# Expanding the functional CD8+ T cell repertoire reduces HSV-1 reactivation from latency in sensory ganglia

by

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**Expanding the functional CD8+ T cell repertoire reduces HSV-1 reactivation from latency in sensory ganglia**

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University of Pittsburgh, 2012

Following corneal infection, herpes simplex virus (HSV)-1 establishes latency in sensory neurons of the trigeminal ganglia (TG). In humans, spontaneous and recurrent reactivation of HSV-1 from latency has the potential to cause lesions on the gums (stomatitis), lips (cold sores, fever blisters), cornea (stromal keratitis), and brain (encephalitis). Latently infected neurons were once thought be largely ignored by the host immune system. Existing evidence shows that not only do HSV-specific CD8 T cells recognize latently infected neurons; they actively maintain viral latency using proinflammatory cytokines and lytic granules containing granzymes. The premise of this study further characterized the nature of the CD8 T cell response. Previous studies displayed that in the C57BL/6 mouse; CD8 T cells infiltrate the TG and become situated in direct apposition to infected neurons. It was known that 50 % of the CD8 T cells recognized the immunodominant epitope on glycoprotein B (gB) while the specificities of the remaining CD8 T cells were undefined. In this study, we observed that the non-gB CD8 T cell repertoire was confined to 18 epitopes on 11 viral proteins. During acute infection, these cells, similar to $gB_{498\text{-}505}$-specific CD8 T cells, readily produce cytokines and release lytic granules upon stimulation. Conversely, during latency, even though these cells remain in the TG, they lose the ability to produce cytokines and release lytic granules upon stimulation suggesting functional compromise, unlike $gB_{498\text{-}505}$-specific CD8 T cells. We show that the immunosuppressive cytokine, IL-10, preferentially suppresses the non-$gB_{498\text{-}505}$-specific CD8 T cell population. Upon

administration of an antibody against the IL-10 receptor, we see a dramatic increase in functional non-$gB_{498-505}$-specific CD8 T cells without apparent effect in the $gB_{498-505}$-specific CD8 T cell population. This increase in functional CD8 T cells leads to a 50% reduction in viral reactivation from latency suggesting the possibility of anti-IL10R as a treatment of recurrent reactivation of HSV-1 from latency.

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# PREFACE

I would first like to express my sincere gratitude towards my mentor, Dr. Robert L. Hendricks, for playing such a crucial role in my development as a scientist. His continual encouragement and enthusiasm has propelled my dedication to scientific research. I will always bring with me his advice to constantly think about my project and its implications for the future.

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# 1.0 INTRODUCTION

## 1.1 HERPES SIMPLEX VIRUS TYPE 1

### 1.1.1 Epidemiology

The human herpes virus family contains nine structurally similar members that impose a significant burden on health systems. The two herpes simplex viruses (HSV) and varicella zoster virus (VZV) infect and enter latency in host sensory neurons. Typically, HSV-1 commonly associates with the orofacial region with a site of latency in the trigeminal ganglia (TG) while HSV-2 most prominently associates with the genital tract and sacral ganglia. As time progresses, these distinctions are becoming less pronounced as demonstrated by the increased cases of genital HSV-1 infection [1]. Herpes simplex virus (HSV) type 1 is estimated to affect at least 58% of the population in the United States while reaching prevalence up to 98% in other areas [2-4]. Asymptomatic loss of viral control in host neurons, which is thought to occur at least once every thirty days in some individuals [5], leads to live virus formation and contributes to high frequencies of transmission in the population. Currently, much effort is directed towards investigating the immunological mechanisms maintaining viral latency and preventing viral spread in hopes of developing a more effective therapeutic treatment.

Pathogenesis associated with HSV infections include herpes labialis (cold sores/fever blisters), genital lesions, herpes simplex encephalitis (HSE) and herpes keratitis. HSE, though rare, affects 1 of every 250,000 individuals per year and is the most common cause of sporadically fatal viral encephalitis in the U.S. This is especially prevalent in neonates. The most severe form of HSV-mediated ocular disease, herpes stromal keratitis (HSK), involves the deep stromal layer of the cornea. Ocular HSV disease is thought to affect 150 out of 100,000 people [4]. In North America and Europe, it is estimated that each year up to 13 people per 100,000 experience their first episode of ocular HSV [4,6-9]. Additionally, it is estimated that 10 million people have been affected by herpetic eye disease with 2 million of those people left with impaired vision. It is thought that more than 95% of these cases are directly attributed to HSV-1 as opposed to other herpes viruses [10]. The corneal thinning, scarring, and neovascularization due to recurrent HSK is the leading cause of corneal blindness due to infectious origin and commonly results in keratoplasty.

### 1.1.2 Virus Structure

HSV-1 is a double stranded DNA virus that has a 152-kbp genome consists of 6 important regions encoding at least 84 viral proteins. During lytic infection, the viral genome is linear and becomes circularized upon entering latency with the help of "a" region located at the end of the linear DNA. Other important regions consist of the 9,000 bp long repeat ($R_L$) (important for immediate early regulatory protein formation and latency associated transcripts), the 108-kbp long unique region ($U_L$) (DNA replication enzymes and capsid proteins), the 6.6-kbp short repeats ($R_S$) (the essential viral protein ICP4), the origins of replication (2 copies), and the 13-kbp unique short region ($U_S$) (viral glycoproteins and response to host defense). The viral

genome is encased by an icosadeltahedral structure of viral capsid proteins. Outside of the capsid, there is another layer, tegument, which contains viral proteins that initiate viral gene transcription upon host cell infection. The outermost component of the virus consists of a lipid bilayer envelope that includes numerous viral glycoproteins [11]. These glycoproteins have functions that aid viral release from the infected host cell and entry into a new target host cell (Figure 1).

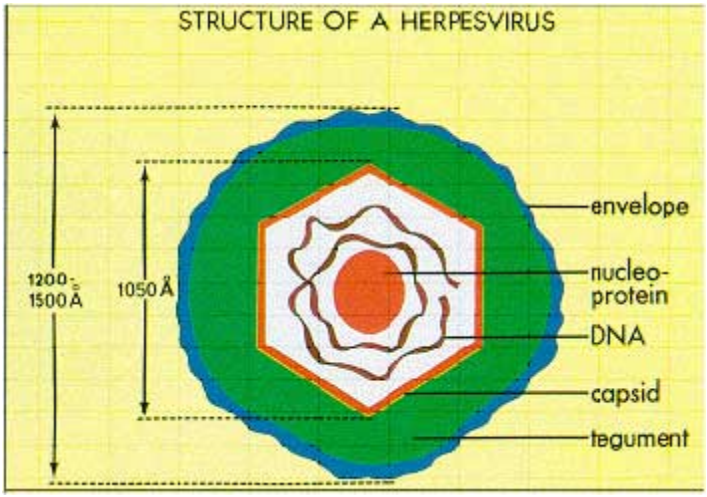


**Figure 1. HSV-1 Structure**

Cartoon depiction of the size and structural components of the HSV-1 virion (Yale website).

### 1.1.3 HSV-1 lytic and latent infection

During lytic infection, herpes viruses are known to sequentially express viral gene products necessary for successful replication of infectious virus. Upon infection of permissive cells, the virus uses the host RNA polymerase II to transcribe immediate early (IE or α) genes within two hours of initial infection. IE genes encode six proteins, ICP0, ICP4, ICP22, ICP27,

ICP47, and $U_S1.5$. All of these genes except ICP47 encode proteins that transactivate and regulate the expression of the next set of genes called early (E or β). ICP47 encodes a protein that inhibits immune recognition of virally infected cells (discussed later). The β gene products act in concert to replicate viral DNA and allow for late (L or γ) gene expression. The γ class of viral genes can be experimentally defined into two categories. Leaky late (γ1) genes are expressed at very low levels prior to DNA synthesis and are rapidly upregulated after viral DNA synthesis. Conversely, γ2 (true late) genes are only expressed after viral DNA synthesis. Late gene products include tegument proteins as well as glycoproteins necessary for viral assembly and egress from the cell [11]. The completion of the sequential expression of lytic genes results in the formation and release of virions ultimately resulting in cell lysis and death (Figure 2). Proteins important for the focus of this dissertation are described in Table 1.

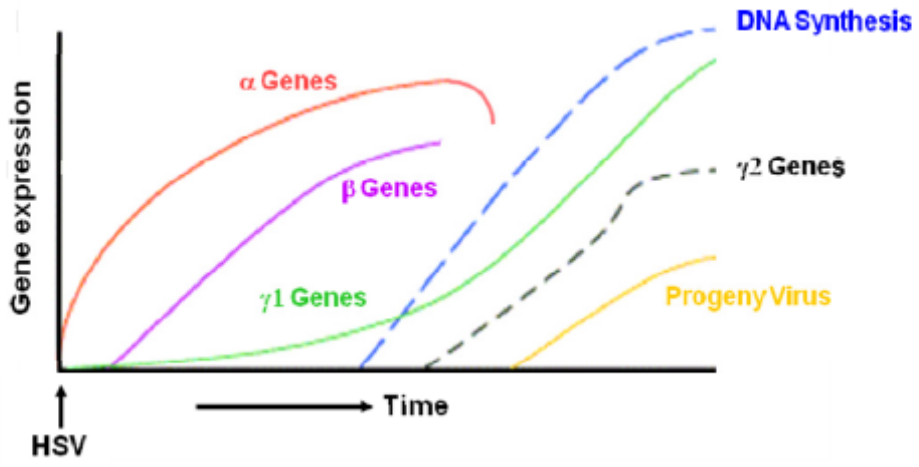


**Figure 2. Kinetics of gene expression during lytic infection**

Cartoon depiction of the kinetics of gene expression after a cell permissive for lytic infection is infected. Courtesy of Brian Sheridan, Ph.D.

Following primary infection in abraded epithelia of the cornea, HSV gains access to sensory neurons and travels via retrograde axonal transport to the neuronal cell bodies in the ophthalmic

branch of the TG [12,13]. In the TG, the virus replicates briefly and enters into latency for the life of the host. The processes that drive HSV into latency are not well understood, but it is thought that the virus, the quiescent nature of the neurons, as well as the immune system play significant roles. HSV latency can be defined as the retention of episomal DNA in neuronal cell bodies without the production of infectious virions [14]. During this time, viral gene expression is largely suppressed except for the abundant expression of latency associated transcripts (LATs) in some neurons, which lack any detectable protein product. Much effort has been devoted to exploring LATs, however, there appears to be no consensus on the role these transcripts play in viral latency [15-17]. Even though these transcripts have been shown to be expendable in the establishment and reactivation from latency, there have been studies showing that LATs inhibit neuronal apoptosis, thus aiding in the survival of the virus in the host [18-21]. LATs also help determine the subsets of neurons HSV will latently infect [22]. Lastly, microRNAs within the LAT gene are thought to regulate viral reactivation from latency by impairing the expression of ICP0 [23-25], which has been shown in culture.

The propensity of HSV to exit latency and enter into a productive lytic infection is also not well understood. This reactivation event produces live virus particles that travel back down neuronal axons and are released at the site of primary infection (cornea) leading to possible disease. Sensitive PCR methods have determined the precise numbers of viral genomes per infected neuron. Numbers range in orders of tens to thousands in mice while humans average 11 HSV DNA copies per neuron [26,27]. These data are of importance to reactivation studies because the number of HSV-1 genome copies per ganglia directly correlates with the virus' ability to reactivate from latency [28,29]. Many factors are attributed to increased rates of viral reactivation from latency which includes immunocompromise, UV-B irradiation, hypothermia,

hyperthermia, invasive surgery, psychological stress, and others [31-33]. It was originally thought that these factors modified neuronal physiology leading to reduced neuronal control of viral reactivation. While the effects of these factors on neuronal physiology have not been discredited and do contribute, our lab and others have shown that these stressors also greatly affect the function of the immune system, a component of the host absolutely essential in controlling viral reactivation from latency [31,34].

Epigenetic modifications of the latent HSV-1 genome that include histone methylation and acetylation influence the transition from latent to lytic infection. During latency, lytic gene promoters are associated with methylated histones, which entail a state of gene inactivity [35]. Conversely, the LAT enhancer region during latency is acetylated suggesting active chromatin [35-38]. When reactivation is induced in ganglionic cultures, the ICP0 promoter region becomes acetylated and allows for production of ICP0 mRNA. Concurrently, the LAT region of HSV-1 becomes deacetylated during this process. To further support this concept, the histone deacetylase inhibitor, sodium butyrate, induces reactivation from latency by modifying DNA-associated histones similar to reactivation from ganglionic cultures [39,40].

**Table 1. Summary of Select Viral Gene Products**

| *Gene Product* | *Class* | *Characteristics* | *Ref* |
|---|---|---|---|
| **ICP0** | α | -A nonessential protein at high MOI<br>-Required for efficient reactivation<br>-Increased expression can induce reactivation from latency<br>-IFN-γ prevents gene expression | [11,30,40,41] |
| **ICP4** | α | -An essential transactivator of β and γ genes<br>-Cleaved by CD8 T cell effector molecule, granzyme B | [11,42,43] |
| **ICP8** | β | -An essential viral protein<br>-Single-stranded DNA binding protein<br>-Binds to the viral DNA and stimulates the viral DNA polymerase | [11,44-47] |
| **ICP47** | α | -An nonessential viral protein<br>-Binds to host TAP proteins to prevent MHC class I presentation of viral antigens<br>-Binds with 100X more affinity in humans compared with mice | [11,48,49] |
| **Ribonucleotide Reductase I** | β | -A nonessential viral protein<br>-Aids in the synthesis of deoxyribonucleotides from ribonucleotides for viral DNA proliferation<br>-Contains a known subdominant epitope<br>-Target for human CD8 T cell response | [11,50-52] |
| **Glycoprotein B** | γ1 | -An essential viral protein<br>-Required for fusion and entry<br>-Expressed at low levels before DNA synthesis and much higher after DNA synthesis<br>-Contains the immunodominant eptiope | [11,53-55] |
| **Glycoprotein C** | γ2 | -A nonessential protein<br>-Involved in cell attachment<br>-Target for antibody generating vaccines<br>-Expression inhibited by IFN-γ | [41,56-59] |
| **LATs** | - | -A nonessential viral transcript<br>-Accumulates to large levels in latently infected neuronal nuclei<br>-Prevents neuronal apoptosis | [18,19,21,60,61] |

## 1.2 THE INITIAL IMMUNE RESPONSE TO HSV-1 INFECTION

### 1.2.1 Cornea

Cells of the innate immune system rapidly respond to the infection from a never-before-seen pathogen. In our mouse model of HSV ocular infection of C57BL/6 (B6) mice, this response begins in the corneal epithelia. While not a classical immune cell, epithelial cells containing toll-like receptors (TLRs) recognize highly conserved patterns on HSV-1 and secrete type I interferons [62,63]. These factors seek to inhibit viral protein translation in infected cells, inhibit infection of neighboring uninfected cells, upregulate MHC class I molecules, attract innate lymphoid cells such as polymorphonuclear (PMN) neutrophils, natural killer (NK) cells, and γδ T cell receptor (TCR) cells. In addition to epithelial cells, corneal resident dendritic cells (DCs) migrate to the site of infection and are thought to secrete chemokines creating a chemotactic gradient for the recruitment of NK cells to the site of initial infection [64]. The role DCs play in the initiation of the adaptive immune response remains controversial and is under extensive investigation, but it is known that the innate immune response is absolutely essential for the initial control of HSV-1 replication in the cornea and the priming of the adaptive response against HSV-1 [65,66].

### 1.2.2 Trigeminal Ganglion

Even though the innate immune response begins in a matter of hours, it is not sufficient to prevent the virus from gaining access to axons innervating the cornea. Once in the axons, the virus reaches the neuronal cell bodies of the ophthalmic branch of the TG. Here, the virus

replicates from 4 to 8 days post infection. Macrophages, γδ T cells, and NK cells infiltrate the TG upon replication of the virus [65,67,68]. Nitric Oxide (NO) and tumor necrosis factor (TNF) produced by macrophages and granzyme A presumably made by NK cells is thought to limit spread of virus to neighboring neurons in the TG [68,69]. During initial infection in the TG, γδ T cells and NK cells produce most of the IFN-γ, which has antiviral properties (discussed below) and can activate macrophages to produce NO and TNF. Depletion of γδ T cells and macrophages during acute infection significantly increases HSV-1 replication. Therefore, innate immune function in the TG is critical for the early control of viral replication in the TG. However, a functional adaptive immune response is required for complete elimination of replicating virus. Mice lacking an adaptive immune system, (severe combined immune deficiency or SCID) establish HSV-1 latency similar to wild-type mice, but eventually succumb to viral encephalitis due to a loss of viral control [70].

## 1.3 ADAPTIVE IMMUNE RESPONSE TO HSV-1

After initial infection, DCs present viral antigens to naïve B cells, CD4 and CD8 T cells in the draining lymph nodes (DLN). While B cells cannot directly prevent viral reactivation, they do help prevent the spread of infectious virus through production of neutralizing antibodies [71]. Studies have shown that the transfer of neutralizing antibodies to a non-immune B cell deficient host will confer better protection compared to control mice. CD4 T cells infiltrate both the cornea and TG after infection [72-75]. In the TG, CD4 T cells do not appear to play a direct role in maintaining viral latency, but aid in the functionality of HSV-specific CD8 T cells before the establishment of latency [76]. In the cornea, however, CD4 T cells are the main adaptive immune

cell that mediates the HSV related immunopathology, HSK [77,78]. While the exact epitopes these CD4 T cells recognize have not been elucidated, evidence suggests cornea-resident CD4 T cells are stimulated to produce chemokines that recruit PMNs and other cells to the cornea. The continual recruitment of inflammatory cells followed by resolution of inflammation eventually leads to the disruption of the corneal collagen stratification and ultimate corneal opacity. Prevention of CD4 stimulation in the cornea is thought to be attributed by the effective control of viral reactivation by CD8 T cells in the TG.

### 1.3.1 CD8 T cell recognition and response to virally infected cells

Host cells constantly break down proteins in the cytosol via proteasomes into small peptides that are transported into the endoplasmic reticulum via the transporter associated with antigen processing (TAP). Once in the endoplasmic reticulum (ER), the peptides are loaded into the peptide groove of MHC class I molecules. MHC class I molecules are shuttled to the surface to allow for recognition by CD8 T cells. During viral infection, viral peptides are loaded into MHC class I molecules and are shuttled to the surface. Each CD8 T cell clone specific for viral peptides bears a unique T cell receptor (TCR) that recognizes a specific peptide/MHC interaction (epitope) [79]. Upon initial recognition of the cognate antigen in the draining lymph node, CD8 T cells respond by proliferating. Interestingly, the epitopes targeted fall into a dominance hierarchy consisting of one or a few dominant epitopes (recognized by a large frequency of virus-specific CD8 T cells) and several other subdominant epitopes (recognized by a smaller frequency of virus-specific CD8 T cells) [80]. Several factors contribute to the dominance hierarchy of viral epitopes. These include the efficiency of peptide generation by proteasomes [81], kinetics of gene expression [80], the peptide binding affinity to MHC [82], the epitope

affinity for TCR, the frequency of epitope-specific CD8 T cell precursors [83,84], and the ability of epitope-specific CD8 T cell precursors to expand and survive. Uniquely, in the B6 mouse model of HSV-1 infection, there is a single immunodominant epitope on HSV-1 glycoprotein B ($gB_{498-505}$). In the flank infection, an estimated 70% of virus-specific CD8 T cells recognize this epitope [53], while 50% of virus-specific CD8 T cells recognize the epitope in the ocular infection [85] suggesting the route of infection may also play a role in the immunodominance hierarchy. Another unique aspect of the immunodominance hierarchy in ocular HSV-1 infection is that a 1:1 ratio of $gB_{498-505}$-specific CD8 T cell:non-$gB_{498-505}$-specific CD8 T cells maintained in the TG throughout latency (Figure 3) while immunodominant CD8 T cells are eventually lost from the ganglia in the flank infection model [85]. Another subdominant epitope was identified on ribonucleotide reductase 1 ($RR1_{822-829}$), which is recognized in both models of HSV-1 infection [50,86].

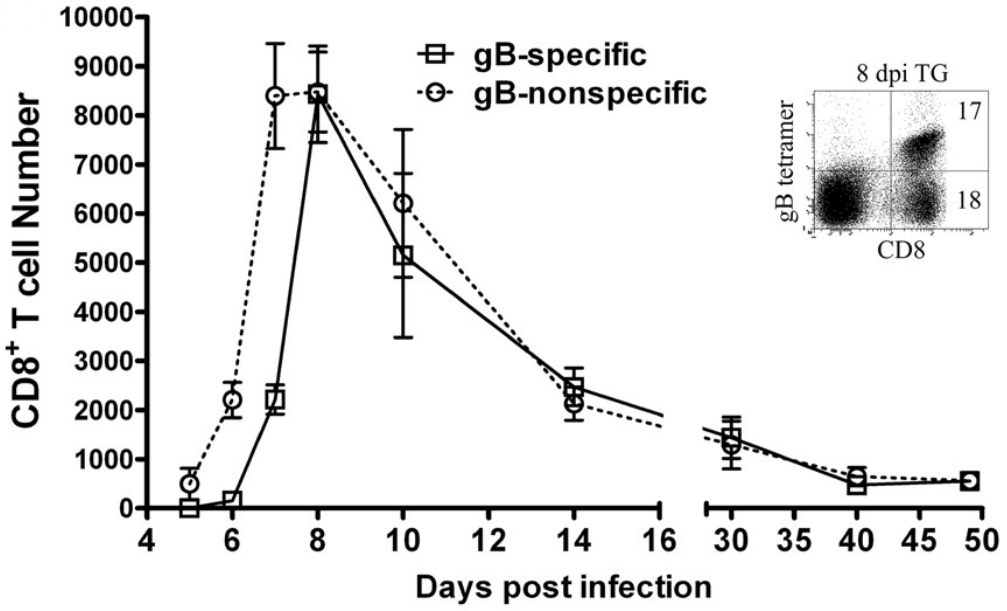


**Figure 3 The 1:1 ratio of $gB_{498-505}$-specifc CD8 T cell:non-$gB_{498-505}$-specific CD8 T cell is maintained in the TG through latency**

The kinetics of the CD8 T cell response was determined using fluorescently conjugated tetramer labeling of gB498-505-specific CD8 T cells [85].

During viral infections, a diverse CD8 T cell response has been attributed to a more effective viral clearance [87,88]. The term "elite controllers" has been used to describe humans that can generate robust T cell responses while also maintaining low viral loads during chronic infections such as HIV [89,90]. A characteristic of these elite controllers is the breadth of their CD8 T cell response. In the mouse model of CMV and macaque model of SIV infection, subdominant T cells directly correlate with better viral control [91]. This is attributed to the potential for mutational loss of immunodominant epitopes. In the event of an immunodominant epitope mutation, a highly functional subdominant CD8 T cell population would provide the best mode of viral control. Additionally, mobilization of the subdominant CD8 T cell response via immunization before lethal viral infections have led to better viral control and less mortality [92,93]. This concept is especially pertinent in our model of HSV-1 infection where at least 50% of CD8 T cell recognizes the immunodominant epitope on glycoprotein B. While subdominant epitopes found in mouse or macaque studies may not directly correlate with human epitopes, subdominant epitopes found in animal models may act as a guide to target specific viral proteins in the development of human vaccines.

A major hurdle to identifying subdominant CD8 T cell specificities is the low frequency among the virus-specific and whole CD8 T cell population. To make matters more complicated, expression of activation markers, CD69 and CD44, have proved to be ineffective at definitively demarcating viral specific CD8 T cells. Therefore, studies detecting T cell epitopes would rely on CD8 T cell stimulation with an array of viral peptides generated by plasmids or synthesized in laboratories. To get an effective assessment of the complete viral-specific CD8 T cell repertoire, overlapping peptide sequences needed to span the entire virus proteome, which becomes costly and time consuming as viruses reach the size of HSV-1, which contains over 80 ORFs [11].

Fortunately, the developments of the Immune Epitope Database and Analysis Resource (IEDB) and other epitope databases have alleviated some of the effort needed to identify CD8 T cell epitopes [94]. These databases keep records of known T- and B- cell epitopes from various infectious and animal models as well as experimental data detailing the specific interactions of peptide/MHC/TCR interactions. Algorithms can predict the probability a viral peptide sequence would also be a T cell epitope by assessing each amino acid's ability to bind to specific parts of the MHC molecule [82,95]. Epitope prediction resources do not eliminate the need for experimental stimulation data, but they do reduce the number of potential candidates. For the studies of this dissertation, with the collaboration of Sette's group, the number of potential epitope candidates was reduced from over 72,000 to just 376 peptides [96].

Upon CD8 T cell recognition of antigen, the TCR transmits a signal to activate effector function. Effector function of CD8 T cells consists of the secretion of antiviral cytokines, IFNγ and TNF α, which inhibit the transcription of viral genes. IFNγ is also known to increase MHC class I expression on host cells allowing for a greater opportunity for CD8 T cell recognition of infected cells. Additionally, CD8 T cell release lytic granules containing perforin and 11 granzymes. The mechanism by which granyzmes enter the cytosol in a perforin dependent manner is not definitively known. Once these serine proteases enter the cytosol, they cleave host proteins leading to programmed cell death or apoptosis. The most well characterized granzyme is granzyme B, which induces apoptosis in a variety of ways. It can directly activate caspase 8 triggering the apoptosis inducing caspase cascade. Also, granzyme B can cleave BID to tBID, which is inserted into the mitochondrial membrane. Insertion of tBID into the mitochondrial membrane allows the release of cytochrome c, which can also activate the apoptosis caspase cascade. Granzyme A possesses capabilities to induce apoptosis by cleaving proteins associated

with the SET complex releasing the protein, Granzyme A-Activated Deoxyribonuclease (GAAD). GAAD then gains access to the nucleus and induces apoptosis by creating single-stranded DNA nicks that cannot be repaired [97].

### 1.3.2 CD8 T cell response to HSV-1 infection

After an ocular infection, around 6 dpi, HSV-specific CD8 T cells begin to infiltrate the TG, peak in numbers by 8 dpi, and contract to a stable memory population for the life of the animal. A requirement for entry into the TG during acute infection is CD8 T cell activation. During this time OT-I specific CD8 T cells, which are superfluous to HSV-1 infection, that are activated by peptide pulsed DCs enter the TG at a high frequency but are eventually lost due to a lack of apparent antigenic stimulation [85]. Human and mouse studies show that TG-resident CD8 T cell are maintained in direct apposition to infected neurons and exhibit an activation phenotype [12,85,98]. Additionally in mice, $gB_{498-505}$-specific CD8 T cells were shown to form immunological synapses with infected neurons, providing confirmation that HSV-1 does not hide from the immune system, but rather is constantly surveyed by CD8 T cells for transition from the latent cycle to lytic cycle [86].

Unique to the TG during HSV latency, CD8 T cells exist independent of memory cytokines IL-7 and IL-15. Typically, in IL-7 and IL-15 KO mice, CD8 T cell memory would be detrimentally affected and effectively lost, which was observed in the spleens of HSV-1 infected IL-15 KO mice. Yet, in the same KO mice, HSV-specific CD8 T cells are maintained and remain functional in the TG [99]. This observation led to the hypothesis that the maintenance and function of TG-resident CD8 T cells is influenced by latent HSV rather than usual memory cytokines. In other words, we proposed that antigenic stimulation of TG-resident CD8 T cells

maintained the population independent of other factors. To support this, our lab showed that cells entering the TG that recognize an irrelevant peptide are eventually lost from the TG during latency while viral specific cells are maintained. We concluded from this that retention of CD8 T cells in the TG requires antigenic stimulation from latent virus [85]. This then led to the hypothesis that the remaining 50% of CD8 T cells (non-$gB_{498-505}$-specific CD8 T cells) in the TG during acute infection and through latency were specific for HSV antigens.

The ability to prevent viral reactivation has been shown to be dependent on the CD8 T cell response. Whenever TGs are explanted and dispersed into ex vivo cultures, HSV [29,100] will spontaneously reactivate from a small number of infected neurons. Depletion of $CD45^+$ cells from these cultures exacerbates reactivation frequency while the addition of recombinant IFN-γ to depleted cultures restores a level of protection equal to 50% of control treated groups [101]. By inhibiting CD8 and/or CD4 T cell function, our lab revealed that the main producer of IFN-γ was CD8 T cells [41]. Later studies indicated that CD8 T cells also prevent viral reactivation in a perforin dependent manner without depleting the pool of latently infected neurons, which suggested an exciting possibility that CD8 T cells use lytic granules in a noncytolytic manner (as compared to the description above) to prevent viral reactivation. Indeed, our lab displayed that CD8 T cells release lytic granules containing granzyme B into latently infected neurons. Rather than inducing apoptosis, it was shown that granzyme B cleaves ICP4, an essential viral protein needed for viral gene expression. These findings suggest a complex interplay between CD8 T cell effector function, sensory neurons, and HSV-1 that permits the retention of latent virus without expanse damage to host neurons [43](Figure 4).

