes to the generation of $CD8^+$ T cell expansions in old mice, then this would be evident following systemic, but not localized infection. This rationale was based on our discovery that the expansions of memory $CD8^+$ T cells depended on ongoing viral replication following systemic, but not localized infection (Chapter 4). Therefore, we compared the incidence of $CD8^+$ T cell expansions between mouse cohorts infected with HSV-1 via those two different routes.

Definition of TCR Vβ $CD8^+$ T cell expansions

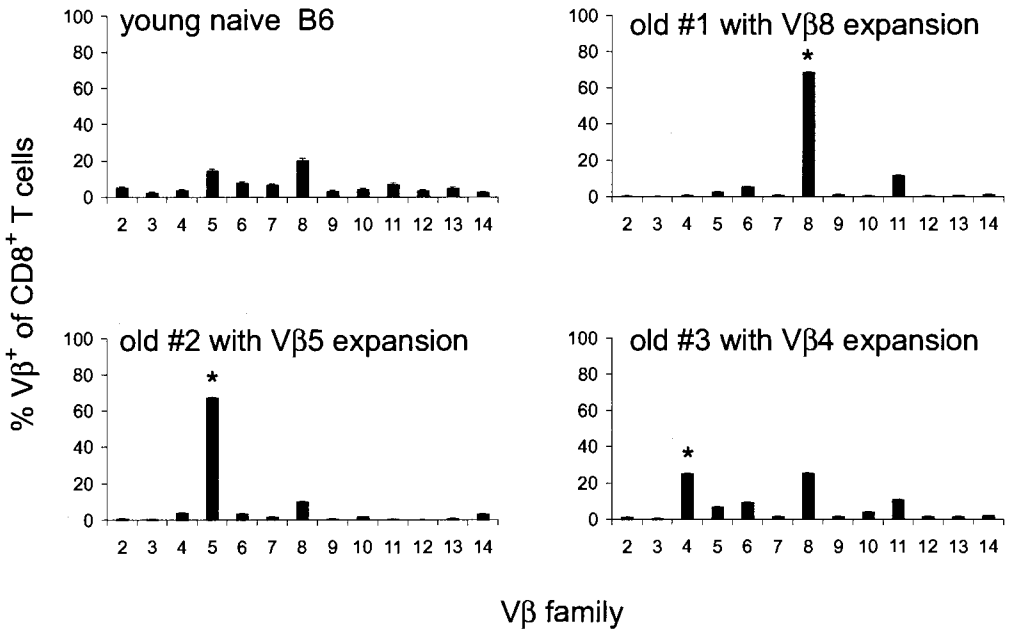
TCE are defined by a variety of methods which often differ between studies. However, the one criterion always considered first is the percentage of $CD8^+$ T cells expressing a TCR belonging to a given TCR Vβ family. The relative contribution of each TCR Vβ family to the overall repertoire of $CD8^+$ T cell pool is remarkably preserved in young mice of a given strain, with very little mouse-to-mouse variation. For the purpose of this study we define a TCR $V\beta^+ CD8^+$ expansions (VBE) to occupy at least double the space that is occupied by the given Vβ family in young mice. Figure 5.1 A shows examples of young diverse TCR Vβ distribution in B6 mice, and examples of old mice with VBE. In Figure 5.1 B, the size of each Vβ family in young

infection status	age (months)	mouse id	expanded Vβ family	% expanded Vβ+ (of CD8+)	avg %Vβ+ in control young mice	fold expansion	% mice with VBE per cohort
naïve	27	8695	4	25.4	4.1	6.2	42%
n = 12	27	8696	8	68.7	20.4	3.4	(5/12)
	27	378	8	43.9	20.4	2.2	
	27	376	5	67.6	14.8	4.6	
	24	5749	8	77.9	20.4	3.8	
avg						**4.0**	
sd						1.5	
							44%
HSV	27	7138	12	30.1	3.9	7.7	(8/18)
(systemic)	27	7166	8	66.2	20.4	3.2	
n = 18	27	7139	13	37.2	4.7	7.9	
	27	7135	8	42.1	20.4	2.1	
	27	7142	11	34.6	7.2	4.8	
	27	7111	10	23.6	4.7	5.0	
	27	7136	14	25.5	2.8	9.1	
	24	5823	10	40.7	4.7	8.7	
avg						**6.1**	
sd						6.1	
							36%
HSV	27	7126	13	13.0	4.7	2.8	(5/14)
(localized)	27	7118	2	23.0	5.6	4.1	
n = 14	27	7129	5	32.4	4.7	6.9	
	27	7127	5	61.7	4.7	13.1	
	27	7124	11	91.9	7.2	12.8	
avg						**7.9**	
sd						4.8	

* only VBE+ mice are listed individually

Table 5.2. Summary of HSV-1-infected and control naïve mouse cohorts. The total mouse number per group (n) is denoted directly underneath the group name in the infection status column. All mice that developed VBE are listed, and the TCR Vβ family, its size (represented as percentage of given $V\beta^+$ cells within total $CD8^+$ T cell pool), the size of the given TCR Vβ family in control young mice, and the fold expansion (determined as fold increase from the percentage of that TCR Vβ family in young mice) are summarized for each VBE. The percentage of mice that developed VBE per each cohort is shown in the last column.

A



B

	Vβ family													
	2	3	4	5	6	7	8	9	10	11	12	13	14	SUM
average %Vβ+ of CD8 in young mice (n=5)	5.6	2.7	4.1	14.8	7.9	6.9	20.4	3.7	4.7	7.2	3.9	4.7	2.8	89.5
standard deviation	0.4	0.2	0.2	0.7	0.6	0.3	0.9	0.3	0.4	0.6	0.3	0.5	0.3	
Vβ expansion cut off (2X avg %Vβ+ in young mice)	11.2	5.5	8.2	29.6	15.9	13.8	40.8	7.4	9.4	14.4	7.8	9.4	5.7	

Figure 5.1 Definition of TCR Vβ CD8$^+$ T cell expansions (VBE) A. Example of diverse repertoire in young B6 mice and of VBE-constricted repertoire in old mice. For young mice, the average percentage of total splenic CD8$^+$ T cells occupied by the given TCR Vβ family (± SD) is shown, based on staining of a cohort of young naïve B6 mice (n = 5) with the available TCR Vβ antibodies. The three examples of VBE were obtained by staining of splenocytes from three naïve 27 m.o. B6 mice. **B.** To be considered a VBE, the Vβ+ T cell population must occupy at least twice the amount of space used by the given Vβ family in young mice. The VBE cut-off values, represented as the minimal percentage that a given Vβ must occupy within total CD8$^+$ T cell pool, are summarized.

mice and the minimum size of that family required to classify a population as VBE are listed.

Other investigators sometimes define VBE as a Vβ population that is at least 3 standard deviations above the average percentage of that Vβ family in young mice (Clambey et al., 2007). However, given the extremely uniform distribution of different Vβ families in young mice, we found that this definition often is not stringent enough and results in isolation of clonotypically and phenotypically heterogeneous populations (Messaoudi et al., 2006c). Therefore we decided to use a more stringent definition of VBE. Another reason to focus on very large VBEs was that the CMV-specific TCE described in humans tended to also be very large (Pawelec et al., 2005), and one of the reasons for this study was to determine if effects of persistent infection in mice can resemble those seen in humans.

The size of the Vβ family was the only criterion we used in this study, therefore it identified VBE, but not necessarily TCE. Confirmation of VBE as TCE would require analysis of TCR sequences expressed by the sorted expanded Vβ populations to determine their clonal composition, which was only rarely possible due to the number of cells required for the assays performed.

Effect of persistent infections on the size of $CD8^+$ and $CD4^+$ T cell pools

First we wanted to know whether persistent infection with MCMV or HSV-1 affects the composition and size of the T lymphocyte compartment. Comparison of percentages of $CD8^+$ and $CD4^+$ T lymphocytes (Figure 5.2 A and B) as well as their numbers (Figure 5.2 C and D) in spleens of the old mice revealed no significant

