

**THE PRESENCE OF LATENT VIRUS INFLUENCES THE MAINTENANCE AND
PHENOTYPE OF THE HSV-SPECIFIC CD8 MEMORY POPULATION**

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HSV-1 establishes latency in sensory neurons of the trigeminal ganglia (TG) following corneal infection. The concept that latently infected neurons are ignored by the host immune response has given way to the notion that CD8 T cells maintained in the TG monitor infected neurons thereby subverting reactivation. The tendency of HSV-1 to periodically reactivate in humans and mediate recurrent disease is associated with significant morbidity. A desire to understand the complex interactions of this pathogen, the neurons that harbor it, and the immune system that monitors latency define this study. Two populations of CD8 T cells rapidly infiltrate the TG coincident with resolution of replicating virus and juxtapose with neurons for the life of the mouse. One population recognizes the immunodominant glycoprotein B (gB) epitope while the other does not (gB-nonspecific). We establish that the homeostatic cytokine IL-15 does not contribute to the maintenance of gB-specific or gB-nonspecific CD8 T cells within the TG during latency. However, IL-15 is crucial for the regulation of gB-specific memory CD8 T cells in noninfected tissues. These findings led us to question whether gB-nonspecific CD8 T cells are important in the HSV-1 response. We demonstrate that gB-nonspecific CD8 T cells upregulate the effector molecule granzyme B, and produce IFN γ and proliferate in response to HSV-1-infected but not gB-transfected targets. This data conclusively shows that gB-nonspecific CD8 T cells in the infected TG are HSV-1 specific. This population is also capable of preventing reactivation following explant of latent TG. Contrary to their gB-specific CD8 counterparts, gB-

nonspecific CD8 T cells have a reduced capacity to produce IFN γ during latency and this reduction in function is associated with increased expression of PD-1. Surprisingly, blockade of PD-L1 did not rescue effector function yet increased the viral burden during latency. We show that a population of neurons expressing PD-L1 contains an enriched reservoir of HSV-1 latency that is highly prone to reactivate.

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PREFACE

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1.0 INTRODUCTION

1.1 HERPES SIMPLEX VIRUS TYPE 1

1.1.1 Disease significance and epidemiology

The human herpes virus (HHV) family is comprised of many structurally related members that represent a considerable health problem throughout the world. It is estimated that 57% of U.S. adults are infected with HSV-1 (1). Infection of the eye, resulting in the disease herpetic stromal keratitis (HSK), is also the most prevalent cause of infectious blindness in developed countries including the U.S. (2). The ability of HSV-1 to establish a latent infection in human sensory ganglia provides numerous challenges for the immune system as it attempts to control infection. HSV antiviral agents curtail reactivation but are not completely effective at preventing viral transmission with intimate contact. Asymptomatic shedding of virus at the site of the initial infection is common following HSV-1 infection in humans and primarily responsible for spread of the disease (3). Due to the difficulties of treating asymptomatic recurrences, many researchers are now focused on the mechanisms employed to maintain the virus in a latent state to provide the basis for more effective therapeutic modalities.

HSV-1 is most commonly associated with orofacial infection, whereas HSV-2 most prominently infects the genital tract. However, this is not a strict dichotomy and either type of

HSV has the potential to infect various mucosal and epithelial surfaces of the body. In fact, HSV-1 is now becoming a significant cause of genital herpes infection in the US (4). Epidemiologic data indicate that the prevalence of ocular HSV disease is $\sim 149/100,000$ individuals with a further 21 – 31/100,000 people newly diagnosed annually in developed nations (2, 5, 6), with HSV-1 being the etiologic agent in $> 95\%$ of ocular HSV infections (7). However, it is thought that clinical surveys grossly underestimate the true prevalence and incidence of HSV infections because approximately two-thirds of primary HSV-1 and -2 infections are subclinical (8). This gross underestimation of prevalence is evident in postmortem human trigeminal ganglia (TG) examined for HSV-1 infection as one study showed that 100% of TGs from individuals 60 years of age or older contained HSV-1 DNA (9). HSV infections are responsible for a variety of pathologic conditions, including herpes labialis (also known as cold sores or fever blisters), genital lesions, herpes simplex encephalitis (HSE) and herpes keratitis. The latter can involve either the superficial corneal epithelium or the deeper stromal layer, with herpes stromal keratitis (HSK) being associated with the most severe disease. Although herpes labialis is often painful and unsightly, HSE and HSK are life- and sight-threatening pathologies, respectively. HSE is the most common cause of sporadically fatal viral encephalitis in the U.S., affecting between 1 in 250,000 and 500,000 individuals/year (10, 11). Moreover, HSK is the most common cause of corneal blindness of infectious etiology in developed nations (2) and the third-leading indication for penetrating keratoplasty (12). Importantly, the visual loss attributed to HSK is not due to primary HSV-1 infection but to the corneal thinning, scarring and neovascularization associated with recurrent disease.

1.1.2 Morphology and genome structure

HSV-1 is a neurotropic large double stranded DNA virus belonging to the *Herpesviridae* family. HSV-1 encodes an estimated 84 viral proteins (13). The 152-kbp HSV-1 genome consists of two segments of unique DNA termed the U_L (unique long) and U_S (unique short) regions surrounded by inverted repeats (13). Its virions have a consistent morphology. The HSV-1 DNA core is surrounded by an icosadeltahedral capsid, a tegument containing proteins necessary for viral transcription initiation, and a lipid envelope derived from the infected cell membrane that contains the viral glycoproteins important for viral budding and entry (Figure 1).

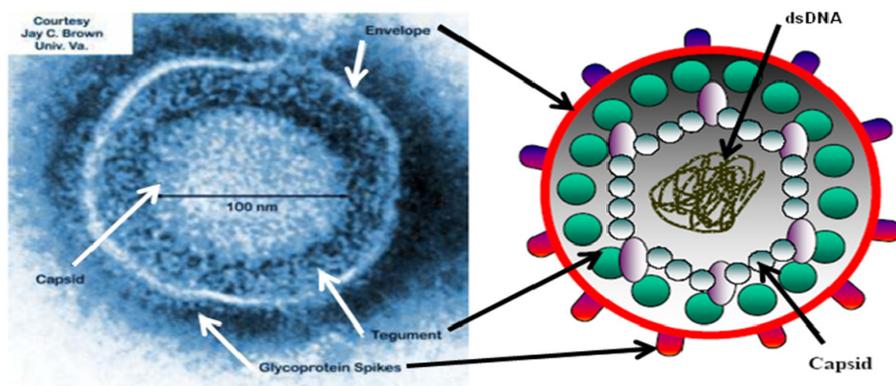


Figure 1. HSV-1 virion structure

An electron micrograph (left) with a corresponding cartoon rendition (right) depicting the five major structural components of the HSV-1 virion.

1.1.3 Viral life cycle

A hallmark of herpes viruses is their sequential expression of gene products during lytic infection. HSV-1 lytic infection in a permissive cell results in cascade regulation of gene expression (Figure 2). Gene products of the immediate-early (IE or α) family primarily function as transactivators of early (E or β) gene expression though they also serve to repress their own

expression. This class of gene products also encodes for the prototypical immune evasion molecule, ICP47. The second class of gene products produced during the lytic infection cycle, the β genes, function in DNA replication and production of substrates for DNA synthesis, providing the framework for late (γ or L) gene expression. There are two distinct late gene families expressed. γ_1 genes are expressed at low levels prior to DNA synthesis but are rapidly elevated following the initiation of DNA synthesis, whereas γ_2 genes are only expressed after DNA synthesis has taken place. This family of gene products encodes the numerous structural and tegument proteins, most notably the large viral family of glycoproteins which stud the viral membrane. Following completion of the gene expression cycle, productive virions bud from the surface of the cell, ultimately resulting in its lysis. A summary describing the function of HSV-1 proteins important to these studies is provided in table 1 (13).

Table 1. Summary of Some Pertinent Viral Gene Products

Gene Product	Kinetic Expression	Proposed Function	Reference
ICP0	α	A nonessential protein best characterized as a promiscuous transactivator of genes. It is required for efficient reactivation from latency and its expression can induce reactivation.	(14, 14)
ICP4	α	An essential protein required for expression of most β and γ genes. Its expression may induce reactivation.	(15, 16)
RR1	β	A nonessential protein involved in deoxyribonucleotide production for viral DNA synthesis. This protein contains a subdominant CD8 T cell epitope.	(17 - 19)
gB	γ_1	An essential glycoprotein required for cell entry. This protein contains the major immunodominant CD8 T cell determinant.	(17, 20 - 22)
gC	γ_2	A nonessential glycoprotein involved in cell attachment.	(22, 23)
gH	γ_2	An essential glycoprotein involved in entry, egress, and cell-to-cell spread.	(24, 25)
LATs (RNA)	--	This nonessential transcript accumulates in most latently infected cells. Promotes neuronal cell survival and plays a role in prevention of apoptosis.	(26, 27)

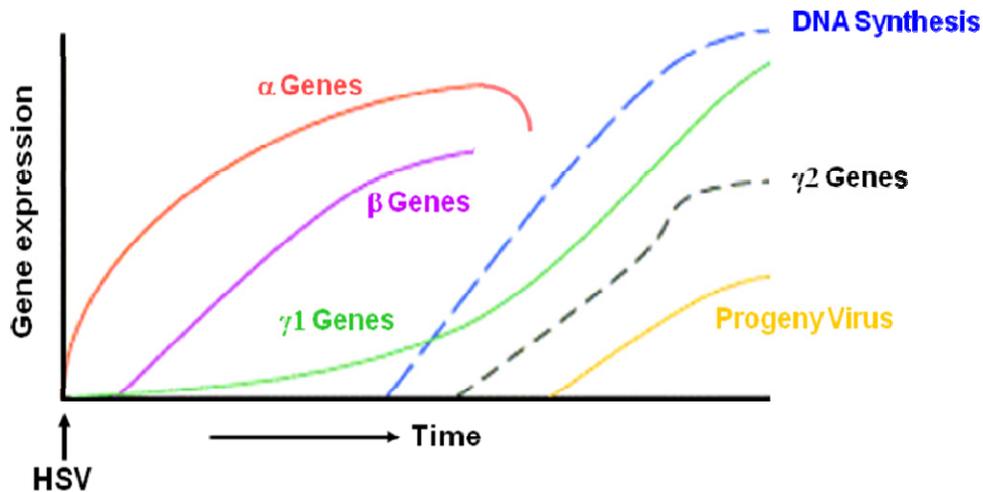


Figure 2. HSV-1 gene expression during lytic infection.

Cartoon illustrates the general trend of gene expression over time following infection of a cell permissive for lytic replication.

There is little known about the pattern of gene expression during reactivation from a stable latent state. This may be explained in part based on the relatively few neurons capable of undergoing reactivation at any given time. Despite this, it is clear that the pattern of gene expression following reactivation from latency is markedly different than lytic gene expression (28 - 30). One study showed that the first gene expressed during reactivation is ICP0 (30), while another study argued that early gene expression precedes immediate-early gene expression (28). It is also apparent that reactivation occurs in the absence of viral tegument proteins, such as VP16, delivered with the virions during lytic infection. Neuronal populations may serve as a reservoir during latency due to the ability of neuronal transcription factors to activate α gene expression in the absence of other viral proteins (31 - 33).

1.2 HSV NEURONAL LATENCY

A hallmark of the herpes family of viruses is their propensity to cause recurrent disease. Upon infection of mucosal or abraded epithelial surfaces, HSV gains access to innervating peripheral sensory nerve termini and travels via retrograde axonal transport to neuronal cell bodies in the connecting sensory ganglia. Thus, following corneal infection, HSV establishes latency within the ophthalmic branch of the TG. HSV replicates briefly within infected sensory neurons and then establishes latency. Latency is operationally defined as the retention of the viral genome within a cell for a prolonged period without production of infectious virions. Indeed, latent HSV persists in sensory ganglia for the life of the individual. In some infected people, latency is periodically interrupted leading to virion formation, anterograde transport to the peripheral site innervated by the neuron, and shedding with or without recurrent lesions. The mechanisms underlying this unique capacity of herpes viruses to establish, maintain and reactivate from a latent state have been the focus of extensive research over the past several decades with much of the emphasis on HSV. The HSV-1 genome is thought to circularize within infected cells during latency based on studies with HSV-1 mutants which establish quiescent infections on cell monolayers (34). The transition from latent to lytic infection during HSV-1 reactivation may be associated with a conversion from this circular genome to a linear form regulated by both host and viral factors. Cross-regulation between host-derived IFN γ and the HSV-1 immediate-early gene product, ICP0, a promiscuous transactivator of HSV-1 lytic genes, appears to influence this transition. ICP0 has been shown to degrade proteins associated with nuclear domain 10 bodies (35), which are discrete nuclear substructures that HSV-1 genomes associate with early during infection, thereby allowing gene transcription and subsequent viral reactivation to occur.

Importantly, IFN γ is able to block HSV-1 reactivation in *ex vivo* cultures of latently infected TG, in part, by inhibiting ICP0 promoter activity (36).

A potential breakthrough regarding our understanding of HSV-1 latency occurred in the mid-1980s when a series of viral transcripts referred to as latency-associated transcripts (LATs) were found to be prominently expressed in latently infected neurons. These transcripts are first detectable as early as 2 days following corneal infection in some models(37); however, much higher levels of LATs are detected within infected TG during latency (38, 39). The possibility that this family of viral transcripts, which appeared to be uniquely expressed during latency, might represent a mechanism for establishing or maintaining HSV latency spurred extensive research into the properties of LATs. Unfortunately, the belief that LATs represented the holy grail of HSV latency was somewhat diminished by studies demonstrating that the LAT region is not absolutely required for HSV-1 establishment and maintenance of latent infections or for reactivation from latency (40 - 42). Nonetheless, recent findings do support a role for LATs in determining the propensity of HSV-1 and HSV-2 to establish latency in different sensory ganglia, and in protecting latently infected neurons from undergoing apoptosis (26, 27, 43). Moreover, recent evidence has also established a role for microRNAs encoded by LAT to impair ICP0 protein expression (44), providing another mechanism by which LATs may direct the establishment and maintenance of latency.

Reactivation is more frequent in immunocompromised individuals such as those infected with HIV, transplant recipients and critically ill surgical patients (45, 46). In addition, stress has long been associated with reactivation of latent herpes virus infections in humans. Stress modulates the immune system by directly affecting immune cells through interactions with neuroendocrine products of the hypothalamic–pituitary–adrenal (HPA) and sympathetic–

adrenal–medullary (SAM) axes. Stress may also indirectly affect the immune system by inducing behavioral changes such as increased smoking and consumption of alcohol, and changes in sleeping, eating and exercise patterns. These indirect effects are often deleterious to the immune system though these pathways are poorly defined. Known stressors such as hyperthermia, societal disruption and physical restraint have all been shown to lead to HSV reactivation in animal models (47 - 49).

These models provide an ideal system to begin exploring the connection between stress and immune dysregulation to determine its impact on reactivation of latent herpes infections. The link among psychologic stress, CD8 T-cell function and HSV-1 reactivation at the level of latently infected neurons is only starting to be understood. A significant and growing body of data demonstrates that CD8 T-cell function is compromised by stress-associated hormones such as glucocorticoids (50, 51). Glucocorticoids modulate the production of various proinflammatory cytokines and chemokines such as IL-1, IL-2, IL-4, IL-5, IL-8, IFN γ and TNF (52, 53). Treatment with the synthetic glucocorticoid dexamethasone may also have a direct effect on the ability of cytotoxic T cells to perform their lytic effector function in part by downregulating levels of the lytic granule component granzyme B (54). The persistence of relatively high levels of IFN γ and TNF maintained in latently infected TG, and the production of these cytokines and expression of granzyme B directly ex vivo by ganglionic CD8 T cells suggests that known inhibitory functions of corticosterone might temporarily compromise the immunosurveillance capability of CD8 T cells thereby promoting HSV-1 reactivation from latency. In accordance with this hypothesis, stress has recently been demonstrated to reduce the number of HSV-specific CD8 T cells in latently infected TG and compromises the function of the remaining cells (55). Further, restraint stress and CD8 T-cell depletion resulted in a comparable increase in the

viral genome copy number in latently infected TG (55). Together these observations strongly support the concept that restraint stress induces HSV-1 reactivation from latency by compromising CD8 T-cell surveillance of latently infected neurons.

1.3 IMMUNITY TO HSV-1

In our murine model of HSV-1 ocular infection, HSV-1 first gains access to innervating neurons following replication in the corneal epithelial surface. Infected cells are capable of eliciting an intrinsic defense against invading pathogens by production of antiviral molecules such as type I interferons. Type I interferons provide numerous benefits to host defense including induction of enhanced resistance to viral replication in the infected cell and nearby cells, upregulation of MHC class I molecules to facilitate expression of viral epitopes, and the migration of PMNs into the central cornea. The primary immune infiltrate into the murine cornea consists predominantly of neutrophils, although macrophages, dendritic cells (DCs), natural killer (NK) cells, and $\gamma\delta$ T cells also infiltrate this site (56 - 59). The innate immune response is vital for initial control of HSV-1 replication and induction of the adaptive immune response to HSV-1.

Despite this, virus inevitably gains access to neuronal cell bodies of the ophthalmic branch of the trigeminal ganglia. Here, the virus undergoes a brief round of replication within 2 – 3 days following corneal infection (60). Macrophages and $\gamma\delta$ T cells infiltrate the TG upon initial viral replication at the site (56, 61). Macrophages function by production of antiviral molecules like nitric oxide (NO) and TNF which limit the lateral spread of virus and may even limit early shedding in the periphery (62). It appears that $\gamma\delta$ T cells provide the majority of IFN γ early

following infection. $\text{IFN}\gamma$ possesses numerous anti-viral properties and also serves to activate macrophages to produce NO and TNF (63). Thus, innate immune control within the ganglia early following infection is likely designed to inhibit viral replication during expansion of adaptive immune cells in the draining lymph node (DLN).

HSV-1 specific T and B cells are generated in the DLN of infected mice by DCs presenting HSV-1 epitopes acquired at the sight of infection. Both arms of the adaptive immune response are important in controlling HSV-1 infection. CD4 T cells appear to be the dominant adaptive immune infiltrate into the cornea and mediate the immunopathologic condition herpes stromal keratitis (HSK). Whether HSK is mediated by CD4 T cells which are HSV-1 specific (64, 65), bystander (66 - 69), specific for self peptides expressed in the cornea (70), or a combination is contentious. Activated B cells produce HSV-1 neutralizing antibodies, which while helpful in preventing spread of virus that has reactivated, are not capable of preventing reactivation. The transition from lytic infection to latency is unclear but LAT expression can be detected as early as 2 days following infection (37). Intrinsic properties of the invaded neurons and the CD8 T cell response control of lytic infection likely both contribute to the establishment of latency. CD8 T cells are the focus of this dissertation and the remainder of this background will focus primarily on them.

Although the studies outlined in section 1.2 identified mechanisms that might contribute to the maintenance of HSV latency and reactivation from the latent state, there is no obvious way to exploit this knowledge in developing a strategy for preventing recurrent herpetic disease. Of greater potential are the exciting observations which emerged in the mid-1990s implicating the host adaptive immune system in maintaining HSV-1 latency in mice. Mice harbor latent HSV-1 in sensory ganglia following infection at peripheral sites, and the virus can be induced to

reactivate from latency *in vivo* by exposure of latently infected mice to stressful stimuli such as hyperthermia, ultraviolet irradiation, and psychologic stressors (47, 48, 50), and can reactivate *in vitro* following explantation and culturing of latently infected sensory ganglia (71, 72). However, HSV-1 does not spontaneously reactivate from latency in murine sensory ganglia *in vivo* as is observed in latently infected sensory ganglia of humans, rabbits and guinea-pigs, suggesting that the mechanisms responsible for maintaining HSV-1 latency are well-developed in mice. Contrasting with the apparent stability of HSV-1 latency in mouse sensory ganglia is the evidence demonstrating a maintained adaptive immune response to HSV-1 within latently infected ganglia. CD69-expressing CD4 and CD8 T cells, inflammatory cytokines including IFN γ , TNF, and IL-6, as well as transcripts for molecules involved in inflammatory cell chemoattraction, such as RANTES, chemokine receptor 5 (CCR5), and CXCR3, are present throughout latency in ganglia of HSV-1-infected mice (56, 61, 73 - 78). Combined with reports of low levels of HSV-1 lytic gene transcripts in latently infected mouse ganglia (79, 80), these findings gave rise to a proposed dynamic model of HSV-1 latency, in which HSV-specific CD8 T cells maintained within latently infected ganglia are continually scanning the microenvironment in an attempt to curtail reactivation and prevent formation of intact virions and shedding in the periphery. Early studies also suggest an important role of CD4 T cells in the progression of acute HSV-1 infection into stable latency (81, 82). However, little is known about the mechanism that CD4 T cells employ in the clearance of virus and establishment of the latent state. Understanding the factors that lead to viral reactivation, the immune mechanisms involved in preventing reactivation, and the fate of latently infected neurons will assist in the design of better therapeutic modalities to treat recurrent herpetic disease.

1.4 CD8 T-CELL-MEDIATED MAINTENANCE OF HSV LATENCY

CD8 T cells appear to be one critical component of the adaptive immune response to HSV-1 within infected ganglia. In general, CD8 T cells exert their effector function either by exocytosis of lytic granules, which contain cytolytic molecules including perforin and granzymes, or by secretion of the antiviral cytokines IFN γ and TNF. Mice deficient in CD8 T cells, either due to genetic manipulation (83) or *in vivo* antibody depletion (84), are unable to control HSV-1 replication and are significantly impaired in their ability to clear the virus from infected ganglionic neurons after primary infection. Further, a significant proportion of neurons undergo cell death in the absence of CD8 T cells (84). These data taken together suggest a nonlytic, protective role for CD8 T cells within infected ganglia. However, certain neurons within infected ganglia are able to support a latent infection, as evidenced by LAT expression, in the absence of an adaptive immune response (83). Thus, it appears that an intricate interplay among viral, neuronal and immune mechanisms mediates establishment and maintenance of HSV latency.

The majority of CD8 T cells found in HSV-1 latently infected ganglia of C57BL/6 (B6) mice are purportedly specific for a single immunodominant epitope within the viral envelope molecule, glycoprotein B (gB₄₉₈₋₅₀₅) (73). Nearly all of the CD8 T cells within latently infected ganglia persistently express the early activation marker, CD69, suggesting recent stimulation (73). Furthermore, a large proportion of gB-specific CD8 T cells also express the lytic granule component, granzyme B (GrB) (85), suggesting these cells receive persistent antigenic stimulation within latently infected ganglia. Importantly, ganglionic gB-specific CD8 T cells are capable of polarizing their T-cell receptors (TCRs) toward neurons within HSV-1 latently infected murine TGs forming an apparent immunologic synapse (73). Such TCR polarization implies the presence of an MHC–gB peptide complex on the surface of latently infected neurons

capable of ligating the gB-specific TCR. Consistent with this observation, depletion of CD8 T cells during latency results in an increase in gH copies present (Figure 3). As noted above, gH is a true late gene and used as a marker to determine the level of viral DNA present. Therefore, increases in gH viral genome copies implies DNA synthesis has occurred and are indicative of reactivation. Interestingly, this loss of control is transient and associated with an increase in CD4 T cells numbers in the latent ganglia suggesting an auxiliary control mechanism which may be CD4 dependent in the absence of CD8 T cell immunosurveillance (Figure 3).

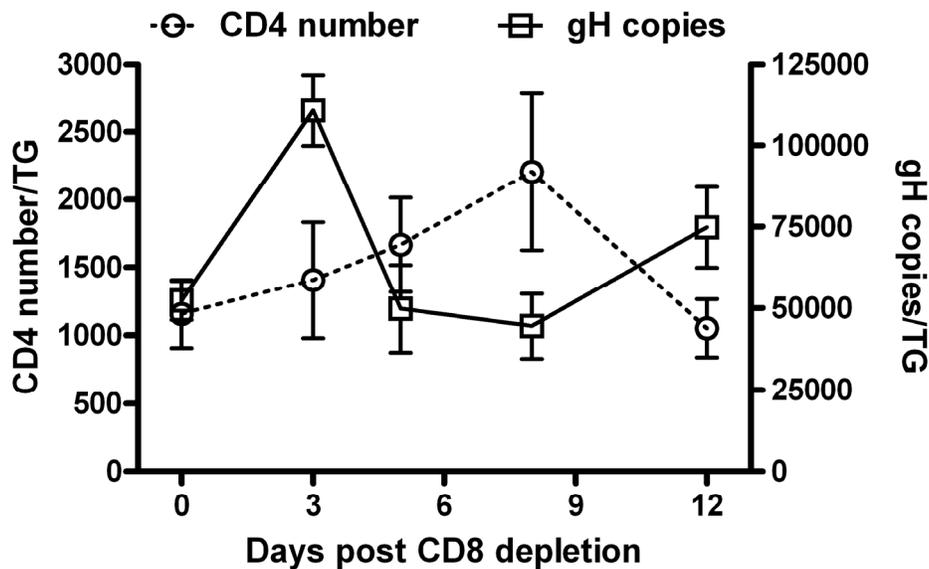


Figure 3. CD8 T cells control latency in vivo

HSV-1 latently infected B6 mice received anti-CD8a mAb injections every other day starting at 30 dpi. Individual TG of mice were harvest at the indicated days post anti-CD8 treatment initiation and examined for CD4 T cell number and gH copies to determine viral burden. (n = 3 – 5/group).

Persistent activation within latently infected sensory ganglia depends on antigen presentation by ganglia parenchymal cells (i.e., neurons and/or their support cells) and not on bone marrow-derived antigen-presenting cells (86). In addition, some neurons within latently infected ganglia demonstrate gB promoter activity suggesting that this protein may be produced in some neurons during latency (Figure 4). These data, combined with the fact that HSV-1

establishes latency only in neurons (87, 88), has led to the hypothesis that infected neurons directly present viral antigen to HSV-specific CD8 T cells during attempted reactivation. Further exploration of the molecular interactions between HSV-1-specific CD8 T cells and HSV-1 latently infected neurons is required.

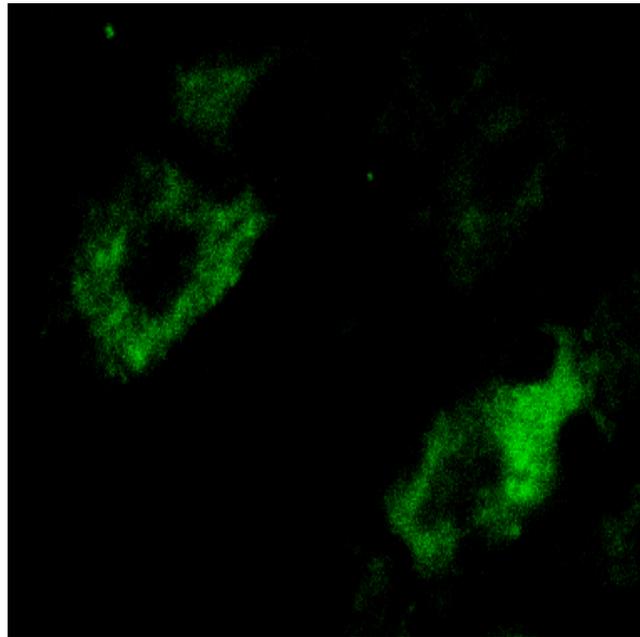


Figure 4. gB promoter activity within neurons of latently infected ganglia

Whole ganglia from mice infected with HSV-1 pgB-EGFP were harvested during latency and imaged for EGFP expression.

Latent HSV-1 can be induced to reactivate following excision and culture of the infected ganglia (74). CD8 T cells from the lymph nodes of HSV-1-infected mice (74), a gB-specific CD8 T-cell clone (2D5) (73), as well as gB-specific CD8 T cells isolated from latently infected ganglia (unpublished observation) are all able to protect HSV-1 latently infected neurons from viral reactivation in *ex vivo* cultures. Further, CD8-mediated protection is antigen-specific, dose-dependent and MHC-restricted (73). IFN γ has been shown to significantly reduce reactivation in *ex vivo* cultures of HSV-1 latently infected TG (36, 89), and CD8 T cells appear to be a significant source of IFN γ in these cultures (89). Furthermore, a yet to be determined perforin-

dependent mechanism also significantly inhibits reactivation in these cultures (unpublished observation). Interestingly, CD8 T cells protect *ex vivo* cultures of latently infected ganglionic neurons without depleting the pool of latently infected neurons (74). Combined with the observations demonstrating significant neuronal death in the absence of CD8 T cells (84), these data strongly suggest that HSV-1-specific CD8 T cells use nonlytic mechanisms to protect HSV-1 latently infected neurons from viral reactivation. As protection is at least partially perforin-dependent, this would suggest the intriguing possibility that lytic granule components can block viral reactivation without destroying the infected neuron.

Consistent with the notion of non-cytolytic CD8 T-cell immunosurveillance of HSV-1 latently infected ganglia is the observation that many gB-specific CD8 T cells also express the CD94-NKG2A inhibitory heterodimer (85, 90). Expression of this receptor on virus-specific CD8 T cells downregulates antigen-specific cytotoxic activity (91) without affecting secretion of the antiviral cytokine, IFN γ (90). Furthermore, a portion of neurons within HSV-1 latently infected TGs were shown to express Qa-1, the major ligand of the CD94-NKG2A heterodimer, and blocking this interaction in *ex vivo* TG cultures led to increased neuronal cell lysis (85). Therefore, it appears that specific neurons can dictate which effector mechanism(s) are employed by CD8 T cells to block HSV-1 reactivation from latency. Neurons that express Qa-1 may restrict CD8 T-cell lytic granule-mediated cytotoxic activity while permitting the use of IFN γ to block HSV-1 reactivation. It is also reasonable to speculate that the antiapoptotic function of LATs has evolved to combat the caspase-mediated effects induced by CD8 T-cell lytic granules.

These findings suggest that a complex tripartite relationship among HSV-1, sensory neurons and CD8 T cells permits long-term retention of latent virus without destruction of neuronal tissue. Indeed, a recent report demonstrated that the reactivation frequency of latently

infected mouse TG can be predicted based solely on the viral genome copy number and the size of the CD8 T-cell infiltrate within the TG (92). Importantly, many of the immunologic phenomena associated with latent HSV-1 infection in mice have been reproduced in human studies examining cadaveric ganglia. CD8 T cells surround HSV-1 LAT⁺ neurons within human TGs (93 - 95). Also, TGs in which HSV-1 LAT⁺ neurons were identified contained significantly more CD8, IFN γ , TNF and the T-cell chemoattractant RANTES mRNA transcripts compared with ganglia whose neurons tested negative for LAT expression (93, 94). It has recently been demonstrated that CD8 T cells resident in human cadaveric ganglia express an effector memory phenotype and cluster around HSV-1 latently infected but not varicella zoster virus latently infected neurons suggesting that clustering is antigen specific (95). Consistent with studies in the murine model, neuronal damage was not observed in latently infected TG despite GrB expression by the CD8 T cells surrounding HSV-1 latently infected neurons (95). These findings in human ganglia, which replicate previous findings in mice, support the relevance of using the murine model of HSV-1 infection to identify mechanisms of HSV-1 latency.

1.5 CYTOKINE MAINTENANCE OF CD8 MEMORY

One major advantage afforded by the adaptive immune system is its ability to develop an anamnestic memory response. The CD8 memory response is stronger and more rapid than the primary response due to functional augmentation and increased frequency of antigen-specific cells within the T cell pool. The CD8 memory pool in both mice and humans are comprised of at least two subpopulations: (i) central memory cells that express the lymph node homing receptors, CD62L and CCR7 and thus reside primarily in secondary lymphoid tissues; and (ii) effector

memory cells that lack lymph node homing receptors, favoring their presence in non-lymphoid tissues (96, 97). It appears that both central and effector memory cells derive from precursors that are generated during the initial expansion of CD8 effector cells. Both of these memory populations express IL-7R α (CD127) and IL-15R α and depend on IL-7 and IL-15 for their maintenance (98 - 102).

In most cases, pathogens are eliminated from the body following a brief acute infection, resulting in a prolonged period in which the CD8 memory pool represents an “armed and waiting” population that is maintained by homeostatic proliferation in the absence of antigen. Homeostasis of this population appears to be dependent on the direct or indirect influence of at least three cytokines. IL-15 acts as a growth and survival factor, while IL-7 maintains viability by inhibiting apoptosis (103). The third cytokine, IL-2 may indirectly inhibit homeostatic proliferation by maintaining the activity of CD4⁺CD25⁺ regulatory T cells (104, 105). The receptors for all three cytokines share a common gamma chain (CD132), but have unique α chains. The IL-15R and IL-2R are heterotrimers, with two shared chains (CD122 and CD132).

IL-15 acts on CD8 memory cells by promoting their homeostatic division and promoting their survival in part by up-regulating anti-apoptotic proteins, such as Bcl-2 (99, 101, 106 - 108). The CD8 memory population generated in response to persistent infections, such as lymphocytic choriomeningitis virus (LCMV) clone 13, is refractory to regulation by IL-15 due to down-regulation of IL-2R β (CD122) (109). It appears that IL-15 is utilized *in vivo* through transpresentation by IL-15R α , which is likely expressed on DCs in lymph tissues, to CD8 memory cells which express the low affinity IL-2R comprised of the IL-2R β and γ C (CD132) chains (102, 110 - 112). Recent evidence suggests that the bioavailability of IL-15 is greatly enhanced by the ability of IL-15/IL-15R α complexes to undergo internalization and

representation on the cell surface of IL-15R α ⁺ cells in the lungs providing a stable intracellular reservoir for sustained IL-15 activity (110, 113). The unique nature of IL-15 sustainability *in vivo* provides a mechanistic explanation for the importance of this cytokine throughout the CD8 T cell lifecycle.

IL-7 is required for the maintenance of naïve and memory CD8 T cells under normal homeostatic conditions (98, 100, 114). Its receptor, IL-7R α (CD127) is expressed by all naïve T cells and further up-regulated on CD8 memory cells (98, 99). IL-7 promotes the maintenance of CD8 memory T cells through upregulation of antiapoptotic molecules like Bcl-2 or Mcl-2 (114 - 116). In fact, IL-7 overexpression can lead to IL-15-independent maintenance of memory-phenotype populations, likely by promoting their survival via up-regulation of anti-apoptotic proteins (100). IL-7R α has been shown to identify memory precursors following LCMV and vesicular stomatitis virus (VSV) infections when expressed on a subset of CD8 effector T cells (98, 117). Although these memory precursors express functional and phenotypic characteristics of effector cells, they are differentiated from true effectors by their expression of CD127, and typically represent less than 10% of the CD8 effector population (117). Interestingly, IL-7R α expression does not correlate with memory precursor generation in a setting of diminished inflammation, such as that of peptide immunizations either through peptide/adjuvant or peptide-pulsed DCs (118). *In vitro* studies have demonstrated that IL-7 down-regulates its own receptor yet regulation of the IL-7R α *in vivo* following infection appears to be IL-7 independent suggesting a distinct mechanism regulating memory precursor formation (119, 120).

