Development of HSV-1 lacking the immunodominant $gB_{498-505}$ epitope and analyses of the alternative $CD8^+$ T cell response in the murine ocular infection model

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ABSTRACT

Development of HSV-1 lacking the immunodominant $gB_{498-505}$ epitope and analyses of the alternative $CD8^+$ T cell response in the murine ocular infection model

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Herpes simplex virus type 1 (HSV-1) establishes latent infections in sensory ganglia such as the trigeminal ganglia (TG), and periodically reactivates to cause repeated corneal infections. These may trigger an immune-mediated corneal disease known as herpes stromal keratitis (HSK), which results in corneal scarring, and eventually blindness. HSK is the most common infectious cause of blindness in the US, affecting over a quarter of a million people annually. Herpes simplex virus type 1 (HSV-1) latent infections in trigeminal ganglia (TG) are associated with persistent $CD8^+$ T cell infiltrate. The ability of such $CD8^+$ T cells to block HSV-1 reactivation from ex vivo ganglionic cultures establish the potential to control reactivation in vivo. As such, targets of $CD8^+$ T cells in the TG will guide vaccination strategies aimed to block reactivation. In latently infected C57BL/6 mice, ~50% of the $CD8^+$ T cell infiltrate in the TG is directed to an immunodominant epitope (residues 498-505) on viral glycoprotein B (gB-CD8). gB-CD8 can block reactivation from latency. Remaining $CD8^+$ T cells in the TG are virus

specific, can block reactivation, but are directed to unknown antigens. Given that $CD8^+$ T cells contribute to maintenance of the HSV-1 latent state, it is important to understand viral antigens targeted by these cells and factors influencing dominance hierarchy. Here, we assessed the $CD8^+$ T cell target specificity in TG of C57BL/6 mice infected with HSV-1 lacking and ectopically restored for the $gB_{498\text{-}505}$ epitope. Epitope mutants with near wild type pathogenicity were isolated, and found to induce TG associated $CD8^+$ T cell infiltrates of size similar to that induced by wild type HSV-1, but with little $gB_{498\text{-}505}$-specificity. The nature of the compensated response reflected increase of $CD8^+$ T cell populations directed to most known subdominant epitopes seen in wild type HSV-1 infection. However, a $gB_{498\text{-}505}$ dominated $CD8^+$ T cell response developed following infection with HSV with $gB_{498\text{-}505}$ epitope-mutation that expressed short $gB_{494\text{-}509}$ peptides at an ectopic (gC) locus. We conclude that loss of the HSV dominant epitope does not alter the size of the HSV-specific $CD8^+$ T cell response nor broaden the TCR repertoire, but rather results in a broader dominance hierarchy of subdominant epitopes rising to co-dominance. We further conclude that immunodominance is not a result of properties of the HSV-1 gB protein itself or its genomic locus.

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1.0 INTRODUCTION

1.1 HERPES SIMPLEX VIRUS TYPE 1

1.1.1 HSV-1 Disease and Epidemiology

Herpes simplex virus type 1 (HSV-1) is a member of the *Herpesviridae* family, and can be further classified as *Alphaherpesvirinae*, along with HSV-2 and varicella zoster virus (VZV). All the human Alphaherpesviruses are neurotropic, infecting multiple cell types during primary and reactivated disease and establishing latency in neurons sensory ganglia that innervate the site of primary infection [1]. It is currently thought that around 80% of the world's population is latently infected with HSV-1 by adulthood [2,3,4]. In the United States, 57% of individuals between the ages of 14 and 49 are seropositive for HSV-1 with the number rising to 90% by the age of 60 [5,6]. Reactivated disease can have variable presentation, ranging from asymptomatic viral shedding to clinical presentation of cold sores, herpetic whitlow, genital lesions, more serious diseases such as neonatal systemic infections, encephalitis as well as blinding stromal keratitis [4].

1.1.2 Herpes Stromal Keratitis Disease

Herpes stromal keratitis (HSK) is caused by Herpes Simplex Virus Type-1 (HSV-1). HSK is the most frequent and serious type of eye infection in the United States causing vision loss in over a quarter of a million people annually [7]. HSK is the result of reactivation of HSV-1 and subsequent viral reactivation at the corneal surface. This reactivation causes an immune infiltrate in the eye that is initially aimed at controlling this HSV-1 replication but subsequently shows lack of specificity to HSV-1 antigens. These immune cells release factors such as interferon-γ (IFNγ) and interleukin-2 (IL-2), and other compounds, including nitrous oxide (NO) that can cause destruction and disorganization of the collagen matrix of the stroma [8,9,10]. There is also a cascade of non-specific responses including neutrophils that recruit macrophages, plasma cells, and $CD4^+$ T cells that regulate proinflammatory cytokines [11,12]. It is the build-up of scar tissue over time that ultimately results in a loss of vision from the cornea [11,13].

Despite advances in understanding immune regulation of HSV-1 infection, there is still no vaccine. Current therapies for HSK include antiviral acyclovir drops and corticosteroids that can help to reduce viral replication and inflammation [14,15]. However, these treatments are only effecting during ongoing viral replication and HSK is still able to develop without the presence of infectious virus in the cornea [16]. Once corneal blindness occurs, a corneal transplant is the single option available with the possibility of restoring vision.

We argue that the only way to prevent HSK is to stop reactivation of HSV-1 and prevent the resultant tissue scarring. The studies in this proposal address the expression of HSV-1

proteins and their immunogenicity during acute infection and latency with the aim of creating vaccines designed to stop HSV-1 from reactivating.

1.2 HSV-1 STRUCTURE

The HSV virion has four compartments: 1) an outer envelope that contains the glycoproteins, 2) the tegument layer that contains about 20-30 viral proteins and some cellular proteins, 3) a capsid layer, and 4) an electron core that encompasses the HSV genome (Figure 1.1) [4]. HSV-1 genome is a linear double stranded DNA of 152 kb genome that is packed inside of the icosahedron shaped capsid. The genome can encode for the expression of about 80 viral proteins [1,17].

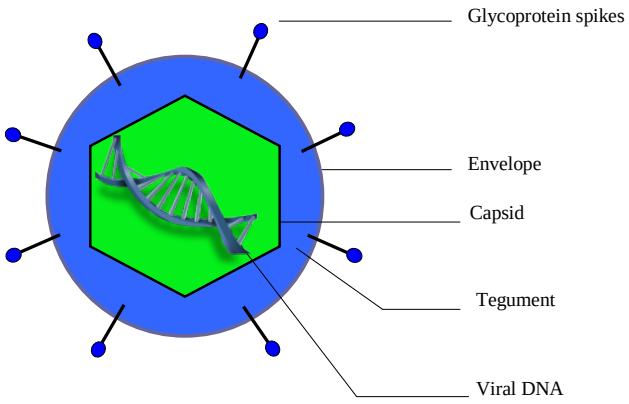


Figure 1.1 Representation of the HSV-1 virion.

The HSV-1 nucleocapsid contains one genome of double stranded viral DNA. The capsid is surrounded by a tegument layer which is enclosed within a host derived envelope containing viral glycoprotein spikes.

1.3 HSV-1 LIFE CYCLE

Most HSV-1 infections occur between the ages of 6 months and 3 years in the human population, usually causing mild or asymptomatic primary infections. The life cycle of HSV-1 is defined in three types: lytic replication, latency, and reactivation. Here lytic infection will be considered first.

1.3.1 Entry

Viral replication begins when HSV-1 is able to infect and enter into a permissible host cell. There are three viral glycoproteins (gB, gC, and gD) that mediate viral attachment to the host cell but others may be involved. Glycoprotein C mediates the initial attachment of the HSV-1 virion by binding to heparin sulfate moieties on the cell surface [18,19]. However, gC is not essential and gB is able to bind in the absence of gC [20]. Once gC binds to the heparin sulfates, gD is now able to bind with better efficiency to other cell surface receptors such as nectin 1, Herpesvirus entry mediator (HVEM) or O-sulfated heparin sulfate. HSV-1 then enters the cells by a fusion event involving gB, gD, and the gH/gL complex [21]. Entry may involve direct

plasma membrane fusion, or uptake as endosomes and fusion from them to gain cytoplasmic entry.

1.3.2 Temporal Gene Expression

During the lytic phase over 80 genes are expressed in kinetic temporal cycles that can be defined experimentally: immediate early, early and late (Fig. 1.2). After the virus enters the hose cell the viral tegument protein, VP16, and the two hos6 transcription factors, host cell factor (HCF) and Oct1 bind immediate early promoters (IE or α) and promote their transcription.

The immediate early genes are the first to be expressed. These can be transcribed in the absence of *de novo* viral protein synthesis, which is distinct from the early and late genes that are expressed only following the expression of early proteins. The IE genes encode proteins that are necessary for the transcription of other HSV-1 genes. Early genes encode proteins responsible for viral DNA replication and late genes are necessary for encoding proteins for glycoproteins, assembly, and maturation. Once the expression of all classes of proteins has occurred, HSV-1 virions are able to be assembled and released from the infected cell [1].

α gene expression peaks around 2-4 hours post infection, with five α proteins being encoded by HSV-1: ICP0, ICP4, ICP22, ICP27, and ICP47. All of these proteins except for ICP47 are needed to regulate gene expression and transcription of the other gene classes. ICP0 is a multifunctional protein with E3 ubiquitin ligase activity that degrades host ND10-PML nuclear domains to prepare the host cell for viral gene expression. It is also important for efficient reactivation from latency [22] and for helping to suppressing the IFNγ response [23,24,25,26,27].

ICP4 is a protein that interacts with the host transcriptional machinery and is necessary for activation of early and late genes [28]. ICP27 is also important for HSV-1 replication, and is also important in inhibiting mRNA splicing and RNA binding [17]. ICP47 is important in allowing HSV-1 to evade the immune system by down regulating antigen presentation by blocking the transporter associated with antigen processing (TAP) [29,30]. One potential role for ICP22 may be altering the localization of cellular proteins into distinct nuclear foci containing ubiquitinated proteins and proteasomal components. [31].

The early or β genes are expressed after accumulation of α proteins. Early gene expression peaks at 5 to 7 h.p.i. These proteins are mainly responsible for further adaptation of the cell and DNA replication. They include DNA polymerase, ribonuclotide reductase, thymidine kinase, the single stranded DNA binding protein, and the viral protein kinases. The late genes (L or γ) are subclassified as γ1 and γ2. γ1 genes are expressed in low amounts during early infection and do not peak until after viral DNA synthesis, while γ2 genes are not transcribed until viral DNA synthesis has initiated. These genes generally encode structural proteins and glycoproteins needed for virion assembly and egress [17].

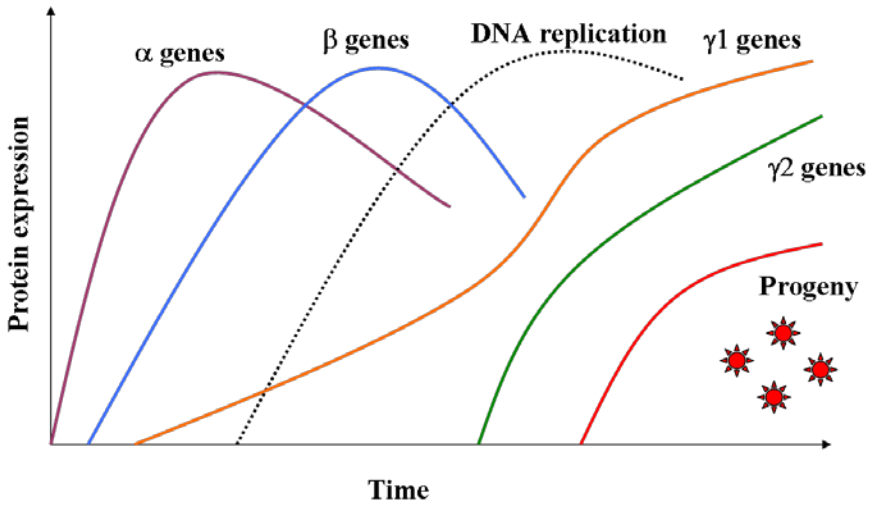


Figure 1.2. HSV-1 gene expression kinetics.

This graph represents the temporal cascade of HSV-1 lytic gene expression. α genes (purple) are expressed immediately after infection is initiated followed by the β genes (blue). After accumulation of β proteins, DNA replication (dotted line) occurs followed by γ gene expression. γ1 genes are made prior to DNA replication while γ2 genes require DNA synthesis before they can be expressed. Progeny virus (red) can be assembled and released from the infected cell once all of the protein classes have accrued.

1.3.3 Latency

After infection of an epithelial cell, HSV-1 gains access to sensory neurons that innervate the initial site of infection. This allows the virion to be transported via retrograde transport to the nerve body of innervating neurons where it may briefly replicate and then establish latency. During latency, HSV-1 lies dormant within the sensory ganglia of the initial site of infection and no productive infectious virus is made.

The mouse model is the preferred method to study HSK and HSV-1 latency. This is due to the fact that mice can be infected through the eye, flank, and nose and also maintain latency

virus in the TG. Mice also rarely spontaneously reactivate, allowing them to be a prime candidate for studying latency [32,33]. After a murine ocular infection, virus is able to access the nerve termini of the neurons that innervate the mouse cornea. HSV-1 is then able to travel by retrograde transport to the neuronal body of the sensory neurons and can be detected in the TG within 2 dpi [34]. Evidence suggests some neurons replicate the virus, while others immediately support a latent state. When latency is established, most lytic gene expression is silenced except for latency (LAT) transcripts that continue to express and accumulate. These LAT transcripts are non poly A, non capped, RNA transcripts that are found in latently infected neurons in the TG [34]. LAT transcripts can be found in both human and murine TG that are latently infected [35,36,37]. LAT null viruses are unable to establish latency with the frequency that wild-type HSV-1 viruses are, although they do enter latency [38]. LAT-null viruses also reactivate with reduced efficiency [39]. LAT null viruses also causes more pathology and induce neuronal death that is not seen in wild-type viruses, leading to the conclusion that one of the functions of LAT is to prevent apoptosis of latency infected neurons and enhance latency establishment [40,41].

Chromatin regulation is also important during latency. The viral genome circularizes and is maintained as an episome within the nucleus [42]. It has been shown that episomal HSV-1 DNA is associated with host nucleosomes and histones [43]. It is thought that latency is controlled through chromatin regulation of these histones. The LAT promoter has increased levels of histone H3 as compared to the ICP0 gene, indicating that the LAT promoter is maintained in an activate state [44] while lytic promoters are associated with heterochromatin [45]. However, current evidence suggest the chromatin state is somewhat in a state of flux, in

which several factors may contribute to changes in its state that leads to de-repression of viral genes [46,47].

1.3.4 Reactivation

Classic reactivation occurs when the viral genome is able to enter the lytic stage after a period of latency. As a result, progeny virus is assembled and released from the latently infected neuron. When HSV-1 reactivates, the envelope proteins and capsids are thought to travel separately via anterograde transport to the nerve termini. It is here that the progeny virus is assembled [48]. The ability of the host to control infection at the site of latency is an important factor in maintaining HSV-1 in latency. However, in some cases limited gene expression has been seen that has been termed “molecular reactivation” and this may correspond to an abortive reactivation attempt by HSV-1 that is able to be quashed before any infectious virus can be produced or which may represent a partial de-repression of gene expression without progression to full virus production.

As such, gene expression during latency was, until recently, thought to be silent except for the expression of LAT’s. However, current studies have shown that lytic gene products are seen in latently infected TGs [49,50]. During reactivation events, it is possible that the “typical” α-β-γ temporal cascade is not followed, and instead there is a two phase gene expression model. A recent study used nerve growth factor depleted TG and showed low levels of deregulated gene expression prior to reactivation events [46]. A second study showed that VP16 may be the limited factor in what initiates the full temporal gene cascade [47]. However, both of these models were *ex vivo*, and need to be substantiated in an *in vivo* model.

In the murine model, several stimuli have been shown to induce full virus reactivation. These include UV, stress, hyperthermia and hypothermia, as well as various chemicals such as sodium butyrate or dexamethasone [51,52,53,54,55]. Physical dislocation of the ganglia also results in reactivation in an *ex vivo* model [56]. In the mouse model, HSV-1 reactivation is limited to only a small number of infected neurons within the infected TG [56] and the rate of reactivation is proportional to the number of genomes that are latent in the infected ganglia [57]. Reactivation *in vivo* results in anterograde axonal transport of infectious virions to the primary site of infection and in the ocular infection model this results in repeated replication in the cornea. Depending on host and viral genetics, subsequent infiltration of the stroma leads to destruction of the corneal matrix and HSK. Gene expression during reactivation is different than during a primary lytic infection. For example, transcriptional activation of HSV-1 genes occurs during reactivation in the absence of VP16 [58]. It has also been suggested that early gene transcripts can be detected before immediately early transcripts in a TG explant model [59]. However, these issues are difficult to resolve and remain quite controversial.

