INFLUENCE OF HERPES SIMPLEX VIRUS TYPE-1 GLYCOPROTEIN B EXPRESSION ON VIRAL PATHOGENECITY AND THE CD8$^+$ T CELL RESPONSE

by

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ABSTRACT

Influence of Herpes simplex virus type-1 Glycoprotein B expression on Viral Pathogenecity and the $CD8^+$ T cell Response

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Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen that establishes a latent infection in sensory ganglia and upon reactivation, can cause severe ocular disease. During lytic replication, viral proteins are expressed in a temporal cascade of immediate early (α), early (β) and late (γ) genes. The γ genes are further sub-classified into γ1 genes which are expressed prior to DNA replication and γ2 genes which are absolutely dependent on DNA replication for their expression. During a latent infection of the trigeminal ganglia (TG), no infectious virus is produced and latency is associated with a persistent virus-specific $CD8^+$ T cell infiltrate that actively block reactivation. In C57BL/6 mice, approximately 50% of these $CD8^+$ T cells are specific to a single epitope on a γ1 protein glycoprotein B ($gB_{498-505;}$ gB-CD8). In the TG, gB-CD8 are retained with an activated phenotype suggesting recent exposure to antigen during abortive reactivation events. While the kinetics of gene expression during lytic infection has been appreciated for some time, little is known about antigen expression during latency and reactivation, and its influence on antigen-specific $CD8^+$ T cells in the TG. Using gB as our model antigen, in this work we show that during reactivation, gB expression occurs much sooner than gC expression, indicating that it expressed fairly early even during reactivation.

Furthermore, by delaying gB expression to after DNA replication we observed a severe impairment in viral replication in the TG and a significant diminishment in the retention and activation phenotype of gB-CD8 in latently infected TG. However delaying gB did not prevent the ability of gB-CD8 to block reactivation indicating they can act very quickly even after DNA replication. Finally we demonstrate that by mutating the gB epitope such that it does not induce a gB-CD8 response, we abrogate the gB-CD8 infiltrate in the TG. These studies all demonstrate that during an HSV-1 infection, only antigen-specific $CD8^+$ T cells infiltrate the TG and antigen exposure during latency is responsible for their retention in the TG. This work has great implications toward designing better immunogens for therapeutic vaccines to prevent HSV-1 reactivation.

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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 human herpesvirus that is classified under the family *Herpesviridae and* sub 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, but establish latency only in the sensory ganglia innervating the sites of infection (135). The ubiquitous nature of HSV-1 makes it such that around 80% of the world's population has been exposed to the virus (152, 184). In the United States, approximately 57% of individuals between the ages of 14 and 49 are seropositive for HSV-1 and this number rises to over 90% after the age of 60 (141, 170). HSV-1 is usually transmitted via oral contact or droplet spread, and is often contracted during early childhood (184). While primary infections are often more serious than reactivated disease, they can often be asymptomatic, leading to many people being unaware of the initial infection. Reactivated disease is of variable clinical presentation ranging from visible disease to asymptomatic shedding. HSV-1 reactivation can cause several diseases including cold sores, gingivostomatitis, herpetic whitlow, genital lesions, encephalitis as well as blinding stromal keratitis (184).

1.1.2 Herpes Stromal Keratitis

HSV-1 can infect the corneal surface and lead to a latent infection in the trigeminal ganglia (TG). Reactivation of the virus from the ophthalmic branch of the TG results in recrudescent disease at the corneal surface that can manifest itself in several forms including blepharitis, conjunctivitis, epithelial keratitis, and herpes stromal keratitis (HSK). HSK is a severe immunopathological disease that results from HSV-1 reactivation and viral replication in the cornea. Approximately 500,000 individuals suffer from HSK in the United States with 300,000 new cases reported every year (86). Reactivation of HSV-1 and subsequent viral replication at the corneal surface results in an immune infiltrate into the eye aimed at controlling viral replication. The infiltrating immune cells release several factors, such as the cytokines γ-interferon (IFNγ) and interleukin-2 (IL-2), and other compounds, including nitrous oxide (NO) and matrix metalloproteinases, that can cause destruction and disorganization of the collagen matrix of the stroma, eventually leading to scar tissue formation and blindness (9, 82, 110). Therapies for HSK include prescription of antiviral drops in conjunction with corticosteroids to reduce viral replication and inflammation respectively (4, 23). However, antiviral treatments are only effective in the face of ongoing viral replication and HSK can develop in the absence of infectious virus in the cornea (43). Treatments with steroidal compounds also have side-effects and cannot reverse the effects of HSK. HSV-1 is the leading cause of infectious blindness in the developed world (56, 85-86). Blindness resulting from HSK is also the leading cause for corneal transplants in the United States alone and approximately 5-10% of corneal transplants are performed on patients with HSK to restore sight (132). Furthermore, recurring HSK can also cause corneal graft rejections amongst corneal transplant patients and alternatively, HSV-1 may be acquired from donor tissue (132-133). The lack of an effective treatment or vaccine for primary or recurrent HSV-1

infections makes it a pathogen of great public health relevance and one that warrants further research.

1.2 HSV-1 STRUCTURE

The HSV virion can be represented as four morphologically distinct compartments: (a) an outer envelope with glycoprotein spikes on its surface, (b) a tegument layer containing 25-30 viral proteins, (c) an icosahedral capsid just beneath the tegument layer and (d) an electron opaque core encompassing the HSV genome (Fig. 1-1) (184).

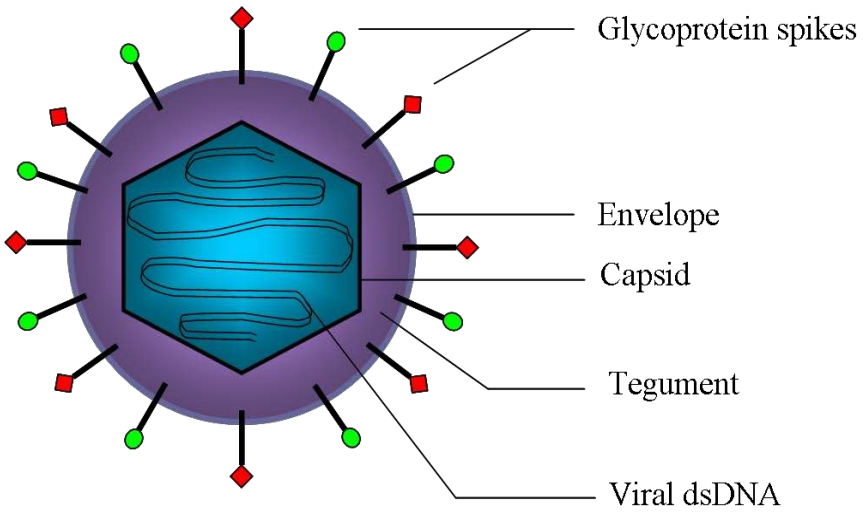

Figure 1-1 Graphical representation of the HSV-1 virion.

The HSV-1 nucleocapsid (blue) contains one genome length of double stranded viral DNA within it. The capsid is surrounded by a proteinaceous tegument layer (purple) which is enclosed within a host derived envelope containing viral glycoprotein spikes (green and red). The diagram is not to scale.

The HSV-1 virion contains a linear double stranded DNA genome of approximately 152 kb that is packaged within the icosahedron shaped capsid. The DNA consists of two unique segments known as the unique long (U_L) and the unique short (U_S) segments (Fig. 1-2). These unique segments are flanked on either end by inverted repeats referred to as R_L and R_s respectively for the long and short repeats. The R_L repeat sequences are denoted as *ab* and *b'a'* and the R_S repeats are denoted as *a'c'* and *ca* (Fig. 1-2). The U_L and U_S segments can invert relative to each other, thus forming four linear isomers which can all produce viable virus particles. The entire HSV-1 genome encodes for the expression of approximately 80 viral proteins (135, 182).

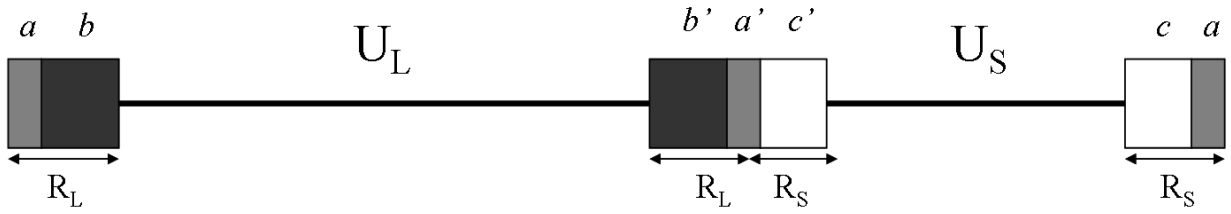

Figure 1-2. Representation of the HSV-1 genome organization.

The HSV-1 genome is separated into a unique long (U_L) and unique short (U_S) segments flanked by inverted repeat sequences *ab, b'a'c'* and *ca*. The diagram is not to scale. Figure is adapted from (184).

1.3 HSV-1 LIFE CYCLE

HSV-1 can adopt two modes of infection, a lytic phase and a latent phase. During the lytic stage, virus amplification leads to the eventual lysis and death of some but not all types of infected cells. The virus can also enter a latent phase, exclusively in neuronal tissue, during which the

viral genome can persist for indefinite periods of time within the infected neuron. HSV-1 can then periodically re-enter the lytic cycle from latency, a phase known as reactivation whereby newly produced virus particles can result in recrudescence of disease.

1.3.1 HSV-1 Entry

Viral replication begins with HSV-1 entry into a permissible host cell. This requires attachment to receptors and the fusion of the virus' membrane and the host cells' lipid membrane. Three viral glycoproteins, glycoproteins B, C and D (gB, gC and gD) mediate viral attachment, binding and entry. Initial attachment of the HSV virion is mediated by the binding of glycoprotein C (gC) to heparan sulfate moieties on cell surface proteoglycans (HSPGs) (53, 148). gC is not essential for entry and in its absence, gB can bind directly to HSPGs (52). However, binding of gC to heparin sulfate confers a higher efficiency for gD to bind to other cell surface co-receptors such as Herpesvirus entry mediator (HVEM), transmembrane adhesion molecules, nectin 1 α or β or 3-O-sulfated heparan sulfate, depending on the cell type. The virus then enters the cell following binding of gD to one of its high affinity receptors followed by a fusion event that requires gB and the gH/gL complex along with gD (172).

1.3.2 Lytic Replication

1.3.2.1 Gene expression pattern

During the HSV-1 lytic cycle, over 80 viral genes are expressed in a tightly regulated temporal cascade (Fig. 1-3). Following entry, the viral nucleocapsid along with some tegument proteins is

transported into the host cell nucleus. Once the nucleocapsid reaches the nucleus, the viral DNA is ejected into the nucleus and transcription of viral proteins can begin.

The immediate early (IE or α) genes are the first to be expressed and can be transcribed in the absence of *de novo* viral protein synthesis, whereas the early (E or β) genes and the late (L or γ) genes are expressed only following α protein expression. The immediate early genes encode proteins involved in transcription of other viral genes. The early genes encode proteins that are responsible for viral DNA replication while the late genes mainly glycoproteins and factors required for virion assembly egress and maturation. Following the expression of all the classes of proteins, infectious virus can be assembled and released from the infected cell (135).

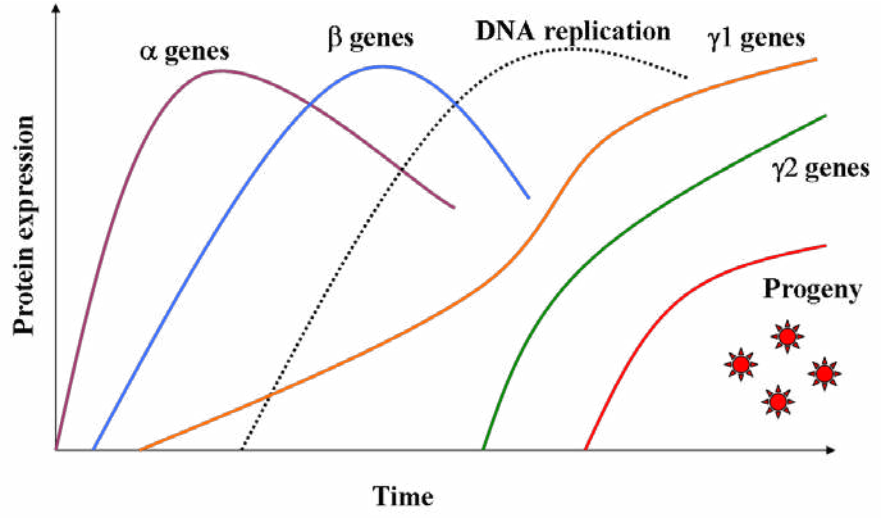

Figure 1-3. HSV-1 lytic gene expression kinetics.

The general pattern of gene expression during HSV-1 lytic replication is shown. The first genes to be expressed are the α genes (purple) followed by the genes (blue). Accumulation of β proteins signifies the beginning of DNA replication (dotted line) following which the γ genes can be expressed. The γ genes are split into two categories, γ1 (orange) and γ2 (green). γ1 genes can be made prior to DNA replication in small amounts while γ2 genes require DNA synthesis before they can be expressed. Once all protein classes have accumulated, progeny virus (red) can be assembled and released from the infected cell.

Immediate early gene transcription begins soon after viral entry and IE proteins expression peaks around 2 to 4 hours post infection (h.p.i). There are five IE proteins encoded by HSV-1 and they are ICP0, ICP4, ICP22, ICP27 and, ICP47. All the IE proteins besides ICP47 can regulate gene expression and functional IE proteins are essential for the transcription of other gene classes. ICP0, is an E3 ubiquitin ligase encoded by HSV-1 and while it is not essential to viral growth, it is important for optimal replication especially in low multiplicity infections and efficient reactivation from latency (49). ICP0 is also involved in counteracting the host defense against viral infection, including suppressing the interferon response and degradation of various cellular proteins (35, 38, 91, 107-108). One of the most important and extensively studied IE proteins is ICP4. ICP4 is indispensable to HSV-1 replication and is required for the activation of both early and late genes (180). ICP4 can also function to repress the expression of some viral genes including its own gene (32, 119). A role for IE protein ICP22 has also been implicated in altering the localization of cellular proteins into distinct nuclear foci containing ubiquitinated proteins and proteasomal components. (6). ICP27 is another IE protein that is indispensable for viral replication. Many important functions have been attributed to ICP27 including its involvement in the switch from early to late gene expression, inhibition of mRNA splicing, RNA binding, and transport of intronless viral mRNA out of the nucleus (182). Finally, ICP47 has been shown to be involved in immune evasion of the virus from the host immune response by downregulating antigen presentation through major histocompatibility complex I (MHC class I) expression by blocking the transporter associated with antigen processing (TAP) protein function (54, 118).

The early or β genes are expressed following the accumulation of α proteins and their expression peaks around 5 to 7 h.p.i. Early genes encode proteins that are responsible for DNA replication including thymidine kinase (tk), DNA polymerase, ribonucleotide reductase and

single stranded DNA binding protein. Expression of early proteins results in the onset of DNA replication following which the γ genes can be expressed. Late gene expression peaks typically between 8 and 12 h.p.i, although some proteins can be detected much earlier during infection. The γ genes can be separated into two subclasses, γ 1 and γ 2. Typical members of the γ 1 gene class such as glycoproteins B and D (gB and gD) are expressed fairly early during infection and their expression is only moderately affected by drugs inhibiting DNA replication. γ 2 genes such as gC and gH are expressed only following DNA replication and their expression is abrogated in the presence of DNA replication inhibitors. The late genes encode mainly structural proteins that are required for assembly of fully functional progeny virus (135).

1.3.2.2 Regulation of gene expression

The temporal regulation of HSV-1 gene expression is dictated largely by the different promoter composition for each gene class. The incoming virus is dependent on a tegument associated protein, VP16 to initiate transcription of IE genes. The VP16 that is brought in with the virion binds to two cellular proteins, HCF and Oct1, which together form the VP16-induced complex. This complex is able to bind to the TAATGARAT sequence uniquely present within the upstream region of each IE promoter to initiate gene transcription. IE gene expression is also further regulated by several *cis*-acting regulatory elements including the presence of several binding sites for a cellular transcription factor SP1, TATA box and in some IE genes, a binding site for ICP4 which can repress gene expression (Fig. 1-4 A).

Early gene promoters do not have elements required for VP16 binding; their promoters contain only sites for SP1 binding and TATA and CAAT elements (Fig. 1-4 B). While early gene promoters appear to have all the *cis*-acting elements required for transcription, they are not transcribed until IE genes have been made. Studies of the early *tk* gene promoter have shown that

functional ICP4 is required for transactivation of this gene (62). While there are no particular features of the TATA element that are specific to early genes, the strength of ICP4 binding to the TATA box seems to confer expression levels rather than kinetics (25).