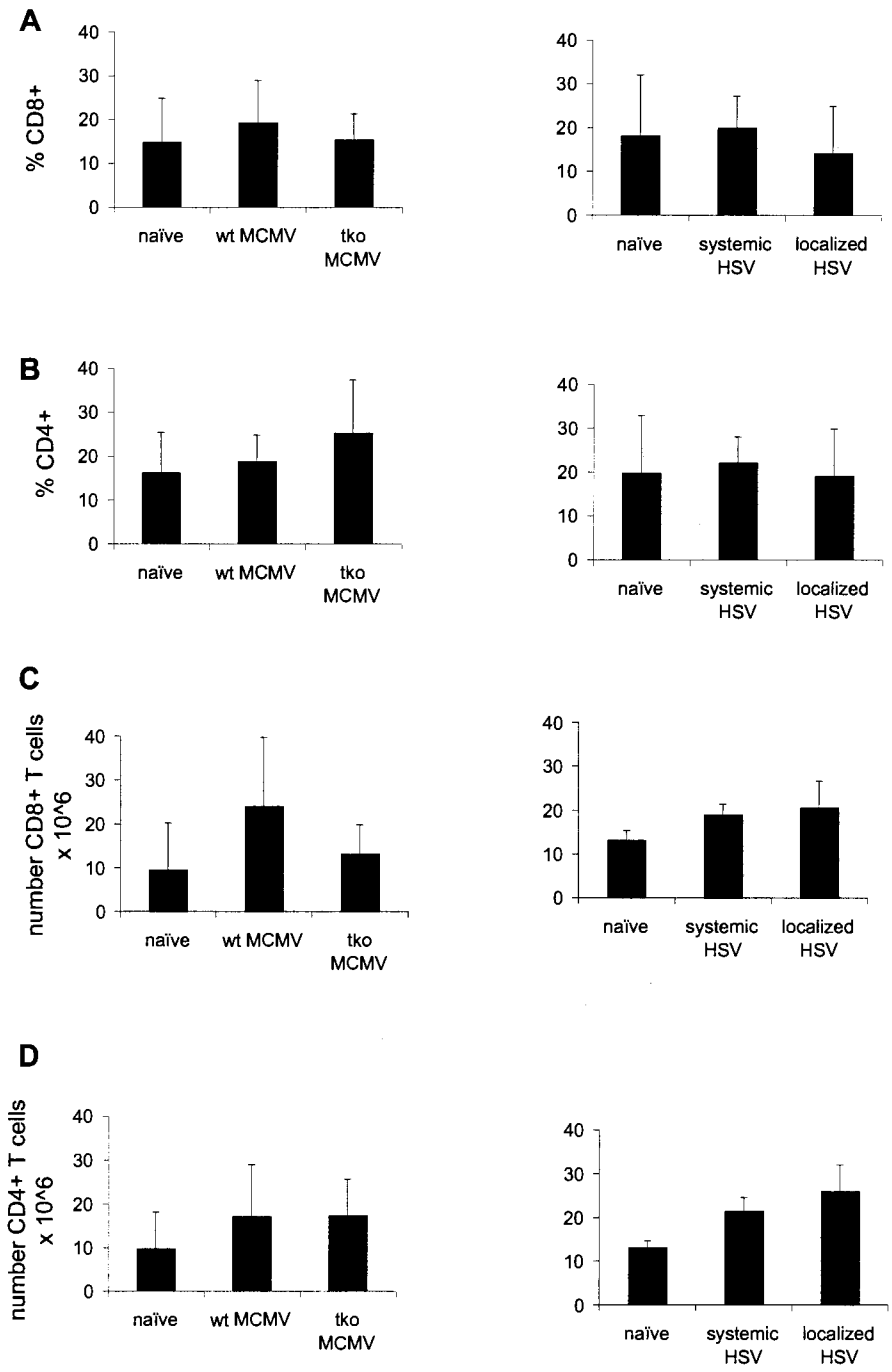


Figure 5.2 Effect of persistent infections on the size of $CD8^+$ and $CD4^+$ T cell pools
The percentage of $CD8^+$ **(A)** and $CD4^+$ **(B)** T cells, as well as the total number of $CD8^+$ **(C)** and $CD4^+$ **(D)** T cells were determined in the spleens of the mouse cohorts listed in Table 5.1 (MCMV cohorts) and Table 5.2 (HSV-1 cohorts) and are represented as average (± SD). No significant differences between cohorts were found (Student's t-test).

differences between infected and naïve animals. In one of the analyzed cohorts, mice infected with wt MCMV tended to have greater percentages and numbers of $CD8^+$ T cells than naïve or tko-infected animals, however these differences did not reach statistical significance and were not observed in another independent cohort. While at this time we conclude that lifelong infection with MCMV and HSV-1 does not distort the composition and size of the T lymphocytes compartment, additional experiments in larger mouse cohorts will be necessary to fully substantiate that conclusion.

Effect of persistent infections on incidence of VBE and diversity of $CD8^+$ T cells in old mice

Using the criterion described above, we compared the incidence of VBE in the experimental mouse cohorts. Mice with VBE were identified in all experimental groups, and detailed information about their Vβ family usage and the size of each VBE are summarized in Table 5.1 and Table 5.2. However, the incidence of VBE, defined as percentage of mice with VBE within each experimental group, was similar in naïve and infected groups (Figure 5.3 A). The incidence of TCE in SPF mice reported in the literature is 30-50% of old mice (Callahan et al., 1993; Ku et al., 1997; Messaoudi et al., 2006b), and the incidence of VBE in our study was within that range both in naïve and infected mice.

Another way to assess the diversity of repertoire is by determining the percentage of total $CD8^+$ T cell pool that remains free of VBE. For example, naïve mouse B2 (Table 5.1) with a $V\beta6^+$ VBE that constitutes 17% of $CD8^+$ T cells has 83% of its CD8 repertoire free of expansions (and presumably polyclonal). A mouse without VBE would

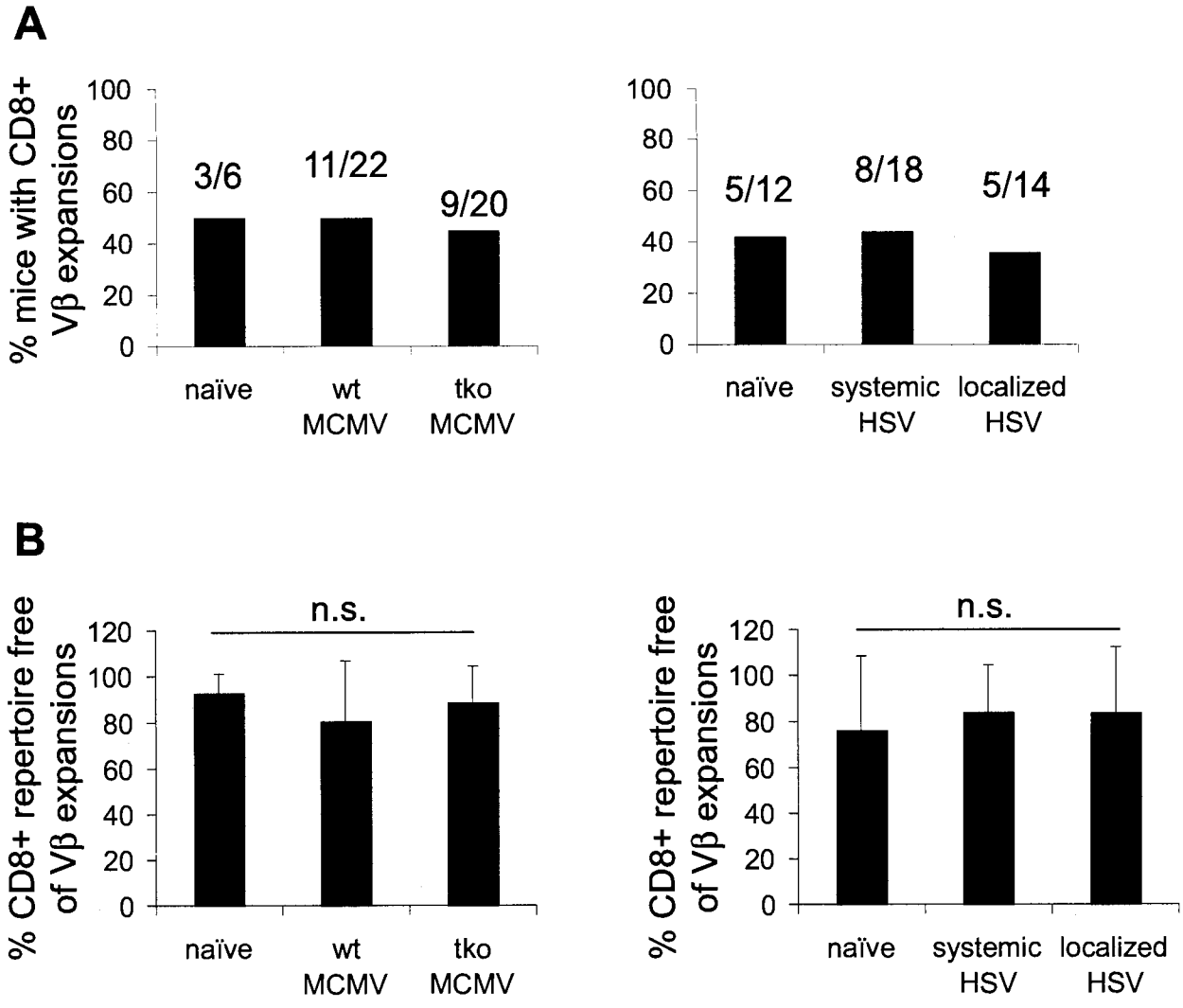


Figure 5.3 Effect of persistent infections on incidence of VBE and diversity of CD8$^+$ T cells in old mice **A.** The percentage of mice with VBE has been determined for each experimental group and is represented by a bar graph. The numbers above each graph show number of VBE$^+$ mice/total mice per group. **B.** The diversity of CD8$^+$ T cell repertoire in naïve, MCMV-infected and HSV-1 infected mice was determined for each mouse by subtracting the percentage occupied by VBE from 100%. The mouse cohorts were as in Table 5.1 (MCMV) and Table 5.2 (HSV-1). The values represent the average percent of the repertoire that is free of VBE (± SD) per group. No significant differences between cohorts were found (Student's t-test).

have 100% of its repertoire free of VBE. The average percentage of VBE-free $CD8^+$ T cells was determined for each experimental cohort. Figure 5.3 B demonstrates that despite presence of large VBE in some mice, on a cohort level infection with persistent virus did not correlate with TCR constriction greater than that observed in naïve mice.

The caveat of the experiments analyzing the impact of MCMV infection on TCR repertoire diversity was the small number (n=6) of naïve mice included in this study. Additional experiments will be necessary to confirm the findings presented here. However, the average percent of TCR repertoire occupied by VBE was very similar in all groups analyzed, leaving on average as much as 80-90% of the repertoire VBE-free (Figure 5.3 B). This included the naïve controls associated with the HSV-infected cohort, suggesting that even when larger number of naïve mice are analyzed, the conclusion regarding the minimal impact of persistent infection on restricting TCR repertoire diversity in old mice would likely stand.

Antiviral specificity of VBE in persistently infected mice

The results so far imply that neither HSV-1 nor MCMV infection strongly influences the diversity of the $CD8^+$ T cell pool in old mice on a cohort level. We next wanted to know whether the VBE that were identified were reactive against viral antigens. It was possible that even though the lifelong infections did not increase incidence of VBE, they could be influencing the nature (AI or AR) of the VBE in the infected mice. Therefore, the identified VBE were tested for their antiviral specificity.