1.4 IMMUNITY TO HSV-1 INFECTION

HSV-1 infection provokes a cellular innate immune response at the initial site of infection. This control is mediated by type 1 interferons (IFN-α and IFN-β) [60]. IFN-α and IFN-β are able to attract neutrophils, macrophages, and natural killer cells, all of which produce antiviral compounds such as NO, IFNs, and interleukin-12 [61,62]. However, while this innate immune

response is able to control the virus in the cornea, HSV-1 can still gain access to nerve termini and infect the TG. In the murine model, HSV-1 reaches the TG within 2 dpi. Macrophages in the TG produce NO and tumor necrosis factor (TNF-α), which helps to reduce the amount of viral spread in the TG initially [63].

However, the innate response is not enough to control viral replication in the TG as evidences by persistent replication in RAG/SCID mice. The adaptive response is one component that drives latency establishment and then maintains the virus in a latent state. The initial response is typified by inflammation caused by $CD4^+$ T cells. These cells produce multiple antiviral cytokines, and are necessary to generate working $CD8^+$ T cells at the site of infection, or in the murine model, in the TG [64]. $CD8^+$ T cells are able to clear virus through cytokine and lytic granule production [65,66,67]. There are neutralizing antibodies made to viral antigens, especially to the glycoproteins (such as gB and gD) [68,69,70]. However, it is as yet unknown how they control latent HSV-1 infections.

In the murine mouse model, HSV-1 can be detected in the TG by 2 dpi and the virus titer declines until latency is established at 8 dpi. Virus specific $CD4^+$ and $CD8^+$ T cells are able to detected at 5 dpi [71]. These cells originate and are expanded in the draining lymph nodes (DLN) where professional antigen presenting cells (APC) are able to deliver HSV-1 antigens from the periphery to cross present to naïve $CD8^+$ T cells [72]. Antigens are thought to be acquired by DCs in the cornea. HSV-1 proteins present within dendritic cells (DC) are processed by a proteosomal pathway into short peptide fragments that are 8-10 amino acids long [73,74,75], which are then transported to the ER lumen by the TAP transporter and processed and assembled into the MHC-I complex. Surface presentation in conjunction with MHC-I is required to

stimulate and expand $CD8^+$ T cells. After activation, HSV-1 specific $CD8^+$ T cells migrate from the DLN into the primary site of infection and the TG, coinciding with the effector phase of the immune response in the TG, which peaks at 8 dpi in the C57BL/6 mouse model. The contraction phase is next, in which most of the $CD8^+$ T cells are lost as most viral antigen is surpressed. However, the cells maintained at low levels after 34 dpi are considered a resident effector memory population [76,77,78,79]. The human TG also sees a memory population, and latently infected neurons are surrounded by $CD8^+$ T cells [80,81].

Concerning $CD8^+$ T in the TG, $CD8^+$ T cells block reactivation through two main activities, namely IFNγ and lytic granules. IFNγ has multiple functions, including its ability to upregulate antigen presentation, help to inhibit viral translation, and also upregulate proteins that can induce apoptosis [82,83,84,85]. Lytic granules are important due to their ability to act through perforin, granulysin, and granzymes, all of which can be cytotoxic to virally infected cells [86,87,88]. Granzyme B is important for inducing caspase-dependent apoptosis [89]. However, evidence suggests it works with perforin to mediate $CD8^+$ T cell blockage of HSV-1 reactivation [66] without causing apoptosis or destruction of any neurons [66,67].

Studies have shown that $CD8^+$ T cells remain in close contact with latently infected TG neurons, suggesting that they may play a significant role in the preservation of the HSV-1 latent state [76]. $CD8^+$ T cell receptors polarize towards neurons in *in situ* latently infected ganglia, indicating that there is recognition of these neurons through MHC-I receptors [90], and these $CD8^+$ T cells also maintain expression of cytokines such as IFNγ, IL-10 and TNF-α [91]. This suggests that there is antigen exposure to retain the TG $CD8^+$ T cell population throughout latency [81,92], and therefore implies that HSV-1 latency is not antigenically silent. Studies have

detected transcripts of α, β, and γ genes in the TG during periods of latency in the absence of infectious virions, supporting spontaneous molecular reactivation events [49,50]. This leads to the hypothesis that antigen expression during periodic attempts by the virus to reactivate can be sensed by surrounding $CD8^+$ T cells, which then act to prevent reactivation.

1.5 C57BL/6 MOUSE MODEL OF LATENCY

Much of the evidence that the latent HSV-1 genome intermittently reactivates to provide sufficient viral antigens for mounting a cellular immune infiltrate has come from work with the C57BL/6 (B6) mouse model. HSV-1 infection in the mouse results in both an ocular disease and latent state that resemble what is observed in humans. Epithelial skin disease is seen initially upon infection in a mouse, but this is resolved quickly. Stromal keratitis develops within 7 days post infection (dpi), and follows through 21 dpi although viral pathogenicity, infection results in virus replication in the corneal epithelial for 1 to 4 days [93].

It is necessary to identify the viral antigens to which $CD8^+$ T cells are targeted in order to design a vaccine. $CD8^+$ T cell populations are usually directed to one or a few immunodominant epitopes that display a hierological order. This may be due to many factors such as the binding affinity of the peptide epitope for MHC-I, the precursor frequency of the $CD8^+$ T cells that are able to react to the epitope, the concentration of the epitope, or the kinetics of expression and the type of infection [94]. These immunodominant populations often are complemented by minor

populations of $CD8^+$ T cells that can recognize subdominant epitopes to a much lower frequency. In the latently infected TG of B6 mice, 50% of the $CD8^+$ T infiltrate is directed to the dominant $gB_{498\text{-}505}$ epitope [76] and 5-20% of $CD8^+$ T cells are directed to residues 822-829 of HSV-1 ribonucleotide reductase subunit 1 [95]. gB is an important γ1 gene, and has been detected as early as 2 hr post infection by $CD8^+$ T cells [96]. $CD8^+$ T cells specific for $gB_{498\text{-}505}$ are selectively retained and depletion of these T cells in an *ex vivo* culture results in reactivation. Studies show that this population has indicators of recent antigen exposure, such as the expression of granzyme B and surface markers of activation (CD25, CD44, CD69) [76,97] These $CD8^+$ T cells are also able to rapidly produce IFNγ after HSV-1 antigen exposure [76,97]. Clones of the $CD8^+$ T cells specific for $gB_{498\text{-}505}$ from the mouse TG are able to block reactivation in *ex vivo*, reactivating ganglionic cultures through MHC-I, establishing that the γ1 protein gB is recognized early and can mediate a $CD8^+$ T cell prevention of HSV-1 reactivation.

Previously HSV-1 latency was considered to be antigenically silent, however there is evidence to show that in latently infected TG, gB-CDs polarize their TCR to the surface of infected neurons to release IFNγ and lytic granules and form an immunological synapse [66,76]. However, it is difficult to quantify how much MHC-I is expressed by these latently infected neurons, since expression becomes so low during the latent phase [98,99]. Granzyme B, a marker of activation, is also seen during latency in these HSV-1 specific $CD8^+$ T cells, suggesting that there may be antigen exposure during latency [71,76], and these cells can also be stimulated to quickly produce IFNγ [76,100]. Together, this suggests that $CD8^+$ T cells recognize antigen and block reactivation before infectious virus is produced. The question of the hierarchical

populations of virus specific $CD8^+$ T cells and the effect of the dominant population on the subdominant population remained at the initiation of this work, undefined.

2.0 SPECIFIC AIMS

2.1 RATIONALE

Herpes simplex virus type 1 (HSV-1) is a neurotropic alphaherpesvirus that causes primary and reactivated secondary diseases. HSV-1 can trigger a severe immune-mediated corneal disease known as herpes stromal keratitis (HSK), which causes corneal scarring and eventually leads to blindness. In C57BL/6 mice, latency is accompanied with a T cell infiltrate in the trigeminal ganglion (TG) that is maintained during the life of the mice. Many of the virus specific $CD8^+$ T cells were specific to an immunodominant protein known as glycoprotein B (gB) which is recognized by $CD8^+$ T cells during lytic and latent phases [96]. During active infection, up to 65% of the cytotoxic T-lymphocyte (CTL) response to HSV-1 is directed against gB [101], and 50% of the $CD8^+$ T cells that remained in the TG during latency were gB specific [76]. Most persisting $CD8^+$ T cells remained in close contact to the neurons during latency, which suggests that they may play a role in blocking reactivation [102,103]. The viral antigens that these $CD8^+$ T cells are targeted to can be manipulated to boost the immune response against existing latent HSV-1 by eliminating the dominant epitope. This forces recognition of an undefined epitope, causing expansion of a different CD8 specific T cell population [101]. As just detailed, knowledge of the subdominant populations was skant and not defined at the start of this work.

However, as will e shown, it appears that the dominant population does affect the subdominant hierarchy, and that there is a complex interplay of the populations of $CD8^+$ T cells that develops in HSV-1 latent infections.

2.2 SPECIFIC AIM 1

Characterize the alternate HSV-1 specific $CD8^+$ T cell response in the trigeminal ganglion when the $gB_{498-505}$ epitope is removed and separate the $gB_{498-505}$ epitope from the expression of gB and place it ectopically in the HSV-1 genome to establish the basis to study of expression of the $gB_{498-505}$ epitope and the proportion of gB-CD8s in latently infected TG.

To better understand which viral antigens $CD8^+$ T cells respond to in order to block reactivation, we will identify the targets of the non gB-CD8s in the absence of the $gB_{498-505}$ epitope. Different HSV-1 viruses with one amino acid change in the gB epitope have been created. These mutant HSV-1s lacking the wild type $gB_{498-505}$ epitope should stimulate a different HSV-1 specific $CD8^+$ T cell response in the murine model than native HSV-1.

Secondly, a peptide virus will be created such that the gB epitope is expressed outside of the gB locus in multiple copies to determine if the gB-$CD8^+$ T cell response can be restored. This would allow the influence of expression kinetics and viral promoter activity on the epitope and its hierarchical dominance to be assessed without modulating the expression of the key gB glycoprotein. We expect this to induce a gB-CD8 infiltrate that is comparable to or greater than that induced by wild type HSV-1. Since mutant-gB HSV-1 does not drive the formation of the $gB_{498-505}$ specific $CD8^+$ T cells in animals, the peptide virus will be created to determine if a $gB_{498-505}$ epitope peptide multimer restores the gB-$CD8^+$ T cell specificity. It is also possible that the expression of the gB plasmid alone may be able to increase the amount of gB-$CD8^+$ T cells infiltrating in the TG beyond 50% after latency. As a result, this may help to prevent reactivation through better vaccine design.

2.3 SPECIFIC AIM 2

Determine the hierarchical order of subdominant CD8$^+$ T cells that are matained during latency, and if they are able to retain an activated phenotype and are capable of protecting against reactivation during latency in the absence of gB specific CD8$^+$ T cells.

The gB mutant HSV-1 can establish latent infections in TG as well as elicit a robust CD8$^+$ T cell response. Therefore, there may be a varying virus-specific CD8$^+$ T cell infiltrate that is blocking reactivation in the TG. The activation state of these CD8$^+$ T cells will be addressed by flow cytometry. Epitope-specific cells can also be selectively expanded out of a latently infected TG to determine if they are able to protect against reactivation events *ex vivo*. This will allow us to further define how the immune response effectively counteracts reactivation using these multiple antigens.

3.0 GENERAL MATERIALS AND METHODS

3.1.1 Virus and cells.

Vero cells (ATCC, Manassas, Virginia), B6WT3 fibroblast cells (MHC-I compatible with C57BL/6 mice; ATCC), gB-Vero (Vero cells transfected with a plasmid expressing gB from the native gB promoter; a kind gift of William Goins, University of Pittsburgh) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), Penicillin-G (100 units/ml), Streptomycin (100 mg/ml) and Fungizone (250 mg/ml). The wild type RE strain of HSV-1 (HSV-1 RE) was used as the basis for all recombinant viruses.

3.1.2 Multistep *in vitro* growth kinetics

Vero cells were infected with different HSV-1 viruses at an MOI of 0.01 PFU/cell, and incubated as stated in each study. Cells and supernatants were combined for harvest, and infectious virus released following three freeze–thaw cycles was detected by standard plaque assay on Vero cells or gB-Veros for any gB-knock out viruses.

3.1.3 Mice

Six to eight week old female C57BL/6 mice were anesthetized by intraperitoneal (i.p.) injection of 2.0 mg ketamine hydrochloride and 0.04 mg xylazine (Phoenix Scientific, San Marcos, CA) in 0.2 mL PBS (Sigma-Aldrich, St. Louis, MO). Anesthetized mice had the epithelial layers of their corneas scratched and then infected with 1 x 10^5 PFU of the respective HSV-1 in 3 µl RPMI (Invitrogen). All animal experiments were conducted in accordance with guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.1.4 Tissue Preparation and ganglia dissociation

At various times post infection as defined in the text, anesthetized mice were injected i.p. with 0.3 ml of 1000 U/ml heparin (Sigma-Aldrich, St. Louis, MO) and euthanized by exsanguination. The trigeminal ganglia were harvested by surgery and digested in 200 µl per two ganglion of RPMI (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) with 400 U/ml collagenase type I (Sigma-Aldich) for 1 hr at 37^{o}C. TG were then triturated into single-cell suspension. Spleens were dispersed mechanically and filtered through a 40 µm nylon cell strainer (BD Biosciences, Bedford, MA). Spleens were treated with red blood cell lysis buffer for three minutes prior to use.

3.1.5 $CD8^+$ T cell expansion and recognition of mutant proteins.

gB-$CD8^+$ T cells used in this work were expanded from TG of day 8 post infection HSV-1 acutely infected mice, as detailed previously [102]. Briefly, collagen dissociated suspensions of latently infected TG were cultured with B6WT3 fibroblasts transfected with plasmids expressing full length WT gB for 10 days, followed by MACS bead purification of $CD8^+$ T cells. Resulting populations were >95% CD3ε, CD8α, and positive for gB498-505/H-$2K^B$ tetramer. To assess the recognition of mutant gB proteins, B6WT3 (1 x 10^5 cells) were transfected with 5μg of plasmids expressing each gB epitope mutant protein or wild type gB under the CMV-IE promoter. At 8 hours post transfection, 5 x 10^4 gB-CD8 were added and cultured for 6h in the presence of Golgiplug^R. T cells were subsequently surface stained for CD45, CD8 and intracellular IFN-γ, as detailed below.

3.1.6 Activation Analysis and staining of T cells

T cell phenotypic characterization was performed essentially as detailed previously [104]. Single cell suspensions of TGs and spleens were stained with anti-CD45, CD8α, and the respective dimer or tetramer for 1 hr at room temperature, and then fixed for 20 minutes with Cytofix/Cytoperm (BD Biosciences, Bedford, MA), stained for intracellular antigens, washed and then analyzed by flow cytometry. $CD8^+$ T cell recognition of target antigens was determined by pulsing B6WT3 fibroblast with the respective peptide at a concentration of 1 μg/ml for 30 min at 37^{o}C/5% CO_2. The dispersed TG or spleen cells were added to peptide-pulsed fibroblasts

in the presence of Golgi-plug (BG Biosciences, Bedford, MA) for 6 hr at 37°C/5% CO_2. After stimulation, cells were stained for surface expression of anti-CD45, CD-107a and CD8α, permeabilized and fixed for 20 minutes using Cytofix/Cytoperm (BD Biosciences) and then subjected to intracellular stain for IFN-γ and TNF-α. The peptides used in this work were detailed previously [104]. For each peptide, TGs from a total of four mice were separately analyzed for reactivity to each peptide.

3.1.7 Reagents and flow cytometry.

Phycoerythrin (PE)-conjugated H-2K^b tetramers complexed with the $gB_{498-505}$, $RR1_{982-989}$, $RR1_{822-829}$, or $ICP8_{876-883}$, peptide and PE-conjugated H-2D^b tetramers complexed with the $ICP8_{168-176}$, $RR1_{372-380}$, or $RR2_{279-287}$ peptide were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Rat anti-mouse Pacific-Blue-conjugated anti-CD8α (clone 53-6.7), APC-conjugated anti-IFN-γ (XMG1.2), PerCP-conjugated anti-CD45 (30-F11), PE-Cy7-conjugated anti-TNFα (MP6-XT22), APC-conjugated anti-granzyme B (anti-GrzB) (GB11), and BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit were purchased from BD Pharmingen (San Diego, CA). The appropriate isotype control Abs were purchased from the same company used for the reactive antibody. All flow cytometry samples were collected on a FACSAria cytometery and analyzed by FACSDiva software (BD Biosciences).