The onset of DNA replication appears to be the major switch from early to late gene expression. The late gene promoters also contain all the *cis*-acting elements needed for gene expression (Fig. 1-4C). However, late genes still require ICP4 interaction with the TATA elements present within the late promoters for their transcription. In contrast to IE and E genes, L genes also contain regulatory elements downstream of the TATA box including an initiator element at the start of the transcription site as well as a downstream activation site that is necessary for maximal expression (Fig. 1-4C). This feature of HSV-1 to temporally regulate various gene classes by virtue of promoter composition differences makes it possible to generate a variety of recombinant viruses in which either viral or non-virally encoded proteins can be driven from an HSV-1 promoter from any gene class (31).

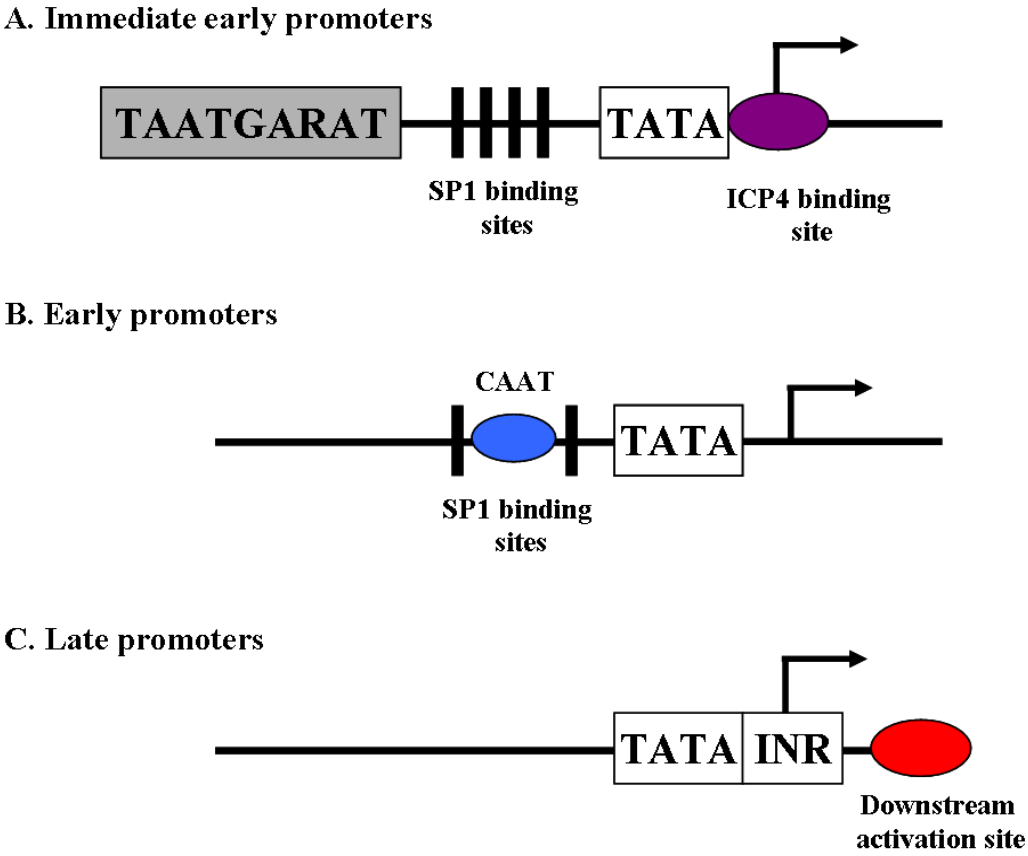

Figure 1-4. Schematic of HSV-1 promoter structures.

A schematic representation of the general arrangement and composition of *cis*-acting regulatory elements within HSV α, β, and γ promoters is shown. The actual number and arrangement of elements varies among promoters of each class. (A) In addition to the TATA element, IE promoters have TAATGARAT elements in the upstream region through which the viral VP16 protein mediates transcriptional activation. Binding sites for eukaryotic transcription factors such as SP1 are also present upstream from the TATA element, and in at least some IE promoters, binding sites for ICP4. (B) Early promoters have binding sites for eukaryotic transcription factors upstream of the TATA element but no identified *cis*-acting regulatory elements further downstream. (C) Late promoters have an initiator element (Inr) at the start of transcription, and in at least some late promoters, a downstream activation site. Figure is adapted from (182).

1.3.3 Latency

Following a productive infection within an epithelial surface, the virus can gain access to the termini of the sensory neurons that innervate the site of infection. This allows the virus to be transported via retrograde transport to the nerve body of these innervating neurons where it can establish latency. Ocular, nasal or oral infections result in the establishment of latency at the TG. During latency, the virus lies dormant within the sensory ganglia and no infectious virus is made.

Our knowledge of HSV-1 latency comes largely from work done in animal models including rabbits and mice. The rabbit model has been used to specifically study ocular infections and has many advantages. Rabbit eyes are similar in size to humans and many stressors that induce herpetic eye disease in rabbits are similar in humans (170). However, once latency has been established in the TG, infected rabbits can spontaneously reactivate and shed HSV-1 ocularly making the rabbit an unreliable model to study true latency in the absence of infectious virus (8, 55). Thus the mouse model is preferred to study HSV-1 latency as mice can be infected via many surfaces including the eye, skin, snout and footpad. Mice maintain latent virus in their sensory ganglia for indefinite periods of time and only very rarely does spontaneous reactivation lead to shedding of infectious virus (45, 99). Studies using the mouse model have significantly increased our understanding the factors that are responsible for and control viral latency.

Following an ocular infection of murine corneas, virus can access the nerve termini of the neurons that innervate the cornea. The virus can travel by retrograde axonal transport to the neuron body of these sensory neurons where it can be detected in the TG within 2 d.p.i (73). Viral proteins of all gene classes are expressed and there is some transient accumulation of IE and E transcripts before establishment of latency (44). HSV-1 and HSV-1 preferentially establish

latency in different neuronal subtypes. HSV-1 (100). While HSV-1 can infect both A5 and KH10 subtypes of neurons, latency occurs predominantly in A5 type neurons. It is thought that following infection, HSV-1 latency is immediately established within A5 fibers while infected KH10 fibers may become subject to death following infection. Upon the establishment of latency, viral gene expression is largely silenced with the exception of one set of transcripts that continues to be expressed and accumulate, known as the latency associated transcripts (LATs). The LATs are untranslated non poly A, non capped, RNA transcripts that are found abundantly in latently infected neurons in the ganglia and their expression coincides with the establishment of latency (73). While lytic viral replication does take place in the infected ganglia, it is not a necessary event preceding the establishment of latency (22, 144). LAT transcripts can be detected both in murine and human ganglia latently infected with HSV-1 (75, 158-159). The full length LAT is transcribed from the latency associated promoter (LAP) and this 8.3 kb transcript is spliced to form two stable 2.0 kb and 1.5 kb introns. These two LAT species accumulate to high abundance in the nuclei of latently infected neurons. No known protein species is encoded for the LAT and LAT functions are attributed to the transcripts themselves. These introns are directly anti-sense to one of the main HSV-1 lytic proteins, ICP0. One of the main functions of LAT is to enable HSV-1 to establish latency. Studies with viruses lacking LAT have been shown to establish latency less well compared to their wild type counterparts (169). Furthermore, LAT-null viruses also reactivate with reduced efficiency (83). LAT gene expression is consistent with reduced lytic gene expression and this seems to be a mechanism by which the virus promotes its own latent state. LAT-null viruses have been shown to cause more severe pathology and induce neuronal death compared to wild type viruses. Thus the function of LAT is to prevent apoptosis of the latently infected neuron and to enhance the establishment of latency (126, 168).

During latency, the viral genome circularizes and is maintained as an episome within the nucleus (Fig. 1-5) (63). Episomal HSV-1 DNA is highly associated with host nucleosomes and histones (33). HSV-1 latency is thought to be controlled through differential chromatin regulation of the histones. A study has shown that that LAT promoter and enhancer show increased levels of acetylated histone H3 compared to the ICP0 gene, which indicates that the LAT promoter is associated with euchromatin and thus maintained in an active state (77). In contrast, another study has shown that lytic promoters are progressively associated with heterochromatin and therefore in a repressed state (179). The LAT transcripts might be involved in promoting heterochromatin modifications on lytic promoters during the establishment of latency as LAT-null viruses show reduced levels of heterochromatin markers (71). CCTC-binding protein (CTCF), a protein which binds to all known vertebrate insulators elements on DNA, has been shown to have binding sites around the LAT promoter (2). CTCF binding to LAT promoter/enhancer elements may prevent the LAT enhancer from activating ICP0 and other lytic genes (2, 17). Furthermore, it has recently been shown that LATs can also exert their effect through and micro-RNA antisense mechanism. A study demonstrated that LAT encodes several distinct micro-RNA species, some of which show complementarity to ICP0 and ICP4 may function by repressing their expression and thereby maintaining latency (173).

1.3.4 Reactivation

Reactivation of HSV-1 occurs when the viral genome re-enters the lytic replication cycle from an extended period of latency and progeny virus is assembled and released from the latently infected neuron (Fig 1-5B). Clinically, reactivation can be symptomatic (detection of virus in the periphery and visible disease) or asymptomatic (detection of virus in the periphery in the absence

of visible disease). On a molecular level, spontaneous reactivation can occur, defined by limited gene expression in the absence of a productive viral infection. When the virus reactivates, capsids and envelope proteins seem to traffic separately, via an anterograde motion to the nerve termini where progeny virus is assembled and released from the reactivating neuron (153). Despite advances in our knowledge in understanding the factors that contribute to viral latency, the exact mechanism by which the virus reactivates is still unknown

Several stimuli have been demonstrated to induce reactivation in mice including exposure to UV-B, psychological stress, transient hyperthermia and treatment with chemical chromatin modulating compounds such as sodium butyrate or dexamethasone (24, 41, 117, 140, 177). These compounds either directly cause changes to the HSV-1 genome to promote transcription or cause dysfunction in the immune response which leads to reactivation. Physical neuronal injury following axotomy also results in viral reactivation (139). How transcriptional activation of HSV-1 genes occurs during reactivation is unknown but can take place in the absence of VP16, although not efficiently (167). The role of immediate early genes during the initiation of reactivation is also unclear. In one study, Tal-Singer and colleagues demonstrated that early gene transcripts could be detected before immediate early transcripts in a TG explant model (164). However, several other studies have demonstrated that recombinant HSV-1 lacking certain immediate early genes such as ICP0 or ICP4 reactivate poorly *in vivo* and express no other viral genes (49, 144). Once reactivation is initiated, DNA replication is necessary for full reactivation to take place as HSV-1 lacking the *tk* gene are unable to reactivate (22). So while IE genes may not be necessary to initiate reactivation, they may be needed for efficient transcription of other viral genes to promote amplification of reactivated virus. In the absence of VP16, cellular proteins have been suggested to compensate for its function to initiate reactivation (94, 164). Cellular stress due to reactivation stimuli causes upegulation of several host transcription factors

including *c-fos, c-jun, c-myc*, and interferon regulatory factor-1 (IRF-1) (81, 164-165). Cellular stress also upregulates cytokines such as IL-6 can regulate LAT and ICP0 genes and lead to reactivation (76). Chromatin remodeling around the LAT region and surrounding lytic genes occurs in response to reactivation stimuli, allowing eurchromatin formation to enhance transcription. Stressors can also cause the loss of CTCF occupancy on LAT promoter elements, thus allowing the LAT enhancer to activate lytic proteins, resulting in reactivation (116).

In the murine model, HSV-1 reactivation events are usually restricted to very few neurons within the infected ganglia (139). The rate of reactivation is directly proportional to the number of genomes in the latently infected ganglia (60). Spontaneous reactivation in mice is rare but has been shown to occur. Transcripts and proteins of multiple kinetic classes have been detected in latently infected ganglia including ICP4 (α), thymidine kinase (β) and gC (γ2) (39). This expression occurs in the TG in the apparent absence of infectious virus production (39, 74). However, other studies have also indicated that in very rare cases, spontaneous reactivation leading to infectious virus in the TG can occur in certain mice strains (99). The host immunity at the site of latency plays an important role in maintaining the virus in a latent state. In most cases, these limited gene expression profiles may represent abortive attempts by the virus to reactivate which are suppressed by the host immunity before the production of infectious virus. However, there are some cases where the host immunity fails to block viral reactivation thus allowing the production of infectious virus and recurrent disease.

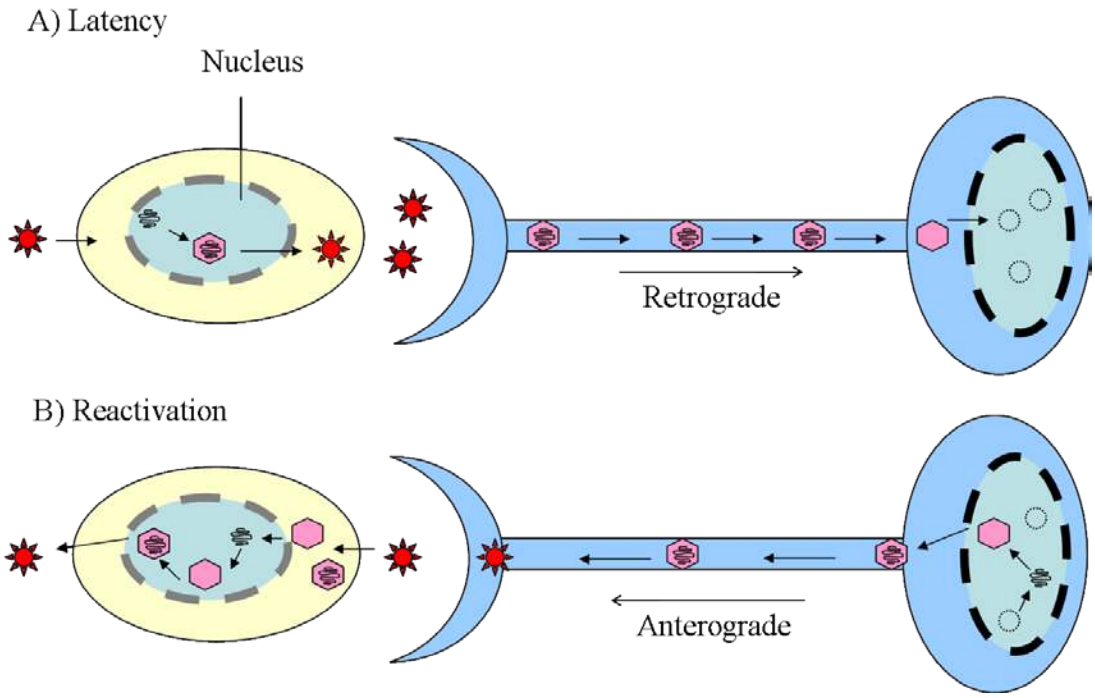

Figure 1-5. Latent and reactivation stages of HSV-1 infection

A) Infection of epithelial cells results in a productive infection whereby progeny virus can access the nerve termini of sensory neurons innervating the epithelial surface. The virus enters the nerve termini and nucleocapsids are transported to the neuronal cell body via retrograde axonal transport. The viral DNA is released into the nucleus and circularizes and persists in this latent state for an indefinite period of time. B) When the virus reactivates, newly formed nucleocapsids are transported in an anterograde fashion and infectious virus is released from the axon whereby it can now infect surrounding epithelial cells, resulting in recurrent infection and disease. Figure is adapted from (71).

1.4 IMMUNITY TO HSV-1

1.4.1 Innate Immune control of HSV-1

Corneal infection with HSV elicits cellular immune responses at the site of infection. Initial control of infection is mediated in part by type I interferons, namely IFN-α and IFN-β, produced by the infected tissue (97). These cytokines attract other cells such as neutrophils, macrophages, and natural killer (NK) cells (184). Neutrophils produce several antiviral molecules such as nitric oxide (NO), tumor necrosis factor α (TNF-α), IFNs and interleukin (IL)-12 (27, 65). IL-12 secretion is thought to activate NK cells that help control infection by producing the type II interferon, IFNγ (34). Even in the face of a robust immune infiltrate in the cornea, the virus is still able to access the nerve termini that innervate the cornea and infect the TG. In the mouse ocular infection model, HSV-1 reaches the TG by retrograde transport within two days of infection where it begins the limited phase of replication (89, 149). Viral replication initiates the infiltration of macrophages and γδ T cell receptor + (γδ TCR^+) cells (89, 150). The macrophages produce NO and TNF-α, and during the first 5 days after corneal infection of HSV-1, experimental depletion of macrophages, blocking NO production, or neutralizing TNF-α all significantly increase ganglionic viral titers ganglionic genome load, and the number of latently infected neurons in the TG (72). The γδ TCR^+ T cells exert their effect by producing IFNγ; experimentally depleting these cells from mice within the first 7 days increases viral titer and spread (72, 143). Thus, innate immune mechanisms appear also to be involved in the reduction of virus from the TG during the early stages of viral infection in the TG before the adaptive immune response comes into play.

1.4.2 Adaptive Immunity to HSV-1

The adaptive immune response follows the innate response and is not only responsible for initial clearance of virus from both the periphery and the ganglion but also to maintain the virus in a latent state. The adaptive immune response is characterized by inflammation of infected tissue by $CD4^+$ T cells, producing antiviral cytokines. $CD4^+$ T cell knockout mice are more susceptible to HSV infection than wild type mice (34, 78, 98). Furthermore, early $CD4^+$ T cell help is required for the generation of functional $CD8^+$ T cells in the TG (40). $CD8^+$ T cells also clear virus via cytokine and lytic granule production (31, 70, 88). The role of antibodies in limiting initial HSV infection remains unclear. No definitive correlation between levels of antibodies and their ability to limit the severity of HSV infection has been made. However, a significant repertoire of neutralizing and non-neutralizing antibodies are generated, particularly to surface viral antigens and the principal viral antigen targets of neutralizing antibodies in mice are glycoproteins, gB, gH and gD (14, 92, 123).