IL-2 has also been implicated in the maintenance of memory CD8 T cells. Until recently, the role of IL-2 was thought to regulate the CD8 memory population indirectly through the action of regulatory CD4 T cells (Tregs) (104, 105, 121 - 123). These studies have recently been

called into question based on the observation that IL-2/anti-IL-2 immune complexes directly stimulate memory CD8 T cells through IL-2R β , providing a mechanism by which IL-2 may have a direct impact on memory T cell maintenance (124, 125). However, the physiologic role of these complexes is questionable. Regardless, Tregs appear to regulate memory CD8 T cells, and their requirement for IL-2 is absolute as IL-2^{-/-}, IL-2R α (CD25)^{-/-}, IL-2R β ^{-/-}, and IL-2 depleted mice all lack Tregs (126 - 130). It has recently been proposed that Tregs may regulate CD8 T cell populations by preventing naïve T cell activation and providing an IL-2 sink during memory homeostasis through the high affinity IL-2R α (131).

In some cases pathogens persist for prolonged periods within the host in the presence of non-sterilizing immunity. Such infections can be of two types: persistent or latent. In a persistent infection typified by the lymphocytic choriomeningitis virus (LCMV) clone 13 infection in mice, replicating pathogens remain in the host. Such infections produce a persistent, high level antigenic load that profoundly influences phenotypic and functional characteristics of the CD8 memory population. The CD8 memory pool generated during persistent infections by LCMV proliferate poorly in response to IL-7 and IL-15 due to down-regulation of their receptors, produce little IL-2, and express the inhibitory receptor PD-1 (109, 132, 133). Prolonged exposure to a high antigenic load during this chronic infection also leads to exhaustion of antigen-specific CD8 memory cells (134).

1.6 PD-1 – PD-L1 IMPACT ON CD8 T CELL BIOLOGY

Following lymphocytic choriomeningitis virus (LCMV) clone 13 infection in mice, CD8 T cells are unable to clear replicating virus and become functionally exhausted in a coordinated fashion

losing the ability to produce IL-2, TNF α , and IFN γ in sequence (132). In this system, exhaustion of the CD8 T cells appears to be a direct consequence of repeated TCR stimulation of LCMV-specific CD8 T cells and not a result of low level but persistent inflammation associated with a consistent low level viremia (135). Similar findings of T cell exhaustion have now been noted in human infections with HIV and HCV (136, 137).

These functionally impaired CD8 T cells express the inhibitory receptor PD-1, a member of the B7-CD28 family. PD-1 is inducibly expressed on many cells of the immune system including CD4 T cells, CD8 T cells, NK cells, B cells, and activated monocytes (138 - 140). While expression of PD-L2 is primarily limited to dendritic cells and macrophages (141), the other known PD-1 ligand, PD-L1, is expressed on a wide range of hematopoietic and nonhematopoietic cells and may be inducibly upregulated by type I and II interferons (141 - 144). This wide range of ligand expression suggests that this pathway may be important in regulating T cell responses in both lymphoid and nonlymphoid tissues. Indeed, the PD-1/PD-L1 pathway has been demonstrated to regulate peripheral tolerance, autoimmunity, and responses to pathogens (145). In the clone 13 model, blockade of PD-1/PD-L1 signaling *in vivo* rescues the number of antigen-specific CD8 T cells, and restores the ability to produce IFN γ and TNF, and kill antigenic targets thereby reducing viral load (133). Moreover, PD-L1 blockade restored and maintained the ability of “helpless” CD8 T cells to proliferate, secrete cytokines, kill infected targets, and lessen viral burden (133). Similar restoration of exhausted CD8 T cells have been noted following HCV infection (146)

Costimulatory molecules on CD8 T cells typically are expressed to modify signals through the TCR thereby affecting the fate of the ligated cell. PD-1 expression on CD8 T cells modifies TCR signaling directly by dephosphorylation of signaling intermediates. PD-1 contains

an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) motif which can recruit SH2-domain containing tyrosine phosphatase 1 (SHP-1) and SHP-2 upon ligation (147, 148). The inhibitory function of PD-1 ligation is lost following mutation of the ITSM alone suggesting that PD-1 functions mainly through recruitment of SHP-2 to the immunologic synapse during TCR signaling (148, 149). Ultimately, PD-1 ligation results in decreased phosphorylation of CD3 ζ , ZAP70, and PKC θ ; all are signaling intermediates necessary for T cell function (150).

Signaling following PD-1 ligation may be bi-directional resulting in signals being transduced into the cell expressing PD-1 ligands. A soluble PD-1-Ig fusion protein (sPD-1) inhibited DC activation and increased IL-10 production (151). This effect was impaired by pretreatment of sPD-1 with an anti-PD-1 which blocks its interaction with PD-L1 and PD-L2 suggesting that PD-1 may induce signals in cells which express their ligands (151). Similar findings have demonstrated that CD4 T cells produce IL-10 following sPD-1 treatment (152). However, no obvious signaling domains are present in the cytoplasmic tail of PD-L1 or PD-L2. The discovery that B7-1 can interact with PD-L1 inducing bidirectional signals which inhibit T cell function through PD-L1 underscore the ability for PD-L1 to transduce its own signal (153).

PD-1 expression on exhausted CD8 T cells following infection with LCMV clone 13 is well documented. LCMV clone 13 infection results in prolonged and elevated levels of antigen exposure. However, little is known about PD-1 expression on CD8 T cells following latent infection in which antigen is periodically exposed to a subset of CD8 T cells. Chapter 6 will focus on PD-1 and PD-L1 expression in the HSV-1 latently infected TG.

2.0 SPECIFIC AIMS

The overall goal of my thesis studies was to determine how HSV-1 latency influences the CD8 T cell memory response. This dissertation will address three main aims of my studies as outlined below. While my thesis studies were organized into these distinct aims for the purpose of clarity, I have chosen to structure the results and discussion of this dissertation according to the story we have generated.

Specific Aim I: Determine the requirements for CD8 T cell homeostatic cytokines in an experimental model of latent HSV-1 infection.

Memory CD8 T cells generated following acute infections which are cleared from the host requires homeostatic signals from IL-15 for their maintenance and are regulated by IL-2. *We hypothesize that gB-specific CD8 T cells will not require IL-15 for their maintenance and will be refractory to IL-2 regulation within the latently infected ganglia.* To address this aim we utilized HSV-1 infected IL-15 KO mice and wild-type mice treated with an anti-IL-2 mAb at various times post infection.

Specific Aim II: Characterize the nature of the gB-nonspecific CD8 T cell response in the acute and latent TG following corneal infection.

As much as 90% of the anti-HSV-1 CD8 T cell response is directed against a single immunodominant determinant encoded within glycoprotein B (gB₄₉₈₋₅₀₅) (154). However, 50% of the CD8 T cells maintained in the latent TG are not specific for this epitope. *We hypothesize that gB-nonspecific CD8 T cells represent a unique phenotype of HSV-1 specific CD8 T cells.* To address this aim, we examined the kinetics of infiltration, phenotype, and effector function of the population of CD8 T cells in the ganglia that fail to recognize gB₄₉₈₋₅₀₅.

Specific Aim III: Determine the contribution of gB-nonspecific CD8 T cells to the immunosurveillance of HSV-1 latency.

Previous studies have demonstrated that gB-specific CD8 T cells are sufficient to prevent reactivation in *ex vivo* models of TG explant (73). However, it has not yet been feasible to show that they are necessary as there is a distinct population of gB-nonspecific CD8 T cells maintained in the TG with them. *We hypothesize that gB-nonspecific CD8 T cells are capable of preventing reactivation from latency.* To demonstrate this, we isolated gB-nonspecific CD8 T cells from lymph tissue early following infection and demonstrate their ability to prevent reactivation in an explant model of reactivation.

3.0 MATERIALS AND METHODS

3.1 MICE AND HSV-1 CORNEAL INFECTIONS

Six- to 10-week-old female C57BL/6J (B6; CD45.2, Thy1.2), B6.SJL-*Ptprc^aPepc^b*/BoyJ (B6.SJL; CD45.1, Thy1.2), B6.PL-*Thy1^a*/CyJ (B6.PL; CD45.2, Thy1.1), B6.129S2-*Cd8a^{tm1Mak}*/J (CD8 KO), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1), Balb/c mice (The Jackson Laboratory, Bar Harbor, ME), gB-TI.1 β Tg (gB-T; kindly provided by Dr. Francis R. Carbone) mice, and C57BL/6NTac-*IL15^{tm1Imx}* (IL-15 KO; Taconic, Hudson, NY) mice were used. Mice slated for infection were anesthetized by intraperitoneal (i.p.) injection of 2.0 mg ketamine hydrochloride and 0.04 mg of xylazine (Pheonix Scientific, St. Joseph, MO) in 0.2 ml of Hanks balanced salt solution (Biowhittaker, Walkersville, MD). The central cornea was scarified 30 – 40 times by forming a perpendicular meshwork using a sterile 30-gauge needle. The abraded central corneas were then infected by topical application of 3 μ L of RPMI (Biowhittaker) containing 1×10^5 plaque-forming units (PFU) of either WT or recombinant HSV-1 strain RE. Infection efficiency was sporadically monitored by topical application of fluorescein (Akorn, Abita Springs, LA) directly onto the cornea 2 days post infection (dpi) and observed under a slit-lamp microscope. All infected mice observed demonstrated routine infection efficiency. All animal experiments were conducted in accordance with guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2 VIRUSES

HSV-1 strain RE, and recombinant HSV-1 strain RE pICP0-EGFP and pgB-EGFP (kindly provided by Dr. Paul R. Kinchington) was grown in Vero cells. Intact virions were isolated from cell supernatants and purified on Optiprep gradients according to manufacturer's instructions (Accurate Chemical and Scientific Corp., Westbury, NY). PFU were determined using a standard plaque assay on a monolayer of Vero cells. Recombinant viruses express EGFP driven by the promoter sequence of the indicated genes and have been described previously (36, 155). In vitro growth kinetics of the recombinant viruses is comparable to the parent strain (36, 155).

3.3 REAGENTS AND ANTIBODIES

The gB₄₉₈₋₅₀₅ (SSIEFARL) and RR1₈₂₂₋₈₂₉ (QTFDFGRL) peptide was purchased from Research Genetics (Invitrogen Corp., Carlsbad, CA). Phycoerythrin (PE)-conjugated H-2K^b tetramers complexed with the gB₄₉₈₋₅₀₅ peptide were kindly provided by the National Institute of Allergy and Infectious Diseases (NIAID) Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). PE-conjugated H-2K^b:Ig dimers were purchased from BD Pharmingen (San Diego, CA) and were complexed with gB₄₉₈₋₅₀₅ peptide at 37°C overnight prior to use. Purified or fluorochrome-conjugated antibodies specific for murine CD16/CD32 (Fc block; 2.4G2), CD8 α (53-6.7), CD8 β (53-5.8), CD3 (145-2C11), CD4 (RM4-5), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), CD62L (MEL-14), CD107a (1d45), CD43 (1B11), and the V β TCR Flow Kit were purchased from BD Pharmingen and used to stain the surface of cells. Fluorochrome-conjugated antibodies specific for the intracellular antigens IFN γ (XMG1.2), TNF

(MP6-XT22), and 5-bromo-2-deoxyuridine (BrdU Flow Kit; 3D4) were purchased from BD Pharmingen. Fluorochrome-conjugated antibodies specific for murine CD3 (17A2), CD127 (A7R34), CD27 (LG.7F9), KLRG1 (2F1), CD86 (GL1), CD80 (16-10A1), CD274 (PD-L1, B7-H1; MIH5), and FoxP3 (FJK-16s) staining set were purchased from eBiosciences (San Diego, CA). The APC-conjugated antibody specific for the intracellular antigen human granzyme B (GB11; cross reacts with murine granzyme B) was purchased from Caltag (Carlsbad, CA). The PE-Cy7-conjugated antibody specific for murine CD279 (PD-1; RMP1-30) was purchased from Biolegend (San Diego, CA). The antibody specific for a neuronal nucleus marker (NeuN; A60) was purchased from Millipore (Billerica, MA). The appropriate isotype control antibodies were purchased from BD Pharmingen, eBiosciences, Biolegend, and Millipore. All flow samples were collected on a FACSAria cytometer and analyzed by FACSDiva software (BD Biosciences).

3.4 TISSUE HARVEST AND PREPARATION

At the indicated dpi, anesthetized mice were injected with 0.3 ml of 1000 U/ml heparin, and euthanized by exsanguination. If the lungs were harvested in the assay then at the indicated dpi mice would be anesthetized i.p. injection of 2.0 mg ketamine hydrochloride and 0.04 mg of xylazine and perfused with 10 ml of PBS. Spleens and draining lymph nodes (DLN) were collected following anesthetization but prior to exsanguinations where indicated. Tissues were digested in 100 μ l per ganglia or 1 ml per lung of DMEM (Biowhittaker) containing 10% FCS (HyClone, Logan, UT or Atlanta Biologicals, Lawrenceville, GA) and 400 U/ml collagenase type I (Sigma, St. Louis, MO) for 1 h at 37°C. TGs were dispersed into single cell suspensions by trituration and passed through a 40- μ m cell strainer cap (BD Labware, Bedford, MA) while

lungs, spleen, and DLNs were mechanically dispersed with the aid of a nylon filter. All tissue cell suspensions were treated with red blood cell lysis buffer (0.16 M NH₄Cl, 0.17 M Tris, in dH₂O, pH 7.2) prior to use in assays. Tissue harvest and preparation was performed under sterile conditions for all adoptive transfers and ex vivo culture assays lasting longer than 6 hours.

3.5 MOUSE TREATMENTS

3.5.1 Antibody treatments

In vivo anti-IL-2 treatment was accomplished during the expansion of HSV-specific CD8 effector T cells by a single injection of 1 mg of anti-IL-2 mAb (clone S4B6-1) i.p. at 6 dpi followed by tissue excision at 8 dpi; or after establishment of HSV-specific CD8 memory by injection of 1 mg of anti-IL-2 mAb i.p. every other day for 1 week prior to excision of tissue. Initial experiments comparing mice treated with an isotype control mAb lacking specificity for mouse proteins to untreated mice revealed no difference in the HSV-specific CD8⁺ T cell response. Therefore, in repetitions of the experiments, anti-IL-2 mAb-treated mice were compared to untreated mice, and data from both groups were pooled.

In vivo anti-PD-1 or anti-PD-L1 treatment was administered after establishment of CD8 memory by injection of 200 µg of anti-PD-1 mAb (clone RMP1-14), anti-PD-L1 mAb (clone 10F.9G2), or rat IgG_{2b} isotype i.p. every third day for 2 weeks prior to excision of tissue.

3.5.2 5-Bromo-2-deoxyuridine (BrdU) administration

BrdU is a synthetic nucleoside analogue of thymidine which incorporates into the DNA of dividing cells and can be readily detected using antibodies specific for BrdU. In this manner, we can readily track dividing cells *in vivo*. Administration of BrdU during latency was accomplished by i.p. injection of 1 mg of BrdU i.p. daily for 1 week prior to tissue excision. Examination of proliferation during acute infection was performed either by single or multiple injections of 1 mg BrdU either 1 day or 2 days prior to tissue harvest at 8 dpi. To examine *in situ* proliferation, 1 mg of BrdU was administered via i.v. injection 4 h prior to tissue harvest.

3.6 PHENOTYPIC ANALYSIS OF T CELLS

3.6.1 Identifying surface expression by flow cytometry

Surface staining was performed on 2×10^6 cells (spleen, lung, and DLN) or an entire TG equivalent from single cell suspensions in FACS buffer (PBS, 1% FCS, 0.1% NaN₃) as described above. All samples were treated with Fc block (anti-CD16/32) for 10 min on ice prior to surface staining to prevent nonspecific binding. For all flow based phenotypic analyses, TG, spleen, DLN, and lung cells were stained for CD45 to permit gating exclusively on infiltrating bone marrow-derived cells. For analysis of gB₄₉₈₋₅₀₅-specific CD8⁺ T cells, cells were additionally stained with anti-CD8 α and gB₄₉₈₋₅₀₅ H-2K^b tetramers or gB₄₉₈₋₅₀₅ H-2K^b dimers. The latter two reagents were used interchangeably in different experiments because in our hands they provided results that were indistinguishable and will be referred to for the remainder of the dissertation as

gB multimers. Cells that did not bind gB multimers are considered to be nonspecific for the gB₄₉₈₋₅₀₅ epitope and will be referred to for the remainder of the dissertation as gB-nonspecific. Following Fc block, antibody and tetramer cocktails were added directly to the cell suspension for 1h on ice in the dark. Cells were then washed in FACS buffer and resuspended in 1% paraformaldehyde (PFA) for flow acquisition.

3.6.2 Ex vivo T cell stimulations

2×10^6 cells (spleen and DLN) or an entire TG equivalent from single cell suspensions were stimulated with 5×10^5 gB-transfected B6/T-350gB fibroblast cell line (156) pulsed with either 10^{-6} M (optimal) or 10^{-11} M (sub-optimal) gB₄₉₈₋₅₀₅ peptide, HSV-1-infected B6/T-350 fibroblasts, B6/T-350 fibroblasts pulsed with either 10^{-6} M gB₄₉₈₋₅₀₅ peptide or RR1₈₂₂₋₈₂₉ peptide, B6/T-350 fibroblasts transfected with a plasmid expressing full length gB, or unstimulated (control) in the presence of RPMI/10% FCS, and GolgiPlug (BD Biosciences) for 6 h at 37°C/5% CO₂. GolgiPlug contains brefeldin A, an inhibitor of protein trafficking through the trans-golgi network. Stimulation in the presence of GolgiPlug allows for the accumulation and detection of cytokines produced during the stimulation period. Where indicated, FITC-conjugated anti-CD107a mAb or its isotype were included for the duration of the culture period. CD107a is a molecule expressed on the interior membrane of lysosomal and lytic granules that is briefly exposed on the surface of CD8 T cells following degranulation. Used in this manner CD107a capture is a sensitive marker of lytic granule exocytosis as previously described (157).

3.6.3 Intracellular staining and lytic granule exocytosis

Intracellular cytokine staining was performed either following stimulations (cytokine production & lytic granule exocytosis) or immediately ex vivo (granzyme B). Regardless, the protocol for staining was nearly identical. Surface staining was performed first on the indicated cell suspensions to identify surface antigens as described above. For intracellular cytokine staining, granzyme B staining, and lytic granule release determination, the BD Cytotfix/Cytoperm kit (BD Biosciences) was used following manufacturer's protocol.

For intracellular staining of the Foxp3 transcription factor to identify regulatory T cells (Tregs), the Foxp3 staining kit (eBiosciences) was used according to manufacturer's instructions. Briefly, cell suspension were permeablized and fixed with the provided reagents for 30 min – 2 h. Cell suspensions were then washed and intracellular staining was performed in the presence of a permeabilization buffer for 30 min on ice. Following a wash in permeabilization buffer, cells were resuspended in FACS buffer for flow acquisition.

Identification of proliferating cells was performed with the BrdU flow kit (BD Pharmingen) according to manufacturer's instruction. Briefly, cell suspensions obtained from mice which had been previously pulsed with BrdU were stained for surface antigens as described above. Following surface staining, cells went through a series of permeabilization and fixation steps. Cellular DNA was digested for 1 h at 37°C with DNase to expose the incorporated BrdU and intracellular staining with an anti-BrdU mAb was performed. Cells were washed and resuspended in FACS buffer for flow acquisition.

3.6.4 Measuring Apoptosis

Cells suspensions were stained for surface expression of various antigens as described above. Following surface staining cells were washed and resuspended in Annexin-V binding buffer. Annexin-V was added to the samples (5 μ l/sample) and acquired on a flow cytometer within 15 min. Annexin-V binding to phosphatidylserine on the outer membrane is an early marker of apoptosis.

Another more sensitive approach to measure apoptosis was performed using the pan-caspase inhibitor, FLICA (FAM-VAD-FMK; Chemicon, Temecula, CA), which selectively bind active caspases and inhibit their function. FLICA is freely permeable through cell membranes and is tagged fluorescently to permit rapid identification of cells undergoing apoptosis. One added benefit of this approach is that cells undergoing apoptosis in the presence of FLICA were prevented from caspase mediated death and therefore would not shift forward and side scatter into a dead cell gate. Cell suspensions were treated with the FLICA reagent for 1 h at 37°C/5% CO₂ in RPMI/10% FCS. Surface staining was performed as described above following this incubation. Ethanol treated samples were used as a positive control in this assay.

3.7 REAL-TIME PCR TO QUANTIFY VIRAL DNA BURDEN

The number of copies of HSV-1 genome in latently infected TG was determined by real-time PCR quantification of the HSV-1 glycoprotein H (gH) gene in DNA extracts of individual TG as previously described (36). Briefly, TGs were excised and dispersed into single cell suspensions with collagenase as described above. DNA was then extracted using DNeasy columns (Qiagen)

according to manufacturers' instructions and quantified spectrophotometrically. 12.5 ng (1 ng/μl) of DNA isolated from TG suspensions was mixed in triplicate with 12.5 μl of TaqMan Universal PCR Master Mix (Roche, Branburg, NJ) and an HSV-1 gH-specific primer-probe set, custom designed and synthesized by ABI Assays-by-Design service (applied Biosystems, Foster City, CA). Samples, standards, and controls (50 μl/well) were assayed in 96-well plates with an ABI Prism 7700 sequence detector. ABI Primer Express v1.5a software default settings were used for instrument control and data analysis. The gH sequences were: forward primer (5'-GACCACCAGAAAACCCTCTTT-3'), reverse primer (5'-ACGCTCTCGTCTAGATCAAAGC-3'), and probe (5'-[FAM]TCCGGACCATTTTC[NFQ]-3'). The number of copies of gH DNA in each sample was determined from a standard curve generated using known concentrations of gH-containing plasmid standards, and the number of copies of viral gH DNA/TG was calculated based on the total DNA extracted from each TG.

3.8 IDENTIFICATION OF NEURONS BY FLOW CYTOMETRY

TGs harvested at the indicated time-points following infection as described above were stained for intracellular expression of a pan neuronal nucleus marker, NeuN. TGs which were to be used for flow based identification of neuron populations were not filtered with a 40-μm cell strainer cap (BD Labware, Bedford, MA) prior to staining.

3.9 CELL SORTING

Spleen or TGs from HSV-1 infected B6 mice were stained with antibodies for 1 h at 4°C prior to cell sorting on a FACSAria cytometer. Under most circumstances, entire pooled spleens or TGs were stained with anti-CD8 (1:500) or anti-CD8 and gB₄₉₈₋₅₀₅ H-2K^b multimers (1:1000) at 1×10^8 cells/ml. A minimum staining volume of 500 μ l was used. Under some circumstances, pooled spleens were first enriched for CD8 T cells to facilitate sorting of a small population of cells by MACS (Millipore) separation following the manufacturer's protocol. Cell sorting for long-term (>6 h) culture assays was performed under sterile conditions whereas sorted cell populations for short-term (\leq 6 h) cultures were not.

Cell sorting of neuronal populations was also performed. Pooled TGs from HSV-1 latently infected B6 mice were harvested and dispersed into single cell suspensions as described above. These assays were performed by staining TG suspensions with anti-CD45 (1:500) and anti-PD-L1 (1:500) for 30 min at 4°C. Since there is no single surface pan-neuronal marker, sorting was performed exclusively on CD45⁺ cells. In subsequent analysis of this population, greater than 90% of them express NeuN (data not shown). Sorted populations were then used for quantification of HSV-1 gH DNA copies.

3.10 EX VIVO REACTIVATION ASSAY

Latent ganglia from mice infected with wild-type or pICP0-EGFP HSV-1 were harvested, dispersed into single cell suspension, and cultured or depleted of CD45 expressing cells by complement fixation as previously described (158). The ganglia suspensions were plated in

IMDM containing 10% FCS, 500 U/ml rIL-2 (R & D Systems, Minneapolis, MN), and 50 μ m 2-mercaptoethanol (2-ME; Fisher Scientific, Fair Lawn, NJ) in 0.2 TG equivalents in a 48-well plate (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ). Sorted populations of CD8 T cells were added to these cultures and incubated for 11 days at 37°C/5% CO₂. At indicated days post explant (dpe), supernatants were harvested for a standard plaque assays on a monolayer of vero cells to determine reactivation. In some experiments, at the indicated dpe the entire supernatant was collected for quantification of viral load by plaque assay. If the cultures were established with latent ganglia from pICP0-EGFP HSV-1 infected mice, concurrent observation was performed for EGFP expression and spread of EGFP to surrounding cells, i.e. reactivation (36). In some experiments, pooled cells from reactivating wells were pooled separate from wells which contained no detectable EGFP spread (nonreactivated) for flow analysis of CD8 T cell populations. In these assays, cells were treated with versene (8 g NaCl, 0.2 g KCl, 1.15 g dibasic Na₂HPO₄, 0.2 g monobasic KH₂PO₄, 0.2 g Na₄EDTA; in 1 L dH₂O, pH 7.5) for 5 min at 37°C to facilitate isolation of the cells.

In a separate set of assays, single cell suspensions from latently infected trigeminal ganglia were depleted of CD45-expressing cells via complement fixation and plated into 1:5 equivalents in a 48-well plate as described above. These cultures were treated with 20 μ g anti-PD-1, anti-PD-L1, or an isotype antibody for the duration of the assay. Supernatants were collected at the indicated times following explant and assayed for the presence of productive virus with a standard plaque assay. Supernatants were replenished with an equal volume of supernatant removed.

3.11 ADOPTIVE TRANSFERS

CD8 T cells were isolated from spleens of naïve or HSV-1 infected mice by cell sorting as described above or MACS column separation according to manufacturer's instructions. This protocol was performed entirely under sterile conditions. Depending on the assay, $10^5 - 3 \times 10^6$ cells were transferred in 200 μ l HBSS via tail vein injection into naïve or previously infected B6 or CD8^{-/-} mice.

3.12 DC-OVA IMMUNIZATION

Bone marrow-derived DCs were generated in GM-CSF (1000 U/ml; Schering-Plough, Kenilworth, NJ)-supplemented cultures, as described (159). On day 6 – 7, CD11c⁺ DCs were isolated using anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech.) and MACSTM separation columns. The isolated DCs were matured for 16 h in 1000 U/mL of GM-CSF, IL-4 (Schering-Plough), IFN γ (Peprotech) and LPS (250 ng/ml, Sigma)-supplemented cultures. DCs were loaded with 1 μ g/ml OVA₂₅₇₋₂₆₄ at 37°C for 2 h, then washed twice and resuspended in PBS. Mice were injected subcutaneously with 3×10^5 ova-loaded DCs.

3.13 CORNEA FLOW CYTOMETRY

Corneas from latently infected Balb/c mice were excised and incubated in PBS-EDTA at 37°C for 10 minutes. Stromas were mechanically separated from overlying epithelium and digested in

84 U collagenase type 1 (Sigma-Aldrich, St. Louis, MO) per cornea for 1.5 hours at 37°C and then were triturated to form a single-cell suspension. Suspensions were filtered through a 40- μ m cell strainer cap (BD Labware, Bedford, MA) and washed. Cell suspensions were stained as described above.

3.14 HSK SEVERITY SCORING SYSTEM

Mice were scored for herpes stromal keratitis (HSK) by slit lamp examination on alternate days for 2 weeks following the initiation of antibody treatment at 30 dpi. A standard scale ranging from 1 to 4, based on corneal opacity, was used: 1⁺, mild corneal haze; 2⁺, moderate opacity; 3⁺, complete opacity; 4⁺, corneal perforation.

4.0 IL-15 AND IL-2 DURING HSV-1 INFECTION

Most studies to date that have described the role of IL-15 and IL-2 in the maintenance of CD8 memory populations have focused on their role following acute infections which are cleared from the host. However, the role of these cytokines has only recently been explored in systems where the CD8 memory population resides in compartmentalized latently infected tissues, such as the TG following ocular HSV-1 infection where T cells are periodically exposed to antigen. Following HSV-1 infection of the corneal surface, the CD8 memory population that is maintained within the TG during latency appears to undergo periodic activation through the TCR (73, 86). Within the same mouse one can examine the role of IL-15, IL-7, and IL-2 in the TG and draw comparisons to CD8 memory T cells found within noninfected tissues, such as the lungs and spleen, in which the T cells are not exposed to their cognate antigen. We use this model to characterize within the same animal the involvement of IL-2 and IL-15 in regulating the generation and maintenance of the CD8 effector and memory pools in non-infected and latently infected tissue.

4.1 IL-15 AND IL-2 REGULATE EFFECTOR CD8 T CELLS

4.1.1 Normal generation of effector CD8 T cells in the DLN

gB-specific CD8 effector T cells accumulate to peak levels (representing about half of the CD8⁺ T cells present) in the TG 8 dpi (Figure 23), with most of the expansion occurring within the DLN between 6 and 7 dpi (160). A single treatment of WT mice with 1.0 mg of anti-IL-2 mAb on 6 dpi had no effect on expansion of gB-specific CD8 effector T cells in the DLN at 8 dpi (Figure 5A). The accumulation of gB-specific CD8 effector T cells in the DLN of IL-15^{-/-} mice was slightly reduced compared to that of untreated WT mice, but no further reduction was observed when IL-15^{-/-} mice were treated with anti-IL-2 (Figure 5B).

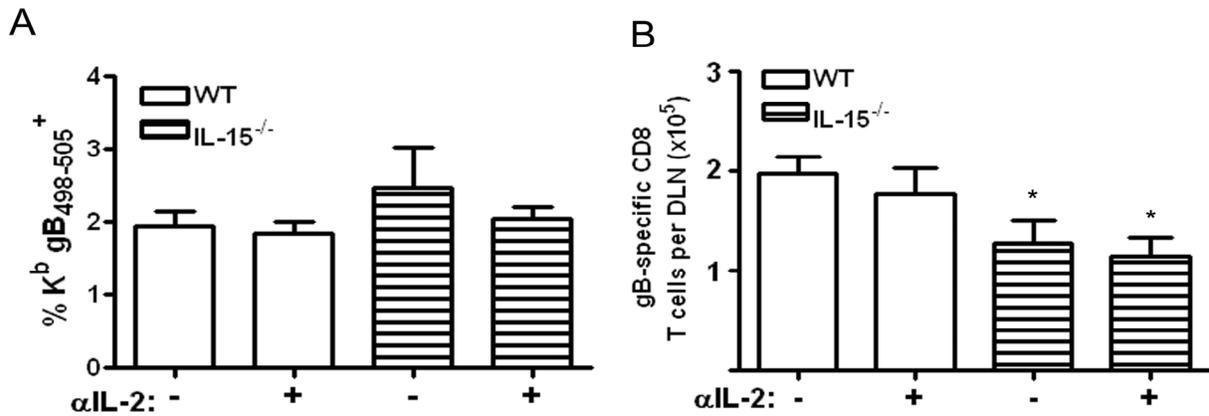


Figure 5. Neither IL-15 nor IL-2 is required for expansion of gB-specific CD8 T cells in the DLN

WT and IL-15^{-/-} mice received an i.p. injection of 1mg anti-IL-2 mAb at 6 dpi and DLN were excised at 8 dpi. DLN cells were simultaneously stained with anti-CD8 and gB₄₉₈₋₅₀₅ multimers. Pooled data are expressed as: (A) the mean (± SEM) percent of CD8⁺ T cells that bind gB₄₉₈₋₅₀₅ multimers; (B) the mean (± SEM) absolute number of gB-specific CD8/DLN. * p < 0.05 comparing the IL-15^{-/-} mice to comparably treated WT mice (n = 6 mice per group).

This slight reduction could be attributed to reduced proliferation, increased apoptosis, accelerated migration out of the DLN, and/or a reduced naïve CD8 T cell pool present in IL-15 deficient mice. The first two possibilities are readily testable. B6 and IL-15^{-/-} mice which received a single injection of anti-IL-2 at 6 dpi were also given daily injections of 1 mg BrdU between 6 dpi and DLN harvest at 8 dpi. DLN from these mice were then split into two groups and examined for annexin-V binding (apoptotic potential) or BrdU incorporation into the DNA of dividing cells (proliferation). IL-15^{-/-} mice, regardless of anti-IL-2 treatment, demonstrated slightly elevated apoptosis levels and slightly reduced proliferation compared to their B6 counterparts (Figure 6). Thus, IL-2 and IL-15 do not appear to significantly regulate the expansion of gB-specific CD8 effector T cells in the DLN of HSV-1 infected mice.

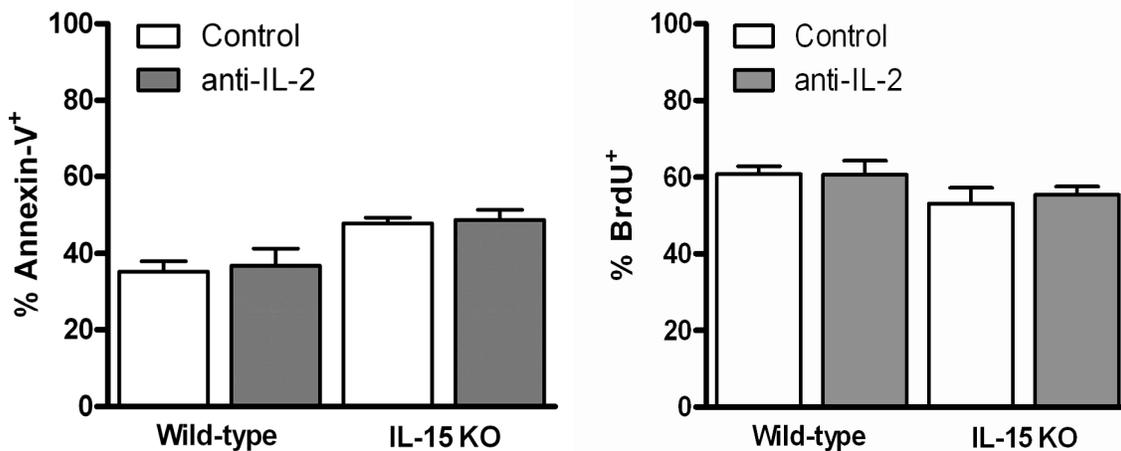


Figure 6. Normal proliferation and apoptosis in DLN of IL-15 KO or anti-IL-2 treated mice
 WT and IL-15^{-/-} mice received an i.p. injection of 1mg anti-IL-2 mAb at 6 dpi and 1mg of BrdU i.p. daily between 6 dpi and DLN excision at 8 dpi. DLN cells were simultaneously stained with anti-CD8, gB₄₉₈₋₅₀₅ multimers, and Annexin-V or intracellularly with anti-BrdU. Pooled data are expressed as: (left) the mean (\pm SEM) percent of gB-specific CD8 T cells that bind Annexin-V; (right) the mean (\pm SEM) percent of gB-specific CD8 T cells that incorporate BrdU during a 48h pulse (n = 5-9 mice/grp).