3.1.8 Quantitative real-time PCR

DNA from latently infected TG was extracted using the Qiagen DNAeasy™ Tissue Kit as per manufacturer's instructions. In short, collagenase-treated TG were resuspended in 200 µl PBS per sample, then treated with 20 µl proteinase K and 180 µl of Buffer ATL, mixed thoroughly, and incubated 10 min at 56ºC on a heating block. Samples were then treated with 200µl of Buffer AL, then 100% EtOH and transferred to a mini-spin column and centrifuged. The columns were treated with 500 µl of Buffer AW1 and Buffer AW2, with spins between each treatment, and finally, samples were eluted in 100 µl Buffer AE (Ambion, Austin, TX). DNA was quantitated using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) using SoftMax Pro 4.3 software (Molecular Devices) and 100 ng DNA per sample was resuspended.

12.5 ng of DNA or water control was mixed in duplicate with 11.25 µl of TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ) and 1.25 µl of the HSV-1 glycoprotein H (gH)-specific primer-probe set, custom designed and synthesized by ABI Assays-by-Design service (Applied Biosystems, Foster City, CA). Samples (25 µl/well) were assayed in 96-well plates with an ABI Prism 7700 sequence detector. ABI Primer Express v1.5a software default settings were used for instrument control and data analysis. The gH sequences were: forward primer (5'-GACCACCAGAAAACCCTCTTT-3'), reverse primer (5'ACGCTCTCGTCTAGATCAAAGC-3'), and probe [5'-(FAM)TCCGGACCATTTTC(NFQ)-3']. The HSV-1 genome contains a single copy of the gH gene; therefore, viral genome copy number can be determined quantitatively by comparing the experimental C_T value observed from

the gH primer-probe assay with C_T values of known concentrations of gH-containing plasmid standards.

3.1.9 *Ex vivo* TG cultures for Reactivation

Single-cell latently infected TG suspensions were plated at one-fifth TG equivalents per well in 48-well culture plates in 400 µl of DMEM containing 10% FBS, 10 mM HEPES buffer (GIBCO), 10 U/ml recombinant murine IL-2 (R and D Systems), and 50 µM 2-mercaptoethanol. Cultures of TG were monitored for reactivation in one or two ways depending on the study. Virus production was assessed by testing culture supernatant fluid for live virus by standard viral plaque assays by sampling 100 µl of the supernatant every other day for use in a standard plaque assay to determine the presence of infectious virus. If a fluorescent virus was used in a study, virus production was monitored by observing the wells under a fluorescence microscope at low magnification (4X objective) for expression of EGFP and/or RFP in neurons and spread to surrounding fibroblasts. Scanning for fluorescence was performed at low magnification to minimize the amount of time the cultures were exposed to uncontrolled conditions outside of the 37 °C, 5% CO_2 incubator, and to minimize UV exposure. Each assessment was conducted every two days for a total of eight to ten days in culture. Data are represented as the percent of wells that were positive for viral reactivation.

In studies requiring depletion of endogenous $CD8^+$ T cells (Chapter 5), latently infected TG suspensions (34 d.p.i) were depleted of endogenous CD8α T cells by antibody/complement mediated lysis using Low-Tox M Rabbit Complement (Cedarlane). Efficiency of depletion was assessed by flow cytometry. Single-cell TG suspensions were plated at one-fifth TG equivalents per well in 48-well culture plates in 400 µl of DMEM containing 10% FBS, 10 mM HEPES

buffer (GIBCO), 10 U/ml recombinant murine IL-2 (R&D Systems), and 50 μM 2-mercaptoethanol. Where indicated, cultures were supplemented with exogenous gB-CD8 at 2 x 10^4 gB-CD8/well. TG cultures were monitored for reactivation by testing culture supernatant fluid for live virus by standard viral plaque assays as described above. Supernatants were tested every two days for a total of ten days in culture. Data are represented as the percent of wells that were positive for viral reactivation.

4.0 GANGLIONIC CD8^{+} T CELL SPECIFICITY INDUCED BY HERPES SIMPLEX VIRUS TYPE 1 LACKING AND ECTOPICALLY RESTORED FOR THE GB$_{498\text{-}505}$ MURINE IMMUNODOMINANT EPITOPE

Sarah Bidula, Srividya Ramachandran, Anthony St. Ledger, Alex Sette, Robert Hendricks, and Paul R. Kinchington

4.1 ABSTRACT

Latency of herpes simplex virus type 1 (HSV-1) in human and murine trigeminal ganglia (TG) is associated with a persistent cellular immune infiltrate, which includes virus specific $CD8^+$ T cells surrounding latently infected neurons. In the HSV-1 ocular infected C57Bl6 mouse latency model, over 50% of the TG associated $CD8^+$ T cells are dominantly skewed to one epitope (amino acids 498-505) on HSV-1 glycoprotein B. Given that CD8 T cells contribute to maintenance of the HSV-1 latent state, it is important to understand viral antigens targeted by these cells and factors influencing dominance hierarchy. Here, we assessed the $CD8^+$ T cell target specificity in TG of C57BL/6 mice infected with HSV-1 lacking and ectopically restored for the $gB_{498-505}$ epitope. Epitope mutants with near wild type pathogenicity were isolated, and found to induce TG associated $CD8^+$ T cell infiltrates of size similar to that induced by wild type HSV-1, but with little $gB_{498-505}$-specificity. The nature of the compensated response reflected increase of $CD8^+$ T cell populations directed to most known subdominant epitopes seen in wild type HSV-1 infection. However, a $gB_{498-505}$ dominated $CD8^+$ T cell response developed following infection with HSV with $gB_{498-505}$ epitope-mutation that expressed short $gB_{494-509}$ peptides at an ectopic (gC) locus. We conclude that loss of the HSV dominant epitope does not alter the size of the HSV-specific $CD8^+$ T cell response nor broaden the TCR repertoire, but rather results in a broader dominance hierarchy of subdominant epitopes rising to co-dominance. We further conclude that immunodominance is not a result of properties of the HSV-1 gB protein itself or its genomic locus.

4.2 INTRODUCTION

Primary mucosal, ocular or skin infections by herpes simplex virus type 1 (HSV-1) invariably lead to infection of axonal termini of innervating sensory neurons, retrograde transport to the sensory ganglionic nuclei and the establishment of a latent state. Latency is characterized by no virus production, repression of lytic gene expression, the entry of the genome into a circular heterochromatin regulated state, and the expression of latency-associated RNA transcripts (LAT) in many HSV-1 genome-positive neurons [96,101,105]. These non-coding RNAs give rise to miRNAs that are thought to contribute to the latency [106]. How the latent state is maintained is not yet clear, but it likely involves both intrinsic activities within the host cell neuron (e.g., chromatin regulation) and extracellular influences such as cellular immunity. Periodically, multiple and varied stimuli trigger release of the latent state and, if complete, virus delivery at the periphery and recurrent disease at or near the site of primary infection. Of particular clinical significance is recurrent infection of the corneal epithelium. This may trigger an immune mediated infiltration of the corneal stroma leading to collagen disorganization and deposition of scarring tissue. Repeated infections progressively opacify the transparent stroma [107,108] to cause herpes stromal keratitis (HSK), a potentially blinding disease that affects some 200,000-300,000 people in the United States annually [7]. HSK is responsible for approximately 10% of corneal transplants in the United States [109].

One approach to prevent HSK is to block HSV reactivation from latency by improving immune surveillance. The TG associated cellular immune infiltrate in the murine ocular infection model peaks near the onset of viral latency, and includes both $CD4^+$ and virus specific $CD8^+$ T

cells. These populations contract as long term quiescence is established, but reach a low persisting level that then remains for the life of the host. Ganglionic $CD8^+$ T cells remain in close apposition to latently infected neurons and show an activated granzyme B^+ memory state [97] as well as polarization of their receptors towards neurons in an apparent immunological synapse that strongly suggests that latency is not antigenically silent. This is in agreement with the finding that rare neurons in the murine latently infected ganglia are positive for HSV mRNA and proteins without virus delivery to the periphery [33,49]. Maintaining the viability and function of the associated $CD8^+$ T cell populations in ex vivo culture TG homogenates reduced or prevented reactivation [76]. Hoshino et al (2007) established that reactivation frequency in ex vivo TG cultures correlates inversely with the number of $CD8^+$ T cells in the ganglia [110]. These observations support a model in which sporadic reactivation events are contained by the cellular immune infiltrate, and has raised the possibility that manipulating the environment to increase number, functionality or retention of ganglionic $CD8^+$T cells could better prevent reactivation. To date, most HSV vaccine strategies reaching clinical trials have been designed to induce protective antibody responses, and these have been largely unsuccessful. Vaccine strategies aimed at inducing T cell responses may be more effective, but only limited information is available on the identity of the HSV-1 epitopes targeted by $CD8^+$ T cells in human disease and how the ganglionic $CD8^+$ T cell population can be manipulated. Obviously, the knowledge of the targets of such T cell populations is important to future vaccine strategies.

The specificity of the HSV-1 $CD8^+$ T cell responses in the murine model has recently been examined. The potential number of targets for cellular immunity is large: HSV-1 is capable of generating some 80 viral proteins in a productive infection, which are expressed in a

coordinated cascade composed of viral immediate early genes (α), followed by early genes (β) and DNA replication, and then late protein synthesis (γ) that peaks after viral DNA replication has begun [111,112]. Similar to other viruses and pathogens, $CD8^+$ T cells to HSV-1 are directed to a very limited number of the potential antigenic pool that typically falls into a dominance hierarchy. C57BL/6 mice infected with HSV-1 have an infiltrate of $CD8^+$ T cells highly skewed (>50%) to a single immunodominant epitope located on the critical glycoprotein B (gB), an HSV-1 early-late gene that functions in entry, membrane fusion and egress, but is nevertheless expressed within two hours of infection. The dominance to $gB_{498\text{-}505}$ [96,101] persists in the CD8 T cell populations associated with both the peak infiltrate in the ganglia and that found at long term latency [76]. Remaining $CD8^+$ T cells in the acutely infected TG are HSV-1 specific and directed to a subdominant epitope repertoire on 11 viral proteins, the majority of which are expressed before viral DNA synthesis. Of significance to this work is that both gB-CD8s and the $gB_{498\text{-}505}$ non-specific populations can block HSV-1 reactivation from latency [102,103].

We have previously sought to manipulate the gB dominant $CD8^+$ T cell response by developing HSV-1 that expressed gB as a true late, viral DNA replication-dependent gene. The CD8 T cell response to this virus contained reduced immunodominance of $gB_{498\text{-}505}$ during latency, without affecting global numbers of the infiltrate, suggesting compensation in the response for the loss of the gB response [113]. A compensatory CD8 T cell response was also seen in dorsal root ganglia following infection with HSV lacking the dominant epitope [101]. Here, we addressed the identity of viral antigens targeted by the $CD8^+$ T cell response in the HSV-1 acutely infected TG in the absence of the immunodominant epitope $gB_{498\text{-}505}$. We show that $gB_{498\text{-}505}$-negative HSV-1 induces a $CD8^+$ T cell response in the trigeminal ganglia

following ocular infection that is similar in size to that induced by wild type virus. The compensated response was accounted for by increase of $CD8^+$ T cell populations directed to most of the 14 subdominant epitopes targeted by the response to a wild type HSV-1 infection [104], suggesting no emergence of a cryptic epitope to dominance. However, expression of the $gB_{498-505}$ peptide sequences alone in the background of HSV-1 S1L $gB_{498-505}$ recombinant virus restored the $gB_{498-505}$ dominated $CD8^+$ T cell response. The broader specificity of the immune response could provide the basis for development of multivalent vaccines. The separation of the epitope from gB expression indicates that the context of the gB protein itself or its genomic locus are not primary drivers of the dominant response.

4.3 MATERIALS AND METHODS

DNA constructs and virus derivatives containing $gB_{498-505}$ mutations

DNAs generated for these studies were amplified by polymerase chain reactions using the proofreading polymerase "Expand" (Roche) under hot start conditions and in reactions containing 4% DMSO, as detailed previously [113]. The plasmid made to derive gB-GFP null virus was modified from a pUC19 based plasmid detailed previously (Ramachandran et al 2010) [113] that contained HSV-1 DNA sequence representing part of the gB promoter and coding region from 54810 to 56801 (with reference to the HSV-1 17 sequence), with EcoR1 site added to 54810, HindIII added to 56801, and a coding-silent AvrII site at HSV-1 bp 58812 upstream of the gB ORF start. The AvrII-EcoRI fragment containing the gB coding sequence was replaced with an EcoRI-AvrII digested PCR fragment (gB residues 507 to the stop codon), generated using the primers gBBackF 5' GCGCCTAGGCTCGGATCCCAGTTTACGTACAAC 3' and gBBackR 5' GAGCGGAATTCATTTACAACAAACCCCCCATCA 3'. (pgBp-gBend"). EGFP was PCR amplified from pEGFP-C3 (Clontech Inc). using the following primers: gBF-EGFP 5' CCC TAG GCT ACC TGA CGG CGG GCA CGA CGG 3' and gBR-EGFP 5' TTG TAC GTA GGA TCC TTA CTT GTA CAG CTC GTC 3' and the BamHI-AvrII digested product was inserted into the first construct to place EGFP under control of the gB promoter (gBp), followed by gB coding sequence after amino acid 507. This construct was used to generate HSV-1 gB-EGFP null virus on gB-Vero cells, selecting based on gain of EGFP, using methods outlined previously [113]. Plaque purified virus was verified for inability to replicate in

non complementing Vero cells, and correct DNA insertion was confirmed by PCR and Southern blotting. Two independently isolated gB null viruses (designated "30" and "G') were isolated that appeared genetically identical.

To develop plasmids with mutations in the $gB_{498\text{-}505}$ epitope, the gBp-EGFP-gB plasmid just outlined was digested with AvrII and SnaBI to remove DNA encoding EGFP, and the gB coding region was restored by placing SnaBI-AvrII digested PCR fragments of the gB protein coding sequence from 1 to 509 with altered epitope sequence. PCR using the forward primers gBFrontF: 5' GCCCTAGGCTACCTGACGGGGGGCACGACGGGCCCCCGTAG 3', and the primers listed in Table 1 to generate the mutations. Each PCR fragment was initially cloned into pGEM-T Easy and DNA sequenced for verification. Resulting constructs contain a full length gB coding sequence with mutations in the $gB_{498\text{-}505}$ region and a coding-silent marker AvrII site to differentiate recombinants from wild type virus. HSV-1 recombinants were obtained by cotransfecting linearized plasmids with HSV-1-gBnull-GFP infectious DNA on Vero cells, followed by isolation and purification of non fluorescent plaques. All viruses were confirmed for gain of the AvrII site and the expected mutations. Two independently isolated viruses containing gB L505A (HSV L8A) and gB S498L (designated HSV S1L) were isolated and found to be similar.

Expression plasmids for each mutant gB protein were created by cloning the AvrII-EcoRI fragment (encoding the gB coding sequence) into the vector EGFP C3, digested with NheI and EcoRI to remove EGFP coding sequence. This allowed expression of the gB proteins from the human cytomegalovirus (hCMV IE) Immediate early promoter.