1.4.2.1 $CD8^+$ T cell control of viral latency

Following ocular HSV-1 infection of mice, virus replicates in the cornea and infectious virus can be detected in the tear film by 1 d.p.i. The virus reaches the TG by 2 d.p.i and peak of viral replication is observed by 4 d.p.i after which virus titers decline till 8 d.p.i, when no infectious virus can be detected. A $CD8^+$ T cell infiltrate ensues which can be observed beginning at 5 d.p.i (145). Initial activation and expansion of $CD8^+$ T cells occurs in the draining lymph nodes (DLN). Professional antigen presenting cells (APC) such as dendritic cells (DC) pick up HSV

antigens in the cornea and migrate to the DLN where LN resident DC's can cross present these viral antigens to naïve $CD8^+$ T cells. Evidence from the murine HSV-1 skin infection model suggests that migratory DCs are able to prime naïve $CD8^+$ T cells through cross-presentation of viral antigens (7). Viral proteins present within DCs are processed through the proteosomal pathway into short peptide fragments of approximately 8-10 amino acids in length (69, 93, 157). Presentation of these peptides on MHC Class I on DCs, along with proper costimulation can activate and expand $CD8^+$ T cells whose TCR can specifically recognize a particular viral peptide (90).

Following activation and expansion, virus-specific $CD8^+$ T cells migrate out of the DLN and into the cornea (site of primary infection), the TG (site of latency) and also other organs and tissues. In the TG of C57bl/6 mice, $CD8^+$ T cell numbers reach their peak at approximately 8 d.p.i, which coincides with the establishment of viral latency. These $CD8^+$ T cells represent the effector phase of the immune response in the TG. This population then undergoes a contraction phase during which 80-90% of the $CD8^+$ T cells are lost from the ganglia starting at around 14 d.p.i till 30 d.p.i. However, a significant population of these cells is still retained in the TG after 34 d.p.i for the life of the animal (memory population) (67, 89, 150, 174). Persistent pools of memory $CD8^+$ T cells are also retained in other tissue besides the TG, such as the lungs and spleens (146). In the mouse, all the $CD8^+$ T cells retained in the TG appear to be virus-specific (145). This retention of $CD8^+$ T cells is also evidenced in human ganglia, where latently infected neurons are surrounded by $CD8^+$ T cells (166, 175). $CD8^+$ T cells force viral latency following the innate response through of both IFNγ production and lytic granule release (70, 89). Actively replicating virus is eventually cleared from the ganglion, leaving only latent viral genomes. The persistent infiltrate of $CD8^+$ T cells in the TG maintain HSV-1 in a latent state. Depletion of $CD8^+$ T cells through direct administration of CD8 antibody *in vivo* or through

immunosuppresion of mice through psychological stress results in reactivation of latent virus as determined by an increase in genome copies (41). In *ex vivo* cultures of dissociated latently infected TG, $CD8^+$ T cells can block reactivation and their depletion also leads to reactivation as evidenced by production of live virus (88).

Using the C57BL/6 mouse model of HSV-1, we have gained an extensive picture on some of the mechanisms that CD8 T cells use to control reactivation. In this mouse strain, a majority of the virus-specific $CD8^+$ T cells (gB-CD8) are specific to a single epitope (amino acids 498-505) on glycoprotein B ($gB_{498-505}$). By using MHC Class I tetramers that bind to the TCR, it has been shown that gB-CD8 constitute up to 70% of the total CD8 T cell infiltrate within latently infected ganglia (67, 178). Classically, gB has been defined as a γ1 regulated gene, expressed with leaky-late kinetics. However recent studies demonstrate that gB-CD8 can recognize infected cell targets within 2 hrs of infection indicating that gB is made very early in infection and reactivation (112). gB-CD8 are able to completely block reactivation in *ex vivo* latently infected TG cultures (70)

$CD8^+$ T cells employ two main effector functions to block reactivation namely IFNγ and lytic granules. IFNγ exerts its effect through several mechanisms which include the upregulation of proteins involved in antigen presentation, antiviral proteins involved in inhibiting viral translation and pro-apoptotic proteins (68, 142, 151, 186). IFNγ can be detected constantly within a latently infected TG (15, 48) and when added alone, it is able to block reactivation in *ex vivo* cultures of latently infected TG (31, 87). More importantly, recent work suggests that IFNγ is able to block even after late gene (gC) expression, indicating that it can act very late in the reactivation process and may block assembly or viral transport down the axon (31). While mice lacking either IFNγ or its receptor are impaired in their initial ability to clear live virus at the site of infection, the virus is nevertheless driven to latency in these mice indicating that IFNγ is not

the only antiviral mechanism employed by CD8 T cells (11, 13, 84). $CD8^+$ T cells contain preformed lytic granules composed several proteins including perforin, granulysin, Fas ligand, and granzymes which are cytotoxic to infected cells (5, 47, 127). Perforin is a calcium dependent pore forming cytolytic protein whose function is necessary for cytolytic activity of $CD8^+$ T cells (64, 102, 128). Granzymes are serine proteases which can cleave both intracellular and extracellular substrates (10). Granzyme B is one of the most extensively studied granzyme and is responsible for the rapid induction of caspase-dependent apoptosis (171). Perforin and granzyme B are essential for $CD8^+$ T cell mediated block of HSV-1 reactivation (70). Mice lacking in either perforin or granzyme B can clear an HSV-1 infection but an increased number of latent genomes are retained in the TG of these mice compared to their wild type counterparts. (70). Furthermore, gB-CD8 lacking either one of these components are impaired in their ability to block reactivation compared to wild type gB-CD8 (70). The most important facet of CD8 T mediated control of viral reactivation is that they are able to do so without causing neuronal destruction (70, 88). In an *ex vivo* study, when $CD8^+$ T cells capable of blocking reactivation were depleted at 5 days post culture initiation, the cultures reactivated, indicating that the neuronal viability was maintained in the presence of $CD8^+$ T cells. Furthermore, $CD8^+$ T cells releasing lytic granules were able to induce the activation of caspases and apoptosis of infected fibroblasts but a similar phenomenon was not observed when $CD8^+$ T cells were cultured along with infected neurons (70). Recent evidence shows that this non-cytolytic activity may be regulated by inhibitor molecules on the surface of neurons, thus protecting them from cytotoxic T-cell mediated killing (162).

While HSV-1 latency has classically been considered to be an antigenically silent state, there is new evidence to show the contrary. In latently infected TG, gB-CD8 polarize their TCR toward the surface of neurons, forming an immunological synapse and release IFNγ and lytic

granules into this synapse (67, 70). The levels of MHC Class I (MHC-I) expressed by latently infected neurons has been difficult to quantify. MHC-I molecules can be readily detected during lytic replication of HSV-1, but their expression becomes undetectable once the virus establishes latency (124-125). However, the presence of $CD8^+$ T cells surrounding latently infected neurons is highly suggestive of MHC-I expression during latency. Furthermore, as mentioned previously, transcripts of lytic genes can be detected within latently infected TG which may represent abortive attempts by the virus to reactivate. Since $CD8^+$ T cells require very low levels epitope expression (as low as one MHC-I/peptide complex) to be stimulated (129, 163), low levels of protein expression during attempted reactivation could be sufficient to stimulate the TG-resident $CD8^+$ T cells to block reactivation. Other evidence pointing to antigen exposure during latency is that TG-resident $CD8^+$ T express high levels of intracellular granzyme B, a marker of activation (67, 145) and IFNγ can be consistently detected in latently infected tissue (67, 80). Furthermore, the $CD8^+$ T cells in the TG are less dependent on homeostatic proliferation signals compared to their counterparts in non-infected tissue consistent with the idea that they are persistently exposed to antigen in the TG (146). Taken together, these studies show that $CD8^+$ T cells are able to provide immunosurveillance by recognizing low levels of antigen and are able to block reactivation before infectious virus production.

1.5 POTENTIAL VACCINES FOR HSV-1

HSV-1 vaccine strategies have been divided into two types: prophylactic and therapeutic. Prophylactic vaccines are aimed at preventing initial infection and must theoretically elicit a sterilizing immunity through a strong antibody response so that no infection or establishment of latency occurs. The concept behind therapeutic vaccines is that it would augment the already existing immunity towards HSV and prevent reactivation. Both prophylactic and therapeutic vaccine designs have focused around viral glycoproteins such as gB and gD, two essential viral proteins. Vaccine strategies include the use of protein subunits, peptides, live virus or DNA vaccines.

While a few prophylactic vaccines have been effective in preventing disease in animal models, the subunit and live attenuated vaccines that have made it to human clinical trials stage did not achieve the level of success to deem these vaccines safe and efficacious (12, 26, 50, 103, 105, 155-156, 181, 189). Several key factors have hindered HSV-1 vaccine development. The first is that natural immunity itself does not prevent infection or disease. It has been well established that prior infection does not prevent subsequent infection or reactivation and the shedding or spread of reactivating virus. Thus such it seems highly unlikely that sterilizing immunity can be feasibly generated. Second, latency occurs quickly and does not require viral replication. Again, it seems highly unlikely that any vaccine will completely prevent the establishment of latency, although it is likely to limit the efficiency by which latency is established. The third factor affecting vaccination for HSV concerns vaccine safety and it is unlikely that a live virus vaccine will be deemed to have achieved a safety rating for widespread use.

Seeing that sterilizing immunity through prophylaxis may be impossible to achieve, it may be more feasible to design therapeutic vaccines to prevent HSV-1 reactivation. The theory behind therapeutic vaccines is to augment the already existing immunity to HSV-1. One such therapeutic vaccine is already effective in preventing reactivation of another alphaherpesvirus, Varicella Zoster Virus (VZV), which cause shingles (121). The shingles vaccine is thought to provide a boost to the already existing immunity to VZV and prevent viral reactivation. Thus far, some HSV-1 subunit and live virus therapeutic vaccines have been tested in human trials with only minimal success (29, 160-161). It is possible that a more potent method of vaccine delivery is needed to improve the efficacy of therapeutic vaccines. Also, a further understanding of how a cellular immune response to HSV-1 is developed during a primary infection and boosted during a secondary challenge is also necessary.

Glycoprotein B has been shown to induce a cellular immune response in both humans and animals (19, 67). In the C57bl/6 model, the strong $CD8^+$ T cell response to this antigen makes it one that can be easily monitored in our mouse model of latency (67). Furthermore, gB is an essential protein that is involved in many aspects of the viral life cycle and any changes to the protein could potentially result in drastic changes in viral replication (52, 113, 185). Understanding how gB contributes to the pathogenecity of the virus could also help us design better and safer vaccines. For these reasons, we can use gB as a model antigen in our system both to understand viral replication and the development of HSV-specific $CD8^+$ T cell responses.

2.0 SPECIFIC AIMS

2.1 RATIONALE

Latent HSV-1 infection of the TG is associated with a persistent virus-specific $CD8^+$ T cell response that can block reactivation. However, despite constant immunosurveillance, HSV-1 can reactivate and cause recurrent disease. An understanding of how HSV-specific $CD8^+$ T cells are maintained in the TG and block reactivation is needed to design an effective therapeutic vaccine for HSV-1. In C57BL/6 mice, half of the virus-specific $CD8^+$ T cells are directed toward an immunodominant epitope on gB (gB-CD8; residues 498-405), a protein expressed early in infection. In the TG, gB-CD8 polarize their TCR toward latently infected neurons and express markers of activation, consistent with persistent exposure to viral antigen. Early expression of gB may be necessary for gB-CD8 to recognize their cognate antigen early during reactivation and thus quickly revert HSV-1 back into a latent state. **The central hypothesis of this thesis work is that early and periodic viral antigen expression during latency influences the gB-CD8 response in the TG.** The specific aims set forth in this thesis are designed to understand whether HSV-1 protein expression can be detected during latency and also how the kinetics of gB expression during lytic replication and reactivation influences the development and maintenance of gB-CD8 in the TG. Furthermore, as a vaccine strategy we intend to determine whether persistent overexpression of the $gB_{498\text{-}505}$ epitope during latency, can induce a greater gB-CD8 response in the TG.

2.2 SPECIFIC AIM 1

To determine whether early and late lytic genes are expressed during latency in the absence of infectious virus.

Hypothesis: Viral antigens are expressed during abortive reactivation events in the absence of infectious virus during latency.

The observation that gB-CD8 within the latently infected TG are persistently activated indicates that low level viral gB expression is recognized by these $CD8^+$ T cells which then act to block reactivation before infectious virus production. In this aim we will design and construct a recombinant HSV-1 containing two fluorescent proteins expressed under lytic promoters. In this virus (RE-pgB-EGFP/pgCRFP), EGFP expression is controlled by the γ1 gB promoter and RFP expression is controlled by the γ2 gC promoter. This allows us to differentiate early gene expression from that occurring beyond DNA replication. We will use this virus to visualize promoter activity during latency and reactivation as well as to determine if lytic replication kinetics are maintained during reactivation.

2.3 SPECIFIC AIM 2

To determine the importance of the kinetics of gB expression for the development and maintenance of a gB-CD8 response in the TG and for the ability of gB-CD8 to block reactivation.

Hypothesis: Low level gB expression during latency is necessary to maintain gB-CD8 in the TG and gB-CD8 T cells need to recognize their antigen early to block reactivation before DNA replication.

During lytic viral infection, gB is expressed as γ1 protein and its expression is mildly sensitive to DNA replication inhibitors. Following infection, gB-CD8 can recognize infected cell targets within two hours of infection, indicating that *de novo* gB expression occurs fairly early in infection. Recognition of low level gB expression soon after initiation of reactivation may be responsible for maintaining high numbers of gB-CD8 in an activated state within the TG. The expression profile of gB may also have consequences during reactivation as early expression may induce a rapid $CD8^+$ T cell response to block reactivation. For this aim, will design and construct a recombinant HSV-1 in which gB is expressed as a γ2 protein under the control of the gC promoter. This eliminates low level gB expression prior to DNA replication. We will use this virus to explore the importance of the appropriate kinetics of gB expression for the development and maintenance of gB-CD8 in the TG and their ability to block reactivation *ex vivo*.

2.4 SPECIFIC AIM 3

To determine whether persistent overexpression of the $gB_{498-505}$ epitope during latency will induce a greater gB-CD8 response in the TG.

Hypothesis: A larger $CD8^+$ T cell infiltrate within the latently infected TG is necessary to block reactivation.

Despite constant immunosurveillance of latently infected TG, HSV-1 is able to reactivate following stimuli that can cause a loss or function in TG-resident $CD8^+$ T cells. A therapeutic vaccine aimed at boosting the immune response in the TG would increase virus-specific $CD8^+$ T cell numbers such that even following a reactivation stimuli, there are still enough functional $CD8^+$ T cells to block reactivation. To test this aim, we will design and generate recombinant HSV-1 in which the $gB_{498-505}$ epitope is expressed as tandem repeats under various viral promoters. We will use these viruses to determine whether overexpression of this epitope generates a greater gB-CD8 response in the TG and whether they are functionally capable of blocking reactivation.

3.0 GENERAL MATERIALS AND METHODS

3.1.1 Cells and viruses

Vero cells (ATCC, Manassas, Virginia), B6WT3 cells (ATCC), gB-Vero (Vero cells stably transfected with a plasmid expressing gB from the native gB promoter; a kind gift of William Goins of the University of Pittsburgh) were grown in Dulbecco's modified Eagle's medium (DMEM) and primary corneal fibroblasts (PCF) were grown as previously described (37) in minimal essential medium (MEM) 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 viruses.

3.1.2 Multistep *in vitro* growth kinetics

Vero or PCF cells were infected with different HSV-1 viruses at an MOI of 0.01 PFU/cell, and incubated as stated in each study for different times. 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.

3.1.3 Analysis of HSV-1 protein expression

Subconfluent Vero cell monolayers were infected with the indicated viruses for each study at an MOI of 10 PFU/cell for 1 h at room temperature (25 °C). Infections were performed in media lacking or containing 150 μg/ml of cycloheximide (CHX; Sigma-Aldrich, St. Louis, MO) or 350 μg/ml phosphonoacetic acid (PAA; Lancaster Synthesis, Pelham, NH) depending on the study. Following media replacement under the same conditions, the monolayers were incubated at 37 °C. CHX-reversal studies were performed at 6 h post-media change as previously detailed (58). Briefly, cells were washed three times with media lacking cycloheximide but containing 15 μg/ml actinomycin-D (AD; Sigma-Aldrich). Cells were incubated for further 2 h in media with AD prior to harvesting. Infected cells incubated in media buffered PAA to block DNA replication, were harvested at either 12 or 24h post-infection. Cells were harvested by washing in ice-cold PBS and the proteins solubilized in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) buffer, boiled, and separated by SDS PAGE. Proteins were detected by staining, or were transferred to Immobilon-P membranes (Millipore, Billerica, MA) for immunoblot analyses. Antibodies to EGFP (Affinity Bioreagents, Golden, CO), RFP (Becton-Dickinson, Franklin Lakes, NJ), gB and gC (Virusys) were detected using horseradish peroxidase and West Dura chemiluminescent reagents (Pierce Biotechnology Inc., Rockford, IL), as detailed previously (36).