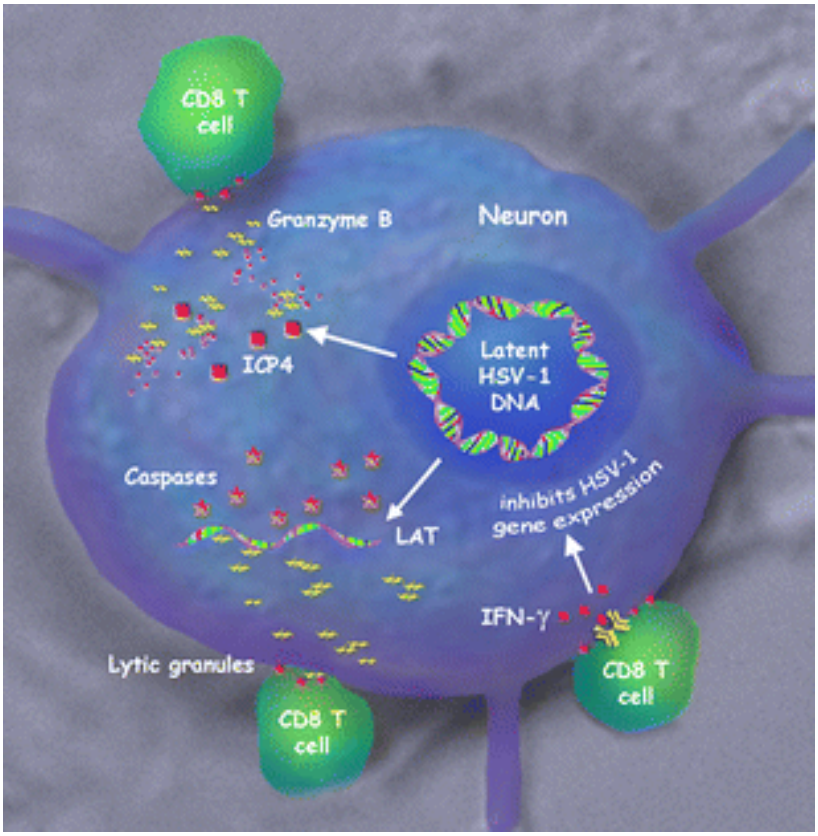


**Figure 4. Cartoon rendition of CD8 T cell maintenance of latency in sensory ganglia**

CD8 T cells recognize viral antigen presented by MHC class I molecules on latently infected neurons. IFN-γ inhibits viral gene expression while granzyme B cleavage of apoptosis inducing caspases is inhibited by ICP4 and LATs.

### 1.3.3 Immune suppression during chronic antigenic stimulation

In chronic viral infections such as LCMV, HCV, and HIV, long term antigenic stimulation from persistent infection correlates with loss of CD8 T cell functionality and a failure to eliminate the virus [102-105]. In LCMV clone 13 infections, loss of CD8 T cell functionality due to overstimulation, considered functional exhaustion, has been characterized by a serial loss of IL-2, TNF α, IFN-γ, and granzyme B [106]. Most severe cases of exhaustion end in CD8 T cell deletion. Loss of functionality typically results from the acquisition of inhibitory receptors and the production of immunosuppressive cytokines.

Functionally exhausted cells express an array of inhibitory receptors such as Tim-3, CD160, cytotoxic T-lymphocyte antigen (CTLA)-4, lymphocyte-activation gene (LAG)-3, and the most extensively studied, programmed death (PD)-1 [103,106-108]. PD-1 expression can be induced on CD4 T cells, CD8 T cells, NK cells, and B cells upon antigenic stimulation as well as activated monocytes. PD-1 has two ligands; PD-L1 (B7-H1), which is expressed on both hematopoietic and non-hematopoietic cells and can be induced with type I and II interferons, and PD-L2, which is selectively expressed on DCs an macrophages [109]. Interaction of PD-1 on T cells and PD-L1 on antigen presenting cells dampens and regulates the T cell response by initiating the recruitment of phosphatases, SHP-1 and SHP-2, to the immunological synapse. This results in the dephosphorylation of the signaling intermediates, CD3ε, ZAP70, and PKCθ, which are necessary for transmitting signals for effector T cell function. The PD-1/PD-L1 pathway continues to be a necessary mechanism in the prevention of autoimmunity, but when this pathway results in a dampened immune response to infection, prolonged pathogen persistence occurs [110]. Active studies in our lab are investigating the nature of CD8 T cell exhaustion due to PD-1/PD-L1 interactions.

In addition to inhibitory receptors, soluble mediators such as IL-10 contribute to the CD8 T cell exhaustion phenotype. Increased levels of IL-10 have been detected in chronic viral infections as well as some cancers. Studies using LCMV have shown that DCs and CD4 T cells are main sources of IL-10 during persistent infection [111-114]. More recent studies have shown that viral specific CD8 T cells have the potential to produce IL-10 as well [115]. After IL-10 binds its receptor, activated Janus Kinase (JAK)-1 and Tyrosine Kinase (TYK)-2 phosphorylate STAT3, which in turn homodimerizes, enters the nucleus, and initiates synthesis of suppressor of cytokine signaling (SOCS)-3. SOCS3 is known to inhibit the translation of TNF α, which is one

of the first effectors lost during functional exhaustion. Additionally, reports show that IL-10 signaling leads to reduced IFN-γ production and reduced expression of the costimulatory molecules, CD80 and CD86 [112,116]. Recently, studies have neutralized the immunosuppressive effects of IL-10 by genetically knocking out the IL-10 allele or have administered antibodies that bind IL-10R preventing IL-10/IL-10R interaction and signaling. Pronounced effects of this treatment on partially exhausted cells include increased functionality and proliferation, which results in reduced or eliminated viral burden [117-119]. However, the effects of this treatment after the establishment of chronic infection and complete functional exhaustion have yet to be investigated.

In herpesvirus infections, IL-10 is an attractive target for therapy due to the presence of IL-10 homologs encoded by Epstein-Barr virus (EBV) and Cytomegalovirus (CMV), which are thought play a role in the persistence of the virus in the host [120,121]. Even though HSV-1 does not contain a gene that encodes a viral homolog of IL-10, IL-10 mRNA is produced in the sensory ganglia during latency [122]. Aspects of this study describe the striking role IL-10 plays in viral persistence during HSV-1 latent infection.

### 1.3.4 Functionality of HSV-specific CD8 T cells

Nearly all studies investigating the TG-resident CD8 T cell response draw on the observations from $gB_{498-505}$-specific CD8 T cells. As stated above, these cells constantly survey the TG for the presentation of their cognate antigen on infected neurons. Interestingly, from the transition from acute infection to latent infection, these cells are considered to increase functionality [76]. In other words, these cells acquire the ability to produce multiple cytokines (IFN-γ and TNF α) contrasting what is observed in chronic infection models such as LCMV where antigen specific

cells lose the ability to produce cytokines and are eventually deleted. Potential explanations for this lack of exhaustion may include low level gene expression during latent infection as opposed to chronic infection. It appears that $gB_{498-505}$-specific CD8 T cells do not receive constant stimulation, but do receive periodic stimulation as seen by *in situ* TCR polarization [86]. This low level stimulation appears to be enough to keep $gB_{498-505}$-specific CD8 T cells from leaving the TG during latency while also preventing exhaustion. Support for this comes from the high level of granzyme B expression and concordant low level of PD-1 expression seen in $gB_{498-505}$-specific CD8 T cells throughout latency [76]. The high functionality of these cells is sufficient to prevent viral reactivation *in vivo* and in ex vivo TG cultures [100], but cannot eliminate the virus from the host. What is not known and what is described in this study is functionality of non-$gB_{498-505}$-specific CD8 T cells and their ability to prevent viral reactivation from latency.

## 1.4 DOWNFALLS OF CURRENT TREATMENTS

Most HSV-1 induced pathology is associated with recurrent viral shedding at the corneal surface induced by psychological and environmental stress, CD8 T cell depletion, ultraviolet light, increased female sex hormone, as well as other factors. Treatment of blindness due to HSV-1 mediated pathology ultimately may require surgical transplantation of a donor cornea. Unfortunately, the success of these grafts is limited, which is thought to be due to subsequent recurrent reactivation events. Current vaccines aimed to prevent infection focus on enhancing the antibody response against HSV-1, but clinical trials have proven futile [123-125]. HSV-1 antibodies help prevent primary infection and extracellular spread of the virus but appear to provide minimal benefits in preventing reactivation and host-to-host spread of the virus. Thus,

the development of a vaccine aimed at bolstering the TG-resident CD8 T cell response has garnered increasing interest. The studies in this dissertation first investigate the efficacy of a CD8 T cell adoptive transfer therapy. We then assess the use of antibody inhibition of suppressive cytokine signaling to prevent HSV-1 viral reactivation from latency.

## 2.0 SPECIFIC AIMS

Many propose that therapeutic therapies for recurrent HSV-1 reactivation events would aim to enhance the CD8 T cell response maintaining viral latency in the trigeminal ganglion (TG). The aims of this study investigate various methods to increase the proficiency of the CD8 T cell response against HSV-1 to prevent viral reactivation from latency. To effectively do this, we first needed to identify the specificities of HSV-specific CD8 T cells. We then assessed the efficacy of an adoptive transfer of CD8 T cells and the manipulation of immune suppression to confer better protection of viral reactivation from latency.

**Identify the subdominant HSV-1 epitopes that are targeted by the CD8 T cells in acutely infected TG and trace their retention in latently infected TG.**

Our lab's previous work displayed that antigenic stimulation was necessary for the retention of CD8 T cells in the latently infected TG. 50% of the TG-resident CD8 T cells from acute infection throughout latency recognize the immunodominant epitope, $gB_{498\text{-}505}$. Therefore, *we hypothesized that the remaining CD8 T cells in the TG would recognize subdominant epitopes on HSV-1 and would be maintained throughout latency.*

**Assess the ability of HSV-specific CD8 T cells to migrate into a latently infected TG.**

Studies show the prevalence of tissue-resident memory ($T_{rm}$) populations in central nervous system tissue such as the brain. $T_{rm}$ populations do not receive replenishment of effector cells from the circulation. From this, *we hypothesized that adoptively transferred effector or memory CD8 T cells would not gain access into the latently infected TG.*

**Investigate the role of host suppression of the immune response in HSV-1 viral persistence.**

During chronic viral infections, IL-10 aids viral persistence by suppressing effector functions of viral specific CD8 T cells. Also, IL-10 has been detected in latently infected trigeminal ganglia leading to our hypothesis that *IL-10 suppresses TG-resident CD8 T cells leading to the retention of HSV-1 in sensory ganglia.*

# 3.0 MATERIALS AND METHODS

## 3.1 MICE AND HSV-1 CORNEAL INFECTIONS

Six- to 10-week-old female C57BL/6J (B6), B6.129S6-Il10$^{tm1Flv}$/J and gB-TI.1β TG (gB-T; kindly provided by Dr. Francis R. Carbone, University of Melbourne) mice were used. Mice were anesthetized by intraperitoneal (i.p.) injection of 2.5 mg ketamine hydrochloride and 0.25 mg of xylazine (Butler-Schein, Dublin, OH) in 0.2 mL of Hanks balanced salt solution (BioWhittaker, Walkersville, MD). The central cornea was scarified 16 times by forming a perpendicular meshwork using a sterile 30-gauge needle. The abraded corneas were then infected by topical application of 3 μL of RPMI (BioWhittaker) containing 1 x $10^5$ plaque-forming units (PFU) of WT HSV-1 strain RE. Infection efficiency was determined by making a 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 animal experiments were conducted in accordance with guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

## 3.2 VIRUS

HSV-1 strain RE 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.

## 3.3 REAGENTS FOR FLOW CYTOMETRY

PE-conjugated H-2K$^b$ tetramers complexed with the $gB_{498–505}$, $RR1_{982–989}$, $RR1_{822–829}$, or $ICP8_{876–883}$, peptides and PE-conjugated H-2D$^b$ tetramers complexed with the $ICP8_{168–176}$, $RR1_{372–380}$, or ribonucleotide reductase 2 ($RR2_{279–287}$) peptide were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Purified or Fluorochrome-conjugated antibodies specific for surface markers, CD16/CD32 (Fc block; 2.4G2), CD45 (30-F11), CD8α (clone 53-6.7), CD4 (RM4-5), VLA-4 (RI-2), LFA-1 (2D7), CD69 (H1.2 F3), CD90.1 (OX-7), CD90.2 (53.21), CD45.1 (A20), CD279 (RPM1-30), CD107a (1d45) and CD45.2 (I04) were purchased from BD Pharmingen, and Biolegend (CD279) (San Diego, CA). Fluorchrome-conjugated antibodies specific for intracellular IFN-γ (XMG1.2), TNF (MP6-XT22), and 5-bromo-2-deoxyuridine (BrdU Flow Kit; 3D4) were purchased from BD Pharmingen. APC-conjugated antibody specific for intracellular antigen human granzyme B (GB11; cross reacts with murine granzyme B) was purchase from Invitrogen (Carlsbad, CA). The appropriate isotype control antibodies were purchased from BD

Pharmingen, Biolegen, and Millipore. All flow samples were collected on a FACSAria cytometer and analyzed by FACSDiva software (BD Biosciences).

## 3.4 TISSUE HARVEST

At the indicated dpi, anesthetized mice were injected with 0.3 ml heparin 1000 U/ml and euthanized by exsanguination or intracardial perfusion with 20 ml of 1X PBS. Trigeminal ganglia (TG) or lungs were harvested and digested in 100 µl (gangion) or 1 ml (lungs) of DMEM (BioWhittaker) containing 10% FCS and 400 U/ml collagenase type I (Sigma-Aldrich) for 1 h at 37°C. TG were dispersed into single-cell suspensions by trituration through a 200 µl pipette tip. Spleens were dispersed mechanically and treated with RBC lysis buffer prior to use. Tissue harvest and preparation were performed under sterile conditions.

## 3.5 MOUSE TREATMENTS

### 3.5.1 TAK-779 treatment

Where indicated, mice were treated subcutaneously with 150 µg TAK-779, a dose previously shown to effectively inhibit the function of CCR5 and CXCR3 in vivo [126]. Control mice received a subcutaneous injection of the vehicle (PBS).

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

At specified dpi, 1 mg of BrdU was given i.p. daily for two days prior to mouse sacrifice (proliferation after stress) or once 4 hours prior to mouse sacrifice (in situ proliferation after antiIL-10R treatment).

### 3.5.3 Corticosterone treatment

For 4 consecutive days beginning at various times after infection, mice were supplied with 400 µg/ml corticosterone (MP Biomedicals, Irvine, CA) in the drinking water. Freshly made corticosterone was re-supplied every other day. Corticosterone was dissolved in 30% (2-hydroxypropyl)-β-cyclodextrin (HBC; Sigma-Aldrich), prepared by dissolving 9 g HBC in 30 ml $H_2O$. 100 mg corticosterone was then dissolved in 5 ml HBC and brought to 250 ml with $H_2O$ per treated group. Thus, if two treated groups were required, 200 mg corticosterone was dissolved in 10 ml HBC and brought to 500 ml with $H_2O$.

### 3.5.4 Antibody treatment

Mice were depleted of $CD4^+$ T cells by i.p. injection of 0.15 mg of anti-CD4 antibody (clone GK1.5) at times described in the text. Systemic IL-10 signaling was inhibited by intraperitoneal (i.p.) injections of 250 ug of anti-IL-10R mAb (1B1.3A) every 3 days for up to 21 days. Control mice were treated with i.p. injection of PBS because we demonstrated in a previous study that similar treatment with a rat antibody of irrelevant specificity did not affect the CD8+ T cell infiltrate in HSV-1 infected TG (Sheridan et al., 2009).

### 3.5.5 Saporin conjugated gB-tetramer

Biotinylated H-2K$^b$ monomers complexed with the $gB_{498-505}$ peptide were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Streptavidin conjugated saporin (SA-ZAP) was purchased from Advanced Targeting (San Diego, CA). To complex the monomers and SA-ZAP, we added 4X molar quantities of monomers to 1X molar quantities of SA-ZAP. Specifically, we would add 1/10 the necessary quantity of toxin to the monomers every 10 min at room temperature for 100 min. To deplete $gB_{498-505}$-specific CD8 T cells *in vivo*, 30 pmol of the complexed toxin was intravenously (i.v.) injected at specified times.

## 3.6 QUANTITATIVE REAL TIME PCR

Total DNA was isolated from tissue using Qiagen DNeasy spin columns (Qiagen, Valencia, CA) and quantified by spectophotometry. Viral DNA was detected using primers specific for the HSV-1 glycoprotein H (gH): forward primer (5'-CGACCACCAGAAAACCCTCTTT-3'), reverse primer (5'ACGCTCTCGTCTAGATCAAAGC-3') and probe [5'(FAM)TCCGGACCATTTTC(NFQ)-3'. The PCR reaction was carried out as previously described [127].

## 3.7 IN VITRO ACTIVATION OF $GB_{498-505}$-SPECIFIC CD8 T CELLS

Naive gBT-I transgenic spleen cells were coated for 1 h at 37°C with 1 μM gB peptide. Cells were then washed twice in HEPES-buffered Earles medium containing 2.5% (vol/vol) FCS before being cultured at a density of $1.7 \times 10^5$ cells per ml in complete medium (mouse tonicity RPMI 1640 medium: RPMI 1640 medium containing 10% (vol/vol) FCS, 2 U/ml IL-2, 50 M -mercaptoethanol, 2 mM L-glutamine, 100 U/ml of penicillin and 100 g/ml of streptomycin). After 2 d, cells were washed and IL-2 was replaced with recombinant human IL-15 (20 ng/ml; R&D Systems). Complete medium containing human IL-15 was replaced every 3-4 d, and cells were used between 10 and 14 d after initiation of the culture [128].

## 3.8 EPITOPE PREDICTION

The entire HSV-1 proteome (GI 9629378) was scanned using algorithms for peptide sequences predicted to have a high-affinity binding capacity for the MHC class I molecules H-2 $K^b$ or H-2 $D^b$. According to the reported peptide length preference of these MHC molecules, peptides comprising 8 and 9 amino acids were analyzed for H-2 $K^b$ and H-2 $D^b$ binding, respectively. For both H-2 alleles, the ANN and SMM binding prediction methods available from the Immune Epitope Database (http://www.iedb.org) were used along with matrices derived from combinatorial peptide libraries reported by Udaka et al. [129]. Each method assigns a score to a peptide based on its sequence that predicts its binding affinity for the respective MHC molecule. For each prediction method, the peptides were ranked corresponding to their predicted binding capacity. To construct a consensus from the prediction methods, the median rank of each peptide

for all applicable prediction methods was taken. Finally, for each allele, the 188 peptides with the highest median ranks, corresponding with the top 0.5% scoring peptides, were selected for screening.

Peptides used in the HSV screening studies were synthesized as crude material by Mimotopes (Clayton, VIC, Australia). Peptides used as radiolabeled ligands for binding assays, were synthesized by A and A Labs (San Diego, CA), and purified to >95% homogeneity by reverse-phase HPLC. Purity of these peptides was determined using analytical reverse-phase HPLC and amino acid analysis, sequencing, and/or mass spectrometry. Peptides were radiolabeled with the chloramine T method, as described elsewhere.

## 3.9 MHC PEPTIDE BINDING ASSAYS

MHC purification, and quantitative assays to measure the binding affinity of peptides to purified H-2 Kb and H-2 Db molecules were performed as previously described [130]. Briefly, 1–10 nM of radiolabeled peptide was co-incubated at room temperature with 1 μM to 1 nM of purified MHC in the presence of 1–3 μM human $\beta_2$-microglobulin (Scripps Laboratories, San Diego, CA) and a mixture of protease inhibitors. After a 2-d incubation, binding of the radiolabeled peptide to the corresponding MHC class I molecule was determined by capturing MHC–peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-one, Longwood, FL) coated with either the Y3 (anti–H-2 Kb) or 28-14-8s (anti–H-2 Db, Ld, and Dq) Ab and measuring bound cpm using the TopCount microscintillation counter (Packard Instrument Co.).

For competition assays, the concentration of peptide yielding 50% inhibition of the binding of the radiolabeled peptide was calculated. Peptides were typically tested at six different concentrations covering a 100,000-fold dose range, and in three or more independent assays. Under the conditions used, where (label) < (MHC) and $IC_{50} \geq$ (MHC), the measured $IC_{50}$ values are reasonable approximations of the true $K_d$ values [131,132].

## 3.10 TETRAMER RELEASE ASSAY

Tetramer release assay was performed as described [133]. Single-cell TG suspensions were stained with $gB_{498–505}$ or $RR1_{982–989}$ for 1 h at 37°C, and the cells were washed and then incubated with anti–H-$2D^b/K^b$ Ab to avoid tetramer rebinding (28-8-6; BD Pharmingen) at 37°C for the designated times. Cells were then stained with anti-CD8 and anti-CD45 and analyzed via flow cytometry to observe loss of tetramer over time.

## 3.11 IN VITRO STIMULATION AND INTRACELLULAR STAINING

B6WT3 fibroblast targets were pulsed with individual or pooled peptides, $RR1_{982-989}$, $RR1_{822-829}$, $RR1_{372-379}$, $ICP8_{171-178}$, $ICP8_{168-174}$ or $ICP_{8876-883}$, at a concentration of 1.0 ug/mL for 45 min at 37$_o$C/5% $CO_2$. Dispersed TG cells were stimulated with peptide pulsed fibroblasts in RPMI 1640 containing 10% FBS, 50 uM 2-Mercaptoethanol (Fisher), anti-CD107a and Golgi-Plug (BD Biosciences) for 6 h at 37$_o$C/5% $CO_2$. After stimulation, cells were stained for surface expression

of CD8α, followed by intracellular staining for IFN-γ after permeabilization and fixation via Cytofix/Cytoperm (BD Biosciences).

For granzyme B expression or BrdU incorporation, dispersed TGs were stained with anti-CD45, CD8α and $gB_{498-505}$ tetramer for 1 h at room temperature. After incubation, cells were permeabilized and fixed with Cytofix/Cytoperm (BD Biosciences) and stained for intracellular granzyme B, or cells were fixed and stained for BrdU per manufacturer's instructions using the BD Phamingen BrdU FITC Flow Kit (BD Phamigen).

# 4.0 DEFINING THE HSV-1 SPECIFIC CD8 T CELL REPERTOIRE IN C57BL/6 MICE

In mice and other species, viruses typically induce a CD8 T cell response that targets a very small fraction of the viral proteins and the potential epitopes they contain. Those viral epitopes that are targeted typically fall into a dominance hierarchy consisting of one or a few dominant epitopes and several other subdominant epitopes. From acute infection throughout latency in the C57BL/6 mouse model of HSV-1 ocular infection, 50% of TG-resident CD8 T cells recognize the immunodominant epitope on HSV-1 glycoprotein B ($gB_{498-505}$) and about 5% of CD8 T cells recognize a previously defined subdominant epitope on ribonucleotide reductase I ($RR1_{822-829}$) [50,85,86]. Upon investigating the kinetics of the CD8 T cell response during HSV-1 infection, our lab has shown that CD8 T cells peak in number by 8 dpi and contract to form a stable memory population by 35 dpi. Our previous studies also strongly suggested that cells infiltrating the TG at 8 dpi and persist through latency are indeed HSV-specific. To better characterize the CD8 T cell response in hopes of making it more effective against HSV-1 reactivation, our initial studies, in collaboration with Dr. Alessandro Sette's laboratory, aimed at identifying the remaining epitopes that are recognized by non-$gB_{498-505}$-specific CD8 T cells.

## 4.1 THE 8 DPI HSV-SPECIFIC CD8 T CELL REPERTOIRE

After scanning the proteome of HSV-1 strain RE, the laboratory of Dr. Alessandro Sette used a combination of MHC binding algorithms as described in the Materials and Methods section of this dissertation to determine potential HSV-1 CD8 T cell epitopes. In total, 376 peptides representing the top 0.5% candidate peptides that bound strongly to $K^b$ and $D^b$ MHC molecules were selected for analysis. We felt the best method of assessing potential candidates was to perform *in vitro* stimulations of tissues thought to contain viral-specific CD8 T cells with peptide pulsed B6WT3 fibroblasts. Eight days post infection (dpi) TG provided an environment highly enriched for HSV-specific CD8 T cells due to our previous studies suggesting that only HSV-specific CD8 T cells enter the TG and peak in numbers at 8 dpi [85]. To initially narrow down the possible candidates, we pooled 25 candidate peptides and loaded B6WT3 fibroblasts with pools of peptides. We performed 6-h *in vitro* stimulations by incubating peptide pulsed fibroblasts with dispersed 8 dpi TG cells. Flow cytometric detection of intracellular IFN-γ identified pools of peptides containing HSV epitopes. After positive pools were identified, we loaded fibroblasts with individual peptides from positive pools. Again, after a 6-h *in vitro* stimulation of TG cells with fibroblasts, flow cytometric analysis resolved the frequency of CD8 T cells responding to a single epitope by detecting the percentage of CD8 T cells with measurable amounts of intracellular IFN-γ. The screening of potential epitope candidates resulted in identifying 19 individual epitopes that included the immunodominant epitope, $gB_{498-505}$, the known subdominant epitope, $RR1_{822-829}$, and 17 previously unidentified subdominant epitopes (Figure 5). In aggregate, 87.77 ± 9.22% of TG CD8 T cells responded to defined HSV-epitopes.

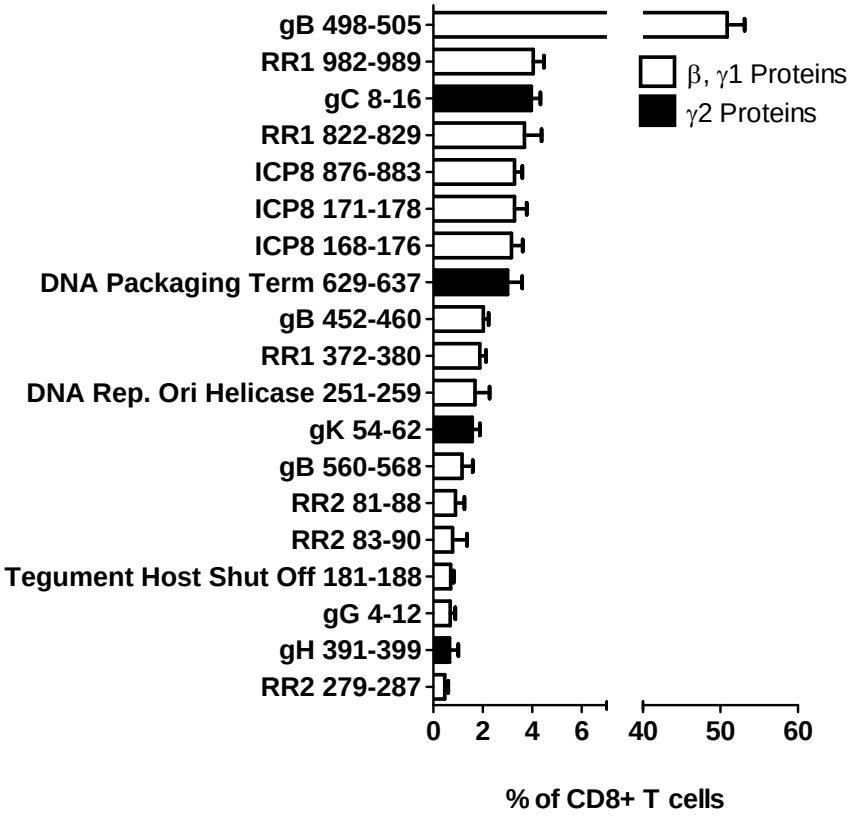


**Figure 5 One dominant and 18 subdominant epitopes define the HSV-specific CD8 T cell repertoire in the trigeminal ganglion (TG) of C57BL/6 (B6) mice**

TG from 8 dpi mice were dispersed into single-cell suspensions. Tissues were then incubated for 6 h with peptide-loaded B6WT3 fibroblasts in the presence of Golgi-Plug, followed by intracellular staining for IFN-γ. Bars represent the cumulative mean ± SEM percentage of TG-resident CD8 T cells that produce IFN-γ (total n=5, two experiments).

To assess the HSV repertoire in lymphoid tissue, we performed *in vitro* stimulations (as described above) of dispersed 8 dpi spleen CD8 T cells with peptide pulsed fibroblasts (Figure 6). In total, 19.84 ± 4.13% of splenic CD8 T cells responded to HSV-epitopes.