The $CD8^+$ T cell response to MCMV is very broad (Munks et al., 2006a; Munks et al., 2006b) and generation of tetramers encompassing all the viral determinants was not

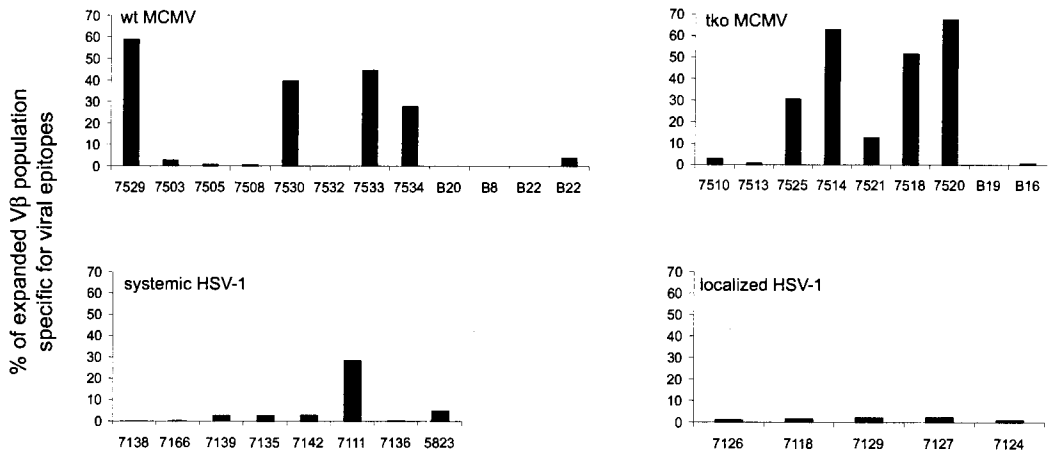
possible. Instead, the identified VBE were tested for their antiviral reactivity in an ex-vivo IFNγ assay, where the splenocytes were stimulated with a panel encompassing all the known MCMV epitopes (Munks et al., 2006b). In case of HSV-1, where the $CD8^+$ T cell response is concentrated on a single epitope gB-8p (Hanke et al., 1991; Wallace et al., 1999), the specificity of each VBE was determined by co-staining with antibody against the expanded Vβ and gB-8p tetramer.

By this method we determined what percentage of each VBE was virus-reactive (Figure 5.4). We found that while some mice had little or no reactivity against the persistent virus in their VBE^+ $CD8^+$ T cell populations, others had VBE largely made up of virus-specific cells. The latter was particularly true for VBE in MCMV infected mice, where virus-reactive $CD8^+$ T cells made up between 40-68% of VBE^+ T cells in some mice (Figure 5.4 A). However, the virus-reactive VBE were not homogenous, since in many cases the VBE contained $CD8^+$ T cells specific for more than one viral epitope (summarized in Figure5. 4 C). Interestingly, $V\beta11^+$ VBE were the most common VBE that reacted against MCMV epitopes, and they were frequently specific for one of the two epitopes derived from IE3 (IE3 416 and IE3 461, Figure 5.4 C).

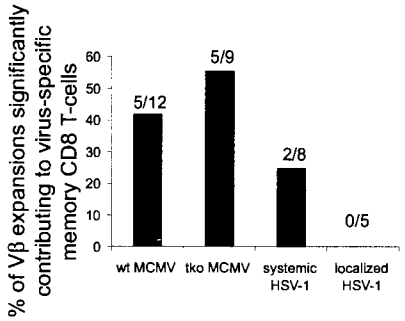
The contribution of virus-specific $CD8^+$ T cells to VBE in HSV-infected mice was less significant (in the most pronounced case 25% of VBE was HSV-specific, mouse 7111, Figure 5.4 A), occurred in fewer mice, and was negligible in the cohort infected via the localized route (Figure 5.4 A and B).

While more mice are needed to reach robust statistical significance, the results presented here do suggest that persistent infections influence the antigenic specificity of VBE, and the extent to which they do so correlates with the extent of virus-specific $CD8^+$

A



B



C

infection	mouse id	expanded Vβ family	% of expanded Vβ population specific for viral epitopes	recognized epitope(s)
wt MCMV	7529	Vb11	59	IE3(416), IE3(461), m78
	7530	Vb11	40	IE3(416), IE3(461)
	7533	Vb11	45	IE3(416)
	7534	Vb11	28	IE3(416), IE3(461)
tko MCMV	7525	Vb11	31	IE3(416), IE3(461)
	7514	Vb11	63	IE3(416), IE3(461)
	7521	Vb2	13	m38.1, m38.2, m102
	7518	Vb11	52	IE3(416), IE3(461)
	7520	Vb11	68	IE3(416), IE3(461)

Figure 5.4 Antiviral specificity of VBE in persistently infected mice. A. The antiviral specificity of the VBE was determined by co-staining the cells with the expanded Vβ-specific antibody and intracellular IFN following 6-hour ex-vivo stimulation with a panel of MCMV-specific peptides (MCMV infected animals, top panels). Alternatively, the cells were co-stained with the Vβ antibody and the tetramer specific for the viral gB-8p epitope (HSV-1-infected animals, bottom panels). The values represent the percentage of cells within each VBE that showed specificity for MCMV (top panels) or HSV (bottom panels). **B.** The percentage of VBEs that contained a significant proportion of virus-specific CD8+ T cells (>5% of VBE specific for a viral determinant) was determined for each experimental group and is represented by a bar graph. The numbers above each bar show the number of virus-reactive VBE/total number of VBE per experimental group. **C.** Examples of epitopes recognized by a proportion of VBE in MCMV-infected mice.

memory inflation driven by the virus. MCMV is a stronger driver of memory $CD8^+$ T cell inflation than HSV-1, judging by the extent of cognate memory inflation induced by these viruses (Chapter 4 and (Karrer et al., 2003)). Similarly, systemic infection with HSV-1, but not localized infection, is associated with virus replication-dependent CD8 memory inflation (Chapter 3 and 4). The hierarchy of the strength of ongoing antigenic stimulation associated with each virus infection seems to be reflected by the percentage of mice with strongly virus-reactive VBE (Figure 5.4 B). MCMV-infected mice had the greatest incidence of virus-reactive VBE (42% of mice in wt-infection, 55% in tko-infection), followed by mice systemically infected with HSV-1 (20% mice), and ending with localized HSV-1 infection where virus-reactive VBE were not detected. One should keep in mind that while localized HSV-1 infection is not associated with elevated generation of large VBE, it can produce age-related Ag-independent expansions of memory $CD8^+$ T cells (Chapter 3). The latter are often small in size, and would not be detected by the screening criteria used in this study, which focused on large VBE, similar to those associated with human CMV infection.

Combined effect of persistent infection and VBE on the repertoire diversity of old mice

To better understand the relative contribution of virus-specific and virus-nonreactive VBE to the overall diversity of $CD8^+$ T cell pool of old mice, we determined what percentage of that pool is occupied by virus-reactive VBE, virus-nonreactive VBE, and VBE^- virus specific memory $CD8^+$ T cells (Figure 5.5). For many mice a large proportion of their repertoire is occupied by a combination of virus-specific and virus-nonspecific $CD8^+$ T cells, reaching up to 91% of total $CD8^+$ T cells in the most extreme

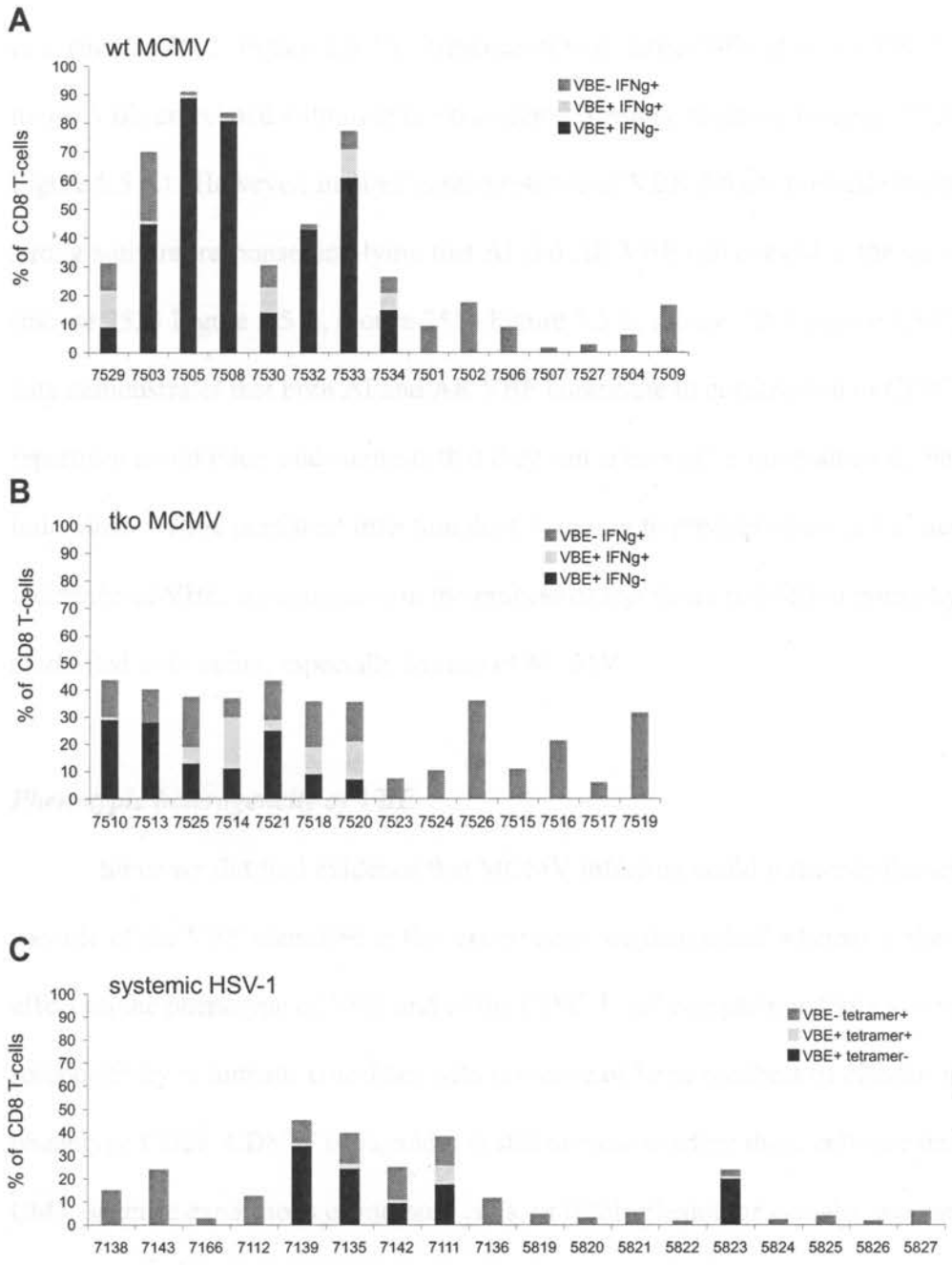


Figure 5.5 Combined effect of persistent infection and VBE on the repertoire diversity of old mice. The percentage of total $CD8^+$ T cell pool occupied by virus-specific $CD8^+$ T cells that were not a part of VBE (VBE^- IFN^+ or VBE^- $tetramer^+$), virus-specific $CD8^+$ T cells that were a part of VBE (VBE^+ IFN^+ or VBE^+ $tetramer^+$) and virus-nonreactive VBE (VBE^+ IFN^- or VBE^+ $tetramer^-$) was determined for wt MCMV infected **(A)**, tko MCMV infected **(B)**, and systemically HSV-1-infected mice **(C)**. The viral specificity was assayed as in Figure 4 (by an intracellular IFN staining following stimulation with viral epitopes in case of MCMV-infected mice, or by staining with gB-8p-specific tetramer in case of HSV-infected mice).