Table 4.4.1. Primer sequences (complementary to the coding sequence) used in PCR to generate mutations in the gB epitope (SSIEFARL) region

Name	***Resulting Mutation***	***Reverse primers used***
WT	SSIEFARL (none)	5’ GTT G**TA CGT A**AA CTG CAG CCT GGC GAA CTC GAT GGA GGA GGT GGT CTT GAT GCG CTC CA 3’
L8A	SSIEFARA	5 ‘ GTT G**TA CGT A**AA CTG agc CCT GGC GAA CTC GAT GGA GGA GGT GGT CTT GAT GCG CTC CA 3’
F5L	SSIELARL	5’ GTT G**TA CGT A**AA CTG CAG CCT GGC cAA CTC GAT GGA GGT GGT CTT GAT GCG CTC CA 3’
S1G	GSIEFARL	5’ GTT G**TA CGT A**AA CTG CAG CCT GGC GAA CTC GAT GGA ccc GGT CTT GAT GCG CTC CA 3’
S1L	LSIEFARL	5’ GTT G**TA CGT A**AA CTG CAG CCT GGC GAA CTC GAT GGA caa GGT GGT CTT GAT GCG CTC CA 3’
S1G/L8A	GSIEFARA	5’ GTT G**TA CGT A**AA CGT agc CCT GGC GAA CTC GAT GGA ccc GGT GGT CTT GAT GCG CTC CA 3’
S1G/I3A	GSAEFARL	5’ GTT G**TA CGT A**AA CTG CAG CCT GGC GAA CTC Ggc GGA ccc GGT GGT CTT GAT GCG CTC CA 3’
L8A/R7K	SSIEFAKA	5’ GTT G**TA CGT A**AA CGT agc CtT GGC GAA CTC GAT GGA GGA GGT GGT CTT GAT GCG CTC CA 3’
S1G/I3N/F5L/E4S	GSNSLARL	5’ GTT G**TA CGT A**AA Ctg CAG CCT GGC cAA gct GtT GGA ccc GGT GGT CTT GAT GCG CTC CA 3’

Ectopic $gB_{498\text{-}505}$ restoration in HSV-1 S1L in gC locus

Head to tail multimers of the gB 494-509 peptide region were generated from the oligonucleotides 5’ P- GAT CCC ACC ATG GCG ATC AAG ACC ACC TCC TCC ATC GAG TTC GCC AGG CTG CAG TTT ACG TAC ACC CAC AAA-3’, which was hybridized to the oligonucleotide (5’P-GATCTTTGTGGGTGTACGTAAACTGCAGCCTGGCGAACTCGATGGAGGAGGTGGTCT TGATCGCCATGGTGG-3’. Resultant double stranded oligonucleotides contain 5’ GATC overhangs representing a cut BamHI at the beginning and BglII at the end of the coding strand. These underwent a series of sequential ligations, followed by digestion with BamHI and BglII to

separate head-to-head and tail-to-tail joins. Multimers were resolved on 2% agarose TBE gels, eluted and cloned into BamHI site of pEGFP N1. A plasmid in which four repeats of the oligonucleotide were in frame with N terminus of EGFP was then digested with BamHI –NotI, and cloned into the BamHI and NotI-cut plasmid pgC-mRFP: gB-EGFP, detailed previously [103]. The resulting plasmid contains the gB promoter driving four in-frame copies of peptide encoding the residues -(asp-pro-thr) -Met-ala-Ile-lys-thr-thr-ser-ser-ile-glu-phe-ala-arg-leu-gln-phe-thr-tyr-thr-his-lys (gB sequence underlined: immunodominant peptide, double-underlined) in frame with EGFP. Recombinant virus containing the plasmid was derived by co transfecting linearized plasmid with infectious HSV-1 S1L DNA, and virus was selected and purified based on gain of mRFP expression from the gC promoter (Figure 1).

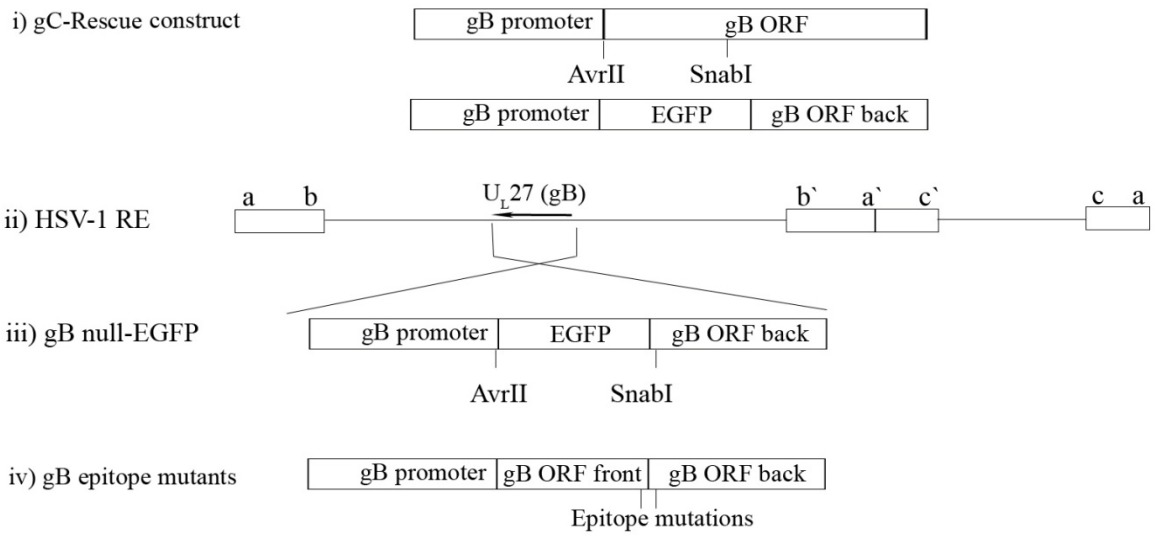


Figure 4.4.1. Construction of gBnull virus and of HSV-1 with $gB_{498\text{-}505}$ mutations. Line I represents the parental plasmid detailed previously (Ramachandran et al 2010), and the replacement of the gB ORF with EGFP followed by the remaining part of the gB ORF from

residue 509 to the end (gB ORF Back). Line ii represents the HSV genome and the approximate coding position and direction of the gene for gB, and line iii represents the recombination into the gB ORF at the GFP locus to give gBnull EGFP viruses. Line iv represents the replacement gB genes and the site of the epitope mutations with respect to the SnabI site used for derivation, as detailed in the text. AvrII and SnaBI are restriction sites used are shown.

4.4 RESULTS

4.4.1 Mutation of the HSV-1 $gB_{498\text{-}505}$ epitope. The primary goals of this work were to determine the antigenic specificity of the $CD8^+$ T cell response to HSV-1 in the absence of the $gB_{498\text{-}505}$ immunodominant epitope in C57BL/6 mice. A secondary goal was to test the feasibility of separating the expression of the immunodominant epitope from that of the gB protein, in order to examine the contribution of viral promoter influence and expression kinetics on the specificity of the $gB_{498\text{-}505}$ $CD8^+$ T response. Both goals required the development of virus that was no longer able to induce a $gB_{498\text{-}505}$ directed CD8 T cell response. Stock et al (2007) detailed a mutation in the gB epitope (L505A, or L8A) that effectively abrogated gB-CD8 development [101]. We generated this change in our virus background, as well as several additional mutants in the gB epitope, for reasons that will become apparent below. Our strategy (Fig, 4.4.1) used mutagenic primers (Table 4.4.1) with select changes in the 498-505 epitope region that amplified the portion of the gB gene from amino acids 1 to 509, which could subsequently be restored into the full gB ORF. As the $gB_{498\text{-}505}$ epitope lies adjacent to an α–helical linker region spanning two regions thought to be important to gB fusogenic activities, it was expected that some changes in this domain would not be compatible with the essential function of gB. Indeed, deleting the 498-505 epitope entirely resulted in an unstable gB protein that did not complement gB null virus (data not shown). However, eight gB proteins with point mutations in the $gB_{498\text{-}505}$ region expressed a gB protein of size indistinguishable from wild type (Fig 4.4.2) that also complemented gB null HSV-1 in culture (data not shown).

To determine if these mutations abrogated gB $CD8^+$ T cell recognition, each mutant gB protein was expressed in B6WT3 plasmid transfected fibroblasts from the HCMV IE promoter, and the ability of gB CD8 T cells (expanded from the TG of an HSV-1 infected B6 mouse) to recognize the cells was assessed by flow cytometry following intracellular IFNγ staining. As expected (Fig. 4.4.2), gB CD8s added to cells expressing wild type gB sequences (gB parental plasmid and one in the gB protein had undergone the entire mutagenesis scheme with WT unmutated sequences) resulted in production of IFNγ by approximately 12-15% of gB-CD8s in a three hour recognition period, indicating MHC-I -antigen restricted recognition by gB CD8s. In contrast, the eight $gB_{498\text{-}505}$ epitope mutant gB proteins were unable to stimulate IFNγ production by gB-CD8s above control levels. We conclude that the changes induced in the peptide region all effectively abrogated gB CD8 T cell recognition and subsequent activation.

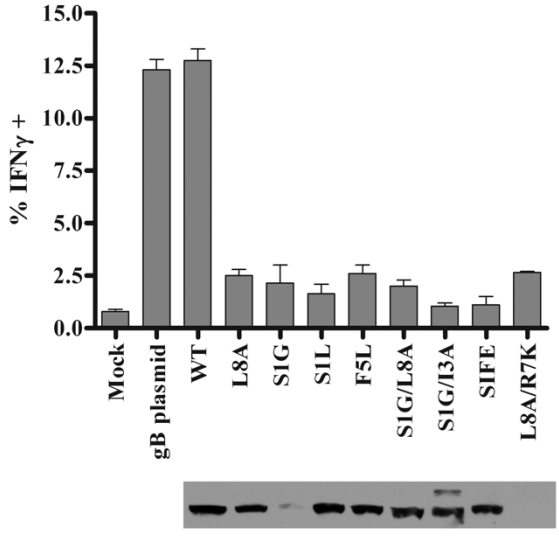


Figure 4.4.2. $gB_{498\text{-}505}$ epitope mutant constructs or wild type were transfected into B6WT3 (1 x 105 cells) at 5μg. 18 hours post transfection, cells were combined with 5 x 10^4 gB-CD8 and stimulated for 6h in the presence of golgiplug. Cells were surface stained for CD45 and CD8,

and stained for intracellular IFN-γ. gB proteins S1G and L8A/R7K showed lower expression levels in this assay, but not others. The graph depicts the mean percent of IFN-γ positive cells (n = 2/group) and standard error of the mean for each stimulation. The bottom panel represents an immunoblot for gB confirming protein expression within each transfection.

Recombinant Virus development and evaluation in vitro.

HSV-1 recombinants containing each mutation were developed by rescue of an HSV-1 gB deficient virus derived on gB Vero cells containing EGFP in place of the N-terminal half of the gB protein (Fig. 4.4.1, i-iii), in a manner similar to that detailed previously [114]. All HSV with mutations except HSV containing gB with the SIFE mutation (Table 1) formed plaques indistinguishable from wild type on non-complementing Vero cells, indicating the mutations were compatible with essential gB functions. HSV with the gB SIFE change (the equivalent of the corresponding VZV gB sequence, residues 397-405) produced very small plaques suggesting impaired functional restoration, so this virus was not characterized further. All viruses replicated in Vero cell culture to the same level of wild type, but this was not the case in vivo. We assessed virus levels in the TG of B6 ocularly infected mice at day 4 post infection, and found only HSV with gB containing S498L (S1L) and L505A (L8A) were as robust as wild type virus (data not shown). HSV-1 KOS-based virus with L8A change, in which an MHC-I anchoring residue was altered, was previously detailed by Stock et al (2007), whereas S1L represents a novel mutation predicted to be at the beginning of the peptide region presented to $CD8^+$ T cells in the MHC-I complex. In both multi-step (infected at MOI of 0.01) and single step (infected at MOI of 10) growth curves in Vero cell culture, these viruses were as robust as wild type (Fig. 4.4.3). No

difference in growth was seen in other cell types, including primary human foreskin and corneal fibroblast culture (data not shown).

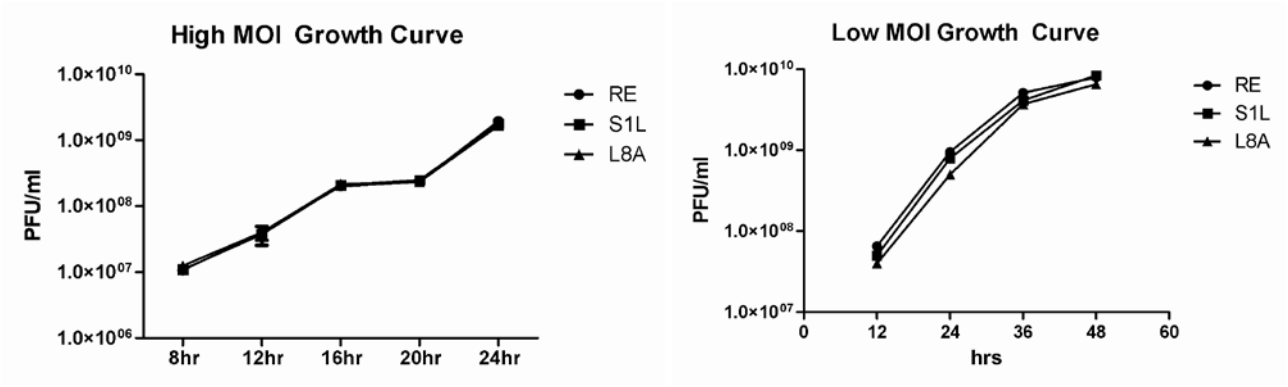


Figure 4.4.3. Monolayer cultures of Vero cells were infected at an MOI of 10 PFU/cell or 0.01 PFU/cell respectively with HSV-1 RE, S1L, or L8A. At the indicated hours post-infection (h.p.i), cells and supernatants were harvested, pooled, subjected to three freeze–thaw cycles and the viral titers were determined. The results are shown as mean numbers of PFU/culture ± standard error of the means (SEM).

HSV $gB_{498\text{-}505}$ mutant virus growth and Pathogenesis *in vivo*.

Inoculation of C57BL/6 mice at the cornea with 1 x 10^5 PFU/eye revealed that both S1L and L8A epitope mutant viruses accessed the ganglia and replicated as efficiently as wild type in the TG at 4 d.p.i (which represents the peak of viral replication). There was no significant difference in the levels observed for each mutant virus or wild-type HSV-1 (Fig 4.4.4A), although a slight trend of L8A to replicate at lower levels was seen. These results indicate that the L8A and S1L mutations in the gB epitope did not impair ganglionic access and replication prior to the onset of latency. We also found ganglionic copy number of the two mutant viruses at day 34 post infection, representing the latent state, were not significantly different from that of wild-type

HSV-1 RE (data not shown).

We also more extensively assessed these viruses for the ability to induce stromal keratitis (HSK), which is highly dependent on both the viral and host genetic backgrounds. As HSK is mild and can be difficult to assess in the B6 mouse due to iris pigmentation, Balb/C mice were utilized, as this strain develops HSK at a high frequency following corneal scarification and HSV-1 inoculation. We recognize that Balb/C have different MHC-I and likely do not exhibit the immunodominance for the $gB_{498-505}$ epitope. HSV-1 RE infected mice developed scores of stromal keratitis that progressed to a level that required sacrifice at day 17 post infection following recommendations of animal care staff. Interestingly, despite an almost equivalent replication in every parameter evaluated, HSV-1 L8A virus isolates were found to be impaired in the ability to induce stromal disease, and did not give clinical scores above background. However, HSV-1 S1L caused moderate HSK that was only marginally delayed as compared to that induced by the wild type parental virus at 17 dpi. These results suggest that HSK is an appropriate evaluation of viral pathogenesis, and that HSV-1 gB S1L virus was more robust than L8A virus in ocular viral pathogenesis.

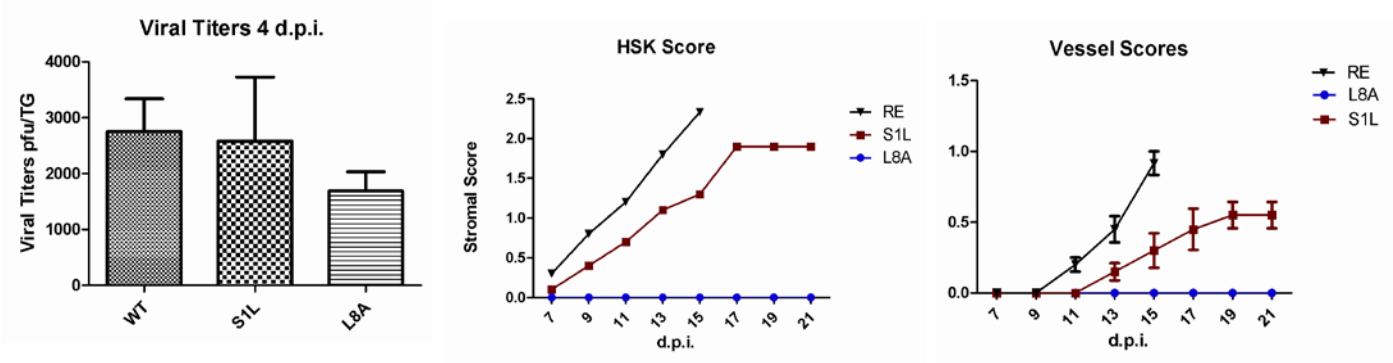


Figure 4.4.4. TG viral growth in B6 Mice (A) and ocular pathogenicity scores in Balb/C mice (B) following ocular scarification and infection with 1 x 10^5 PFU with HSV-1 WT (RE), HSV-

1 L8A, or HSV-1 S1L. A) TG were harvested and subjected to three freeze thaw cycles and infectious virus released into the supernatant was titrated on Vero cells. The graph represents the mean virus titer for each virus ± SEM of the mean (n = 5 mice). B) mice were monitored for 21 dpi for the development of stromal disease. The data are represented as the mean +/- SEM of the mean. (n=5 mice).