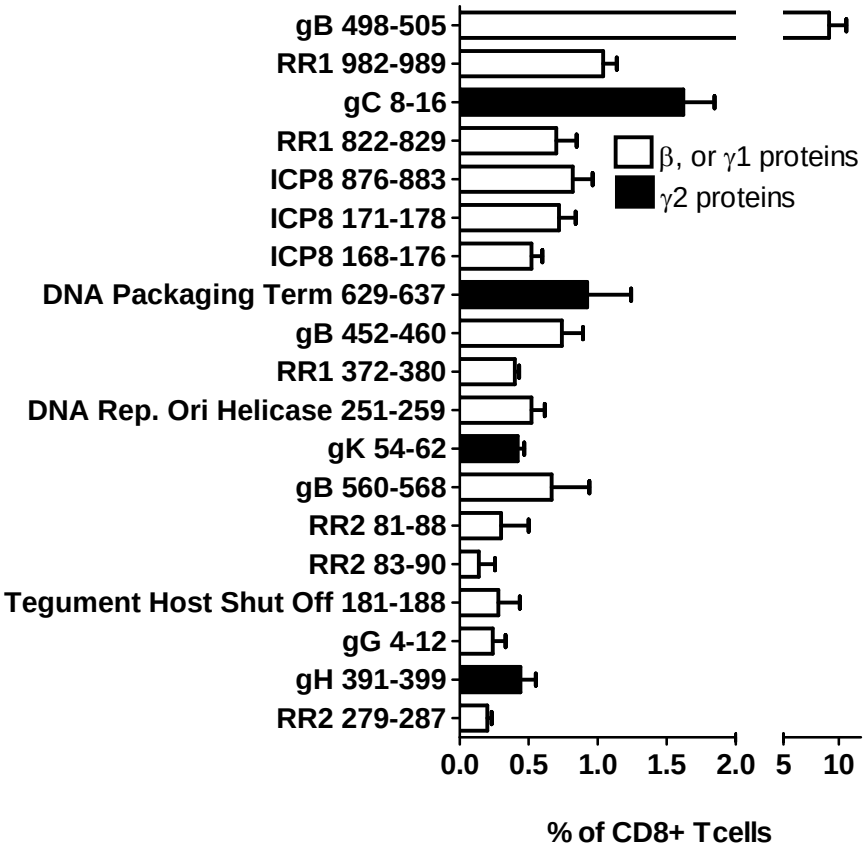


**Figure 6 One dominant and 18 subdominant epitopes define the HSV-specific CD8 TCR repertoire in the spleen of C57BL/6 (B6) mice**

Spleens from 8 dpi mice were dispersed into single-cell suspensions. Tissues were then incubated for 6 h with peptide-loaded B6WT3 fibroblasts in the presence of Golgi-Plug, followed by intracellular staining for IFN-γ. Bars represent the cumulative mean ± SEM percentage of TG-resident CD8 T cells that produce IFN-γ (total n=5, two experiments).

We next wanted to compare the frequencies of subdominant epitopes in the TG and the spleen to assess the possibility of preferential expansion or exclusion of CD8 T cells specific for subdominant epitopes in the TG. To start, 8 dpi TG and spleens were stimulated with HSV-infected fibroblasts. After stimulation with HSV-infected fibroblasts, as many as 80% of CD8 T cells in TG produced IFN-γ. Additionally, 17.3 ± 2.0% of splenic CD8 T cells responded to HSV-1 infected fibroblasts. Due to the high frequency of TG-resident CD8 T cells stimulated by HSV-1 infected fibroblasts, and the fact that HSV-1 infected fibroblasts stimulated a nearly

identical frequency of splenic CD8 T cells as the cumulative frequency of CD8 T cells responding to HSV-epitopes, we presumed that the fibroblasts presented all the HSV-epitopes. Therefore, we could calculate the frequency splenic HSV-specific CD8 T cells specific for individual epitopes (Figure 7).

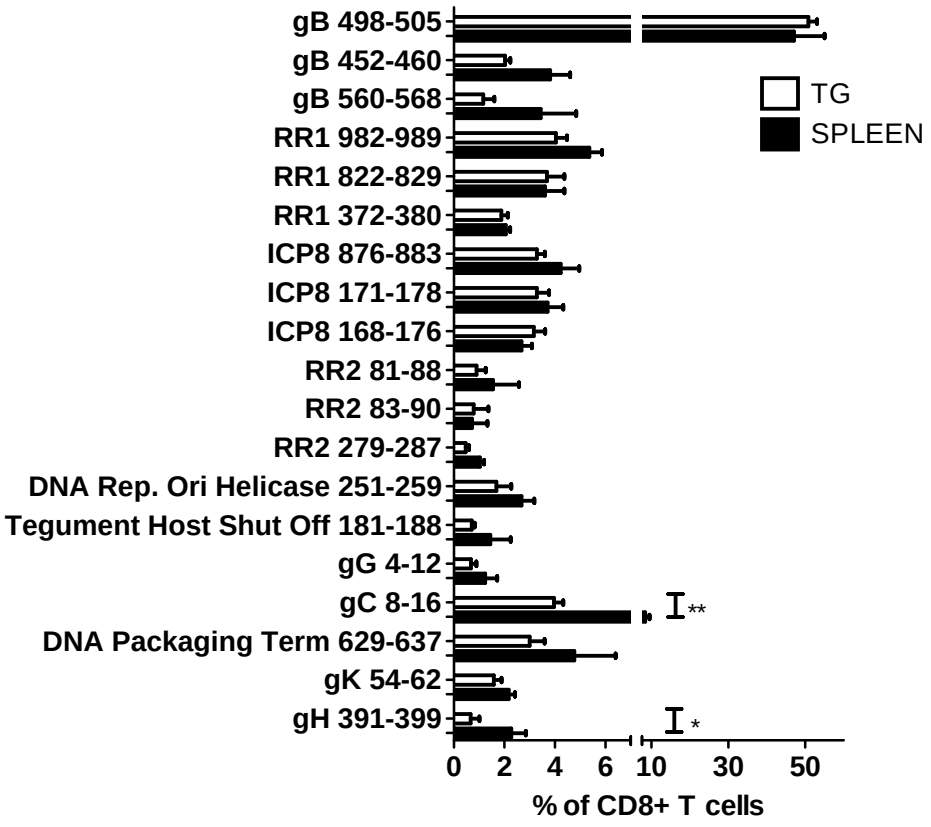


**Figure 7 Comparison of HSV-specific CD8 T cells in the TG and spleen that respond to HSV-1 epitopes**

Frequencies were calculated by dividing the % stimulation via HSV-infected fibroblasts by the % stimulation via peptide pulsed fibroblasts (Figure 4 &5) in the TG and spleen.

With these analyses (Figure 5-7), we were able to make several conclusions that directed our future observations. First, we concluded that we identified the entire CD8 T cell repertoire during acute HSV-1 infection. Importantly, the repertoire is consistent between the site of infection (TG) and the peripheral lymphoid tissues (spleen), suggesting there is universal expansion of CD8 T cell specificities in the TG.

The HSV-specific CD8 T cell repertoire targets a restricted array of HSV-1 proteins that are derived from only 11 of approximately 84 HSV-1 proteins (Table 1). There appears to be no α gene products targeted, but 11 of 19 epitopes and 4 of 19 epitopes are derived from β or γ1 gene products respectively. Thus, nearly 80% of the epitopes are derived from viral proteins that are expressed prior to DNA synthesis, which further supports the role these cells play in preventing viral reactivation from latency. Even though HSV-specific CD8 T cells recognize 19 epitopes on 11 proteins, nearly 75% of the CD8 T cell response is directed toward epitopes on three viral proteins: gB (53.18% spleen/61.58% TG), RR1 (10.79% spleen/10.93% TG), and ICP8 (10.38% spleen/11.08% TG).

**Table 2 Epitopes recognized by HSV-specific CD8 T cells in spleens and TG**

| | | | | | | SPLEEN | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sequence** | **Pro** | **Type** | **Pos.[1]** | **MHC (H-2)** | **Binding Capacity ($IC_{50}$, nM)** | **CD8 (%)** | **HSV-CD8+ (%)[2]** | **TG Percentage of CD8[3]** | **TG Percentage of Spleen[4]** |
| SSIEFARL | gB | γ1 | 498 | $K^b$ | 0.78 | 9.14 | 46.07 | 57.9 | 125.81 |
| YQPLLSNTL | gB | γ1 | 452 | $D^b$ | 5153 | 0.74 | 3.73 | 2.30 | 61.62 |
| SARMLGDVM | gB | γ1 | 560 | $D^b$ | 9206 | 0.67 | 3.38 | 1.32 | 39.05 |
| Total gB | | | | | | 10.55 | 53.18 | 61.58 | 115.80 |
| GAMRAVVPI | gG | γ1 | 4 | $D^b$ | 499 | 0.24 | 1.21 | 0.78 | 64.46 |
| Total γ1 | | | | | | 10.79 | 54.39 | 62.36 | 114.65 |
| FAPLFTNL | RR1 | β | 982 | $K^b$ | <0.5 | 1.04 | 5.24 | 4.60 | 87.79 |
| QTFDFGRL | RR1 | β | 822 | $K^b$ | 2.1 | 0.7 | 3.53 | 4.19 | 118.70 |
| FGLLNYALV | RR1 | β | 372 | $D^b$ | 1.0 | 0.4 | 2.02 | 2.14 | 105.94 |

[1] Pos., starting amino acid position; Pro., protein; UL9, DNA replication origin-binding helicase; UL28, DNA replication origin-binding helicase; UL41, tegument host shutoff protein.

[2] The % of CD8 T cells stimulated by the epitope divided by the total % of CD8 T cells stimulated with all HSV epitopes (19.84%).

[3] The % of $CD8^+$ T cells stimulated by the epitope divided by the total % of $CD8^+$ T cells stimulated with all HSV epitopes (87.77%).

[4] % TG divided by % spleen.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total RR1 | | | | | | 2.14 | 10.79 | 10.93 | 101.30 |
| GAINFINL | ICP8 | β | 876 | $K^b$ | 6.2 | 0.82 | 4.13 | 3.74 | 90.56 |
| INNTFLHL | ICP8 | β | 171 | $K^b$ | 2.0 | 0.72 | 3.63 | 3.74 | 103.03 |
| AVCINNTFL | ICP8 | β | 168 | $D^b$ | 450 | 0.52 | 2.62 | 3.60 | 137.41 |
| Total ICP8 | | | | | | 2.06 | 10.38 | 11.08 | 106.74 |
| SFYRFLFA | RR2 | β | 81 | $K^b$ | 64 | 0.3 | 1.51 | 1.03 | 68.21 |
| YRFLFAFL | RR2 | β | 83 | $K^b$ | 5.4 | 0.14 | 0.71 | 0.89 | 125.35 |
| AAIENYVRF | RR2 | β | 279 | $D^b$ | 38 | 0.2 | 1.01 | 0.52 | 51.49 |
| Total RR2 | | | | | | 0.64 | 3.23 | 2.44 | 75.54 |
| FLPRLGTEL | UL9 | β | 251 | $D^b$ | 3049 | 0.52 | 2.62 | 1.91 | 72.90 |
| LGYAYINS | UL41 | β | 181 | $K^b$ | 10 | 0.28 | 1.41 | 0.80 | 56.74 |
| Total β | | | | | | 5.64 | 28.43 | 27.16 | 95.53 |
| LAVVLWSLL | gC | γ2 | 8 | $D^b$ | 2781 | 1.62 | 8.17 | 4.51 | 55.20 |
| YSVENVGLL | UL28 | γ2 | 629 | $D^b$ | 1.9 | 0.93 | 4.69 | 3.42 | 72.92 |
| FAFVNAAHA | gH | γ2 | 391 | $D^b$ | 4.9 | 0.44 | 2.22 | 0.75 | 33.78 |
| WMKMNQTLL | gK | γ2 | 54 | $D^b$ | 131 | 0.42 | 2.12 | 1.80 | 84.91 |
| Total γ2 | | | | | | 3.41 | 17.20 | 10.18 | 59.20 |
| Total subdom. | | | | | | 10.70 | 53.95 | 36.90 | 68.40 |
| Total | | | | | | 19.84 | 100.02 | 99.7 | |

## 4.2 EFFECTOR FUNCTIONS OF NON-$GB_{498-505}$-SPECIFIC CD8 T CELLS

Optimal control of viruses, including HSV-1, has been associated with CD8 T cell expression of granzyme B and production of IFN-γ and TNFα. Consistent with previous reports [76,85], nearly all of the CD8 effector T cells in the TG at 8 dpi that are specific for the immunodominant $gB_{498–505}$ epitope express granzyme B (Figure 8 A&B). To evaluate granzyme B expression in non-$gB_{498-505}$-specific CD8 T cells, the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA) provided us with fluorescently

tagged tetramers complexed with 8 different subdominant HSV-1 epitopes. These reagents allowed us to measure intracellular granzyme B levels directly ex vivo in non-$gB_{498-505}$-specific CD8 T cells using flow cytometric analysis. Our results showed that consistent with $gB_{498-505}$-specific CD8 T cells, non-$gB_{498-505}$-specific CD8 T cells express high levels of granzyme B. Even though these cells express high levels of granzyme B during acute infection, the frequency of cells expressing granzyme B is slightly reduced compared to the immunodominant CD8 T cells ($p \leq 0.025$).

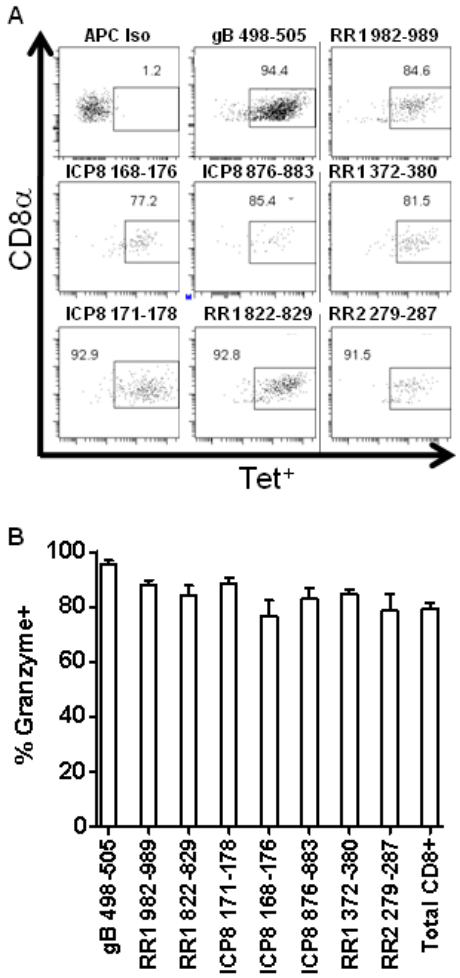


**Figure 8 CD8 T cells specific for subdominant epitopes from 8 dpi TG express granzyme B.**

(**A&B**) Dispersed TG were stained for 1 h at room temperature with αCD45, CD8α, and tetramers loaded with subdominant epitopes followed by intracellular staining for granzyme B expression. (**A**) Representative dot plots of granzyme B expression of select HSV-specific CD8 T cells. (**B**) Bars represent cumulative mean ± SEM percentage of peptide-specific CD8 T cells that express granzyme B (total n=4, 2 experiments). Granzyme B expression is lower in non-gB-specific CD8 T cells compared with $gB_{498\text{-}505}$-specific CD8 T cells. The *p* value for all specificities is <0.025.

Studies propose that CD8 T cells producing TNFα in coordination with IFN-γ are CD8 T cells best suited to combat viral infections. This is especially true for $gB_{498\text{-}505}$-specific CD8 T cells that gain the ability to produce TNFα from acute infection to latency. With this, we wanted to assess the ability of non-$gB_{498\text{-}505}$-specific CD8 T cells to produce multiple cytokines upon recognition of their cognate antigen. To compare the level of functionality of the CD8 T cells

specific for immunodominant and subdominant epitopes, 8 dpi TG cells were stimulated for 6 h with targets pulsed with each of the 19 defined epitopes and stained for intracellular IFN-γ and TNFα. The frequency of multifunctional CD8 T cells that produced both IFN-γ and TNFα (as opposed to IFN-γ only) reactive to subdominant epitopes varied widely. CD8 T cells specific for some subdominant epitopes had a high frequency of multifunctional cells (≥60%), which is similar to the frequency of multifunctional cells among CD8 T cells specific for the immunodominant $gB_{498–505}$ epitope (Figure 9A). The frequency of multifunctional cells among CD8 T cells specific for other subdominant epitopes were as low as 21.85%. After plotting the percent of peptide-specific cells that were multifunctional against the frequency of that peptide-specific cell, we observed a significant correlation between the frequency of CD8 T cells specific for a given subdominant epitope in the TG and the frequency of multifunctional cells within that population (Figure 9B).

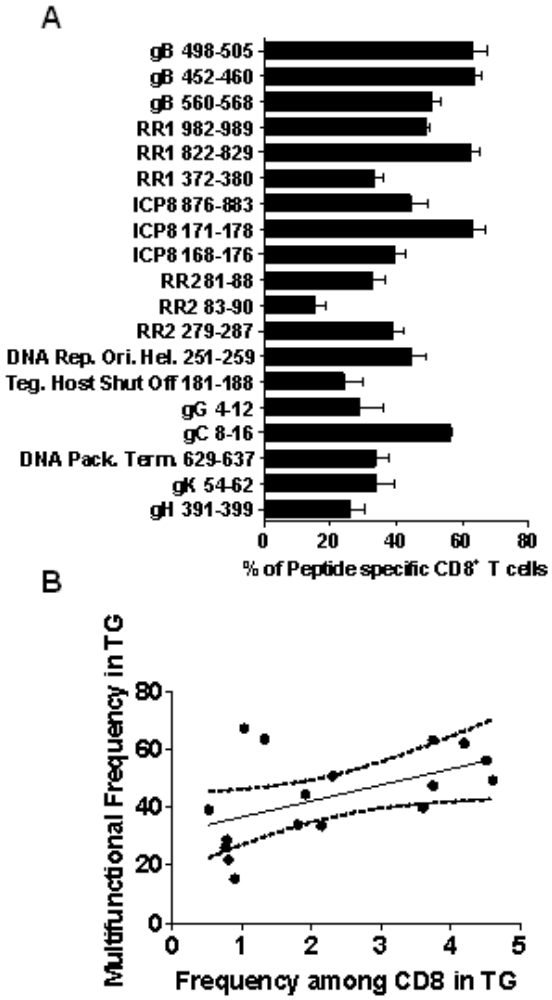


**Figure 9 Non-gB-specific CD8 T cells are multifunctional; multifunctionality correlates with immunodominance**

(**A&B**) TG from 8 dpi B6 mice were dispersed into single-cell suspensions. Tissues were then incubated for 6 h with peptide-loaded B6WT3 fibroblasts in the presence of Golgi-Plug, followed by intracellular staining for IFN-γ and TNFα. (**A**) Bars represent the cumulative mean ± SEM percentage of peptide-specific CD8 T cells that secrete IFN-γ and TNF-α when stimulated in vitro (total n=4, 2 experiments). (**B**) Data points represent percentage abundance of individual epitopes in the TG at 8 dpi versus the percentage of epitope-specific cells that are multifunctional. Linear regression shows a significant correlation ($p$ = 0.0337) between the prevalence in the TG and multifunctionality.

## 4.3 IMMUNODOMINANCE OF HSV-1 CD8 T CELL EPITOPES IS NOT DETERMINED SOLELY BY TCR AFFINITY

The correlation of multifunctionality with immunodominance directed our next set of studies looking at the determinants of immunodominance. Factors influencing the immunodominance hierarchy among T cell epitopes include MHC binding affinity of the epitope, TCR binding affinity, efficiency of peptide processing and loading on MHC, and the frequency of CD8 T cell precursors specific for a specific epitope. We ruled out MHC binding affinity as a crucial factor determining immunodominance, based on the fact that several peptides representing subdominant epitopes had MHC binding affinities that were as high as or higher than that of the immunodominant $gB_{498–505}$ epitope (Table 2). To determine if TCR binding affinity is a critical element determining immunodominance, we used a tetramer release assay to compare the TCR affinity of the immunodominant $gB_{498–505}$ epitope with that of one of the subdominant epitopes ($RR1_{982–989}$). The two epitopes exhibited comparable TCR binding affinity as indicated by the fact that the slopes of the tetramer release curves were not significantly different (Figure 10).

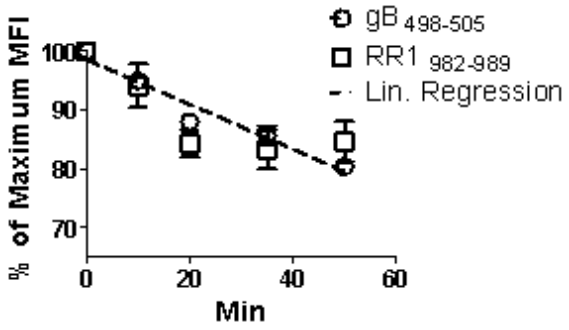


**Figure 10 Immunodominance is not solely determined by TCR affinity for MHC–peptide complexes**

Dispersed TG were stained with $gB_{498–505}$ or $RR1_{982–989}$ tetramer for 1 h at 37°C. Anti–H-$2K^b$/H-$2D^b$ blocking Ab was added, and TG were incubated for the designated times at 37°C to observe tetramer dissociation. Data represent the cumulative mean ± SEM fluorescence intensity (MFI) of $CD8^+$ $tetramer^+$ cells as a percentage (n=3 representing data from 3 independent experiments) of the maximum MFI observed at time zero. Linear regression represents the slopes of both $gB_{498–505}$ and $RR1_{982–989}$. Data are representative of three independent experiments.

## 4.4 PRECURSOR FREQUENCY INFLUENCES IMMUNODOMINANCE

Along with the affinity of peptide for MHC class I and TCR affinity of peptide/MHC class I complexes, precursor frequency has been shown to influence the immunodominance hierarchy of CD8 T cells. It is well documented that fluorescently conjugated tetramers effectively label antigen specific CD8 T cells after priming and expansion of the population from immunization or viral infections. Until recently, these reagents were somewhat poor at detecting antigen-specific CD8 T cells in naïve mice due to the low frequency of precursors and the high background noise from analysis. Upon enrichment of these cells prior to analysis, resolution of antigen specific cells in naïve mice can occur [134]. Due to the drastic disparity in immunodominance during B6 HSV-1 infection, we hypothesized that precursor frequency played a role in this disparity. Because previous studies have shown the greatest results with CD8 T cells that are higher in the immunodominance hierarchy [83,84,134] and are specific for peptides that bind strongest to MHC class I, we chose to compare $gB_{498\text{-}505}$(immunodominant)- and $RR1_{982\text{-}989}$(subdominant)-specific CD8 T cell precursor numbers. To do this, we first dispersed the spleen, cervical lymph nodes (LN), brachial LN, axillary LN, mesenteric LN, and inguinal LN from a naïve B6 mouse into single cell suspensions. We labeled whole cells from naïve mice with either $gB_{498\text{-}505}$ or $RR1_{982\text{-}989}$ tetramers conjugated to the fluorochrome PE. Magnetic isolation of the $PE^+$ population and addition of APC-conjugated tetramer allowed for resolution of antigen specific CD8 T cells from unspecific cells using flow cytometric analysis (Figure 11A & B). CD8 T cell precursors specific for $gB_{498\text{-}505}$ outnumber $RR1_{982\text{-}989}$-specific CD8 T cell precursors by 3.7 fold (Figure 11B), which is less than the 10 fold difference observed during acute infection (Figure 4). The average number of $gB_{498\text{-}505}$-specific CD8 T cells per mouse equates to 685 cells per mouse and a precursor frequency of 0.0052% (1 gB-cell in

every 19,124 CD8 T cells). There are 186 $RR1_{982-989}$-specific CD8 T cells per mouse resulting in a precursor frequency of 0.0014% (1 RR1-cell in every 70,430 CD8 T cells).

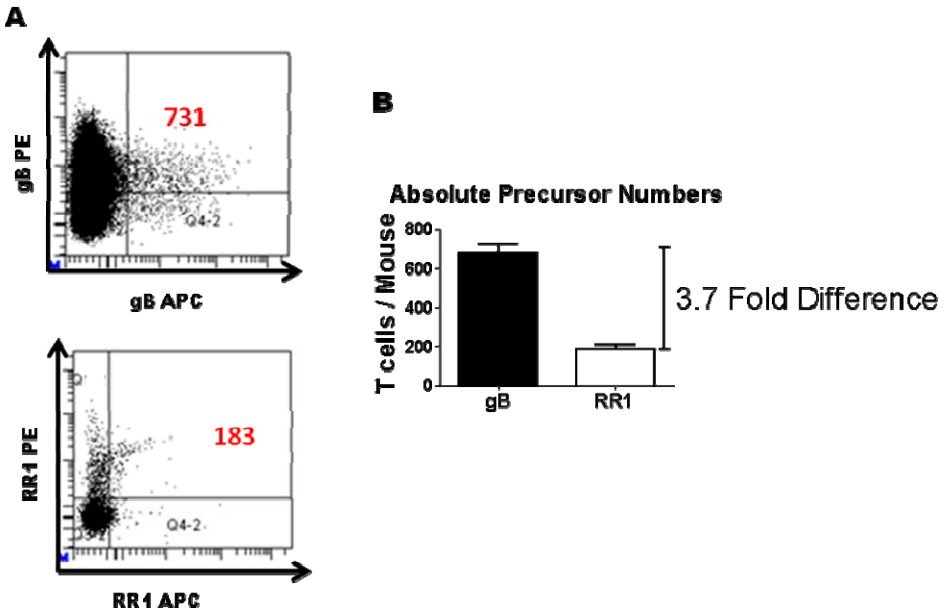


**Figure 11 CD8 T cell precursor numbers per naive mouse**

(**A&B**) CD8 T cells specific for $gB_{498-505}$ or $RR1_{982-989}$ were enumerated by magnetic separation for $PE^+$ cells and flow cytometric analysis. (**A**) Representative dots plots showing precursor numbers. Cells binding PE and APC tetramer are considered precursors. (**B**) Data represent the cumulative mean ± SEM number of antigen-specific precursors per mouse (n = 3 per specificity). Experiment was repeated with similar results. p = 0.0019.

## 4.5 DISCUSSION

After infection at mucosal sites, HSV-1 is transported to the nuclei of sensory neurons where it establishes a latent infection. In humans and mice, CD8 T cells appear to play essential roles in preventing viral reactivation [12,41,43,98,100], and theories for future vaccines center around the idea of bolstering the TG-resident CD8 T cell population [125,135]. The studies of this dissertation sought to explore and enhance the nature of the CD8 T cell response in the B6 mouse to better protect the host from viral reactivation from latency. Our initial study was

undertaken to identify the specificities of CD8 T cells in acutely infected TG as well as attempt to identify factors influencing the CD8 T cell immunodominance hierarchy. We and others have demonstrated that only activated CD8 T cells infiltrate the TG of mice during the acute phase of corneal HSV-1 infection and remain closely associated with neuronal cell bodies as the virus enters a life-long latent state [86]. Half of the CD8 T cells in the infected TG throughout infection are specific for a strongly immunodominant epitope on HSV gB, and the remaining cells in the TG were thought to also be HSV-specific. Based on our previous findings, we anticipated that CD8 T cells in the acutely infected TG would represent a highly enriched source of HSV-specific CD8 T cells that could provide sensitive detection of HSV-1 CD8 T cell epitopes.

The HSV-1 genome encodes at least 84 open reading frames [136]. Thus, the CD8 T cell response is highly selective, targeting only 19 epitopes on 11 viral proteins. The selective targeting of a minute portion of thousands of available peptide sequences is commonly seen in viral infections and other systems [82,83,137-139]. The mechanism of such selective targeting is unknown, but certain patterns emerge. For instance, viral immediate early genes are infrequent targets of CD8 T cells in mouse models of infection [80], consistent with our observation that no HSV-1 immediate early proteins are targeted. Previous studies revealed that 87% of vaccinia virus epitopes and a majority of CD8 T cell epitopes in human lesions are on proteins that are produced early in the viral life cycle [140,141]. This is consistent with our observation that ~80% of the HSV-1 CD8 T cell epitopes are on proteins that are produced before viral DNA synthesis, and nearly 80% of HSV-specific CD8 T cells target these epitopes. The kinetic class of proteins that is most frequently targeted is the Early proteins that contain 58 and 65% of CD8 T cell epitopes in HSV-1 and vaccinia virus, respectively. This is not a simple reflection of the

frequency of this class of genes in the viral genome because Early genes represent only 14.2% [11] and 35% of HSV-1 and vaccinia virus genes, respectively. Although the mechanism responsible for selective targeting of early genes is not known, the advantages to the host are obvious. Viral DNA can be infectious, and the HSV-1 genome copy number in latently infected sensory ganglia is directly correlated with reactivation frequency [29].

Recent studies have established that initial expansion of HSV-specific CD8 T cells in the draining LN of mice is induced by lymph node resident $CD8\alpha^+$ dendritic cells (DCs) [142-145]. Presumably these $CD8\alpha^+$ DCs cross-present viral Ags acquired from migratory DCs from the site of infection [146]. Both mature and immature DCs express HSV receptors nectin-1 and nectin-2 and herpesvirus entry mediator and are susceptible to HSV infection [147-149]. However, only immature DCs are susceptible to productive infection. Moreover, productive infection of immature DCs leads to apoptosis that is dependent on both early and late HSV gene products [150]. Infection of mature DCs results in an abortive infection in which HSV-1 α, β, and γ1 genes are expressed, but no infectious virions are produced [151]. HSV-1 infection of mature DC results in downregulation of CD83 expression and impaired T cell stimulatory capacity. These findings are consistent with a model in which DCs that are matured at sites of infection before being infected with HSV, are primarily responsible for transporting viral Ags to the lymph nodes for cross-presentation by $CD8\alpha^+$ DCs. Because these DCs permit only abortive HSV infections, the viral proteins they produce appear to be primarily those produced prior to viral DNA synthesis. Such a model would explain the fact that 80% of HSV-1 CD8 T cell epitopes are derived from viral proteins that are produced before HSV-1 DNA synthesis. At later stages of infection, DCs that infiltrate sites of the lesion might begin to phagocytose apoptotic or necrotic

parenchymal cells containing γ2 gene products, accounting for the small but significant response to several of these proteins.