case (mouse 7505, Figure 5.5 A). Presence of very large (80% of total $CD8^+$ T cells or more) VBE correlated with little or no antiviral memory response (mouse 7505 and 7508, Figure 5.5 A). However, in other cases presence of VBE did not preclude maintenance of strong antiviral response, implying that AI and AR VBE can coexist in the same animal (mouse 7533 Figure 5.5 A, mouse 7514 Figure 5.5 B, mouse 7111 Figure 5.5 C). This data demonstrates that both AI and AR VBE contribute to constriction of $CD8^+$ T cell repertoire in old mice, and suggests that they can arise and be maintained in the same individual. While persistent infection does not seem to predispose mice for increased incidence of VBE, it contributes to the process of repertoire restriction normally associated with aging, especially in case of MCMV.

Phenotypic heterogeneity of VBE

Since we did find evidence that MCMV infection could influence the antigenic specific of the VBE identified in this experiment, we then asked whether it also had an effect on the phenotype of VBE and of the $CD8^+$ T cell compartment as a whole. CMV seropositivity in humans correlates with presence of large numbers of effector memory phenotype $CD28^-$ $CD8^+$ T cells, and it is still unclear whether these cells are uniquely CMV-specific expansions of memory cells, or if this phenotype can also be associated with CMV-nonreactive VBE, induced indirectly by ongoing persistent infection, possibly via influence of inflammatory cytokines.

First we determined the phenotype of the total $CD8^+$ T cell pool in naïve and MCMV-infected mice (Figure 5.6 A). Analysis of expression of CD62L, CD127, CD122, CD27 and CD28 by $CD8^+$ T cells in naïve and infected mice demonstrated great

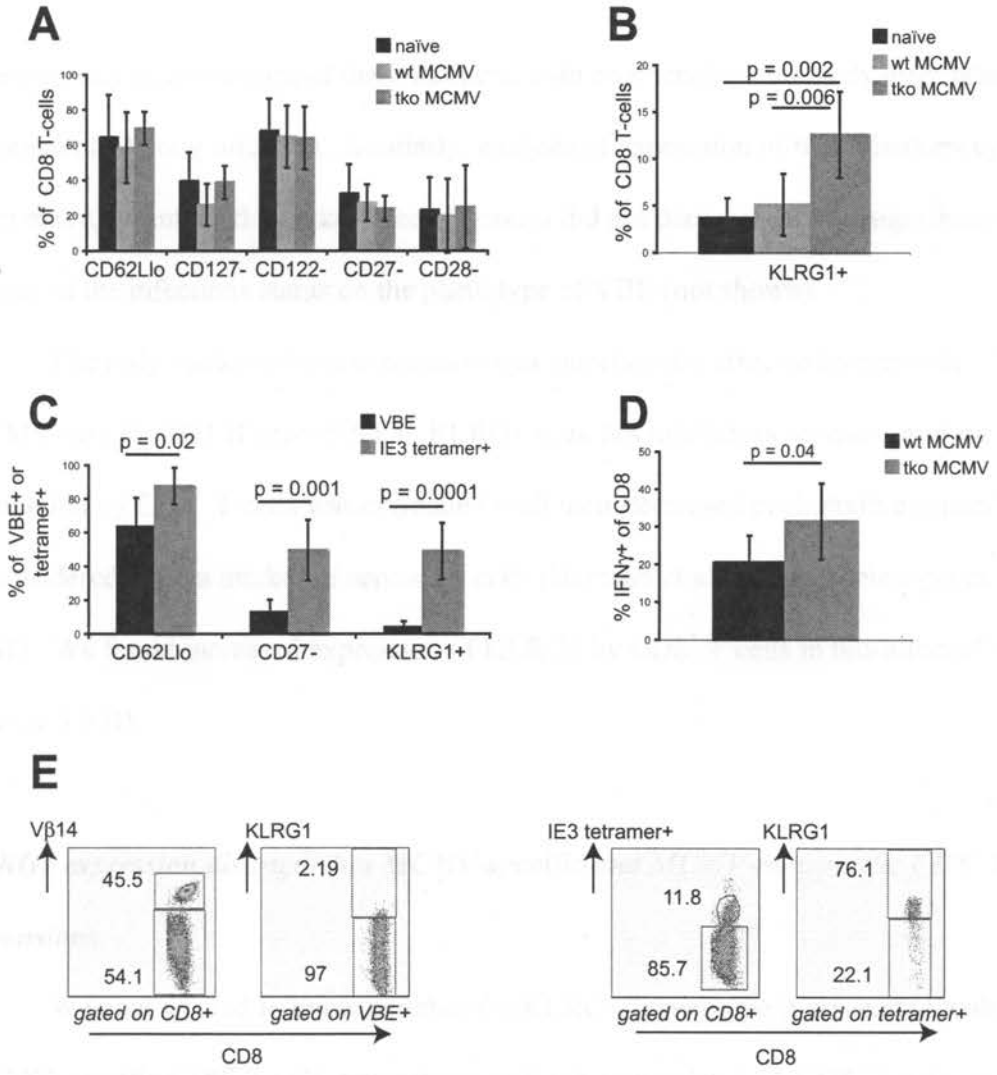


Figure 5.6 KLRG1 expression distinguishes MCMV-specific and MCMV-nonspecific CD8$^+$ T cell expansions. A. Surface expression of CD62L, CD127, CD122, CD27 and CD28 was determined within total CD8$^+$ T cell pool in naïve, wt-MCMV infected and tko-MCMV infected mice. The average expression (± SD) of the phenotype indicated at the bottom of the graph is shown. No significant differences were found between the three experimental groups for any of the markers tested (Student's t-test). **B.** Percentage of KLRG1$^+$ CD8$^+$ T cells in tko MCMV-infected mice is greater than in wt-infected or naïve mice. The difference was statistically significant (Student's t-test). **C.** Comparison of expression of CD62L, CD27 and KLRG1 by VBE and IE3-tetramer$^+$ CD8$^+$ T cells. The average percent of VBE or IE3-tetramer$^+$ cells that were CD62L^{lo}, CD27$^-$ and KLRG1$^+$ is shown (± SD). VBE and IE3-tetramer+ populations from both wt MCMV-infected and tko MCMV-infected mice were included in the analysis, allowing to compare the phenotype of 6 VBEs and 8 IE3-tetramer$^+$ cell populations. No significant differences were observed in phenotypes of VBE from wt and tko-infected mice, or between IE3-tetramer$^+$ cells from wt and tko-infected mice. **D.** Sum of IFN responses to all tested viral epitopes is greater in tko-infected (n = 6) than in wt (n =7) MCMV-infected mice. The graph represents the average total percent of IFN $^+$ cells (± SD) obtained by adding the responses to all tested MCMV epitopes.

heterogeneity in expression of these markers, with no phenotype uniquely associated with presence of lifelong infection. Similarly, analysis of expression of these markers by VBE from naïve, wt-infected and tko-infected groups did not demonstrate any significant impact of the infections status on the phenotype of VBE (not shown).

The only marker whose expression was significantly affected by presence of MCMV was KLRG1 (Figure 5.6 B). KLRG1 is an NK-inhibitory receptor, and its expression by $CD8^+$ T cells was correlated with their decreased proliferative capacity and is considered to be a marker of senescent cells (Hamann et al., 1997; Voehringer et al., 2001). We found increased expression of KLRG1 by $CD8^+$ T cells in tko-infected mice (Figure 5.6 B).

KLRG1 expression distinguishes MCMV-specific and MCMV-nonspecific $CD8^+$ T cell expansions

We next wanted to know whether the $KLRG1^+$ phenotype is associated with MCMV-specific $CD8^+$ T cell expansions, or if it is upregulated on $CD8^+$ T cells in infected mice regardless of their specificity. To this effect, expression of KLRG1 was compared between virus-nonreactive VBE and virus-specific memory $CD8^+$ T cells identified by staining with p:MHC tetramers. During MCMV-infection, $CD8^+$ T cell response to certain epitopes continues to increase in size (memory inflation). We used tetramers specific for three such inflationary epitopes, derived from viral IE3, m38 and m139 genes. Phenotypes of VBE and virus-specific inflating memory $CD8^+$ T cells are compared in Figure 5.6 C, using phenotype of IE3-epitope specific cells as an example. The same phenotypes were documented for m38 and m139-specific memory $CD8^+$ T

cells. We found that significantly higher proportion of virus-specific memory $CD8^+$ T cells expressed KLRG1, as compared to VBE. In addition, increased proportion of virus-specific cells was $CD62L^{lo}$ and $CD27^-$ as compared to VBE, indicating their effector memory phenotype. An example of KLRG1 staining is shown in Figure 5.6 E.

Since memory cells specific for the MCMV epitopes associated with memory inflation expressed the same $KLRG1^+$ $CD62L^{lo}$ $CD27^-$ phenotype in wt as well as tko MCMV-infected mice, we wondered why the increased expression of KLRG1 on the total $CD8^+$ T cell pool level was observed only in tko-infected mice (Figure 5.6 B). One explanation for that could be the fact that the overall antiviral memory $CD8^+$ T cell response, as measured by addition of responses to all individual epitopes in an ex-vivo IFNγ assay, was greater in tko than in wt-infected mice (Figure 5.6 D and results in (Munks et al., 2007)). This would correlate with presence of more virus-specific $KLRG1^+$ memory $CD8^+$ T cells in tko than in wt-infected mice. However, staining with KLRG1 was not done in conjunction with intracellular IFNγ staining, therefore the data available at this time cannot directly test this hypothesis.