3.1.4 Mice and ocular infections

Three to five-week old female wild type C57BL/6 (Jackson Laboratories, Bar Harbor, ME) were anesthetized by the intraperitoneal injection of 2.0 mg of ketamine hydrochloride and 0.04 mg of

xylazine (Phoenix Scientific, St. Joseph, MO) in 0.2 ml of Hanks buffered salt solution (BioWhittaker, Walkersville, MD). Both corneas were scarified and infected topically with purified HSV-1 viruses. Unless otherwise indicated, the infectious dose used was at 1×10^5 PFU/eye in 3 µl of RPMI (BioWhittaker). All animal studies were carried out under University of Pittsburgh IACUC approved protocols in AALAAC approved facilities and in accordance with the ethical treatment of animals, as defined by the ARVO Statement for the Use of Animals in Ophthalmic and Vision research.

3.1.5 Viral replication and spread *in vivo*

Viral replication in the eye was determined in tear film samples obtained using a foam-tipped applicator. The foam tips were transferred to DMEM, vortexed and virus titrated on Vero cells by a standard plaque assay. To determine viral titers in the ganglia, TG were surgically excised at 4, 5 or 8 days post-infection (d.p.i) from euthanized animals, homogenized using trituration and subjected to three freeze–thaw cycles prior to titration on Vero cells.

3.1.6 Single-cell tissue and sample preparation

At the indicated times after infection, mice were euthanized by exsanguination. TG and draining lymph nodes (DLN) were excised. TG were resuspended in DMEM containing 10% FBS and 400 U/ml collagenase Type 1 (Sigma-Aldrich) per TG for 1 h at 37 °C. Cells were then dissociated into single-cell suspensions by trituration. Single-cell TG suspensions were passed through a 40 µm filter-cap tube (BD Pharmingen) to remove debris and stained for flow cytometric analysis as described below. Draining lymph nodes (DLN) were excised and

mechanically dispersed with the use of a nylon filter and treated with red blood cell lysis buffer (0.16M NH_4Cl, 0.17M Tris in dH_2O, pH 7.2) prior to staining. Single-cell TG or DLN suspensions were stained for flow cytometric analysis as described below.

3.1.7 Flow cytometry

Fluorochrome-conjugated Abs against CD8α (clone 53-6.7), CD45 (clone 30-F11) and IFN-γ (clone XMG1.2), and the proper isotype control Abs were purchased from BD Pharmingen (San Diego, CA). Antibodies against granzyme-B (clone GB11) and the respective isotype control were purchased from Caltag. PE-conjugated H-$2K^b$ dimers (BD Pharmingen) were incubated with SSIEFARL peptide at 37°C overnight prior to use to identify the H-$2K^b$-restricted HSV-1 $gB_{498–505}$-specific $CD8^+$ T cell population. Cells prepared for flow cytometric analysis as described above 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. Cells were then fixed with 1% paraformaldehyde (PFA) and resuspended in FACS bufferFor intracellular stainining the BD Cytofix/Cytoperm kit with GolgiPlug™ was used following manufacturer's protocol. Briefly, following surface staining, cells were permeabilized with Cytofix/Cytoperm solution for 20 min on ice. Intracellular Abs were diluted in 1X BD Perm/Wash solution and incubated with samples for 30 min on ice. 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).

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. Briefly, In short, collagenase-treated TG were resuspended in 200 µl PBS per sample, then treated with 20 µl proteinase K and 200 µl Buffer AL, mixed thoroughly, and incubated 10 min at 70ºC on a heating block. Samples were then treated with 200 µl 100% EtOH and transferred to a mini-spin column and centrifuged. The columns were treated with Buffer AW1 and Buffer AW2, with spins between each treatment, and finally, samples were eluted in 100 µl Nuclease-Free H_2O (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 to a 1 ng/ml concentration in nuclease-free H_2O.

25 ng of DNA or water control was mixed in duplicate with 25 µl of TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ) and an 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 (50 µl/well) were assayed in 96-well plates with an ABI Prism 7700 sequence detector. ABI Primer Express v1.5a software default settings were used for instrument control and data analysis. The gH sequences were: forward primer (5'-GACCACCAGAAAACCCTCTTT-3'), reverse primer (5'ACGCTCTCGTCTAGATCAAAGC-3'), and probe [5'-(FAM)TCCGGACCATTTTC(NFQ)-3']. The 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 $CD8^+$ T cell stimulation assay

For this assay, $CD8^+$ T cells were used directly from infected TG or purified $gB_{498-505}$-specific $CD8^+$ T cells were used. At 8 d.p.i, TG were excised and dissociated into single-cell suspensions by treatment with collagenase type I as just described. One-half TG equivalent of cells were then combined and with 5×10^5 B6WT3 cells 18 h following transfection with plasmids specific to each study. For chapter 4, 8 d.p.i. infected TG were used and the conditions represented transfected cells expressing EGFP (EGFP-N1), RFP (mRFP-1), glycoprotein B (pPEP98) or untransfected cells. For chapter 6 purified gB-CD8 were used and the conditions involved cells transfected with pPEP98 plasmid, EGFP-N1 or plasmids expressing gB epitope mutations from the CMV promoter: gB-WT, gB-L8A, gB-F5L, gB-S1L, gB-S1G, gB-S1G/L8A, gB-S1G/I3A, gB-L8A/R7K and gB-S1G/I3N/F5L/E4S or mock transfected. Incubation was performed in the presence of GolgiPlug™ (BD Biosciences). Following a 6 h stimulation at 37 °C, cells were fixed with 4% paraformaldehyde and surface stained using fluorochrome-conjugated antibodies against CD8α and CD45, as described above. The cells were then permeabilized using Cytofix/Cytoperm (BD) and stained for intracellular IFN-γ prior to flow cytometric analysis.

3.1.10 *Ex vivo* TG cultures

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 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 (2X 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 DEVELOPMENT AND PATHOGENIC EVALUATION OF RECOMBINANT HERPES SIMPLEX VIRUS TYPE 1 EXPRESSING TWO FLUORESCENT REPORTER GENES FROM DIFFERENT LYTIC PROMOTERS.

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4.1 ABSTRACT

To develop means to explore viral gene expression in ganglia without laborious histological sectioning and staining, we created a two color fluorescent recombinant HSV-1, in which enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) are expressed from the glycoprotein B (gB) and glycoprotein C (gC) promoters respectively. We show that this virus retained growth and pathogenic capacity both *in vitro* and *in vivo* compared to wild type HSV-1; established latent infections with similar genome copy number in trigeminal ganglia (TG); induced a similar HSV-specific $CD8^+$ T cell infiltrate; did not induce $CD8^+$ T cells reactive to EGFP or RFP; and reactivated from latency with normal kinetics in *ex vivo* TG cultures. Fluorescent EGFP expression in plaques surrounding neurons preceded RFP expression and provided highly sensitive detection of reactivation and different stages of infection in *ex vivo* TG cultures. Expression of both EGFP and RFP in neurons was readily detectable in whole mounts of TG excised during acute infection and following *invivo* sodium butyrate-induced reactivation from latency. This virus constitutes a useful reagent for monitoring lytic viral promoter activity in sensory neurons *in vivo* and *in vitro*.

4.2 INTRODUCTION

Herpes simplex virus type-1 (HSV-1) adopts two modes of infection in humans and in animal models: a productive lytic infection and a latent infection in which the viral genome can persist for prolonged periods without virion formation. Lytic infections occur in most non-neuronal cell types and involve the coordinated and highly regulated temporal expression of approximately 80 viral genes to yield progeny virus. Temporal phases of viral gene expression can be experimentally defined into at least four classes. The immediate early (IE or α), genes are transcribed first without requiring *de novo* protein synthesis. The transcription of early (E or β) and late (L or γ) genes requires the expression of functional α proteins. Maximal β gene transcription rates occur prior to viral DNA replication, whereas γ gene transcription peaks only after DNA replication has initiated. The γ genes are sub-classified into γ1 and γ2; γ1 gene expression can occur prior to viral DNA synthesis, while γ2 gene expression absolutely requires initiation of viral DNA synthesis (58). The γ1 genes include glycoprotein gB, an essential glycoprotein which is an important immunogenic target of adaptive immunity in humans and animal models (67, 187). However, recent reports have detected gB expression at very early stages of viral infection (112).

In the well-characterized murine animal model of HSV-1 corneal infection following scarification, virus undergoes a short productive replication in the corneal epithelium that enables access of infectious virions to the termini of sensory neurons that innervate the cornea. Virions travel by retrograde axonal transport to the neuronal nuclei within the ophthalmic branch of the trigeminal ganglion (TG), where viral genomes enter a latent state. Productive infection in the TG is limited by innate immunity, may amplify the viral load and permit lateral spread to surrounding neurons (72, 101). Latency in neurons is accompanied by silencing of most lytic

protein expression and restriction of most transcription, except that from the non- protein-encoding latency-associated transcripts (LATs) (30, 131, 159). Silencing lytic gene expression is thought to involve epigenetic mechanisms, as the latent circular episomal viral genome becomes histone-associated (33, 106). Latency enables HSV-1 to coexist with the host for prolonged periods with the capacity to intermittently reactivate from latency leading to cause recurrent disease and transmission to new host.

The once accepted paradigm that HSV latency as an antigenically silent state that is ignored by the host immune system has now changed to one in which sporadic antigenic expression occurs, permitting immune surveillance of latently infected neurons (31). Like many other herpesviruses, HSV-1 expresses a limited array of lytic proteins in the context of a latent infection in the murine model, even though spontaneous viral reactivation from latency occurs rarely if ever (99). Both transcripts and proteins of multiple kinetic classes have been detected in latently infected ganglia. including infected cell protein 4 (ICP4- α), thymidine kinase (TK- β) and gC (γ2) (39) Expression occurs in the TG in the apparent absence of any infectious virus (39, 74). This either represents normal viral gene expression during rare aborted spontaneous reactivation events, or may represent isolated gene expression events outside of the accepted kinetics of the lytic cascade (164). Low or intermittent lytic protein expression during latency correlates with the retention and maintenance of an activated HSV-specific $CD8^+$ T cell population in latently infected TG in close apposition to latently infected neurons, in both mouse and human TG (61, 67, 166, 175). The activated phenotype of these cells is not shared by their counterparts in non-infected tissue such as the lungs or spleen (61, 67, 175), further supporting persistent or sporadic antigen expression and presentation in the context of MHC-I in neurons.

Several HSV-1 recombinants expressing reporter genes from viral promoters have been developed to detect viral lytic gene expression. These viruses have incorporated *Escherichia* coli

LacZ and firefly luciferase reporters to detect viral gene expression *in situ* (51, 96). Such reagents are also potentially useful for examining lytic gene expression during HSV-1 reactivation from latency *in situ* or in *ex vivo* ganglionic cultures. A significant disadvantage of these methods is that their detection requires substrate cofactors or extensive tissue processing. Moreover, the long term stability of β-galactosidase may errantly indicate persistent gene expression in a transcriptionally silenced environment (79). We previously employed a recombinant HSV-1 expressing EGFP, driven by either the ICP0 or gC promoter, to track viral gene expression during ocular infection and reactivation from latency in explanted TG (31). While providing detection of viral gene expression at the level of sensitivity of real time RT-PCR (31), these viruses were limited to detection of activity of a single viral promoter. Here, we describe the construction of a novel dual fluorescent reporter HSV- 1 recombinant that permits simultaneous detection of gene expression from two different kinetic classes of viral promoters within single infected cells including neurons.

4.3 MATERIALS AND METHODS

4.3.1 Construction of recombinant HSV-1 RE-pgB-EGFP/pgC-RFP.

The recombinant virus generated for this study RE-pgB-EGFP/pgC-RFP (pgB/pgC) was derived on the HSV-1 RE background and is a null mutant for the non-essential gC gene. Cloning was based on a previously detailed plasmid that contained the gC promoter driving expression of EGFP, followed by part of the gC ORF (31). This contained an NheI/XbaI fragment of EGFP obtained from the plasmid EGFP-N1 expressed in frame with the eighth amino acid of gC, but which is translationally stopped before the gC ORF. A 500 bp DNA fragment of the gB promoter was generated by PCR amplification from HSV-1 RE DNA using primers and the proofreading polymerase Expand (Roche, Indianapolis, IN) to amplify sequences from immediately upstream of the gB initiating ATG to aposition 500 bp upstream (Forward 5′-GGGATCCGTCGTGCCCCCCGTCAGGTAGC-3′ and Reverse, 5′-GGGAAGCTTTGACGAAGCGGTCGTTGGCCAGCCT-3′). The primers were designed to add unique HindIII and BamHI sites to the distal and proximal ends, respectively, to facilitate cloning into unique HindIII and BamHI sites inside the polylinker sequences immediately N-terminal to the EGFP gene in pgC-EGFP, to give pgC-pgB-EGFP. The second fluorescent gene, RFP was PCR amplified using mRFP-N1 with primers that added flanking NheI sites to facilitate in-frame cloning into the unique NheI site downstream of the gC promoter in the pgC-pgB-EGFP plasmid. The final plasmid, pgC-RFP/pgB-EGFP, contains the gC promoter driving mRFP followed by the gB promoter driving EGFP. HSV-1 RE infectious DNA was co-transfected with SspI linearized pgC-RFP/pgB-EGFP into Vero cells, using Lipofectamine 2000

(Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Homologous recombination between the flanking gC promoter and the gC coding sequences enabled insertion of the dual fluorescent cassette into the gC locus. Fluorescent progeny plaques were identified by microscopy under short-term exposure to a xenon lamp, with excitation narrowed to the peak range of excitation of RFP and subjected to several rounds of plaque purification until pure. The correct insertion of the cassette into the virus as well as genetic purity was confirmed by Southern blotting and by absence of expression of gC protein, using a commercial specific antibody to gC (Virusys, Skykesville, MD). Purified virus was obtained from Vero cells using OptiPrep® gradients, according to the manufacturer's instructions (Accurate Chemical and Scientific Corp., Westbury, NY). Purified virus was aliquoted, stored at − 80 °C and quantified on Vero cell monolayers.

4.3.2 Analysis of HSV-1 RNA transcription

Subconfluent Vero cell monolayers were infected with pgB/pgC at an MOI of 10 PFU/cell for 1 h at room temperature (25 °C) in normal media or media containing 350 µg/ml PAA. Following media replacement under the same conditions, the monolayers were incubated at 37 °C. Cells were harvested at 6, 8 and 12 h post-infection and whole cell RNA was extracted using the RNeasy Midi Kit (Qiagen, Valencia, CA). RNA samples containing formamide were run on a 1% agarose gel containing formaldehyde, and then transferred to positively charged nylon membrane (Genescreen, Perkin Elmer, Boston, MA). Identical blots were probed for EGFP using a 900 bp fragment from a NheI/BglII restriction digest of EGFP-C3 and for RFP, an 850 bp fragment from NheI/HindIII digest of RFP. Probes were labeled using random primer labeling extension with the incorporation using dCTP-[α-^{32}P] (3000 Ci/mmol: Perkin Elmer).

Autoradiographs exposed to be in the linear range of the film were then quantified using MetaMorph software.

4.3.3 *In vivo* induction of reactivation

Latently infected mice (33 d.p.i) were treated with a single intraperitoneal injection of 1200 mg/kg of sodium butyrate in PBS (final volume, 100 µl) (117). TG for confocal imaging were harvested 3 days post-injection as described below.

4.3.4 Confocal imaging of undissociated TG mounts

Mice were euthanized on days 4, 15, 35 or 36 post-infection by cardiac perfusion, using freshly made fixative containing sodium *m*-periodate (0.001 M), lysine (0.075 M) and paraformaldehyde (1%) in 0.1 M Phosphate buffer. TG were excised and further incubated overnight in the same fixative at 4 °C in round bottom 96-well plates. Fixed TG were then squash mounted onto glass slides with Aqua-mount and imaged using confocal microscopy (Olympus).

4.4 RESULTS

4.4.1 Development of a recombinant dual fluorescent HSV-1

Single fluorescent reporters driven by viral promoters in HSV-1 were detailed previously for the study of pathogenesis (3, 31) These enabled rapid assessment of viral gene expression and growth, but were limited to single viral temporal classes of promoters. We were interested in developing viruses capable of assessing gene expression at the single-cell level. We developed a virus in which the expression of two temporal classes of viral promoters could be simultaneously assessed using two fluorescent genes (Fig. 4-1). The virus was derived using a plasmid that contained a DNA fragment of the sequences upstream of the gB ATG. This plasmid was placed in the polylinker of pEGFP-N1 to drive EGFP expression in a manner similar to that used to derive HSV-1 in which the ICP0 or gC promoter controlled EGFP expression (Fig. 4-1B) (31). The plasmid also contained the RFP gene immediately downstream of the gC promoter but upstream of the gB promoter (Fig. 4-1C). Recombinant virus (pgB/pgC) was derived through homologous recombination between the flanking gC sequences and the gC gene in the HSV-1 RE genome to contain the insertion of the two genes in the non-essential gC gene, placing the RFP gene under the control of the native gC promoter at its native site and same direction (Fig. 4-1D). The RFP gene contained eight amino acids of gC fused to its N-terminus. EGFP expression is directed by the inserted upstream gB promoter sequences and is translationally stopped before the rest of the gC ORF. Thus gC is not expressed in this virus (data not shown). RNAs for both fluorescent genes are in the same direction as the native gC transcript and are presumably terminated by the native gC polyadenylation motif downstream. While the two RNAs share the same polyadenylation signal, this is a common occurrence in HSV-1. The

recombinant virus could readily be differentiated from wild type virus using fluorescence microscopy, and virus was isolated using excitation filters to illuminate RFP signals to minimize UV-induced DNA damage. Correct insertion and disruption of the gC gene was confirmed using Southern blotting and western blotting for gC expression (data not shown).