A third assessment for robustness was assessed for S1L, the more robust HSV-1 mutant, by determining its ability to reactivate from latency, using a modification of the ganglionic explant reactivation system detailed by Decman et al 2005 [115]. In this assay, B6 mice latently infected with parental HSV-1 or HSV-1 S1L virus through the ocular route were sacrificed at day 34 post infection, and then depleted of endogenous CD8 T cells as detailed previously [102]. Ganglionic equivalents at $1/5^{th}$ latent infected TG/well were cultured in 48 well plates and monitored for reactivation by sampling the supernatant for infectious virus every two days. A portion of these cultures were also treated with 2×10^4 gB CD8 T cells/well, which has previously been shown to prevent reactivation from latency [102]. The prediction was that gB CD8 T cells would be able to prevent reactivation frequency of HSV-1 but not HSV-1 S1L. Indeed, in the absence any CD8 T cells, similar levels of cultures reactivated parental virus and the S1L, with over half of the cultures at day 10 showing reactivation. The addition of gB CD8 T cells prevented most of the reactivation of wild type virus, and at 10 days post explant, only 8% of the cultures had undergone reactivation. In contrast, HSV-1 S1L in the presence of gB CD8s reactivated at the same level as either virus without gB CD8 T cells present, consistent with the expectation that the S1L change fully abrogated recognition of targets by gB CD8 T cells. We conclude that HSV S1L is robust and near wild type in most aspects of pathogenesis, and yet is completely lacking the ability to be recognized by gB CD8 T cells (Figure 4.4.5).

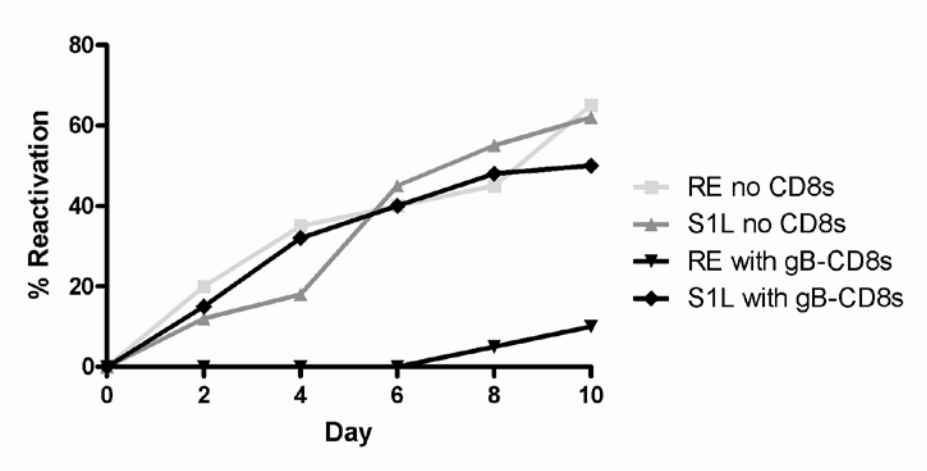


Figure 4.4.5. Ganglionic reactivation of HSV-1 parental virus (RE) and HSV-1 containing S1L in $gB_{498-505}$. Day 34 latently infected B6 mice infected following ocular scarification were sacrificed, the TGs obtained and dispersed. Endogenous CD8 T cells were depleted, and $1/5^{th}$ equivalents of ganglia/well were incubated under conditions to maintain T cell viability and function, either alone or with 2 x 10^4 gB CD8s/ well. Samples of culture fluid were harvested every two days and titrated for the presence of virus.

HSV lacking the $gB_{498-505}$ epitope induce a compensatory CD8 T cell infiltrate in the TG at day 8 post infection that lacks gB Specificity The HSV-1 L8A and S1L viruses were used to address the consequences of mutation of the $gB_{498-505}$ immunodominant epitope on the ganglionic $CD8^+$ T cell response following ocular infection. Stock et al [101] reported that flank infection of B6 mice with an HSV-1 KOS gB L8A mutant resulted in an equivalent $CD8^+$ T cell response in the dorsal root ganglion that was of size similar to that induced by wild type virus, but the nature of the change was not assessed. In ocular infected B6 mice at day 8 post infection (the peak level of infiltrate in the TG), total $CD8^+$ T cell infiltrates to HSV-1 L8A and S1L were not statistically different from wild type infection (Fig 4.4.6a). However, simultaneous staining of $CD8^+$ T cells with $gB_{498-505}$ H2-K^B tetramers revealed that while wild type virus induced a

mean $gB_{498-505}$ specific $CD8^+$ T cell response of 52%, responses to HSV-1 L8A and S1L infection lacked almost any $gB_{498-505}$ specificity. Tetramer bound gB-CD8 to HSV-1 L8A was 4.8%, while that to S1L was only 3.2% (Fig 4.4.6b). Analyses of the spleen revealed a near absence of $CD8^+$ T cells marked by the gB tetramers (data not shown), suggesting virus S1L and L8A mutants were unable to stimulate a $gB_{498-505}$ specific $CD8^+$ T responses. This work indicates a compartmental compensation in CD8 T cell response which must therefore have altered specificity.

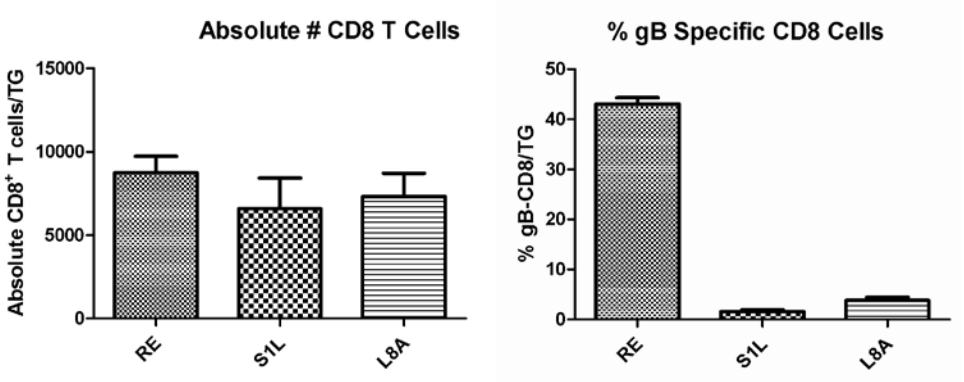


Figure 4.4.6. Mice were infected with 1 x 10^5 PFU/eye with HSV-1 WT, HSV-1 L8A, or HSV-1 S1L. TG were excised at 8 d.p.i., dissociated into single cell suspensions and surface stained the expression of $gB_{498-505}$. Cells were analyzed by flow cytometry. The data are represented as the mean +/- SEM of the mean. (n=5 mice)

Nature of the compensatory $CD8^+$ T cell response

That HSV-1 S1L induces an equivalent absolute number of $CD8^+$ T cells infiltrating the ganglia with almost no reactivity to the gB epitope suggests that the $gB_{498-505}$ compartment is replaced. Possibilities included 1) a $CD8^+$ T cell response developed to a previously unrecognized epitope, 2) the $CD8^+$ T cell response increased to a previously minor HSV-1

subdominant epitope which rose to immunodominance, 3) that $CD8^+$ T cells recognize the mutant S1L gB epitope LSIEFARL, or 4) that some or many of the subdominant epitopes rose to co-dominance. We first addressed the recognition of the mutated epitope, by pulsing B6 fibroblasts with either the S1L, L8A or WT peptides (LSIEFARL, SSIEFARA SSIEFARL, respectively) and then assessing the ability of ganglionic CD8 cells to produce IFNγ (as a marker of activation). Ganglionic CD8 T cells induced by a wild type HSV-1 ocular infection expressed IFNγ following stimulation with SSIEFARL peptide pulsed fibroblasts, but did not become activated by B6 cells pulsed with S1L and L8A peptides. The HSV-1 S1L induced ganglionic $CD8^+$ T cell population did not produce IFNγ to fibroblasts pulsed with any of the 3 peptides, S1L, L8A, or wild-type (data not shown). As such, the alternative response is not due to compensation of $CD8^+$ T cells to the mutant peptides due to recognition of them.

We then evaluated the subdominant epitopes for recognition, by HSV-1 S1L day 8 induced ganglionic $CD8^+$ T cells, and compared the levels to wild type. The epitope repertoire was recently determined in the B6 background, and shown to be restricted to 18 HSV-specific epitopes (out of 376 predicted targets evaluated) on 11 viral proteins [116] [104] simultaneous comparisons confirmed that a wild type HSV-1 infection induced half of the $CD8^+$ T cells in the TG at day 8 as $gB_{498\text{-}505}$ specific, while S1L did not. However, using the 18 subdominant peptides individually to pulse B6 fibroblasts, we found the fraction of ganglionic $CD8^+$ T cells responding to each target from the TG of S1L infected mice at 8 dpi (by IFNγ staining) were almost all increased over the levels from wild type infections (Fig 4.4.7). The degree of increase was variable, with less than a two fold increase for some peptides to well over 11 fold for $UL25_{338\text{-}345}$ over those levels seen following a wild type HSV-1 infection. Only a few (such as

gG and RR2 83-90) remained relatively unaffected in the S1L induced response. In total, the increase in the fractions to these 18 epitopes accounted for more than the total compensated $CD8^+$ T cell response (119.7% ± 32.6%), suggesting there was not an additional epitope to account for a large fraction of the compensatory response. Thus the compensation is due to a broad increase of those populations directed to most of the subdominant epitopes and does not involve a dominant response to a new cryptic or single subdominant epitope.

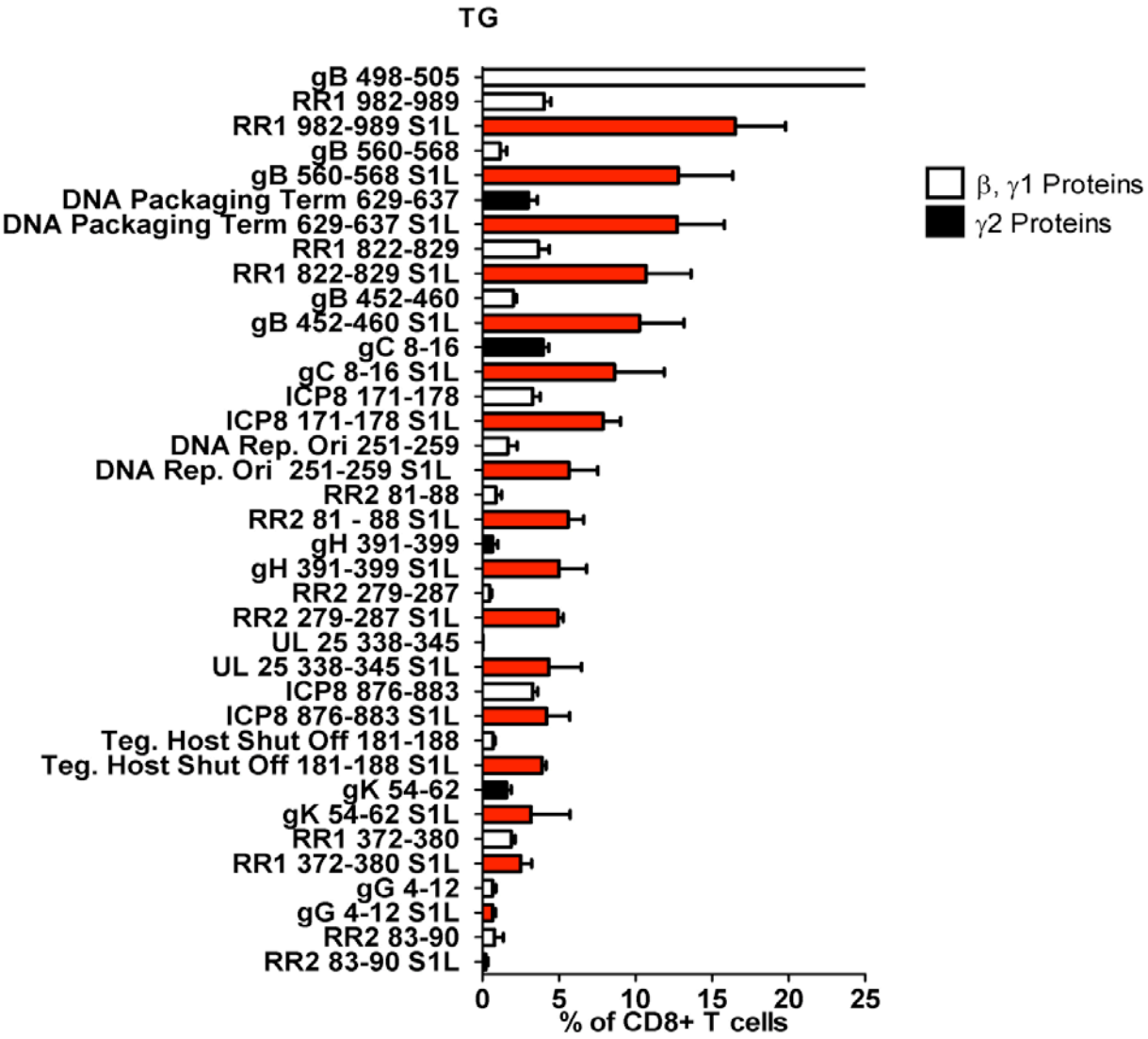


Figure 4.4.7. 18 subdominant epitopes were tested to try and define the phenotype of the TCR in the absence of the $gB_{498-505}$ dominant epitope. TG were excised at 8 d.p.i., dissociated into single cell suspension, and then incubated for 6 h with peptide-loaded B6WT3 fibroblasts in the presence of Golgi-Plug. This was followed by an intracellular stain for IFNγ. The data are represented as the mean of $CD8^+$ T cells producing IFNγ +/- SEM of the mean. (n=3 mice).

Ectopic restoration of the immunodominant epitope independent of gB. One influence known to affect immunodominance hierarchy of $CD8^+$ T cells is the kinetics and levels of expression as well as the structural properties of the protein target. Of note is that most of the subdominant epitopes seen in wild type infections are on viral proteins belonging to the early and gamma 1 classes [104]. Indeed, delay of gB to a true late gene reduces gB498-505 dominance in the TG [113]. However, changing the expression of gB (and the $gB_{498-505}$ epitope) changes viral pathogenicity significantly. Glycoprotein B has multiple roles in the efficient replication of HSV-1 in vivo, mediating not only virus entry and fusion, but also influencing blockade of the endoplasmic reticulum stress sensor 'protein kinase RNA-like endoplasmic reticulum kinase' (PERK) [117] and in the egress of nucleocapsids across the nuclear membrane [118]. Delay of gB expression affected not only the ganglionic infiltrate of gB-CD8s but also ganglionic replication and thus, the efficiency of latency establishment. Ectopic expression of the peptide in the HSV-1 S1L background would allow manipulation of expression of the $gB_{498-505}$ epitope independent of gB expression. Such viruses were generated using the scheme outlined in Fig 4.4.8. The construction resulted in the generation of HSV-1S1L. 4gBpep, which expressed, at the gC locus, 4 multimers of a 14 mer peptide containing gB residues 494-509 and an initiating methionine, all in frame with EGFP and driven by the gB promoter. Virus was selected on gain of mRFP, driven by the gC promoter.

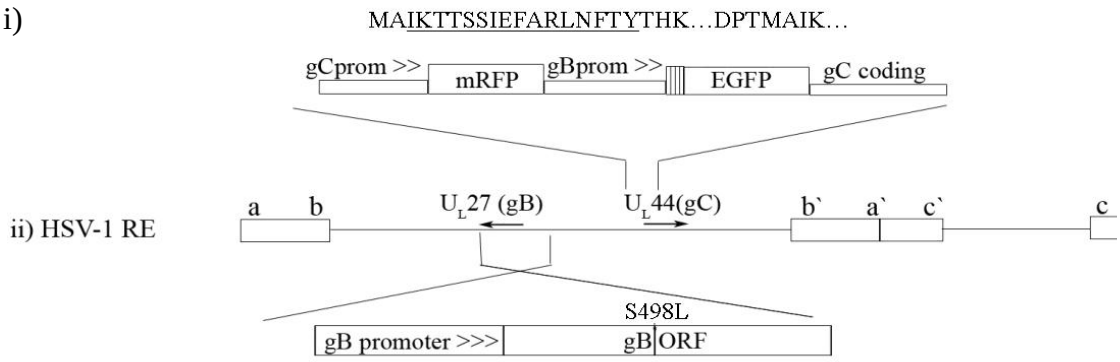


Figure 4.4.8. Construction of gBnull virus and of HSV-1 with $gB_{498\text{-}505}$ mutations. Line I represents the parental plasmid detailed above, and the insertion of the gC promoter in frame with mRFP followed by the gB promoter in frame with four $gB_{494\text{-}509}$ repeats and EGFP. Line ii represents the HSV genome and the approximate coding position and direction of the plasmid.