Several factors can potentially contribute to the dominance hierarchy of viral epitopes. These include the efficiency of peptide generation by proteasomes [81], viral gene expression [80], the peptide binding affinity for MHC [83], the epitope affinity for TCR, the frequency of epitope-specific $CD8^+$ T cell precursors [83], and the ability of epitope-specific $CD8^+$ T cell precursors to expand and survive. The strong immunodominance of the $gB_{498–505}$ epitope cannot be attributed solely to the peptide affinity for MHC as several of the subdominant epitopes have similar or higher affinities. Moreover, a tetramer release assay demonstrated a similar TCR affinity for the immunodominant and subdominant $RR1_{982–989}$ epitopes. Furthermore, magnetic bead enrichment and flow cytometric analysis of $tetramer^+$ precursors from naïve B6 mice revealed that $gB_{498-505}$-specific CD8 T cell precursors out number $RR1_{982-989}$-specific CD8 T cells by 3.7 fold. Interestingly, the difference in precursor numbers does not account for the exaggerated immunodominance of $gB_{498-505}$-specific CD8 T cells. These findings suggested that the determining factor in the dominance of $gB_{498-505}$ over the $RR1_{982-989}$ epitope is related either to efficiency of peptide generation or the proliferative capacity or survival characteristics of epitope-specific CD8 T cell precursors. Our results showing a consistent CD8 T cell repertoire between the acute TG and spleen as well as the latent TG suggest that priming of these cells should be investigated. For example, different clonotypes of CD8 T cell precursors may possess more proliferative potential compared to others, thus our precursor studies may conceal a greater divide between epitope-specific CD8 T cells. Also during priming, evidence shows that immunodominant CD8 T cells become armed with immunosuppressive cytokines such as interleukin (IL)-10 to suppress the proliferation/survival of subdominant CD8 T cells [115].

Addressing the potential of $gB_{498-505}$-specific CD8 T cells to suppress the subdominant CD8 T cell response may illuminate immunosuppressive targets to diversify the HSV-specific repertoire, which would confer better protection from viral reactivation from latency.

Targeting 50% of the HSV-specific CD8 T cell repertoire to a single immunodominant epitope would seem to place the host at significant risk should that peptide be mutated. However, a recent study showed that a recombinant virus in which the $gB_{498–505}$ epitope was mutated failed to expand $gB_{498–505}$-specific CD8 T cells, but the magnitude of the HSV-specific CD8 T cell response and viral clearance were not altered [152]. In this study, we show that CD8 T cells specific for most of the subdominant epitopes exhibit functional characteristics similar to those reactive to the dominant epitope during acute infection. In other words, subdominant HSV-specific CD8 T cells possess the mechanisms necessary to combat viral reactivation.

Our initial studies have important implications for the development of HSV vaccines. Murine studies show that immunization with CD8 T cell epitopes generates effector and memory CD8 T cells and reduces HSV lethality and viral load in the CNS [153,154]. Although the exact epitopes recognized by human CD8 T cells will likely be different [52,155], our findings and the study from Laing et al. [52] suggest that generating CD8 T cells targeting β and γ1 proteins before infection might prove to be a good strategy for vaccine development. Moreover, although epitopes from HSV-1 γ2 proteins are recognized by only 20% of the HSV-specific CD8 T cell repertoire in mice, our previous findings suggest that IFN-γ can block HSV-1 reactivation from latency even at a point after γ2 gene expression [41]. Thus, targeting these gene products might also be useful in controlling both lytic infections and reactivation from latency.

The practicality of immunizing humans prior to primary infection remains questionable due to the inability to diagnose primary infection. Therefore, a paramount vaccine would be a

therapeutic treatment enhancing the latent TG-resident CD8 T cell population either by increasing CD8 T cell numbers or by increasing functionality of the TG-resident CD8 T cell population. Knowing the specificities of all the CD8 T cells in the TG of B6 mice, we directed our next studies towards characterizing the latent CD8 T cell response.

# 5.0 THE HSV-SPECIFIC CD8 T CELL REPERTOIRE DURING LATENCY

The ratio of $gB_{498-505}$-specific CD8 T cells: non-$gB_{498-505}$-specific CD8 T cells remains constant from acute infection throughout latency [85]. After our initial studies we recognized that even though the $gB_{498-505}$-specific CD8 T cell population remains stable throughout infection, the non-$gB_{498-505}$-specific CD8 T cell population, comprised of 18 different specificities, may be altered during latency as seen in other models [137]. Additionally, $gB_{498-505}$-specific CD8 T cells become more functional over time, producing multiple cytokines during latency, and can prevent viral reactivation *in vivo* and in ex vivo TG cultures. The extent of non-$gB_{498-505}$-specific CD8 T cell functionality and the ability these cells to prevent viral reactivation has not been well defined. In the current study, we aimed to characterize the latent non-$gB_{498-505}$-specific CD8 T cell population by identifying the TCR specificities, assessing CD8 T cell function, and the ability of non-$gB_{498-505}$-specific CD8 T cells to prevent viral reactivation from latency.

## 5.1 CD8 T CELLS SPECIFIC FOR SUBDOMINANT HSV-1 EPITOPES LOSE FUNCTIONALITY IN LATENTLY INFECTED TG

CD8 effector T cells accumulate to peak levels in TG of C57BL/6 mice by 8 days post HSV-1 corneal infection (dpi), and contract to form a stable memory population by 30 dpi [85]. Half of the CD8 T cells are specific for a single immunodominant epitope on gB as assessed by tetramer

staining, and all of these immunodominant cells maintain the ability to produce IFN-γ when stimulated with the $gB_{498-505}$ epitope directly ex vivo [76]. Two subdominant epitopes on HSV-1 ribonucleotide reductase1 ($RR1_{982-989}$ and $RR1_{822-829}$) and two on HSV-1 infected cell protein 8 ($ICP8_{171-178}$ and $ICP8_{876-884}$) together represent 27.8% of the subdominant population at 8 dpi based on tetramer staining (Figure 12A). Tetramer staining tended to underestimate the frequency of epitope reactive cells during acute infection since the frequency based on tetramer staining was lower than the frequency based on intracellular staining for IFN-γ following epitope stimulation, though the difference was statistically significant only for $ICP8_{876-883}$. Although there was some individual variation, the frequency of the subdominant CD8 T cell populations remained fairly constant during latency as assessed by tetramer staining, representing in aggregate 23.9% of the subdominant population at 30 dpi (Figure 12A).

The TG-resident subdominant CD8 T cells did, however, exhibit a rather dramatic loss of functionality during latency as indicated by a significantly reduced frequency of IFN-γ producing cells relative to tetramer staining cells in latently infected TG (Figure 12A). This comparison might underestimate the functional impairment of the subdominant CD8 T cells, as some appear refractory to tetramer staining as noted above and discussed in Appendix A. Moreover, the frequency of multifunctional subdominant CD8 T cells (capable of producing IFN-γ, TNFα, and releasing lytic granules when stimulated directly ex vivo) was also significantly reduced in latently infected TG compared to acutely infected TG (Figure 12B). No functional compromise was observed in the corresponding subdominant CD8 T cells in the spleen (data not shown). In our model, HSV-1 latent infections are restricted to the TG and brainstem, suggesting that the functional compromise of TG-resident subdominant CD8 T cells likely requires antigenic exposure. Functional impairment was restricted to the subdominant population, since the TG-

resident immunodominant $gB_{498-505}$-specific cells showed a slight increase in multifunctional cells during latency in agreement with previous findings [76].

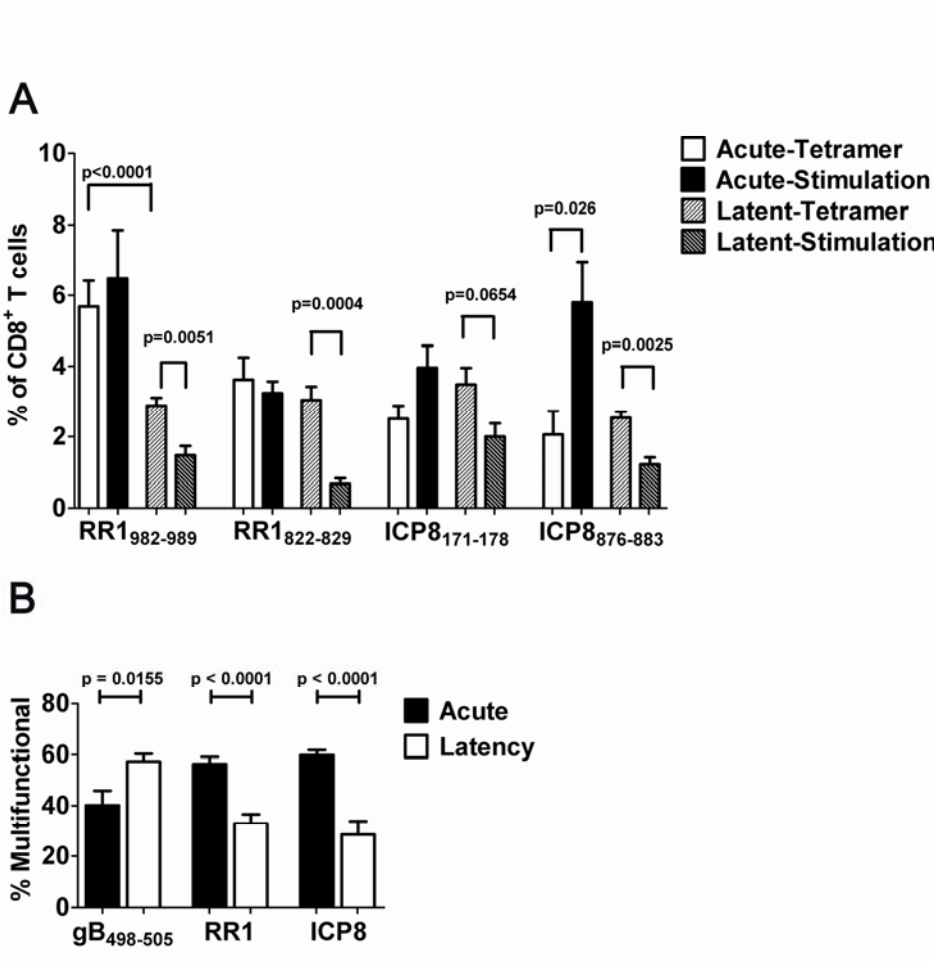


**Figure 12 Subdominant CD8 T cells become functionally compromised during latent HSV-1 infection**

(**A&B**) TG were obtained from HSV-1 infected mice during acute (8 dpi) or latent (30-35 dpi) infection, and the dispersed cells (**A**) were stained with H2-$K^b$ tetramers containing peptides corresponding to subdominant epitopes on HSV-1 ribonucleotide reductase 1 (RR1) or infected cell protein 8 (ICP8); or were stimulated with B6WT3 fibroblasts pulsed with the same peptides, stained for intracellular IFN-γ, and analyzed by flow cytometry. The bars represent the cumulative mean ± SEM percent of CD8 T cells that are tetramer positive during acute (2 experiments, total n = 8-10 per peptide) and latent (4 experiments, total n ≥ 20/peptide) infection, or frequency of CD8 T cells that are IFN-γ positive during acute or latent infection (2 experiments, n ≥ 8/peptide). (**B**) TG cells were stimulated with B6WT3 fibroblasts pulsed with 3 subdominant epitopes on RR1 ($RR1_{982-989}$, $RR1_{822-829}$, and $RR1_{372-380}$) or on ICP8 ($ICP8_{171-178}$, $ICP8_{168-176}$ , and $ICP8_{876-883}$) and monitored for surface CD107a (lytic granule release) and intracellular IFN-γ and TNFα by flow cytometry. Bars represent the cumulative mean ± SEM frequency of multifunctional (IFN-$\gamma^+$, TNF$\alpha^+$ and CD107$a^+$) cells (2 experiments, n ≥ 9/peptide pool). The p values were determined by a Student's T test.

## 5.2 $GB_{498-505}$-SPECIFIC CD8 T CELLS EXPRESS MORE GRANZYME B COMPARED TO SUBDOMINANT CD8 T CELLS

Another anti-viral mechanism $gB_{498-505}$-specific CD8 T cells use to prevent HSV-1 reactivation from latency is releasing lytic granules containing granzymes. With our next experiment, we examined *in situ* granzyme B expression in the non-$gB_{498-505}$-specific CD8 T cell population. Similar to the results in Figure 12, there exists a disparity in the expression of the effector molecule, granzyme B, between $gB_{498-505}$-specific CD8 T cells and non-$gB_{498-505}$-specific CD8 T cells. With 70% of $gB_{498-505}$-specific CD8 T cells expressing granzyme B and only 30% of non-$gB_{498-505}$-specific CD8 T cells expressing the serene protease, it initially appeared that $gB_{498-505}$-specific CD8 T cells were better equipped to prevent viral reactivation (Figure 13). However, other studies have used granzyme expression as a marker of stimulation rather than a marker of virucidal activity [98].

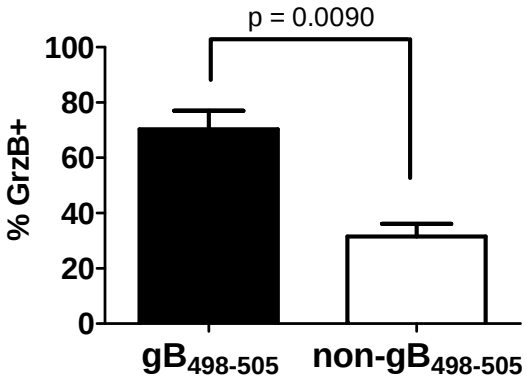


**Figure 13 Subdominant CD8 T cells express lower amounts of granzyme B during latent HSV-1 infection.**

TG cells from latently infected mice were stained for CD8**α**, $gB_{498-505}$ TCR, and granzyme B (GrzB) expression and analyzed by flow cytometry. Bars represent the cumulative mean ± SEM frequency of GrzB+ cells (1 experiment, n=3). This experiment was repeated 3 times with similar results.

## 5.3 NON-GB$_{498-505}$-SPECIFIC CD8 T CELLS PROLIFERATE AT A HIGHER RATE AND EXPRESS HIGHER LEVELS OF PD-1 COMPARED TO GB$_{498-505}$-SPECIFIC CD8 T CELLS

As stated above, CD8 T cells respond to antigenic stimulation by proliferating. In an attempt to identify the degree of antigenic stimulation of non-gB$_{498-505}$-specific CD8 T cells, we next evaluated the rate of proliferation of the non-gB$_{498-505}$ population as well as select specificities in the population, which include RR1$_{982-989}$, RR1$_{822-829}$, ICP8$_{171-178}$, and ICP8$_{876-883}$. Unexpectedly, after 2 daily injections of BrdU, we observed that the non-gB$_{498-505}$-specific CD8 T population as a whole (22%) incorporated 2 times the amount of BrdU compared to their gB$_{498-505}$ counterparts (11%). The four subdominant CD8 T cell specificities tested also incorporated BrdU at a higher rate compared to the immunodominant population albeit at varying degrees of incorporation (Figure 14A). Indeed, from these results, it appeared that subdominant CD8 T cells during latent infection are stimulated to a higher extent than gB$_{498-505}$-specific CD8 T cells.

We next investigated the expression of an activation marker as well as a known marker of exhaustion, programmed death (PD)-1 on subdominant CD8 T cells. When bound to its ligand, PD-L1, PD-1 recruits factors to the TCR synapse to dampen signaling through the TCR [110]. During acute viral infections, PD-1 upregulation is common due to the high degree of stimulation during the expansion phase of the CD8 T cell response [156]. After a virus is cleared and the memory population is fully developed, PD-1 will then only be upregulated in times of antigenic exposure such as secondary infections. Moreover, in models such as LCMV infection, continual antigenic stimulation correlates with increased levels of PD-1 and retention of live virus [102,157]. Combining our previous functional data with the fact that latent HSV-1 genomes are never fully eliminated from the host, we hypothesized that subdominant CD8 T

cells are functionally compromised and thus would have increased expression of PD-1. Indeed, our results show that all specificities of subdominant CD8 T cells have increased levels of PD-1 expression compared with the $gB_{498-505}$-specific CD8 T cells. Similar to the BrdU incorporation data, subdominant CD8 T cells express varying levels of PD-1. Additionally, the non-$gB_{498-505}$ population (20.5%) expressed 3 fold more PD-1 as compared to the $gB_{498-505}$ population (6.7%). Degrees of CD8 T cell exhaustion can also be characterized by comparing the mean fluorescence intensity of cells expressing PD-1. Non-$gB_{498-505}$-specific CD8 T cells consistently express 33% more surface PD-1 expression per cell when compared with $gB_{498-505}$-specific CD8 T cells from same TG (Figure 14B-D)

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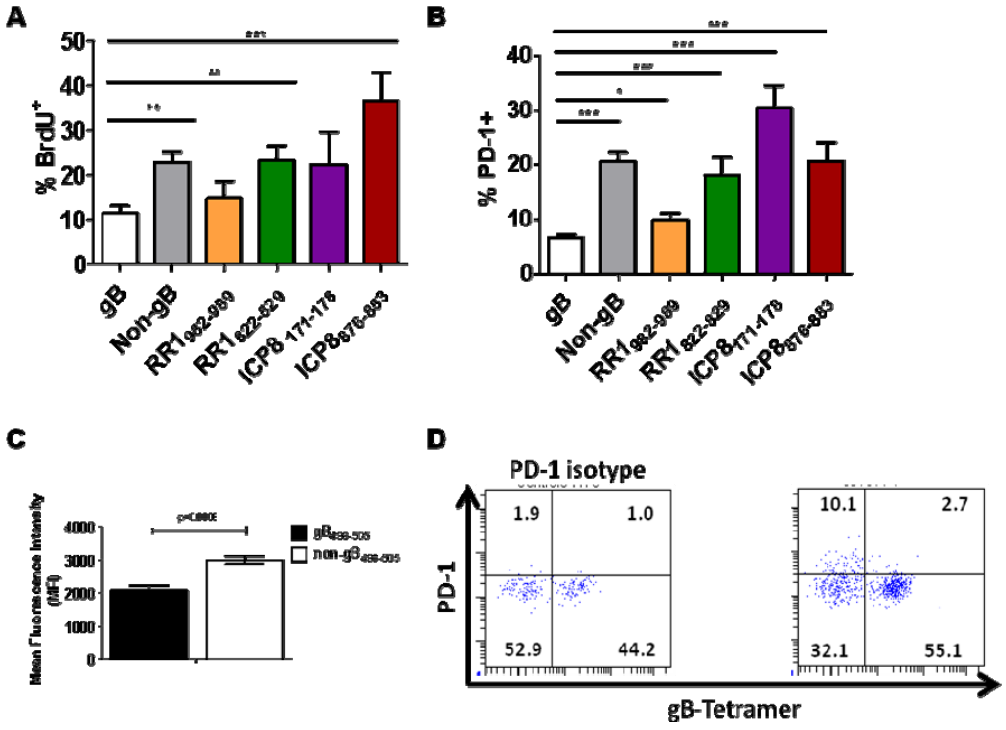


**Figure 14 Non-gB$_{498-505}$-specific CD8 T cells incorporate more BrdU and express more PD-1 when compared with gB$_{498-505}$-specific CD8 T cells**

(**A-D**) Latently infected mice were given 100mg intraperitoneal (i.p.) injections of BrdU on 36 and 37 dpi. TG were dispersed into single-cell suspensions, stained for CD8**α**, gB$_{498-505}$, RR1$_{982-989}$, RR1$_{822-829}$, ICP8$_{171-178}$, ICP8$_{876-883}$ TCR, (**A**) BrdU and (**B-D**) PD-1 and analyzed by flow cytometry. (**A**) Bars represent the cumulative mean ± SEM percentage of BrdU positive cells (2 experiments, total n=4-9). (**B**) Bars represent the cumulative mean ± SEM percentage of PD-1 positive cells (4 experiments, total n=19-24). (**C**) Bars represent the cumulative mean ± SEM mean fluorescence intensity of PD-1 positive cells (2 experiments, total n=7) (Contributed by Sophia Jeon). (**D**) Representative dot plots displaying PD-1 expression on gB$_{498-505}$ and non-gB$_{498-505}$-specific CD8 T cells. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$

## 5.4 GB$_{498-505}$-TOXIN COUPLED TETRAMER DEPLETES GB$_{498-505}$-SPECIFIC CD8 T CELLS *IN VIVO*

The functional compromise of non-gB$_{498-505}$-specific CD8 T cells suggested an inability of these cells to prevent viral reactivation from latency. Described in an earlier study, we proposed to deplete gB$_{498-505}$-specific CD8 T cells using tetrameric MHC class I molecules complexed with

$gB_{498-505}$ peptide and conjugated to the toxin saporin. After conjugation, the toxin-coupled tetramer was then injected intravenously (i.v.) into latently infected mice. Upon specifically binding the $gB_{498-505}$ TCR, the toxin/TCR complex was endocytosed giving saporin the ability to inactivate ribosomes, which led to cell death. Due to the specificity of TCR for MHC/peptide, the toxin depletes antigen specific CD8 T cells with minimal unspecific damage. We administered several doses of toxin-coupled tetramer and observed the most effective dose to be 30 pmols. At this dosage, we achieved a two-fold reduction in TG-resident $gB_{498-505}$-specific CD8 T cell numbers combined with a concurrent two-fold increase in non-$gB_{498-505}$-specific CD8 T cell numbers three days after administration of treatment (Figure 15A&B). We observed no change in the subdominant CD8 T cell population specific for the $RR1_{822-829}$ peptide. By 6 days after dosage, the $gB_{498-505}$-specific CD8 T cell numbers fully recovered, in fact, $gB_{498-505}$-specific CD8 T cell numbers expanded to 1900 total cells per TG equating to a five-fold increase over the $gB_{498-505}$-specific CD8 T cell numbers observed at 3 days after treatment. Non-$gB_{498-505}$-specific CD8 T cell numbers did not change significantly between 3 and 6 days after treatment (Figure 15C).

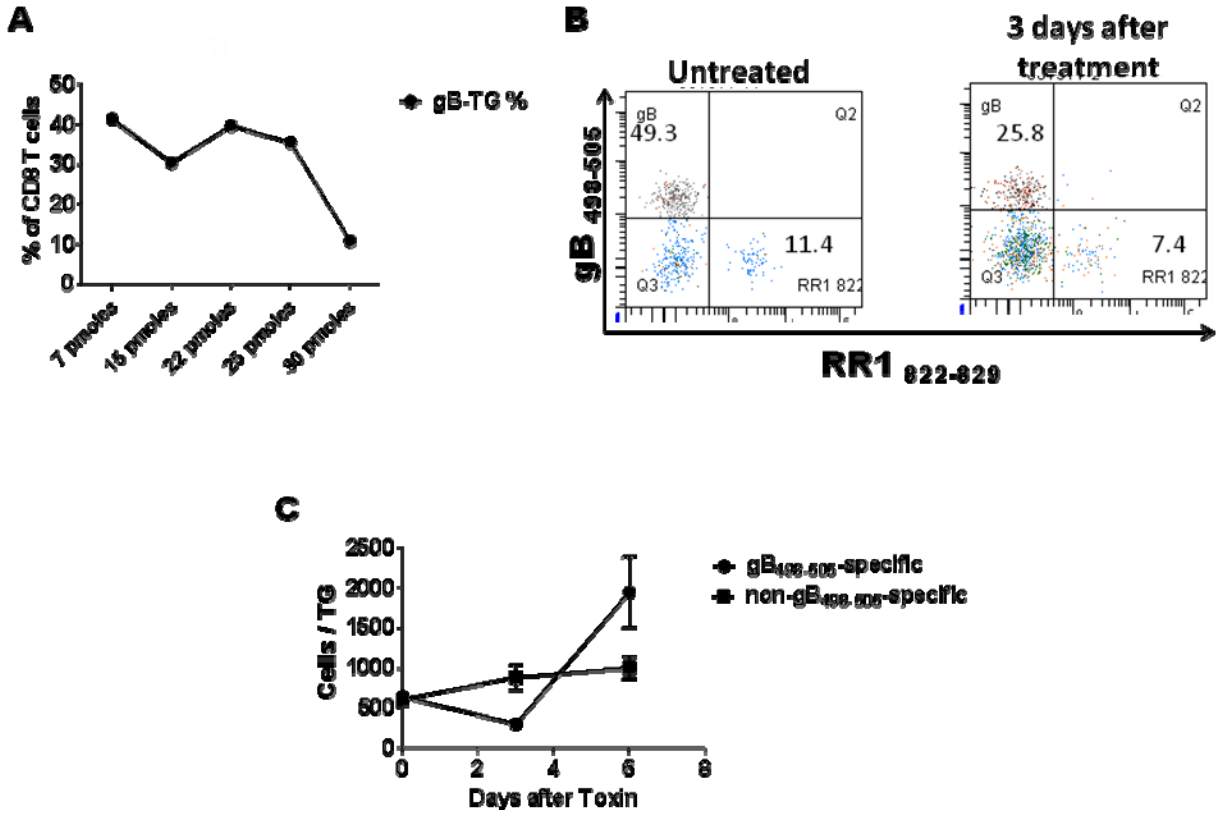


**Figure 15 $gB_{498-505}$ toxin-coupled tetramer specifically depletes $gB_{498-505}$-specific CD8 T cells *in vivo***

(**A-C**) Latently infected mice were given intravenous (i.v.) injection of (**A**) 7 pmol, (**A**) 15 pmol, (**A**) 22 pmol, (**A**) 25 pmol, (**A-C**) 30 pmol of $gB_{498-505}$ tetramers conjugated to saporin. After (**A-C**) 3 days and (**C**) 6 days, TG were dispersed into single-cell suspensions and stained for CD8**α**, $gB_{498-505}$ TCR, and $RR1_{822-829}$ TCR and analyzed by flow cytometry. (**A**) Dots represent individual mice treated with $gB_{498-505}$ tetramers conjugated to saporin (1 experiment, n=1). (**B**) Representative dot plots displaying reduced TG-resident $gB_{498-505}$-specific CD8 T cell frequency and maintained TG-resident $RR1_{822-829}$-specific CD8 T cell frequency. (**C**) Symbols represent the cumulative mean ± SEM number of $gB_{498-505}$-specific or non-$gB_{498-505}$-specific CD8 T cells (2 experiments, n=6-7). This experiment was repeated once with similar results.

## 5.5 REDUCTION IN $GB_{498-505}$-SPECIFIC CD8 T CELL NUMBERS COMPROMISES MAINTENANCE OF VIRAL LATENCY

After observing a reduction in $gB_{498-505}$-specific CD8 T cell numbers, we next asked if this reduction correlates with viral reactivation from latency. Due to the rapid effects of toxin-tetramer treatment, the most accurate assessment of viral reactivation would be to quantify viral

genomes from infected ganglia between untreated and toxin-tetramer treated mice. After three days of treatment, when $gB_{498-505}$-specific CD8 T cell numbers are lowest, we observed a 60% increase in viral genome copy number. Re-establishment of viral control was achieved once $gB_{498-505}$-specific CD8 T cell numbers were restored (Figure 16A). Due to the high limit of detection of live virus formation and/or the high affinity HSV-1 neutralizing antibody make detecting live virus difficult, we decided to support our claim of viral reactivation with CD8 T cell activation phenotypes. We hypothesized that loss of viral control would correlate with increased viral gene expression as well as increased antigenic stimulation of CD8 T cells. As mentioned earlier, CD8 T cells proliferate upon antigenic stimulation. Therefore, we assessed BrdU incorporation into proliferating CD8 T cells as a measure of increased antigenic stimulation. Concurrent with increased copy number after toxin treatment, we observed significant increases in the frequency of CD8 T cells incorporating BrdU three days after toxin treatment. Immunodominant $gB_{498-505}$-specific CD8 T cells had a 5.5 fold increase in the rate of BrdU incorporation while non-$gB_{498-505}$-specific CD8 T cells as a population only increased BrdU incorporation by a factor of 2.4. However, the largest change in BrdU incorporation occurred in the $RR1_{822-829}$-specific CD8 T cell population, which increased by a factor of 6.2 (Figure 16B). This would suggest different populations of CD8 T cells receive varying degrees of stimulation during a reactivation event. Furthermore, we supported our claim by assessing PD-1 expression on different specificities of CD8 T cells as a dynamic marker for antigenic stimulation. Indeed, the frequencies of PD-1 expressing cells in the $gB_{498-505}$ and non-$gB_{498-505}$ CD8 T cell populations increased from the starting frequencies of 10% and 20% respectively to 30% at 3 days after toxin-couple tetramer administration. Levels were restored to steady-state

latency levels by 6 days after treatment. This pattern paralleled with the amplified then contracted viral genome copy numbers observed after treatment administration (Figure 16C).