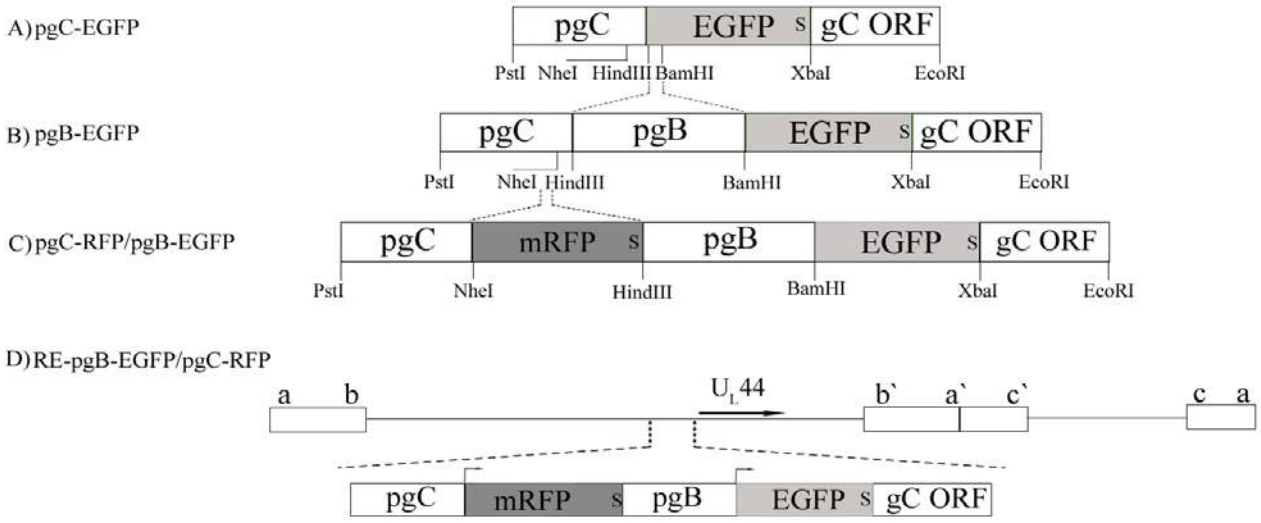

Figure 4-1. Construction of HSV-1 RE-pgB-EGFP/pgC-RFP virus.

A) Representation of the pgC-EGFP construct, in which expression of EGFP is driven by the gC promoter (pgC), followed by the rest of the gC open reading frame (gC ORF). The 's' at the end of EGFP denotes the stop codon. Restriction sites referred to in the text are also indicated below the diagram. B) The subsequent construct is shown in which the gB promoter (pgB) was inserted at the BamHI and HindIII sites in EGFP in order to drive EGFP expression. C) The final construct in which mRFP was inserted at the NheI and BamHI sites so as to be driven by the gC promoter. D) Representation of the recombinant virus showing the final plasmid that was recombined into the HSV-1 RE genome at the U_L44 locus.

4.4.2 Analysis of HSV-1 protein and RNA transcript kinetics *in vitro*

The gB promoter was chosen as it is expressed very early during infection, even though it is classified as a leaky-late (γ1) gene, whereas gC is a true-late (γ2) gene that requires DNA replication for transcription. As such, EGFP expression was expected to occur in the presence of DNA replication inhibitors, while RFP expression should be restricted. Comparisons of EGFP and RFP proteins expressed from cells infected with pgB/pgC to native gB and gC protein expression from HSV-1 RE are shown in Fig. 4-2A. Both EGFP and gB were expressed by 8 h after high multiplicity infection, whereas gC and RFP were not efficiently detected until 12 h post-infection. Both EGFP in the recombinant virus and gB in RE were detected in the presence of the DNA replication inhibitor, phosphonoacetic acid (PAA). Neither gene was expressed in the presence of cycloheximide (CHX) or following a cycloheximide reversal by actinomycin-D (95), establishing that the genes were not errantly transcribed in the absence of *de novo* viral protein synthesis. Expression of RFP and gC was blocked by PAA, cycloheximide, and after cycloheximide reversal, consistent with true-late gene expression. We also assessed expression of EGFP and RFP RNAs (Fig. 4-2B). RFP and EGFP transcripts were of the size expected for RNAs terminating at the polyadenylation site downstream of the gC gene, with the EGFP transcript being smaller, as expected from its initiation downstream of the RFP gene. While EGFP mRNA could be detected as soon as 6 h post-infection, RFP mRNA was barely visible at 8 h post-infection. More importantly, levels of EGFP mRNA were detected in the presence of PAA, while no RFP transcripts were seen in the presence of PAA. While the exposure level of the northern blots shown in Fig. 4-2B was normalized to the 12 h time point, extensive overexposure of RFP-probed northerns did not reveal any RFP transcripts in PAA-treated samples. Quantification of autoradiographs exposed in the linear range of the film indicated that

PAA reduced the EGFP transcripts to 36% of the untreated control at 12 h, whereas PAA reduced RFP transcripts to 2% of the untreated control. These data would be expected for a DNA replication-facilitated and a DNA replication-dependent expression, respectively, and establish that RFP and EGFP expression are regulated differentially as predicted from their true-late and leaky-late promoters.

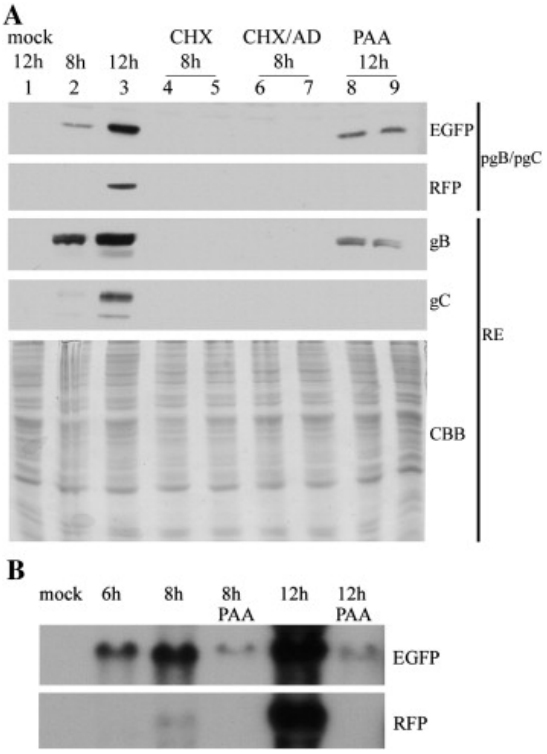

Figure 4-2. Kinetics of viral protein and RNA transcript expression in vitro.

Vero cell monolayers were infected at an MOI of 10 PFU/cell with HSV-1 RE or the pgB/pgC recombinant. The drug treatments (150 µg/ml cycloheximide, CHX; 15 µg/ml actinomycin-D, AD; 350 µg/ml phosphonoacetic acid, PAA) and the times of harvest post-infection, shown in hours, are detailed at the top of each figure part. A) Proteins were harvested and analyzed by immunoblotting for EGFP, RFP, gB, and gC expression using specific antibodies detailed in Materials and methods and indicated to the right of the figure. The top two blots show expression from the pgB/pgC dual fluorescent virus, whereas the bottom blots show a parallel infection with RE. Coomassie brilliant blue (CBB) staining was performed for RE to show equal loading of samples in individual lanes. B) RNA from pgB/pgC-infected cells was analyzed by northern blot using DNA probes specific to EGFP and RFP. Autoradiographs for each fluorescent gene were normalized at the 12 h time point to enable the relative levels of gene expression at earlier times and in the presence of PAA to be compared.

4.4.3 *In vitro* growth kinetics

Replication of the recombinant virus was compared to wild type RE in a multi-step growth curve in Vero cells infected at MOI of 0.01. Peak titers of virus progeny were observed at 24 h post-infection for both wild type and recombinant viruses (Fig. 4-3), and the difference in mean titers was not statistically significant ($p > 0.05$) at any time points tested. Thus, the recombinant virus does not appear to be growth impaired in Vero cells.

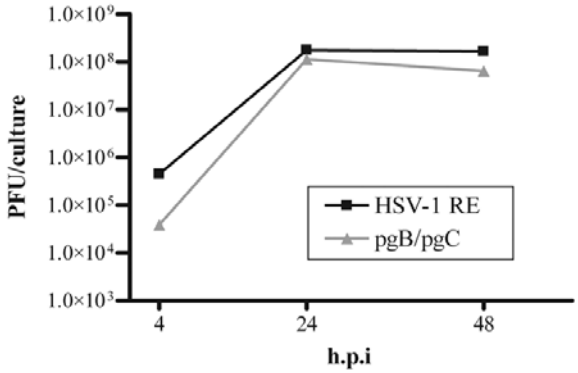

Figure 4-3. Multistep viral replication kinetics.

Monolayer cultures of Vero cells were infected at an MOI of 0.01 PFU/cell with HSV-1 RE or pgB/pgC. 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). Viral yields were not significantly different ($p > 0.05$) at any time tested as assessed by a Student's *t* test, suggesting similar rates of replication.

4.4.4 Viral replication and spread in mice

Because the recombinant virus is defective in the expression of gC, and some reports have suggested that replication of gC-null viruses may be attenuated in mouse models (95), we assessed *in vivo* growth in infected corneas and TG of C57Bl/6 mice following topical corneal infection. Titers of both wild type and recombinant viruses peaked in the tear film at 1 day post-

infection (d.p.i) and decreased over time at rates which did not differ statistically (Fig. 4-4A). Similarly, peak TG titers of pgB/pgC and HSV-1 RE (observed at 4 d.p.i) did not differ significantly (Fig. 4-4B). Both viruses were largely eradicated from the TG by 8 d.p.i (Fig. 4-4B), although a low level of residual virus (both HSV-1 RE and pgB/pgC) could be recovered from some mice. These data indicate the recombinant virus exhibits normal corneal virulence and neurovirulence in mice.

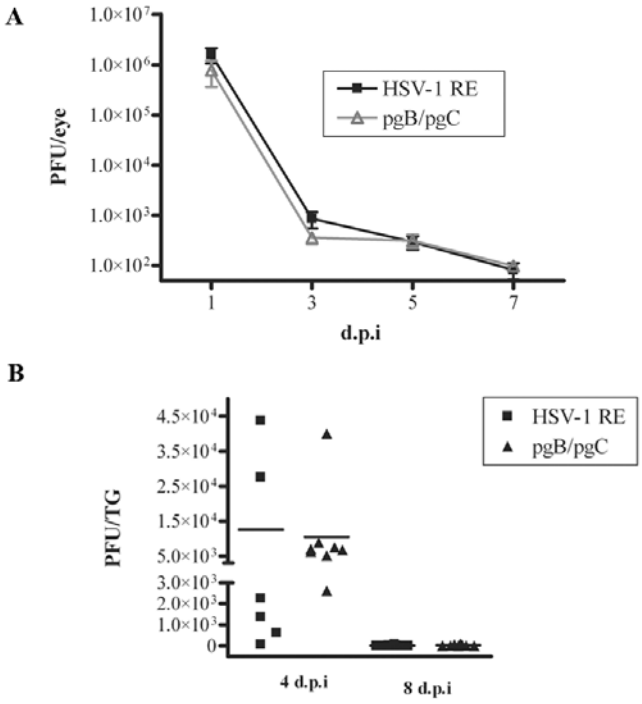

Figure 4-4. Viral titers in tear films and TG of infected mice.

Mice were infected with HSV-1 RE or pgB/pgC at 1×10^5 PFU/eye. A) Eye swabs were obtained at the indicated days post-infection (d.p.i) and titered on Vero cells. The viral titers are shown as means ± standard error of the means (SEM). B) TG were excised, homogenized and subjected to three freeze–thaw cycles prior to determination of viral titers. Titers of the two viruses in eye swabs or TG were not significantly different ($p > 0.05$) at any time point as assessed by a Student's *t* test.

4.4.5 Viral load and CD8$^+$ T cell infiltrates within latently infected TG

Previous reports have established the capacity of CD8$^+$ T cells specific for the immunodominant $gB_{(498–505)}$ epitope to inhibit HSV-1 reactivation from latency both *in vivo* and in *ex vivo* TG cultures of C57BL/6 mice (41, 67, 88, 120). Another recent report has demonstrated that HSV-1 reactivation frequency in *ex vivo* TG cultures is determined by the load of latent virus and the size of the CD8$^+$ T cell infiltrate (60) Therefore, we compared the parameters of CD8$^+$ T cell infiltrate and ganglionic viral load in C57BL/6 TG harboring latent pgB/pgC to those harboring WT HSV-1 RE following parallel corneal infections. Quantitative real-time PCR assessment of viral genome copy number in the TG obtained 33–36 days after HSV-1 corneal infection with recombinant and WT virus was statistically identical (Fig. 4-5). Moreover, neither the size of the overall CD8$^+$ T cell pool, nor the size of the HSV-specific CD8$^+$ T cell pool varied significantly in TG colonized by the pgB/pgC or WT RE (Fig. 4-6).

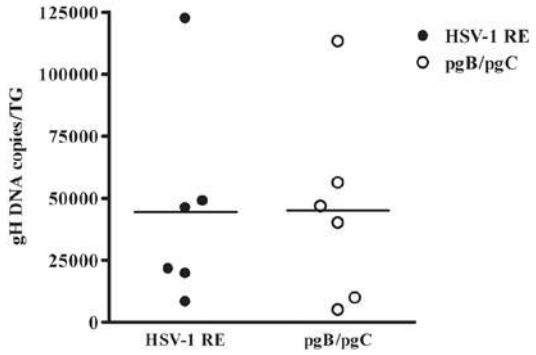

Figure 4-5. HSV-1 genome copy number in latently infected TG.

TG were obtained 33–36 days after corneal infection with WT HSV-1 RE or pgB/pgC. Each data point represents the number of gH DNA copies per TG as determined by real-time PCR analysis of purified TG DNA. Data are combined from two independent experiments. The difference in HSV-1 genome copy number between WT HSV-1 RE and the recombinant is not significant as assessed by Student's *t* test ($p \geq 0.05$).

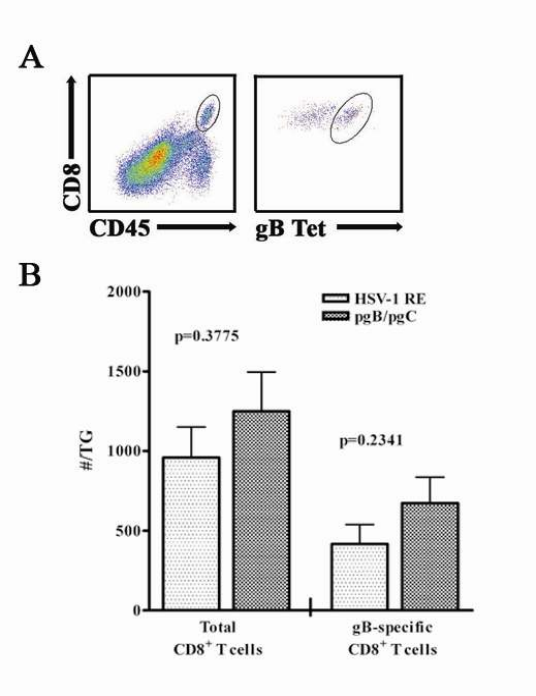

Figure 4-6. Immune infiltrate in latently infected TG.

TG were obtained 33–34 days after corneal infection with either pgB/pgC or HSV-1 RE, dispersed with collagenase, stained for CD45, CD8, and $gB_{498–505}$ -specific T cell receptor, and analyzed by flow cytometry. A) Representative dot plots show separated populations of total $CD8^+$ T cells (circled, left plot), and $CD8^+$ $gB_{498–505}$ $H2\text{-}K^b$ $tetramer^+$ (gB Tet) T cells (circled, right plot). B) Data are presented as the mean ± SEM of the total numbers of $CD8^+$ T cells/TG or the total number of gB-specific $CD8^+$ T cells/TG. Data are pooled from two independent experiments with $n \geq 8$ TG for all conditions. The p values were obtained with a Student's t test comparing the number of cells in TG infected with WT or pgB/pgC.