DISCUSSION

This study assessed the impact of two herpesviruses, MCMV and HSV-1, on the size, diversity and phenotype of $CD8^+$ T cell pool of old infected mice. We found that neither MCMV nor HSV-1 lifelong infection affected the overall composition and size of the T lymphocyte pool. The impact of these infections on the diversity of the $CD8^+$ T cell T cell pool, as judged by the incidence and size of VBE, was also not significant on a

cohort level. However, some subtle effects of the presence of the persisting viruses were observed. This was mostly evident in MCMV-infected mice, in which a proportion of their VBE, originally identified only by staining with antibodies against various TCR Vβ chains, was made up of MCMV-specific $CD8^+$ T cells. This effect was less pronounced in mice infected with HSV-1. Presence of partially virus-specific VBE correlated with the extent of virus-specific CD8 memory inflation associated with each infection or infection route, with MCMV infection being associated with the most pronounced memory inflation, and localized HSV-1 infection with the least.

Additional finding was that infection with tko MCMV, a virus lacking three known evasion genes, resulted in an increased percentage of $CD8^+$ T cells expressing KLRG1 in the old infected mice. All three of the immune evasion genes deleted in the tko virus are known to interfere with MHC I-associated antigen presentation pathway and to prevent CD8-mediated lysis of infected cells *in vitro* (Kavanagh et al., 2001; Pinto et al., 2006; Reusch et al., 1999; Ziegler et al., 1997). The *in vivo* role of these immune evasion genes is less well understood. While *in vivo* infection with tko MCMV was not associated with marked differences in the size and epitope hierarchy of $CD8^+$ T cell response in adult animals (Gold et al., 2004; Munks et al., 2007), two independent cohorts of old mice demonstrated an overall larger MCMV-specific memory $CD8^+$ T cell population in tko than in wt-infected old mice ((Munks et al., 2007) and Figure 5.6 D). We do not know the mechanism by which lifelong infection with tko MCMV leads to increased antiviral memory in the old mice at present time. However, it can be speculated that the reason for the increased frequency of $KLRG1^+$ $CD8^+$ T cells in tko-

infected mice could be related to the increased number of $KLRG1^+$ MCMV-specific $CD8^+$ T cells.

The implications of the results discussed here fall into two major categories. First, they increase our understanding of the mouse infection models themselves. Second, they allow us to compare the results obtained from mouse models to the data on infection, immune response and immune senescence to the same (HSV-1) or highly homologous (HCMV) viruses in humans . Persistent viral infections were implicated to play a part in development of old age-associated changes in the human T cell compartment, particularly by driving the development of the very large $CD28^-$ $CD8^+$ T cell expansions (Pawelec et al., 2005). It would be pertinent to develop a suitable animal model to test this prediction, since design of experiments with human subjects is limited by ethical as well as technical constraints, such as the great diversity of the human HLA haplotypes and the viral determinants presented by them. Being able to account for the antigenic specificity of the T cell expansions seen in elderly humans is important in addressing the possible mechanism by which the persistent infection drives development of T cell expansions.

Human infections with HSV-1 have not been correlated with increased incidence of TCE in circulation, although presence of clonally expanded $CD8^+$ T cells was recently reported in latently infected human ganglia (Derfuss et al., 2007). In concordance with that, we did not notice a significant impact of lifelong localized HSV-1 infection on VBE development in mice. This most likely reflects the nature of this lifelong infection, which is characterized by latency with periodic viral reactivation. While the reactivation incidents provide enough antigenic stimulation to drive inflation of the HSV-specific

memory $CD8^+$ T cells, its impact is likely not pronounced enough to affect the overall diversity of the $CD8^+$ T cell pool, neither by establishment of very large clonal virus-specific $CD8^+$ T cell population, nor by providing inflammatory environment affecting bystander lymphocytes.

In contrast, CMV infection in humans and in mice is associated with very strong virus-specific CD8 memory inflation, affecting cells of several antiviral specificities (Sierro et al., 2005). While CMV is capable of establishment of latency, the analysis of memory $CD8^+$ T cells both in humans and in mice suggests that the virus may be continuously providing antigenic stimulation, as inferred by memory inflation and the effector phenotype of memory $CD8^+$ T cells. While studies with more mice are needed to confirm our findings, the data presented here suggests that in the mouse model lifelong MCMV infection does not lead to increased incidence of $CD28^-$ $CD8^+$ VBE. Further studies must be performed to decide whether the MCMV model can be used as a model to study the impact of CMV on development of age-associated $CD8^+$ T cell expansions in humans. These would involve large cohorts of mice, including infected and naïve groups, housed under identical conditions. In addition to performing experiments done in this study (analysis of TCR Vβ expression and phenotype of $CD8^+$ T cells, determination of antiviral specificity of VBEs), experiments addressing the clonal composition, differentiation status (i.e. telomere length) and functional responses of VBE (i.e. proliferation in response to homeostatic cytokines or CD3 stimulation) in infected and naïve mice would be needed. In conjunction with that, the serum levels of inflammatory cytokines between naïve and persistently infected mice should be compared. Finally, one should test whether persistent infections in mice lead to decreased responses upon

vaccination against novel pathogens, or to increased susceptibility to infection (i.e. flu, West Nile Virus), as was suggested for CMV-seropositive humans, and whether this correlates with presence of VBE.

On the other hand, the ability to test for antigenic specificity of VBE in the mouse model allowed us to identify several markers which can be useful in identifying CMV-specific and CMV-unresponsive $CD8^+$ T cell expansions. Expression of KLRG1 provided the best distinction between virus-nonresponding VBE and virus-reactive expanded memory $CD8^+$ T cell populations. This finding can be potentially useful in human studies, where identification of the specificity of VBEs is not always possible. However, before this can be done routinely, studies comparing expression of KLRG1 on VBE of known CMV-specificity status need to be performed, to test whether the correlation between KLRG1 expression and CMV-specificity found in mice holds true in humans.

Even though the large Vβ expansions identified in our screen were never 100% MCMV-specific, virus-specific memory $CD8^+$ T cells partially contributed to some VBE. Therefore, even though we did not specifically test whether the VBE we found were clonal, these data suggest they were not. Rather, they often consisted of T cells with two or more different antigenic specificities (i.e. the Vβ11+ VBE in mouse 7529 in Figure 5.5 C consisted of T cells specific for three different MCMV epitopes and cells specific for at least one more non-MCMV determinant). This finding calls for another look at the human data. While a number of studies did determine that the CMV-specific TCE in the elderly humans were indeed clonal or oligoclonal, other studies did not. The fact that the VBE we found here were relatively heterogeneous in their antigenic

specificity strongly suggests that the mechanism behind their development differs from that described for some murine TCE, which were very homogenous both in their clonotypes and phenotypes (Ku et al., 2001; Messaoudi et al., 2006c).

Finally, based on our data the inflammation-driven expansion of bystander (not virus-specific) $CD8^+$ T cells does not appear to be a factor in development of VBE in persistently infected mice. First, we did not observe greater incidence or increased size of VBE in infected mice, although this remains to be verified in a larger study. More importantly, we did not notice an increase in percentage of $CD28^-$ $CD8^+$ T cells in infected mice. Conversion of human T cells to this phenotype was associated with ongoing type I IFN stimulation (Borthwick et al., 2000; Fletcher et al., 2005). Therefore, it is not likely that inflammation is an important contributor to VBE development in persistently infected mice. However, we did not test for it directly, and to formally demonstrate it we would need to 1) determine levels of inflammatory cytokines in infected and naïve mice, and 2) verify the effects of ongoing type I IFN stimulation on the phenotype of murine $CD8^+$ T cells.

In conclusion, we have undertaken the first steps to assess the impact of lifelong viral infections on the process of TCE formation in mouse models where the biology of the viruses and the specificity of the antiviral $CD8^+$ T cell response is known. These studies may prove useful in elucidating the mechanisms behind generation of AR and AI TCE in mice and in humans. While the impact of MCMV infection on the $CD8^+$ T cell compartment in mice did not corroborate all of the characteristics of HCMV's impact on the aging $CD8^+$ T cell compartment in humans, some of our findings, such as identification of KLRG1 as a surface marker that clearly distinguishes between virus-

specific and virus-nonreactive $CD8^+$ T cell expansions may be useful if verified in human studies.

Summary

In this chapter we have analyzed the impact of lifelong viral infections on the homeostasis of $CD8^+$ T cell pool with respect to its size, phenotype and TCR diversity. We used knowledge gained from the literature as well from studies of the maintenance of memory $CD8^+$ T cells following HSV-1 infection described in this thesis (Chapters 3 and 4) to analyze this issue. Despite inducing significant Ag-driven expansion of memory $CD8^+$ T cells, systemic infection with neither HSV-1 nor MCMV resulted in generation of very large virus-specific VBE, and did not affect the size of the $CD8^+$ T cell pool. However, the Ag-specific memory $CD8^+$ T cells contributed in part to the VBE found in some persistently infected mice. Large MCMV-specific memory $CD8^+$ T cell expansions could be distinguished from MCMV-unreactive VBE by elevated expression of KLRG1. What we learned from this study, as well as from Chapters 3 and 4, is that both age and persistent viruses do contribute to changes in the $CD8^+$ T cell phenotype and diversity over lifetime. However, our data show that a variety of often independent mechanisms lead to development of immunosenescence-associated phenomena, such as TCE and loss of naïve-phenotype T cells. Persistent infections do play a part in these processes, but at least in mice, their role appears to contribute to, rather than drive, these processes.

CHAPTER 6: DISCUSSION

In this dissertation I have examined the relationship between lifelong viral infections and effector and memory $CD8^+$ T cells. Below the findings of the experiments described in the dissertation are discussed in the context of the current literature on each subject.