4.1.2 Impaired accumulation of effector CD8 T cells in the TG

A single treatment of B6 mice with 1.0 mg of anti-IL-2 mAb on 6 dpi reduced the accumulation of gB-specific CD8 effector T cells in the TG by 60% (Figure 7). The accumulation of gB-specific CD8 effector T cells in the TG of IL-15^{-/-} mice was reduced by 76% compared to that of untreated B6 mice, but no further reduction was observed when IL-15^{-/-} mice were treated with anti-IL-2 (Figure 7). The effect of anti-IL-2 treatment and of IL-15 deficiency was limited to accumulation of CD8 T cells in non-lymphoid organs since gB-specific CD8 effector T cells expanded normally in the DLN of both IL-15^{-/-} and anti-IL-2 treated mice (Figure 5). A slight reduction in the overall number of gB-specific CD8 T cells was observed in the DLN of IL-15^{-/-} mice. However, this reduction was slight compared to the overall reduction observed in the acutely infected ganglia and is likely attributed to the reduced naïve CD8 T cell population in IL-15^{-/-} mice.

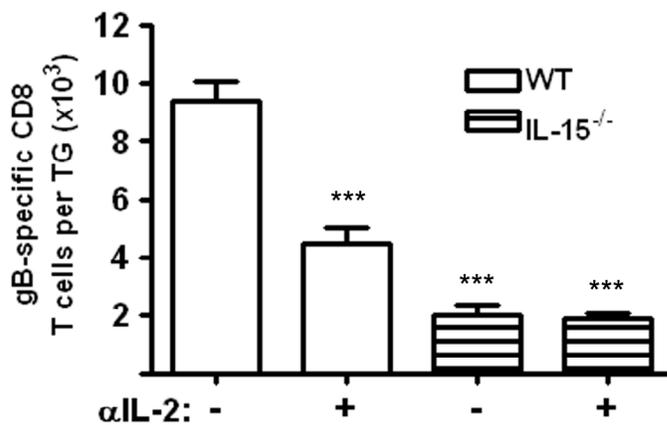


Figure 7. Both IL-2 and IL-15 are required for optimal generation of the gB-specific CD8 effector T cells in the acute TG.

WT and IL-15^{-/-} mice received an i.p. injection of 1 mg anti-IL-2 mAb at 6 dpi and TGs were excised at 8 dpi. TG cell suspensions were simultaneously stained with anti-CD45, anti-CD8, and gB₄₉₈₋₅₀₅ H-2K^b multimers. The entire TG sample was analyzed by flow cytometry, and pooled data are expressed as the mean (\pm SEM) absolute number of gB-specific CD8/TG. *** $p < 0.001$ comparing WT anti-IL-2 treated to WT control, or IL-15^{-/-} mice to comparably treated WT mice ($n = 12-16$ mice per group).

The large reduction observed in the number of gB-specific CD8 T cells in the TG of IL-15^{-/-} mice and WT mice treated with anti-IL-2 could be attributed to reduced proliferation, increased apoptosis, and/or impaired migration into the TG. Since, the reduction in absolute numbers in the IL-15^{-/-} TG are far greater than the reduction observed in the DLN, it is unlikely that the two are directly related. However, this does not rule out a role of impaired gB-specific CD8 T cell generation in the DLN of IL-15^{-/-} mice in contributing to the impaired accumulation in the TG. To examine proliferation, B6 and IL-15^{-/-} mice which received a single injection of anti-IL-2 at 6 dpi were also given daily injections of 1 mg BrdU between 6 dpi and TG harvest at 8 dpi. TG from these mice were examined for BrdU incorporation into the DNA of dividing cells. No difference was observed following a 2-day pulse of BrdU in gB-specific CD8 T cell proliferation in the acute TG (Figure 8). CD8 T cells isolated from the TG incorporate 10% more

BrdU than gB-specific CD8 T cells isolated from the DLN at the same time-point. This suggests that a proportion of these cells are undergoing proliferation in the TG in situ. This topic will be revisited in chapter 5.

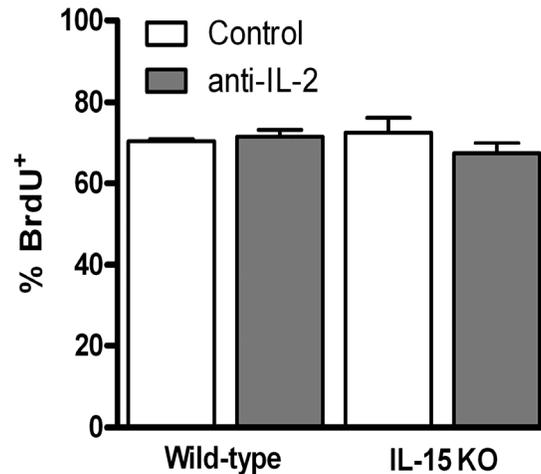


Figure 8. IL-15 and IL-2 do not regulate proliferation of gB-specific CD8 effector T cells in the acute TG.

WT and IL-15^{-/-} mice received an i.p. injection of 1 mg anti-IL-2 mAb at 6 dpi and 1mg of BrdU i.p daily between 6 dpi and TG excision at 8 dpi. TG cells were simultaneously stained with anti-CD45, anti-CD8, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly with anti-BrdU. Pooled data are expressed as the mean (\pm SEM) percent of gB-specific CD8 T cells that incorporate BrdU during a 48h pulse (n = 2-3 mice/grp).

To test whether IL-15 regulated apoptosis in the acutely infected TG, ganglia from B6 and IL-15^{-/-} mice were harvested at 8 dpi. Since there is no evidence in the literature that IL-2 regulates CD8 T cell apoptosis in vivo this assay was only performed on B6 and IL-15^{-/-} mice. TG from these mice were then split into two groups and examined for apoptosis. Two assays were employed to measure apoptosis in this setting. First, the traditional annexin-V binding assay was performed. Secondly, since the TG requires collagenase treatment which may affect T cell death, the caspatag assay was used as well. This assay takes advantage of the FLICA reagent which freely enters cells and binds all active caspases thereby inactivating them and preventing

cell death. This reagent is also tagged with a fluorescent marker allowing ready detection of cells which have been induced to undergo apoptosis. Both populations of CD8 T cells undergo comparable apoptosis as observed with annexin-V binding (Figure 9). Interestingly, IL-15^{-/-} mice tend to undergo slightly reduced apoptosis by this measurement, particularly among the gB-nonspecific population.

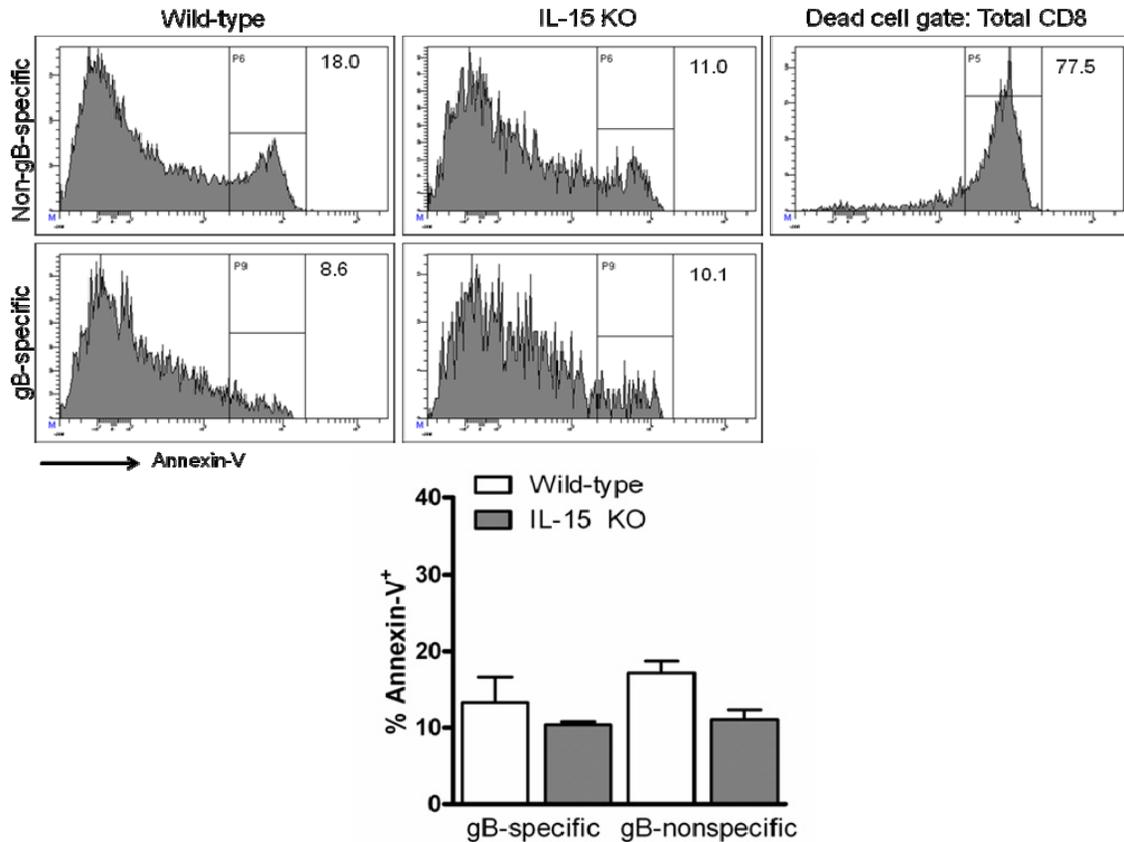


Figure 9. IL-15 KO does not appear to regulate apoptosis in the acute TG

TGs of WT and IL-15^{-/-} mice were harvested at 8 dpi. TG cell suspensions were stained with anti-CD45, anti-CD8, gB₄₉₈₋₅₀₅ H-2K^b multimers, and with Annexin-V. Samples were immediately analyzed by flow cytometry and representative histograms are shown. Numbers within histograms indicate the percent of the indicated population positive for Annexin-V binding. Gates are based on the Annexin-V binding among cells which are low on forward and side scatter. Pooled data are expressed as the mean (\pm SEM) percent of CD8 T cells that bound Annexin-V (n = 3 mice/grp).

This observation is more pronounced when the FLICA reagent is used to examine apoptosis. IL-15 does not appear to regulate apoptosis except in the gB-nonspecific CD8 T cell population that demonstrates reduced apoptosis in the absence of IL-15 (Figure 10). Lack of IL-15 on the CD8 population resulted in diminished or unchanged apoptosis levels and could therefore not explain the diminished CD8 T cell response in the acute TG. The elevated apoptosis observed with this assay compared to annexin-V binding is likely attributable to this reagents ability to impair caspase-dependent cell death in cells which have been induced to undergo apoptosis.

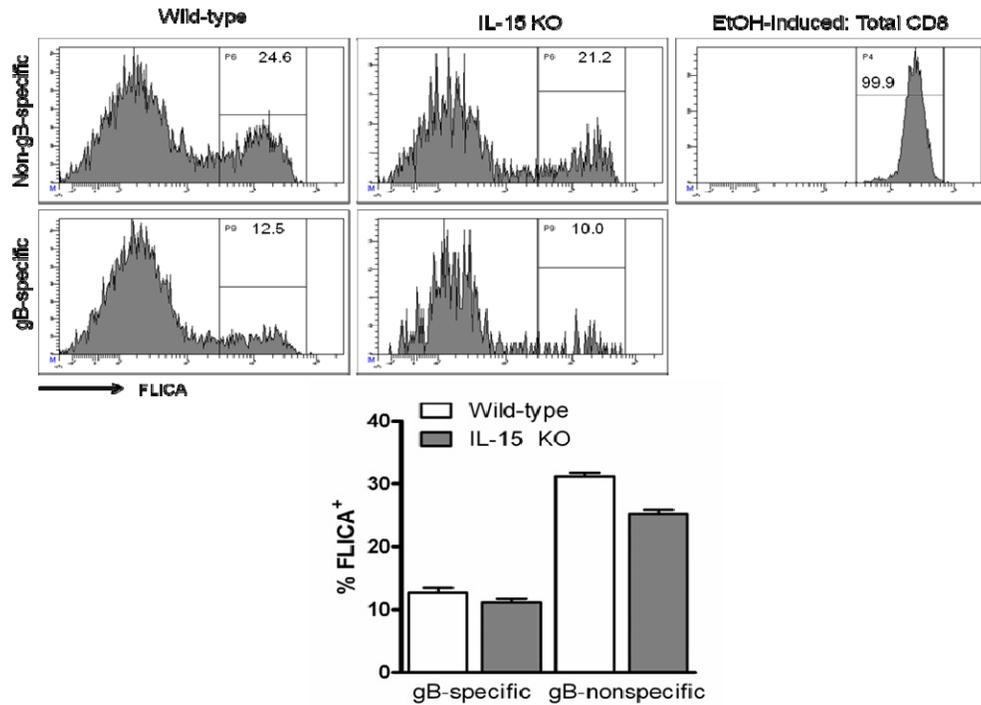


Figure 10. Absence of IL-15 does not promote T cell death in the acute TG.

TGs of WT and IL-15^{-/-} mice were harvested at 8 dpi. TG cell suspensions were incubated with FLICA for 1 h at 37°C prior to surface staining with anti-CD45, anti-CD8, and gB₄₉₈₋₅₀₅ H-2K^b multimers. Samples were analyzed by flow cytometry and representative histograms are shown. Numbers within histograms indicate the percent of the indicated population positive for FLICA labeling. Gates are based on a TG suspension treated with 10% EtOH under identical culture conditions. Pooled data are expressed as the mean (± SEM) percent of CD8 T cells that bound Annexin-V. p <0.001 comparing wild-type gB-nonspecific with IL-15^{-/-} gB-nonspecific (n = 3 mice/grp).

Thus, IL-2 and IL-15 function in an overlapping or sequential manner to regulate the accumulation of gB-specific CD8 effector T cells in the infected TG. Failure to establish a role for impaired proliferation or accelerated apoptosis suggests that the decreased accumulation in the acute TG is due to an impaired ability for the CD8 T cells to infiltrate the acute ganglia.

4.2 CD8 MEMORY PRECURSOR GENERATION IN THE TG

4.2.1 IL-15 but not IL-2 is required for memory precursor generation

Within the gB-specific CD8 effector population present in the TG of WT mice at 8 dpi is a small subpopulation (approximately 5%) that expresses CD127. In an LCMV infection model this CD127⁺ subpopulation was shown to represent the CD8 memory precursor pool (117). Although a single treatment of WT mice with 1 mg of anti-IL-2 mAb at 6 dpi dramatically reduced the size of the gB-specific CD8 effector population in the TG (Figure 7), it did not influence the size of the CD127⁺ CD8⁺ subpopulation (Figure 11). In contrast, the CD8 memory precursor pool was significantly reduced in IL-15 KO mice (Figure 11). This data, combined with the notion that IL-

2 and IL-15 impair infiltration into the TG, suggest that IL-2 may regulate a chemokine that selectively recruits effectors but not memory precursors into the TG.

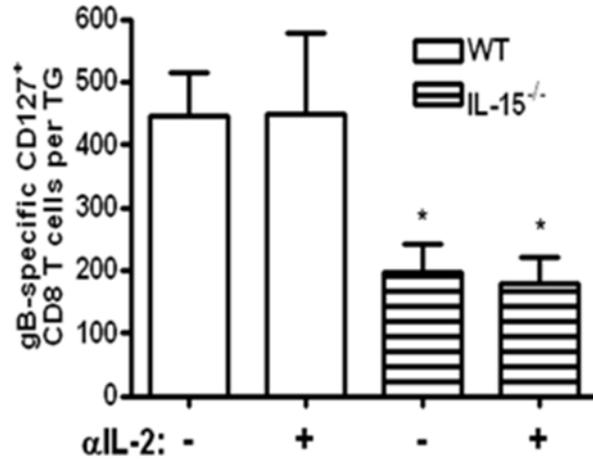


Figure 11. IL-15 is required for gB-specific memory precursors in the acute TG.

WT and IL-15^{-/-} mice received a single i.p. injection of 1 mg anti-IL-2 mAb at 6 dpi and TGs were excised at 8 dpi. TG cell suspensions were simultaneously stained for CD45, CD8, CD127, and gB₄₉₈₋₅₀₅ H-2K^b multimers, and the entire TG sample was analyzed by flow cytometry. Pooled data are expressed as the mean (\pm SEM) absolute number of gB-specific CD8 T cells that express CD127 per TG. * p < 0.05 comparing IL-15^{-/-} mice to comparably treated WT mice (n = 3-8 mice per group).

4.2.2 Memory precursor size but not effector burst size corresponds to the pool of CD8 T cells maintained during latency

As noted above, the size of the CD127⁺ subpopulation of gB-specific CD8 effector T cells was reduced in IL-15 KO mice but not in anti-IL-2 treated mice even though the overall gB-specific CD8 effector T cell population was dramatically reduced in both groups. Therefore, it was of interest to determine if either the size of the overall gB-specific CD8 effector population or the

size of the CD127⁺ subpopulation correlated with the size of the gB-specific CD8 memory pool that was retained in the latently infected TG. As noted in Figure 11, in all four treatment groups (WT and IL-15 KO untreated or treated with anti-IL-2) the size of the CD127⁺ gB-specific CD8 present in the TG at 8 dpi varied significantly, but in all cases correlated closely with the size of the gB-specific CD8 memory pool present at 65 dpi (Figure 12). In contrast, the size of the overall gB-specific CD8 pool in the TG at 8 dpi (Figure 7), which also varied widely among the groups, did not correlate with the size of the gB-specific CD8 memory pool present at 65 dpi (Figure 12). These findings are consistent with the notion that the gB-specific CD8 memory population retained in latently infected TG derive from a subpopulation of gB-specific CD8 effector T cells that express CD127 and are dependent on IL-15.

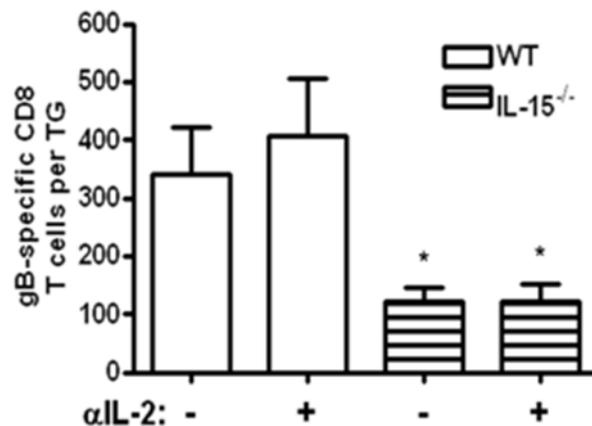


Figure 12. gB-specific CD8 memory precursor pool corresponds to the size of the gB-specific memory pool in the latent TG.

WT and IL-15^{-/-} mice received a single i.p. injection of 1 mg anti-IL-2 mAb at 6 dpi and TGs were excised at 65 dpi. TG cell suspensions were simultaneously stained with anti-CD45, anti-CD8, and gB₄₉₈₋₅₀₅ H-2K^b multimers. The entire TG sample was analyzed by flow cytometry. Pooled data are expressed as the mean (± SEM) absolute number of gB-specific CD8 T cells per TG. * p < 0.05 comparing IL-15^{-/-} mice to comparably treated WT mice (n = 3-8 mice per group).

4.3 IL-15 AND IL-2 DURING LATENCY

4.3.1 IL-15 is not required to maintain the CD8 pool in the latent ganglia

The restriction of latent HSV-1 to nervous tissue permits a comparison within the same animal of factors that regulate the size of the gB-specific CD8 memory pool in the presence or absence of latent virus and presumably low level antigenic exposure. Following contraction of the CD8 effector pool, a CD8 T cell infiltrate consisting of 40 – 50% gB-specific cells is established within the latently infected TG of both WT and IL-15 KO mice (Figure 13A). The reduced size of the CD8 memory population in TG of IL-15 KO mice relative to WT mice resulted in a significantly reduced absolute number of gB-specific CD8 memory T cells (Figure 13B). However, once established the gB-specific CD8 memory pool was maintained at a constant level in the latently infected TG of both WT and IL-15 KO mice, demonstrating that IL-15 is not required to maintain the CD8 memory pool within latently infected tissue.

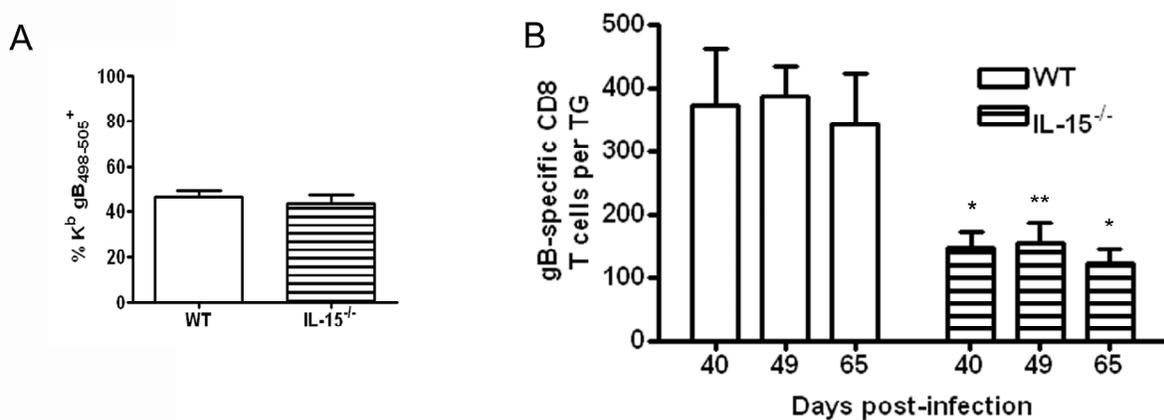


Figure 13. IL-15 is not required to maintain the CD8 memory pool at the site of latency

The TG of WT and IL-15^{-/-} mice were excised at (A) 40-65 dpi, or at the designated time (B) and simultaneously stained with anti-CD45, anti-CD8, and gB₄₉₈₋₅₀₅ H-2K^b multimers. The entire TG sample was analyzed by flow cytometry. Pooled data are expressed as (A) the mean (\pm SEM) percentage of cells in the CD8 gate that were specific for gB₄₉₈₋₅₀₅; or as (B) the mean (\pm SEM) absolute number of gB-specific CD8 T cells per TG. * $p < 0.05$, ** $p < 0.01$ comparing the IL-15^{-/-} mice to WT mice ($n = 4-8$ mice per group).

IL-7 was established as an important cytokine required for the maintenance of CD8 memory populations established in infection models which are cleared by the host. The expression of the IL-7 receptor α chain was evaluated on the population of CD8 T cells that is maintained in the latently infected TG to determine whether IL-7 signals play an important role during CD8 memory T cell maintenance. As observed in other persistent or latent infection models (161, 162), CD127 was not expressed on the all the CD8 T cells maintained in the latently infected TG of HSV-1 infected mice (Figure 14). Whether the lack of the persistently maintained CD8 T cell population to fully upregulate CD127 expression during latency is due to TCR stimulation of the gB-specific CD8 T cell population is unclear. While gB-specific CD8 T cells isolated from the spleen of a latently infected mouse also fail to fully upregulate CD127 (data not shown), their expression is consistently higher suggesting that TCR stimulation may modulate CD127 expression but it is not the only factor. However, a higher percentage of gB-

specific CD8 T cells isolated from the latently infected TG of IL-15 KO mice express CD127 (Figure 14). Whether this increase is due to preferential survival of these cells in the absence of IL-15 signals and whether IL-7 contributes to their maintenance is an area of future investigation.

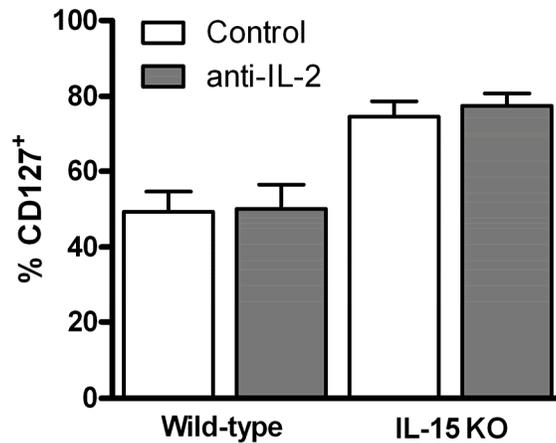


Figure 14. A higher proportion of CD8 T cells from latently infected IL-15 KO TG express CD127.

The TG of WT and IL-15^{-/-} mice were excised after the establishment of latency (49 dpi). TG cell suspensions were simultaneously stained for CD45, CD8, CD127, and gB₄₉₈₋₅₀₅ H-2K^b multimers. The entire TG sample was analyzed by flow cytometry. Pooled data are expressed as the mean (\pm SEM) percentage of gB-specific CD8 T cells that express CD127 per TG. $p < 0.05$ comparing the IL-15^{-/-} mice to WT mice with their respective treatments ($n = 5$ mice/grp).

The gB-specific CD8 memory pool was also reduced in the lungs of IL-15 KO mice relative to WT mice (Figure 15). Furthermore, the gB-specific CD8 memory pool diminished to nearly undetectable levels in the lungs by 42 dpi, demonstrating that IL-15 is necessary for the homeostatic maintenance of HSV-specific CD8 memory cells in the absence of latent virus. It would be interesting to determine whether CD127 expression is also upregulated on gB-specific CD8 T cells in the lungs of IL-15 KO mice during latency.

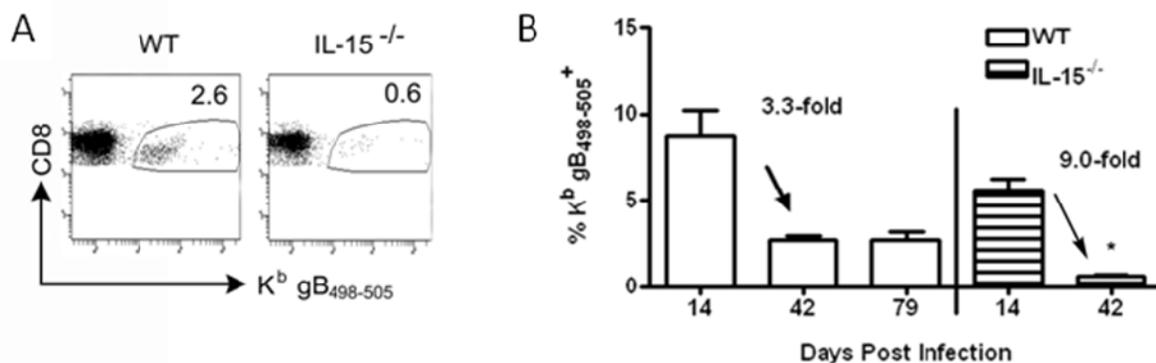


Figure 15. IL-15 is required for maintenance of gB-specific CD8 memory in the noninfected lungs.

Lungs were excised at 42 dpi (A), or the designated time (B), simultaneously stained with anti-CD8 and gB₄₉₈₋₅₀₅ H-2K^b multimers, and analyzed by flow cytometry. (A) Representative dot plots with the percentage of gB-specific CD8 T cells indicated. (B) Pooled data are expressed as the mean (\pm SEM) percentage of cells in the CD8 gate that were gB-specific. * $p < 0.05$ comparing the IL-15^{-/-} mice to WT mice at each time (n = 4-6 mice per group).

4.3.2 IL-2 regulates the CD8 memory pool in noninfected tissues

The presence of latent virus also appears to influence the regulatory effect of IL-2 on proliferation of gB-specific CD8 memory cells. Treatment with anti-IL-2 after memory establishment had no effect on the size of the gB-specific CD8 memory pool in latently infected TG of WT or IL-15 KO mice (Figure 16).

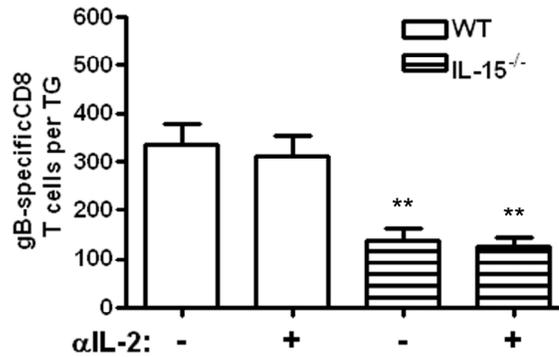


Figure 16. IL-2 does not regulate the gB-specific memory pool after its establishment

WT and IL-15^{-/-} mice received injections of 1 mg anti-IL-2 mAb every other day for one week prior to TG excision at 40 or 49 dpi. TG cells were simultaneously stained with anti-CD8 and gB₄₉₈₋₅₀₅ H-2K^b multimers, and analyzed by flow cytometry. Pooled data are expressed as the mean (± SEM) absolute number of gB-specific CD8/TG. ** p < 0.01 comparing the IL-15^{-/-} mice to comparably treated WT mice (n = 7–11 mice per group).

Although, anti-IL-2 treatment after memory establishment had no effect on the proliferation of the gB-specific CD8 memory pool in the latently infected TG, it did significantly increase the proliferation in the TG of memory CD8 T cells that were not specific for gB₄₉₈₋₅₀₅ (Figure 17). This suggests that a compartment of gB-nonspecific CD8 T cells is regulated by IL-2. Whether this is a specific and direct regulation of a proportion of this population through immune complexes formed by anti-IL-2 and IL-2 or an effect on the function of Tregs in the latent ganglia is unclear. The role of Tregs in the latent ganglia will be addressed below.

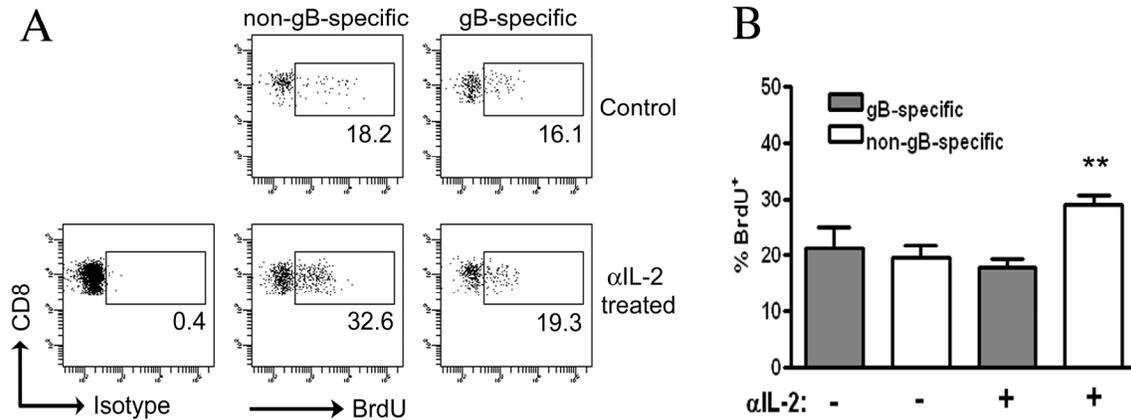


Figure 17. Anti-IL-2 treatment in latent TG induces proliferation of gB-nonspecific CD8 T cells.

WT mice received injections of 1 mg BrdU daily, and 1 mg of anti-IL-2 mAb every other day for one week prior to TG excision (40-49 dpi). TG cells were simultaneously stained with anti-CD45, anti-CD8, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly with anti-BrdU and analyzed by flow cytometry. (A) Representative dot plots of TG cells from WT mice with the percentage of gB-specific and non-gB-specific CD8 T cells that incorporated BrdU into cellular DNA indicated. (B) Pooled data showing the mean (\pm SEM) percentage of gB-specific and non-gB-specific CD8 T cells that incorporated BrdU into cellular DNA. ** $p < 0.01$ comparing anti-IL-2 treated non-gB-specific to control non-gB-specific ($n = 5-6$ mice per group).

The basal rate of proliferation of the gB-specific CD8 T cell population in the latently infected TG was approximately 2-fold higher than that of their counterparts in the lungs, and 1.5 fold higher than those in the spleen (Figure 17B & 18). Anti-IL-2 treatment after memory establishment significantly increased the proliferation of the gB-specific CD8 memory pool in the lungs and spleen (Figure 18). The increased proliferation of gB-specific CD8 memory cells in the lungs and spleen was particularly notable in the IL-15 KO mice, suggesting antagonistic roles for IL-2 and IL-15 in regulating homeostatic proliferation of the gB-specific CD8 memory population. This latter observation demonstrates that anti-IL-2 treatment was effective in the TG, and further supports the notion that persistent antigen stimulation abrogates the regulatory effect of IL-2 on proliferation of the gB-specific CD8 memory T cells within latently infected tissue.