4.4.6 RE-pgB/pgC does not induce a $CD8^+$ T cell response to EGFP or RFP in C57Bl/6 mice

EGFP expressing vectors can induce $CD8^+$ T cell responses to EGFP in BALB/c mice (1). Such reactivity could alter the response of $CD8^+$ T cells to target cells infected with recombinant viruses that express fluorescent proteins. Therefore, we investigated the possible immunogenicity of EGFP and RFP proteins expressed from pgB/pgC virus. For these studies, gB, EGFP, or RFP

was expressed from plasmids under the control of the CMV IE promoter in B6WT3 cells. Expression of transfected proteins was confirmed by western blot or fluorescence microscopy (data not shown). Transfected or untreated cells were used to stimulate IFN-γ production by $CD8^+$ T cells within dispersed C57BL/6 TG harboring latent pgB/pgC (Fig. 4-7). Only gB-transfected cells induced a significant IFN-γ response. Thus, pgB/pgC does not induce a $CD8^+$ T cell response to EGFP or RFP that is detectable in the latently infected TG.

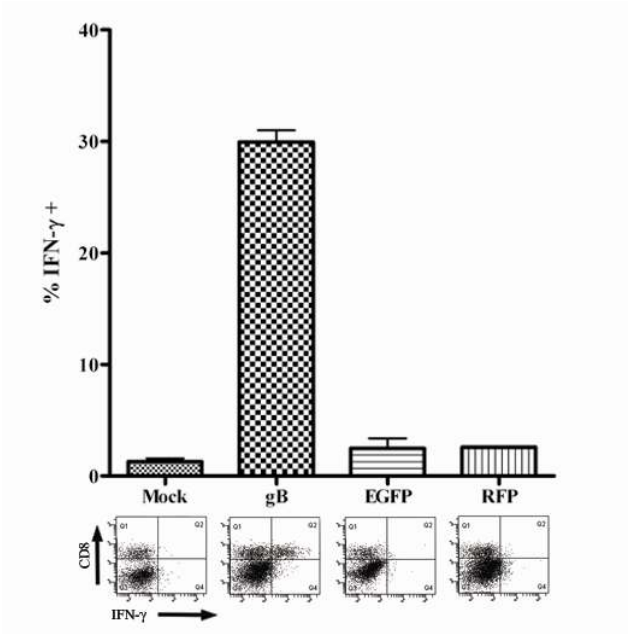

Figure 4-7. Antigen specificity of $CD8^+$ T cells in latently infected TG.

TG were obtained 8 days after corneal infection with pgB/pgC, dispersed with collagenase, and stimulated for 6 h with syngeneic B6WT3 cells that were mock transfected or transfected with plasmids expressing gB, EGFP, or RFP. Cells were surface stained for CD45 and CD8, and stained for intracellular IFN-γ. Representative dot plots show cells that were gated on CD45 and analyzed for expression of CD8 and IFN-γ. The graph depicts the mean percent of IFN-γ positive cells (n = 2/group) and standard error of the mean for each stimulation. The IFN-γ response of gB stimulated $CD8^+$ T cells is significantly ($p < 0.05$) different from the response of the other three groups as assessed by an ANOVA with Tukey's post-test.

4.4.7 RE-pgB/pgC reactivates normally in *ex vivo* TG cultures

Fluorescent protein expression in neurons and surrounding fibroblasts has previously enabled rapid assessment of HSV-1 reactivation from latency in *ex vivo* TG cultures (31) The pgB/pgC virus was designed to offer the advantage of being able to distinguish expression of the γ1 gB promoter from expression of the γ2 gC promoter within the same cell. Examination of *ex vivo* cultures of TG latently infected with pgB/pgC at low magnification revealed fluorescent plaques that initially expressed only EGFP, but then expressed both EGFP and RFP by day 4 (Fig. 4-8A). RFP expression could be detected at earlier stages (2 days post-explant; d.p.e), but this required higher magnification and more intense UV exposure, and we were concerned for the effects on culture viability. These studies establish that the dual fluorescent virus reactivates from latency, and that gB and gC promoters are temporally regulated following reactivation. We then compared the reactivation kinetics within TG latently infected with either pgB/pgC or WT HSV-1 RE. Dispersed cells from TG harboring recombinant or wild type latent virus were cultured, and reactivation was determined by sequential analysis of supernatant fluids for infectious virus using a standard viral plaque assay. Reactivation frequencies as determined by either spread of fluorescence or infectious virus in culture supernatants were nearly identical for recombinant and wild type viruses (Fig. 4-8B). To insure that reactivating pgB/pgC did not revert to wild type during latency, supernatant fluids from reactivated TG cultures were added to Vero cell monolayers and developing plaques were monitored with a fluorescence microscope. As illustrated in Fig. 4-9, gB promoter-driven EGFP fluorescence was easily detected in single or small foci of indicator cells by 24 h in culture, and gC promoter-driven RFP was first detected in Vero cells by 30 h in culture. The extended time to detect fluorescent gene expression as compared to the kinetic studies detailed in Fig. 4-2A reflects the requirement for sufficient

amounts of both proteins to accumulate to enable detection following presumably much lower multiplicities of infection from viral reactivation. However, all cells ultimately expressed both promoters, and no non-fluorescent plaques were observed, demonstrating that reversion to wild type virus did not occur within latently infected neurons.

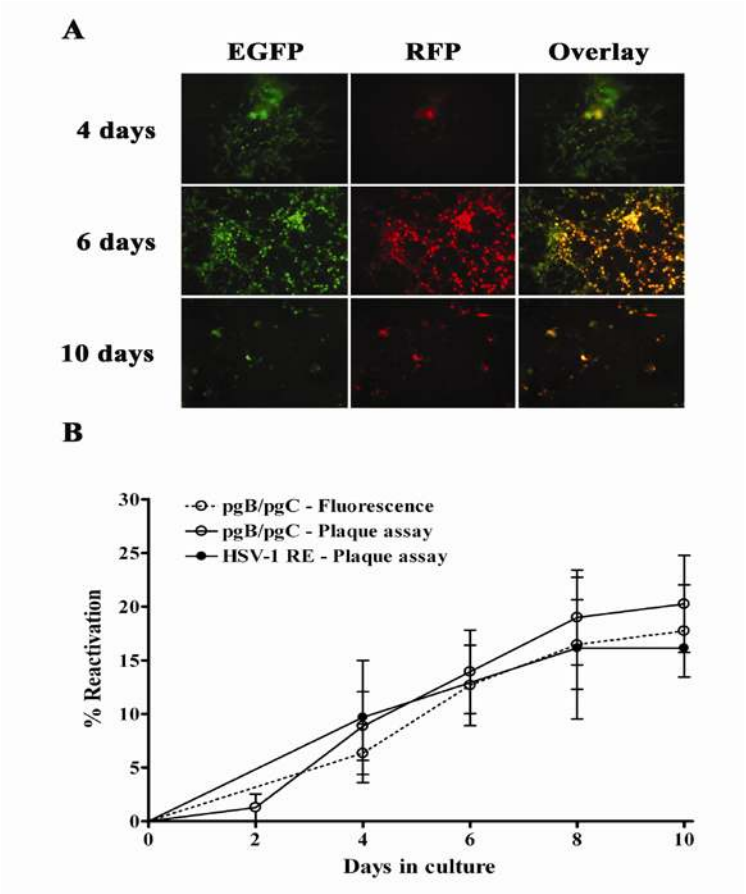

Figure 4-8. Detection of viral promoter activity in ex vivo TG cultures by fluorescence microscopy.

TG were excised 32–47 days after corneal infection with HSV-1 RE or pgB/pgC, dispersed with collagenase, and cultured (0.2 TG/well) at 37 °C. A) Images of pgB/pgC-infected TG cultures were acquired at the indicated times following culture initiation. Magnification, 10×; BF, brightfield. B) At the indicated times following culture initiation, HSV-1 reactivation from latency was detected based on spread of fluorescence from neurons to surrounding fibroblasts (pgB/pgC-infected TG), and on the presence of infectious virus in culture supernatants (plaque assay). The mean percent of cultures exhibiting HSV-1 reactivation from latency and standard error of the mean is shown at each time point. Based on a survival curve analysis the reactivation frequency of pgB/pgC was not significantly different ($p > 0.05$) when assessed by fluorescence or plaque assay; nor was the reactivation frequency of pgB/pgC as detected by either method significantly different ($p > 0.05$) than that of HSV-1 RE as assessed by plaque assay.

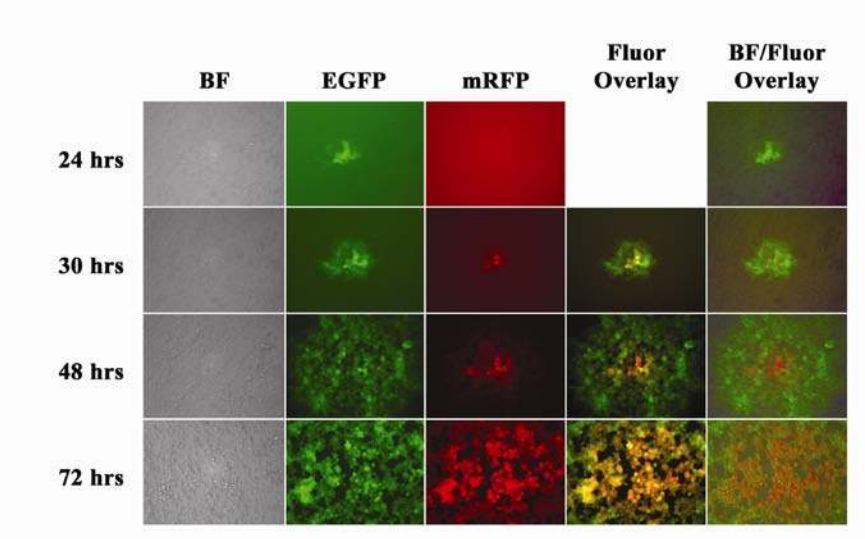

Figure 4-9. Detection of viral promoter activity within pgB/pgC-infected Vero cells by fluorescence microscopy.

Supernatant fluid from reactivated *ex vivo* cultures of TG infected with pgB/pgC was placed on monolayers of Vero cells and incubated at 37 °C. Images were acquired at the indicated times. Magnification, 20×; BF, brightfield; Fluor, fluorescence.

4.4.8 Confocal imaging of fluorescent gene expression in TG during acute infection and induced reactivation

We next used the dual fluorescent virus in whole mounts of TG tissue to image viral gene expression *in situ*. Both EGFP and RFP expression were consistently detected in whole mounts of TG that were excised at 4 d.p.i (Fig. 4-10B). Fluorescence was detected in both neurons and non-neuronal cells, and was restricted to the ophthalmic branch of the TG (not shown). The coexpression of EGFP and RFP indicated that lytic gene expression from both γ1 and γ2 was ongoing in these neurons. Fluorescence was not detected in non-infected TG (Fig. 4-10A). While not conducted in the same study, examination of TG whole mounts from HSV-1 RE (non-fluorescent) infected mice did not reveal any fluorescence at acute or latent time points. We then

explored viral promoter expression in the TG following the induction of reactivation with sodium butyrate, a histone deacetylase inhibitor that has recently been shown to efficiently induce HSV-1 reactivation from latency (117). Within three days post-intraperitoneal injection of a non-lethal dose of sodium butyrate, several regions of the ganglia contained multiple neurons expressing both EGFP and RFP, as detected in TG whole mounts (Figs. 4.10E and F). The numbers of these EGFP and RFP regions varied from 1 to 5, suggesting that several neurons in local regions of the TG were undergoing reactivation and lytic gene expression. High magnification revealed punctate speckles of fluorescence that was restricted to neuronal cell bodies (Fig. 4-10F). In two separate studies involving 8 mice each, 100% of mice showed dual fluorescent protein expression. Fluorescent gene expression was never detected in the maxillary or mandibular branches of the TG, consistent with the presence of latent genomes being restricted to the ocular branch, as previously reported (67). Parallel swabbing of the ocular tear films at day 3 following the induction of sodium butyrate failed to reveal any virus in treated or untreated mice (data not shown). We did not detect any fluorescence in uninfected but sodium butyrate-treated mice (Fig. 4-10D) nor in latently infected untreated mice (Fig. 4-10C). While vehicle (phosphate buffered saline; PBS)-treated latently infected mice controls were not performed in this work, parallel studies have indicated that PBS injection alone does not have any effects on latent genome copy number (unpublished observations). Fluorescent gene expression is not detectable in day 15 infected TG (data not shown). As such, the dual fluorescence seen in latently infected sodium butyrate-treated animals suggests that viral gene expression of both γ1 and γ2 classes in the TG reflects reactivation from latency. Virus is not seen at the periphery indicating that lytic gene expression can occur without necessarily resulting in virus delivery to the periphery. This is consistent with recent models suggesting there is

immune control of reactivation and peripheral virus delivery following onset of lytic gene expression from the latent state.

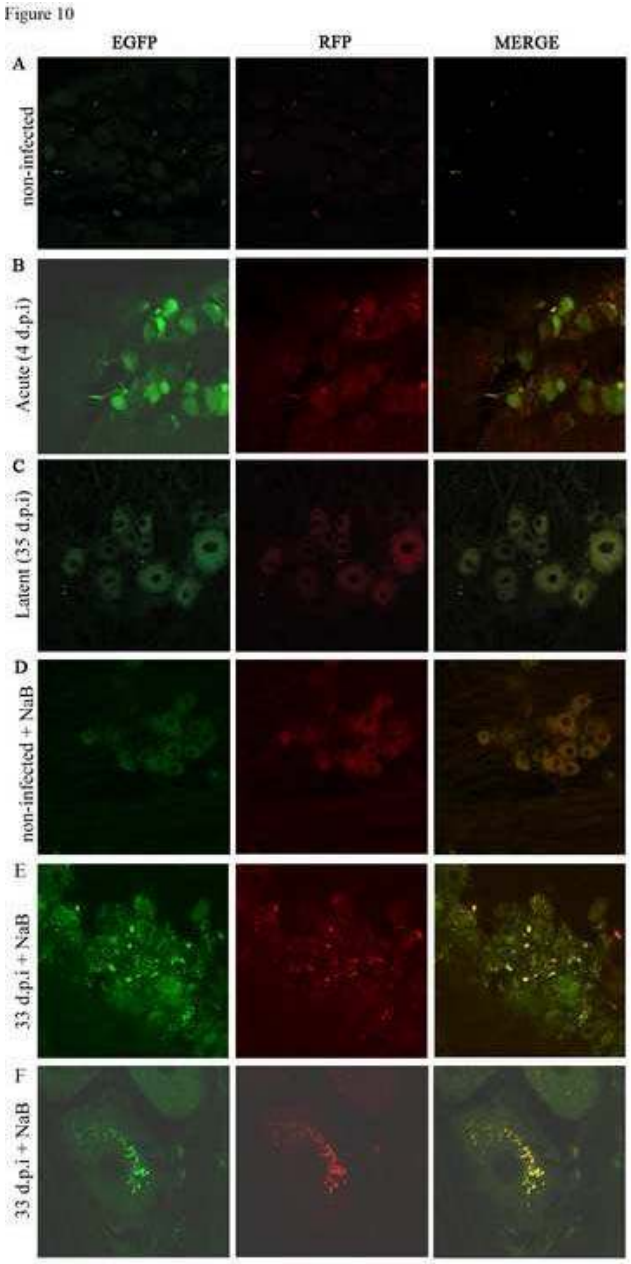

Figure 4-10. Detection of in situ promoter activity in whole fixed TG tissue by confocal microscopy.

TG were excised from mice at the indicated days post-infection (d.p.i), fixed and mounted onto glass slides prior to imaging by confocal microscopy. A and D) non-infected; B) acute infection (4 d.p.i); C, E, F) latent infection (> 35 d.p.i). C–F) Some of the non-infected and latently infected mice were treated with 1200 mg/kg sodium butyrate (109) 3 days before TG excision. F) Zoomed image of a single neuron showing both EGFP and RFP expressions. Magnification 60X.

4.5 DISCUSSION

The advancement of fluorescence imaging equipment combined with the availability of genes encoding proteins that fluoresce at different wavelengths has greatly enhanced our ability to visualize gene expression *in situ*. In previous studies, we developed single fluorescent viruses under the ICP0 and or gC promoters to examine acute infection in mice as well as reactivation from latency (31). Another group has also developed a recombinant fluorescent HSV-1 to study acute infection and reactivation (3). However, in this virus, EGFP is expressed from the human cytomegalovirus major immediate early promoter (HCMV-MIEP) and not an HSV-1 promoter. Here we describe the construction and characterization of a recombinant HSV-1 that expresses EGFP and RFP from different kinetic classes of HSV-1 promoters. To our knowledge, the use of recombinant HSV-1 to simultaneously visualize expression of two or more genes within a single cell in the context of HSV-1 promoters has not been explored. This dual fluorescent reporter virus demonstrated the expected kinetics of EGFP and RFP protein expression regulated by the native promoters for gB and gC, respectively. The virus had growth phenotypes indistinguishable from wild type HSV-1 RE in mice, including parameters of acute corneal growth, acute TG replication, establishment of and reactivation from latency. Importantly, we show that latency was associated with a $CD8^+$ T cell immune infiltrate in the TG that was equivalent to that seen in TG latently infected with wild type virus. Further, we detected no $CD8^+$ T cell reactivity to the fluorescent proteins, EGFP or RFP. The fluorescent reporters allowed rapid visualization and quantification of reactivation events following TG explant. We were also able to demonstrate multiple reactivation events *in situ* in ganglia of latently infected mice treated with sodium butyrate to induce reactivation.