B6 mice inoculated by the ocular route using 1 x 10^5 PFU/per eye were compared in parallel to mice infected with either the parental RE strain, or with the S1L mutant (Fig 4.4.9). The ganglionic titers for each virus were statistically insignificant, as were total absolute number of $CD8^+$ T cells infiltrating the ganglia at day 8 post infection. However, gB-CD8s were detected in the HSV-1 S1L-4gBpep infected animals that were not statistically significant from the $gB_{498\text{-}505}$ specific $CD8^+$ T cell response to wild type HSV-1 RE in the same experiment. This data indicates the ectopic expression of the epitope, independent of gB, is sufficient to drive the development of the $gB_{498\text{-}505}$ dominated $CD8^+$ T cell response in the C57Bl6 mouse. Thus the dominance of the epitope is not a result of their structural features of the gB protein itself.

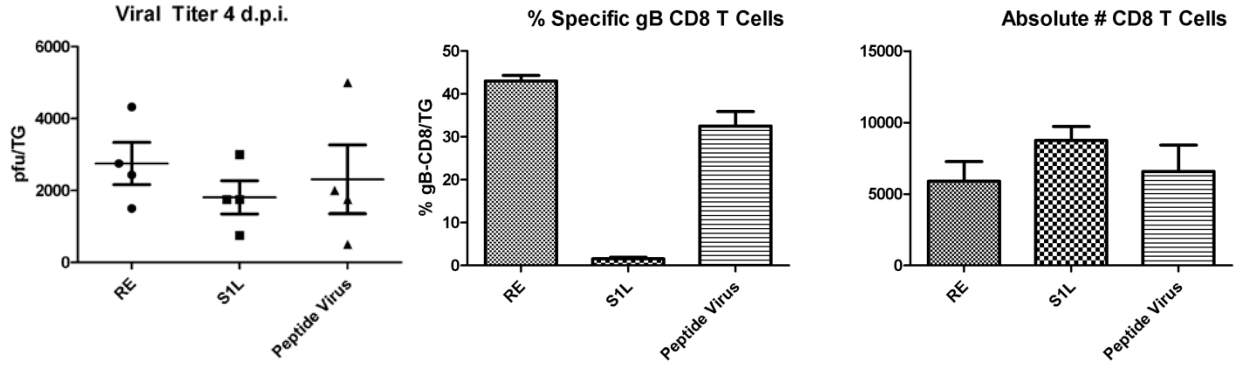


Figure 4.4.9. Characterization of the ectopic gB virus

(A) TG viral growth in B6 Mice (B) Absolute number of $CD8^+$ T cells at 8 d.p.i. (C) %gB Specific $CD8^+$ T cells at 8 d.p.i. A) TG were harvested 4 d.p.i. and subjected to three freeze thaw cycles and infectious virus released into the supernatant was titrated on Vero cells. The graph represents the mean virus titer for each virus ± SEM of the mean (n = 5 mice). (B) and (C) Mice were infected with 1 x 10^5 PFU/eye with HSV-1 WT, HSV-1 S1L, or the peptide virus. TG were excised at 8 d.p.i., dissociated into single cell suspensions and surface stained the expression of $gB_{498-505}$. Cells were analyzed by flow cytometry. The data are represented as the mean +/- SEM of the mean. (n=5 mice)

4.5 DISCUSSION

This work is designed to understand aspects of the development of the $CD8^+$ T cell repertoire to HSV-1 in the B6 murine model, particularly pertaining to the immune infiltrate into the trigeminal ganglion that is concomitant with the establishment of the latent state. The immunodominance of the $gB_{498\text{-}505}$ epitope, to which a majority (>50%) of HSV-1 specific $CD8^+$ T cells are directed following HSV-1 infection of the B6 mouse, has been well established, and reagents that identify this population have allowed the probing of many aspects of the cellular immune response following HSV infection and the latent state. This includes the observations that $gB_{498\text{-}505}$ $CD8^+$ T cells express granzyme B and surface markers of activation (CD25, CD44, CD69) [76,97] during latency, and rapidly produce IFNγ, IL10 and TNFα after HSV-1 antigen exposure [76,91,97]. The receptor polarization towards neurons during latency in B6 mice [90] has strongly suggested that gB is sporadically expressed during latency. Importantly, clones of the $CD8^+$ T cells specific for $gB_{498\text{-}505}$ from the mouse TG are able to block reactivation in *ex vivo* models establishing that the γ1 protein gB is recognized early and can mediate $CD8^+$ T cell blockade of the HSV-1 reactivation process. More is becoming known of the nature of the remaining $CD8^+$ T cells and their role in infection. The $CD8^+$ T cell population specific to subdominant epitopes can also block reactivation from latency [116]. The studies described here were initiated to address the nature of the $CD8^+$ T cell response to infectious viruses lacking this dominant epitope. From this work we can draw several important conclusions.

The S1L mutant did not induce a $gB_{498\text{-}505}$ dominated response, and yet was robust in pathogenesis. This was deemed important as virus replication in the TG could affect the antigens

presented and the recruitment and retention of the $CD8^+$ T cells in the ganglia. Many mutations in this region were developed in culture, but were attenuated in mice suggesting that the region defined functions *in vivo* that are not important *in vitro*. The natures of these specific roles are not yet known. Aside from its role in fusion and entry, gB is also involved in the modulation of the PERK activity and has roles in promoting nuclear egress of the nucleocapsid that may affect pathogenensis in vivo. However, S1L and L8A viruses showed near wild type levels in most respects. L8A mutants have been evaluated previously in the flank skin infection model C57BL/6 mice, and no differences were found when compared to wild type infection [101]. However, these authors did not assess their virus in the ocular infection model, where we found a very low induction of stromal disease in a different mouse strain where the dominance of the epitope would not contribute. This suggests further, as yet unknown, roles of this region of gB affecting pathogenesis.

The loss of the $gB_{498\text{-}505}$ immunodominant epitope results in the development of a $CD8^+$ T cell response equivalent in size infiltrating the TG that compensates for the loss of half of the $CD8^+$ T cells seen in HSV-1. This confirms the observation of Stock et al (2007), who found a similar ganglionic compensation in the $CD8^+$ T cell response following flank skin infection with HSV-1 containing the L8A mutation that also blocks development of $CD8^+$ T cells with $gB_{498\text{-}505}$ specificity [101]. Here we have established the nature of this compensation in the ganglia, which intriguingly suggests that the TG has mechanisms to regulate the $CD8^+$ infiltrate at the initial infection. The nature of this is not yet known: it is possible that gB $CD8^+$ T cells regulate the subdominant $CD8^+$ T cell populations, and the epitope loss results in other T cell populations filling the ganglionic compartment. The nature of this compensation is an increase of the

fraction of $CD8^+$ T cells directed to minor subdominant epitopes targeted by the natural developing $CD8^+$ T cell response to HSV-1. This repertoire is directed to a very limited subset of HSV-1 proteins. The majority of these were of the early or early late class [104]. This work shows that loss of the epitope does not result in an antigenic expansion seen by $CD8^+$ T cells or emergence of one or a few epitopes to dominance. The S1L infection resulted in upregulation of most of the 18 epitopes without the presence of a novel subdominant epitope that appear to identify the entire repertoire specific to HSV S1L in C57BL/6 mice. From this we can conclude that the $CD8^+$ T cell response is being compensated for by a broad increase of the subdominant populations. Since it has been shown that the non-$gB_{498\text{-}505}$ specific population induced by wild type HSV-1 infection can block reactivation [116], we consider it highly probable that this alternative $CD8^+$ T cell TG infiltrate will block reactivation efficiently.

The $CD8^+$ T cell response seen in an HSV-S1L infection also seems to be selective for proteins that are produced prior to HSV DNA synthesis, which is also seen in the wild-type infection [104]. Early proteins are the most frequently targeted epitopes. There is no known mechanism for why there is a dominance hierarchy, but there are many factors that may play a role in selection such as viral gene expression during reactivation events, [119] precursor frequency, and the ability of the peptide to bind MHC [94]. Further characterization will develop our understanding on how $CD8^+$ T cells specific to immunodominant epitopes influence the infiltration and retention of those specific to non-immunodominant epitopes. It will also help us understand the dynamics of the development and maintenance of $CD8^+$ T cell responses in the murine ganglia. Furthermore, the identity of the alternate $CD8^+$ T cell epitopes will also shed light on which viral antigens $CD8^+$ T cells respond to and the mechanisms involved in blocking

reactivation. The identity of these viral antigens may be the basis for the development of a new multivalent vaccine to induce protection against reactivation to HSV-1.

In this work, we also effectively separated the epitope from the gB protein by expressing the epitope ectopically from at the gC locus in the S1L background. This work leads to the conclusion that the immunodominance of the $gB_{498\text{-}505}$ in this mouse strain is not the consequence of the gB protein itself, nor the genomic locus from which it is expressed. It is conceivable that the immunodominance was the result of a structural definition of the protein that may have rendered the dominant peptide more easily generated from the gB protein. The gB locus and the epigenetic conditions that regulate its expression could have also contributed to immunodominance, but this does not seem to be the case. Ectopic expression of a peptide multimer alone resulted in a $gB_{498\text{-}505}$ dominated response that was identical to that of the wild type virus. Ruling out these scenarios, it is possible that the immunodominance is dictated in part by the precursor frequency of the $CD8^+$ T cells or another unknown mechanism.

While these studies will help us understand the dynamics of how $CD8^+$ T cell responses develop and are maintained in the murine ganglia, translation of this work to human studies will prove to be more challenging. In the murine model we show a proof of principle that by altering expression of the immunodominant $CD8^+$ T cell epitope, we can augment the immune response to that antigen and subsequently prevent reactivation. However, since humans may not react to the same $CD8^+$ T cell epitopes, the challenge facing scientists is to be able to predict or identify viral epitopes that are reactive in humans. Also, since therapeutic vaccines would need to be delivered locally to the site of latency, an effective and safe method of delivery is needed. The use of live virus may not be a viable option due to safety concerns. While the use of a replication

defective HSV-1 viral vector would be useful in that there would be constant antigen presentation to surrounding $CD8^+$ T cells, there is still the risk of viral reactivation from the vaccine virus, which could exacerbate any existing disease. Use of an appropriate adjuvant plays a critical and important part in the efficacy of a vaccine. While peptide vaccines with a strong adjuvant would boost the immune response temporarily, repeated vaccinations may be necessary. However, knowledge of the importance of the cellular immune response in the control of HSV-1 infection and reactivation has led to several animal studies involving vaccines aimed at eliciting such a response, such as DNA vaccines and vaccines encoding cytokines. The future of HSV-1 therapeutic vaccines may lie in these new techniques and targets.

5.0 SUBDOMINANT CD8 $^{+}$ T CELL ACTIVATION PHENOTYPE DURING LATENCY IN THE ABSENSE OF GB SPECIFIC CD8^{+} T CELLS

5.1 ABSTRACT

Latency of HSV-1 in human and murine trigeminal ganglia (TG) is associated with a persistent virus specific cellular immune infiltrate. It has been shown that in the C57BL/6 mouse model, mice infected with HSV-1 lacking the $gB_{498-505}$ epitope develop an alternative $CD8^+$ T cell repertoire and this repertoire is still able to block viral reactivation from latency. In chapter 4 we identified the global nature of the $CD8^+$ T cell repertoire at the peak response. However, the activation phenotype of CD8s recognizing these subdominant epitopes at latency is unknown. Here, we assessed the activation phenotype of these CD8s and their specificity. CD8s specific to select subdominant epitopes maintained hierarchy that was somewhat different from the responses at the peak of the infiltrate. They also showed ratios of IFNγ staining at 30 dpi as seen in an acute infection, as well as an activated state interpreted through GrzB and PD-1 staining. We conclude that loss of the HSV dominant epitope results in a retained functionality of the HSV-specific $CD8^+$ T cell repertoire during latency, and contrasts to the exhausted state seen for subdominants in the presence of the gB population.

5.2 INTRODUCTION

Latency is initially established when replicating virus is cleared from the ganglion, leaving only latent HSV-1 genomes in a chromatin regulated gene expression repressed state. In the C57BL/6 mouse, the persistent infiltrate of $CD8^+$ T cells maintain, in part, these genomes in latency. $CD8^+$ T cell numbers reach their peak at 8 dpi, coinciding with the onset of viral latency, but contract to a memory population of virus-specific $CD8^+$ T cells that remain in the TG for the life of the animal [71,76,77,78,79]. There are many questions about how these cells maintain latency. This chapter focuses on whether or not these cells are able to remain functional in the absence of the $gB_{498\text{-}505}$ epitope and how their specificity alters to that of the peak response. We examine if the subdominant infiltrate remains, and if the HSV-1 SIL peptide virus can ectopically restore a gB-CD8 population during latency.

It is as yet unclear why the $CD8^+$ T cell population in C57BL/6 mice dominates on the $gB_{498\text{-}505}$ dominant epitope. Immune dominance can be dictated by multiple factors, such as the epitope concentration presented, the binding affinity of the peptide to the MHC-I complex, the precursor frequency of the $CD8^+$ T cells that are able to react to $gB_{498\text{-}505}$ as well as the kinetics and level of expression. We have shown that in the absence of this epitope the subdominant population is able to rise and fill the void for the missing $CD8^+$ T cell population. However, how this subdominant population changes or how the dominant population can be manipulated in order to help prevent reactivation is as yet undetermined.

$CD8^+$ T cells act through IFNγ production and lytic granule release [66,78]. Consistent with others' observations (10,12,32), gB-$CD8^+$ T cells that produce IFNγ were found during all

stages of latency, and this population showed indications of recent antigen exposure through the expression of granzyme B, the ability to quickly produce IFNγ following antigen exposure, and through surface markers of activation such as CD44 and CD69 (40,95). gB-$CD8^+$ T cells contain lytic granules that are composed of multiple proteins, including perforin, Fas ligand, granulysin, and granzymes, all of which are all cytotoxic to infected cells [86,87,88]. Granzymes are important because they can cleave the proteins of intracellular and extracellular pathogens [120]. Granzyme B also induces caspase-dependent apoptosis, and with the help of perforin can induce $CD8^+$ T cell blocking of HSV-1 reactivation without apoptosing a neuron [66,67,89].

There is evidence to suggest that HSV-1 latency is not antigencally silent. In a latently infected TG, gB-CD8s polarize their TCR to the surface of neurons, and through this immunological synapse release IFNγ and lytic granules [66,76]. This indicates *in vivo* recognition of neurons through an MHC-1 manner and suggests that low level antigen exposure retains the TG $CD8^+$ T cell population during latency. Studies have also shown that $CD8^+$ T cells only need one MHC-I to peptide complex in order to be stimulated, so it is possible that low levels subdominant protein epitope expression during abortive reactivation events can be enough stimulus for $CD8^+$ T cells to block reactivation. [121,122].

5.3 MATERIALS AND METHODS

5.3.1 BrdU Staining

A FITC BrdU Flow Kit was purchased from BD Pharmingen (San Diego, CA). Phycoerythrin (PE)-conjugated H-2K^b tetramers complexed with the $gB_{498-505}$, $RR1_{982-989}$, $RR1_{822-829}$, or $ICP8_{876-883}$ peptide and PE-conjugated H-2D^b tetramers complexed with the $ICP8_{168-176}$, $RR1_{372-380}$, or $RR2_{279-287}$ peptide were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). All other antibodies were purchased as described previously.

Latently infected mice with either wild type HSV-1 or the S1L HSV-1 were pulsed for two days prior to harvest with 1mg/mouse of BrdU in 0.1ml PBS via i.p. injection. At 34 days post infection the TG were harvested and cells were prepared for flow cytometric analysis. TG cells were washed twice with FACS buffer (1% FBS, 0.1% sodium azide in PBS) prior to staining. Cells were incubated for 10 minutes with F_C block prior to surface staining with the appropriate antibodies. Following surface staining, cells were permeabilized with Cytofix/Cytoperm solution for 20 min on ice and then incubated with Cytofix/Cytoperm plus for 10 minutes on ice. After being washed with 1X BD Perm/Wash solution, cells were treated with 30 μg of DNAse for 1hr at 37°C. Cells were washed again and then incubated for 30 minutes in BrdU antibody diluted in 1X BD Perm/Wash. After labeling, suspensions were washed with 1X Perm/Wash and resuspended in FACS buffer for analysis by flow cytometry. Flow cytometry

was performed on a BD FACSAria Cell Sorter using FACSDiva 4.1 or 5.0.2 software (BD Pharmingen).

5.4 RESULTS

5.4.1 Subdominant Epitope Hierarchy of the $CD8^+$ T cell response during latency

Eight days post infection, HSV-1 S1L induces an equivalent absolute number of $CD8^+$ T cells infiltrating the ganglia with almost no reactivity to the gB epitope. It was determined that this response was not due to compensation of $CD8^+$ T cells to the mutant $gB_{498\text{-}505}$ peptides, but instead was compensated for by an increase in the number of CD8s specific to subdominant epitopes as described in Chapter 4.