Protective function of $CD8^+$ T cells

$CD8^+$ T cells combat viral infections by eliminating infected cells or blocking viral replication. However, they constitute only one component of the complex immune response contributing to the elimination of the virus, and it is often difficult to discern their relative contribution in providing protection from infection. By careful analysis of the $CD8^+$ T cell response following ocular HSV-1 infection, we demonstrated specific functions of these CTLs in mediating antiviral protection. A critical factor enabling CTL functions is the timing of their activation and recruitment to the infected tissues. We showed that activated virus-specific $CD8^+$ T cells could first be seen at the site of infection on day 6 p.i. (Chapter 2 Figure 2.2), which was 3-4 days after the virus has already spread there (Chapter 2 Figure 2.6). Viral access to the nervous system is crucial in allowing the virus to establish latent infection. A study performed concurrently with ours (van Lint et al., 2004) was in agreement with our results. They also found that in a flank scarification model the gB-8p-specific CTLs were activated too late to prevent viral spread from the inoculation site into the nervous system. Similarly, in an intranasal (i.n.) HSV-1 infection model activated $CD8^+$ T cells arrived in the brain after the virus (Anglen

et al., 2003); this was not surprising, as the virus can access the brain directly via the olfactory nerve. All of the above studies demonstrate that without pre-existing virus-specific memory $CD8^+$ T cells, the kinetics of HSV-1 spread are too fast compared to the kinetics of naïve precursor T cell activation, and the $CD8^+$ T cells simply cannot participate in controlling viral dissemination.

Despite not being able to prevent viral spread, we and others demonstrated that once activated, $CD8^+$ T cells play a protective role during HSV-1 infection. We have shown that in the absence of $CD8^+$ T cells (CD8α knockout mice), mice were unable to control viral replication in the nervous system and succumbed to encephalitis (Chapter 2 and (Lang and Nikolich-Zugich, 2005)). The protective capacity of antiviral $CD8^+$ T cells was also demonstrated by Anglen et al., where mice with pre-existing memory gB-8p-specific $CD8^+$ T cells survived the stress-induced HSV-1 encephalitis (Anglen et al., 2003). Interestingly, in that study the mice were immunized by systemic infection with recombinant Vaccinia Virus expressing the gB-8p epitope (rVV-gB), and the protective memory $CD8^+$ T cells were present in brains of these mice prior to infection with HSV-1, indicating that migration of activated cells into the brain can take place in the absence of brain infection. This is in line with results from Lefrancois group, which demonstrated almost unlimited tissue access of activated cells (Masopust et al., 2004b). Van Lint et al. (van Lint et al., 2004) confirmed that if introduced by adoptive transfer prior to infection, activated virus-specific $CD8^+$ T cells can completely eliminate viral replication and spread, whereas adoptive transfer of activated CTLs into already infected $Rag^{-/-}$ mice at day 4 p.i. terminated viral replication both in the skin and the nervous system and prevented death from encephalitis.

It is important to note that while activated virus-specific CTLs efficiently control viral replication, they cannot easily prevent establishment of latency, although by limiting viral replication they may decrease the size of latent viral load (LeBlanc et al., 1999) and, likely, the number of latent sites (Sawtell et al., 1998), although the latter was not tested. Memory $CD8^+$ T cells present in brains of mice prior to HSV-1 challenge were unable to prevent viral entry as demonstrated by presence of viral DNA in the brains by PCR (Anglen et al., 2003). Since no infectious virus was detected in these mice and they survived the challenge, it implies that memory cells limited the extent of viral replication, but could not prevent it. The only instance where gB-8p-specific CTLs were able to prevent viral migration to the nervous system was in the event of adoptive transfer of activated cells, allowing them to be completely armed and present at the site of infection before viral challenge (van Lint et al., 2004). This situation is not likely to ever arise under natural conditions *in vivo*, and even in the presence of memory $CD8^+$ T cells primary infection with HSV-1 is likely going to result in establishment of latency. The only efficient means of preventing establishment of latency seems to be aggressive treatment with antiviral drugs (LeBlanc et al., 1999). We have shown that treatment with famvir prior to and during ocular infection with HSV-1 prevented establishment of latency in the trigeminal ganglia (TG, Chapter 3). Whether this treatment would be effective in other infection models remains to be tested.

In summary, the experiments presented in this thesis along with recent data from the literature confirmed the importance of protective role that gB-8p-specific CTLs play during HSV-1 infection. In the absence of pre-existing immunity, the $CD8^+$ T cells are

unable to prevent viral spread, but once activated, are crucial in controlling viral replication *in situ* and preventing encephalitis.

Development of CD8$^+$ T cell response in lymphoid and non-lymphoid organs

Interestingly, the kinetics of virus spread from all the different epithelial inoculation sites tested in the above described studies (cornea, skin, intranasal mucosa) are the same, with HSV-1 reaching the neurons innervating the site by day 2 p.i. However, the kinetics of CD8$^+$ T cell activation differ slightly between the models used. Evidence of CD8$^+$ T cell activation in the draining LN (proliferation, cytotoxic function) was found as early as day 2 p.i. in the flank scarification model (Coles et al., 2002; Mueller et al., 2002b), whereas in the ocular infection used in our studies we did not see virus-specific CD8$^+$ T cell proliferation until day 5 (Chapter 2 Figure 2.1). The kinetics of gB presentation were analyzed in great detail in the Carbone laboratory (Mueller et al., 2002b). To determine how early gB is presented, the authors digested the LNs with collagenase in order to isolate DCs presenting the Ag, and used them to stimulate gB-8p-specific hybridoma *in vitro*. They found that LN DCs isolated as early as 4-6 hours p.i. were able to stimulate the hybridoma. In another experiment, this time using adoptive transfer of CFSE-labeled TCR Tg T cells prior to infection, they demonstrated that virus specific CD8$^+$ T cells present in the LN upregulate CD69 within 4-6 hours p.i., but their proliferation was not detected until 48 hours p.i. In the latter experiments, the LNs were simply dispersed into single cell suspensions, as was done in our experiments (Chapter 2), allowing us to directly compare the kinetics of T cell activation following footpad and ocular infection.

The exact kinetics of $CD8^+$ T cell activation were not examined in the intranasal model, however the authors determined the kinetics of infiltration of activated gB-8p-specific $CD8^+$ T cells into the brain, which begun on day 5 p.i. (Anglen et al., 2003) Similarly in the flank scarification model CTLs were detected in the nervous system on day 5, whereas in our experiments this did not happen until day 6. In all of these experiments the analysis of migration of activated gB-8p-specific $CD8^+$ T cells was done in the same way (staining with tetramer), and it is not likely that technical differences factored into obtaining slightly different results. The differences in the timing of infiltration of the infected organs appear to be a direct result of the different kinetics of priming. Those in turn are likely affected by the particular conditions of each infection model used, and reflect the time needed for Ag to reach the LN.

Experiments in i.n. flu infection model provided compelling evidence that the timing of $CD8^+$ T cell activation in the LN depends on the rate of influx of DCs from the infection site (Yoon et al., 2007). It is at present not known how viral Ag reaches the LN following ocular infection. Liu et al. (Liu et al., 2002) demonstrated that cornea-derived DCs migrate to the draining LN within 24 hours post allogeneic corneal transplant in mice. It appears that despite that, following ocular HSV-1 infection it takes several days for the Ag to reach the LN. Migration of Ag-loaded DCs from cornea may be more restricted than from skin.

Regardless of how the Ag reaches the LN, studies presented here (Chapter 2) and those of others demonstrate that $CD8^+$ T cell priming takes place predominantly in draining LNs, and not outside of lymphoid tissues (Marten et al., 2003; Mendez-Fernandez et al., 2005; Walter and Albert, 2007) . This is not a new concept, however

recent data provided some new insights regarding the specific events that take place in the LNs. Our work (Chapter 2 and Lang 2005) and other studies (Coles et al., 2002; Mueller et al., 2002b) demonstrated that following localized infections virus-specific $CD8^+$ T cells can first be detected exclusively in the draining LNs. Only after extensive proliferation and acquisition of activated phenotype (Chapter 2) and effector functions (IFNγ production, cytotoxic function) they are simultaneously released to other tissues. Our results are in agreement with those of others, which demonstrate that only cells that have proliferated extensively and acquired the activated phenotype (i.e. $CD43^{hi}$ cells in our studies) and effector functions are found in the infected non-lymphoid organs. However, unlike Coles et al. (Coles et al., 2002), who also found that only highly differentiated CTLs can be found in the spleen, we demonstrated presence of virus-specific $CD8^+$ T cells in early stages of proliferation and differentiation into CTL (i.e. $CD43^{int}$) in spleens of ocularly infected mice. At present we do not know the reason behind this difference. The caveat of the above studies (both ours and those of Coles et al.) is that they used an adoptive transfer of large number ($1 - 2 \times 10^6$) of TCR Tg cells to study the *in vivo* anti-HSV $CD8^+$ T cell response. Careful analysis of the impact of number of cells used in such experiments revealed that providing too high a frequency of Tg precursors skews the kinetics, proliferation and the phenotype of transferred cells following Ag challenge (Badovinac et al., 2007). Transfer of high number of Tg cells (5×10^5) resulted in fewer divisions per cell and in earlier peak of the expansion following challenge, compared to transfer of low number of cells (5×10^3 or fewer). Importantly, transfer of 5×10^3 or fewer cells better approximated the kinetics of the endogenous response than transfer of higher cell numbers. The literature on $CD8^+$ T cell activation

following HSV-1 infection should be revisited in light of these new findings. However, in our studies of the ocular infection, the kinetics of expansion of the transferred TCR Tg (Chapter 2 Figure 2.2) and endogenous gB-8p-specific precursors in mice that did not receive transfer of Tg T cells (Chapter 4 Figure 4.3 B) were the same.