The use of fluorescence to identify viral reactivation in *ex vivo* cultures of latently infected TG saves both time and resources compared to using standard viral plaque assays, and it enables dynamic studies of reactivation events *ex vivo*. The impetus for this work was the challenging issues surrounding the dynamic analysis of the expression multiple viral genes at the single-cell level, especially during reactivation from latency. There are no other simple, reproducible and sensitive assays for such analyses. The widely used approach of *in situ* hybridization (131) and/or PCR to address RNA expression, and/or immunohistochemistry (IHC) to identify protein synthesis (137-138) suffer from practical and dynamic limitations. While ISH, PCR and IHC approaches enable identification of gene expression events at the single cell level, they require time-consuming visual inspection of hundreds of sections per ganglia at any one time point and highly specific immunological reagents to enable protein detection. Analyses of two proteins expressed by the same cell requires either complex staining schemes or serial sectioning and identification by different antibodies, followed by location of the same cell in subsequent sections, which can be difficult. The dual fluorescent approach with a virus such as that detailed here allows expression from two viral promoters to be easily visualized at the single-cell level in a dynamic fashion.

Several reporter genes, including β-galactosidase and luciferase, have been used to assess gene expression during the various stages of HSV-1 latency (101). The use of β-galactosidase has the disadvantage that its detection requires fixation and treatment with substrate compounds for detection. While whole organs can be analyzed, it is also a static assay. It has also been suggested that β-galactosidase is quite stable and can be detected for long periods after transcription is halted in the TG, possibly leading to the errant conclusion that transcription is ongoing (101). Following corneal infection with our recombinant virus, expression of EGFP and RFP drastically decreased from four to eight d.p.i. The rapid loss of

EGFP and RFP detection renders these reporters more accurate for detection of ongoing viral gene expression.

Use of luciferase in conjunction with image visualization and infrared spectroscopy (IVIS) system has enabled dynamic parameters of infection to be addressed, but this technology has not yet enabled activity to be correlated at the single-cell level. The pgB/pgC virus enabled easy and dynamic examination of viral gene expression in single-cell suspensions of dissociated TG in *ex vivo* cultures. The dual fluorescence is an extension of our previous studies in which single fluorescent viruses were used to show that IFN-γ, a $CD8^+$ T cell effector molecule, can block HSV-1 reactivation. IFN-γ inhibited reactivation in part by blocking expression of the ICP0 α gene, which is required for efficient reactivation of HSV-1 from latency (49), and also by blocking a late step following expression of the gC γ2 gene (31). However, these studies required infecting different mice with the different recombinant viruses. The virus described herein permits such an analysis examining expression of two different temporally regulated genes within single neurons of the same ganglia from the same infected mouse. HSV-specific $CD8^+$ T cells were shown to block HSV-1 reactivation prior to expression of gC (88). This virus will allow a more definitive investigation of the step in the viral life cycle at which $CD8^+$ T cells block reactivation from latency in neurons.

The pgB/pgC virus is an extremely valuable tool in studying various aspects of HSV-1 latency and reactivation as the fluorescent proteins are expressed from different HSV-1 promoters. This virus has applications in molecular virology and viral immunology and may lead to a better understanding of HSV-1 latency and reactivation both *in vivo* and in *ex vivo* experiments.

5.0 DELAYING THE EXPRESSION OF HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEIN B TO A TRUE LATE GENE ALTERS NEUROVIRULENCE AND INHIBITS THE gB-CD8$^+$ T CELL RESPONSE IN THE TRIGEMINAL GANGLION.

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5.1 ABSTRACT

Following an HSV-1 ocular infection of C57BL/6 mice, activated $CD8^+$ T cells specific for an immunodominant epitope on HSV-1 glycoprotein B (gB-CD8) establish a stable memory population in HSV-1 latently infected trigeminal ganglia (TG) whereas non-HSV-specific $CD8^+$ T cells are lost over time. The retention and activation of gB-CD8 appears to be influenced by persistent viral antigenic exposure within the latently infected TG. We hypothesized that the low level expression of gB from its native promoter before viral DNA synthesis is critical for the retention and activation of gB-CD8 in the TG during HSV-1 latency, and for their ability to block HSV-1 reactivation from latency. To test this, we created a recombinant HSV-1 in which gB is expressed only after viral DNA synthesis from the true late gC promoter (gCp-gB). Despite minor growth differences compared to its rescuant in infected corneas, gCp-gB was significantly growth impaired in the TG and produced a reduced latent genome load. The gCp-gB and rescuant infected mice mounted a similar gB-CD8 effector response, but the size and activation phenotype of the memory gB-CD8 was diminished in gCp-gB latently infected TG, suggesting that stimulation of gB-CD8 requires gB expression before viral DNA synthesis. Surprisingly, late gB expression did not compromise the capacity of gB-CD8 to inhibit HSV-1 reactivation from latency in *ex vivo* TG cultures, suggesting that gB-CD8 can block HSV-1 reactivation at a very late stage in the viral life cycle. This data has implications toward designing better immuogens for vaccines to prevent HSV-1 reactivation.

5.2 INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen that is responsible for repeated corneal infections that can induce a blinding keratitis. The murine model of ocular HSV-1 infection has elucidated the role of the host immunity in the establishment and maintenance of viral latency in trigeminal ganglia (TG). HSV-1 infection of a scarified mouse cornea leads to a short lived and self resolving epithelial lesion caused by viral replication in and destruction of corneal epithelial cells. During replication, viral genes are expressed in tightly regulated temporal cascade characterized by sequential expression of immediate early (α) genes and early (β) genes before viral DNA synthesis. The late γ genes are maximally expressed after viral DNA replication and can be subdivided into γ1 genes, that are expressed in low amounts before viral DNA replication and γ2 genes that are absolutely dependent on DNA replication for expression (57). The γ1 gene product glycoprotein B (gB), a multifunctional structural glycoprotein, contains an immunodominant epitope ($gB_{498-505}$) that is recognized by a majority of $CD8^+$ T cells (gB-CD8) in C57BL/6 mice within 2 hours of target cell infection (67, 112, 178).

Replicating HSV-1 in the corneal epithelium accesses the termini of interdigitating sensory neurons and travels via retrograde axonal transport to the neuronal soma in the TG. The viral genome is maintained in sensory neurons in a latent state in which no infectious virus is produced. Latency is characterized by repression of most viral lytic cycle genes, and abundant expression of viral RNAs known as latency associated transcripts (LATs) with no known protein products (73, 75). Repression of viral protein synthesis during latency has led to the prevalent view of latency as a quiescent and antigenically silent infection that is ignored by host immunity. However, very low levels of gene transcripts and proteins from all kinetic classes have been detected in latently infected murine TG (39, 74). Furthermore, the findings of recent

immunologic studies of HSV-1 latency in mice are inconsistent with the notion that latent virus is ignored by the host immune system. $CD8^+$ T cells infiltrate the TG during acute HSV-1 infection, with peak accumulation occurring coincident with elimination of replicating virus and establishment of latency (67, 178). In C57BL/6 mice gB-CD8 represent about half of the $CD8^+$ T cell infiltrate in the TG (67). Most if not all of the remaining $CD8^+$ T cells in infected TG appear to recognize as yet undefined HSV-1 proteins (145). The effector $CD8^+$ T cell population in acutely infected TG undergoes contraction as latency is established, giving rise to a small but stable memory population with the same 50:50 ratio of gB-specific to gB non-specific cells (67).

In both mice and humans, $CD8^+$ T cells are found in the HSV-1 latently infected TG in direct apposition to neurons (67, 70, 166). Taking advantage of tetramers that bind to the T cell receptor of gB-CD8, we have demonstrated that these cells form an immunologic synapse with neurons in latently infected TG, and even release lytic granules into the synapse. Thus, $CD8^+$ T cells can detect and respond to latent virus during immunesurveillance (67, 70). The TG-resident gB-CD8 population exhibits a more activated phenotype (CD69 and granzyme B expression) and is less dependent on homeostatic proliferation signals than its counterparts in non-infected tissue, such as the lungs and spleen (145). These data are consistent with persistent antigenic exposure within the latently infected TG. gB-CD8 can employ the cytokine interferon gamma (IFN-γ) and lytic granules to prevent HSV-1 reactivation from latency *in vivo* and in *ex vivo* TG cultures without inducing neuronal apoptosis (31, 70).

The importance of appropriate kinetic expression of HSV-1 genes for virulence and the generation of host immunity has not been explored. Here we constructed a recombinant HSV-1 that expresses the γ1 gB gene as a γ2 gene, eliminating low level expression prior to viral DNA synthesis. We investigated whether this kinetic change of gB expression would influence HSV-1

virulence, the generation and homing of gB-CD8 to the TG, and the ability of gB-CD8 to inhibit the reactivation from latency.

5.3 MATERIALS AND METHODS

5.3.1 Construction of recombinant HSV-1.

The recombinant virus containing gB under the late glycoprotein C (gC) promoter (HSV-1 gCp-gB) in the HSV-1 RE background, and its corresponding rescuant (HSV-Rescue), were constructed as shown in Figure 5-1. The sequence of gB and its promoter were PCR amplified from 54817 to 56640 bp in two sections; gB was amplified using primers gB-E (HindIII) 5’ ; gB-S (BamHI)

5’ ; and the promoter was amplified using 28-E (NheI/BamHI)

5’ and

28-S (EcoRI) 5’-GAGAATTCTGACGAAGCGGTCGTTGGCCAGCC.

All DNAs were amplified from the RE genomic template using the proofreading polymerase Expand (Roche) under hot start conditions and in reactions containing 5% DMSO. Each PCR fragment was cut with the terminal engineered restriction sites, and triple ligated into the vector pUC19 as a HindIII-BamHI-EcoRI fragment to generate plasmid pK1968 which contained a unique BamHI and NheI site just upstream of the gB ATG start codon. A portion (508 bp) of the region immediately upstream of the gC initiating ATG (96,227-95,820) containing the gC promoter (104) was similarly PCR amplified using the primers gCp-S (BamHI) 5’ and gCp-B (Nhe-Bgl2-AflII) 5’ . Restriction sites in the primers are underlined. The PCR product was then cut with BamHI and NheI, inserted into the corresponding sites engineered upstream of gB in pK1968. The resulting construct was digested with NheI and AflII and an NheI-AflII fragment of pEGFP-C1 containing EGFP and its polyadenylation signal were inserted to be upstream of the gC promoter and downstream of, and driven by the gB promoter.

Plasmid derivative for the rescuant was developed by PCR amplifying a portion of the gB gene and the promoter/U_L28 region using the primers 54810F -EcoRI 5' AND and 55812R AvrII-5' GTCCTCCAGCACCTCGCCCC**T**AGGCTACCTGACG: and 55812F AvrII-5' GTCAGGTAGCCT**A**GGGGCGAGGTGGAGGAC with 56801R HindIII GCCCAAGCTTACGACGGGGACCGTGTCGCCGT (mutations in bold and underlined). Each PCR fragment was digested with the terminal end restriction sites, combined and triple ligated into EcoRI and HindIII digested pUC19. The single mutation resulted in the insertion of a silent non-coding unique AvrII restriction site. All DNA inserts were sequenced for integrity.

HSV-gCp-gB was derived by co-transfection of infectious wild type HSV-1 RE viral DNA with plasmids linearized with SspI into Vero cells, using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA). GFP positive virus plaques were picked and plaque purified to homogeneity, as detailed previously (Chapter 4). The rescuant (HSV-Rescue) was derived by co-transfecting viral DNA from the EGFP positive gCp-gB virus with the promoter-gB plasmid containing the novel AvrII site. Rescuant plaques were selected and picked based on loss of fluorescence followed by subsequent titration of the mixed progeny from the transfection. (Fig. 5-1 i-iii). The recombinants were positively identified for inserted DNA sequences (gCp-gB) or for the inserted AvrII site (Rescue) by Southern blot analysis of restricted viral DNA. Viral DNA from both viruses were cut with NcoI, AvrII and NcoI - AvrII double digests, and probed with 5' ^{32}P-radiolabelled oligonucleotides derived from the gB coding sequence or the U_L28 coding sequence (Data not shown).

A recombinant with U_s3 deleted in the HSV-1 RE background was also derived for this study (Fig. 5-1 iv). Sequences of part of the U_S3 protein were PCR amplified from the HSV-1 RE genomic template using the GC-RICH PCR System (Roche) under hot start conditions and primers $U_S3.5F$ 5' - GGG AAT TCA TGT ACG GAA ACC AGG ACT AC - 3'

and U_S3.5R 5' - GGA AGA TCT TCA TTT CTG TTG AAA CAG CGG CAA - 3'. The PCR product was digested with BglII and EcoRI and ligated to pEGFP-C2 digested with BamHI and EcoRI. Collapse of this construct with Xho I resulted in removal of the N terminal part of the U_S3 coding sequence encoding residues 1 to 169. The remaining part lacks ATG, is out of frame with respect to EGFP and lacks domains required for kinase activity. DNA containing the promoter sequence upstream of the U_S3 gene was PCR amplified using U_S3PF primer 5' - GCG CCC TAG GGC TAG CTC GCC GCA CCG TGA GTG CCA - 3' and U_S3PR primer 5' - GCC ATT AAT ATT AAT GCC GCG AAC GGC GAT CAG AGG GTC AGT - 3'. This PCR product was digested with inserted to replace the CMV IE promoter. The resulting construct contained EGFP flanked by U_S3 promoter and the distal end of U_S3 (Fig. 5-1 iv). Viruses were selected from co-transfections of HSV-1 RE viral DNA with the linearized plasmid and were identified and plaque purified based on gain of EGFP fluorescence. Insertion of GFP and deletion of the N-terminus of the U_S3 coding sequences was confirmed by Southern blot analysis of viral DNA (data not shown).

5.3.2 Analysis of eIF2α phosphorylation in primary corneal fibroblasts

Confluent primary corneal fibroblasts (PCF) were infected at an MOI of 5 with either Rescue or gCp-gB for 1 hour at 25°C either in the presence or absence of PAA (350 µg/ml). Media was replaced following 1 hr under the same conditions and cells were incubated at 37°C for 4, 8 or 12 hours. Thirty minutes prior to harvest, cells were incubated with 5µM of Thapsigargin (Tg). Cells were then washed and the proteins were solubilized in SDS PAGE buffer, boiled, and separated by SDS PAGE. Immunoblot analysis was then performed using antibodies to eIF2α (Abcam), phosphorylated eIF2α (phospho-S51;Abcam) and gB.

5.4 RESULTS

5.4.1 Construction and characterization of recombinant HSV-1.

We created HSV-1 gCp-gB that exhibits gB expression with true-late kinetics and complete dependence on viral DNA replication. Construction of the virus and its recscuant (Rescue) are

depicted in Figure 5-1. In place of its native promoter, gB is driven by a γ2 gC promoter in gCp-gB so that the protein is only expressed following DNA replication. The strategy required maintenance of the gB promoter in the genome, because it is contiguous with the upstream UL28 essential ORF encoding a terminase subunit. As such, HSV-1 gCp-gB was engineered so the gB promoter drove expression of EGFP followed by a polyadenylation motif to terminate RNA made from the gB promoter (Fig. 5-1 ii). The rescuant (Rescue) engineered on the gCp-gB background restored gB expression back to its native promoter and could be differentiated from parental virus (HSV-1 RE) by the insertion of a silent and novel AvrII restriction site (Fig. 5-1 iii).

An immunoblot analysis performed at 8 hours post infection (h.p.i.) revealed abundant expression of gB in cells infected with wild-type HSV-1 RE (WT RE) and Rescue virus, but only trace expression in cells infected with gCp-gB virus (Fig. 5-2A). This was similar to the low levels of gC protein expressed by all the viruses (Fig. 5-2B). By 12 h.p.i similar levels of gB expression were detected in WT RE, Rescue, and gCp-gB. As expected, the presence of the HSV-1 DNA replication inhibitor phosphonoacetic acid (PAA) blocked expression of gC in all viruses and of gB protein in gCp-gB (Fig. 5-2A and B, lane 6), while permitting low level expression of gB from its native promoter in WT RE and the Rescue virus (Fig. 5-2A lane 6). These data confirm that gB expression kinetics in gCp-gB is regulated as a γ2 late gene, eliminating the low level gB protein expression prior to DNA replication observed when expression is regulated by its native promoter.