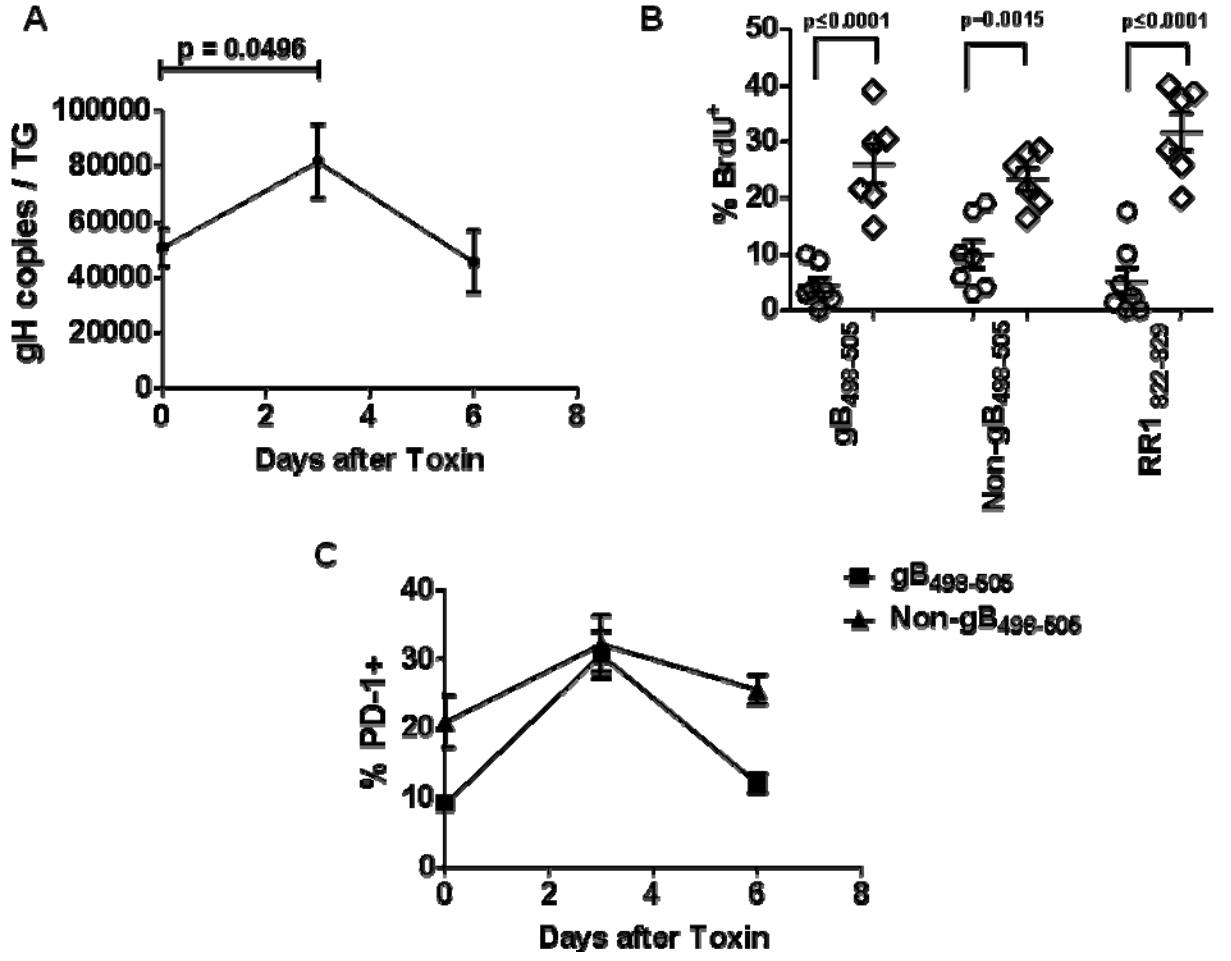


**Figure 16 $gB_{498-505}$ toxin-coupled tetramer induces viral reactivation from latency**

(**A-C**) Latently infected mice were given intravenous (i.v.) injection of 30 pmol of $gB_{498-505}$ tetramers conjugated to saporin. Two days after toxin injection, mice were given an intraperitoneal (i.p.) injection of 100mg of BrdU. On days (**A-C**) 3 and (**A&C**) 6 days after toxin injection, TG were dispersed into single-cell suspensions and (**A**) were assessed for viral genome copies by performing Real Time-PCR of DNA extracted from ganglia or (**B&C**) were for CD8**α**, $gB_{498-505}$ TCR, (**B**) $RR1_{822-829}$ TCR, (**B**) BrdU (**C**) PD-1 and analyzed by flow cytometry. (**A**) Symbols represent the cumulative mean ± SEM number of gH copies per ganglion (2 experiments, total n=6-8). (**B**) Symbols represent individual mice and error bars represent cumulative mean ± SEM frequency of individual T cell specificities positive for BrdU incorporation (1 experiment, n=6-7). (**C**) Symbols represent cumulative mean ± SEM frequency of $gB_{498-505}$ or non-$gB_{498-505}$ CD8 T cells that were PD-1 positive (2 experiment, n=6-7). The p values were determined by a Student's T test.

## 5.6 DISCUSSION

Recent evidence from several laboratories suggests that strategies aimed at augmenting the TG-resident CD8 T cell population could inhibit HSV-1 reactivation from latency, potentially reducing the frequency of recurrent disease. To ensure proper development of this type of strategy, it is essential to fully characterize the CD8 T cell response. My initial studies defined the acute HSV-1 CD8 T cell repertoire, but it is well documented that in chronic viral infections, the CD8 T cell repertoire is prone to changes [103]. Since the likelihood of identifying a primary HSV-1 infection is quite rare, characterization of the latent CD8 T cell response was necessary for the development of a CD8 T cell therapy.

The unusually strong immunodominance of the $gB_{498-505}$ epitope in B6 mice is well documented [53,85,152]. These cells represent 50% of the HSV-1 specific CD8 T cell repertoire in spleens as well as in both acutely and latently infected TG. The remaining 50% of CD8 T cells in acutely infected TG are also HSV-1 specific as demonstrated by their ability to produce cytokines (IFN-γ and TNF α) when stimulated with 18 different HSV-1 subdominant epitopes [50,96]. Using tetramers containing four of the subdominant epitopes that together constitute approximately 30% of the subdominant population; we demonstrate little if any change in the CD8 T cell repertoire within the memory population that is retained in the TG during viral latency. However, in contrast to the immunodominant CD8 T cells that retain full functionality during latency, those specific for subdominant epitopes exhibit a dramatic loss of functionality.

We next explored the cause for functional compromise in subdominant CD8 T cells. It was possible that cells were losing functionality due to the lack of antigenic stimulation or over stimulation resulting in functional exhaustion. A previous study from our lab suggested that only cells stimulated in the TG are maintained in the TG during latency [85]. Therefore, we

hypothesized that subdominant CD8 T cells in the TG are over simulated during latency leading to exhaustion and an inability to produce effector molecules. Reliably detecting lytic viral gene expression remains elusive due to the limits of detection of current assays, so we were unable to correlate the level of gene expression to the degree of functional compromise CD8 T cells. However, our previous study exhibiting the polarization of the $gB_{498-505}$ TCR on CD8 T cells towards latently infected neurons [86] displayed heightened sensitivity of CD8 T cells. Likewise, we used CD8 T cell responses to antigen as a measure of the degree of stimulation received during latency. In our study, we assessed proliferation rates and the levels of PD-1 expression on CD8 T cells during latency. We observed that during latency, non-$gB_{498-505}$-specific CD8 T cells incorporate more BrdU over time and express more PD-1 than their $gB_{498-505}$-specific CD8 T cell counterparts. This would suggest that even in the absence of detectable viral gene expression, non-$gB_{498-505}$-specific CD8 T cells do receive more antigenic stimulation than $gB_{498-505}$-specific CD8 T cells. When the non-$gB_{498-505}$-specific CD8 T cell population was further delineated into individual peptide-specific populations, we observed that specificities appear to receive varying levels of stimulation during latency.

Though no substantial evidence exists showing viral gene expression during the transition from latent to lytic infection in neuronal tissue, our results suggest that the stochastic model of viral gene expression may apply [158,159]. From our results, RR1- and ICP8-specific CD8 T cells appear to be stimulated to a higher degree than $gB_{498-505}$-specific CD8 T cells. Furthermore in the stochastic model of gene expression, it is well documented that the β genes coding the RR1 and ICP8 proteins are abundantly expressed before DNA synthesis [50] while gB expression is fully expressed only after DNA synthesis [160]. This would suggest that if HSV-1 persistently attempts to escape latency while CD8 T cells constantly prevent escape from latency,

early gene CD8 T cells (RR1 and ICP8) would encounter more antigenic stimulation, while leaky-late CD8 T cells (gB) would encounter less antigenic stimulation. From this, we propose a possible "Goldilocks" model of CD8 T cell stimulation during latency. Cells that receive little to no antigenic stimulation during latency are eventually lost during latency, as described in our previous studies. Cells (RR1- and ICP8-specific CD8 T cells) overly stimulated during latency are maintained in the TG, but eventually lose the ability to produce effector molecules capable of preventing viral reactivation from latency. Lastly, cells that receive optimal antigenic stimulation ($gB_{498-505}$-specific CD8 T cells) are maintained in the TG and increase functionality through latent infection.

Functionally exhausted cells are significantly impaired in the ability to control viral infections [103,106,110]. We knew that the TG-resident non-$gB_{498-505}$-specific CD8 T cell population contained a large number of functionally exhausted cells, so we wanted examine if the limited number of functional cells in the population could maintain viral latency *in vivo*. To do this, we used a recently developed technique that uses saporin-conjugated tetramers complexed with a viral antigen of interest to systemically deplete antigen-specific CD8 T cells [161]. Initial assessments of depletion showed that we do get effective depletion of up to 50% of $gB_{498-505}$-specific CD8 T cells without observable effects on the non-$gB_{498-505}$ population. In fact three days after treatment administration, we observed a significant increase in the non-$gB_{498-505}$-specific population bringing the total number of TG-resident CD8 T cells to typical levels noticed during latent infection. By 6 days after toxin administration, $gB_{498-505}$-specific CD8 T cell numbers recovered to levels 3 fold higher than expected. We concluded from this that the recovery could be due to residual amounts of toxin-coupled tetramer binding the TCR of $gB_{498-505}$-specific CD8 T cells in amounts that were not enough to cause apoptotic death but was

enough to stimulate CD8 T cell function. It was also possible that viral reactivation from latency in response to the drastic reduction in functional CD8 T cell numbers could stimulate the remaining cells increasing the CD8 T cell population days after toxin-treatment.

Inhibition of CD8 T cell function and depletion of CD8 T cells *in vivo* results in viral reactivation from latency 3 days after inhibition/depletion. Even though we did not alter total CD8 T cell numbers, the 50% reduction in $gB_{498\text{-}505}$-specific CD8 T cell numbers resulted in a 2-fold increase in viral genome copy number. Additionally, the increased antigenic stimulation of CD8 T cells led to higher rates of proliferation as well as upregulation of PD-1 in all populations of CD8 T cells. Importantly, the observed increase in viral genome copies resolved once the $gB_{498\text{-}505}$-specific CD8 T cell population was restored to steady state latency numbers. We concluded from these observations that during the depletion of $gB_{498\text{-}505}$-specific CD8 T cells, non-$gB_{498\text{-}505}$-specific CD8 T cells essentially replace the lost population through proliferation. However, due to the high functionality of $gB_{498\text{-}505}$-specific CD8 T cells and the compromised nature of non-$gB_{498\text{-}505}$-specfic CD8 T cells, we actually observed a net loss in the total numbers of functioning cells in the latent ganglia. After 3 days of treatment, the virus finally overcame CD8 T cell control progressing to the stage of DNA synthesis. Three days later, the $gB_{498\text{-}505}$-specific CD8 T cell population was restored and reclaimed control over the virus reducing copy number and the level of CD8 T cell stimulation (reduced PD-1 expression).

The impacts of these studies provided important observations that should be taken into consideration when considering therapies to prevent HSV-1 reactivation. First, we have shown from this study that non-$gB_{498\text{-}505}$-specific CD8 T cells do not possess the antiviral power that their immunodominant counterparts own. This poses a problem in the event of a mutation in the immunodominant epitope that renders immunodominant CD8 T cells useless. Therefore, a

possible strategy would be to develop a therapy that targets exhaustion markers to increase functionality (actively studied in our lab). Additionally, the development of a tactic that boosts the subdominant response while maintaining the $gB_{498-505}$-specific CD8 T cell response may provide benefit. As we examined in our study, whenever we sacrifice immunodominant CD8 T cells to increase subdominant CD8 T cells, viral reactivation occurs. Lastly, due to the efficiency of $gB_{498-505}$-specific CD8 T cells at preventing viral reactivation, a therapy that aims to increase the efficacy or numbers of those cells may be beneficial. Rather than attempting to resuscitate subdominant CD8 T cells from an exhausted state, our next study focused on increasing the pool of TG-resident $gB_{498-505}$-specific CD8 T cells.

# 6.0 CIRCULATING HSV-1-SPECIFIC CD8 T CELLS DO NOT ACCESS HSV-1 LATENTLY INFECTED TG

Recurrences of herpetic disease are associated with exposure to stressors known to compromise T cell function. Therapeutic vaccines can be designed to enhance existing T cell memory populations for increased protection against re-infection. In the case of HSV-1, recurrent disease results from reactivation of latent virus in sensory ganglia, which is controlled in part by a ganglia-resident HSV-specific memory CD8 T cell population. Recent data suggested that the CD8 T cell population in HSV-1 latently infected sensory ganglia may be compartmentalized and not replenished from the periphery, a feature ascribed to tissue resident memory ($T_{rm}$) CD8 T cells. Evidence to support this comes from the establishment of an HSV-specific memory population in IL-$15^{-/-}$ mice, suggesting that the population can be maintained without replenishment from the periphery [99]. Also, when DRG from 2 different congenic strains of mice infected with HSV-1 are transplanted under the kidney capsule of another mouse, CD8 T cells are first lost inducing HSV-1 reactivation from latency. Following reactivation, the CD8 T cell populations were restored expressing the congenic marker of the original DRG donor with no apparent infiltration of blood circulating CD8 T cells. The goal of this study was to investigate the maintenance of the TG–resident memory CD8 T cell population at the true orthotopic site as well as bolster it through adoptive transfer of effector and memory HSV-specific CD8 T cells.

Based on our previous studies, non-$gB_{498-505}$-specific CD8 T cells did not appear to be a worthwhile target for an adoptive cell transfer vaccine due to the significant loss of functionality and ability to prevent viral reactivation during latent infection. Therefore, we focused our efforts on boosting the immunodominant $gB_{498-505}$-specific CD8 T cell population. These cells retain high levels of functionality through latent infection and can protect from viral reactivation from latency without the help of subdominant CD8 T cells. These studies were aided by the availability of gBTI-1 mice that allowed for the harvesting of a large number of $gB_{498-505}$-specific CD8 T cells.

## 6.1 BLOCKING THE CHEMOKINE RECEPTORS CXCR3 AND CCR5 REDUCES CD8 T CELL INFILTRATION INTO GANGLIA DURING ACUTE HSV-1 INFECTION

Mice received a single subcutaneous injection of TAK-779 at 6 dpi to inhibit the activity of both CXCR3 and CCR5 [162] during the initial CD8 T cell infiltration of the TG that occurs 6-8 days after HSV-1 corneal infection. Total CD8 T cells and $gB_{498-505}$-specific CD8 T cells were quantified by flow cytometry on dispersed cells from individual TG at 8 dpi (Figure 17 A & B). The TAK-779 treatment significantly reduced the CD8 T cell population in the TG compared to mock treated (PBS) controls, while not altering the frequency of $gB_{498-505}$-specific CD8 T cells. The reduced CD8 T cell population in TAK-779 treated mice did not affect the HSV-1 viral burden in the TG (Figure 17C), which is largely controlled by the innate immune system prior to $CD8^+$ T cell infiltration [68].

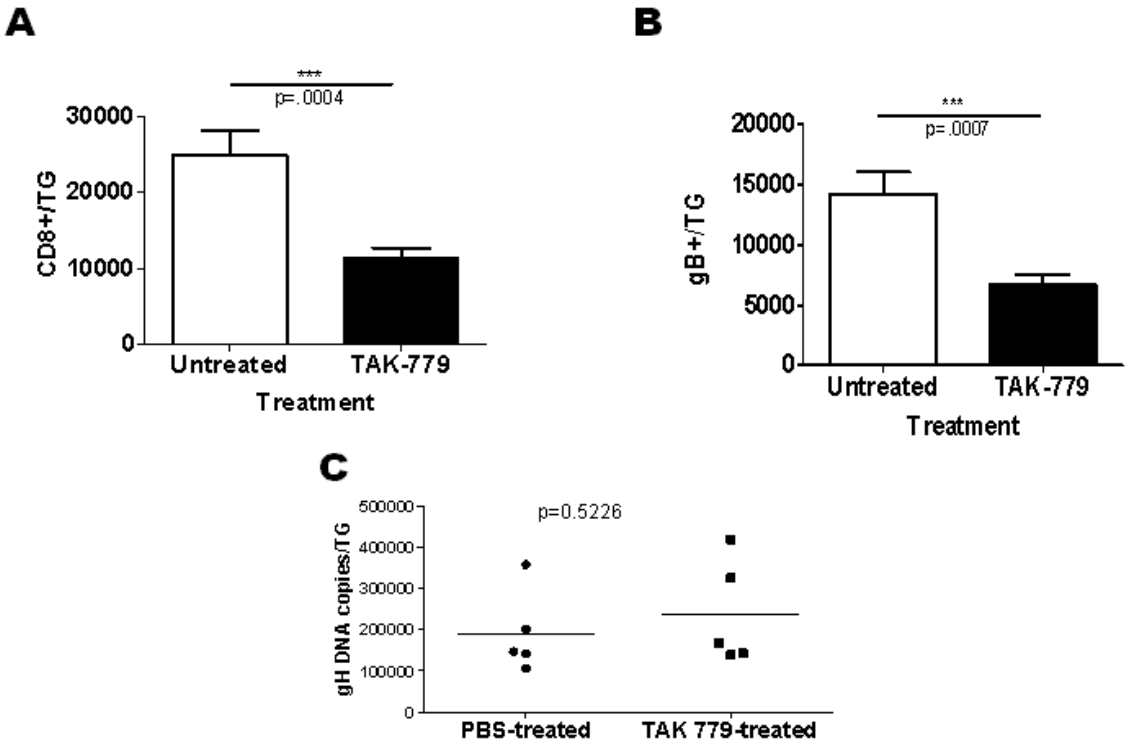


**Figure 17 Effect of systemic TAK-779 treatment on CD8 T cell infiltration into HSV-1-infected ganglia**

(**A-C**) Six days following corneal HSV-1 infection, B6 mice were treated subcutaneously with 150 µg/mouse TAK-779 or with an equal volume of PBS. Two days following treatment, TG were excised and dispersed into single cells. The cells were stained for surface CD8α, CD45, and gB-specific T cell receptor, and analyzed by flow cytometry. Data represent the cumulative mean ± SEM of total CD8 T cells **(A)** or gB-CD8 **(B)** per TG from three experiments involving a total of 6 PBS-treated and 11 TAK-779 treated mice. (**C)** DNA extracted from dispersed TG cells and analyzed by real-time PCR for the number of viral glycoprotein H (gH) gene copies revealed no significant group differences. A two-tailed Students t test was used for all statistical analyses. Experiments were performed by Susanne Himmelein, PhD with repeats performed by Anthony St. Leger.

## 6.2 CXCR3 AND CCR5 ARE NOT REQUIRED TO MAINTAIN THE MEMORY CD8 T CELL POPULATION WITHIN LATENTLY INFECTED TG

The CD8 effector T cells in the acutely infected TG undergo contraction and establish a stable memory population by 30 dpi [86]. To interrogate the role of CXCR3 and CCR5 in maintaining the memory CD8 T cell population in latently infected TG, mice were treated with TAK-779 or PBS as a control at 30, 32, and 34 dpi, and total CD8 T cells and $gB_{498-505}$-specific CD8 T cells were quantified in the TG at 36 dpi. As illustrated in Figure 18, TAK-779 treatment did not influence the size of the total CD8 T cell population (Figure 18A) in the TG, or the frequency of $gB_{498-505}$-specific CD8 T cells (Figure 18B). Thus, the chemokine receptors CXCR3 and CCR5 do not appear to be required to maintain the CD8 T cell population within HSV-1 latently infected TG, at least for the 6 day observation period employed. CD8 T cells in latently infected TG of both mice and humans tend to cluster around latently infected neurons, some forming immunological synapses with the neurons, and most expressing an activated phenotype. Mechanisms governing the attraction of CD8 T cells to infected neurons and their activation status are unknown. Here we show that blocking CXCR3 and CCR5 with TAK-779 for 6 days did not alter the activation phenotype of TG-resident CD8 T cells (Figure 18C).

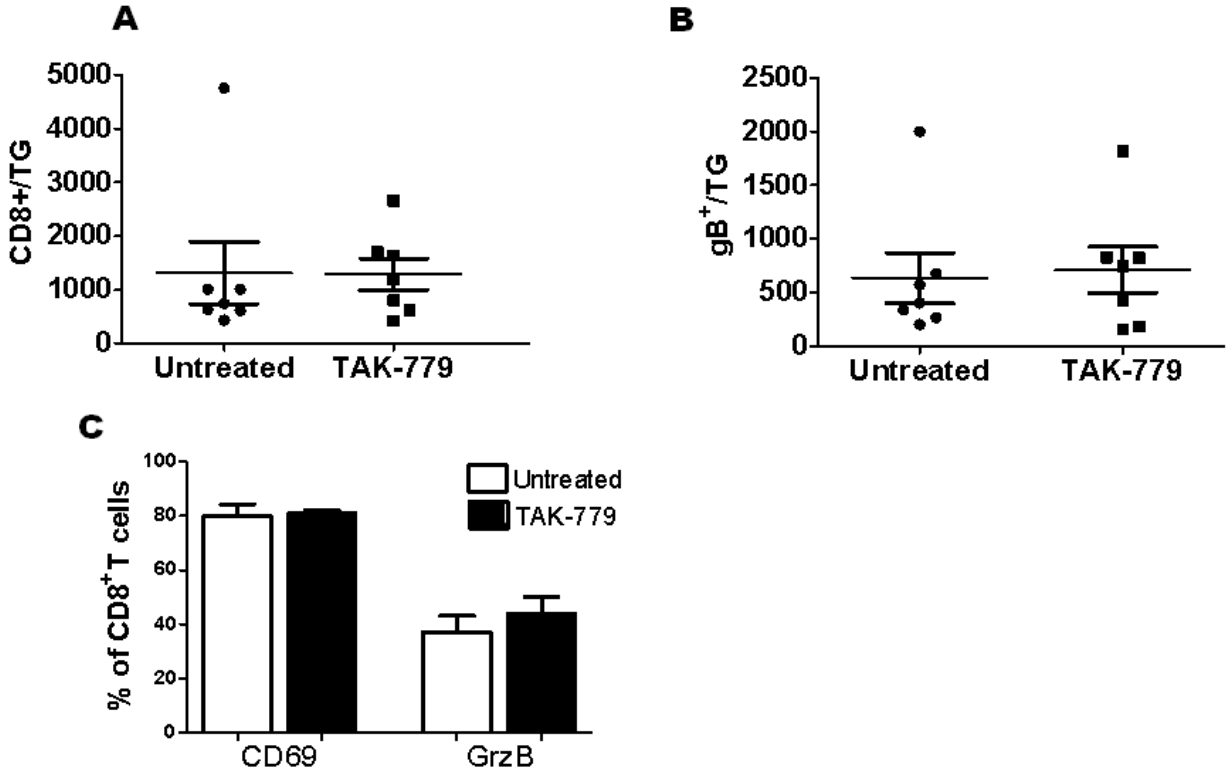


**Figure 18 Effect of TAK-779 treatment during HSV-1 latency on size and activation of memory CD8+ T cell population**

(**A-C**) HSV-1-infected mice were treated with TAK-779 or PBS at 30, 32 and 34 days post-infection (dpi). TG were excised 36 dpi, dispersed into single cells, stained for CD45, CD8α, and $gB_{498-505}$-specific T cell receptor, total CD8 T cells **(A)** or $gB_{498-505}$-specific CD8 T cells **(B)** per TG are presented. Data represent the cumulative mean ± SEM of cells in the TG (total n=8-9, 2 experiments performed by Anthony St. Leger). **(C)** Alternatively dispersed TG cells were stained for activation markers, CD69 and granzyme B (GrB). Bars represent cumulative mean ± SEM frequency of CD8 T cells that expressed CD69 and GrzB (n=4 from experiments performed by Susanne Himmelein, PhD). Students t test revealed no statistical difference between the groups.

## 6.3 HSV-SPECIFIC CD8 T CELLS IN THE PERIPHERAL BLOOD DO NOT MIGRATE INTO HSV-INFECTED GANGLIA DURING VIRAL LATENCY

It has recently been suggested that the CD8 T cell population within HSV-1-infected ganglia is compartmentalized, self-sustainable, and does not require replenishment from the circulating

lymphocyte pool [163]. We employed adoptive transfer studies in which CD45.2$^{+}$ $gB_{498\text{-}505}$-specific CD8 memory or effector T cells were transferred to latently infected mice and their migration into the TG was monitored. In a first set of experiments $gB_{498\text{-}505}$-specific CD8 T cells were obtained from the spleens of CD45.2$^{+}$ gBTI1.1 mice 30 days after HSV-1 corneal infection, and transferred to latently infected CD45.1$^{-}$ mice. The transferred and endogenous $gB_{498\text{-}505}$-specific CD8 T cells were quantified in the spleen, blood, and TG of the recipient mice 2.5 and 4.5 weeks after transfer. As illustrated in Figure 19, the $gB_{498\text{-}505}$-specific CD8 T cells in the spleen and blood of recipient mice contained an equal or greater frequency of donor cells at all times tested, but no donor cells were detected in the TG. Thus there is no detectable infiltration of HSV-1 specific CD8$^{+}$ T cells into the TG during stable latency.

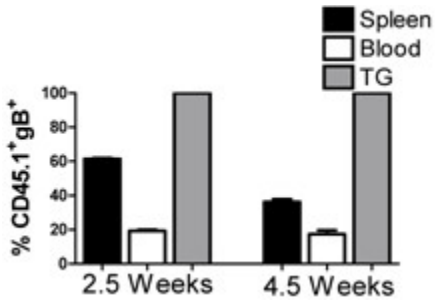


**Figure 19 Adoptively transferred $gB_{498\text{-}505}$-specific CD8 T cells are excluded from HSV-1-infected TG during stable latency**

CD8 T cells were isolated from 30 dpi gBT I.1 (CD45.2) spleens and $10^6$ cells were intravenously (i.v.) injected into latently infected wild-type mice (CD45.1). At indicated times after transfer, spleens, blood, and TG were harvested and stained for CD8α, CD45, CD45.1, CD45.2, and $gB_{498\text{-}505}$-specific T cell receptor. Data represent the percentage of gB-specific cells that are CD45.1+ (endogenous). Bars represent cumulative mean ± SEM frequency of $gB_{498\text{-}505}$-specific CD8 T cells that were donor derived (n=3 from experiments performed by Susanne Himmelein, PhD).

## 6.4 REPOPULATION OF THE TG FOLLOWING DISRUPTION VIA STRESS AND CORTICOSTERONE IS NOT DEPENDENT ON PROLIFERATION

We previously established that exposure of latently infected mice to corticosterone or restraint stress reduces the CD8 T cell population in the TG, and exposure to restraint stress additionally induces HSV-1 reactivation from latency [31] (and Freeman and Hendricks, unpublished data). Here we show that simultaneous exposure of latently infected mice to stress and corticosterone on 4 consecutive days (30-34 dpi) reduced the CD8 T cell population in the TG by >80% (Figure 20A), but both the total CD8 T cell and the $gB_{498-505}$-specific CD8 T cell populations fully recovered within 4 days. It is interesting to note the original 1:1 ratio of $gB_{498-505}$-specific CD8 to non-$gB_{498-505}$-specific CD8 T cells was maintained following recovery of the population. Treatment also resulted in HSV-1 reactivation from latency as assessed by a significant increase in HSV-1 genome copy number in the TG and detection of HSV-1 genomes in the ipsilateral cornea (Figure 20B).

We hypothesized that recovery of the CD8 T cell population in the TG after stress and corticosterone treatment would involve proliferation of the remaining cells, infiltration of CD8 T cells from the blood, or a combination of both. We favored proliferation as re-establishing the original frequency of $gB_{498-505}$-specific CD8 T cells through infiltration from the blood would seem unlikely. However, the level of proliferation in the recovered population as assessed by BrdU incorporation was similar to that of the unperturbed population in mock treated mice that were not exposed to corticosterone and stress (Figure 20C), suggesting that recovery was not accomplished through proliferation of remaining cells.