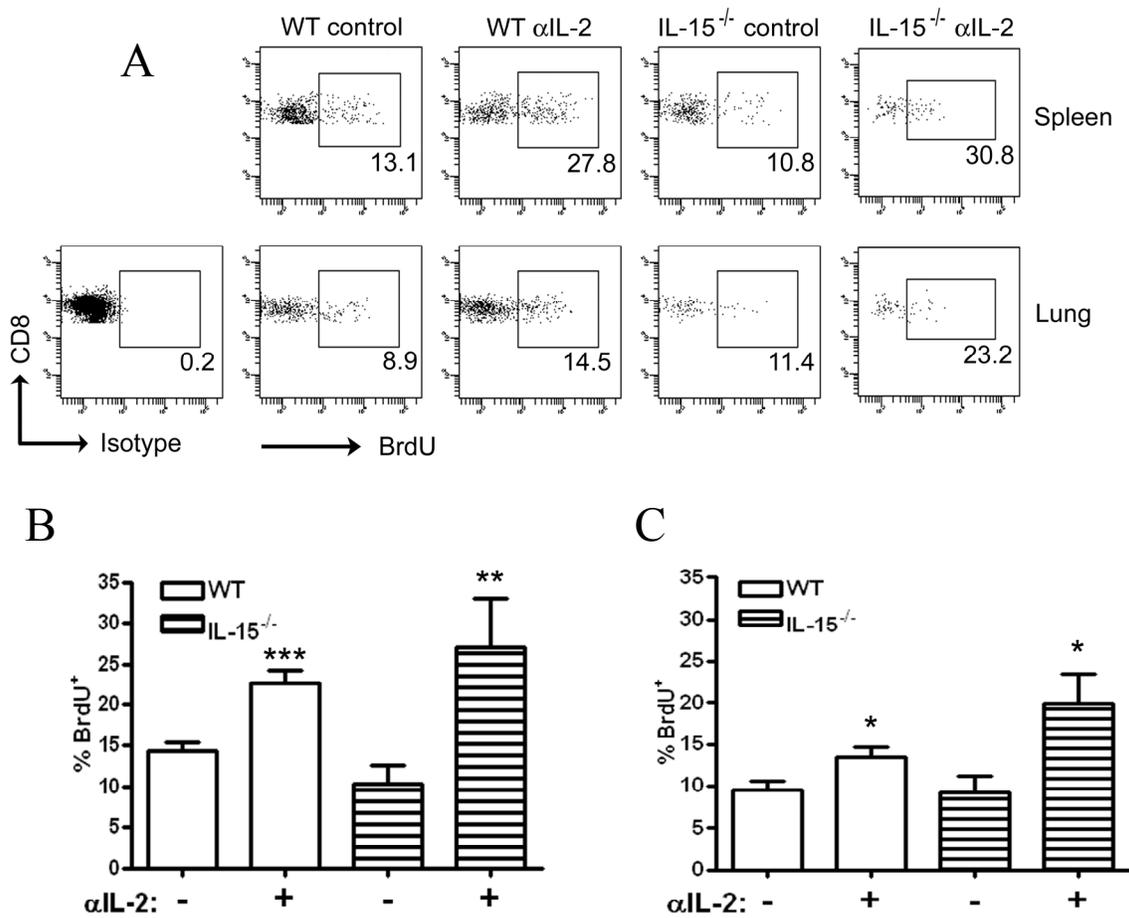


Figure 18. IL-2 regulates proliferation of gB-specific CD8 memory in noninfected tissues during latency.

WT and IL-15^{-/-} mice received injections of 1 mg BrdU daily, and 1 mg of anti-IL-2 mAb every other day for one week prior to spleen and lung excision (40-49 dpi). Cells were simultaneously stained with anti-CD45, anti-CD8, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly with anti-BrdU and analyzed by flow cytometry. (A) Representative dot plots of spleen and lung cells from WT and IL-15^{-/-} mice with the percentage of gB-specific CD8 T cells that incorporated BrdU into cellular DNA indicated. (B, C) Pooled data of the mean (\pm SEM) percentage of gB-specific CD8 T cells that incorporated BrdU into cellular DNA in the spleen (B) and lungs (C) is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing anti-IL-2 treated to controls for each group ($n = 4-10$ mice per group).

4.3.3 Anti-IL-2 treatment does not reduce the size of the Treg population in the latent ganglia

The expression of the transcription factor FoxP3 defines a population of CD4⁺ Treg cells, and these cells have been shown to regulate the homeostatic proliferation of the CD8 memory pool (104, 105). Thirty days after HSV-1 corneal infection, Foxp3⁺ cells represented 17% and 15% of CD4⁺ T cells in the spleen and lungs, respectively, but only 4% of the CD4⁺ T cells in the TG (Figure 19). Thus, the higher basal rate of proliferation of gB-specific CD8 memory cells in the TG is associated with a lower frequency of CD4⁺ FoxP3⁺ Treg cells. Injections of anti-IL-2 mAb every other day for 7 days prior to sacrifice significantly reduced the overall frequency of FoxP3⁺ cells in the spleen and lungs, while not influencing their frequency in the TG (Figure 19). These findings are consistent with the hypothesis that IL-2 negatively regulates the homeostatic proliferation of gB-specific CD8 memory T cells in non-infected tissue (spleen and lungs) by maintaining a resident population of Foxp3⁺ Tregs. However, a differential direct effect of IL-2 immune complexes on proliferation of HSV-specific CD8 memory in latently infected and non-infected tissue is also possible and will be discussed.

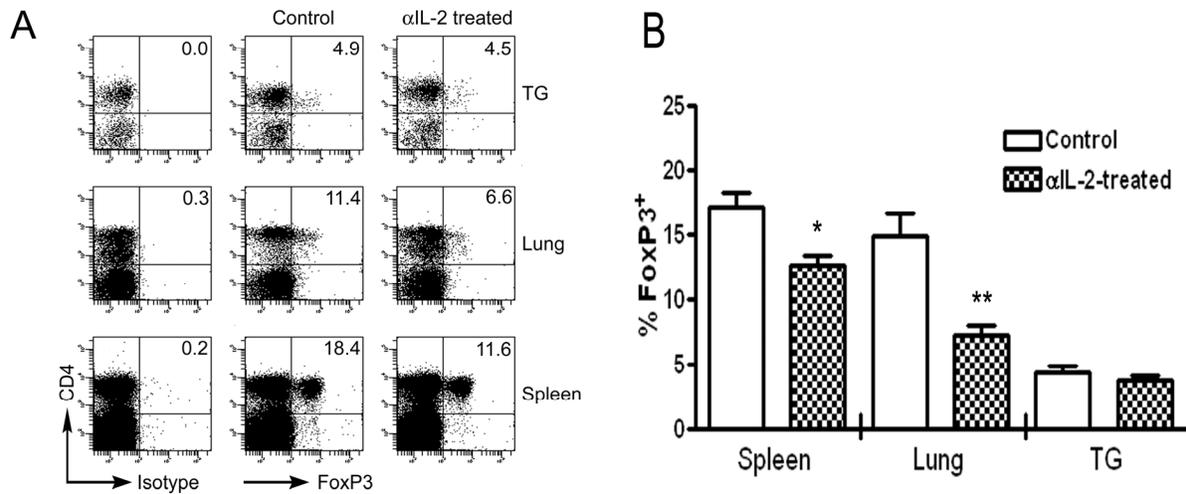


Figure 19. IL-2 differentially regulates the Treg population in latently infected and non-infected tissue.

WT mice were left untreated or received injections of 1 mg of anti-IL-2 mAb every other day for one week prior to TG, spleen, and lung excision. Single cell suspensions of each tissue were stained for surface expression of CD45 and CD4, followed by intracellular staining for FoxP3. (A) Representative dot plots of tissue excised at 37 dpi show that FoxP3 expression is limited almost entirely to CD4⁺ T cells, and the percentage of CD4⁺ T cells that express FoxP3 is indicated in the upper right quadrant. (B) Pooled data from two experiments are expressed as the mean (\pm SEM) percentage of CD4⁺ T cells that express FoxP3. * $p < 0.05$, ** $p < 0.01$ comparing the anti-IL-2 treated mice to untreated mice for each tissue ($n = 3-8$ mice/group).

4.3.4 Anti-IL-2 treatment does not impair T cell function in the latent ganglia

We tested whether anti-IL-2 treatment during latency or the persistent absence of IL-15 would impact the functional program of the CD8 memory pool that is retained in the latently infected TG. WT or IL-15 KO mice received injections of anti-IL-2 mAb every 48 h for 1 week prior to harvest. CD8 T cells were obtained from their TG at 31 dpi and tested for their capacity to produce the cytokines IFN γ and TNF, or to release lytic granules (CD107a surface expression) in response to gB₄₉₈₋₅₀₅ peptide-pulsed targets. In nontreated, WT mice approximately 45% of CD8 T cells produced IFN γ and degranulated. Under every instance examined all cells which

produced IFN γ also released lytic granules. Anti-IL-2 treatment did not alter the functional program of the gB-specific CD8 memory pool (Figure 20).

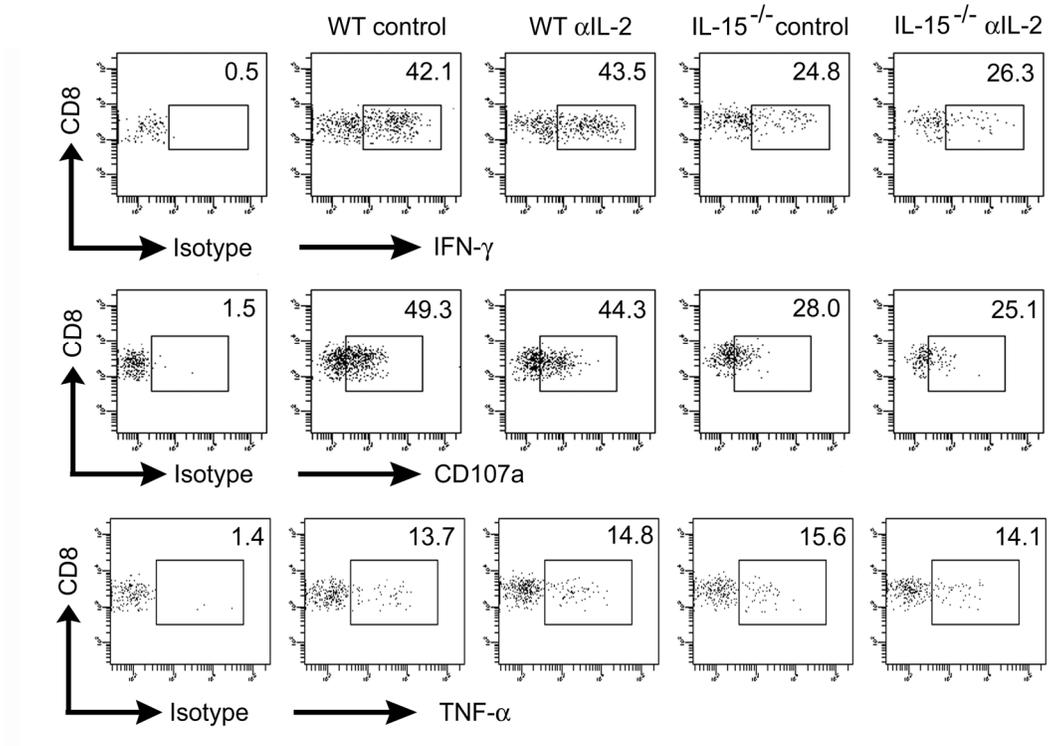


Figure 20. Anti-IL-2 treatment during latency does not affect T cell function in the TG.

WT and IL-15^{-/-} mice received injections of 1 mg of anti-IL-2 mAb every other day for one week prior to TG excision at 31 dpi. TG cells were optimally stimulated with gB-transfected B6/T-350gB fibroblasts pulsed with 10⁻⁶ M gB₄₉₈₋₅₀₅ peptide in the presence of FITC-conjugated anti-CD107a mAb and Golgi-plug for 6 h. Following stimulation the cells were stained for intracellular expression of IFN γ and TNF. Representative dot plots gated on CD8 T cells with the percentage of cells showing surface CD107a, or intracellular expression of IFN γ or TNF indicated.

4.3.5 IL-15 deprivation impairs CD8 T cell function in the latent ganglia

In contrast, the gB-specific CD8 memory pool in the TG of IL-15 KO mice showed a significant functional compromise. In response to optimal stimulation (targets pulsed with 10⁻⁶ M gB₄₉₈₋₅₀₅ peptide) the gB-specific CD8 memory pool in the TG of IL-15 KO mice exhibited a significantly

diminished production of the cytokine IFN γ and reduced lytic granule release, but normal production of TNF (Figure 20 & 21).

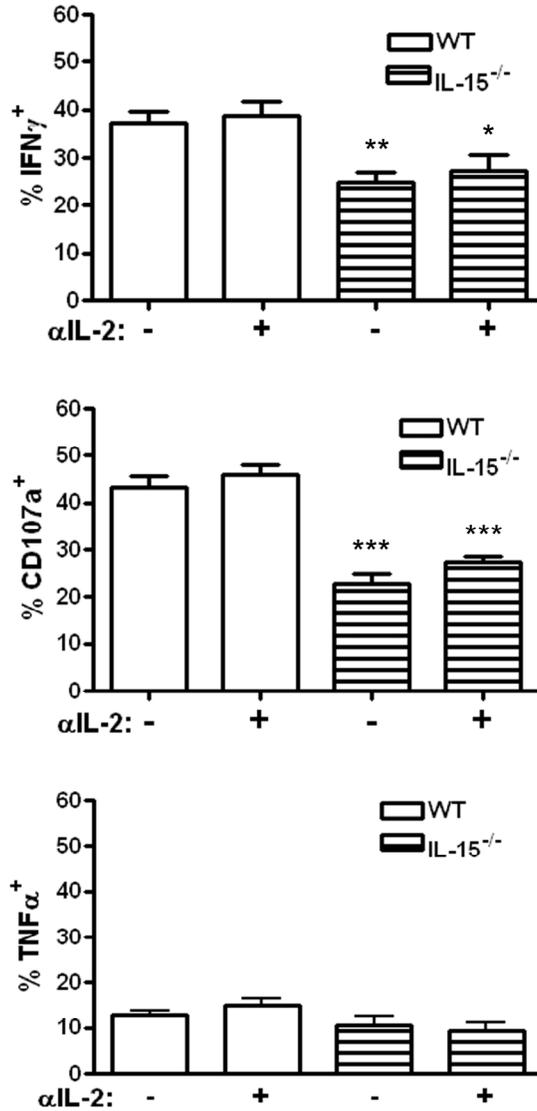


Figure 21. Impaired IFN γ production and degranulation in latent TGs of IL-15 KO mice.

Pooled data from 4 similar analyses as described in the preceding figure are expressed as the mean (\pm SEM) percentage of CD8 T cells that express intracellular IFN γ , surface CD107a, or intracellular TNF. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing IL-15^{-/-} mice to comparably treated WT mice (n = 4 mice per group).

The pattern of cytokine production was of interest. Following optimal stimulation, approximately 37% of CD8⁺ T cells in the TG of WT mice produced IFN γ (Figure 22). Of these, approximately 2/3 produced IFN γ only, while 1/3 produced both IFN γ and TNF. Virtually all CD8⁺ T cells that produced TNF also produced IFN γ . In contrast, following suboptimal stimulation (targets pulsed with 10⁻¹² M peptide) overall production of IFN γ was reduced to 30%, and the reduction was entirely within the population that produced IFN γ but not TNF. Following optimal stimulation of CD8⁺ T cells from TG of IL-15 KO mice only 26% of CD8⁺ T cells produced IFN γ (Figure 22). The reduced IFN γ production relative to comparably stimulated WT CD8⁺ T cells was largely accounted for by cells that produced IFN γ but not TNF. The proportion of CD8 T cells in TG of IL-15 KO mice that produced only IFN γ following optimal stimulation was comparable to that of WT mice following suboptimal stimulation. These findings are consistent with the notion that cells that produce both IFN γ and TNF are more capable of responding to a reduced stimulus than those that produce IFN γ alone. These data demonstrate that the reduced gB-specific CD8 memory population that develops in the TG of IL-15 KO mice is comprised of a higher proportion of the CD8 T cells capable of responding to low doses of antigen by producing both IFN γ and TNF, suggesting that they are of a higher functional avidity.

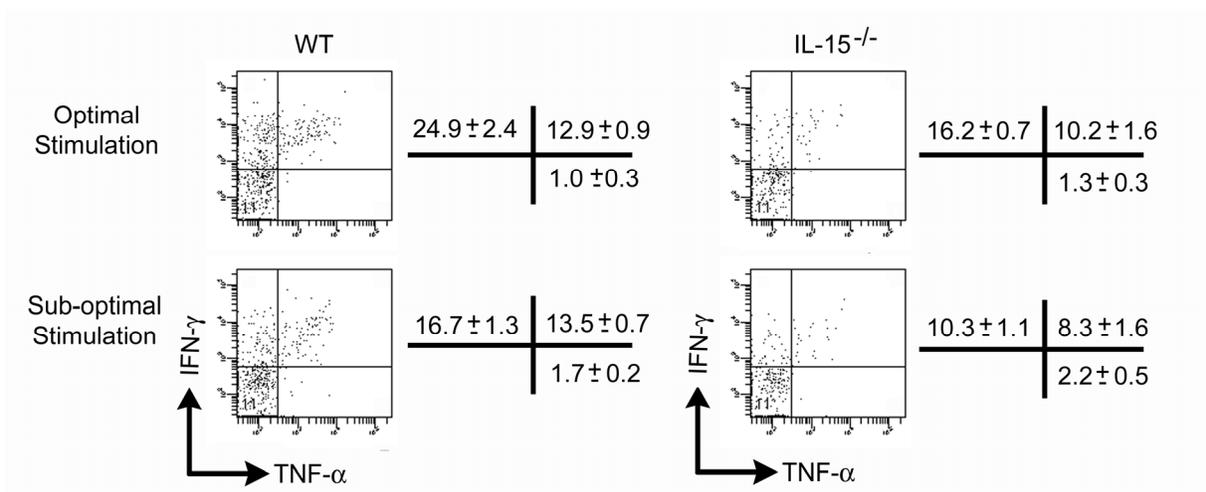


Figure 22. IL-15 is required for maintaining optimal function in the latent TG.

TG were excised from WT and IL-15^{-/-} mice at 31 dpi and TG cells were stimulated with 5×10^5 gB-transfected B6/T-350gB fibroblasts pulsed with either 10^{-6} M (optimal stimulation) or 10^{-12} M (sub-optimal stimulation) gB₄₉₈₋₅₀₅ peptide for 6 h in the presence of Golgi-plug. Following stimulation the cells were stained for surface expression of CD45 and CD8, and for intracellular expression of IFN γ and TNF. Representative dot plots are gated on CD45⁺ CD8⁺ T cells. Pooled data from 4 such analyses are shown to the right of each dot plot and expressed as the mean (\pm SEM) percentage of gated cells that express IFN γ only (upper left quadrant), IFN γ and TNF (upper right quadrant), or TNF only (lower right quadrant).

4.4 DISCUSSION

A hallmark of the herpesviruses is their capacity to induce recurrent disease, particularly in immunocompromised individuals. Recurrent disease results from the reactivation of these viruses from a latent state and subsequent viral proliferation resulting in tissue destruction. Accumulating evidence supports a dynamic view of herpesvirus latency in which CD8 T cells play an important role in monitoring viral gene expression in latently infected cells and preventing virion formation (36, 73, 74). In mouse models of HSV-1 infection, latency is

maintained in sensory neurons. CD8⁺ T cells have been shown to interact closely with latently infected neurons *in vivo* (56, 73), to form an apparent immunologic synapse with multiple neurons within each latently infected TG (73), and to prevent HSV-1 reactivation from latency in neurons in *ex vivo* TG cultures at least in part through production of IFN γ (36, 89). In addition, recent findings in humans validate the murine HSV-1 model by demonstrating the persistence of a chronic immune infiltrate in the TG of infected individuals that is nearly identical to that seen in the TG of HSV-1 infected mice (93). Thus, the establishment and maintenance of an effective and persistently reactive HSV-specific CD8 T cell population within latently infected sensory ganglia might constitute an important protective mechanism to prevent viral reactivation and recurrent disease.

Distinct factors appear to contribute to the development and maintenance of the CD8 memory pool in different models of infectious disease. Within lymphoid organs, CD8 T cells undergo an expansion, contraction, and homeostatic memory phase. These three phases are recapitulated within infected tissue. However, different cytokines appear to regulate the three phases of the CD8⁺ T cell response in lymphoid and non-lymphoid tissue (101, 163 - 165). Here we establish that IL-2 and IL-15 are of no or marginal importance in the initial expansion of gB-specific CD8 T cells in the DLN following a localized HSV-1 corneal infection. However, treatment with anti-IL-2 mAb and/or deprivation of IL-15 had a dramatic inhibitory effect on the establishment of an HSV-specific CD8 T cell population in the infected TG.

A recent study demonstrated that injection of certain anti-IL-2 mAb including the one used in these studies either alone or pre-complexed with IL-2 directly augmented proliferation of memory phenotype CD8 T cells in the spleens of treated mice (124). The IL-2/anti-IL-2 immune complexes apparently bound to Fc receptors and efficiently presented IL-2 to CD8 memory

phenotype T cells that express high levels of the IL-2 receptor beta chain (CD122), but inefficiently to naïve CD8⁺ T cells that are CD122^{lo}. Our observation that anti-IL-2 treatment did not influence the initial expansion of gB-specific CD8 T cells in the lymph nodes of infected mice (Figure 5) is consistent with that finding. However, the failure of anti-IL-2 treatment to augment the proliferation of gB-specific CD8 effector T cells in the TG 8 dpi is not consistent with direct stimulation by IL-2 immune complexes, as effector CD8 T cells would be expected to express high levels of CD122 and thus be susceptible to direct stimulation by IL-2 immune complexes. Indeed, treatment with anti-IL-2 led to a dramatic reduction in the accumulation of gB-specific CD8 effector T cells in the TG. Whether the accumulation of gB-specific CD8 effector T cells in the TG is influenced by increased stimulation by IL-2 immune complexes or reduced IL-2 stimulation through neutralization remains to be determined.

Little is known of the factors that influence the generation of the CD8 memory precursor population during and shortly after the effector phase of the response to acute infection. However, identification of these factors might be aided by the recent observation that CD127 expression on activated CD8 T cells marks a CD8 memory precursor subpopulation. Within lymphoid organs, the size of the memory precursor pool is proportional to the size of the effector pool (117). Based on this observation, one might predict that factors that influence the initial expansion of CD8⁺ T cell effectors would also influence the generation of the memory precursors. Our findings establish that this is not necessarily true in non-lymphoid organs. In the TG 8 dpi, IL-15 deprivation did proportionally reduce the size of the overall gB-specific CD8 effector pool and of the CD127⁺ gB-specific CD8 memory precursor pool. However, anti-IL-2 treatment also dramatically reduced the size of the gB-specific CD8 effector pool, but did not influence the size of the CD127⁺ gB-specific CD8 memory precursor pool. Thus, CD127⁻ CD8⁺

effector T cells and CD127⁺ CD8 memory precursor T cells appear to be separate populations with distinct regulatory requirements.

Previous studies have suggested that the size of the CD8 effector T cell population determines the size of the CD8 memory pool that survives contraction (166). Here we show that within the infected TG mice developed a normal CD8 memory pool in the presence of a CD8⁺ effector T cell pool that was dramatically reduced by anti-IL-2 treatment. In contrast, mice whose gB-specific CD8 effector population and gB-specific CD8 memory precursor pools were both reduced by IL-15 deprivation had significantly reduced numbers of gB-specific CD8 memory cells in their latently infected TG. Moreover, in four different treatment groups (WT and IL-15 KO untreated or treated with anti-IL-2) the number of gB-specific CD8 memory cells retained in the TG following contraction of the gB-specific CD8 effector cells varied widely, but in all cases closely approximated the number of gB-specific CD8 memory precursor cells present at the peak of gB-specific CD8 effector T cell expansion. These data are consistent with the notion that in infected peripheral tissue, as in lymphoid organs, the size of the CD127⁺ memory precursor population and not the size of the overall effector population determine the size of the pool of antigen-specific memory CD8 T cells that survive contraction.

This conclusion is predicated on the assumption that the gB-specific CD8 memory pool that is retained in the TG is not greatly influenced by infiltration from the lymphoid organs. Two observations support this hypothesis. First, as noted above the number of CD127⁺ gB-specific CD8 memory precursors in the TG at 8 dpi closely correlates with the number of gB-specific CD8 memory in the TG following contraction of the gB-specific CD8 effector pool. The second observation favoring the notion that the CD8 memory pool in the latently infected TG is maintained independent of infiltration is that in IL-15 KO mice gB-specific CD8 memory is

diminished to nearly undetectable levels in the lungs and spleen between 14 and 65 dpi, while remaining at constant levels in the TG.

The homeostatic proliferation of memory CD8⁺ T cells following removal of antigen is dependent on IL-15 (99, 101, 102, 164). However, CD8 T cells generated during a chronic infection are refractory to IL-15 induced homeostatic proliferation due to reduced expression of IL-15R (109). This difference in responsiveness to IL-15 following acute and chronic infection is presumed to be due to differences in TCR signaling. Other factors, including differences intrinsic to the pathogens, might also contribute. During HSV-1 latency, persistent TCR stimulation in the TG is strongly suggested by the activation phenotype of the gB-specific CD8 T cells surviving contraction, the apparent formation of an immunologic synapse between gB-specific CD8 T cells and neurons, and the persistent presence of inflammatory cytokines (56, 73, 75 - 77, 93, 167). Our observation that IL-15 is required to maintain the gB-specific CD8 memory pool in the non-infected lungs, but not in the latently infected TG within the same mouse is consistent with the notion that periodic TCR stimulation can supplant the need for IL-15 in maintaining the gB-specific CD8 memory pool.

Although a small population of gB-specific CD8 memory cells was maintained in the latently infected TG of IL-15 KO mice, these cells were functionally impaired. The HSV-1 specific CD8⁺ T cells in the latently infected TG of wild type mice fall into two functionally distinct populations; one that responds to a low concentration of antigenic peptide with production of both IFN γ and TNF; and a second that responds only to a high concentration of antigenic peptide with production of IFN γ but not TNF. Thus, although the number of IFN γ ⁺ gB-specific CD8 memory is significantly reduced in the TG of IL-15 KO mice, those that do remain are sensitive to low concentrations of HSV-1 antigen and produce both IFN γ and TNF.

This functional population of gB-specific CD8 memory cells might be particularly critical in a latently infected tissue in which low levels of viral antigens are likely to be encountered.

Stimulation of dispersed TG cells with targets pulsed with an optimum concentration of gB₄₉₈₋₅₀₅ peptide resulted in three functionally distinct populations of CD8⁺ T cells: (1) IFN γ ⁻ and TNF⁻, (2) IFN γ ⁺ and TNF⁻, and (3) IFN γ ⁺ and TNF⁺. When optimally stimulated, fewer CD8⁺ T cells from TG of IL-15 KO mice produced IFN γ compared to those of WT mice. The reduction was almost exclusively in the IFN γ ⁺ TNF⁻ population. This population appears to represent gB-specific CD8 T cells capable of responding to a reduced stimulus as these cells isolated from WT mice failed to respond when stimulated with targets pulsed with a sub-optimal concentration of gB peptide. Therefore these data suggest that within latently infected tissue anti-viral CD8 memory cells capable of producing IFN γ alone depend on IL-15 for optimal function. IL-15 appears to promote functional maturation of CD8⁺ T cells following acute infection by enhancing co-receptor expression and favoring the homeostatic proliferation of high avidity cells by virtue of their elevated expression of IL-15R α (168). These selective forces would not operate in an IL-15 deficient mouse. However, within latently infected tissue of IL-15 KO mice persistent low level expression of viral proteins might provide a selective force favoring maintenance of a functionally distinct IFN γ ⁺ TNF⁺ CD8⁺ T cell population.

We observed that anti-IL-2 treatment during the latent phase of the HSV-1 infection resulted in increased homeostatic proliferation of gB-specific CD8 T cells in the non-infected lungs and spleen, while not affecting the proliferation of those maintained within the latently infected TG. Interestingly, anti-IL-2 treatment during latency did augment proliferation of the non-gB-specific CD8⁺ T cells within the TG. Our previous studies revealed that these non-gB-specific CD8⁺ T cells in latently infected TG lack detectable reactivity to HSV-1 proteins (73).

These observations suggest that the rate of homeostatic proliferation of gB-specific CD8 memory cells is differentially regulated in latently infected and non-infected tissue. Since IL-2 differentially regulates proliferation of HSV-specific and non-HSV-specific CD8⁺ T cells within the same TG, a likely explanation would be that low level TCR stimulation in the TG renders gB-specific CD8 memory cells refractory to regulation by IL-2.

A subpopulation of CD4⁺ T cells expressing the forkhead transcription factor FoxP3 has been identified as regulatory T cells capable of inhibiting proliferation and function of effector T cells (105). These CD4⁺ Treg cells have also been implicated in controlling the homeostatic proliferation of classical memory CD8 T cells (104). The survival and function of the CD4⁺ Treg cells is dependent on IL-2 (126, 130, 169, 170). Thus, the negative regulation by IL-2 of the homeostatic proliferation of memory CD8⁺ T cells is thought to be mediated indirectly through CD4⁺ Treg cells. However, the above interpretation was based on the assumption that anti-IL-2 mAb neutralizes IL-2 function *in vivo*, an assumption that is now called into question by the recent observation that IL-2/anti-IL-2 immune complexes actually enhance IL-2 utilization by CD8 memory-phenotype cells, directly enhancing their proliferation. Arguing against direct stimulation of CD8 memory in our model is the observation that treatment of IL-15 KO mice with anti-IL-2 also resulted in enhanced proliferation of gB-specific CD8 memory in the spleen and lung. A direct stimulatory effect of IL-2 immune complexes on IL-15 KO CD8 memory cells is unlikely since these cells have been shown to be CD122^{lo} (171).

During HSV-1 latency we observed significantly higher levels of Treg cells in the non-infected lungs and spleen when compared to the latently infected TG. After 1 week of anti-IL-2 treatment in HSV-1 latently infected mice, the size of the Treg population was dramatically reduced in the lungs and spleen, and this correlated with a significant increase in the homeostatic

proliferation of the gB-specific CD8 memory pool. In contrast, the Treg population in the latently infected TG of the same mice was not diminished by anti-IL-2 treatment, and the homeostatic proliferation of gB-specific CD8 memory cells in the TG was also unaffected. These findings seem to suggest that the homeostatic proliferation of gB-specific CD8 memory in the lungs and spleen are a direct consequence of the reduction in numbers of Tregs following anti-IL-2 treatment. However, the alternative possibility that IL-2 immune complexes directly stimulated the proliferation of memory CD8 T cells in the lungs and spleen cannot be ruled out. In either case, it would appear that the homeostatic proliferation of gB-specific, but not non-gB-specific CD8 memory in the latently infected TG is refractory to IL-2 regulation.

The available evidence in mouse models of HSV-1 infection suggest that quantitative and qualitative differences in the HSV-specific CD8 memory pool maintained within latently infected sensory ganglia might influence susceptibility to HSV-1 reactivation from latency and recurrent disease. Our findings demonstrate unique regulatory requirements for generation and maintenance of this HSV-specific CD8 T cell pool in tissue that harbor latent virus. These findings have important implications for the design of immunology-based intervention in recurrent herpetic disease.

5.0 gB-NONSPECIFIC CD8 T CELLS IN HSV INFECTION

Following HSV-1 infection, it is estimated that 70 - 90% of the CD8 T cell response is directed against a single immunodominant epitope in glycoprotein B; a surprising finding for a virus with a genome containing at least 84 open reading frames (13). The remaining HSV-specific CD8⁺ T cells are thought to be directed against a subdominant epitope on the viral ribonucleotide reductase (17). These conclusions derived from studies characterizing the specificity of CD8 T cell at the peak of the effector response in lymphoid tissue. Interestingly, a recombinant HSV-1 in which the immunodominant gB₄₉₈₋₅₀₅ epitope was eliminated induced an HSV-specific CD8 T cell response of normal magnitude, and the RR1₈₂₂₋₈₂₉ epitope remained subdominant (172). The authors of that study proposed that previously cryptic epitopes became dominant.

Both the effector and memory CD8⁺ T cell populations in the HSV-1 infected TG are comprised very consistently of a 1:1 ratio of cells either specific for the dominant gB₄₉₈₋₅₀₅ epitope or reactive to neither the gB₄₉₈₋₅₀₅ epitope nor the sub-dominant RR1 epitope. The gB-specific CD8⁺ T cells have been shown to inhibit HSV-1 reactivation from latency in the TG. Whether the gB-nonspecific population is HSV-specific and contributes to control of viral latency was previously unknown. This chapter will focus on the gB-nonspecific CD8 T cell population and their role following HSV-1 infection.

5.1 PHENOTYPE OF gB-NONSPECIFIC CD8 IN THE TG

5.1.1 Infiltration and maintenance in the TG

The failure of a naïve TG to harbor T cells allows us to examine the populations of CD8 T cells which are maintained within that tissue for the life of the mouse. gB-specific CD8 T cells reach peak levels in the trigeminal ganglia at 8 days post infection (dpi) and expand within the DLN between 5 and 8 dpi (Figure 23) (160, 173). Given that most of the CD8 T cells generated against HSV-1 are purportedly specific for the strongly immunodominant gB₄₉₈₋₅₀₅ epitope (73, 154) and that a population of CD8 T cells is proportionately maintained at a 1:1 ratio within the TG for the life of the animal led us to question how these cells contribute to the anti-HSV-1 response. Prior to peak accumulation of gB-specific CD8 T cells in the TG, a population of CD8 T cells which does not bind gB₄₉₈₋₅₀₅-loaded tetramers first migrates into the TG and reaches peak levels at 7 dpi, 1 day prior to the gB-specific population (Figure 23).

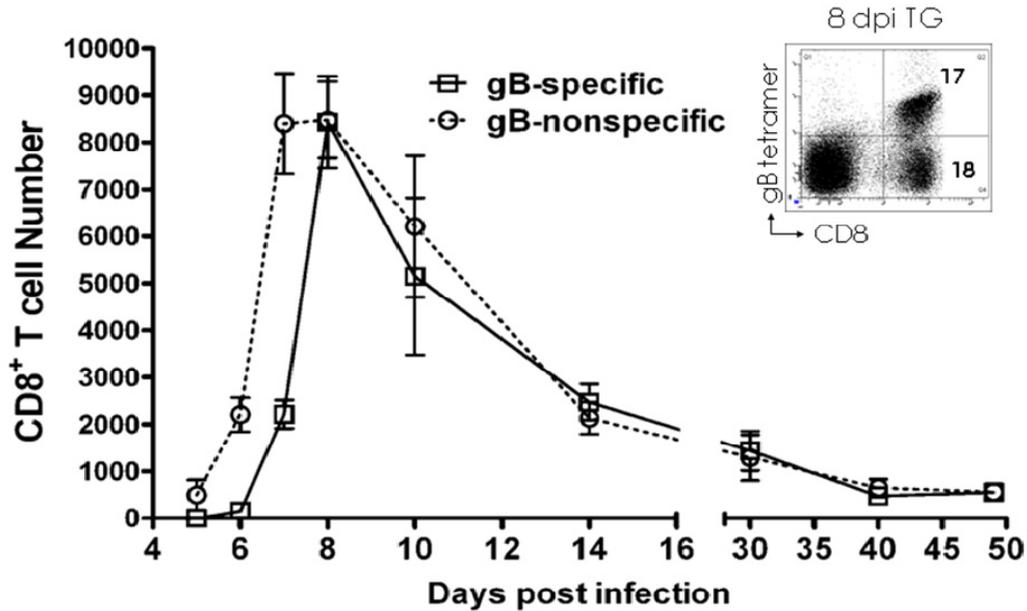


Figure 23. Expansion, contraction, and maintenance of the CD8 T cell populations in the TG.