Viral replication and generation of $CD8^+$ T cell response

Another factor regulating the availability of the Ag for T cell priming is the extent of viral proliferation, although that is not true under all experimental conditions. Localized HSV-1 infections, such as ocular infection used in our experiments, or the flank scarification model, required viral proliferation to allow T cell priming. In Chapter 3, we demonstrated that completely blocking viral replication by treatment with famciclovir prior to and during localized infection prevented generation of gB-8p-specific response. Similarly, limiting the extent of viral replication during flank scarification model affected CTL priming (Stock et al., 2004). Specifically, by surgically removing the primary infection site at various times p.i. but preceding viral spread into the sensory ganglia, the authors demonstrated that presence of virus was required for at least 8 hours in order to generate CTL response, and >24 hours of infection were required to generate optimal numbers of CTLs (Stock et al., 2004). This result is in seeming conflict with another study from the same group which demonstrated activation of gB-8p-specific $CD8^+$ T cells in the draining LN as early as 4-6 hours p.i. (Mueller et al., 2002b). The difference can be in part explained by different readouts of the two studies. Mueller et al. analyzed activation of gB-specific cells in the LN, and were able to detect some, but very small amount of gB-specific cytotoxic activity early post infection. In contrast,

Stock et al. measured the size of gB-specific CD8 response in spleen on day 7 p.i. It is likely that T cell activation induced in the first 8 hours of infection was not enough to stimulate a robust enough proliferative response to generate a detectable virus-specific $CD8^+$ T cell population. Alternatively, the data suggests that generating a CTL response of an optimal size in this model requires prolonged period of Ag presentation. The latter seems to be the case, as transfer of naïve gB-specific TCR Tg cells into infected mice as late as day 7 p.i. resulted in their activation (Stock et al., 2004). Similarly, in our experiments we observed presence of gB-specific $CD8^+$ T cells in various stages of proliferation as late as day 12 p.i., indicating continued recruitment of new precursors into the CTL response (Chapter 2). Therefore, following localized infection CTL priming occurs over prolonged period of time, and this extended Ag presentation is required for generation of response of optimal size. These results are in agreement with study by Yoon et al. (Yoon et al., 2007), which demonstrated that activation of $CD8^+$ T cells during i.n. flu infection occurs in a "conveyer belt" fashion, that is, naïve T cells enter the program of activation sequentially, depending on the time of their encounter with antigen. This in turn depends on the timing of APC arrival from the site of infection, which takes place continuously for several days p.i. However, this study is in contrast to results obtained from *Listeria monocytogenes* (LM) infection model, where termination of bacterial replication as early as 24 h.p.i. did not affect the size of CD8 response (Badovinac and Harty, 2007). These results need to be reconciled, but are not mutually exclusive. The differences are likely due to differences between the pathogens' replication cycle, their systemic spread and infection routes used. One must also be cautious with the interpretation of the results. For example, both in case of HSV-1 and

LM infection, 24 hours of pathogen replication were sufficient to generate a strong CTL response (Badovinac and Harty, 2007; Stock et al., 2004), however this response could have been further enhanced in case of HSV-1 if viral replication was allowed to proceed. Therefore, presence of prolonged Ag presentation during localized HSV-1 infection does not exclude the possibility that "programming" of activated $CD8^+$ T cells takes place there. Of note, the infection route differed between the two studies, with the HSV-1 being administered via localized route (scarified skin), and LM being injected systemically (intravenously, or i.v.).

Our studies of systemic HSV-1 infection provide evidence that infection route can affect the kinetics of CTL priming (Chapter 4), as well as the dependence of priming on viral replication. Unlike during localized infection, antiviral treatment during primary systemic infection with HSV-1 did not prevent generation of robust CTL response (Chapter 4 Figure 4.7), although its overall size was decreased. Similar results were obtained with systemic infection with replication deficient HSV-1 mutants, lacking viral thymidine kinase (TK) function (Brehm et al., 1997). The TK^- virus generated a protective CTL response *in vivo*. Priming of cells in the absence of viral replication appears to proceed due to the ability of infected cells to synthesize gB despite the terminated viral lifecycle. gB synthesis proceeded to comparable levels in B6/WT3 cells infected with wt and TK^- HSV-1 (Brehm et al., 1997). We did not test expression of gB in mice treated with famciclovir, but one could expect that it will proceed just as it does during TK^- viral infections, since viral replication is inhibited at the same stage (TK-dependent DNA synthesis) in both cases. These studies are also in agreement with the result that cells infected with UV-inactivated HSV-1 are unable to present gB (Mueller et

al., 2003), since the ability of this virus to express gB *de novo* is abrogated, owing to the UV-induced damage to the DNA template.

Influence of Ag on maintenance of memory CD8$^+$ T cells during latent infection

Infection with HSV-1 leads to development of protective memory CD8$^+$ T cell response (Anglen et al., 2003; Brehm et al., 1997). We have investigated the longevity of this response and the factors involved in its maintenance. We found that the gB-8p-specific memory cells form a long-lived population, and can be detected for the lifetime of the mouse (Chapter 3). While other studies focused on the study of HSV-specific memory CD8$^+$ T cells in the latently infected ganglia (Khanna et al., 2003; Sheridan et al., 2006; Suvas et al., 2006), we were interested in the maintenance of systemic response. In particular, we wanted to investigate whether the lifelong infection results in periodic stimulation of the systemic memory CD8$^+$ T cell pool. This seemed a likely possibility in light of evidence of spontaneous viral reactivation (Feldman et al., 2002; Margolis et al., 2007) and the ongoing stimulation of TG-resident memory CD8$^+$ T cells throughout the latent period (Khanna et al., 2003).

We found evidence for antigenic stimulation of systemic memory CD8$^+$ T cells in latently infected mice in form of memory inflation (Chapter 4), however the extent of that inflation was dependent on several factors. First, extensive systemic viral spread correlated with the magnitude of memory CD8$^+$ T cell expansion. We believe that the greater viral spread leads to establishment of more reservoirs of latent virus, which would lead to more frequent incidence of reactivation. Second, blocking viral reactivation

prevented or decreased the extent of memory inflation, depending on the extent of initial viral replication and spread. We interpreted this as evidence for the presence of antigenic stimulation during latency, and concluded that the ability of the virus to reactivate from latency is a strong factor in driving memory inflation. The fact that limited memory inflation took place in some mice despite ongoing famciclovir treatment may reflect the kinetics of gB expression discussed above. Specifically, while famciclovir prevents full viral reactivation, the fact that it only blocks the replication cycle at the DNA synthesis stage means that in cells undergoing spontaneous reactivation some gB synthesis would take place, and this might be enough to stimulate the T cells.

Alternatively, by continuous treatment with the antiviral drug, we may have selected a drug-resistant viral mutant. We do not believe it likely, as our viral stock is made from a plaque-picked clone of HSV-1 strain 17, however viral mutation could have occurred. Such resistant mutants were isolated from HSV-positive humans (Lebel and Boivin, 2006; Strick et al., 2006) , but the incidence of acyclovir-resistance is very rare (<1% of immunocompetent subjects, <5% HIV^+ subjects) among patients who regularly use the drug. Presence of drug-resistant virus could be tested by sequencing viral genomes recovered from latently infected mice, however at present we are unable to obtain such viral isolate. Unlike in the ocular model, where the anatomical sites of viral replication have been mapped, the exact locations of latent virus after i.p. infection are not known. While during acute infection the replicating virus can be recovered from the spinal cord, associated sensory ganglia and brain, detection of virus during latency is more difficult without knowing the precise location(s) of the latent reservoir.

Up to now very little has been known about maintenance of systemic memory $CD8^+$ T cells during latent viral infections. In that respect the best studied latent virus is murine cytomegalovirus (MCMV). Recent immunological evidence demonstrated strong ongoing stimulation of virus-specific $CD8^+$ T cells during this lifelong infection (memory inflation, (Karrer et al., 2003; Karrer et al., 2004)), and this prompted some investigators in the field to consider MCMV to be a chronic rather than latent virus. Work in this thesis uncovered some similarities between maintenance of memory $CD8^+$ T cells during systemic HSV-1 and MCMV infection. In both cases, following resolution of the acute infection and contraction of the effector CTL response, the virus specific memory $CD8^+$ T cell pool increases over time from the set-point, and the memory $CD8^+$ T cells are characterized by predominantly effector memory phenotype ($CD62L^{lo}$ $CD127^-$ $CD27^-$). This phenotype is indicative of recent or repeated contact with Ag, and our studies provide evidence that it is a result of viral stimulation during lifelong infection (Chapter 4). However, unlike during MCMV infection, where the expansion of virus-specific memory $CD8^+$ T cells continues throughout the latent period (Karrer et al., 2003; Karrer et al., 2004) the memory inflation following HSV-1 infection reaches a plateau (Chapter 4). This implies that either the strength or the frequency of $CD8^+$ T cell stimulation during MCMV infection is more pronounced than in case of HSV-1.

While our results show that Ag can influence the size and phenotype of memory $CD8^+$ T cells during HSV-1 latency, one should remember that it is not always the case, and that the nature of that influence differs from that described for persistent chronic infections. During localized HSV-1 infection, associated with limited systemic virus spread, the antiviral memory is maintained at a stable, low frequency and virus-driven

memory inflation does not take place, as demonstrated by comparison of ocularly infected mice that were either left untreated or were continually treated with famciclovir (Chapter 3). In mice that do undergo virus-driven memory inflation, such as is the case following systemic HSV-1 infection (Chapter 4), the maintenance of that memory seems to be accomplished by different mechanisms than those described for chronic viruses. First, chronic viral infections (chronic LCMV, HIV, HCV) are associated with driving the memory $CD8^+$ T cells into state of functional exhaustion (rev. in (Shin and Wherry, 2007). This does not happen over the course of HSV-1 infection (Chapter 3 and data not shown). Second, while Ag was needed to maintain the frequency of expanded memory T cell populations during HSV-1 infection, it was not absolutely required for their survival, unlike memory cells specific for chronic LCMV and polyoma virus, which required Ag for survival (Shin et al., 2007; Vezys et al., 2006). Third, Ag-driven proliferation of memory $CD8^+$ T cells is involved in maintenance of expanded memory $CD8^+$ T cell populations during lifelong HSV-1 infection, however this proliferation is not nearly as robust as the Ag-driven proliferation of memory $CD8^+$ T cells during chronic viral infection (Shin et al., 2007). Finally, infection with chronic viruses is not associated with memory inflation (Shin et al., 2007; Wherry et al., 2004).

Factors influencing $CD8^+$ T cell homeostasis in old age – influence of persistent viruses and homeostatic mechanisms

One of the still unanswered questions regarding maintenance of HSV-specific memory $CD8^+$ T cells is whether the phenotypically different memory $CD8^+$ T cells are maintained by the same mechanisms, or if their maintenance is regulated independently.