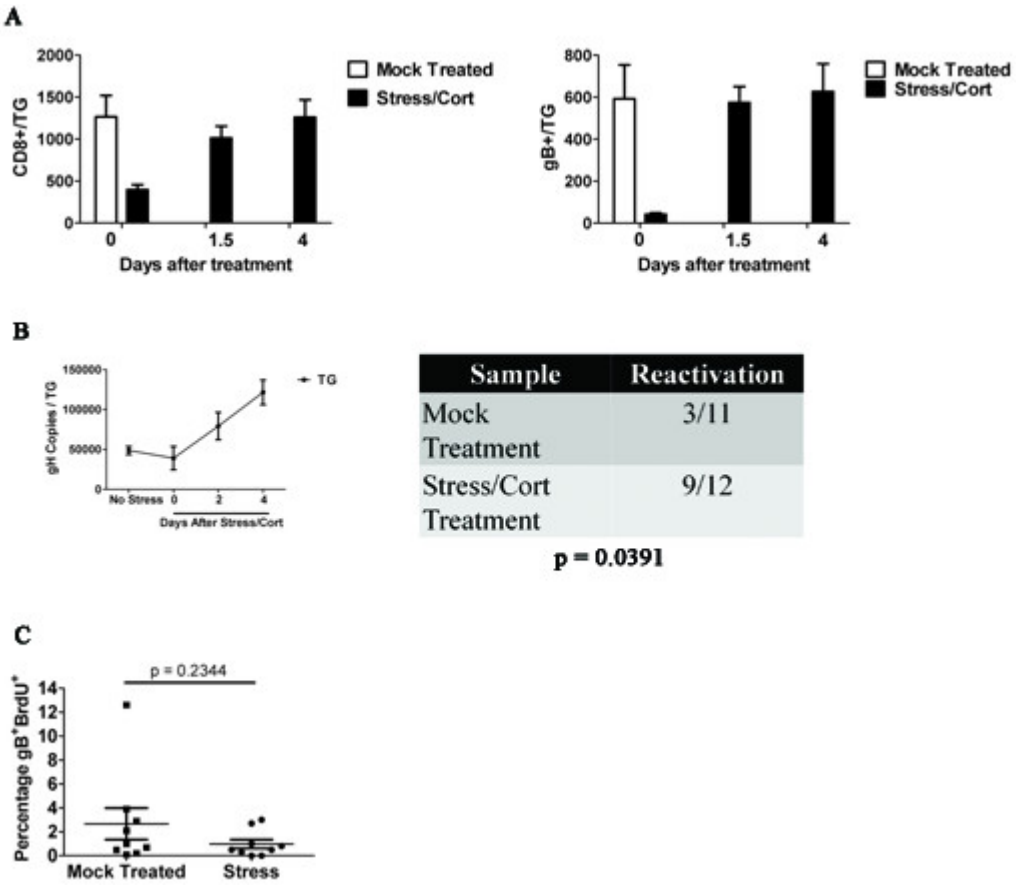


**Figure 20 Following disruption of the TG-resident CD8 T cell population via stress and corticosterone treatment, repopulation occurs independent of proliferation**

Latenly infected mice received 4 days of restraint stress and corticosterone-treated drinking water (400 µg/ml), followed by 1.5, 2, and 4 days of fresh water. TGs were harvested and stained with CD8α, CD45, and gB-specific T cells receptor. **(A)** The numbers of $CD8\alpha^+$ cells and $CD8\alpha^+gB^+$ were quantified via flow cytometry. Data shown are the means and standard errors for 7 (time point 0), 5 (time point 1.5), and 12 (time point 4) mice from two independent experiments performed by Anthony St. Leger. **(B)** RT-PCR of DNA from ganglia and corneas shows increased gH copies after stress and corticosterone treatment. gH copies peak in the TG at 4 days after stress and corticosterone while detectable viral burden in the cornea peaks at 2 days after fresh water (Fisher's exact test). RT-PCR of DNA from corneas shows increased detectable gH copies 2 days after stress and corticosterone treatment ended. TG data represents means and standard errors from 6 mice (No stress and day zero) or 2 mice (day two and four). Experiments performed by Alexander Rowe, PhD and Anthony St. Leger. **(C)** BrdU was given intraperitoneally (i.p.) 12 hours after fresh water. TG were then harvested 1.5 days after stress and stained for CD8α, CD45, gB-specific T cell receptor, and BrdU incorporation. Data represent means and standard error of 9 mice from 2 experiments performed by Anthony St. Leger.

## 6.5 HSV-SPECIFIC CD8 T CELLS IN THE PERIPHERAL BLOOD DO NOT MIGRATE INTO HSV-INFECTED GANGLIA FOLLOWING DISRUPTION OF THE TG-RESIDENT CD8 T CELLS

We explored the alternative possibility that recovery of the CD8 T cell population in the TG following corticosterone and stress treatment was accomplished by infiltration of CD8 T cells from the blood. Memory and effector $gB_{498-505}$-specific CD8 T cells were generated *in vitro* by pulsing gBT-1 Thy $1.1^{+}$ spleens with the gB peptide (SSIEFARL) in the presence of IL-2 for three days (effector cells), followed by culture with IL-15 for 10 days (memory cells) as previously described [128]. The effector cells were $CD44^{high}$ $CD69^{+}$ $LFA\text{-}1^{low}$ $VLA\text{-}4^{low}$ whereas the memory cells were $CD44^{high}$ $CD69^{-}$ $LFA\text{-}1^{high}$ $VLA\text{-}4^{high}$ (Figure 21A). Latently infected Thy1.2 mice were exposed to stress and corticosterone for 4 days followed by adoptive transfer of effector or memory $gB_{498-505}$-specific CD8 T cells. The TG, blood, spleens, and lungs were obtained 4 days after transfer, and the donor (Thy1.1) and recipient (Thy1.2) $gB_{498-505}$-specific CD8 T cells were quantified. As illustrated in Figure 21B&C the donor effector and memory cells comprised a significant portion of the $gB_{498-505}$-specific CD8 T cells in the spleen, blood, and lungs of recipient mice, but were undetectable in the TG. These findings do not support the concept that recovery of the CD8 T cell population in the TG is through infiltration of HSV-1 specific CD8 T cells from the blood.

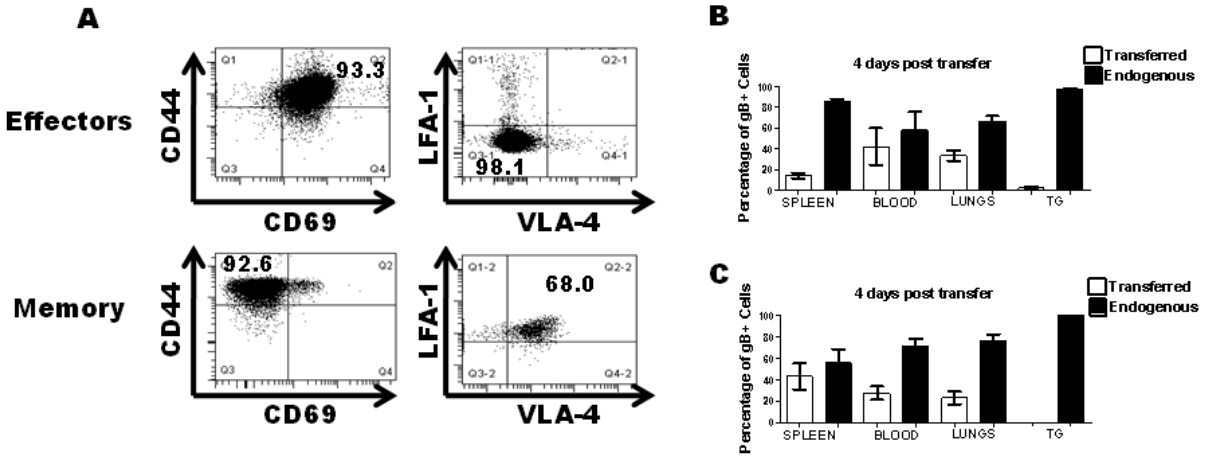


**Figure 21 Following disruption of the TG-resident CD8 T cell population via stress and corticosterone treatment, in vitro generated effector or memory cells do not enter the TG**

(**A-C**) Spleens from naïve gBT I.1 Thy 1.1 mice were pulsed with SSIEFARL ($gB_{498-505}$) and incubated in IL-2 for three days (effector cells) and some cells were further incubated for 10 days with IL-15 replacing IL-2 to generate memory cells. **(A)** Cells were stained for CD44, CD69, LFA-1, and VLA-4 and analyzed by flow cytometry. Latently infected wild type mice ($Thy1.2^+$) were subjected to 4 consecutive days of restraint stress and corticosterone. After the final treatment mice received an adoptive transfer of $10^6$ effector **(B)** or memory **(C)** $CD8^+$ T cells ($Thy1.1^+$). Four days after the adoptive transfer, spleens, blood, lungs, and TGs were harvested and stained for CD8α, CD45, Thy1.1 (Donor), Thy1.2 (Recipient), and gB-specific T cell receptor. Data represent means and standard errors of the percentages of $gB_{498-505}$-specific CD8 T cells that are of donor (Thy1.1) or recipient (Thy1.2) origin in the specified tissues (n = 4 mice per group). The experiments were performed by Anthony St. Leger and were repeated with similar results.

## 6.6 REPOPULATION OF THE TG FOLLOWING STRESS AND CORTICOSTERONE DOES NOT DEPEND ON CD4 T CELLS

A previous study demonstrated that CD4 T cells are required for recovery of the CD8 T cell population in latently infected TG following transplantation under the kidney capsule of recipient mice [164,165]. To determine if this is also true for recovery of the CD8 T cell population in latently infected TG at the orthotopic site, latently infected mice were treated with stress and

corticosterone on 4 consecutive days (30-34 dpi) and received anti-CD4 mAb on the first and last day of treatment. As shown in Figure 22A, the TG were completely depleted of CD4 T cells at the end of treatment and 4 days later (Figure 22B) when the CD8 T cell population had fully recovered. However, CD4 T cell depletion had no effect on recovery of the CD8 T cell population in the TG (Figure 22C).

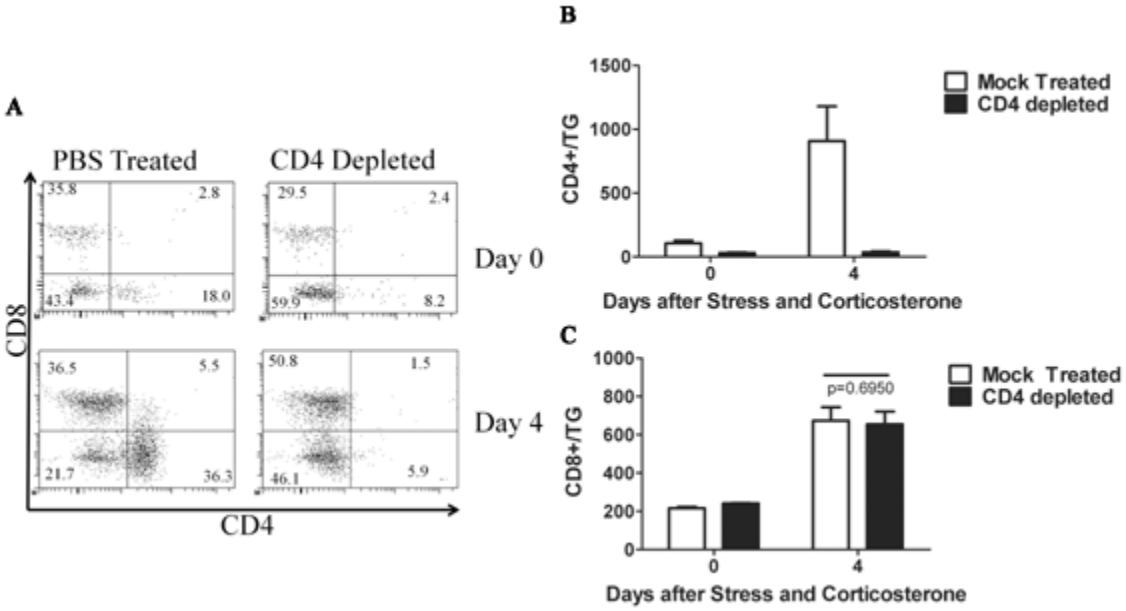


**Figure 22 CD4 T cells are not required for the recovery of CD8 T cells within corticosterone-treated ganglia**

(**A-C**) Mice latently infected with HSV-1 received 4 days of restraint stress and corticosterone treatment (30-34 dpi). At 27 and 30 dpi mice received intraperitoneal injections of 0.75 mg/ml α-CD4 monoclonal antibody (clone GK1.5) or a control antibody. TG were excised at 38 dpi and stained for CD8α, CD4, CD45, and gB-specific T cell receptor. **(A)** Represenative dot plots demonstrate effective depletion of CD4 T cells in the TG at 34 and 38 dpi. **(B)** Quantification of the CD4 depletion in mock depleted and CD4-depleted groups. **(C)** TG were excised at 38 dpi and analyzed for recovery of the CD8 T cell population. Data are presented as means and standard errors of 15 (mock depleted) or 16 (CD4-depleted) mice from 3 independent experiments performed by Anthony St. Leger and Susanne Himmelein, PhD. Students t test revealed no significant differences in the number of $CD8^+$ T cells within TG from the mock depleted or CD4-depleted groups.

## 6.7 DISCUSSION

The frequency of HSV-1 reactivation from latency in ex vivo TG cultures is inversely proportional to the size of the ganglionic CD8 T cell population [29]. Based on these findings some have advocated that all future HSV-1 vaccines be evaluated based on their capacity to enhance the HSV-specific CD8 T cell population in latently infected sensory ganglia. Such an approach would only be feasible if the HSV-specific CD8 T cells expanded in peripheral lymphoid organs through immunization have access to latently infected ganglia. With our initial studies identifying HSV-specific CD8 T cells, we decided to investigate the ability of expanded HSV-specific CD8 T cells to enter the TG from the circulation.

Evidence obtained in a model system in which latently infected DRG are transplanted under the kidney capsule of recipient mice suggest that HSV-specific CD8 T cells do not infiltrate latently infected DRG even when the resident CD8 T cell population is substantially disrupted [165]. However, a number of factors that may be unique to the transplanted tissue could have influenced CD8 T cell migration in that model. The authors suggested that the loss of CD8 T cells from the DRG following transplantation was likely due to death resulting from the trauma of transplantation. Such trauma could also induce other changes within the microenvironment of the latently infected ganglion such as alterations in the chemokine and cytokine milieu that could influence CD8 T cell infiltration. Moreover, revascularization of the transplanted tissue could influence T cell migration.

Based on these concerns we sought a more physiological model in which to examine the HSV-specific CD8 T cell infiltration into latently infected TG. We concluded that even though we had identified all the CD8 T cell specificities, we decided to first investigate the ability of

expanded $gB_{498-505}$-specific CD8 T cells to enter the TG from the circulation. This was due to the availability of a large pool of $gB_{498-505}$-specific CD8 T cells from the gBT-I transgenic mouse.

We first identified chemokine receptors that are used to direct CD8 T cell into acutely infected TG and asked if blocking these receptors during latency would result in diminution of the CD8 T cell population in latently infected TG. Activated CD8 T cell in human and mouse TG highly express the chemokine receptors CCR5 and CXCR3 and their corresponding ligands, CCL5 and CXCL10, have also been detected in infected ganglia [166-170]. These receptors play important roles in other viral models especially HIV, but the role these receptors play in CD8 T cell recruitment into the TG during HSV infection had not been established. In this study, we demonstrated using systemic treatment with the chemical non-peptide inhibitor of CXCR3 and CCR5, TAK-779, significantly reduced the infiltration of CD8 effector T cells into acutely infected TG, but did not influence the size of the CD8 memory T cell population within latently infected TG. Important to our studies involving subdominant CD8 T cells, we observed no apparent preferential influence of these receptors on various CD8 T cell specificities due to the unchanged frequency of $gB_{498-505}$-specific CD8 T cell during and after treatment. Our latency studies suggest that even though these receptors aid migration into the TG during acute infection, CD8 T cell do not need these receptors for retention in the TG throughout latency. However, our results do not describe if the effects of inhibiting these receptors during acute infection persists into latency. If the effect of CXCR3/CCR5 inhibition becomes less apparent during latency after acute treatment, more support would be given to the hypothesis that once CD8 T cells enter the TG, they are a self-supporting population that requires no assistance from peripheral blood during latency.

We next hypothesized that, if CXCR3 and CCR5 did not act to retain or recruit CD8 T cells into the TG during latent infection, an adoptive transfer of memory cells would infiltrate the TG and increase HSV-specific CD8 T cell numbers. Unfortunately, CD8 T cells were shown to not migrate into latently infected TG came from the observation that adoptively transferred $gB_{498-505}$-specific CD8 T cells that are retained in the blood, spleen, lymph nodes, and lungs of recipient mice over an extended period of at least 4.5 weeks are not detectable in the TG during the same period. The above findings strongly suggest that TG containing an established CD8 memory T cell population and latent HSV-1 are not permissive to CD8 T cell infiltration. We next asked if diminution of the resident CD8 T cell population through elevated serum corticosterone levels and reactivation of HSV-1 from latency through exposure of mice to restraint stress would change the microenvironment of the TG, rendering it permissive to CD8 T cell infiltration. However, adoptively transferred $gB_{498-505}$-specific CD8 T cells did not enter the TG from the blood even when reactivation and diminution of the TG-resident CD8 T cell population was induced by exposure of latently infected mice to stress and corticosterone (Figure 22).

A caveat to these findings is that the adoptively transferred CD8 T cells obtained from gBT1.1 mice may not accurately reflect the migratory capability of the endogenous $gB_{498-505}$-specific CD8 T cell memory population in the lymphoid organs and blood of the host. This seems unlikely given that neither effector cells generated through *in vitro* stimulation for 3 days with the $gB_{498-505}$ peptide, nor memory cells obtained by incubating the effector cells for an additional 10 days in medium containing IL-15 were able to enter the TG. The transferred memory population expressed high levels of the LFA-1 and VLA-4 adhesion molecules typically used by CD8 T cells to enter infected tissue. Moreover, CD8 T cells that were isolated from spleens of

HSV-1 latently infected mice (30 dpi) also failed to enter the TG during recovery of the CD8 T cell population following stress and corticosterone treatment (data not shown). These findings combined with observations made with transplanted HSV-1 latently infected DRG, and with vesicular stomatitis virus infected brains strongly suggest that once a $T_{rm}$ CD8 T cell population is established in nervous tissue, further infiltration of cells from the blood is effectively blocked [163,165].

Since infiltration of CD8 T cells from the blood did not seem to account for recovery of the CD8 T cell population in the TG of stress and corticosterone treated mice, we concluded that recovery likely resulted from proliferation of the remaining cells in the TG. However, our results showed that proliferation of TG-resident cells after stress and corticosteron treatment was lower compared to homeostatic proliferation of control mice not exposed to stress or corticosterone. Conversely, it is possible that the burst of proliferation was missed, the level of proliferation in both the $gB_{498-505}$-specific and non-$gB_{498-505}$-specific CD8 T cell populations measured after termination of treatment could not account for the rapid recovery of the populations three days after treatment.

The lack of a requirement of CD4 T cell help in the repopulation of the TG with CD8 T cells remains an interesting observation in comparison to the requirement of CD4 T cells in the transplanted DRG model. We hypothesize that the trauma associated with DRG excision and transplantation leads to extensive cell death, which would then require donor CD4 T cells to help the proliferation and re-establishment of DRG-resident CD8 T cells. Alternatively, our model entails no trauma and relies on female sex hormones and psychological stress to deplete the TG of CD8 T cells. Therefore, rather than causing death of immune cells, our treatment, we hypothesize, induces emigration of CD8 T cells from tissues into the blood, followed by

immigration back into the TG 4 days over the ensuing 4 days. This reasoning would explain the lack of proliferation and necessity of CD4 T cell help during repopulation of the TG. It would also explain the identical frequency of $gB_{498\text{-}505}$-specific and non-$gB_{498\text{-}505}$-specific CD8 T cells in the endogenous and recovered population of CD8 T cells. Moreover, the failure of adoptively transferred CD8 T cells to enter the TG during recovery would suggest selective re-entry of the original TG-resident CD8 T cells. This would suggest acquisition, by the TG-resident CD8 T cells, of a homing receptor that permits selective re-entry into the latently infected TG.

Our findings suggest that at some point after establishment of an HSV-specific CD8 T cell population the infected TG becomes resistant to further T cell infiltration. We have shown that the $gB_{498\text{-}505}$-specific CD8 T cells exhibit a progressively higher functional avidity (ability to detect a low epitope density) over time in latently infected TG, whereas their counterparts in the spleen and lungs showed decreased functional avidity over the same period [76]. The ability to detect very low levels of MHC/ peptide complexes on latently infected neurons would likely enhance the ability of CD8 T cells to provide immunesurveillance of latently infected ganglia, but be of lesser importance in the periphery. Therefore, there might be a selective advantage to the host to restricting infiltration of HSV-specific CD8 T cells into the TG. However, restricting entrance of CD8 T cells into the latently infected TG will complicate the development of therapeutic vaccines designed to bolster the TG-resident CD8 T cell population.

# 7.0 MANIPULATION OF HOST SUPPRESSION LEADS TO INCREASED CD8 T CELL FUNCTIONALITY

Unfortunately, our findings indicated that adoptively transferred CD8 T cells into the circulation do not gain access to the TG. Since $gB_{498-505}$-specific CD8 T cells remain fully functional through latent infection, we concluded that enhancing the $gB_{498-505}$ response is not currently feasible. Our next step concentrated on the improvement of the non-$gB_{498-505}$-specific CD8 T cell response. Results from chapter 5 demonstrated that non-$gB_{498-505}$-specific CD8 T cells appear chronically stimulated through latent infection. During chronic viral infections, viral persistence leads to chronic antigenic stimulation of CD8 T cells. Effects of constant stimulation through the CD8 T cell receptor leads to upregulation of inhibitory receptors such as programmed death (PD)-1, loss of effector function, and in extreme cases physical deletion of the CD8 T cell population. An area of study gaining momentum is the development of techniques aimed towards reversing the effects of exhaustion, which increases CD8 T cell functionality to better protect from viral infections. Current strategies inhibit PD-1/PD-L1 (currently studied in our lab), Tim-3/galectin-9 ([171,172] and currently studied), and CTLA-4/CD28 interactions. Also, immunosuppressive cytokines such as IL-10 and TGF-β produced by the host have also been targeted to improve the immune response towards viral infections.

By administering an antibody to block the IL-10 receptor, we aimed to better understand the state of functional compromise in the non-$gB_{498-505}$-specific CD8 T cell population.

Additionally, we wanted to assess how the immunosuppressive cytokine, IL-10, was inhibiting the CD8 T cell response during latent infection. Lastly, we desired to observe whether this treatment, as seen in LCMV infection, correlates with reduced viral burden and presented itself as a possible therapy for recurrences of HSV-1 reactivation.

## 7.1 IL-10R BLOCKADE ALTERS THE IMMUNODOMINANCE HIERARCHY IN LATENTLY INFECTED TG BY SELECTIVELY AUGMENTING EXPANSION OF SUBDOMINANT CD8 T CELLS.

It was possible that the functional compromise of the subdominant population during latency (Chapter 5) represented a partial exhaustion as is seen in chronic viral infections [173], where the exhaustion can be reversed by treating mice with antibodies to the IL-10 receptor (IL-10R) [117-119,174]. To test this possibility, mice that had established a latent HSV-1 infection (35 dpi) were systemically treated every three days with anti-IL-10R mAb or PBS for a period of 3 weeks. After treatment, the TG were excised, the cells dispersed with collagenase, and the number of total or immunodominant CD8 T cells was determined by flow cytometry. The treated mice showed a dramatic increase in total CD8 T cells during treatment (Fig. 23A), and the increase was almost entirely accounted for within the subdominant CD8 T cell population (Fig. 23B). As expected, the control mice did not show an increase in TG-resident CD8 T cells during treatment, and maintained an approximate 1:1 ratio of $gB_{498\text{-}505}$-specific to non-$gB_{498\text{-}505}$-specific cells. The increased number of non-$gB_{498\text{-}505}$-specific CD8 T cells was associated with a significant increase in their rate of proliferation as indicated by BrdU incorporation (Fig. 23C). The increase in subdominant CD8 T cells in anti-IL-10R treated mice appears to be restricted to

the TG since the number of $RR1_{982-989}$-specific CD8 T cells in the TG of anti-IL-10R-treated mice increased approximately 2-fold, while their numbers in the spleen remained constant (Fig. 23D). Since HSV-1 is confined to the TG during latency, these data are consistent with the notion that IL-10 regulates proliferation resulting from antigenic exposure rather than homeostatic proliferation of the subdominant CD8 T cells in latently infected mice.

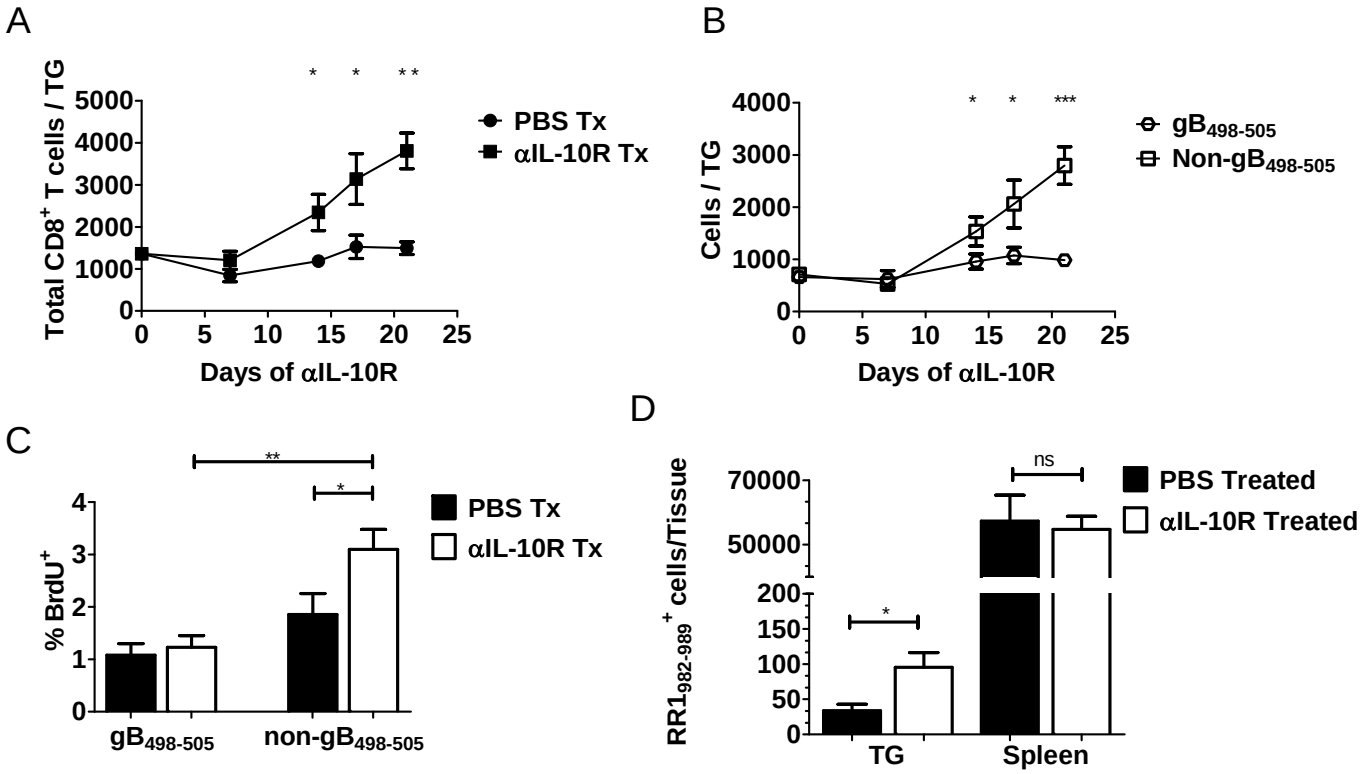


**Figure 23 Anti-IL-10R treatment preferentially increases proliferation of non-$gB_{498-505}$ specific CD8 T cells.**

(**A&B**) Mice latently infected with HSV-1 were given intraperitoneal (i.p.) injection of PBS or anti-IL-10R for up to 21 days. TGs were harvested and dispersed into single-cell suspensions and stained for CD8α (**A**) and $gB_{498-505}$ TCR (**B**) and analyzed by flow cytometry. Symbols represent the cumulative mean ± SEM number of $CD8^+$ T cells per TG at 7, 14, 17, and 21 days of treatment (at least 2 independent experiments, total n = 6-15). (**C&D**) Latently infected mice were treated with PBS or anti-IL-10R mAb for 17 days, and then given an intraperitoneal (i.p.) injection of 2mg of BrdU. (**C**) After 4 hours, TGs were dispersed into single-cell suspensions, stained for CD8α, $gB_{498-505}$ TCR, and BrdU, and analyzed by flow cytometry . Bars represent the cumulative mean ± SEM percentage of BrdU positive cells (2 experiments, total n = 10). (**D**) TGs and spleens were dispersed into single-cell suspensions, stained for CD8α and $RR1_{982-989}$ TCR and analyzed by flow cytometry. Bars represent the cumulative mean ± SEM number of $RR1_{982-989}$ tetramer positive cells (2 experiments, total n = 10). Statistical significance was determined using a Student's T test. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$.