TG were harvested at indicated dpi and stained with gB₄₉₈₋₅₀₅ tetramers for quantification of CD8 infiltrate into the TG. gB-specific CD8 T cells are considered CD8 T cells which bound tetramer while gB-nonspecific CD8 T cells are considered those which do not bind tetramer. Inset is example staining of CD8 T cells from an 8 dpi TG with gB₄₉₈₋₅₀₅ tetramer demonstrating 50% tetramer positivity. Quantification of CD8 T cells within the graph is depicted as the mean (\pm SEM) absolute number (n = 3-18 mice/group).

5.1.2 gB-nonspecific CD8 T cells are phenotypically comparable to their gB-specific counterparts

Between 7 – 8 dpi, the gB-nonspecific CD8 T cells express similar levels of granzyme B (grzB), CD27, CD69, and KLRG1 as gB-specific CD8 T cells suggesting that the majority of CD8 T cells which enter the TG following HSV-1 infection have recently been stimulated through their TCR (Figure 24). In addition, the majority of gB-nonspecific CD8 T cells downregulate CD127 and CD62L, also consistent with recent TCR stimulation (Figure 24).

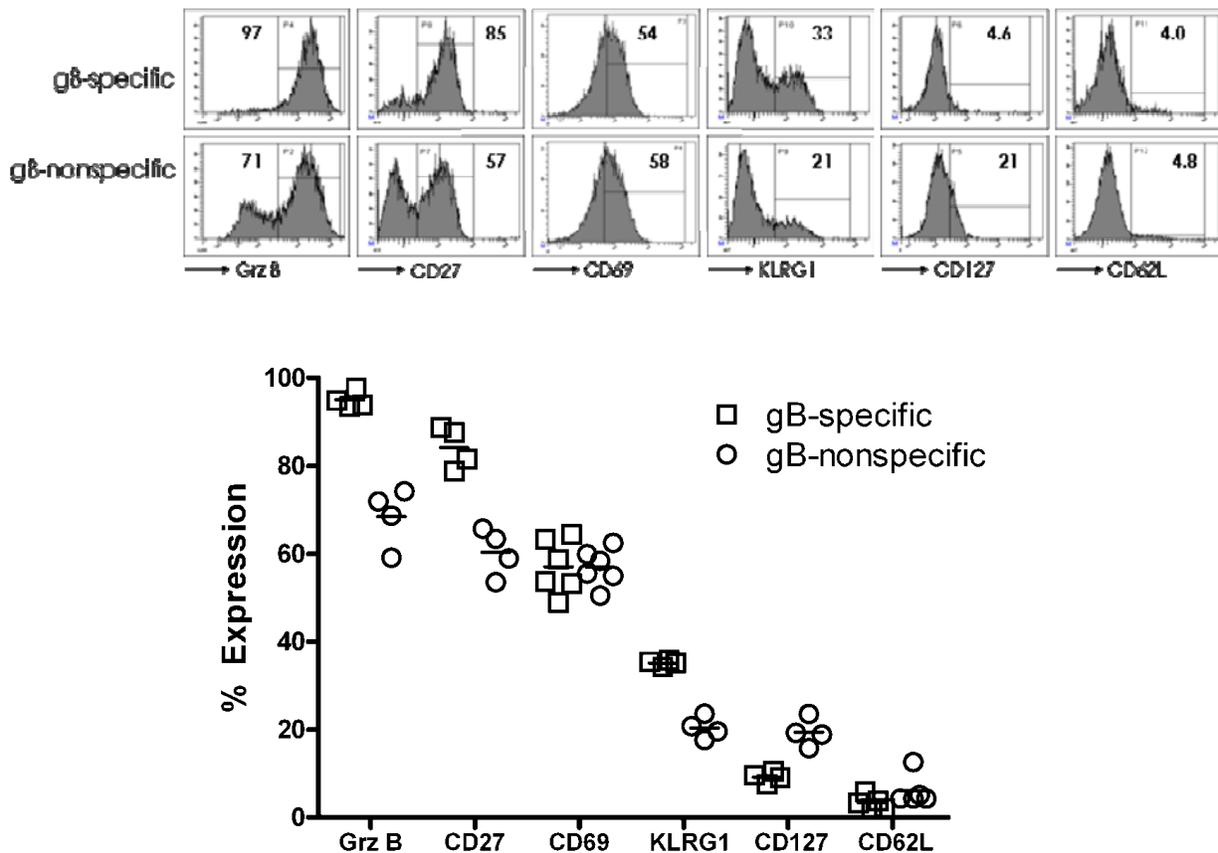


Figure 24. gB-nonspecific CD8 T cells display an activated phenotype in the acute TG.

TG were excised from B6 mice at 7 or 8 dpi and stained with antibodies specific for the indicated receptors. Representative histograms are gated on either gB-specific or gB-nonspecific CD8 T cells as demonstrated in (A) inset and gates are based on appropriate isotype controls. Number within histograms corresponds to percentage of cells within the positive gate. Quantification of individual mice is represented graphically as percent expression of indicated receptor per population of CD8 T cells with a solid line indicating the mean. $p < 0.05$ for grz B, CD27, KLRG1, and CD127 staining.

Consistent with this observation, during acute infection the majority of gB-nonspecific CD8 T cells have proliferated within 72 h prior to TG harvest as indicated by BrdU incorporation following a 3 day pulse (Figure 31). Furthermore, we can identify proliferating T cells *in situ* by i.v. injection of BrdU 4h prior to tissue harvest. Using this BrdU pulsing technique, we can detect proliferation of both gB-specific and gB-nonspecific CD8 T cells within the ganglia at day 7 pi but not in circulation (Figure 25) suggesting that T cells are undergoing further rounds of

division following infiltration into the infected ganglia. A large number of gB-nonspecific CD8 T cells are proliferating within the TG at 7 dpi despite a decreased percentage of BrdU incorporation within this population (Figure 26). This discrepancy is consistent with the imbalance of absolute CD8 T cell numbers of each population at 7 dpi (Figure 23). This data strongly suggests that this population of CD8 T cells which infiltrates the infected ganglia have recently been stimulated through their TCR and is likely responding to HSV-1 antigens other than gB₄₉₈₋₅₀₅. This data, taken together, strongly suggest that the majority of the CD8 T cell population which infiltrates the ganglia following HSV-1 infection have experienced recent TCR stimulation.

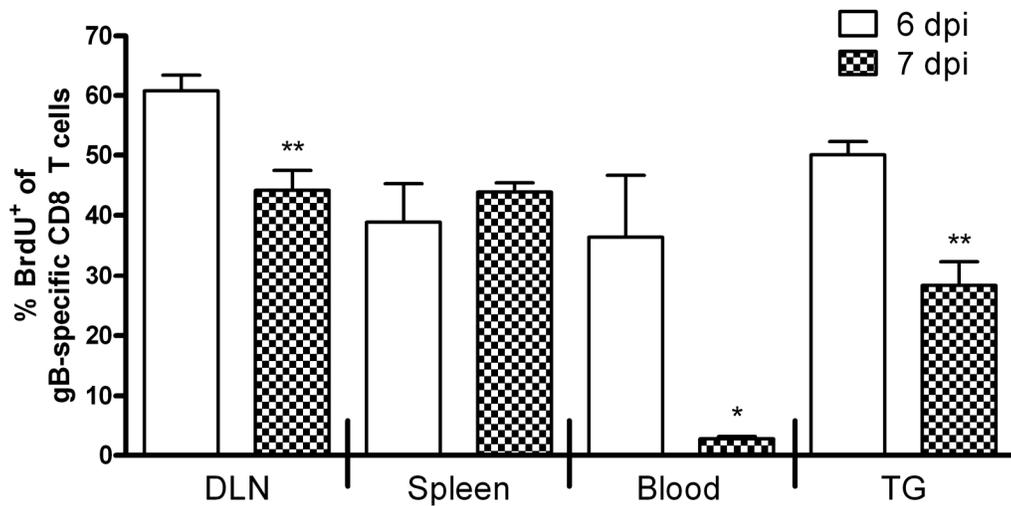


Figure 25. In situ proliferation following 4 h BrdU pulse with iv administration.

HSV-1 infected mice were given 1 mg BrdU iv 4h prior to DLN, spleen, blood, and TG harvest at indicated time to measure proliferation of CD8 T cell populations. Cell suspensions were simultaneously stained with anti-CD45, anti-CD8, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly with anti-BrdU and analyzed by flow cytometry. Pooled data showing the mean (\pm SEM) percentage of gB-specific CD8 T cells that incorporated BrdU into cellular DNA during a 4 h pulse. * $p < 0.05$, ** $p < 0.01$, comparing 6 and 7 dpi groups from respective tissues ($n = 3-5$ mice/group).

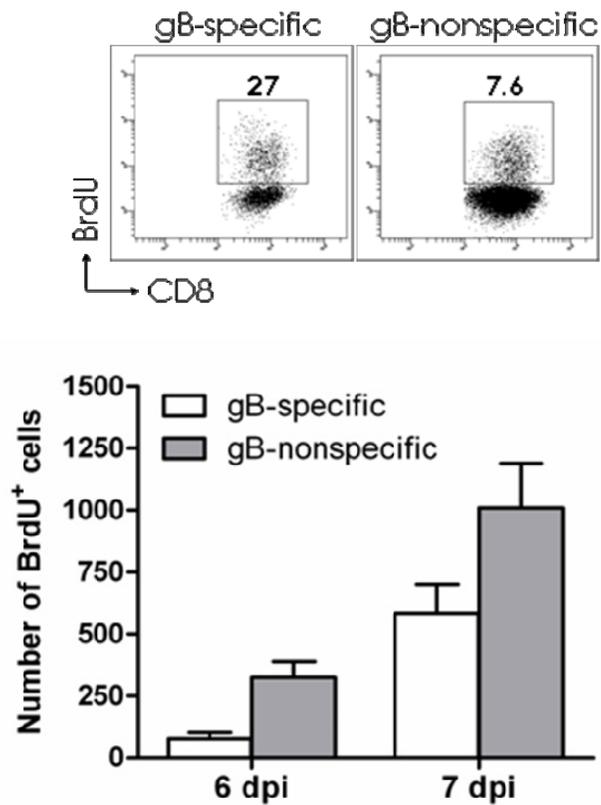


Figure 26. gB-nonspecific CD8 T cells proliferate in the TG

HSV-1 infected mice were given 1 mg BrdU iv 4h prior to TG harvest at indicated time to measure in situ proliferation of CD8 T cell populations. Cell suspensions were simultaneously stained with anti-CD45, anti-CD8, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly with anti-BrdU and analyzed by flow cytometry. Representative dot plots from a 7 dpi mouse are gated on either gB-specific or gB-nonspecific CD8 T cells as demonstrated in (A) inset and gates are based on appropriate isotype controls. Number within histograms corresponds to percentage of cells within the positive gate. Quantification of individual mice is represented graphically as the mean (\pm SEM) absolute number of CD8 T cells which incorporated BrdU during a 4h pulse. $p < 0.05$ comparing the gB-specific CD8 T cells and the gB-nonspecific CD8 T cells at 6 dpi ($n = 4-5$ mice/group).

5.1.3 gB-nonspecific CD8 T cells have a more diverse TCR V β repertoire

One unique characteristic of the gB₄₉₈₋₅₀₅-specific CD8 T cell population maintained within the acute ganglia is the narrow V β repertoire they employ. Approximately 80% of the gB₄₉₈₋₅₀₅-specific response is dominated by two particular V β TCRs, V β 10^b and V β 8.2 (Figure 27A & B) (174). Examination of the gB-nonspecific CD8 T cell population reveals a much more diverse TCR V β utilization than their gB-specific counterparts. However, 40% of the gB-nonspecific population is also dominated by two particular V β TCRs, V β 5.1 and/or 5.2, and V β 8.1 and/or 8.2. The remaining 60% of the V β usage is divided amongst the other V β TCRs (Figure 27A & C). These results are not unexpected as the gB-nonspecific CD8 T cell population most likely represents a much more heterogeneous population of cells than the CD8 T cells that respond to the gB₄₉₈₋₅₀₅ epitope.

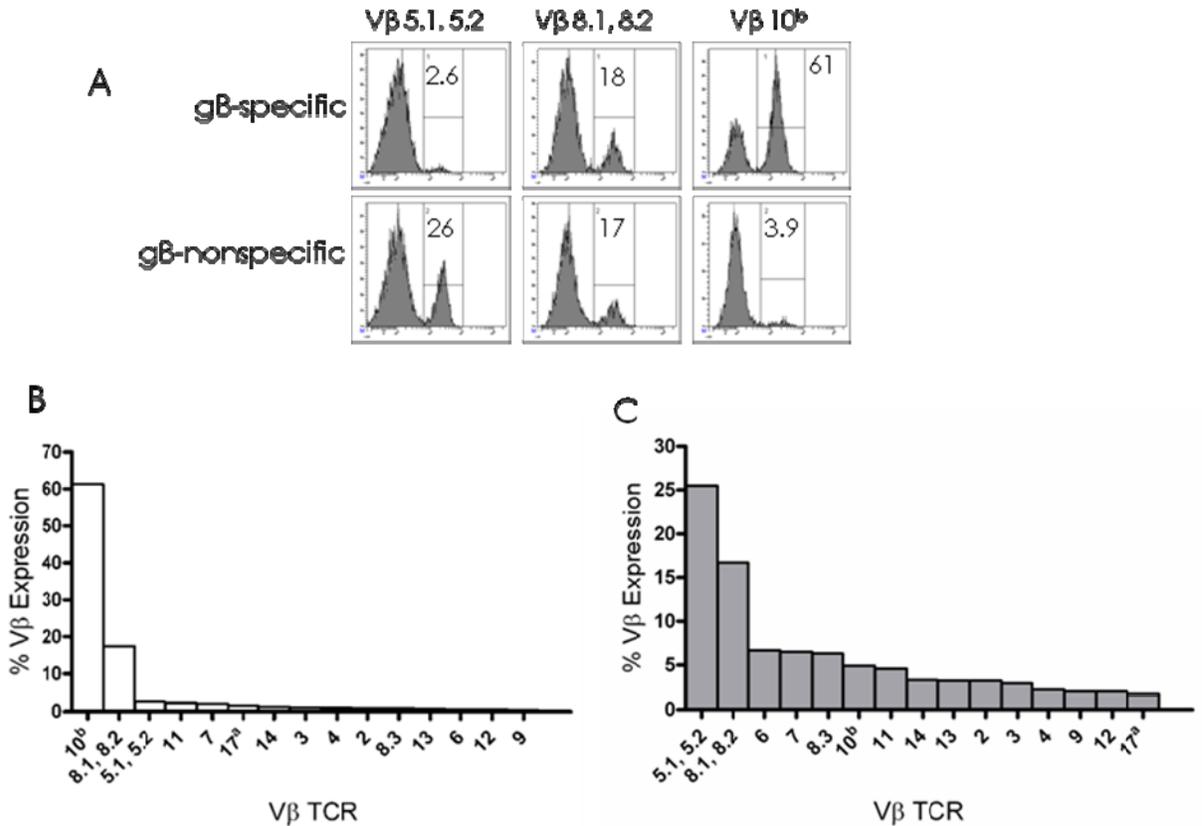


Figure 27. The gB-nonspecific CD8 T cell population displays a diverse Vβ TCR usage.

TG were harvested at 8 dpi and pooled prior to staining with gB₄₉₈₋₅₀₅ tetramers to identify specificity and different Vβ TCR antibodies. (A) Representative histograms for the three prominent Vβ (Vβ 5.1, 5.2, Vβ 8.1, 8.2, and Vβ 10^b) TCR utilized by the entire TG resident CD8 T cell population are gated on either gB-specific or gB-nonspecific CD8 T cells. Number within histograms corresponds to percentage of cells within the positive gate. Quantification of pooled ganglia is represented graphically as percent expression of indicated Vβ TCR per (B) gB-specific CD8 T cells or (C) gB-nonspecific CD8 T cells.

Analysis of the three major CD8 TCR Vβs utilized by the entire TG resident CD8 population reveals that they remain stable with the establishment of latency (Figure 28). At 30 dpi, the TCR Vβ repertoire of gB-specific and gB-nonspecific CD8 T cell populations remains largely unchanged from the established hierarchy at 8 dpi. Occasionally, the gB-specific CD8 T cell repertoire flips with Vβ 8.2 becoming co-dominant with Vβ 10^b (data not shown) past 30

dpi. However, this finding is not consistent and since these observations cannot be followed longitudinally in the TG it is difficult to say whether this is due to outgrowth at certain times of a V β 8.2 or the initial establishment of a dominant V β 8.2.

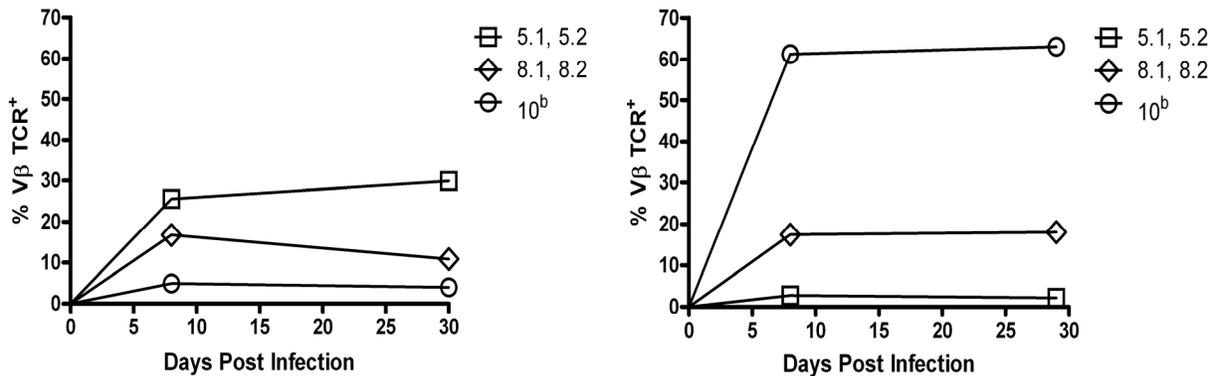


Figure 28. Stable V β TCR expression in the latent TG.

TG were harvested at indicated dpi and pooled prior to staining with gB₄₉₈₋₅₀₅ tetramers to identify specificity and different V β TCR antibodies. Quantification of pooled ganglia is represented graphically as percent expression of indicated V β TCR per (left panel) gB-specific CD8 T cells or (right panel) gB-nonspecific CD8 T cells for the three prominent V β (V β 5.1, 5.2, V β 8.1, 8.2, and V β 10^b) TCR utilized by the entire TG resident CD8 T cell population.

5.1.4 Bystander activation of HSV-1 nonspecific CD8 T cells does not contribute to CD8 T cell infiltration

To exclude the possibility that the gB-nonspecific CD8 T cells which infiltrate the TG following corneal HSV-1 infection are not cells which have undergone bystander activation and enter due to upregulation of migratory receptors we tested the ability for OT-1 CD8 T cells to infiltrate the TG. Bulk CD8 T cells isolated from spleens of naïve gB-T and OT-1 transgenic mice were adoptively transferred into a naïve CD8 deficient mouse and allowed to acclimate for 1 day prior to infection. This strategy allows us to ensure that all gB-nonspecific CD8 T cells are not specific

for other HSV-1 antigens. The TG and DLN of these mice were harvested 7 days following infection, the peak of the gB-nonspecific CD8 T cell response in the TG, and examined by flow cytometry for infiltration into the TG and activation phenotype. Low level bystander activation of OT-1 CD8 T cells was observed in the DLN based on intracellular grz B expression 7 days following infection (Figure 29). Virtually all of the gB-T CD8 T cells in the DLN expanded and upregulated grz B at this time-point (Figure 29).

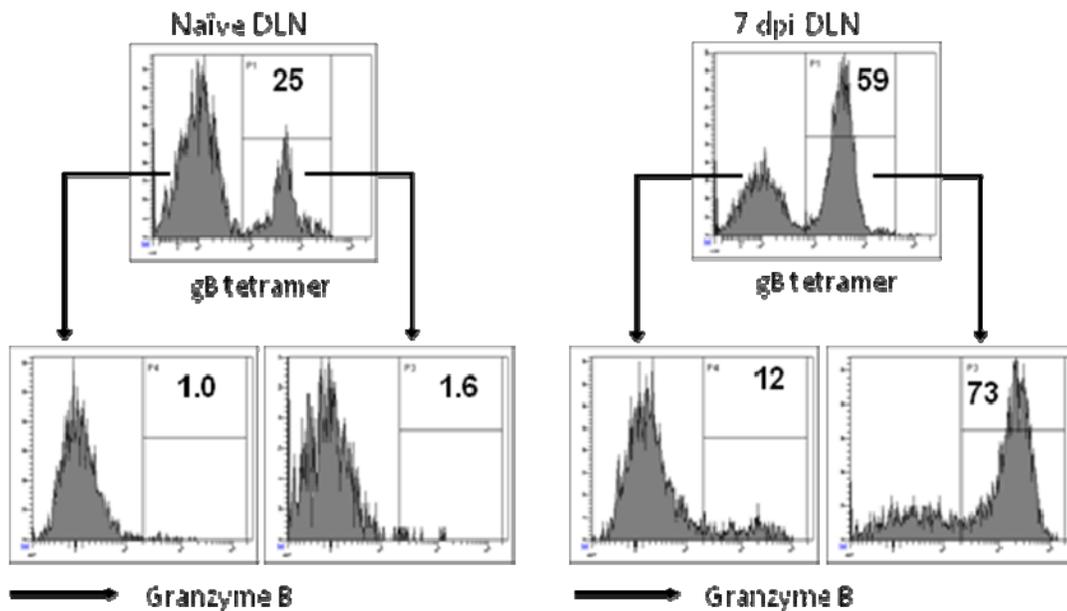


Figure 29. OT-1 CD8 T cells do not upregulate granzyme B

gB-T and OT-1 CD8 T cells were isolated from naïve spleens and transferred into $CD8^{-/-}$ mice 1 day prior to infection with HSV-1. DLN of these mice was harvested 7 days later for characterization of the expanding and populations. Mice which received cells but were not infected were used as controls (labeled naïve). Representative parent histograms are gated on CD8 T cells with the gating strategy depicted. Number within histograms corresponds to percentage of cells within the positive gate.

Despite approximately 10% of the OT-1 CD8 T cells undergoing bystander activation indicated by upregulation of grz B in the DLN, these cells failed to infiltrate the TG to any significant degree (Figure 30). Moreover, gB-T CD8 T cells which infiltrate the ganglia are

phenotypically identical based on the markers examined to endogenous gB-specific CD8 T cells which infiltrate the ganglia of replete mice (compare Figure 24 and 30). This data together suggests that bystander CD8 T cells do not significantly contribute to the CD8 T cell response in the normal acutely infected TG.

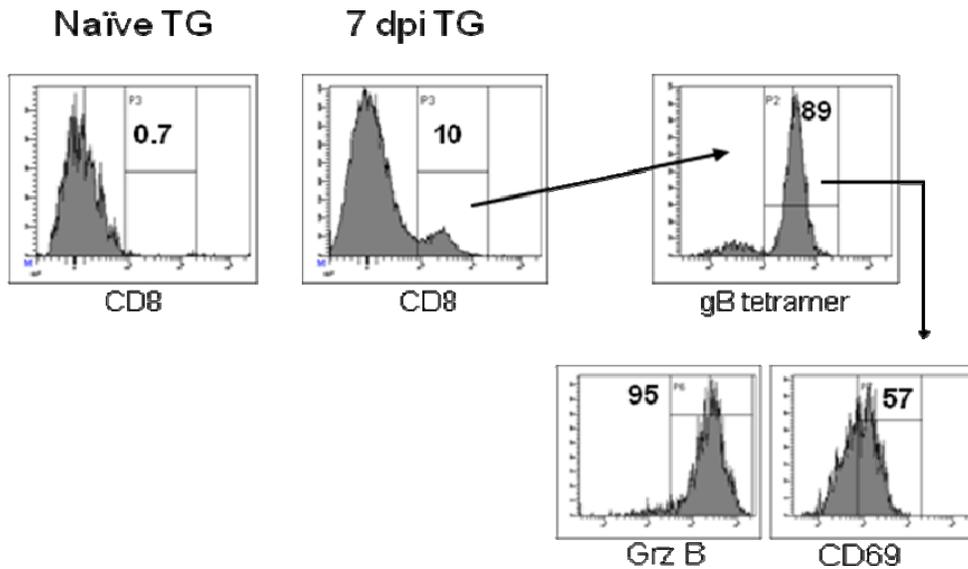


Figure 30. OT-1 CD8 T cells fail to infiltrate the ganglia of CD8 KO mice

gB-T and OT-1 CD8 T cells were isolated from naïve spleens and transferred into CD8^{-/-} mice 1 day prior to infection with HSV-1. TG of these mice was harvested 7 days later for characterization of the infiltrating populations. Mice which received cells but were not infected were used as controls (labeled naïve). Representative parent histograms are gated on CD45 cells with the gating strategy depicted. Number within histograms corresponds to percentage of cells within the positive gate.

Since bystander activation does not appear to promote migration of CD8 T cells that are not HSV-1 specific into the infected TG, we asked if any CD8 T cell that is stimulated with cognate antigen at the time of HSV-1 corneal infection can enter the infected TG. To address this point, CD45.1⁺ B6 mice received an adoptive transfer of CD45.2⁺ OVA-specific OT-1 CD8 T cells 1 day before simultaneous HSV-1 corneal infection and subcutaneous immunization with OVA peptide pulsed DCs. At 8 dpi the TG were excised, single cells suspensions were stained

for CD45.1, CD45.2, gB tetramers, and CD8 α , and analyzed by flow cytometry. The recipient CD45.1⁺ CD8 T cells in the TG showed the expected 1:1 ratio of gB-specific to gB-nonspecific cells (Figure 31A), but there was also an equivalent population of donor OVA-specific CD45.2⁺ cells. This provided a model in which to determine if CD8 T cells that specifically do not recognize HSV-1 antigens can be retained in the latently infected TG. As depicted in Figure 23, the gB-specific and gB-nonspecific CD8 T cell populations in the TG undergo contraction between 8-30 dpi and then maintain populations of constant and similar size thereafter. This pattern is maintained by recipient CD8 T cells in the current model (Figure 31B). However, the donor OVA-specific CD8 T cells that were present in equivalent numbers to their recipient gB-specific and gB-nonspecific counterparts at 8 dpi were dramatically reduced in frequency by 40 dpi. Thus, while any CD8 T cells that recently received TCR stimulation can migrate into the acutely infected TG, those known to lack HSV-1 specificity are lost from the TG during latency. The fact that gB-nonspecific CD8 T cells are retained in similar proportion to gB-specific cells lends further support to the notion that these cells are indeed HSV-specific.

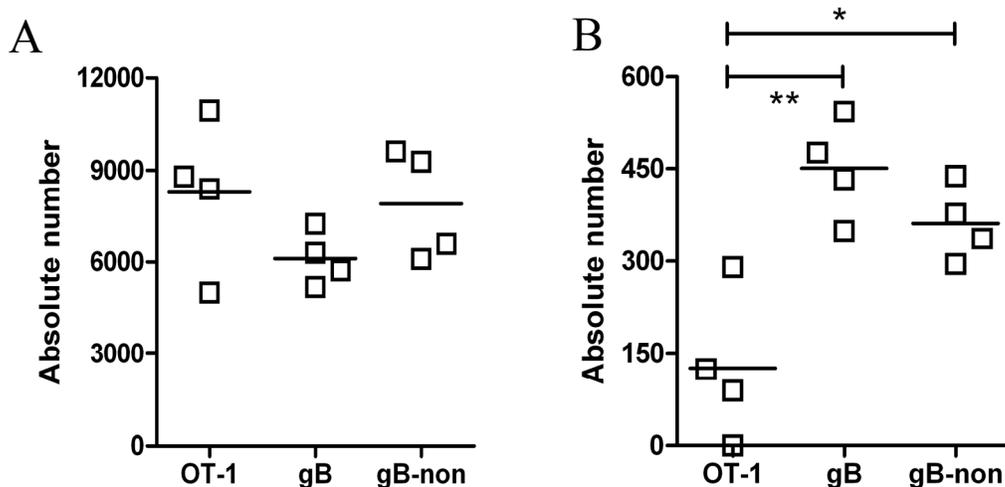


Figure 31. Known HSV-1-nonspecific CD8 T cells are not retained in the latent TG.

CD45.2⁺ OT-1 CD8 T cells were isolated from naïve spleens and 10⁵ were transferred into B6.SJL (CD45.1) mice 1 day prior to simultaneous infection with HSV-1 and DC-Ova immunization. TG of these mice was harvested 8 (A) and 40 (B) days later for characterization of the infiltrating populations. Host and donor populations were identified based on congenic markers and host populations are further delineated by recognition with gB multimers. Quantification of individual mice is represented graphically as absolute number of the indicated population of CD8 T cells with a solid line indicating the mean. ** p < 0.01, * p < 0.05.

5.2 GB-NONSPECIFIC CD8 RECOGNIZE HSV-1 ANTIGENS

5.2.1 30% of IFN γ production is accounted for by gB-nonspecific CD8 T cells

To directly test whether gB-nonspecific CD8 T cells could respond to targets presenting their cognate antigen, gB-nonspecific and gB-specific CD8 T cells were sorted from the TG of mice infected 8 days previously and given twice daily BrdU injections starting at 5 dpi. Cells were sorted on their ability to bind gB₄₉₈₋₅₀₅ multimers. Sorted cells were stimulated with HSV-1

infected targets, or gB-pulsed, RR1-pulsed, or nonpulsed targets in the presence of brefeldin A for 6h; following stimulation cells were stained for intracellular IFN γ and incorporation of BrdU. A large proportion of gB-nonspecific CD8 T cells sorted from the TG which have incorporated BrdU also produce IFN γ in response to HSV-1-infected but not gB- or RR1-pulsed targets (Figure 31). When compared directly, the gB-nonspecific CD8 T cell IFN γ response accounts for approximately 30% of the total anti-HSV-1 response. In addition, approximately 70% of the gB-nonspecific CD8 T cells have recently incorporated BrdU into dividing DNA suggesting that the vast majority have experienced TCR stimulation. This data conclusively shows that a large contribution of the overall anti-HSV-1 CD8 T cell response is provided by CD8 T cells which do not recognize the immunodominant gB₄₉₈₋₅₀₅ epitope within the acute TG.

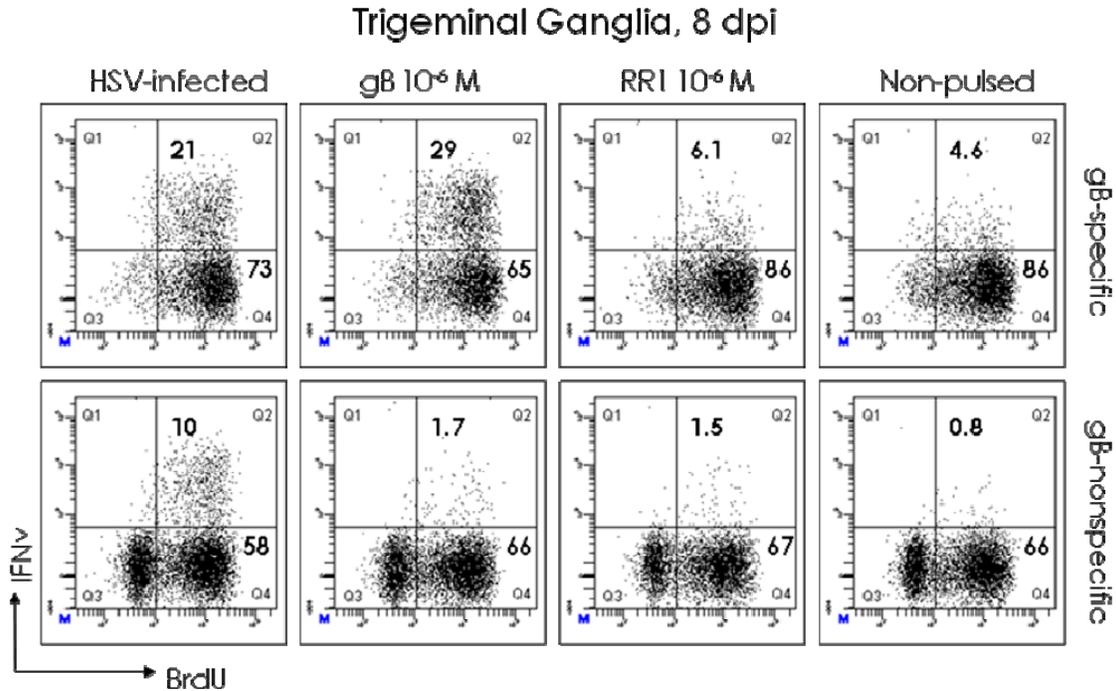


Figure 32. A proportion of gB-nonspecific CD8 T cells recognize other HSV-1 epitopes in the acute TG.

B6 mice were infected with HSV-1 and given 1 mg BrdU ip daily beginning at 5 dpi. Pooled TG were harvested 8 dpi (3 days of daily BrdU), sorted into either gB-specific or gB-nonspecific CD8 T cells (~95% purity), and then stimulated with indicated targets for 6h at 37°C/5% CO₂ in the presence of GolgPlug. Cell suspensions were stained for surface expression of CD45 and CD8, and then intracellularly for IFN γ and BrdU following stimulation. Dot plots show the percentage of cells within the respective quadrant. IFN γ staining in the gB-specific T cell population in response to RRI- or non-pulsed targets is likely due to stimulation through the gB-tetramer.

The identical study as described above was performed in the DLN and spleen of mice infected 7 days previously, but these mice received a single injection of BrdU 1 day prior to harvest. BrdU incorporation was employed to facilitate identification of a small population of HSV-1-specific CD8 T cells amongst the majority of HSV-1-nonspecific CD8 T cells which would be isolated from an acutely infected DLN given that a majority of gB-nonspecific CD8 T cells proliferate in the ganglia following a 4 h BrdU pulse (Figure 32).