We wanted to evaluate the nature and hierarchy of the $CD8^+$ T cell response to the subdominant epitopes for recognition by HSV-1 S1L in a latently infected animal to determine if these subdominant $CD8^+$ T cells remained in the same hierological order. It has been seen in studies with our collaborator, Anthony St. Leger, that CD8+ T cells responding to subdominant epitopes in a wild-type infection show signs of exhaustion, such as high levels of PD-1 and low levels of IFNγ secretion. However, in an S1L infection, it seemed that subdominant epitopes were still able to maintain some functionality, and this needed to be studied further. Due to constraints in the number of CD8+ T cells in the gangalia at latency, only a select representative population of the peptides was assayed. We found the fraction of ganglionic $CD8^+$ T cells responding to each peptide from the TG of S1L infected mice at 30 dpi (by IFNγ staining) remained increased over the levels from wild type infections (Fig 5.4.1). The ratio of cells responding to each target also appeared to remain consistent with the relative ratios from 8 dpi infected animals suggesting a similar hierarchical order. We compared this with data from a wild type infection (A. St. Leger and R Hendricks, unpublished data). This showed that the $CD8^+$ T

responding to the subdominant epitopes in an S1L infected ganglion were more active as seen by IFNγ production.

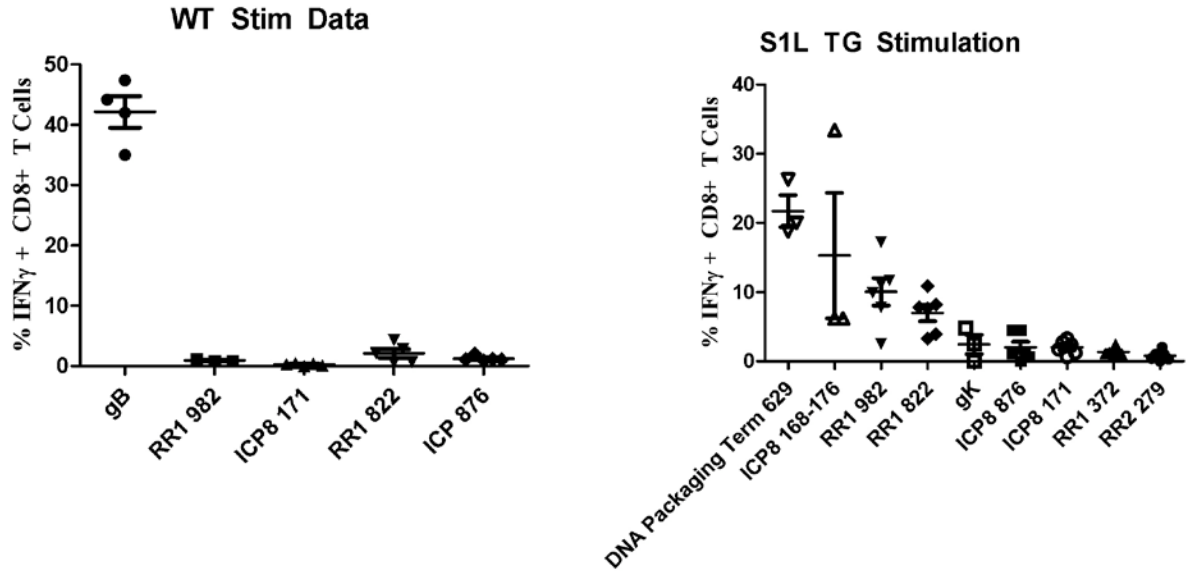


Figure 5.4.1. 18 subdominant epitopes were tested to try and define the phenotype of the TCR in the absence of the $gB_{498-505}$ dominant epitope. TG were excised at 30 d.p.i., dissociated into single cell suspension, and then incubated for 6 h with peptide-loaded B6WT3 fibroblasts in the presence of Golgi-Plug. This was followed by an intracellular stain for IFNγ. **A)** Data from a wild type infection from A. St. Leger and R. Hendricks. **B)** Data from an S1L infection. The data are represented as the mean of $CD8^+$T cells producing IFNγ +/- SEM of the mean. (n=3 mice).

5.4.2 BrdU Staining of Subdominant Epitopes

We hypothesized that the $CD8^+$ T cell population in the HSV-1 S1L infected TG would have more proliferation of the subdominant population because they are seeing more antigen than in a wild-type infection. It was possible that the reason we were seeing more subdominant cells in the S1L infected TG was that they were proliferating at a higher rate. However, proliferation of the subdominant cells also occurs in a wild type infection, but more apoptosis is seen. Therefore, this needed to be addressed. After staining for tetramer and analyzing by flow

cytometry, we determined how many tetramer specific $CD8^+$ T cells were also positive for BrdU. The level of proliferation in the population as assessed by BrdU incorporation in the HSV-1 S1L $CD8^+$ T cells was similar to that of the HSV-1 WT infected mice (Fig. 5.4.2). This suggested that proliferation may not be an important factor in distinguishing the subdominant population or their activation state.

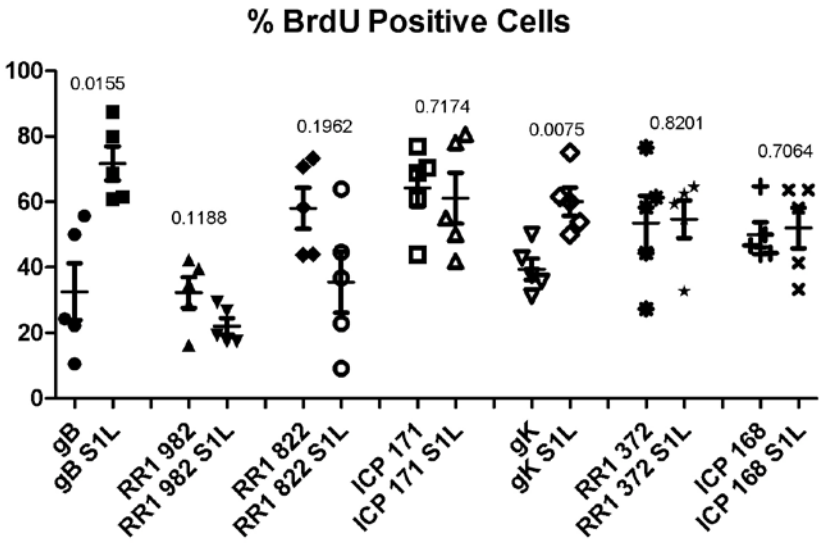
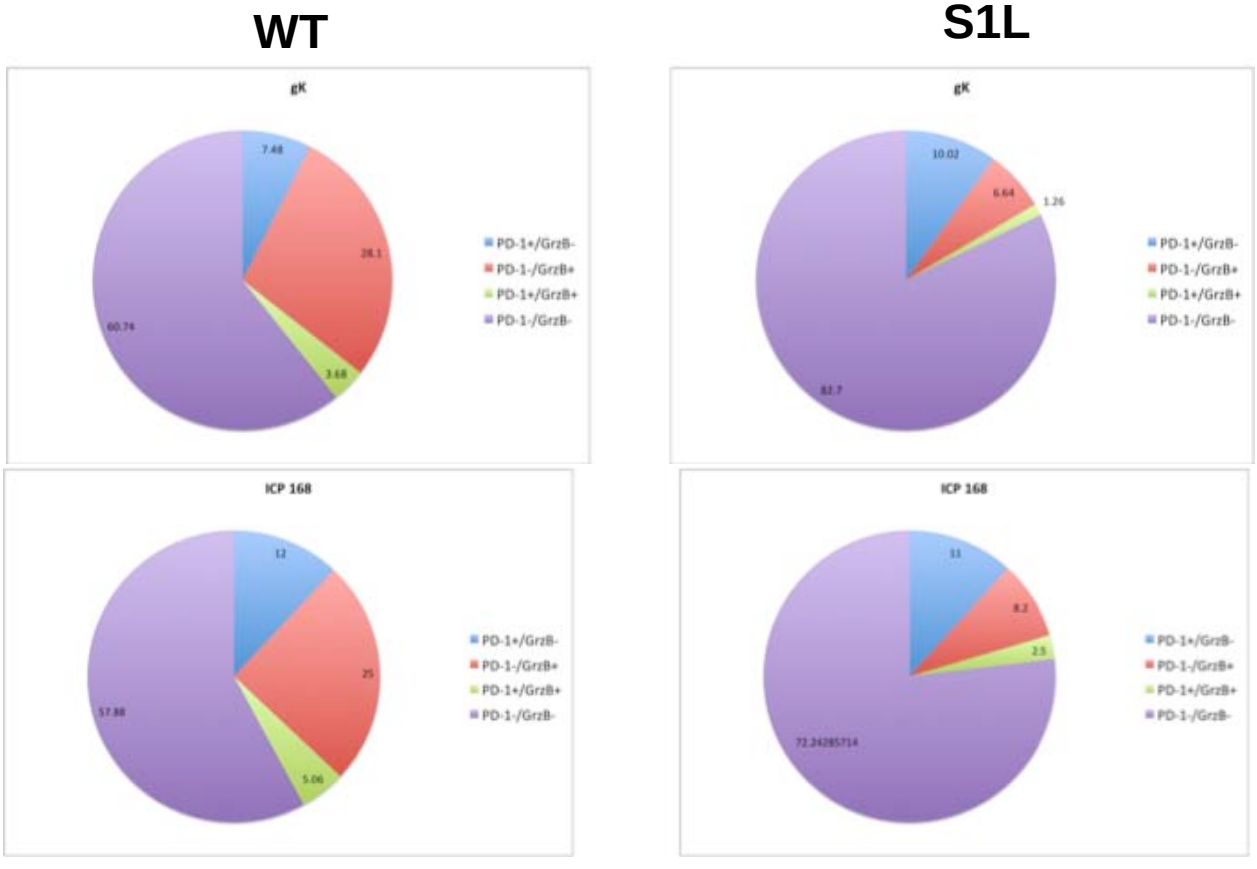


Figure 5.4.2. BrdU was given intraperitoneally (i.p.) 48 and 24 hours before harvest. TG were then harvested and stained for CD8, CD45, tetramer specific T cell receptor, and BrdU incorporation. There is only a 1% gB population seen in the S1L infected TG as compared to 50% seen in the WT infected TG. Data represent each specific animal and standard error of 6 mice from 2 experiments. Significance was determined by one way ANOVA.

5.4.3 PD-1/GrzB Activation State of Epitopes at Latency

Studies have shown that a $CD8^+$ T cell can act through gB and block reactivation through a non-cytolytic pathway, which may involve IFNγ and GrzB [102,115]. Determining which cells from a HSV-1 S1L infection are GrzB positive will identify which antigens are being presented and recognized in the absence of the dominant epitope. Programmed death receptor-1, or PD-1,

is another important marker that can indicate both recent exposure of $CD8^+$ T cells to antigen but can also indicate exhaustion. We wanted to compare the activation state of the $CD8^+$ T cells in latently infected mice with either wild type HSV-1 or HSV-1 S1L. B6 mice were inoculated using 1 x 10^5 per eye of either wild type HSV-1 or HSV-1 S1L (Figure 5.4.3). At 30 dpi, TG were then excised and stained for CD8, CD45, GrzB and PD-1 and analyzed by flow cytometry to determine the activation state of the $CD8^+$ T cell population. There appears to be a large amount of variation in the activation states between the WT subdominant epitopes as compared to the F5L subdominant epitopes. Although there is more GrzB in the WT $CD8^+$ T cells, there also seems to be more PD-1, which may mean that there is more exhaustion in these cell populations or that they have recently been exposed to their antigen at higher levels since it has been shown that these $CD8^+$ T cells are able to protect against reactivation. Further studies need to be performed to determine what determines differences in the dominant versus subdominant cells.



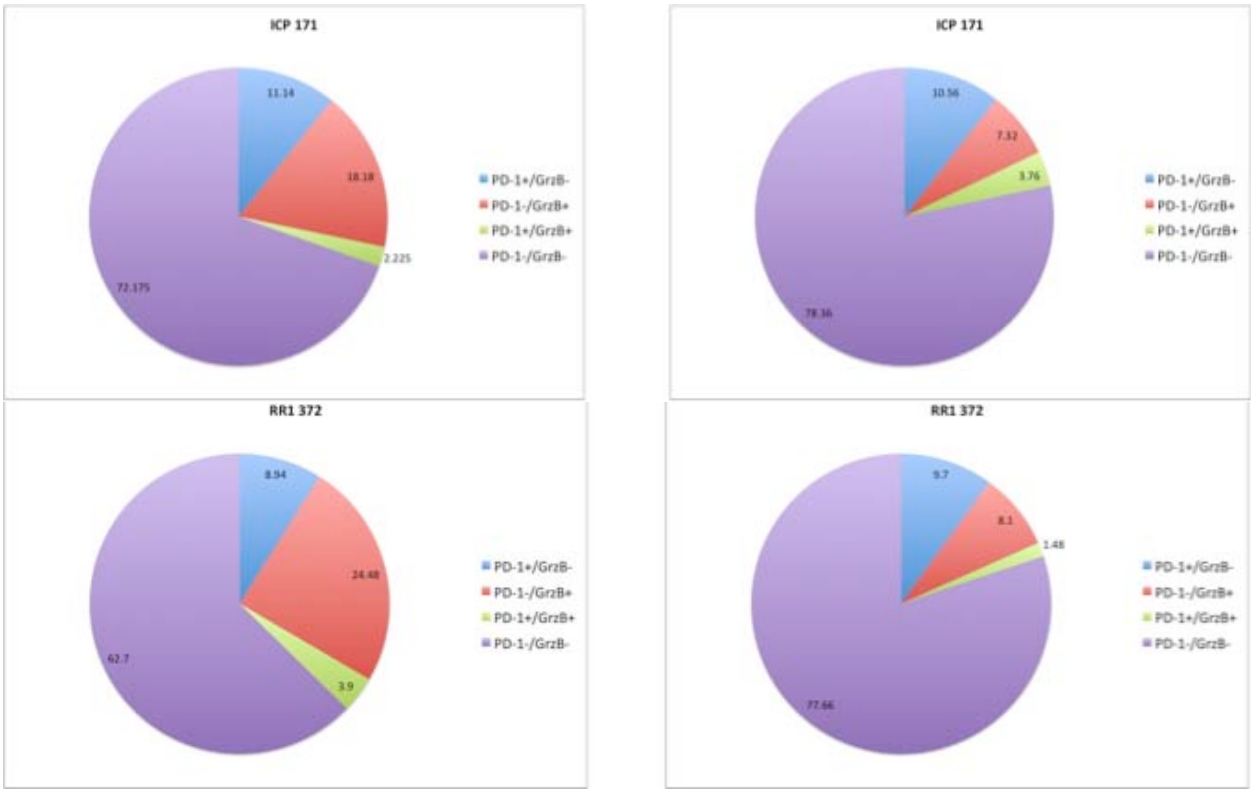


Figure 5.4.3. Characterization of the activation state of subdominant epitopes

Mice were infected with 1 x 10^5 PFU/eye with HSV-1 WT or HSV-1 S1L. TG were excised at 30 d.p.i., dissociated into single cell suspensions and surface stained for the expression of a respective subdominant epitope. Cells were analyzed by flow cytometry. The data are represented as the mean of the total tetramer positive population (n=3 mice). Four examples are shown.

5.4.4 Response to the ectopically restored Peptide Virus at latency

One stimulus affecting immunodominance hierarchy of $CD8^+$ T cells is the kinetics and levels of expression of the protein target. As our lab has shown, delay of gB expression to a true late gene effectively leads to reduced $gB_{498\text{-}505}$ dominance in the TG [113] at latency changing the expression of gB (and the $gB_{498\text{-}505}$ epitope) significantly changes viral pathogenicity. We showed in Chapter 4 that ectopic expression of the $gB_{498\text{-}505}$ peptide in the HSV-1 SIL epitope-

negative background allows for manipulation of expression of the $gB_{498-505}$ epitope independent of gB expression during acute infection. It was deemed important to verify that this expression was maintained through a latent infection. B6 mice were inoculated using 1 x 10^5 per eye and compared to mice infected with either the WT strain or the S1L mutant (Figure 5.4.4). The absolute CD8 count is similar in all viruses and all viruses are similarly virulent as shown in chapter 4. Not only does the total $CD8^+$ T cell count reach similar numbers but the number of gB-CD8s detected in the HSV-1 S1L-4gBpep infected mice were also similar to that of the WT infected animal. This signifies that ectopic expression of the $gB_{498-505}$ epitope maintains the dominant $CD8^+$ T cell response in the B6 mouse model throughout latency as compared to wild type.

The specificity at the subdominants was also assessed by comparing two subdominant epitopes, RR1 982 and RR1 882, between these three viruses. In animals infected with HSV-1 S1L-4gBpep, the ability to respond to subdominant epitopes seems to restore to that of a WT infected animal, while animals infected with HSV-1 SIL still respond to subdominant epitopes at an increased level. Both of these studies show that dominance of the epitope is not a result of the structural features of the gB protein itself or its locus.