## 7.2 IL-10R BLOCKADE EXPANDS BOTH FUNCTIONAL AND NON-FUNCTIONAL SUBDOMINANT CD8 T CELLS IN LATENTLY INFECTED TG

To determine if the function of the subdominant CD8 T cells in latently infected TG was also rescued by IL-10R blockade, latently infected mice were treated for up to three weeks with anti-IL-10R mAb as described above. TG cells of treated and control mice were dispersed and CD8 T cells were either stained directly ex vivo for GrzB or the cells were stimulated with B6WT3 cells pulsed with pools of peptides corresponding to the three epitopes on RR1 or on ICP8 in the presence of anti-CD107a antibody (to detect lytic granule release) and then stained for intracellular IFN-γ and TNFα. Consistent with previous findings, the immunodominant $gB_{498\text{-}505}$-specific CD8 T cells expressed more GrzB than the non-$gB_{498\text{-}505}$-specific cells prior to treatment (Fig. 24A). However after a 1 week lag period, GrzB expression increased dramatically in the non-$gB_{498\text{-}505}$-specific cells, reaching comparable levels to the $gB_{498\text{-}505}$-specific cells by the end of the 3-week anti-IL-10R treatment. The anti-IL-10R treatment increased the mean number of $GrzB^+$ $gB_{498\text{-}505}$-specific during the first two weeks of treatment, but the difference was not statistically significant (Fig. 24A). The number of IFN-$\gamma^+$ subdominant CD8 T cells was significantly elevated in anti-IL-10R mAb treated mice relative to controls following stimulation with RR1 or ICP8 epitopes, but the frequency of IFN-$\gamma^+$ cells was not affected (Figs 24B&C). Moreover, anti-IL-10R mAb treatment increased the number (not shown) but not the frequency (Fig. 24D) of multifunctional cells capable of producing IFN-γ, TNFα, and releasing lytic granules when stimulated with subdominant epitopes. Together our findings suggest that anti-IL-10R mAb treatment does not rescue the function of impaired subdominant CD8 T cells, but rather increases the rate of proliferation of both functional and non-functional cells.

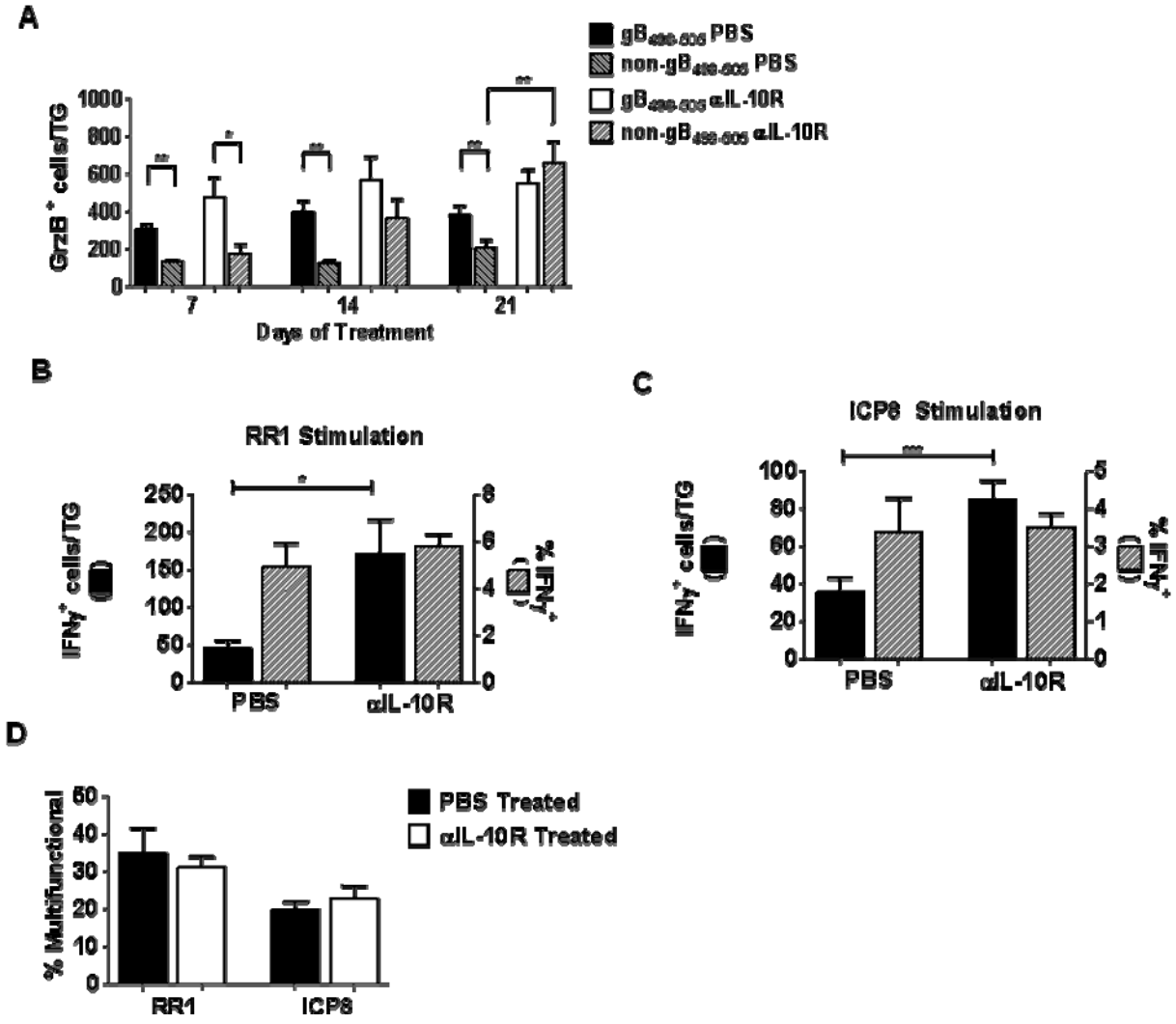


**Figure 24 Treatment with anti-IL-10R mAb increases the total number of functional non-gB$_{498-505}$-specific CD8 T cells.**

Latently infected mice were treated with PBS or anti-IL-10R mAb for 7-21 days (**A**), or 14 days (**B-D**). After treatment, TG cells were stained for CD8α, gB$_{498-505}$ TCR, and granzyme B (GrzB) expression and analyzed by flow cytometry (**A**). Bars represent the cumulative mean ± SEM number of GrzB+ cells (2 experiments, total n = 6). (**B-D**); TG cells were incubated for 6 hours with B6WT3 fibroblasts pulsed with three RRI epitopes (**B&D**) or three ICP8 epitopes (**C & D**). Cells were monitored for surface CD8α and IFNγ (**B&C**), or surface CD8α and CD107a and intracellular IFN-γ and TNFα (**D**) and analyzed by flow cytometry. Solid black bars represent cumulative mean ± SEM number of IFN-$\gamma^+$ cells and hashed bars represent cumulative mean ± SEM frequency of CD8$^+$ T cells that were IFN-$\gamma^+$ (**B&C**, 3 experiments, n = 11) , or percentage multifunctional (IFN $\gamma^+$ , TNF$\alpha^+$, and CD107a$^+$) CD8$^+$ T cells (**D**, 3 experiments, n = 7-12). Statistical significance was determined using Student's T test. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$.

## 7.3 THE EFFECT OF ANTI-IL-10R TREATMENT PERSISTS FOR AT LEAST 3 WEEKS

It was of interest to determine if the augmented functionality of subdominant CD8 T cells persists after anti-IL-10R treatment is terminated. TG were excised 3 weeks after PBS or anti-IL-10R treatment was discontinued and the number of total and $GrzB^+$ immunodominant and subdominant CD8 T cells was determined by flow cytometry. Although the total number of TG-resident subdominant CD8 T cells and the number of $GrzB^+$ subdominant CD8 T cells declined somewhat during the 3 weeks after termination of anti-IL-10R treatment (compare Fig. 22B with Fig. 25A, and Fig. 24A with Fig 25B), both populations remained significantly elevated compared to the controls. Again, the $gB_{498\text{-}505}$-specific CD8 T cells were not affected by treatment.

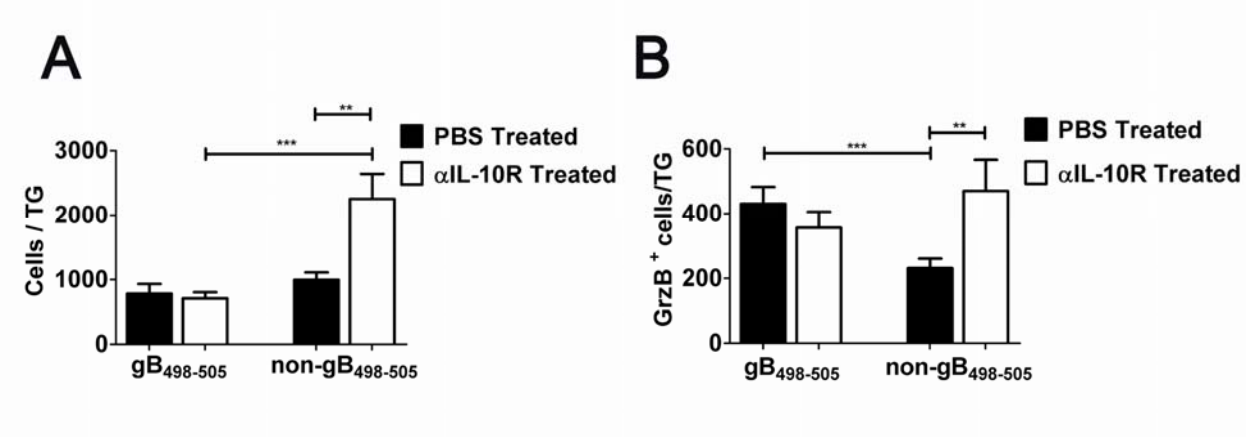


**Figure 25 The effects of anti-IL-10R mAb are maintained for at least 3 weeks after terminating treatment.**

(**A&B**) Latently infected mice were treated with PBS or anti-IL-10R mAb for 3 weeks and were rested for an additional 3 weeks. After rest, TG cells were stained for CD8α, $gB_{498-505}$ TCR, and granzyme B (GrzB) and analyzed by flow cytometry. (**A**) Bars represent the cumulative mean ± SEM number of CD8 T cells per TG (2 experiments, n = 9 – 16). (**B**) Bars represent the cumulative mean ± SEM number of $GrzB^+$ cells (3 experiments, n=16-20). Statistical significance was determined by a Student's T tests. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$.

## 7.4 IN VIVO ANTI-IL-10R MAB TREATMENT DURING LATENCY REDUCES VIRAL REACTIVATION

In untreated latently infected mice, HSV reactivation frequency in ex vivo TG cultures is directly proportional to the viral genome copy number, and inversely proportional to the number of CD8 T cells in the TG [29]. The anti-IL-10R mAb treatment did not significantly alter the HSV-1 genome copy number in latently infected TG (data not shown). Since treatment did significantly increase the number of functional HSV-specific CD8 T cells, we predicted treatment would reduce the HSV-1 reactivation frequency in ex vivo TG cultures. TG were excised from anti-IL-10R treated and control mice at the end of treatment or 3 weeks after treatment was terminated, the cells from each TG were dispersed into cultures (the equivalent of 0.15 TG/culture), and supernatants of each culture were sampled every other day for 10 days and assayed for infectious

virus as previously described [41,43,100]. The data are presented either as the frequency TG cultures that reactivated (roughly an estimate of the number of neurons that reactivated in each TG); or as the frequency of TG with any neurons that reactivated. Control mice that received PBS treatment in vivo showed a low frequency of reactivated cultures that was significantly increased when the function of the CD8 T cells was compromised by addition of anti-CD8 mAb to the cultures (Fig. 26A). Thus, CD8 T cells that were in the TG of control mice at the time of excision could partly, but not completely block HSV-1 reactivation from latency in ex vivo TG cultures. Compared to controls, TG that were excised immediately after 3 weeks of anti-IL-10R treatment showed a trend toward reduced reactivation in cultures with or without anti-CD8 mAb, but the differences were not statistically significant. In contrast, TG that were excised 3 weeks after termination of anti-IL-10R treatment showed a significantly lower frequency of reactivation in cultures with and without anti-CD8 mAb compared with TG from control mice.

Expressing the data as a percentage of TG with HSV-1 reactivation (i.e., at least one well of a given TG showed reactivation), all TG from control mice exhibited viral reactivation even in cultures where CD8 T cell function was intact (Fig. 26B). In contrast, CD8 T cells were able to completely block HSV-1 reactivation in 40% of TG that were excised immediately after anti-IL-10R mAb treatment and 45% of TG that were excised 3 weeks after treatment was terminated. Moreover, even when CD8 T cell function was compromised in TG cultures, we observed no virus reactivation in 10% of the TG that were removed immediately after anti-IL-10R mAb treatment and 25% of those removed 3 weeks after treatment was terminated. Thus, the increased number of functional subdominant CD8 T cells in TG of anti-IL-10R mAb treated mice provided significantly increased protection from reactivation in ex vivo TG cultures, and

also appear to have completely eliminated neurons capable of reactivating virus in vivo in up to 25% of TG.

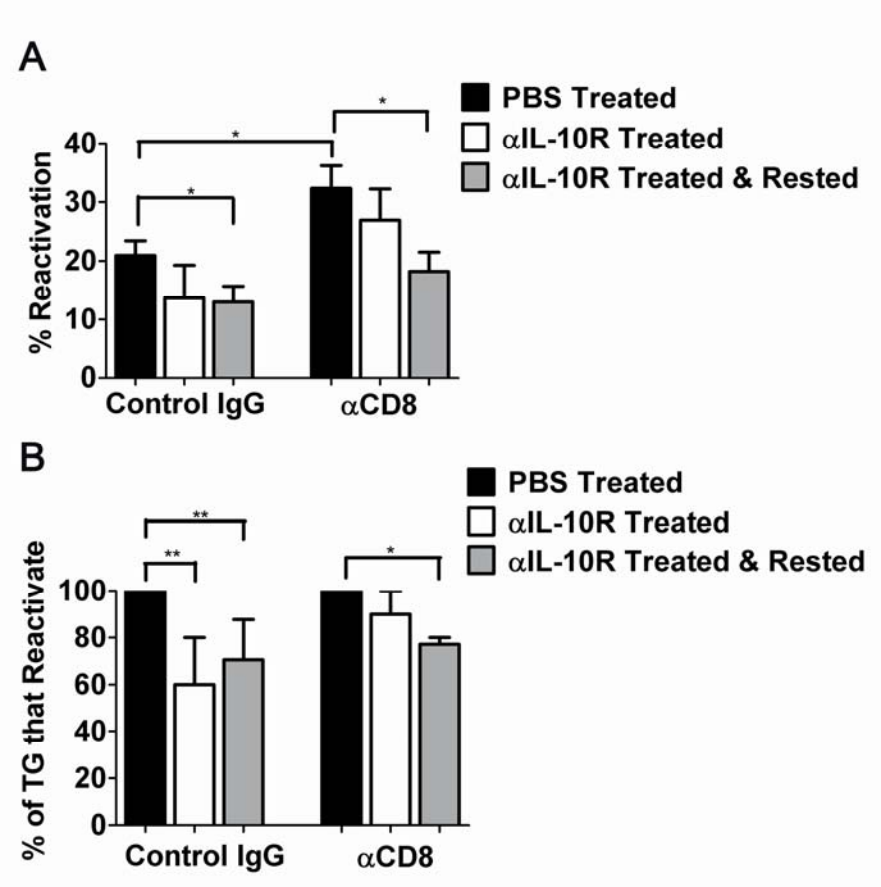


**Figure 26 Treatment with anti-IL-10R mAb reduces viral reactivation from latency.**

(**A&B**) Latently infected mice were treated with PBS or anti-IL-10R mAb for 3 weeks. Immediately after or 3 weeks after termination of treatment, TG cells from 2 mice were pooled and cultured (0.15 TG/well). Half of the wells were treated with either antiCD8α mAb or control IgG. Culture supernatants were serially sampled every 2 days, and assayed for infectious virus on monolayers of Vero cells. Bars represent the cumulative mean ± SEM percentage of virus positive wells per pooled TG (**A**) (2-4 experiments, total n = 10-24) or the mean ± SEM percentage of TG pools that produced detectable virus (**B**). Statistical significance was determined using an unpaired Student's T test (**A**) or Fisher's Exact Test (**B**). * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$.

## 7.5 DISCUSSION

The successful design of a therapeutic or prophylactic treatment for HSV infection remains elusive. Recent evidence from several laboratories suggests that strategies aimed at augmenting the TG-resident CD8 T cell population could inhibit HSV-1 reactivation from latency, potentially reducing the frequency of recurrent disease [34,41,43,86,100,165]. HSV-1 therapeutic vaccines could be designed to increase the number and function of circulating CD8 T cells, but clinical trials of HSV vaccines to date have been restricted to genital infections and have established limited efficacy in preventing viral transmission or the frequency of recurrent disease [125,175,176]. Failure of therapeutic HSV vaccines might be related in part to their focus on increasing antibody rather than T cell responses, but our study from Chapter 6 demonstrated limited if any access of circulating HSV-specific CD8 T cells to latently infected TG [9]. Therefore, alternative approaches to augmenting the TG-resident CD8 T cell population may be required.

The unusually strong immunodominance of the $gB_{498\text{-}505}$ epitope in B6 mice is well documented [53,85,152]. These cells represent 50% of the HSV-1 specific CD8 T cell repertoire in spleens as well as in both acutely and latently infected TG. The remaining 50% of CD8 T cells in acutely infected TG are also HSV-1 specific as demonstrated by their ability to produce cytokines (IFN-γ and TNF-α) when stimulated with 18 different HSV-1 subdominant epitopes that together with the immunodominant $gB_{498\text{-}505}$ epitope define the entire HSV-1 specific CD8 TCR repertoire [50,96]. Using tetramers containing four of the subdominant epitopes that together constitute approximately 30% of the subdominant population; we demonstrate little if any change in the CD8 T cell repertoire within the memory population that is retained in the TG during viral latency. However, in contrast to the immunodominant CD8 T cells that retain full

functionality during latency, those specific for subdominant epitopes exhibit a dramatic loss of functionality.

Retention of CD8 T cells in the latently infected TG appears to require antigenic exposure [85]. Moreover, proliferation of the CD8 T cells in latently infected TG is less dependent on homeostatic proliferation signals from IL-15 than their counterparts in the spleen [99]. Together these findings suggest that proliferation of HSV-specific CD8 T cells in the TG is driven at least in part by antigenic exposure. Our current study demonstrates anti-IL-10R mAb treatment dramatically and selectively increases proliferation of CD8 T cells specific for subdominant HSV-1 epitopes in latently infected TG, while not affecting their proliferation in the spleen. This finding could indicate selective expression of IL-10R on subdominant CD8 T cells in the TG, and/or selective regulation of antigen driven, but not homeostatic proliferation of subdominant CD8 T cells by IL-10.

Similarly, the functional compromise of CD8 T cells in the latently infected TG is not seen in their counterparts in the spleen, suggesting that functional compromise is also driven by antigenic exposure. The similarity between the functional compromise of subdominant CD8 T cells in latently infected TG and functionally exhausted CD8 T cells during chronic viral infections is striking. One might predict that the very low and usually undetectable levels of HSV-1 protein expression in latently infected TG would not elicit CD8 T cell exhaustion. However, the ability to produce antigens without detectable gene products via defective ribosomal products (DRiPs) and immunoribosomes [80,81,177], and the proposed transient derepression of HSV-1 genes during latency [178], may result in persistent antigenic exposure to HSV-specific CD8 T cells in latently infected TG. The subdominant epitopes could be

expressed at a higher level than the immunodominant $gB_{498-505}$ epitope, leading to selective exhaustion of subdominant CD8 T cells.

During chronic LCMV infections, persistent exposure to viral antigens can lead to loss of functionality and ultimately to deletion of the exhausted cells [103,106,179,180]. There appears to be progressive functional impairment beginning with impairment of TNFα production followed by a loss of IFN-γ, which is seen only in cells that are at an advanced stage of exhaustion that occurs prior to deletion [103,157]. In these chronic viral infection models, functional exhaustion can be rescued by IL-10R blockade, with those cells at early stages of exhaustion being most readily rescued [117,118,174,181,182]. In HSV-1 latently infected TG, subdominant CD8 T cells exhibit a significant loss of IFN-γ, TNFα, and lytic granule release suggesting a late stage of exhaustion. Treatment with anti-IL-10R mAb results in an increase in the number of IFN-γ producing and IFN-γ impaired subdominant CD8 T cells, such that the frequency of functional cells was unchanged. It appears, therefore, that anti-IL-10R treatment does not rescue the function of cells that are functionally impaired, but rather induces the proliferation of functional and non-functional CD8 T cells in the TG.

The in vivo anti-IL-10R treatment increased the number of subdominant CD8 T cells in the TG that loaded GrzB into their lytic granules in vivo, and produce IFN-γ when stimulated ex vivo. The increased number of functional subdominant CD8 T cells persisted for at least 3 weeks after anti-IL-10R treatment was terminated. Since TG resident CD8 T cells use GrzB and IFN-γ to inhibit HSV-1 reactivation from latency, we predicted that the expanded number and TCR repertoire of functional TG resident CD8 T cells would improve the ability of these cells to block HSV-1 reactivation from latency in ex vivo TG cultures. Indeed, there was a significant reduction of reactivation in cultures of TG from anti-IL-10R treated mice. All of the TG from

control mice reactivated the virus as expected since over the past 16 years we have performed hundreds of these cultures and have never seen a latently infected TG that failed to reactivate the virus. In contrast, nearly half of TG from anti-IL-10R treated mice failed to reactivate the virus when CD8 T cell function was intact in ex vivo TG cultures. Protection from reactivation was partially eliminated when anti-CD8α mAb was added to cultures, demonstrating that the enhanced number of functional subdominant CD8 T cells in TG following anti-IL-10R treatment provided better protection from reactivation. However, there also appears to be an in vivo effect of treatment in that a significant proportion of TG from anti-IL-10R treated mice failed to reactivate the virus even when CD8 T cell function was compromised in ex vivo TG cultures. This effect was most pronounced in latently infected TG from mice that were rested for three weeks following 3 weeks of anti-IL-10R treatment. Since these mice retained high numbers of functional subdominant CD8 T cells during this time, we hypothesize that neurons attempting to reactivate the virus during and after treatment were either pushed into a very stable state of latency, or perhaps more likely were killed by the expanded number of functional CD8 T cells. We saw a slight, but not statistically significant reduction in viral genome copy number in TG of anti-IL-10R treated mice (data not shown). Given the small number of neurons that appear to require CD8 T cell protection from reactivation at any given time (approximately 6 neurons/TG) [183], it would likely require a very large number of TG from αIL-10R treated mice to demonstrate a significant reduction of viral DNA resulting from destruction of these few neurons.

Based on current and previous findings we propose the following theoretical framework for CD8 T cell control of HSV-1 latency. In C57BL/6 mice HSV-1 latently infected neurons in the TG are surrounded by HSV-specific CD8 T cells that maintain a strict 1:1 ratio of cells

specific for an immunodominant and subdominant epitopes. The immunodominant CD8 T cells maintain full functionality during latency [14], whereas the subdominant CD8 T cells become functionally compromised. The functional $gB_{498-505}$ specific CD8 T cells prevent HSV-1 reactivation from latency through production of IFN-γ and release of lytic granules, while sparing the infected neuron [41,43,100]. Treatment of latently infected mice with anti-IL-10R mAb dramatically increases the number of functional, $GrzB^+$ subdominant CD8 T cells in the TG. Under these circumstances attempted HSV-1 reactivation results in attack of the host neuron by both immunodominant and subdominant $GrB^+$ CD8 T cells that either overwhelm the neuron's antiapoptotic mechanisms leading to neuronal death, or push the virus into a stable latency in which $CD8^+$ T cell protection is no longer required. Over time this leads to a latently infected TG that is completely refractory to HSV-1 reactivation even if CD8 T cell function is subsequently compromised. It is not clear if this strategy would be effective in latently infected humans because the functional state of CD8 T cells in HSV-1 latently infected human TG is not known. However, our data provide proof of principal that strategies aimed at expanding the repertoire of functional TG-resident CD8 T cells can reduce the likelihood of a reactivation event.

# 8.0 SUMMARY AND CONCLUSIONS

The studies of this dissertation first outline the CD8 T cell repertoire of the C57BL/6 mouse from acute infection through latent infection. We showed that even though the CD8 T cell repertoire is maintained throughout infection, immunodominant CD8 T cells increase functionality and can effectively prevent viral reactivation from latency while subdominant CD8 T cells lose functionality and the ability to control viral reactivation. Our next study attempted to increase the number of functional cells through adoptive CD8 T cell transfer. It became evident during these studies that circulating CD8 T cells cannot gain access to the latently infected TG, suggesting that TG-resident CD8 T cells are a self-sustaining immune population. Therefore, we focused our efforts on attempting to enhance the TG-resident subdominant CD8 T cell response. We completed this task by administering anti-IL-10R mAb that blocks the IL-10R. This treatment increased the number of functional non-$gB_{498\text{-}505}$-specific CD8 T cells. The increase in functional cells correlated with better protection from viral reactivation from latency. Therefore, diversification and expansion of the functional CD8 T cell repertoire leads to better protection from viral reactivation.

We came to several conclusions from the results of these studies that can aid the advancement of therapies to prevent recurrent reactivations in humans. Attractive methods for prospective therapies have been through subunit vaccination before infection aimed at generating an effective antibody response. Even though clinical trials have proved these vaccinations as

ineffective, we feel the subunit vaccine, if aimed at generating a CD8 T cell response may still be efficacious. Specifically, our studies have shown that a vaccine should be directed towards creating a CD8 T cell response during the phase when viral proteins are expressed before DNA synthesis. Furthermore, it appears as if humans generate responses against the early protein, RR1, and other early proteins [52,140,184]. Since our studies show that increasing the subdominant CD8 T cell response, vaccinations that increase the diversity of functional HSV-specific CD8 T cells may help humans prevent recurrent reactivation events.

Conversely, the above vaccination strategy would most certainly need to be modified to effectively inhibit HSV-1 reactivation. Evidence for this comes from our studies showing the functional exhaustion of HSV-specific CD8 T cells and the exclusion of TG-resident CD8 T cells from being replenished from peripheral memory cells. Generation of memory cells prior to infection may indeed allow cells to enter the TG earlier; however, these cells appear destined for exhaustion. Therefore, once the TG becomes isolated from the circulating memory CD8 T cells during latent infection, vaccine-derived TG-resident CD8 T cells appear destined to lose functionality and the ability to prevent viral reactivation. To further complicate the generation of a prophylactic vaccine therapy, the diagnostic identifier of primary HSV-1 infection remains difficult to determine for some people. Therefore, we conclude that in tandem with vaccine development, there should also be development of therapeutic strategies that prevent or eliminate the effects of exhaustion on TG-resident CD8 T cells.

With our observation of functional exhaustion in subdominant CD8 T cells, we were also able to substantiate the exceptional sensitivity of CD8 T cells. In combination with our previous studies, we corroborated the observation that viral-specific CD8 T cells receive antigenic stimulation throughout latent infection. With our results, we were able to deduce that different

CD8 T cell specificities receive varying levels of stimulation in the absence of detectable lytic gene expression during latency. In fact, results suggest that the level of gene expression and antigen generation, though not detectable, leads to functional exhaustion. It may be difficult to accept that an absence of gene expression would result in the vast functional exhaustion we observed; however, we concluded from this that the small size of the TG as well as the absence of CD8 T cell replenishment from the periphery significantly reduces the amount of antigen needed to induce exhaustion. The exquisite sensitivity of CD8 T cells has the potential to be a valuable tool in examining the pattern of gene expression as HSV moves in and out of latent infection. For example in Chapter 5, we used markers of stimulation to support our observations of increased copy number during a reactivation event. The dynamics of these markers on peptide-specific CD8 T cells may allow for detection of individual viral genes expressed as the virus exits latency.

The unfortunate circumstance that prevents the entry of circulating CD8 T cells from entering the TG keeps the advent of adoptive CD8 T cell therapies for the prevention of HSV-1 reactivation elusive. Due to this observation, we concluded from our studies that more investigation of the TG-resident CD8 T cell population must take place. For example, examination of tissue homing receptors on TG-resident CD8 T cells may illuminate strategies to manipulate the phenotype of effector/memory cells to allow for entry of CD8 T cells into the TG. Also, examination of the TG microenvironment such as the network of blood vessels may provide some much needed information that would aid understanding of factors preventing recruitment of viral-specific CD8 T cells into the TG.

Lastly, we concluded from our studies that increasing and diversifying the numbers of functional CD8 T cells leads to a reduction in reactivation from latency. We accomplished this

through antibody inhibition of IL-10 signaling. We concluded that there appears to be a dysregulated response to IL-10 through our selective augmentation of the subdominant CD8 T cell population compared to the immunodominant CD8 T cell population after treatment. Additionally, we concluded that the increased response led to better control of viral reactivation from latency that can be maintained for extended lengths of time. Combining these observations with lack of response in uninfected tissue led the determination that increasing and broadening the functional repertoire of CD8 T cells results in indefinite protection from viral reactivation from latency. This brings us to our ultimate conclusion that the most effective therapy to protect from recurrent reactivation events would be a treatment that broadens and expands the functional CD8 T cell repertoire without having effects on the peripheral immune response such as treatment with anti-IL-10R mAb.