Draining Lymph Node, 7 dpi

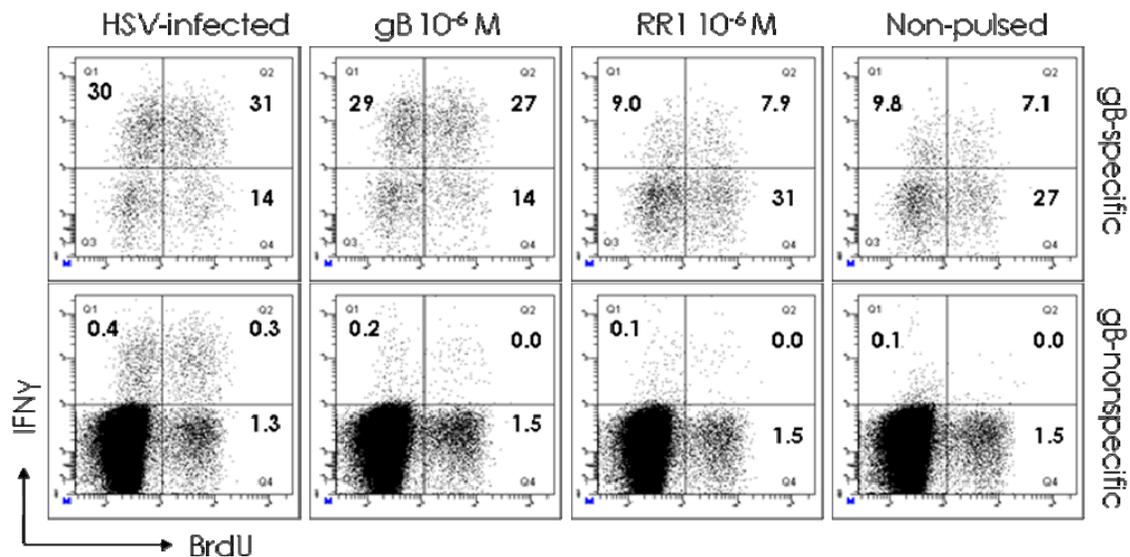


Figure 33. A proportion of gB-nonspecific CD8 T cells recognize other HSV-1 epitopes in the acute DLN.

B6 mice were infected with HSV-1 and given 1 mg BrdU ip daily beginning at 6 dpi. Pooled DLN were harvested at 7 dpi (single injection of BrdU), sorted into either gB-specific or gB-nonspecific CD8 T cells, and then stimulated with indicated targets for 6h at 37°C/5% CO₂ in the presence of GolgiPlug. Cell suspensions were stained for surface expression of CD45 and CD8, and then intracellularly for IFN γ and BrdU following the stimulation. Dot plots show the percentage of cells within the respective quadrant.

5.2.2 gB-nonspecific CD8 T cells proliferate in response to HSV-infected targets

As a further measure of HSV-1 specificity, the gB-nonspecific CD8⁺ T cells sorted from the infected TG at 8 dpi were stained with CFSE, stimulated for 72 h with HSV-infected, gB peptide pulsed, or non-pulsed targets, and analyzed by flow for proliferation as measured by CFSE dilution. As shown in Figure 34, approximately 72% of gB-nonspecific CD8⁺ T cells from infected TG underwent one or more rounds of proliferation in response to HSV-infected targets, whereas only 25% and 27% proliferated in response to gB-pulsed and non-pulsed targets,

respectively. Thus, at least 50% of gB-nonspecific CD8⁺ T cells can respond to HSV-1 antigens. Furthermore, proliferation of the gB-nonspecific CD8 T cell population in response to HSV-1-infected targets was more extensive than the gB-nonspecific proliferation in response to gB-pulsed or non-pulsed targets as determined by a 50% reduction in CFSE MFI of the gB-nonspecific CD8 T cell population.

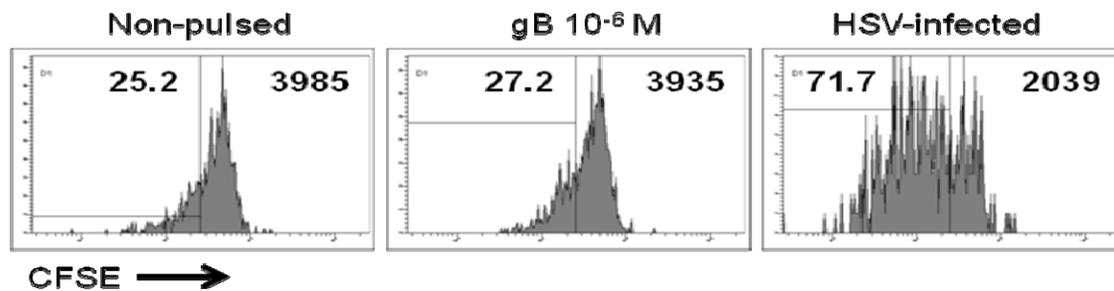


Figure 34. gB-nonspecific CD8 T cells divide in response to HSV-1-infected targets.

B6 mice were infected with HSV-1 and pooled TG was harvested 8 dpi. A pure population of gB-nonspecific CD8 T cells was obtained by sorting, labeled with 1.0 μ M CFSE, and stimulated with indicated targets for 3 d at 37°C/5% CO₂. Cell suspensions were collected and stained for surface expression of CD45 and CD8, and analyzed for CFSE dilution. Histograms show the percentage of cells within the respective quadrant on the left and the CFSE MFI of the entire CD8 population on the right.

5.2.3 gB-nonspecific CD8 T cells do not recognize other gB epitopes

Targets were transfected with a plasmid expressing full length gB to determine if the gB-nonspecific CD8 T cell population within the ganglia was responding to a different non-gB₄₉₈₋₅₀₅ epitope contained within gB. TG were harvested 8 dpi, dispersed into a single cell suspension and either used directly or incubated with gB₄₉₈₋₅₀₅ tetramer for 1h at room temperature prior to a 6h stimulation assay to measure IFN γ production. Bulk CD8 T cells from the 8 dpi ganglia produce IFN γ in response to HSV-infected and gB-pulsed targets (Figure 33). Targets transfected with plasmids expressing full length gB stimulated a pronounced yet reduced level of

IFN γ , likely a result of low transfection efficiency. However, gB-nonspecific CD8 T cells isolated from an 8 dpi TG responded to stimulation with HSV-infected targets but failed to produce IFN γ in response to gB-transfected targets (Figure 33). These results suggest that while a proportion of these cells are HSV-1 specific, they are not specific for any gB encoded epitopes.

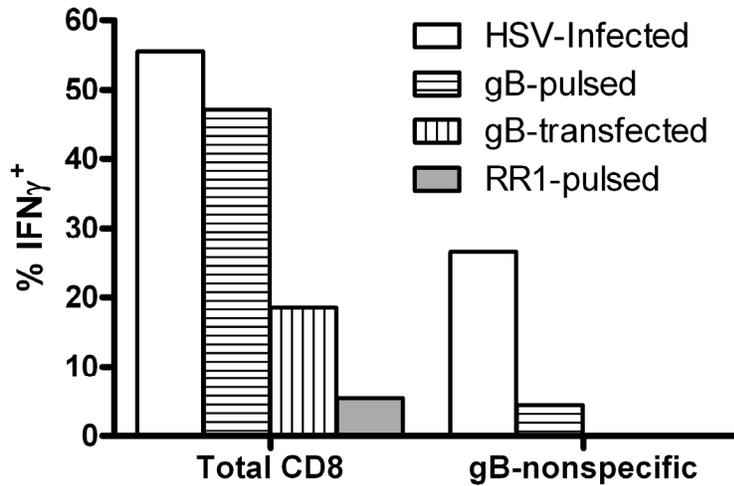


Figure 35. gB-nonspecific CD8 T cells do not recognize other gB epitopes.

Pooled 8 dpi TG were preincubated with gB₄₉₈₋₅₀₅ tetramer or left untreated for 1 h at ambient temperature prior to 6 h stimulation with indicated targets in the presence of GolgiPlug. Following stimulation, cell suspensions were stained for surface expression of CD45 and CD8, and then intracellularly for IFN γ . Preincubation with tetramer is a rapid approach to identify gB-nonspecific CD8 T cell function. The graph depicts the percentage of total CD8 or gB-nonspecific CD8 T cells which produce IFN γ following 6 h stimulation with indicated targets normalized to the non-pulsed control stimulation.

5.3 GB-NONSPECIFIC CD8 T CELLS PREVENT REACTIVATION

5.3.1 gB-nonspecific CD8 T cells prevent viral spread

To ascertain whether gB-nonspecific CD8 T cells are an important component of the HSV-1 response, we sought to determine whether they were capable of preventing reactivation following TG explant. Due to the low numbers of endogenous gB-nonspecific CD8 T cells in the HSV-1 infected TG, it is not feasible to use this tissue as the source of these cells in *ex vivo* culture models of reactivation. To navigate this issue, we used gB-nonspecific CD8 T cells isolated from the spleen of mice infected 8 days previously. gB-specific and gB-nonspecific CD8 T cells were sorted from 8 dpi spleens and added to latently explanted TG cultures depleted of CD45 expressing cells. Control wells received no CD8 T cells and served as a positive control for reactivation. gB-specific CD8 T cells are capable of preventing reactivation and impairing viral spread in wells which reactivate virus (Figure 34). In some cases, the gB-specific CD8 T cells appear to drive reactivation back into a silent state or at least below the level of sensitivity of detection. However, gB-nonspecific CD8 T cells were unable to prevent reactivation of virus in cultures depleted of endogenous CD45 expressing cells. Interestingly, they were able to prevent spread of the virus once reactivation had occurred (Figure 34). One interpretation of these findings is that the gB-nonspecific population was not initially large enough to prevent reactivation, but following exposure to antigen proliferated extensively enough to control viral spread. Another possible interpretation is that gB-nonspecific CD8 T cells recognize an antigen at a point too late in viral replication to prevent reactivation.

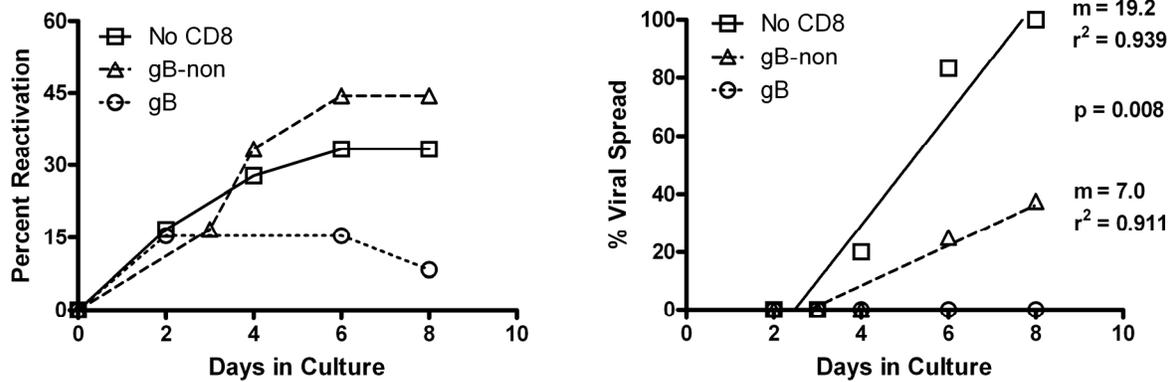


Figure 36. gB-nonspecific CD8 T cells prevent viral spread.

Pooled spleens from an 8 dpi mouse were stained with anti-CD8 and gB₄₉₈₋₅₀₅ tetramer. Cells were then sorted into 2 populations: gB-specific CD8 T cells and gB-nonspecific CD8 T cells. HSV-1 latently infected TG cultures depleted of CD45 expressing cells via complement fixation were plated in 1:5 TG equivalents in 48-well plates (n = 12-18 wells/group). Each well received approximately 2.5×10^5 sorted gB-nonspecific CD8 T cells or 5×10^3 sorted gB-specific CD8 T cells. Cultures were then monitored for reactivation by collecting supernatants at indicated time points for a standard plaque assay on a monolayer of veros (left graph). Viral spread was determined subjectively by the percent of wells in which the vero monolayer was >85% destroyed/total number of reactivating wells.

5.3.2 CD86 expression enriches for HSV-1-specific cells on the gB-nonspecific population

In an attempt to normalize the numbers of HSV-specific CD8 T cells per well, we sought a method to enrich for HSV-1 specific CD8 T cells among the gB-nonspecific CD8 population isolated from an 8 dpi spleen. In studies of HIV infected individuals CD86 expression may identify antigen-specificity in a non-TCR-specific manner (175). We sought to enrich for HSV-1 specific CD8 T cells from the gB-nonspecific splenocyte population in an acutely infected spleen by CD86 expression. To validate this approach, latently infected TGs were also examined for CD86 expression. The majority of CD8 T cells in the latently infected TG expressed high levels

of CD86 suggesting that this may be an ideal marker for identifying antigen specific CD8 T cells in the absence of known antigen specificity (Figure 35).

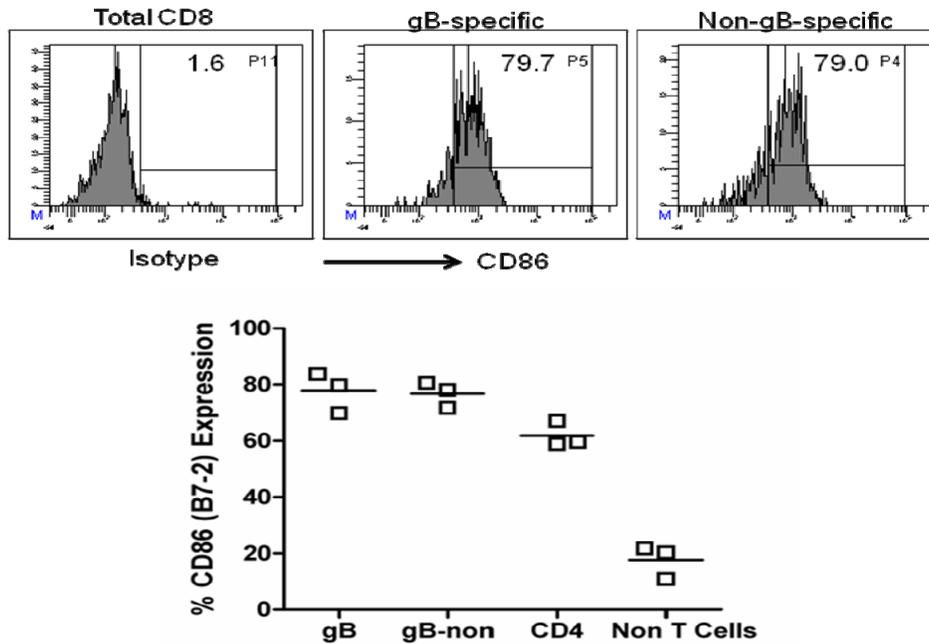


Figure 37. CD8 T cells express high levels of CD86 in the latent TG.

TGs were harvested from HSV-1 latently infected B6 mice and dispersed into single cell suspension. Cell suspensions from individual ganglia were simultaneously stained with anti-CD45, anti-CD8, anti-CD4, anti-CD86, and gB₄₉₈₋₅₀₅ H-2K^b multimers and analyzed by flow cytometry. (Upper panel) Representative histograms with the percentage of indicated populations that express CD86. (Lower panel) Data displaying individual mice and mean (solid line) percentages of indicated populations that express CD86.

To confirm that CD86 expression enriched for HSV-1 specificities among the gB-nonspecific population spleens harvested from 8 dpi mice were sorted into 3 distinct CD8 T cells populations: (1) gB₄₉₈₋₅₀₅ tetramer bound cells, (2) gB-nonspecific CD86⁺ cells, and (3) gB-nonspecific CD86⁻ cells. All 3 populations of cells were approximately 90% pure based on post sort gating (Figure 36A). The sorted populations were then incubated with either HSV-1 infected or gB-pulsed targets for 6 h in the presence of brefeldin A to measure IFN γ production. We

found that selection of CD86⁺ gB-nonspecific CD8 T cells enriched for HSV-specific CD8 T cells approximately 5-fold (Figure 36B).

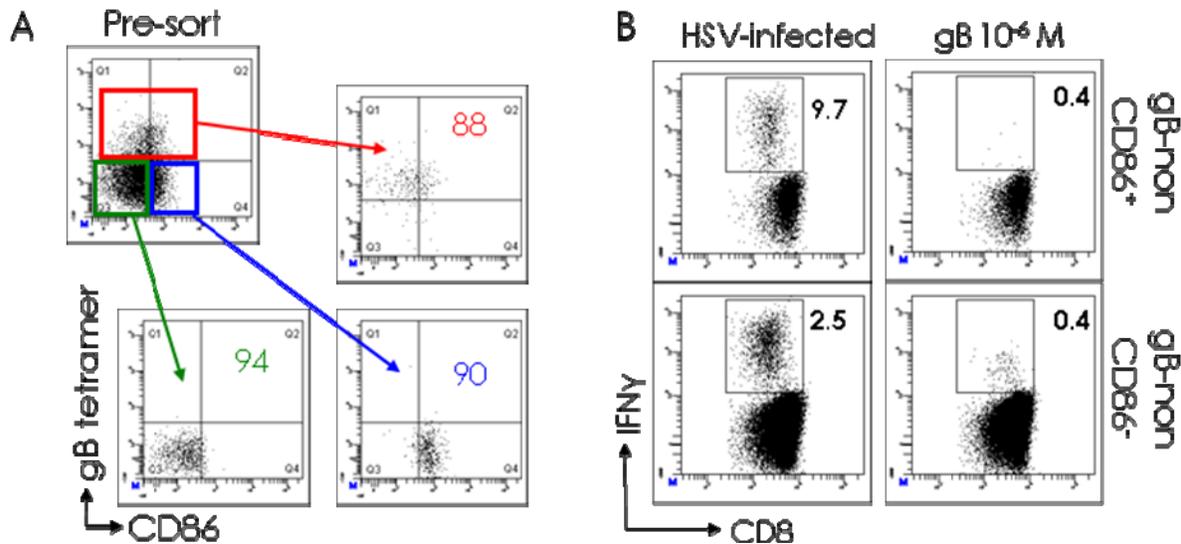


Figure 38. CD86 expression enriches for HSV-1 specific CD8 T cells amongst the gB-nonspecific CD8 population in lymphoid tissue.

Pooled spleens from an 8 dpi mouse were stained with anti-CD8, anti-CD86, and gB₄₉₈₋₅₀₅ tetramer. Cells were then sorted into 3 populations: (1) gB-specific CD8 T cells, (2) gB-nonspecific CD86⁺ CD8 T cells, and (3) gB-nonspecific CD86⁻ CD8 T cells. Pre- and post-sort dot plots are shown in (A) with respective purities shown. Sorted populations were then stimulated for 6 h in the presence of GolgiPlug with indicated targets. Following stimulation, cell suspensions were stained for surface expression of CD45 and CD8, and then intracellularly for IFN γ . Dot plots are shown from a representative assay with the percentage of IFN γ producing cells presented.

5.3.3 gB-nonspecific CD86⁺ prevent reactivation and limit viral spread

The remainder of the cells were cultured with pICP0-EGFP HSV-1 latently-infected TG explants depleted of CD45-expressing cells. Explants were monitored for reactivation for 11 days to evaluate their capacity to prevent HSV-1 reactivation from latency. The addition of either gB-specific or gB-nonspecific CD86⁺ T cells prevented reactivation in approximately 50% of cultures examined (Figure 37). Moreover, quantification of replicating virus by standard plaque

assay revealed that wells in which gB-specific or gB-nonspecific CD8 T cells were reconstituted and had undergone reactivation had approximately 5-fold less virus per well (Figure 37). These data together suggest that gB-nonspecific CD8⁺ T cells isolated from an acute spleen prevent reactivation comparably to gB-specific CD8 T cells isolated in the same manner.

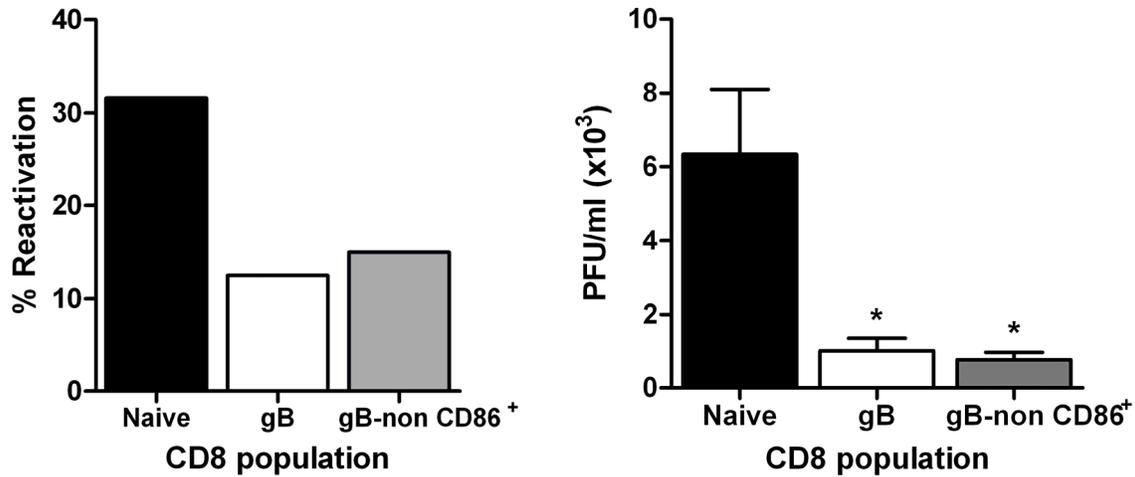


Figure 39. gB-nonspecific CD8⁺ CD8 T cells prevent reactivation and limit viral spread.

pICP0-EGFP HSV-1 latently infected TG cultures depleted of CD45 expressing cells via complement fixation were plated in 1:5 TG equivalents in 48-well plates. Cells sorted as described above were added to the cultures and the cultures were monitored for reactivation by collecting supernatants at 8 dpe (left panel) and 11 dpe (right panel) for a standard plaque assay on a monolayer of veros (n = 20-38 wells/group). Naïve CD8 T cells were obtained by MACS purification of naïve B6 splenocytes. (Left panel) Percent reactivation is determined by the percentage of wells which contain viral plaques. (Right panel) PFU/ml is based on quantification of the wells which reactivated and displayed as the mean (\pm SEM) PFU/ml. * p < 0.05 comparing either population to the control wells which received naïve CD8 T cells.

5.3.4 T cell outgrowth of select populations during culture

Use of the pICP0-EGFP HSV-1 virus allows us to harvest the remaining T cells at the end of the assay from wells which demonstrated enhanced green fluorescence protein (EGFP) spreading (reactivation) and wells which did not demonstrate EGFP expression (non-reactivated wells)

(36). Interestingly, all wells which received gB-specific CD8 T cells rapidly expanded a population of cells which were not specific for gB₄₉₈₋₅₀₅. On the contrary, wells which received gB-nonspecific CD8⁺ T cells only had gB-specific outgrowth from wells which had reactivated denoted by spread of EGFP in the cultures (Figure 38). Outgrowth of the gB-nonspecific population in wells which did not reactivate in combination with gB-specific outgrowth only in wells which did reactivate strongly suggests that gB-nonspecific CD8 T cells may be encountering antigen prior to and more often than gB₄₉₈₋₅₀₅-specific CD8 T cells during latency.

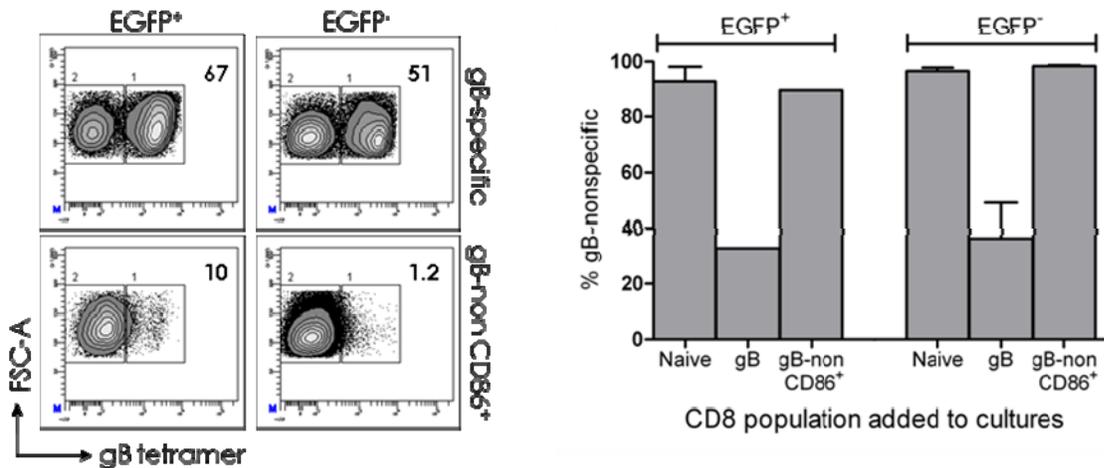


Figure 40. gB-nonspecific CD8 T cells expand in TG explant cultures.

At 11 dpe CD8 T cells were collected from pooled wells which expressed EGFP spread or contained no discernible EGFP expression for flow analysis. Pooled wells were stained with anti-CD8, anti-CD45, anti-CD3, and gB₄₉₈₋₅₀₅ tetramers. Representative contour plots show the CD3⁺ CD8 T cells isolated from pooled wells which received either gB-specific or gB-nonspecific CD8⁶⁺ CD8 T cells from wells which demonstrated EGFP spread or did not. Number within contour plot shows the percentage of CD8 T cells which bound gB₄₉₈₋₅₀₅ tetramer. Quantification of pooled wells is represented graphically as the mean percentage of CD8 T cells which did not bind gB₄₉₈₋₅₀₅ tetramers (\pm SEM).

CD3 staining was performed on the retrieved T cell populations to ensure that tetramer negative cells had not simply downregulated their TCR in response to activation. Wells which reactivated, denoted by EGFP spread, had considerably decreased CD3 mean fluorescence

intensity (MFI) compared to EGFP negative wells. However, gB-nonspecific CD8 T cells which were isolated from wells which received gB-specific CD8 T cells had comparable CD3 expression per cell indicating that the appearance of a tetramer negative population is not due to TCR downregulation (Figure 39).

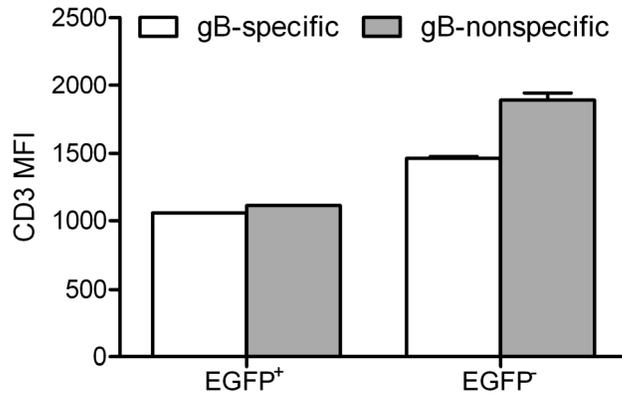


Figure 41. TCR downregulation does not account for gB-nonspecific population in TG explants cultures.

At 11 dpe CD8 T cells were collected from pooled wells which expressed EGFP spread or contained no discernible EGFP expression for flow analysis as described above. Pooled wells were stained with anti-CD8, anti-CD45, anti-CD3, and gB₄₉₈₋₅₀₅ multimers. Quantification of pooled wells is represented graphically as the mean (\pm SEM) MFI of indicated specificity CD8 T cells.

5.4 DISCUSSION

A strong and efficacious CD8 T cell response allows the host to combat many viral pathogens without the need for therapeutic interventions. Following exposure to an infectious agent, host APCs at the site of infection capture and process foreign proteins. These APCs then traffic to lymph nodes and either present antigen directly to T cells or pass antigen to a distinct lymph node resident DC subset for antigen presentation in the context of MHC class I (176). These

antigen-specific CD8 T cells then undergo a period of robust division and differentiation into an effector population armed to eliminate the invading pathogen.

Typically, CD8 T cell responses against particular pathogens are generated within a defined hierarchy of magnitude. The largest CD8 T cell response directed at a single epitope is referred to as the immunodominant epitope or response while lesser responses are referred to as subdominant. A third category of dominance occurs following a manipulation of the system in which an immunodominant epitope is eliminated and a normally silent or 'cryptic' determinant is expanded (177). Cryptic epitopes are particularly important following expansion of deleterious T cell populations in the context of heterologous immunity (178). The many factors which govern robust expansion of a particular subset of T cells while establishing limited or undetectable accumulation of others is well known. However, how these diverse factors interact and influence one another for the establishment of a public hierarchy is less clear.

These studies have taken advantage of new technologies in an attempt to clarify the HSV-1-specific CD8 T cell response following corneal infection in mice. Previous work has suggested that the majority of the CD8 T cell response against HSV-1 is directed against a single immunodominant epitope within gB (73, 154). Use of MHC I multimers complexed with this epitope in conjunction with efficient sorting of distinct T cell populations has allowed a closer look in tissues which have previously been difficult to examine by flow cytometric analysis. Early limiting dilution assays (LDAs) showed that up to 25% of HSV-1 specific CD8 T cells generated in a B6 mouse were specific for gB₄₉₈₋₅₀₅ (20, 179). More sensitive approaches later suggested that those studies greatly underestimated the contribution of gB-specific CTL to the overall HSV-1-specific pool (154). Our current study challenges the findings which argued that CD8 T cells specific for the gB₄₉₈₋₅₀₅ epitope represent almost 90% of the HSV-1 specific CD8 T

cells (154). This study had the added benefit of examining the CD8 T cell response at the site of infection, the sensory ganglia which innervates the eye, as opposed to lymphoid tissues. In a naïve mouse, few if any CD8 T cells reside in the latently infected ganglia. Thus, allowing an examination of CD8 T cell infiltration following infection uncomplicated by the presence of a large pool of contaminating naïve CD8 T cells. Our data suggest that as much as 40% of the CD8 T cell population resident in the infected ganglia is specific for other non-gB or non-RR₈₂₂₋₈₂₉ epitopes. Moreover, approximately 30% of the IFN γ produced by CD8 T cells in the ganglia early following infection is produced by CD8 T cells which do not recognize the immunodominant gB epitope (Figure 31). We believe that this is not a surprising result given the size and complexity of HSV-1 and its lifecycle.

Measuring IFN γ production following stimulation with infected targets is likely not the most accurate way to determine antigen specificity as HSV-1 encodes multiple immune evasion genes which may limit T cell recognition (180, 181). Peptide stimulation with an optimal dose produces a more robust response than stimulating gB-specific CD8 T cells with HSV-1 infected targets (data not shown). The functional capacity of these cells, regardless of stimulation, also fails to achieve 100% IFN γ production despite the certainty that cells sorted based on gB tetramer specificity contain TCR specific for that epitope. Coupled with the evidence that 75% of gB-nonspecific CD8 T cells upregulate granzyme B in the acute TG, while naïve OT-1 CD8 T cells fail to infiltrate the TG or upregulate granzyme B in the DLN, it is unlikely that bystander activation contributes to the HSV-1 response in the TG (Figure 24, 29, and 30). Moreover, the CD8 T cell granzyme B expression is in exact concordance with BrdU incorporation regardless of gB specificity within the acutely infected TG (Figure 24 and 31).

Of particular interest is our observation that the previously unappreciated gB-nonspecific CD8 T cell population can prevent reactivation and limit viral spread in TG explant models of reactivation in the absence of their gB-specific counterparts (Figure 34 and 37). Using CD86 expression, we were able to enrich the percent of HSV-1 specific CD8 T cells among the gB-nonspecific CD8 compartment in the spleen 4-fold. This enrichment allowed us to normalize the numbers of gB-specific CD8 T cell with those of gB-nonspecific CD8 T cells specific for other HSV-1 epitopes in an *ex vivo* reactivation assay to accurately determine that this population of cells were also capable of preventing reactivation.

While these cells are clearly not specific for RR1₈₂₂₋₈₂₉ or epitopes contained within gB, it is difficult to ascertain their specificity. Data demonstrating that this population of cells within the latent ganglia is the only T cell population to express the inhibitory receptor PD-1 (discussed in chapter 6) suggests these cells may be recognizing an antigen expressed during latency more abundantly than gB or prior to gB expression during reactivation. This would suggest the gB-nonspecific CD8 T cell population may be a “first responder” to reactivating neurons. Evidence for this hypothesis is demonstrated with gB-nonspecific outgrowth from explanted TG cultures depleted of CD45 expressing cells and reconstituted with pure (~90%) populations of gB-specific CD8 T cells. After 11 days in culture 40% of the recovered CD8 T cells no longer recognized gB-bound tetramers. Lack of tetramer binding was not associated with downregulation of TCR as CD3 expression levels were comparable between T cell populations. Astonishingly, these gB-nonspecific CD8 T cells expanded regardless of reactivation state; expansion occurred comparably in the presence or absence of reactivation. However, gB-specific CD8 T cells only expanded from wells which reactivated when pure (~90%) populations of gB-nonspecific CD8 T cells were added. Wells which did not reactivate latent virus did not expand

gB-specific CD8 T cells. This data taken together would suggest that gB-nonspecific CD8 T cells recognize an HSV-1 determinant that is expressed earlier than gB in the course of reactivation and possibly at some level during latency.

6.0 PD-1 AND PD-L1 EXPRESSION DURING LATENCY

Somewhere between the dichotomy of viral clearance and high level persistence associated with LCMV clone 13 infections an intermittent level of TCR stimulation occurs during herpes viral latency. PD-1 expression on CD8 T cells following LCMV clone 13 infection mediates T cell exhaustion in this setting (133). This chapter will examine the consequence of PD-1 expression on a narrow subset of CD8 T cells and expression of its ligand on the neuron population in the ganglia of HSV-1 latently infected mice.

6.1 PD-1 EXPRESSION DURING LATENCY

6.1.1 gB-nonspecific CD8 T cells express reduced CD127 during latency

The population of CD8 T cells found within a latently infected mouse ganglia can be roughly divided into two distinct subpopulations based on their ability to either bind multimers loaded with the immunodominant gB₄₉₈₋₅₀₅ epitope (gB-specific) or not bind the same tetramer (gB-nonspecific) (demonstrated gating strategy in Figure 42A). The population of gB-nonspecific CD8 T cells was recently identified to contain a proportion of HSV-1-specific CD8 T cells. We examined CD127 expression on the gB-nonspecific CD8 T cell population during latency to see if they resembled a mature memory pool. This population of CD8 T cells fails to fully

upregulate CD127 during latency. On average 70% of the gB-specific CD8 T cell population express CD127, whereas approximately 50% of their counterparts do. This data suggests that a proportion of them may be continually exposed to antigenic stimulus (Figure 40).