The phenotype of memory $CD8^+$ T cells in systemically infected mice undergoing memory inflation was heterogeneous, consisting predominantly of effector memory phenotype cells, but also including a significant proportion of cells with central memory phenotype (Chapter 4). At this point we do not know whether both of these phenotypically different subsets require Ag for their maintenance, or if they are maintained independently.

We also do not know whether both of these memory cell populations will survive equally well in old mice. Our analysis of memory responses in old mice following ocular infection (Chapter 3) indicates that in old age, memory $CD8^+$ T cells of central memory phenotype, particularly those with elevated expression of receptors involved in T cell homeostasis (IL-7 and IL-15) may be selected to survive and/or expand better than other memory phenotype ($CD44^+$) $CD8^+$ T cells. This old age-associated memory cell expansion was not related to viral stimulation, as mice treated with famciclovir also had increased frequency of HSV-specific memory $CD8^+$ T cells in old age. Similar results were recently obtained in an acute viral infection model, where memory $CD8^+$ T cells specific for influenza and parainfluenza virus significantly increased in frequency and phenotype in old mice (Ely et al., 2007), however in that study expansions of both central- and effector memory phenotype were identified. It will be of interest to find out the relative contribution of Ag and homeostatic cytokines in maintenance of the phenotypically different memory $CD8^+$ T cell subsets. This could be accomplished by adoptive transfer experiments using sorted populations of memory $CD8^+$ T cells and a variety of recipients, including naïve, infected, and cytokine-deficient ($IL\text{-}7^{-/-}$, $IL\text{-}15^{-/-}$).

The study of lifelong maintenance of Ag-specific $CD8^+$ T cell subsets not only increases understanding of immunobiology of a particular viral infection, but also has a potential to shed light on the mechanisms leading to lymphocyte population changes associated with immunosenescence. One of the hallmarks of immunosenescence is development of large clonal expansions of $CD8^+$ T cells (TCE), leading to constriction of TCR repertoire within that lymphocyte subset (Clambey et al., 2007; Clambey et al., 2005). Both persistent viral infections and disturbance of homeostatic mechanisms have been implicated as potential causes behind TCE development. TCEs can be divided into Ag-reactive (AR) and Ag-independent (AI), with the AR TCE referring to T cell expansions of known specificity (mainly large expansions of HCMV-specific $CD8^+$ T cells in humans), and AI TCE referring to T cell expansions of unknown antigenic specificity (for example those found in SPF mice). Data in Chapter 3 of this thesis and the above mentioned study by Ely et al. provide the first documentation of development of T cell expansions from pre-existing memory T cell pool in absence of antigenic stimulation. Both of these studies showed that in some mice memory $CD8^+$ T cell populations that persisted at a constant and low frequency throughout life increased in old age, and this increase was Ag-independent and not associated with loss of functional responses (Chapter 3 and (Ely et al., 2007)). These studies suggest that one of the mechanisms by which old age-associated TCE arise is expansions of cells stochastically selected from the existing pool of Ag-experienced memory $CD8^+$ T cells.

The mechanism that allows these TCE to outgrow other memory cells is unknown, but one of the possible causes could be their increased responsiveness to IL-7 or IL-15, resulting in increased survival or proliferation. This prediction can be tested by

comparing proliferation, survival and expression of relevant molecules (i.e. antiapoptotic Bcl-2) by expanded and control non-expanded memory $CD8^+$ T cells in presence of titrated amounts of IL-7 and IL-15 *in vitro* and *in vivo*. Alternatively, they could have a defect in perceiving homeostatic signals, and instead follow an independent program of survival and proliferation, possibly regulated by cell-intrinsic factors. Messaoudi et. al recently demonstrated that some AI TCE detected in SPF mice have skewed response to normal homeostatic signals, including abnormal proliferative response upon transfer into lymphopenic or full compartment (Messaoudi et al., 2006c). These cells had the same phenotype (central memory with elevated expression of IL-7R and IL-15R) as the expanded HSV-specific memory cells found in old mice (Chapter 3), suggesting that perhaps they are maintained by similar mechanism. This could be tested by adoptive transfer of both types of TCE (HSV-specific and TCE of unknown specificity from an SPF mouse) into lymphopenic or eulymphoid hosts and comparing their survival and proliferation.

Somewhat surprisingly, we did not find evidence for increased incidence of TCE in old mice infected long term (>2 years) with either of two persistent viruses, HSV-1 and MCMV (Chapter 5). HCMV seropositivity has been linked with increased incidence of large $CD8^+$ T cell expansions exhibiting $CD28^-$ phenotype, some of which were CMV-specific. A popular view in the field is that at least in humans, persistent infections, dominantly HCMV, are the main cause of development of TCE and the CD28- phenotype in large proportion of $CD8^+$ T cells in the elderly (Pawelec et al., 2005). Both viral Ag-driven and bystander inflammatory cytokine-driven stimulation of T cells has been proposed to contribute to this process (Akbar and Fletcher, 2005). Type

I IFN was demonstrated to drive both $CD8^+$ (Borthwick et al., 2000) and $CD4^+$ (Fletcher et al., 2005) human T cell conversion to $CD28^-$ phenotype, and stimulation of plasmacytoid dendritic cells (pDCs) with CMV lysates induced IFNα production by these cells (Fletcher et al., 2005). Since both MCMV and systemic HSV-1 infections are also associated with inflation of memory cells specific for certain viral epitopes, and appear to undergo relatively common reactivation, we expected these lifelong infections to affect the overall TCR repertoire diversity by one of the above mentioned mechanisms (Ag-driven expansion or inflammation-driven expansion). By screening the $CD8^+$ T cell pool of old infected mice, we did not find a significant effect of presence of either infection on the overall size, phenotype, and diversity of the $CD8^+$ T cell repertoire (Chapter 5). However, we did detect both virus-specific and virus-unreactive $CD8^+$ T cell expansions (VBE), and together they significantly contributed to TCR repertoire restriction in some mice.

The fact that we did not detect any large VBE that were uniquely specific for a viral epitope in old systemically infected mice was somewhat surprising in light of our results demonstrating expansion of HSV-specific memory $CD8^+$ T cells in old ocularly infected mice (Chapter 3). Data in Chapter 3 and in Ely et al. (Ely et al., 2007) imply that TCE can be stochastically chosen to develop from pre-existing Ag-experienced memory pool, and this would imply that the more memory cells of given specificity there are, the more likely some of them will be selected to become a TCE. In other words, one could expect that a mouse in which 20% of $CD8^+$ T cell pool is gB-8p-specific would be more likely to develop an old-age associated gB-8p-specific $CD8^+$ T cell expansion than a mouse where the normal frequency of HSV-specific memory cells is only 2%. At present

we do not know what determines the selection of memory cells for their old-age associated expansion, but perhaps the memory $CD8^+$ T cells generated as a result of systemic infection lack the yet unidentified factor that would promote their development into TCE. The phenotypic heterogeneity of the memory $CD8^+$ T cells generated by systemic HSV-1 infection suggests that this population may also be heterogeneous in its responsiveness to antigenic and homeostatic stimuli, and perhaps not all of these cells have the characteristics necessary to become a TCE. Instead, they continue to be maintained in the same fashion in an old organism as they were during adulthood.

The studies of maintenance of T cell pool discussed here show that no single determinant (persistent infection or aging) is responsible for disturbance of the T cell population balance associated with immunosenescence, however they both contribute to it. Therefore one way in which to counteract the old age-associated defects in T cell homeostasis would be by a combination of 1) improving maintenance of "young" naïve T cell pool and 2) curtailing the extent of persistent pathogen infections. Immune reconstitution has been discussed as a possible method of "rejuvenating" the immune system of elderly humans (Capri et al., 2006; Virts et al., 2006), and could be accomplished eater by improving thymic output or maintenance of naïve T cells. The mouse model is very suitable for experimentally testing that prediction by well-designed adoptive transfer and thymus graft experiments. Caloric restriction is one of the possible treatments that can improve maintenance of diverse, naïve T cell pool. It was recently demonstrated to delay onset of T cell senescence by lengthening the period of T cell production and improving maintenance of naïve-phenotype, diverse T cells in non-human primates (Messaoudi et al., 2006a). With respect to limiting the impact of

persisting pathogens on the decreased diversity and altered phenotype of T cells over time, two main strategies are possible: vaccination and therapeutic antiviral drug treatment. The problems regarding vaccination are that most of the lifelong infections are acquired very early in life, therefore only the very young could potentially be vaccinated, as the majority of the adult population already carries persistent viruses such as HSV-1 and HCMV. In addition, at present such vaccines do not exist, and in many cases, owing to the cell tropism of the virus, vaccination is not likely to be successful. This was discussed in reference to HSV-1 infection in Chapter 1. Finally, prophylactic antiviral treatment of seropositive patients (i.e. for CMV or HSV-1) is a possible strategy, although these drugs are not always completely successful at controlling viral replication, and are only available for few of the known persistent pathogens. The potential of all of the above strategies for preventing age-associated disturbances in the homeostasis of T cell pool can be experimentally tested.

Summary

In conclusion, in this thesis I have examined several aspects of the interaction between lifelong viral infections and $CD8^+$ T cells. In examining the primary and memory $CD8^+$ T cell response to HSV-1 I found that the *in vivo* relationship between the virus and the T cells is complex and dependent on many factors, such as the inoculation route, the extent of systemic viral spread, and extent of viral replication. The work yielded a number of novel findings. The major discoveries related to the process of maintenance of HSV-specific memory cells. I have shown that unlike previously thought, HSV-1 infection in mice can be associated with viral reactivation, resulting in

Ag-driven expansion of HSV-specific memory $CD8^+$ T cells. Another important finding of this thesis was the discovery that age-related expansions of $CD8^+$ T cells can arise from Ag-experienced memory pool by an Ag-independent process. Finally, the experiments in old HSV- and MCMV-infected mice begun to experimentally address the hypothesis that persistent viral infections affect the homeostasis of T cells in old age. Many questions still remain regarding the mechanisms of maintenance of persistent virus-specific memory $CD8^+$ T cells and their relationship to the old-age associated disturbances in T cell homeostasis , but the work presented in this thesis contributes to our understanding of these issues and provides leads for future investigation. The findings discussed here have an important biological significance, as the homeostatic disturbance of the T cell pool is a major factor that correlates with increased morbidity and mortality in the elderly.

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