# 9.0 FUTURE DIRECTIONS

## 9.1 EXAMINING EXHAUSTION

The nature of functional exhaustion remains an interesting topic in the resolution of chronic and latent infections. Our studies in chapter 5 illustrate that by latent infection a great majority of subdominant CD8 T cells become exhausted. We still do not know at what point between acute infection and latent infection these subdominant CD8 T cells lose functionality. Therefore, a description of the kinetics of functional compromise during infection would shed light on the degree of functional exhaustion. Also, defining the role viral gene expression plays in functional exhaustion is critical for the complete understanding of the process during latency. Our collaboration with the Kinchington laboratory has provided the ability to express viral epitopes from varying viral promoters [185]. The unique properties of the $gB_{498\text{-}505}$ epitope make it an attractive target for these studies. By moving the $gB_{498\text{-}505}$ epitope to different promoters, one could produce epitopes resulting in exhausted CD8 T cells. Functionality of $gB_{498\text{-}505}$-specific CD8 T cells could then depict the role viral gene expression plays in exhaustion. Similarly, moving exhausted epitopes to the gB promoter leading to alleviated exhaustion phenotypes would confirm observations.

Our most recent data also help detail the nature of exhaustion in latently infected TG (Figures 29&30 in Apendix A). Results illustrate that functional subdominant CD8 T cells in the

TG during latency do not bind detectable amounts of fluorescently conjugated tetramers suggesting these cells either express TCR that binds with low affinity to peptide/MHC or express low amounts of surface TCR. Conversely, a large percentage of cells that do not function bind detectable levels of TCR. We concluded from these data that the ability to bind MHC/peptide directly correlates with the state of exhaustion. We can identify variations in the composition of the TCR by isolating functional and non-functional subdominant CD8 T cells from mice that express Thy1.1 concurrently with IFN-γ. Single-cell PCR analysis of TCR may show that higher affinity clonotypes of protein-specific CD8 T cells are more likely to become exhausted.

## 9.2 CHARACTERIZING THE TG MICROENVIRONMENT DURING LATENCY

We concluded from our CD8 T cell adoptive transfer studies that TG-resident CD8 T cells appear "stamped" with a surface marker that maintains their retention in the TG, which is absent on peripheral or circulating memory CD8 T cells. Studies by others investigating $T_{rm}$ CD8 T cells suggests a possible tissue resident marker would be CD103. Our studies discount this as a unique marker of $T_{rm}$ due to its expression on only 50% of CD8 T cells in the TG regardless of specificity. A marker of interest could be VLA-1 (CD49a), which is an integrin known to be expressed on CD8 T cells specific for HSV antigens [165]. Also, this surface marker is known to be important for retention of CD8 T cells in infected lung tissue during influenza infection [186]. Analysis of the TG microenvironment could provide insight into factors that allow entrance of CD8 T cells into the TG. A drawback to these studies and other studies examining the latently infected TG is the low numbers of CD8 T cells in the tissue. This complicates comparison of these cells to peripheral memory CD8 T cells. A possible way to better study TG-resident CD8 T

cells would be to incubate dispersed TG cells with in vitro generated memory cells. If entry is determined by a soluble factor in the TG, we may be able to provide exogenous cells with the marker capable of allowing entrance into the TG.

## 9.3 FURTHER DEFINING THE EFFECTS OF ANTI-IL-10R MAB TREATMENT

### 9.3.1 Are neurons dying or is HSV pushed into a more latent state?

An important question in our studies involving the administration of anti-IL-10R mAb treatment asks whether neurons harboring virus capable of reactivation are killed or if HSV-1 is pushed into a more latent state. Our studies show that increased numbers of granzyme B expressing CD8 T cells correlates with a lasting reduction in viral reactivation from latency. It is well characterized that the main role of granzyme B is to cleave and activate caspases leading to apoptosis of the host cell [97]. In HSV-1 infection, granzyme B has been shown to prevent viral reactivation not by inducing apoptosis, but by cleaving the immediate early protein, ICP4 [43]. With the observed increase in the number of granzyme B expressing CD8 T cells, we would hypothesize that the increased lytic hit would override the anti-apoptotic capabilities of ICP4 and LATs resulting in neuronal apoptosis. Additionally, IL-10 is a known cytokine that promotes neuronal survival during stress [187], so IL-10R inhibition may not only affect the immune system, it may also increase the neuronal susceptibility to granzyme B mediated apoptosis.

Alternatively, granzyme B is known to bind a protein associated with heterochromatin in the nucleus [188]. The status of heterochromatin of viral DNA is thought to play a role in the reactivation from latency. Therefore, it is possible that the genetic modifications in

heterochromatin by granzyme B may not be inducing apoptosis but may be re-ordering the viral heterochromatin to a more stable form. To visualize this, it would be necessary to identify neuronal apoptosis during treatment. This can be accomplished using the TUNEL reagent, which effectively labels apoptotic cells, and confocal microscopy. The caveat of this process would be the extremely low frequency of infected neurons that can reactivate the virus. Confocal microscopy could aid in the search for apoptotic neurons due to the ability to mark hubs of immune cell interactions. The most effective time to glimpse apoptotic neurons would be after three weeks of treatment. At this point, the TG harbors the largest numbers of CD8 T cells, and it will be straightforward to detect clusters of CD8 T cells surrounding infected neurons. To confirm the first hypothesis, we would expect to observe apoptotic neurons in the centers of these clusters while TGs from PBS treated mice would contain clusters of CD8 T cells surrounding non-apoptotic neurons. Testing the state of heterochromatin would consist of infecting neurons *in vitro* in the presence of acyclovir to induce a "latent" state. We could then treat these neuronal cultures with perforin and granzyme B to assess changes in host heterochromatin as well as heterochromatin of viral DNA.

### 9.3.2 What cell produces IL-10?

In LCMV models, several cell types have been shown to produce IL-10 such as CD4 T cells, macrophages, DCs, B cells, and others [112,181]. Additionally, other viral models have recently shown that CD8 T cells can, in fact, produce IL-10 during infection to limit the inflammatory response slowing the kinetics of viral clearance. The role of IL-10 in viral control has not been investigated, so the producer of IL-10 remains unclear. The TG contains several types of cells that have the potential to produce IL-10 such as neurons, CD4/CD8 T cells, macrophages, B

cells, and DCs. Due to the difficulties of stimulating IL-10 production in immune cells. The use of reporter mice would be the best step to identify the main IL-10 producer during HSV-1 infection. Specifically, performing flow cytometry on TG cells or imaging whole TG with confocal microscopy would allow detection of IL-10 producers in mice that express GFP concurrently with IL-10.

### 9.3.3 What are the specificities of the increased TG-resident CD8 T cells?

We currently hypothesize that all CD8 T cells before and after anti-IL-10R mAb treatment recognize HSV antigens. However, our assays have only accounted for a fraction of the HSV-specific CD8 T cell repertoire during latency. This is primarily due to the limitation in fluorescently labeled tetramer technology. We have been unable to use tetramer based assays to identify CD8 T cell specificities due to the low level of TCR expression and/or ineffective labeling with the tetramer. We do have evidence that subdominant CD8 T cells that are tetramer$^+$ for an HSV antigen and CD8 T cells that produce IFN-γ when stimulated with that same antigen are mutually exclusive (Figure 29). In other words, it appears as if tetramer$^+$ cells are functionally exhausted while the functional counterparts have either low affinity TCR or express low amounts of surface TCR. This would suggest that we have underestimated the known frequency of HSV-specific CD8 T cells in a latently infected TG. To reach an appropriate frequency of HSV-specific CD8 T cells, we should stain latently infected TG cells with tetramer followed by a 6 hour stimulation with fibroblasts pulsed with peptides used in the tetramer pretreatment protocol. We are still faced with the problem of ineffective tetramers (for specificities other than RR1 and ICP8), but this process will at least provide a more accurate assessment of the frequency of RR1- and ICP8-specific CD8 T cells.

To better assess the specificities that have proven incapable of being fluorescently labeled with tetramer, manipulation of the peptide may allow for better binding to MHC or the TCR may be required. Alternatively, there has been a recent report detailing the use of photocrosslinkable tetramers that can label with very high efficiency [189]. These reagents have been shown to be able to detect peptide-specific CD8 T cells with very low numbers of surface TCR or with low affinity TCR.

### 9.3.4 The effects of acute anti-IL-10R treatment

We were intrigued to find a study that shows IL-10 in cooperation with IL-21 during acute LCMV infection actively induces memory development in the viral-specific CD8 T cell population [190]. Without these cytokines, the host is left with a pool of CD8 T cells retaining a terminal effector phenotype rather than a self-renewing memory population. While the effect of this on chronic viral burden was not explored, we sought to investigate the effects of acute IL-10 neutralization in our model of HSV-1 infection due to the necessity of self-renewing memory cells in preventing viral reactivation.

For these studies, we gave two i.p. injections of anti-IL-10 mAb on 0 and 5 dpi. We then sacrificed mice at the peak of the response (8 dpi) and latency (35 dpi) and assessed the numbers and phenotypes of the infiltrating lymphocytes. We notice marked effect of treatment at both 8 and 35 dpi in regards to total CD8 and CD4 T cells in the TG. In the CD8 T cell compartment, we observed a 50% increase in total cells during acute infection that is maintained through contraction and latency. Interestingly, we saw a 200% increase in total CD4 T cells in the TG during acute infection and latency (Figure 27A). These data give support to our hypothesis that CD4 T cells are not just bystanders in the prevention of viral reactivation but do play a yet to be

determined role. We find it interesting that in these studies CD4 T cells are preferentially regulated by the immunoregulatory cytokine, IL-10.

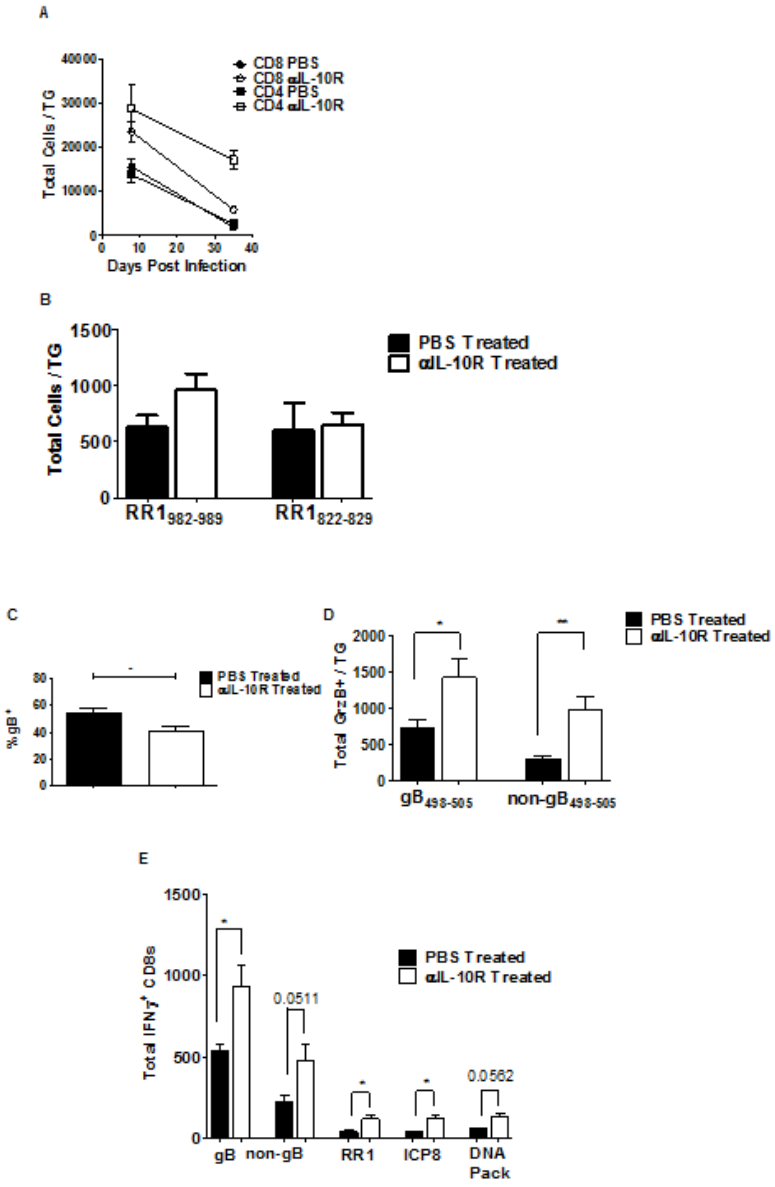


**Figure 27 The effects of acute anti-IL-10R mAb treatment**

(**A-D**) C57BL/6 mice were infected with HSV-1 RE and given an i.p. injection of anti-IL-10R mAb on 0 and 5 dpi. TG were excised and dispersed into single-cell suspensions on (**A&**B) 8 and (**A&C**-E) 35 dpi. (**A-D**) Cells were stained for (**A-D**) CD8α, (**A&C&D**) $gB_{498-505}$ TCR, (**B**) $RR1_{982-929}$ TCR, (**B**) $RR1_{822-829}$ TCR and (**D**) granzyme B (GrzB) and analyzed by flow cytometry. (**A**) Symbols represent the cumulative mean ± SEM of total numbers of cells in the TG at 8 and 35 dpi (total n=10-14, 3 experiments). (**B**) Bars represent the cumulative mean ± SEM of the total numbers of $RR1_{822-829}$ or $RR1_{982-989}$ TG-resident CD8 T cells at 8 dpi. (total n=8-9, 2 experiments) (**C**) Bars represent the cumulative mean ± SEM frequency of $gB_{498-505}$-specific CD8 T cells at 35 dpi. (total n=7-8, 2 experiments) (**D**) Bars represent the cumulative mean ± SEM total numbers of granzyme $B^+$ $gB_{498-505}$-specific or non-$gB_{498-505}$-specific CD8 T cells in the TG at 35 dpi. (total n=7-10, 2 experiments). (**E**) TG cells were incubated for 6 hours with HSV-infected fibroblasts or B6WT3 fibroblasts pulsed with three RRI epitopes, three ICP8 epitopes, or 1 DNA Packaging Terminase epitope in the presence of Golgi-Plug. Cells were monitored for surface CD8α and interacellular IFN-γ and analyzed by flow cytometry. Bars represent the cumulative mean ± SEM number of IFN-$\gamma^+$ at 35 dpi (total n=4-10, 1 experiment).

We further examined the effects of CD8 T cells by characterizing the effects of acute antibody treatment on the immundominance hierarchy. While we did not observe any change in select subdominant specificities (Figure 27B), we did notice a slight reduction in the immunodominance of $gB_{498-505}$-specific CD8 T cells (Figure 27C). Similar to our latent anti-IL-10 mAb treatment, we could not identify a specificity of CD8 T cells that was preferentially expanded. As stated earlier, granzyme B and IFN-γ secretion are absolutely required for the prevention of viral reactivation, so we assessed the ability of cells to produce these effector molecules during latency. Again, we noticed that the latent subdominant CD8 T cell population is significantly affected by acute anti-IL-10R mAb treatment. In the subdominant CD8 T cell population we detected more than a 2 fold increase in the numbers of cells expressing granzyme B directly ex vivo and in the numbers of cells that secrete IFN-γ upon antigenic stimulation. Contrary to latent anti-IL-10R mAb treatment, $gB_{498-505}$-specific CD8 T cells also increased approximately 2 fold increase in effector function in mice treated with anti-IL-10R mAb during primary infection (Figure 27 D&E).

Even though our results do show a marked increase in the cellular response to HSV-1 after IL-10 inhibition, much is needed to identify the exact role IL-10 plays in the generation of the cellular immune response against HSV-1. Phenotypically characterizing the increased pool of cells using KLRG-1, CD127, CD27, Tbet, and Eomesodermin would aid in the demarcation of these cells as effector or memory cells similar to previous studies. Additionally, proliferation assays would explain the proliferative potential of the expanded population. If the expanded population does exhibit an effector phenotype, it would be interesting to observe the size of the population for extended periods of time such as 90, 120, and 180 dpi. Due to the eventual loss of effector-like cells, we would hypothesize that the cells will eventually be lost from the TG. If

indeed these cells are lost, quantification of viral burden and spontaneous reactivation from ex vivo cultures would convey if the boosted population was reduce the possibility of viral reactivation from latency. If the effector-like cell population is retained at high levels for long periods of time after infection, our studies would then be able to describe another unique aspect of the TG microenvironment that prevents the eventual contraction of effector-like cells.

## 9.4 WHAT ROLE DO CD4 T CELLS PLAY DURING LATENCY?

Even though CD4 T cells play a key role in mediating HSK, CD4 T cells enter TG, similar to CD8 T cells. TG CD4 T cell numbers peak between 10-12 dpi and contract to form a stable latency population equivalent to the TG-resident CD8 T cell population. Early in infection, TG-resident CD4 T cells have the ability to produce IFN-γ (data not shown), but their phenotype during latent infection has yet to be investigated. Recently, we acquired a transgenic mouse that possesses CD4 T cells bearing a TCR that recognizes an HSV antigen on glycoprotein D. By adoptively transferring CD4 T cells of irrelevant specificities, we can determine if, similar to CD8 T cells, only HSV-specific CD4 T cells infiltrate and are retained in infected TG. If only HSV-specific CD4 T cells enter the TG, then we can assume that, similar to CD8 T cells, CD4 T cells require antigenic stimulation for retention in the TG. Recently, we excised TG from latently infected IL-10-eGFP mice and analyzed dispersed cells with flow cytometry. Intriguingly, we found that as many as 25% of CD4 T cells in the TG express GFP directly ex vivo (Figure 28). We can infer from these results that roughly a quarter of CD4 T cells are stimulated at any point during latency.

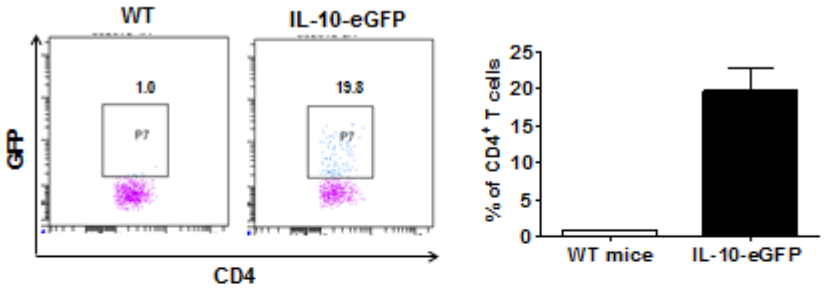


**Figure 28 TG-resident CD4 T cells produce IL-10 in latently infected ganglia**

TG from latently infected WT or IL-10-eGFP mice were excised and dispersed into single-cell suspensions and were immediately stained for CD45 and CD4 and analyzed by flow cytometry. Dot plots represent GFP expression in WT and IL-10-eGFP mice. Data represents the cumulative mean ± SEM frequency CD4 T cells that express GFP (total n = 1 (WT) or 3 (IL-10-eGFP) from 1 experiment).

These results give critical and exciting evidence for further characterizing the dynamics of latency. Our current theory suggests that during latency, a small amount of viral gene expression occurs that produces peptides, which stimulate HSV-specific CD8 T cells. Our theory then would not support antigenic stimulation of CD4 T cells due to the apparent absence of MHC class II molecules on host neurons. Detection of MHC class II on latently infected neurons using confocal microscopy would help support our theory; however, the inability to detect MHC class II would require a new hypothesis. If confocal microscopy revealed that CD4 T cells clustered around satellite cells or other APCs such as macrophages, it would suggest that viral proteins have made way out of the cell to allow for cross-presentation to CD4 T cells by surrounding cells. Therefore, we would hypothesize that during latency, rather than intermittent viral gene

expression stimulating CD8 T cells, viral gene expression would produce full proteins that are released into the TG microenvironment to be picked up and presented to CD4 T cells. This process could either be due to exocytosis of viral products or the exciting possibility of DC networks extending nanotubes into host neurons that pick up viral peptides for antigen presentation to CD4 T cells. The nature of antigenic stimulation of CD4 T cells during HSV infection would not only help develop hypotheses related to HSV associated latency, but it will also help describe the dynamics of antigen presentation and the immense sensitivity of CD4 T cells during a viral infection.

Our investigation of in situ production of IL-10 by TG-resident CD4 T cell response during HSV infection also demonstrates a role that CD4 T cells play during latency. From our studies, it seems as if CD4 T cells act to suppress the CD8 T cell response as described by our IL-10 neutralization studies in Chapter 7. Therefore, we hypothesize that TG-resident CD4 T cells act to prevent the destruction of non-regenerating neurons that harbor latent HSV. These IL-10 producing CD4 T cells resemble a Th2 CD4 T cell subset. Defining whether the rest of the CD4 T cells are the same or different subsets remains an intriguing avenue of research. This can be done by examining cytokine production and transcription factor expression after stimulation with antigen pulsed DCs.

Similarly, studies should determine whether the subtypes of CD4 T cells are stable during various transitions of infection such as the transition from acute to latent infection, latent infection to reactivation/lytic infection, and reactivation/lytic infection to latent infection. It would be interesting to determine if CD4 T cells display a more anti-viral phenotype during a time of lytic infection to limit viral spread while the phenotypes are more suppressive during latent infection when the chance for viral spread is low or non-existent. To accomplish this, we

could induce reactivation using our psychological stress model and use intracellular cytokine detection to effector molecules produced. Additionally, flow cytometric detection of transcription factors would aid the delineation of CD4 T helper subsets.

# APPENDIX A

# SUPPLEMENTAL DATA

## A.1.1 Exhaustion of RR1-specific CD8 T cells

After our studies showing exhaustion of subdominant CD8 T cells, we wanted to begin to further delineate the extent of exhaustion in the subdominant CD8 T cells. We focused our efforts on CD8 T cells specific for epitopes on the HSV-1 protein RR1. During latency, RR1-specific CD8 T cells lose a significant amount of functionality compared to acute infection. Nevertheless, the entire population does not become functionally compromised, which led to our hypothesis that high levels of TCR expression would lead to increased stimulation and eventual exhaustion. Conversely, RR1-specific CD8 T cells with little to no detectable TCR expression would be stimulated to a lesser degree and be less prone to exhaustion. To test this hypothesis, we first pretreated latently infected TG cells with pools of tetramers complexed to the 3 RR1 epitopes followed by a 6 hour *in vitro* stimulation with fibroblasts pulsed with the same 3 RR1 epitopes. After stimulation, we analyzed TG cells by flow cytometry for TCR expression and IFN-γ production.

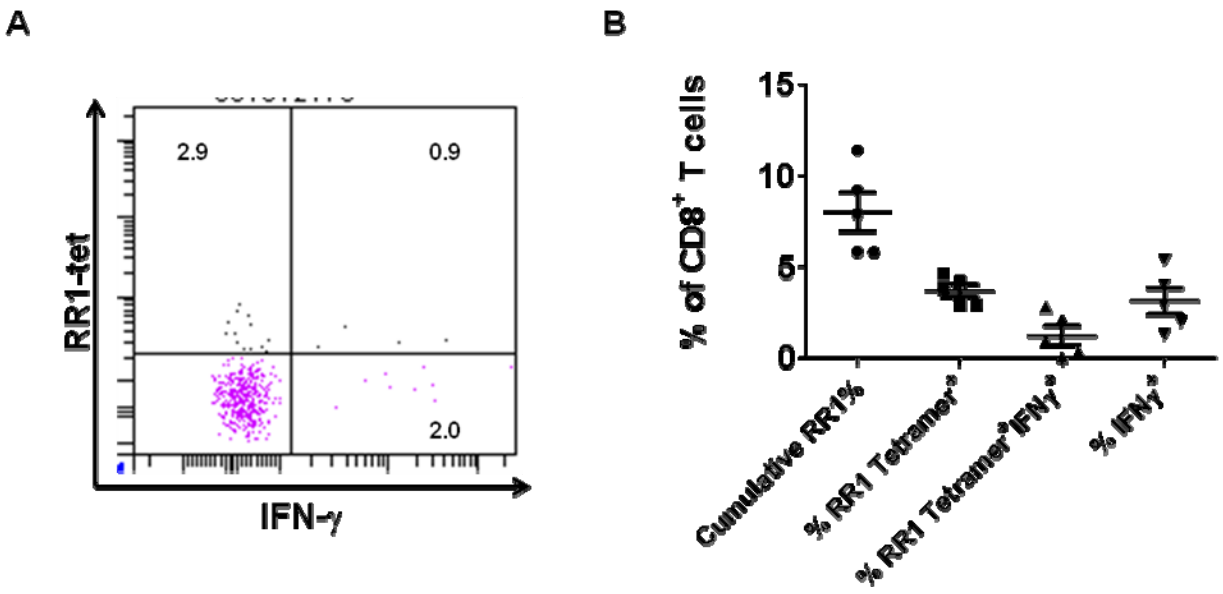


**Figure 29 Functioning subdominant CD8 T cells in the TG express lower amounts of TCR than non-functional subdominant CD8 T cells**

**(A&B**) TGs from latently infected mice were harvested and dispersed into single-cell suspensions and were pretreated with pooled tetramers complexed with $RR1_{822-829}$, $RR1_{982-989}$, and $RR1_{372-380}$ epitopes for 1 h at room temperature. TG cells were incubated for 6 h with B6WT3 fibroblasts loaded with 3 RR1 peptides in the presence of Golgi-Plug, followed by intracellular staining for IFN-γ. Samples were analyzed for tetramer positivity and IFN-γ production by flow cytometry. (**A**) Representative dot plots displaying tetramer staining and IFN-γ production in the CD8 T cell population after pretreatment and stimulation. (**B**) Data represents the cumulative mean ± SEM frequency of cells that were positive for the noted parameters (total n = 5, 1 experiment).

These results suggest that functional exhaustion in a latently infected TG relates to either how well a CD8 T cell TCR can bind peptide/MHC or the level of expression of TCR. Results from chapter 4 show that the TCR affinity for MHC/peptide between cells that do not become exhausted ($gB_{498-505}$) and cells that do become exhausted (RR1) are identical. Therefore, unless a transition occurs between acute infection and latent infection, we are left to assume that the level of TCR expression directly correlates to functional exhaustion during latency. Evidence to support this comes from data comparing stimulation and tetramer staining from acute and latent infection.

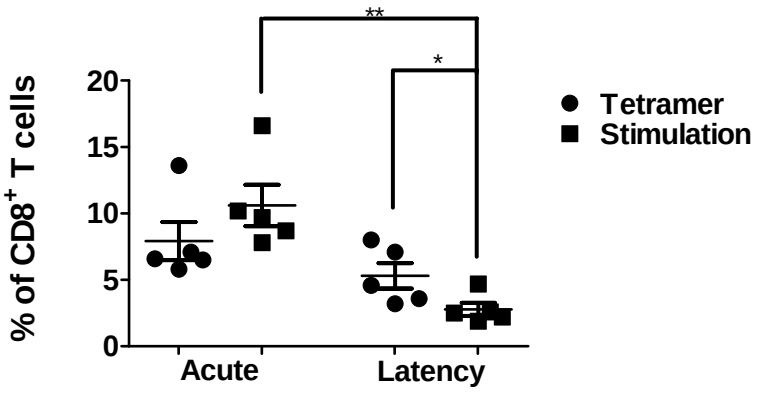


**Figure 30 RR1 tetramer staining does not appear to be strictly correlative to stimulation data.**

TG cells from acute or latent TGs were either stained with pooled RR1 tetramer or stimulated with B6WT3 fibroblasts pulsed with pooled RR1 epitopes for 6 h in the presence of Golgi-Plug. Cells were then analyzed for RR1-TCR expression or IFN-γ production by flow cytometry. Data represents the cumulative mean ± SEM frequency of CD8 T cells that were either tetramer+ or IFN-γ+.

During acute infection, we observe that tetramer staining of RR1-specific CD8 T cells underestimates the cells that respond to peptide pulsed fibroblasts. Conversely, during latency we see that tetramer staining overestimates the cells that respond to peptide pulsed fibroblasts. In other word, there are RR1-specific CD8 T cells during acute infection that respond to peptide but do not express enough TCR to be detectable by flow cytometry. We feel that these tetramer negative cells that respond to peptide are cells that retain functionality in the latently infected TG. Further characterizing the differences between tetramer- cells that function and tetramer + cells that do not function during latency is an essential task to better protect from viral reactivation from latency.

## APPENDIX B

## LIST OF WORKS

1) **St. Leger AJ**, Jeon S, Hendricks RL. Expanding the functional CD8+ T cell repertoire reduces HSV-1 reactivation from latency in sensory ganglia. (*submitted to PLoS Pathogens).*
2) Bidula SM, Ramachandran S, **St. Leger AJ**, Sette A, Hendricks RL, Kinchington PR. Ganglionic CD8+ T cell specificity induced by herpes simplex virus type 1 lacking and ectopically restored for the $gB_{498\text{-}505}$ C57BL/6 murine immunodominant epitope (*in preparation).*
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