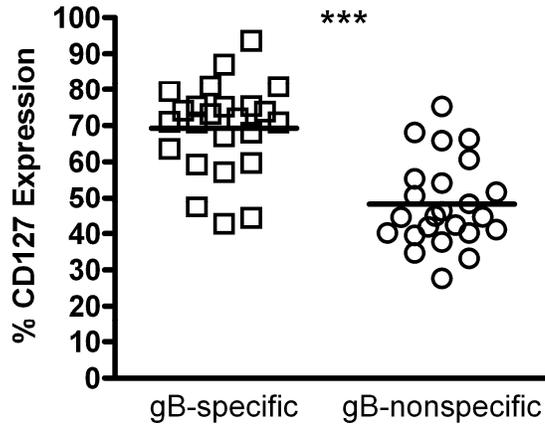


Figure 42. CD127 expression is reduced on gB-nonspecific CD8 T cells.

TGs were harvested from HSV-1 latently infected B6 mice (47-88 dpi) and dispersed into single cell suspension. Cell suspensions from individual ganglia were simultaneously stained with anti-CD45, anti-CD8, anti-CD127, and gB₄₉₈₋₅₀₅ H-2K^b multimers and analyzed by flow cytometry. Data displaying individual mice and mean (solid line) percentages of indicated populations that express CD127 is shown. Representative dot plots displaying the staining pattern are shown below. *** $p < 0.001$.

6.1.2 PD-1 expression is confined to gB-nonspecific CD8 T cells

We sought to determine whether either population of CD8 T cells or CD4 T cells express PD-1. Surprisingly, PD-1 expression was confined to the gB-nonspecific population of CD8 T cells (Figure 41). This observation is consistent with the recent report that this population of cells contains an HSV-1-specific pool of CD8 T cells which appear to encounter antigen prior to their gB-specific counterparts.

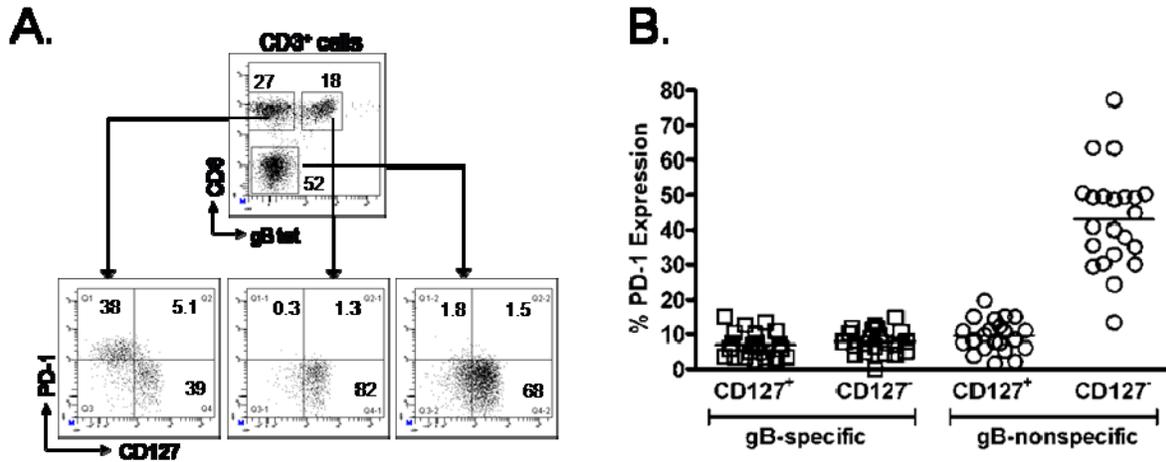


Figure 44. PD-1 expression is confined to CD127⁻ gB-nonspecific.

TGs were harvested from HSV-1 latently infected B6 mice (47-88 dpi) and dispersed into single cell suspension. Cell suspensions from individual ganglia were simultaneously stained with anti-CD45, anti-CD8, anti-CD3, anti-PD-1, anti-CD127, and gB₄₉₈₋₅₀₅ H-2K^b multimers and analyzed by flow cytometry. (A) Representative dot plots displaying the gating strategy and PD-1 and CD127 staining in indicated populations are shown. Percent of T cells contained with gates or quadrants are indicated. (B) Data displaying individual mice and mean (solid line) percentages of indicated populations that express PD-1 is shown. $p < 0.001$ comparing the CD127⁻ gB-nonspecific population to all other groups.

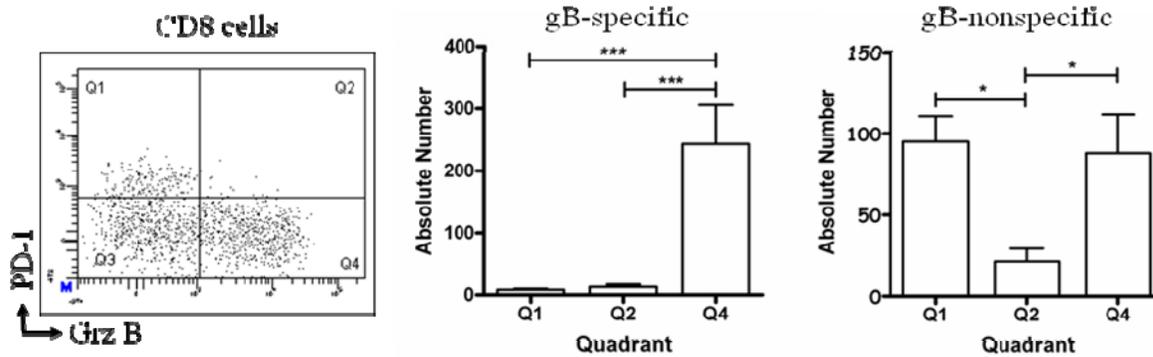


Figure 45. PD-1 expression excludes granzyme B containing cells.

TGs were harvested from HSV-1 latently infected B6 mice and dispersed into single cell suspensions. Cell suspensions from individual ganglia were simultaneously stained with anti-CD45, anti-CD8, anti-PD-1, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly for granzyme B and analyzed by flow cytometry. Representative dot plot is shown gated on total CD8 cells. Data displaying mean absolute number (\pm SEM) of indicated populations from corresponding gating strategy depicted in the representative dot plot. * $p < 0.05$, *** $p < 0.001$ comparing indicated groups.

6.1.4 PD-1 expressing gB-nonspecific CD8 T cells undergo elevated proliferation *in situ*.

PD-1 expression on CD8 T cells following persistent infection such as LCMV clone 13 is thought to identify CD8 T cells which are functionally exhausted and no longer capable of proliferation (133, 182). We sought to determine if the population of PD-1 expressing gB-nonspecific CD8 T cells in the latent ganglia proliferated *in situ*, and how their proliferation compared to the gB-nonspecific CD8 T cells which did not express PD-1. B6 mice were treated with 1 mg BrdU ip daily for 1 week prior to TG harvest during latency and examined by flow cytometry for incorporated BrdU. No differences were observed comparing gB-specific CD8 T cells with the total gB-nonspecific CD8 T cell population in the latent TG. However, we observed a 2-fold increase in proliferation of PD-1⁺ gB-nonspecific CD8 T cells compared to gB-nonspecific CD8 T cells which did not express PD-1 (Figure 44). PD-1 expression is thought

to impair proliferation of exhausted CD8 T cells but the increased proliferation observed may reflect increased exposure to antigen in this system. For example, PD-1 negative gB-nonspecific CD8 T cells may not be exposed to antigen during latency (or similar levels of antigen exposure as gB-specific CD8 T cells).

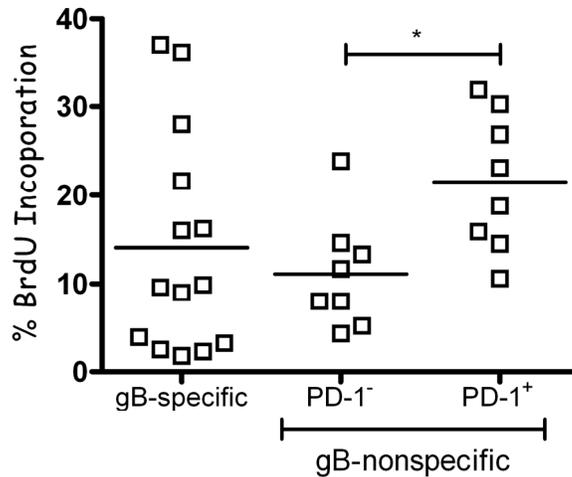


Figure 46. PD-1 expressing gB-nonspecific CD8 undergo more extensive proliferation *in vivo*. HSV-1 latently infected B6 mice (40-61 dpi) received injections of 1 mg BrdU daily for one week prior to TG excision. TG cells were simultaneously stained with anti-CD45, anti-CD8, anti-PD-1, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly with anti-BrdU and analyzed by flow cytometry. Data displaying individual mice and mean (solid line) percentages of gB-specific and gB-nonspecific CD8 T cells that incorporated BrdU into cellular DNA is shown. * p < 0.05 comparing PD-1 subsets of the gB-nonspecific CD8 T cell population.

6.2 IMPAIRED GB-NONSPECIFIC FUNCTION DURING LATENCY

6.2.1 gB-nonspecific CD8 T cells demonstrated reduced function during latency.

The best method to identify HSV-1-specific CD8 T cells which do not recognize the gB₄₉₈₋₅₀₅ epitope is to sort the TG T cell population into those which are gB₄₉₈₋₅₀₅-specific and those that

are not, and stimulate them with HSV-1 infected or gB-pulsed targets in the presence of brefeldin A to measure IFN γ production. With this approach, we can identify the contribution of both populations of T cells either during the acute infection or following the establishment of latency. We observed that the gB-specific IFN γ response is stable over time yet the gB-nonspecific HSV-1 response wanes with the establishment of latency (Figure 45). This loss of function over time is consistent with the upregulation of PD-1 on a proportion of this population of cells (Figure 41) and with the published observation of CD8 T cell impairment during chronic infections (109, 132).

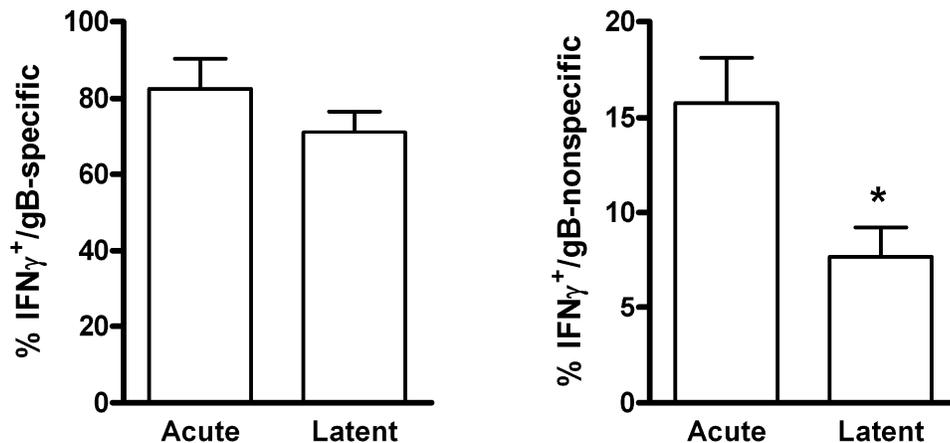


Figure 47. gB-nonspecific CD8 T cell function is reduced in the latent ganglia.

Pooled TG cell suspensions from HSV-1 acutely (8 dpi) or latently infected B6 mice were simultaneously stained with anti-CD8 and gB₄₉₈₋₅₀₅ H-2K^b multimers prior to cell sorting. Suspensions were sorted into two distinct (> 95% purity) populations based on gB-specificity as indicated. Sorted populations were optimally stimulated with B6/T-350 fibroblasts pulsed with 10⁻⁶ M gB₄₉₈₋₅₀₅ peptide in the presence of Golgi-plug for 6 h at 37°C. Following stimulation the cells were stained for intracellular expression of IFN γ . * p < 0.05 (n = 2-6 assays/group)

However, in vivo blockade of either PD-1 or PD-L1 for 2 weeks prior to harvest fails to show consistent rescue of the IFN γ response in the gB-nonspecific population of CD8 T cells despite the vast majority being HSV-1 specific during the effector phase (Figure 46). In fact, our

data demonstrate a reproducible impairment of both populations of CD8 T cells following anti-PD-1 treatment *in vivo* despite the observation that gB-specific CD8 T cells express insubstantial levels of PD-1 (Figure 46).

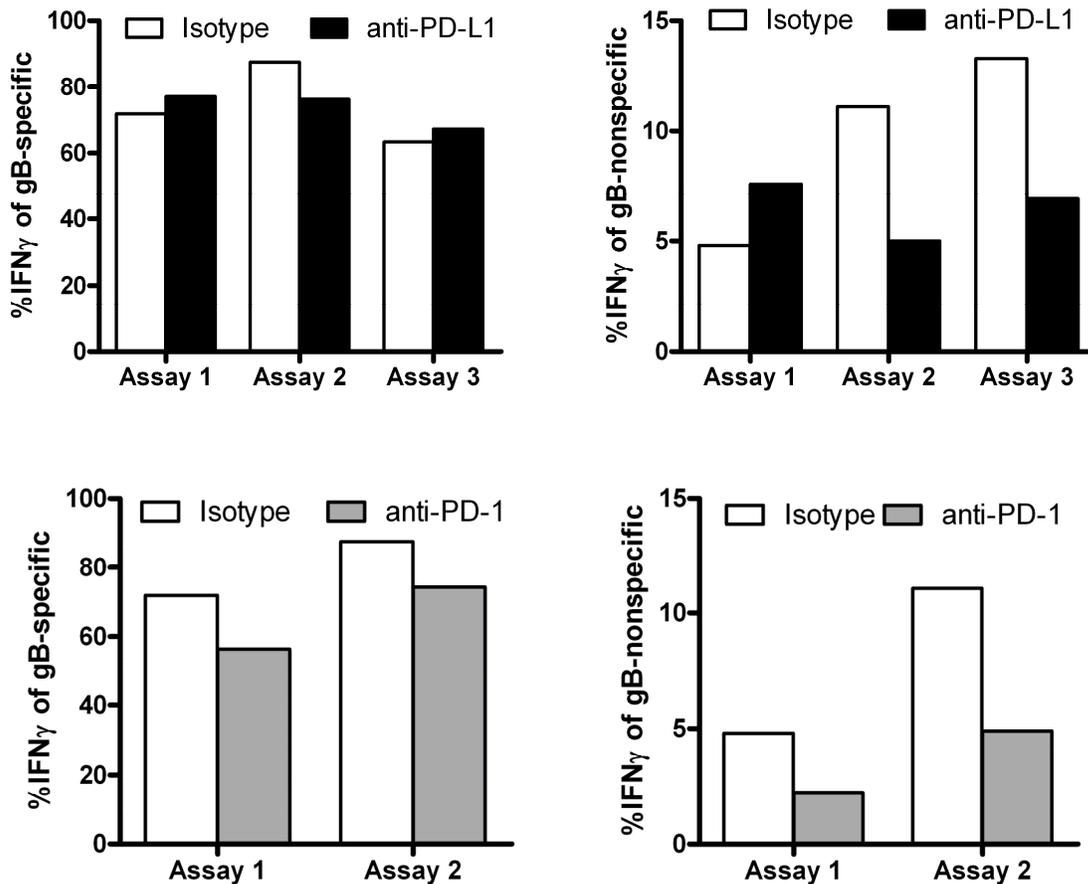


Figure 48. Blockade of PD-1 signaling.

Pooled TG cell suspensions from HSV-1 latently infected B6 mice treated with an isotype antibody, anti-PD-L1 (upper panel), or anti-PD-1 (lower panel) for every 3 days for 2 weeks prior to harvest were simultaneously stained with anti-CD8 and gB₄₉₈₋₅₀₅ H-2K^b multimers prior to cell sorting. Suspensions were sorted into two distinct (> 95% purity) populations based on gB-specificity as indicated. Sorted populations were optimally stimulated with B6/T-350 fibroblasts pulsed with 10⁻⁶ M gB₄₉₈₋₅₀₅ peptide in the presence of Golgi-plug for 6 h at 37°C. Following stimulation the cells were stained for intracellular expression of IFN γ . Individual assays show the mean (\pm SEM) percentage of the indicated CD8 T cell population that expresses intracellular IFN γ .

The failure to rescue gB-nonspecific function can also be observed after *ex vivo* granzyme B staining suggesting that these cells may be exhausted past the point of rescue (Figure 47A). An alternative hypothesis would be that these cells are not HSV-1 specific yet their maintained expression of PD-1 throughout latency would suggest otherwise.

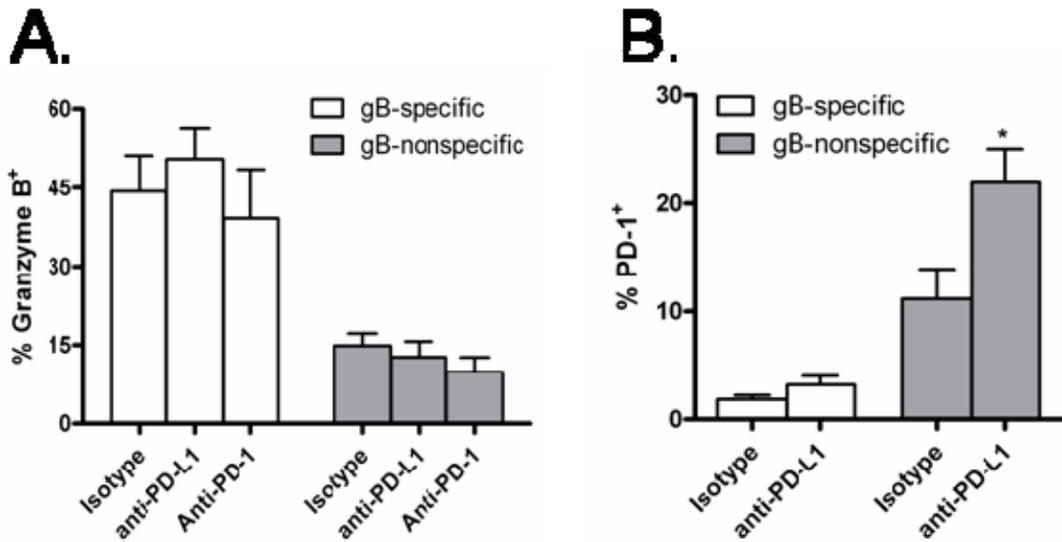


Figure 49. Granzyme B and PD-1 expression is consistent with lack of functional rescue.

TG cell suspensions from individual HSV-1 latently infected B6 mice (>35 dpi) treated with an isotype antibody, anti-PD-L1, or anti-PD-1 for every 3 days for 2 weeks prior to harvest were simultaneously stained with anti-CD45, anti-CD8, anti-PD-1, gB₄₉₈₋₅₀₅ H-2K^b multimers, and intracellularly with anti-granzyme B and analyzed by flow cytometry. Pooled data showing the mean (\pm SEM) percentage of indicated groups which express granzyme B (A) or PD-1 (B) is shown. * $p < 0.05$ comparing the gB-nonspecific population of anti-PD-L1 treated to isotype treated mice (n = 8-10 mice/group).

6.2.2 CD8 T cell number following *in vivo* treatment

However, we do observe a trend of increasing cell numbers following 2 wk administration of anti-PD-L1 or anti-PD-1 suggesting that PD-1 and PD-L1 signaling may preferentially regulate proliferation over functional rescue (Figure 48). These subtle changes in cell number and the

inability to rescue functional capacity of this population are not inconsistent with the wide range of PD-1 expression observed in the gB-nonspecific population of CD8 T cells (Figure 41) and a marked increase in PD-1 expression following PD-L1 blockade (Figure 47B).

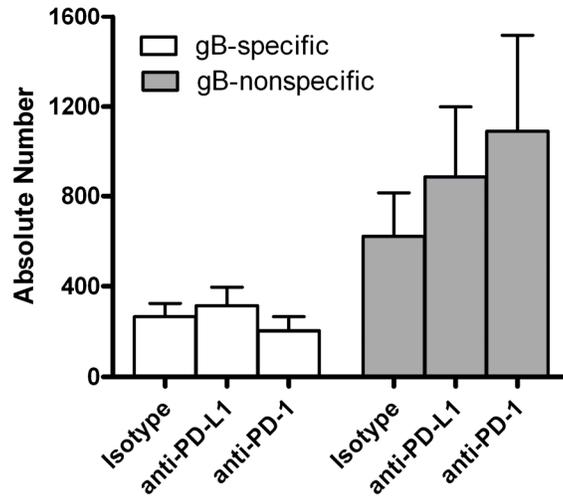


Figure 50. PD-1 signaling blockade indicates a trend of increasing gB-nonspecific cell numbers in the latent TG.

TG cell suspensions from individual HSV-1 latently infected B6 mice (>35 dpi) treated with an isotype antibody, anti-PD-L1, or anti-PD-1 for every 3 days for 2 weeks prior to harvest were simultaneously stained with anti-CD45, anti-CD8, and gB₄₉₈₋₅₀₅ H-2K^b multimers. The entire TG sample was analyzed by flow cytometry for quantification. Pooled data showing the mean (\pm SEM) absolute number of indicated groups is shown (n = 8-10 mice/group).

6.3 EFFECT OF IN VIVO PD-1 OR PD-L1 BLOCKADE ON REACTIVATION

6.3.1 Increased levels of viral gH gene copies following treatment

The increased PD-1 expression observed following PD-L1 blockade *in vivo* and the decreased effector function observed in the HSV-1-specific CD8 T cell population led us to ask whether the

latent burden as denoted by levels of viral gH gene copies increased following antibody treatment. Increased gH copies could explain the increased levels of PD-1 expression on the gB-nonspecific population following antibody blockade as this would be suggestive of reactivation occurring *in vivo* resulting in increased antigenic stimulation. Levels of the true late gH gene copies in nonmanipulated C57BL/6 mice is extremely stable overtime (data not shown). Therefore, increases in gH gene copies in manipulated mice is suggestive of reactivation *in vivo*. However, whether or not observed increases in gH gene copies results in production of infective virions is unclear. Latently infected mice were treated with isotype, anti-PD-L1 or anti-PD-1 antibodies for 2 weeks prior to harvest. Individual ganglia were isolated from these mice and DNA was isolated for real-time PCR analysis of gH copies. There is a substantial increase in gH copies in mice which received either anti-PD-L1 or anti-PD-1 suggesting that these mice have reactivated latent virus (Figure 49). Increases in viral gene copies following PD-1 blockade is consistent with the increase observed when CD8 T cell function is impaired as demonstrated in a number of other recently published methods (55, 158).

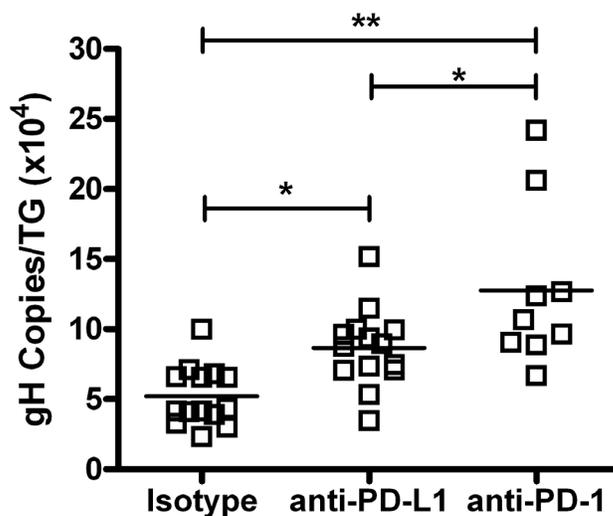


Figure 51. Increased gH copies is evidence of reactivation following PD-1 signaling blockade. TG cell suspensions were obtained from individual HSV-1 latently infected B6 mice (>35 dpi) treated with an isotype antibody, anti-PD-L1, or anti-PD-1 every 3 days for 2 weeks prior to harvest. DNA obtained from individual ganglia of treated mice was subjected to quantitative real-time PCR to determine viral genome copies present. Data displaying individual mice and mean (solid line) gH copies/TG of indicated treatment groups. * $p < 0.05$ and ** $p < 0.01$ for indicated comparisons.

6.3.2 CD80 does not likely play a role following PD-L1 blockade

PD-L1 has two known receptors to which it may bind and transduce a signal. The anti-PD-L1 antibody used in this study blocks both the PD-L1:PD-1 and the recently identified inhibitory PD-L1:B7-1(CD80) interaction (153). We examined CD80 expression levels in the latently infected TG to determine if B7-1 may contribute to the observed phenotypes following anti-PD-L1 blockade. CD80 is not expressed in the latently infected ganglia to any appreciable levels on any cell population examined (Figure 50). While this does not rule out a role for CD80 expression following PD-L1 blockade *in vivo*, it suggests that the observed T cell phenotypes following treatment are a result of blocking the PD-L1:PD-1 pathway.

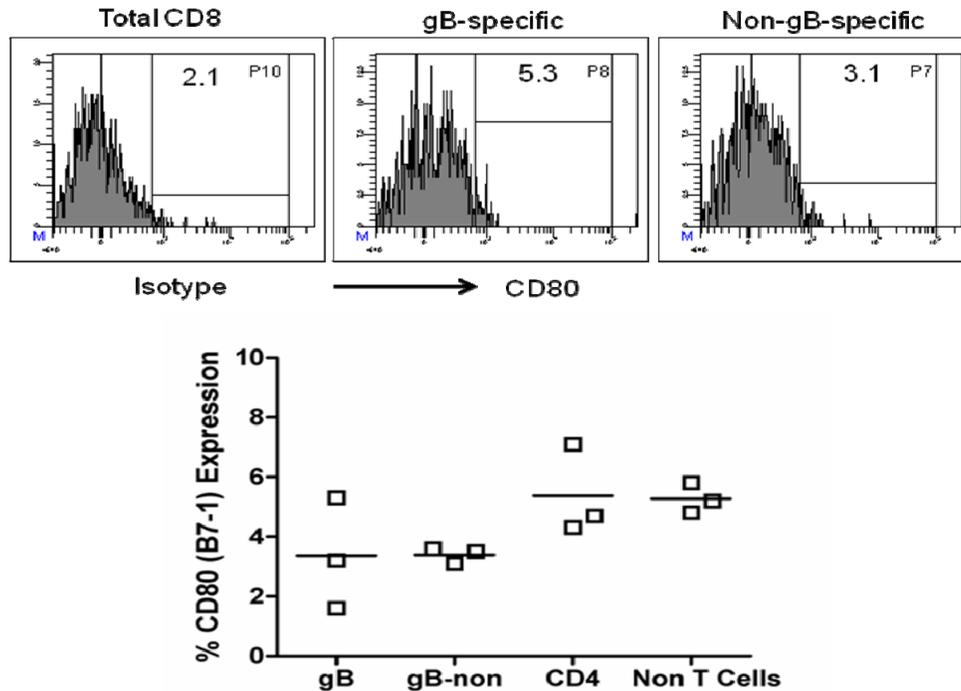


Figure 52. CD80 expression does not appear to play a role in the PD-L1 pathway in the latent ganglia.

TGs were harvested from HSV-1 latently infected B6 mice and dispersed into single cell suspension. Cell suspensions from individual ganglia were simultaneously stained with anti-CD45, anti-CD8, anti-CD4, anti-CD80, and gB₄₉₈₋₅₀₅ H-2K^b multimers and analyzed by flow cytometry. (Upper panel) Representative histograms with the percentage of indicated populations that express CD80. (Lower panel) Pooled data displaying individual mice and mean (solid line) percentages of indicated populations that express CD80.

6.3.3 Neuronal PD-L1 expression is rapidly upregulated following HSV-1 infection and maintained on a subpopulation during latency

Previous data has shown that CD8 T cells necessary to monitor latently infected neurons and prevent reactivation can interact with neurons following HSV-1 infection (73). Here, we sought to determine whether neurons could regulate CD8 T cell responses following HSV-1 corneal

infection by expression of costimulatory receptors. Surprisingly, neurons rapidly upregulate PD-L1 following infection (Figure 51A). The kinetics of upregulation is consistent with the level of inflammation in the ganglia (Figure 51B). Early PD-L1 expression is not likely due to HSV-1 infection of those neurons as upregulation of PD-L1 is not consistent with the level of neuronal infection. We obtained thick sections of naïve, acute, and latent HSV-1 infected mice ganglia to confirm the findings of our flow analysis of neuronal PD-L1 expression. PD-L1 expression was found on neurons of all sections of murine ganglia consistent with the levels determined from flow analysis (data not shown).

PD-L1 expression was also noted on CD8 and CD4 T cells within the latent ganglia (data not shown). However, T cells are rarely seen in close opposition to each other within the latently infected ganglia while interactions with neurons occur more frequently (73). This hypothesis will be further tested in experiments using bone marrow chimeras generated in WT or PD-L1^{-/-} mice with WT or PD-L1^{-/-} bone marrow to determine whether neuronal PD-L1 expression or T cell PD-L1 expression regulates gH copy load in the latent ganglia. PD-L2 expression was not detected on any cell subset examined in the latently infected TG including APCs, T cells, and neurons (data not shown).

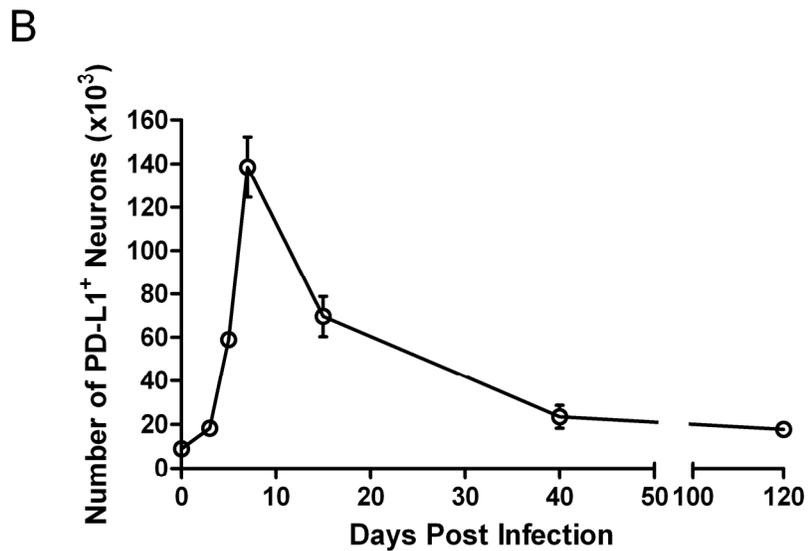
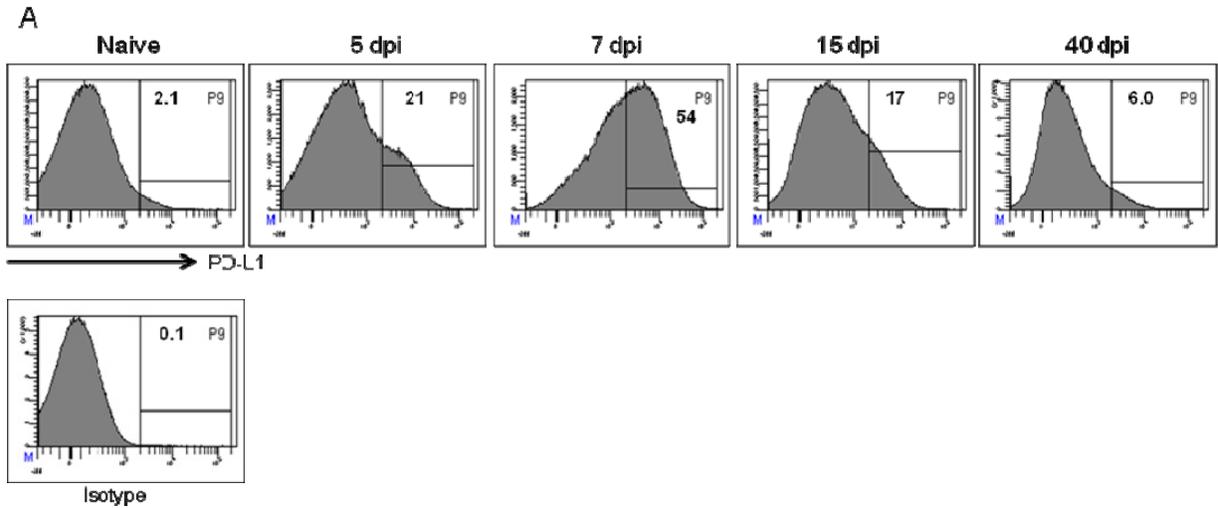


Figure 53. PD-L1 is expressed on neurons following HSV-1 infection

TGs were harvested from HSV-1 infected B6 mice at the indicated time-points and dispersed into single cell suspension. Cell suspensions from individual ganglia were simultaneously stained with anti-NeuN, anti-CD45, and anti-PD-L1. The entire TG sample was analyzed by flow cytometry for quantification. (Upper panel) Representative histograms are gated on the NeuN⁺ population with the percentage of cells expressing PD-L1 shown. (Lower panel) Pooled data showing the mean (\pm SEM) absolute number of PD-L1⁺ neurons is shown ($n = 3$ mice/group).

6.3.4 Treatment of TG cultures with anti-PD-L1 induces reactivation

The increased viral burden following PD-L1 blockade could be explained by a direct interaction between the antibody and neurons which express PD-L1. To test this hypothesis, ganglia from latently infected mice were explanted, depleted of CD45⁺ cells, and cultured in 1:5 TG equivalents for 8 days. Cultures were treated with isotype, anti-PD-L1 or anti-PD-1 antibody throughout the culture period and supernatants were harvested periodically for quantification of replicative virus in a standard plaque assay. Treatment with anti-PD-L1 antibody induced reactivation above that observed in the absence of a protective CD8 T cell response suggests that this antibody is inducing reactivation in a CD45-independent fashion in neurons (Figure 52). Moreover, anti-PD-1 treatment had no effect on the level of reactivation consistent with the notion that impaired IFN γ production from CD8 T cells directly results in elevated gH copies. An alternative approach is to deplete PD-L1 expressing neurons via complement fixation. To do this we obtained latently infected ganglia and depleted CD45⁺ and PD-L1⁺ cells by complement fixation prior to culture initiation. Subsequent harvesting of culture supernatants for quantification of virus in a standard plaque assay revealed loss of reactivating neurons in the cultures which had PD-L1 expressing neurons removed (Figure 6B). We further used dymal beads to isolate PD-L1⁺ cells from CD45 complement depleted ganglia suspensions for culturing and reactivation monitoring. In this assay the majority of the reactivatable neurons were housed in the PD-L1 enriched fraction (Figure 53).