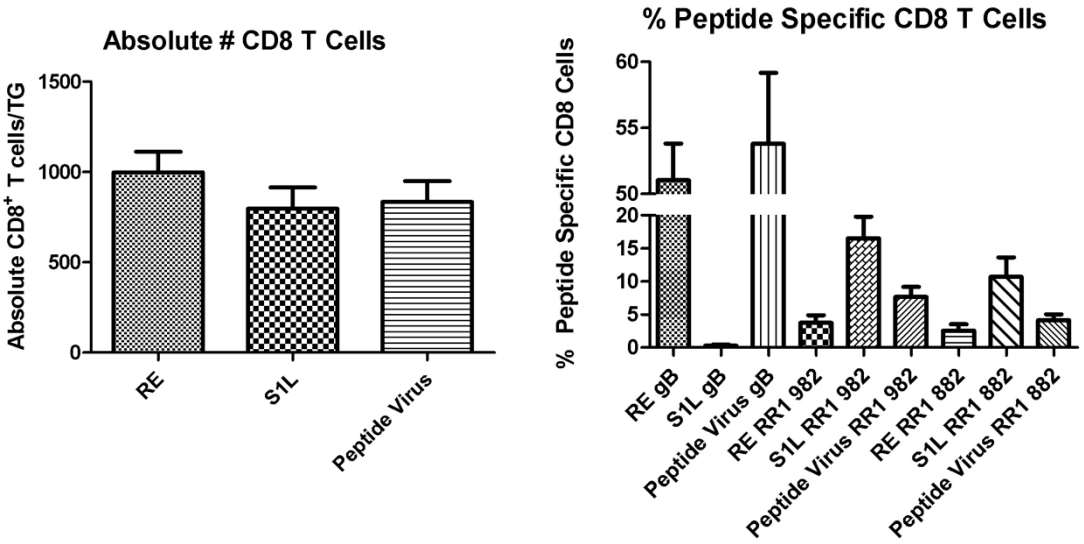


Figure 5.4.4. Characterization of the ectopic gB virus at latency

(A) Absolute number of $CD8^+$ T cells at 30 d.p.i. (B) %gB specific $CD8^+$ T cells at 30 d.p.i. (A) and (B) Mice were infected with 1 x 10^5 PFU/eye with HSV-1 WT, HSV-1 S1L, or the peptide virus. TG were excised at 30 d.p.i., dissociated into single cell suspensions and surface stained for the expression of $gB_{498\text{-}505}$. Cells were analyzed by flow cytometry. The data are represented as the mean +/- SEM of the mean. (n=6 mice)

5.5 DISCUSSION

Recurrent HSK resulting from reactivation of latent HSV-1 in neurons of the TG lead to progressive corneal scarring and loss of vision. Strategies to block HSV-1 reactivation from latency before virus is delivered to the periphery can prevent inflammation and scarring, but to date, no vaccine is available. Until recently, HSV-1 latency was believed to be antigenically silent and invisible to the immune system, making vaccine development difficult. However, there has been a shift in paradigm and our data, along with multiple other studies, show that the interaction between the infected ganglion, the sporadically reactivating HSV-1 genome, and the $CD8^+$ T cells control, at least in part, the reactivation process [123,124]. Understanding and manipulating this interaction may be the key to controlling HSV-1 reactivation and preventing HSK.

Previous to this work, a strong role for $CD8^+$ T cells being able to control viral latency was established but it was unclear how HSV-1 antigen expression influenced the way these cells responded to the virus during this period [67,125,126]. We show that $CD8^+$ T cells responding to subdominant epitopes are retained in similar ratios during latency as they are found during an acute infection, and that they are able to act quickly as demonstrated by their ability to produce IFNγ. This shows that antigen exposure is important for the retention and functionality of these cells during latency. This work has major implications towards designing a better therapeutic vaccine to prevent HSV-1 reactivation in the trigeminal ganglia.

We determined that HSV-1 S1L induced a similar $CD8^+$ T cell infiltrate at latency as compared to a wild type virus. The next step was to perform *ex vivo* proliferation assays as well

as stain for GrzB and PD-1 to determine if these subdominant epitope populations were still functionally competent in an HSV-1 S1L infected TG. While more GrzB was seen in the WT $CD8^+$ T cells, there also seems to be more PD-1, which may mean that there is more exhaustion in these cell populations.

Using the SIL peptide virus, we compared the number of gB-CD8's retained in a peptide virally infected TG in parallel with an S1L infected animal and a wild type infected animal. We showed that ectopic expression of the $gB_{498\text{-}505}$ epitope can maintain the dominant $CD8^+$ T cell response in the mouse model throughout latency. We also compared two subdominant epitopes, RR1 982 and RR1 822 between these three viruses. In animals infected with HSV-1 S1L-4gBpep, the ability to respond to subdominant epitopes seems to return to that of a WT infected animal. This work leads to the conclusion that the immunodominance of the $gB_{498\text{-}505}$ in this mouse strain is not the consequence of the gB protein itself, nor the genomic locus from which it is expressed.

It was conceivable that the immunodominance was the result of a structural definition of the protein that may have rendered the dominant peptide more easily generated from the gB protein. The gB locus and the epigenetic conditions that regulate its expression could also have contributed to immunodominance, but this does not seem to be the case. The separation and ectopic expression of the epitope now establishes the feasibility of this approach to assess the contribution of the kinetics of viral gene expression to dominance of the gB-CD8 T cell responses, since we can now use different classes of viral promoters to drive the multipeptide. For example, we have previously found the true late expression of gB (from the gC protein) results in the development of an altered dominance in that the 50% population in the TG is

reduced, and depletes over time [113]. It is known that immunodominance can be influenced by the kinetics of viral gene expression as well as the load of antigen presented at the priming stages as well as the level of antigen expressed at persistent stages of infection [127]. For example, with vaccinia virus it was recently demonstrated that altered expression of a late antigen to an immediate early antigen resulted in the enhancing of acute and memory $CD8^+$ T cell responses [128]. It may be that specific promoters are able to more effectively express a viral antigen. This may induce a more effectively retained infiltrate in the ganglia that can better block reactivation if they are functional. T cells at latency to subdominant populations appear to express considerably less granzyme B [71], and it has been suggested that periodic sporadic reactivation events may drive abnormal inflation of $CD8^+$ T cells following systemic administration of HSV -1 [129]. Studies on the T cell specificity of the retained $CD8^+$ T cell infiltrate and their functionality during latency are in progress for both HSV -1 and HSV-1 lacking the dominant epitope. However, the low levels of $CD8^+$ T cells that are retained in the ganglia at latency confound the assessment of T cell specificity.

This work has given us considerable insight into the retention of antigen specific $CD8^+$ T cells in the latently infected TG. Translating this work to human studies will require a large amount of work; however, this gives us a solid groundwork for which immunogens to focus on, such as the immediate early and early proteins that seem to be seen more frequently by $CD8^+$ T cells, and will allow us to begin development of an effect vaccine against HSV-1 reactivation.

6.0 GENERAL DISCUSSION AND CONCLUSIONS

The $CD8^+$ T cell response in the HSV-1 infected ganglia is an important component of host control of reactivation during latency. The immunodominance of the $gB_{498-505}$ epitope in $CD8^+$ T cells in the C57BL/6 mouse model is impressive, accounting for half of the HSV-1 specific systemic $CD8^+$ T response, the $CD8^+$ T cell infiltrate in the acutely infected TG [125], and the persisting contracted $CD8^+$ T population associated with latency [130].

gB-CD8s in the HSV-1 latently infected TG express surface markers of activation (CD25, CD44, CD69) [76,97], are granzyme B positive, and rapidly produce IFNγ and TNFα upon HSV-1 antigen exposure [82,91,125,131]. They also demonstrate T cell receptor polarization towards neurons during latency in an immunological synapse [90] and the ability to block reactivation *ex vivo* [102,103]. It is likely that these gB-CD8s respond to sporadic gB antigen expression during latency and block progression to reactivation. This is consistent with reports that show HSV-1 gene and protein expression in the latently infected mouse and human TG without any productive virus production [34,49].

Recent work has focused more on the HSV-1 specific non-$gB_{498-505}$ CD8 T cell populations, since these also have the potential to prevent HSV-1 reactivation [116]. They recognize a very limited repertoire of 18 epitopes on 14 viral proteins [104]. This study

investigated the viral specificity of $CD8^+$ T cells that infiltrate the acutely infected TG in the absence of the dominating gB epitope in order to understand the relationship of $CD8^+$ T cell repertoires responding to HSV-1 ocular and ganglionic infection. The hierarchy of the immune response is affected by multiple factors, including MHC affinity, T cell precursor frequency, and antigen expression timing and structure [90,132,133].

We developed two mutant HSV-1 viruses that lack the gB epitope but which retained near wild type pathogenicity levels in the mouse model. Full pathogenicity was deemed necessary as reduced viral replication in the TG could affect the level of antigens presented and the recruitment of the $CD8^+$ T cell population in the ganglia, as seen in the LCMV model [127]. HSV-1 L8A had been shown in the flank skin infection model in C57BL/6 mice to be similar to wild type in pathogenesis and did not generate a dominating gB-CD8 population [101]. However, we favored HSV-1 S1L in our studies because L8A showed a reduced CD8 infiltrate in the mouse ganglia over that generated to S1L and wild type (Fig 4.4.6), which was also seen by Stock et. al (2007). Interestingly, neither mutation resulted in development of $CD8^+$ T cell populations to the mutant epitopes, suggesting they prevented stimulation and maturation of the $gB_{498\text{-}505}$ precursor populations that account for part of the immunodominance [104].

The loss of the dominant $gB_{498\text{-}505}$ epitope resulted in an equivalent $CD8^+$ T cell response infiltrating the TG that compensated for the loss of half of the $CD8^+$ T cells seen in an HSV-1 infected TG. It is intriguing how this compensatory response is controlled since its size is almost identical to that of the wild type. Factors that may contribute include the antigen presenting cells that, without the dominant epitope, may better present the subdominant epitopes and more effectively stimulate the T cell populations. It may also be that gB-CD8s control the infiltration,

as it has long been known that IFNγ production is one factor that regulates the $CD8^+$ T cell hierarchy, and gB-CD8s rapidly express IFNγ upon stimulation [76,102].

Our work now shows the nature of the compensation in the TG occurs by parallel increase of the fraction of $CD8^+$ T cells directed to most of the minor subdominant epitopes targeted by the natural developing $CD8^+$ T cell response to HSV-1. In our data, there was no emergence of one or only a few peptides or a new epitope to dominance or co-dominance, and as the total $CD8^+$ T cell numbers in the ganglia is similar to the sum of the fractions of the total compensation to each epitope, we consider it unlikely that the compensation is due to significant antigenic expansion of $CD8^+$ T cells to peptides outside of the tested targets. Thus the loss of the immundominant epitope results in a broader T cell repertoire, which may be more effective at sensing the sporadic reactivation events that occur during latency and preventing reactivation.

There are some consistencies in the hierarchy of dominance between wild type and S1L infections, but some changes as well. For example, one of the highest subdominant targets to wild type infection is the ribonucleotide reductase, of which two epitopes 982 and 822 are among the highest in the S1L infection as well. In particular, we observed a 4-5 fold increase in the ribonucleotide reductase 982 subdominant epitope (RR1 982), which contrasts with the absence of an increase in the subdominant epitope in the study of Stock et. al. [101]. The reason for this is not clear, as we have also found with our L8A that the CD8s to the RR1 822 epitope also increase (data not shown). It is also possible that the site of infection between the two studies may influence subdominant alternative responses. Stock et al. argued that the compensatory response was due to the emergence of cryptic epitopes, which is in direct contrast to our conclusion that it is upregulation of the next best ranking epitopes that seems more likely to

occur in the acutely infected TG. It is clear that some subdominant populations arise to codominance from while other higher ranking epitopes show much less of an increase following the loss of the gB epitope, such as that to ICP8 976, which shows only a modest 1.2 fold increase, while a few epitopes that have very minor populations in a wild type infection (UL25 for example) show relative large fold increases. This is inconsistent with the emergence of cryptic epitopes seen for Influenza and Listeria, but in some ways is similar to the pattern seen in other systems. For example, loss of epitope specific responses in LCMV resulted in compensatory responses against other subdominant epitopes, without changing the hierarchy and compensated responses leading to a rise in hierarchical importance of other viral targets can still mediate effective targeting by $CD8^+$ T cells responses [134]. Serial upranking of minor subdominant epitopes following sequential deletion of the dominant epitopes has also been seen for HIV epitopes delivered by vaccinia vectors [134]. The broadening of the $CD8^+$ T cell repertoire following loss of the dominant epitope has also been seen with other pathogens [135]. The dominant epitope is often replaced by several subdominant epitopes. The drivers of the HSV dominance hierarchy and the changes induced by the S1L mutation are not yet clear, but are likely complex. It is known that a specific population of $CD8^+$ T cells can affect others in such a way that dominance hierarchy is a contextual series of events in which the dominance of a specific epitope is dependent upon other epitopes and the response to them [136]. Thus it is possible that gB-CD8 development may suppress the development and infiltration of non gB-CD8s in our HSV model. Since gB-CD8s express IFNγ in the ganglia, and IFNγ is predicted to influence $CD8^+$ T cell hierarchy and functionality [137], it is possible that the loss of the dominating gB-CD8s relieves the suppressor effect on other epitope-specific populations and

allows the increased proliferation of the subdominant-specific $CD8^+$ T cell precursors [138]. Recent work suggests IFNγ release by gB-CD8 may up regulate PD-L1 expression in neurons that may drive exhaustion of PD-1 positive subdominant CD8s (S Jeong and RL Hendricks, unpublished data). If so, the rise in subdominant CD8s may be a direct result of the loss of the major CD8 dominant population.

It is interesting that the $CD8^+$ T cell response seen in an HSV-S1L infection also seems to be selective for proteins that are produced prior to HSV DNA synthesis, which is also seen in the wild-type infection [104]. Most of the epitope targets are directed to early proteins, with the strongest compensatory increase being directed to epitopes on ribonucleotide reductase (an early protein), and two gamma1 regulated proteins, gB and the DNA terminase subunit. It is notable that there is very little evidence of any immediate early protein recognition and only a few epitopes from proteins expressed only after DNA replication are seen. Levels and timing of antigen are known to greatly influence memory cell development and functionality [138]. The contribution of viral kinetics to dominance hierarchy is largely unexplored in HSV-1, but recent evidence has strongly suggested the classical cascade of HSV-1 seen in lytic infected cells does not necessarily apply to sporadic reactivation events [46,47]. There are many factors that may play a role in selection of targets for $CD8^+$ T cell development, such as kinetics and levels of viral gene expression during reactivation events [119], precursor frequency, and the ability of the peptide to bind MHC [94]. Further characterization of the contribution to $CD8^+$ T cell development will help us develop our understanding of how $CD8^+$ T cells specific to immunodominant epitopes influence the infiltration and retention of those directed to non-immunodominant epitopes. The identity of the alternate $CD8^+$ T cell epitopes may also reveal

viral antigens to which $CD8^+$ T cells can respond to in blocking reactivation, which could then be developed as T cell generating vaccines.

This work also has additional relevance to vaccine design, since it adds to the wealth of information that indicates $CD8^+$ T cell induction is an important goal of any vaccine. Most current HSV-1 vaccines are based on viral-encoded surface glycoprotein subunits designed to boost the neutralizing antibody response, and have largely failed to meet their criteria in clinical trials for prevent infection or disease. We argue that an effective vaccine strategy that boosts $CD8^+$ T cell levels in the ganglia and surrounding the sensory neurons could prevent spread in the ganglia upon infection, or prevent reactivation from latency if used therapeutically. In the murine model, our goal was to show proof of principle that manipulating the $CD8^+$ T cell response by removing the dominant epitope and expressing multiple copies of it expands the immune response to a particular antigen. While these studies will help us understand the dynamics of how $CD8^+$ T cell responses develop and are maintained in the murine ganglia, translation of this work to human studies will prove to be more challenging, because the diverse HLA types will dictate that the identity of specific antigens to which $CD8^+$ T cells are directed. This might make it difficult to predict which viral epitopes are reactive in a human HSV-1 infection. Interestingly, recent genome wide screening of $CD8^+$ T cell targets for seven diverse individuals suggest that some HSV-1 proteins appear to be targeted more frequently than others, with the large subunit of ribonucleotide reductase showing a prominence in six individuals to both CD4 and $CD8^+$ T cells [139]. This correlates with the main dominant epitope seen in the S1L infected B6 mice. Thus it is likely that additional proteins outside of the usual glycoprotein

candidates are valid potential vaccines for induction of a protective T cell immune response that is optimally intended to prevent reactivation.

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