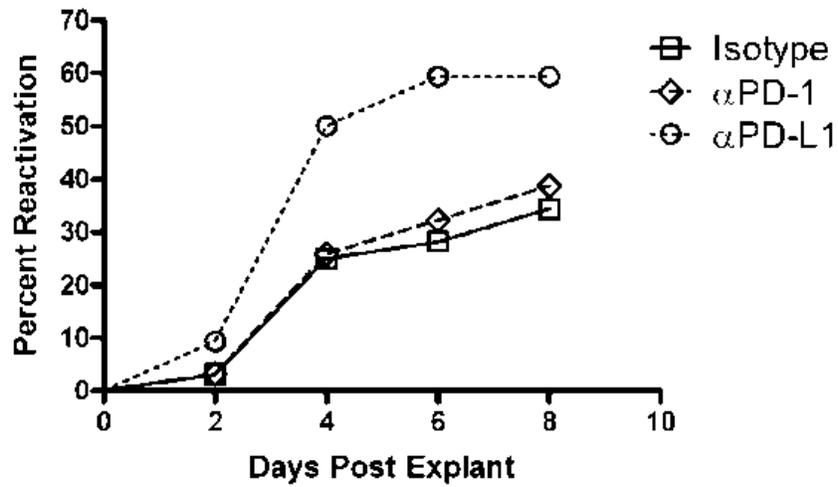


Figure 54. Anti-PD-L1 treatment of CD45-depleted latent TG cultures induces reactivation. HSV-1 latently infected TG cultures (>50 dpi) depleted of CD45 expressing cells via complement fixation were plated in 1:5 TG equivalents in 48-well plates and treated with ___ ug of indicated antibodies. The cultures were monitored for reactivation by collecting supernatants at the indicated time-points for a standard plaque assay on a monolayer of veros. Percent reactivation is determined by the percentage of wells which contain viral plaques. A representative assay is shown.

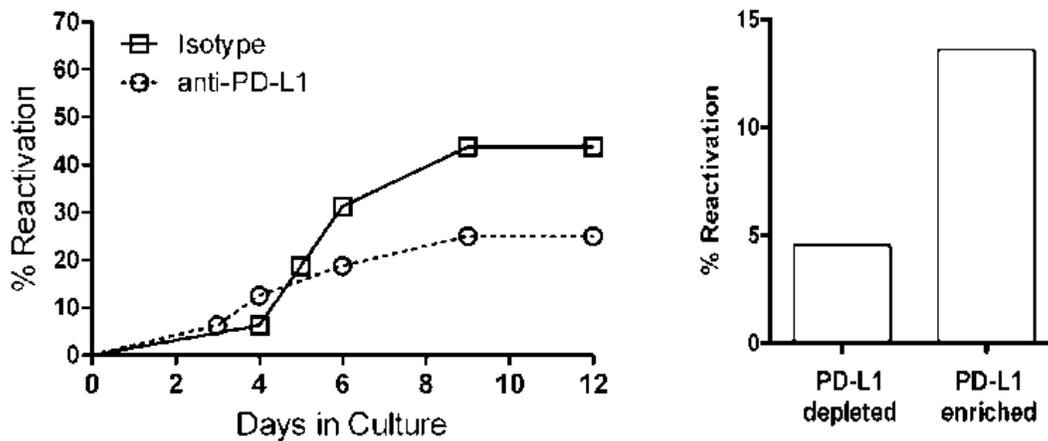


Figure 55. PD-L1 expression identifies a population of neurons prone to reactivate

(Left panel) HSV-1 latently infected TG cultures (>50 dpi) depleted of CD45 and PD-L1 expressing or isotype treated cells via complement fixation were plated in 1:5 TG equivalents in 48-well plates. The cultures were monitored for reactivation by collecting supernatants at the indicated time-points for a standard plaque assay on a monolayer of veros. (Right panel) HSV-1 latently infected TG cultures (>50 dpi) depleted of CD45 expressing cells via complement fixation were then stained with anti-PD-L1 mAb. Cell suspensions were incubated with anti-rat IgG dynal beads to selectively deplete or enrich for PD-L1 expressing cells and plated in 1:5 TG equivalents in 48-well plates. The cultures were monitored for reactivation by collecting supernatants at 8 days following culture initiation for a standard plaque assay on a monolayer of veros. Of note, use of dynal beads to deplete cells often results in lower reactivation frequencies. Percent reactivation is determined by the percentage of wells which contain viral plaques.

6.3.5 PD-L1 expression on neurons during latency identifies a population enriched for HSV-1 latency

One possible explanation of this observation is that the PD-L1⁺ population of neurons contains neurons which harbor multiple copies of HSV-1 genome and are therefore more prone to reactivate (183). To determine if this is true, CD45⁻ cells were sorted into PD-L1⁺ and PD-L1⁻ fractions and gH gene copies were determined. While the PD-L1⁻ fraction contained the majority

of latent genomes, when normalized for the number of cells or DNA isolated the PD-L1⁺ fraction was greatly enriched for latent burden (Figure 54).

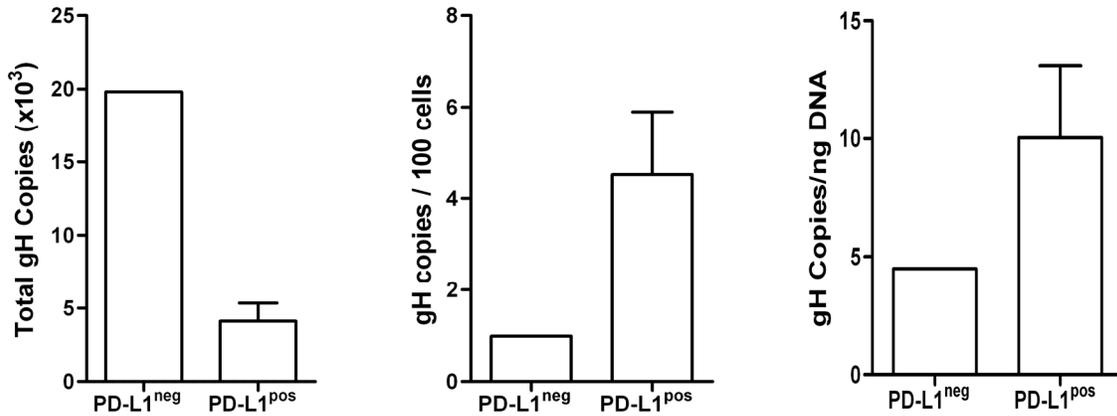


Figure 56. PD-L1 expression denotes a neuronal population enriched for HSV-1 latency

Pooled TG cell suspensions from HSV-1 latently infected B6 mice were stained with anti-CD45 and anti-PD-L1 and cells which did not express CD45 were sorted into two distinct populations (> 95% purity) as indicated above. DNA was isolated from these samples and analyzed for levels of viral gH DNA copies. Representative data of two such assays is shown.

6.4 PD-1 IN BALB/C DISEASE MODEL

To determine whether PD-1 – PD-L1 signaling plays a role in the outcome of HSV-1 induced corneal immunopathology, we employed the use of latently infected Balb/c mice for studies involving the manipulation of this signaling pathway. Balb/c mice first develop corneal scarring from immune infiltrates in the eye consisting predominately of CD4 T cells and neutrophils at approximately 7 dpi. This disease lasts for the life of the animal. In addition to examining the immune infiltrate of the TG, the development of disease in these mice allows us to evaluate the immune infiltrate at the site of initial infection during latency. A previous study demonstrated that administration of a PD-L1-specific blocking antibody to Balb/c mice resulted in the

increased proliferation and survival of HSV-specific corneal CD4⁺ T cells as well as the exacerbation of herpetic stromal keratitis (184).

To further examine this observation, TG and cornea from HSV-1 latently infected Balb/c mice were harvested to ascertain whether PD-1 expression occurred in T cells infiltrating these tissues. Almost all the T cells in the cornea of a mouse infected with HSV-1 strain RE are composed of CD4 T cells (185). During latency, approximately 77% of the CD4 T cell population isolated from diseased corneas expressed CD127. Of these about 40% express the inhibitory receptor PD-1 (Figure 55). This is in contrast to CD8 T cells isolated from the TG of Balb/c and B6 mice where the majority of PD-1 expression is confined to cells which do not express CD127 (Figure 42B and 55). However, PD-1 expression appears to be more variable in the Balb/c HSV-1 model as CD8 T cells from only 1 in 4 TGs expressed any appreciable levels of PD-1.

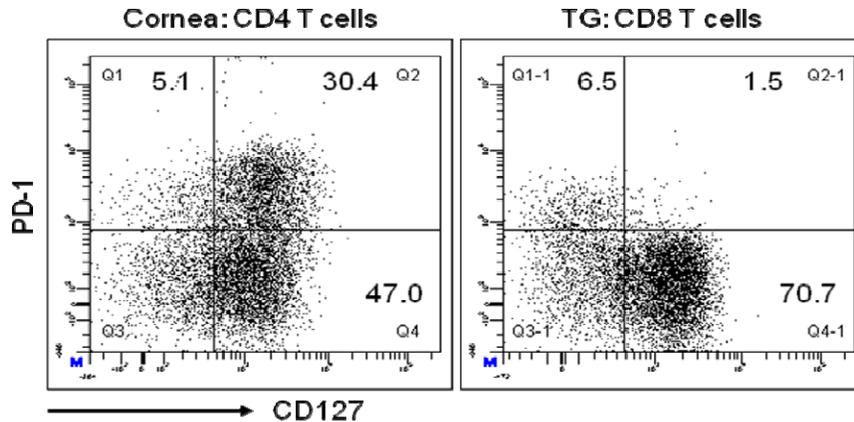


Figure 57. PD-1 is expressed by CD4 T cells in the cornea and CD8 T cells in the TG of latently infected Balb/c mice and CD8.

TGs and corneas were harvested from HSV-1 latently infected Balb/c mice (> 35 dpi) and dispersed into single cell suspension. Cell suspensions from individual ganglia and pooled corneas were simultaneously stained with anti-CD45, anti-CD8, anti-CD4, anti-PD-1, and anti-CD127 and analyzed by flow cytometry. Representative dot plots gated on the indicated tissues and populations are shown. Percent of T cells contained within quadrants are indicated. PD-1 expression was only observed on CD8 T cells in 1 of 4 TGs analyzed from latently infected Balb/c mice.

Due to PD-1 expression on CD4 T cells in the cornea of diseased mice and on CD8 T cells in at least some latently infected mice TGs, we sought to determine the effect of blocking PD-1 signaling by treating latently infected Balb/c mice with anti-PD-L1 after initiation of disease during stable latency. Approximately 40 – 50% of the CD4 T cells isolated from a latently infected Balb/c cornea express Foxp3 (Figure 56) and Tregs have been reported to express PD-1 in intracellular compartments (186 - 188). Approximately 67% of PD-1 expressing CD4 T cells isolated from the cornea of latently infected mice express CD127 and the transcription factor Foxp3 (Figure 56). Foxp3⁺ Tregs upregulate PD-1 expression by approximately 10% following PD-L1 blockade. This increase was not particular to Foxp3⁺ cells as PD-1 expression was nearly doubled in CD4 T cells which do not express Foxp3 (Figure 56).

It is unclear whether this change in PD-1 expression is due to elevated levels of antigen in the sensory ganglia or direct regulation of PD-1 through PD-L1 signaling.

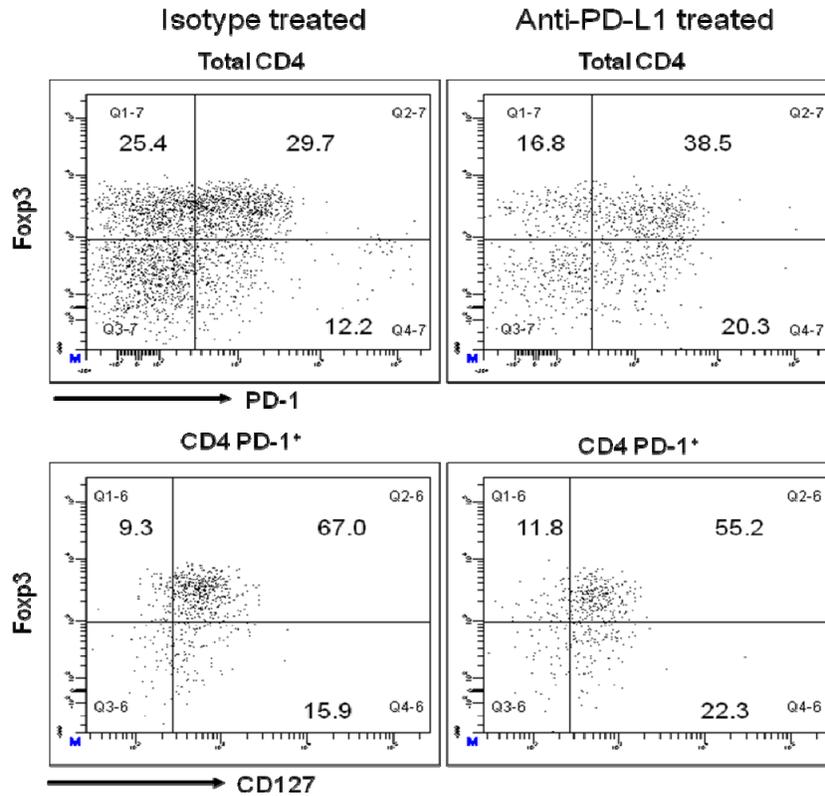


Figure 58. The majority of PD-1⁺ CD4 T cells in the cornea of Balb/c mice express Foxp3 and CD127.

Corneas were harvested from HSV-1 latently infected Balb/c mice (> 35 dpi) treated with an isotype antibody or anti-PD-L1 every 3 days for 2 weeks prior to harvest and dispersed into single cell suspension. Cell suspensions of pooled corneas were simultaneously stained with anti-CD45, anti-CD4, anti-PD-1, anti-CD127, and intracellularly with anti-Foxp3 and analyzed by flow cytometry. Dot plots gated on the indicated populations from the indicated groups are shown. Percent of T cells contained within quadrants are indicated.

TG from these same mice were harvested to determine if any functional changes could be detected in CD8 T cells in mice treated with anti-PD-L1. Blockade of PD-1 signaling lead to a slight increase in IFN γ and TNF production in response to HSV-1 infected MKSA (class I matched) targets (Figure 57). Whether this increase is meaningful is not clear. However, it may

be consistent with a rescued functional capacity of CD8 T cells during periodic antigen stimulation associated with a latent infection.

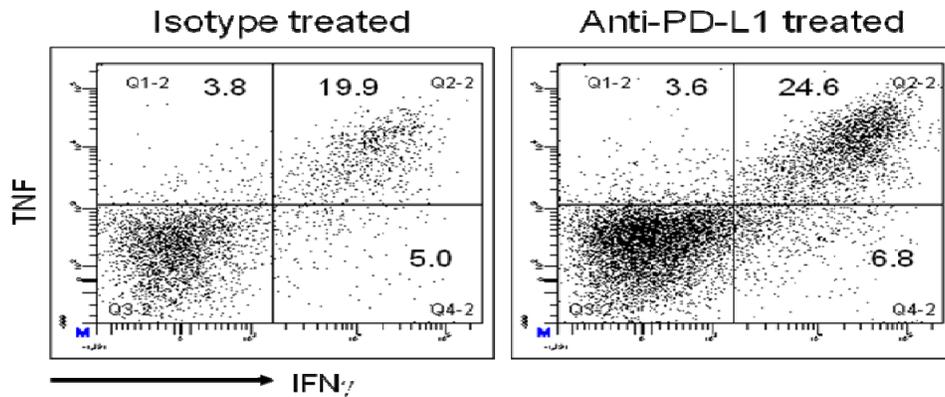


Figure 59. Anti-PD-L1 treatment induces a slight increase in CD8 T cell function in the latent ganglia.

TG cell suspensions from HSV-1 latently infected Balb/c mice were treated with an isotype antibody, or anti-PD-L1 every 3 days for 2 weeks prior to harvest. TG cell suspensions were stimulated with HSV-1 infected MKSA fibroblasts in the presence of Golgi-plug for 6 h at 37°C. Following stimulation the cells were stained for intracellular expression of IFN γ and TNF. Individual assays show the mean (\pm SEM) percentage of the indicated CD8 T cell population that expresses intracellular IFN γ . Dot plots gated on CD8 T cells from the indicated groups are shown. Percent of T cells contained within quadrants are indicated.

The ultimate reason to examine PD-1 expression and PD-L1 blockade in this model was to determine whether treatment with anti-PD-L1 could alleviate or worsen HSV-1 induced corneal scarring. However, contrary to published literature, mice treated with anti-PD-L1 and monitored on alternating days until tissue harvest demonstrated no appreciable change in HSK severity throughout the course of treatment (Figure 58). The decision to not pursue these assays further was made on the limiting evidence that PD-1 or its ligand play an important role in disease maintenance. However, the observation that the PD-1 expressing CD4 T cells in these corneas are almost entirely composed of regulatory T cells deserves further investigation.

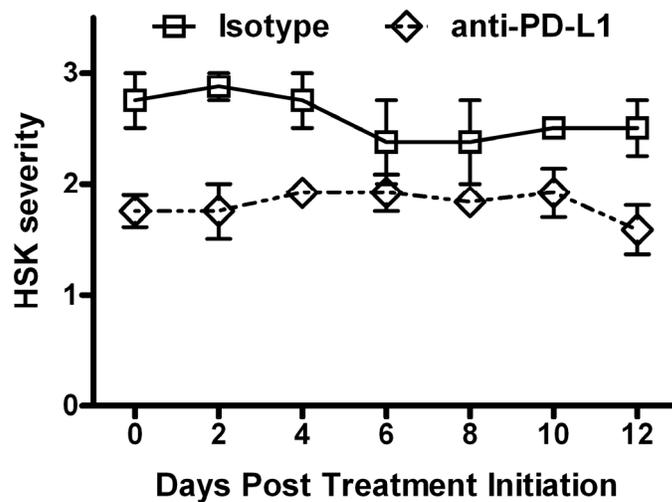


Figure 60. Anti-PD-L1 treatment has no apparent effect on the severity of HSK.

Corneas from diseased eyes of HSV-1 latently infected Balb/c mice were observed every other day after initiation of isotype or anti-PD-L1 treatment for disease severity on a slit lamp scope. Disease is scored by a 4-point system.

6.5 DISCUSSION

The previous chapter demonstrated that a large percentage of the gB-nonspecific CD8 T cell population that initially infiltrates the HSV-1 infected ganglia is specific for HSV-1 antigens and are capable of preventing reactivation in a TG explant model of reactivation. Throughout this chapter we followed up on those initial observations by examining the gB-nonspecific CD8 T cells which are maintained within the latently infected ganglia. This population of CD8 T cells does not require IL-15 for their homeostatic maintenance within the TG during latency suggesting exposure to antigen during this time period (Figure 13) (189). Persistent, high level exposure of CD8 T cells to their cognate antigen, as observed in LCMV clone 13 infection, results in upregulation of PD-1 and impaired functional capacity and proliferation. We sought to

determine the outcome of low level, intermittent stimulation on T cells of PD-1 expression and function in the HSV-1 corneal infection model. Consistent with models of chronic infection, PD-1 was upregulated on a population of CD8 T cells not specific for the immunodominant gB₄₉₈₋₅₀₅ epitope within the latently infected TG. As a whole, this population appeared functionally compromised and in particular, lacked granzyme B expression among its PD-1⁺ subset suggesting that they may be HSV-1 specific yet functionally exhausted (Figure 43 and 47). The same population of CD8 T cells does not express CD127 suggesting that this population of cells may experience periodic antigenic exposure. This is consistent with the increased proliferation observed in this population of cells during latency homeostasis (Figure 44).

Surprisingly, blockade with either anti-PD-1 or anti-PD-L1 antibodies fail to restore function of the gB-nonspecific CD8 T cell population or granzyme B expression during latency. This phenomenon may be indicative of a CD8 T cell population which is beyond the point of rescue yet maintained indefinitely for the life of the animal. An alternative explanation is this population of cells is not HSV-1-specific. We find the latter possibility unlikely as the majority of CD8 T cells which infiltrate the TG early are specific for HSV-1 antigens and all evidence to date supports the concept that the TG is compartmentalized during latency (unpublished observations). Moreover, blockade of PD-1 inhibits the ability of the gB-nonspecific CD8 T cells population to further produce IFN γ . Interestingly, this inhibition in function was also noted in the gB-specific subset of CD8 T cells, a population which does not appear to express any appreciable levels of PD-1. Whether this inhibition is mediated by PD-1 expressing CD8 T cell regulation of HSV-1-specific CD8 T cells is unclear but supports the notion that the gB-nonspecific PD-1⁺ T cells are HSV-1 specific. The observation that PD-1 expression is confined to CD8 T cells which do not express CD127 also implies recent TCR stimulation as effector CD8

T cells downregulate CD127. In this situation it seems unlikely that bystander activation mediates CD127 downregulation as the total population of CD8 T cells would be expected to be phenotypically similar in this regard. Ongoing experiments are attempting to demonstrate synapse formation between PD-1 expressing cells in the latent ganglia and PD-L1 expressing neurons to further support the claim that they are antigen-specific.

This study is the first to describe PD-L1 expression on a neuronal population. PD-L1 expression is often upregulated during inflammation and infection which acts as a dampening mechanism for the immune response (145). Early following HSV-1 infection, the majority of neurons upregulate PD-L1 expression suggesting this may be a mechanism to prevent overt inflammation in a vital tissue incapable of regeneration. Dampening the immune response and its associated inflammation would be critical in the TG. This pattern of PD-L1 expression early following infection is consistent with levels of type I and II interferons following HSV-1 infection, molecules shown to modulate PD-L1 expression (143, 144).

Maintenance of elevated PD-L1 expression in neurons during latency suggests a continued role for this molecule during latent infection. The intriguing possibility that PD-L1 expression denoted the pool of latently infected neurons was explored. CD45 negative cells from latently infected ganglia were sorted based on PD-L1 expression and analyzed for HSV genome copies. While the majority of HSV-1 genomes were maintained in neurons which lacked PD-L1 expression during latency, we observed that genome copies were increased on a per cell basis. This suggests that this population of neurons contained a greater proportion of latently infected neurons or neurons which contained multiple copies of viral genome. To distinguish between these possibilities, we postulated that depletion of PD-L1 expressing neurons from latently infected TG explant cultures would reduce reactivation, consistent with the findings that elevated

viral genome copies is directly proportional to reactivation rates (183). In corroboration of this hypothesis, reactivation was reduced when PD-L1 expressing cells were removed from CD45-depleted TG cultures. Moreover, we show by selective enrichment of PD-L1 expressing nonhematopoietic cells that this population contains the majority of reactivable neurons.

Treatment of CD45 depleted TG explant cultures with anti-PD-L1 antibody induced reactivation of neurons from latency. This finding was surprising given that the clone used in these studies is purportedly antagonistic, PD-1 expressing cells were removed by CD45 depletion, and CD80 is not expressed in the latent ganglia. Our observation would suggest that anti-PD-L1 is mediating signal transduction into neurons which express PD-L1 thereby inducing reactivation. An alternative hypothesis is that PD-L1 may bind another receptor regulating latency that is blocked with this antibody. However, we find this hypothesis unlikely as the most likely source of another receptor, CD45 expressing cells, have been removed from these cultures. This finding has tremendous impact on the potential use of anti-PD-L1 treatment in ongoing clinical trials; particularly those aimed at treating HIV infected individuals as they are more likely to harbor HSV infection.

Why do gB-nonspecific CD8 T cells express PD-1 and neurons which readily reactivate express PD-L1? Initially PD-L1 expression may have served to prevent overt neuronal destruction associated with the large infiltration of activated T cells and possibly to inhibit T cell responses providing a feasible means to periodically reactivate and spread disease. It seems that as latency progresses the role of PD-L1 expression may evolve since blockade of PD-1 signals fails to restore functional capacity of PD-1 expressing cells in this system. We propose that one possible reason for the expression of PD-1 and PD-L1 during latency may be to periodically induce reactivation in a small proportion of neurons. The benefit of this model is to provide a

source of periodic antigen stimulation for the dominant CD8 T cell population in the ganglia to promote their retention in this tissue. CD8 T cells in the ganglia do not express granzyme B and blockade of PD-1 signaling fails to augment production of cytokines. This suggests that these cells are completely incapable of performing typical CD8 effector mechanisms required to prevent reactivation. However, they are the only population of cells in the latent ganglia that expresses any appreciable levels of PD-1 and therefore the only known cells capable of ligating PD-L1 on neurons which results in reactivation. These studies demonstrate the dynamic nature of the immune response during latency.

We further sought to determine if PD-1 expression regulated HSK disease in the Balb/c model of corneal HSV-1 infection. A previous study demonstrated that anti-PD-L1 treatment exacerbated HSK and was associated with an increase in CD4 T cells in the cornea (184). Our results indicate that PD-L1 blockade does not regulate HSK progression following the establishment of HSK during latency. However, we demonstrate that PD-L1 blockade may regulate Foxp3 expressing regulatory CD4 T cells in the cornea of diseased mice. During latency approximately 50% of the CD4 T cells in the cornea express Foxp3. Moreover, the majority of these express both CD127 and PD-1 on their surface. The role of PD-1 expression on CD4 Tregs has yet to be explored and requires further investigation. It may suggest that CD4 Tregs can regulate CD8 T cell responses by interacting with PD-L1 expressing T cells.

7.0 FUTURE DIRECTIONS

Numerous avenues of future exploration still need to be undertaken to fully define the observations outlined throughout this dissertation. This section will define possible future directions to be undertaken for each major section.

Chapter 4: IL-15 and IL-2 during HSV-1 Infection

The most notable omission from these observations is the outcome of IL-15 deficiency on the maintenance of viral latency. Experiments to determine whether IL-15 deficiency results in comparable establishment and maintenance of HSV-1 infection need to be performed by examining levels of gH viral genome copies during the acute infection (8 dpi) and during latency (>34 dpi). Similarly, it would also be interesting to determine whether anti-IL-2-treatment early and its associated decrease in HSV-specific CD8 effectors in the TG results in increased viral burden. However, early anti-IL-2 treatment had no observable effect on the memory pool maintained in the latent TG. Therefore, it would be of interest to compare gH viral copies during acute infection and latency.

While the data strongly support that the HSV-1-specific population is being maintained by antigenic exposure in the absence of IL-15 this has yet to be definitively demonstrated. Currently, a recombinant HSV-1 with a single point mutation in an MHC class I anchor residue of the gB epitope is under development. Using this virus we could infect one eye of IL-15^{-/-} mice

with the parent strain and the contralateral eye with the recombinant HSV. In this manner, we could monitor whether antigen is truly responsible for HSV-specific CD8 memory maintenance in the absence of IL-15 by following both gB-specific and gB-nonspecific populations in the TG and lungs. As latency progresses, I would expect to observe decreased gB-specific CD8 T cells in the contralateral TG similar to the loss of ova-specific OT-1 during latency and maintained gB-specific CD8 T cells in the ipsilateral TG.

Notably, the role of IL-7, another critical cytokine required for the maintenance of memory CD8 T cells, has yet to be explored in this model. The worked outlined in chapter 4 would serve as a blueprint for future studies examining how IL-7 influences the CD8 T cell population in the latent TG. The use of mixed bone marrow chimeras (WT and CD127^{-/-}) would greatly enhance the feasibility of these studies.

Chapter 5: gB-nonspecific CD8 T cells in HSV-1 infection

This data clearly and conclusively demonstrates that a large proportion, if not all, of the CD8 T cells maintained in the latent ganglia are HSV-1 specific. However, a substantial portion of this story has yet to be elucidated in the antigen specificity of the gB-nonspecific CD8 T cell population. The only conclusive data has demonstrated that these cells respond to a non-gB encoded epitope that is produced prior to gB and/or at higher levels during the reactivation process. However, ruling out gB as a candidate gene product narrows the field to approximately 80 other proteins, a sizable task. Chances for success can be enhanced by examining CD8 T cell responses to likely candidates, such as those that have been shown to be crucial for reactivation or detected in latent ganglia.

It would also be interesting to determine whether these gB-nonspecific CD8 T cells are capable of protecting in a vaccine model of HSV-1 infection. Determining the relative contribution of distinct HSV-1-specificities and at what point in the reactivation cascade do individual specificities contribute to protection could ultimately help us understand HSV gene expression and progression from latency to reactivation.

Chapter 6: PD-1 and PD-L1 expression during latency

The focus of this body of work is the expression of PD-L1 on a subset of neurons that is prone to reactivate. While we demonstrate that the gB-nonspecific population of CD8 T cells maintained in the latent TG expresses the receptor for PD-L1, we have yet to demonstrate a direct link between PD-1 expression on gB-nonspecific CD8 T cells with PD-L1 expression on reactivation prone neurons. In the absence of known HSV-specificity for this subset of gB-nonspecific CD8 T cells, the most likely methodology to demonstrate a direct interaction is to visualize PD-1⁺ CD8 T cell interactions with PD-L1⁺ neurons using *in situ* imaging. Using this approach we can also use either V β TCR staining or gB tetramer staining to demonstrate that this PD-1⁺ cell interaction is definitively with gB-nonspecific CD8 T cells. This data would also strongly suggest that the PD-1 expressing CD8 T cell is HSV-1-specific.

It will also be important to demonstrate that PD-L1 expression by neurons or their support cells and not PD-L1 expression by hematopoietic cells regulate the observed phenomena outlined in detail in chapter 6. This would best be determined using bone marrow chimeric mice in which WT mice are reconstituted with PD-L1 deficient bone marrow and vice versa. Since all CD8 T cells isolated from the latent TG express PD-L1, this approach may also provide a means to determine how PD-1 blockade is impairing CD8 T cell function. This would be detectable if

PD-1 blockade is impairing CD8 T cell function by directly interacting with PD-L1 and delivering a negative signal into PD-L1 expressing cells. A bone marrow chimeric mouse generated with wild-type bone marrow in PD-L1 deficient mice may also help address whether PD-1⁺ gB-nonspecific CD8 T cells can induce reactivation in vivo. If this is a mechanism for gB-specific retention by providing a periodic source of antigen for this protective T cell population we would expect that gB-specific CD8 T cell numbers would decline over time. Alternatively, viral gH copies could be monitored for gradual decline in latent burden. Additional studies could utilize gB-specific CD8 T cells deficient in both IFN- γ and granzyme B in ex vivo reactivation assays as these cells upregulate PD-1 after 1 – 2 weeks in culture. These cells would therefore express PD-1, have a TCR capable of forming an immunologic synapse, but be unable to prevent reactivation. We would expect increased reactivation frequency if T cell mediated PD-L1 ligation is capable of inducing reactivation.

PD-1 expression by the majority CD4 regulatory T cells in the cornea of Balb/c mice is an interesting finding which should be further explored. Whether these Tregs regulate T cell responses by PD-1 expression would be a novel finding. Tregs may also regulate gB-nonspecific function in the latent TG of B6 mice as suggested following anti-IL-2 treatment.

Determining what regulates PD-L1 expression in the small subset of neurons would also be potentially important. Clearly, the presence of latent virus is not the only factor as the majority of latent DNA is housed within PD-L1- neurons. However, what impact latent virus has on PD-L1 expression is unclear. Cytokines may also play a crucial role in PD-L1 expression during initial viral replication within the TG. One possibility is that LATs may regulate PD-L1 expression as both LATs and PD-L1 appear to be elevated in neurons with high viral genome

copy number. Delineating the impact of viral, inflammatory, and neuronal factors which regulate PD-L1 expression will be crucial for future studies.

8.0 SUMMARY AND CONCLUDING REMARKS

The concept that latently infected neurons are ignored by the host immune response has given way to the notion that CD8 T cells maintained in the TG monitor infected neurons thereby subverting reactivation. This dissertation has added to our understanding of how CD8 T cells function to combat HSV-1 infection and reactivation by exploring how these cells are maintained in the ganglia and how the environment and virus influence their maintenance and behavior. We establish that the homeostatic cytokine IL-15 does not contribute to the maintenance of CD8 T cells within the TG during latency. Moreover, it appears that the presence of antigen can function to promote their retention and activation in the latent ganglia. We demonstrate that a CD8 T cell population thought to be nonspecific for HSV-1 antigens is HSV-1-specific and contributes to HSV-1 control. Finally, we show that a population of neurons expressing PD-L1 represents an enriched cellular reservoir of latent virus that is highly prone to reactivate.

Although many individuals acquire HSV-1 very early in life, the incidence of seroconversion increases into adulthood. Thus, a prophylactic vaccine might protect a portion of the population from acquiring latent HSV-1 infections. However, universal protection from HSV by a prophylactic vaccine appears unlikely. Therefore, a critical challenge facing herpes virologists and viral immunologists is to exploit the available knowledge of HSV-1 latency to develop new therapeutic strategies for maintaining HSV-1 in a latent state. It is our belief that at present the best hope for accomplishing this goal lies in our evolving understanding of the

interaction between HSV-specific CD8 T cells and latently infected neurons. Development of a therapeutic vaccine targeting the viral proteins recognized by CD8 T cells that are retained within latently infected ganglia would seem to be a useful approach. Although unsuccessful human vaccine studies have included gB as an immunogen, these studies have largely targeted CD4 T-cell and antibody responses (190). Our findings would suggest that vaccine formulations designed to additionally activate CD8 T cells would be more efficacious at preventing reactivation of latent virus and shedding at the periphery. In fact, a recent report concluded that evaluation of future HSV vaccines in animal models should include the effect of the vaccine on the ratio of the number of CD8 T cells to the number of copies of viral genome within the latently infected sensory ganglia (92). Although there remains scant hope of eradicating latent virus from host neurons, the recognition of a role for the host immune system in monitoring and controlling HSV-1 latency provides a new approach to the design of better therapeutic modalities for reducing the morbidity and mortality associated with HSV infections.

APPENDIX

LIST OF PUBLICATIONS

Sheridan BS, Khanna KM, Frank GM, and RL Hendricks. (2006). Latent virus influences the generation and maintenance of CD8⁺ T cell memory. *J. Immunol.* **177**:8356-8364.

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Cherpes TL, **Sheridan BS (co-first author)**, and RL Hendricks. The impact of PD-L1 expression on neurons within HSV-1 latently infected sensory ganglia. (Manuscript in preparation).

APPENDIX A

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