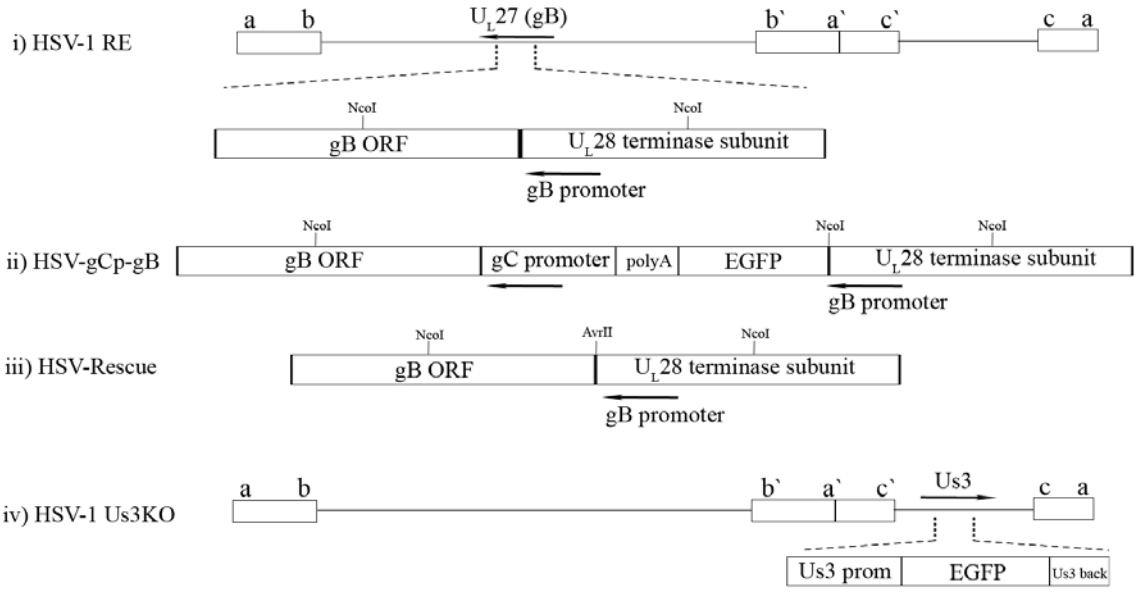

Figure 5-1. Construction of HSV-gCp-gB, HSV-Rescue and HSV-1 U_S3KO viruses.

i) Representation of the HSV-1 genome showing the U_L27/U_L28 locus which contains the gB promoter (arrow) in the U_L28 coding sequences upstream of the gB ORF. The restriction sites were used to identify the insertion, as detailed in the text, are indicated on the gene locus at their approximate positions. **ii)** Expansion of the corresponding region in recombinant HSV-1 gCp-gB virus, showing that in place of its native promoter, gB is driven by the well characterized γ2 gC promoter in gCp-gB so that the protein is only expressed following DNA replication. The strategy required maintenance of the gB promoter in the genome, because it is concurrent with the upstream UL28 ORF encoding an essential terminase subunit. As such, HSV-1 gCp-gB had the gB promoter driving EGFP followed by a polyadenylation motif to terminate RNA made from the gB promoter. **iii)** Representative structure of the HSV-Rescue virus, showing that gB expression is restored to its native promoter, but that a unique non coding AvrII site distinguishes it from the parental strain. **iv**) Representation of the genome showing the position of the U_S3 locus, which was modified such that the U_S3 promoter drives the expression of EGFP, followed by an untranslated and nonfunctional portion (amino acids 170-481) of U_S3 ORF.

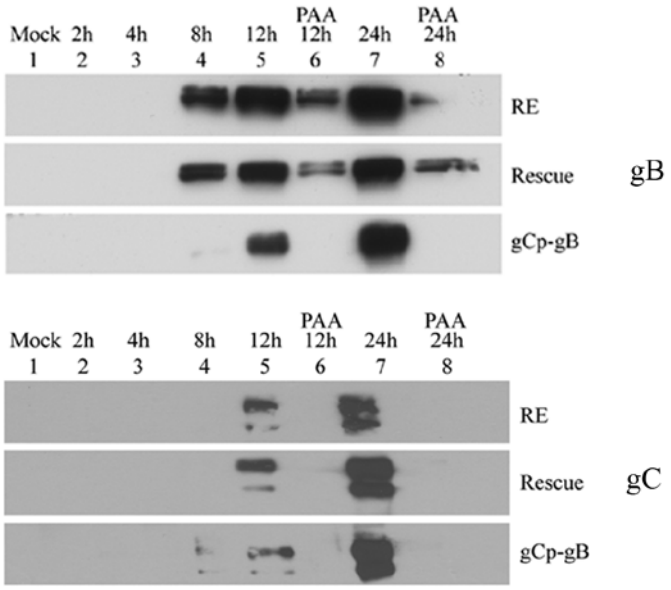

Figure 5-2. HSV-gCp-gB expresses gB with true-late kinetics.

Confluent monolayers of Vero cells were infected with HSV-1 RE, gCp-gB or Rescuant at an MOI of 10 with or without the addition of 350μg of phosphonoacetic acid (PAA). Total SDS PAGE separated proteins were analyzed by immunoblotting for gB (A) or gC (B) using pools of monoclonal antibodies. The times of harvest are shown above each figure and lane designation in hours (h). The top panel of each figure represents infection with HSV-1 RE, the middle panel is HSV-Rescue and the bottom panel is HSV-gCp-gB. Only the region corresponding to the main glycoprotein products are shown.

5.4.2 Viral replication of gCp-gB is impaired *in vivo* but not in culture.

Following infection of Vero cell monolayers at a low multiplicity of infection (MOI = 0.01 PFU/cell), gCp-gB exhibited a growth curve that was nearly identical to those of the rescuant (Rescue), demonstrating normal replication efficiency for gCp-gB *in vitro* (Fig. 5-3A). However, gCp-gB virus showed some growth impairment *in vivo* in mice. Following corneal infection of C57BL/6 mice, we observed a significant (approximate 2-fold) decrease in tear film titer for gCP-gB compared to its rescuant at 1 day post infection (d.p.i.) (Fig. 5-3B), but titers equalized thereafter. By 7 d.p.i., infectious virus could not be detected in the tear film of mice infected with

either virus demonstrating that gCp-gB virus is cleared with normal kinetics in the cornea. We next assessed acute viral replication in the TG. Replication of gCp-gB was severely impaired (5-fold) at 4 d.p.i. (peak of virus replication) compared to the rescue virus (Fig. 5-3C) and wild type virus (not shown). Infectious gCp-gB and its rescuant were cleared from the TG by 8 d.p.i. Thus, HSV-1 virulence is compromised in the cornea and more profoundly in the TG when gB expression prior to viral DNA replication is prevented.

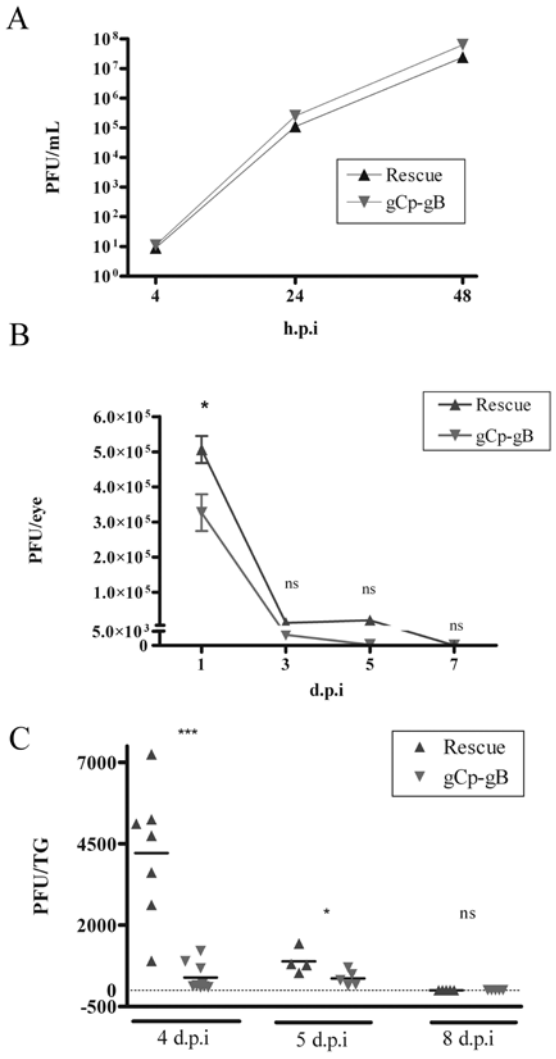

Figure 5-3. Viral replication titers are reduced in corneal tear films and TG of gCp-gB infected mice but not in culture.

A) Vero cell monolayers were infected at an MOI of 0.01 with gCp-gB or Rescue viruses. Cells and supernatants were collected at the designated hour post infection (hpi), subjected to three freeze-thaw cycles and plaque forming units (PFU)/ml of HSV-1 were measured on Vero cells. The difference between the viral titers of gCp-gB and Rescue was not significantly different at any time tested ($p>0.01$). The experiment was repeated two independent times with similar results. **B&C)** Mice were infected with Rescue or gCp-gB at 1 x 10^5 PFU/eye. **B)** Eye swabs were performed at the indicated days post infection (d.p.i.) and HSV-1 was titered on Vero cells. The viral titers are shown as means ± standard error of the means (SEM). *Titers were significantly different as assessed by a Students' *t*-test ($p<0.05$) **C)** Infected TG were excised, homogenized and subjected to three freeze–thaw cycles and HSV-1 titered on Vero cells. Each data point represents the mean viral titer from a single TG as determined by plaque assay. The data are combined from two independent experiments. The significance of differences in TG titers was assessed by a Student's *t*-test (*** $p = 0.0008$), (* $p= 0.0573$). At 8 d.p.i., no infectious virus could be detected from TG infected with either virus.

5.4.3 Establishment of HSV-1 gCp-gB latency is impaired.

Previous studies have demonstrated that replication-impaired HSV-1 viruses establish latency with reduced efficiency as determined by genome copy load per ganglia (59). Since replication of gCp-gB was impaired in the TG, we wanted to test whether it was also impaired in its ability to yield latent genome loads equivalent to its rescuant. Corneal infection of mice with 1 x 10^5 PFU/eye with gCp-gB indeed resulted in a reduced latent viral load in the TG compared to its rescuant when assessed at 14, 34, and 64 d.p.i. (Fig. 5-4). However we were able to equalize the latent load of gCp-gB and its rescuant by reducing, three-fold, the infectious dose of the Rescue virus (Fig. 5-4).

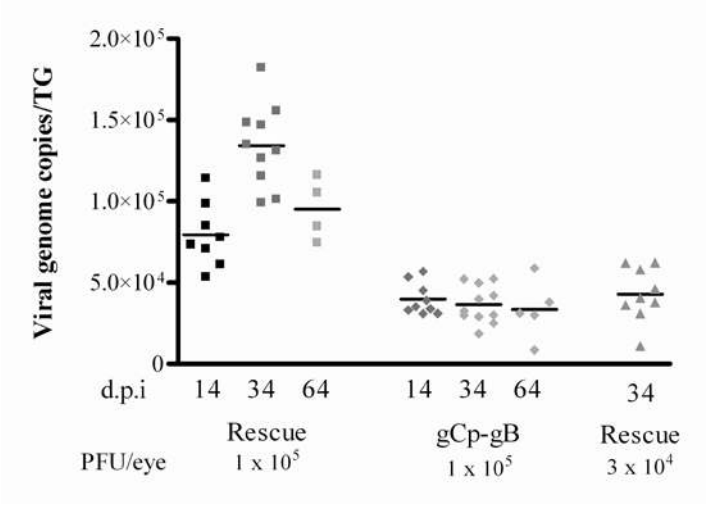

Figure 5-4. HSV-gCp-gB establishes latency with fewer genome copies compared to the Rescue.

Corneas of mice were infected at an infectious dose of $1x10^5$ or $3x10^4$ PFU/eye. TG were excised at 14, 34 and 64 days post infection (d.p.i.) and genome copies were determined by real time PCR. Each data point represents the viral genome copy from a single TG. The data are combined from two independent experiments. At a similar infectious dose ($1x10^5$) the rescue virus induced a significantly higher ($p<0.05$) latent viral load than the gCp-gB virus at all times tested. Reducing the infectious dose of rescue virus 3-fold relative to that of gCp-gB resulted in latent viral loads that were not significantly different ($p>0.05$). Data were analyzed by a Student's *t*-test.

5.4.4 Delayed onset of gB expression does not influence the initial expansion of gB-CD8 in the lymph nodes, but does reduce their accumulation and retention in the infected TG.

We next asked whether the altered kinetics of expression of gB would influence the initial expansion of gB-CD8 in the draining lymph nodes following corneal infection. At 8 d.p.i a similar total number (Fig. 5-5 A left) and frequency of gB-CD8 (Fig. 5-5A right) were observed in lymph nodes of mice infected with gCp-gB and rescuant, indicating that delayed gB expression did not impair priming of naïve $CD8^+$ T cells. Concordant with lower replication levels of gCp-gB in the TG, the total numbers of $CD8^+$ T cells infiltrating gCp-gB and rescuant infected TG were significantly reduced at all time points observed (Fig 5-5B left panel). Analysis of the gB-CD8 population in the TG revealed the expected expansion (8 d.p.i.), contraction (14-30 d.p.i.) and stable memory population (>30 d.p.i) with both gCp-gB and rescue virus (Fig. 5-5B right panel). However, the gB-CD8 effector population was diminished in gCp-gB acutely infected TG at 8 d.p.i. and 14 d.p.i (Fig. 5-5B right panel) and while both viruses established a stable gB-CD8 memory population by 34 d.p.i., the frequency of these cells was significantly reduced in gCp-gB (18%) infected TG compared to its rescuant (49%) (Fig 5-5B right panel).

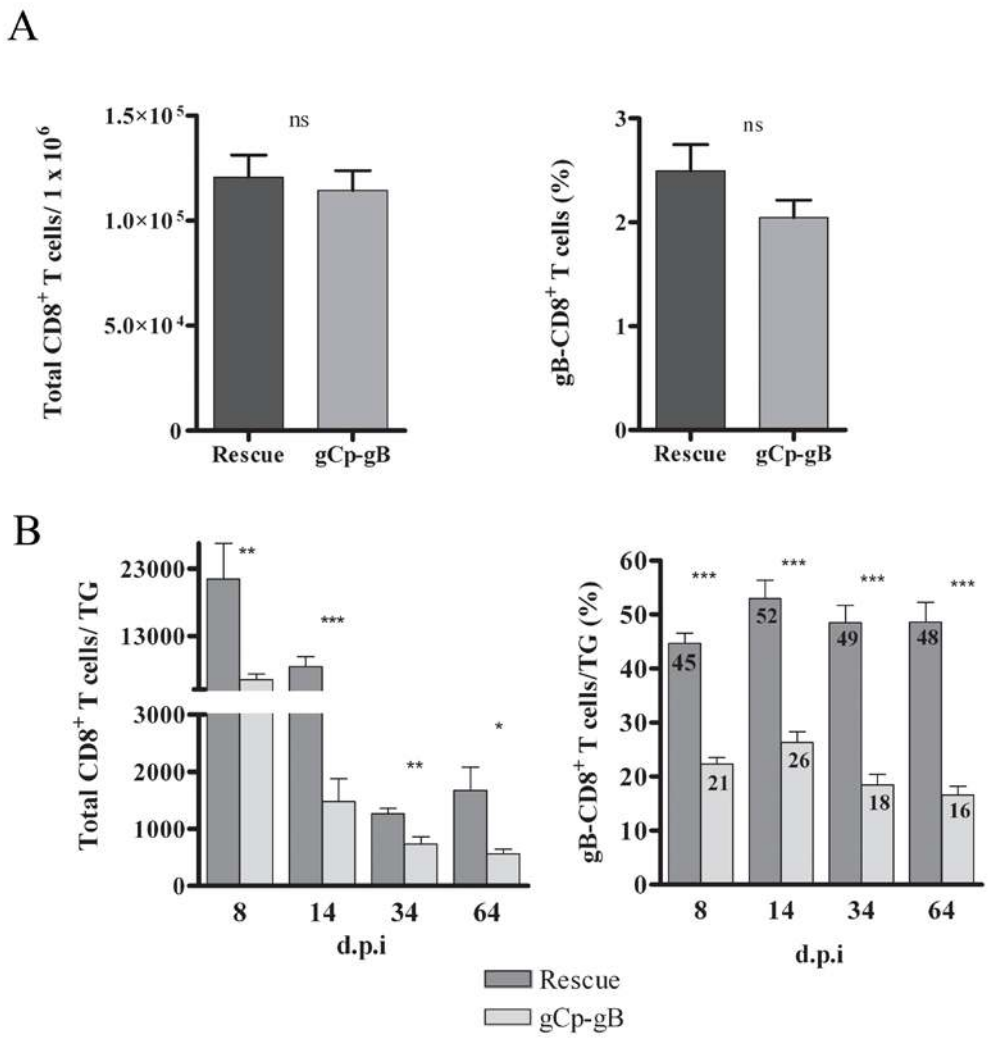

Figure 5-5. HSV-gCp-gB infected mice contain fewer gB-CD8 in their TG but not lymph nodes.

TG or lymph nodes were excised and dispersed into single cell suspensions and stained for CD45, CD8, $gB_{498-505}$-T cell receptor or intracellular granzyme-B expression, and analyzed by flow cytometry. The data are represented as the mean +/- SEM of the mean. **A)** The graph represents the total number of $CD8^+$ T cells (left) and the percentage of gB-$CD8^+$ T cells (right) in draining lymph nodes of mice infected with 1 x 10^5 PFU/eye of either Rescue or gCp-gB at 8 d.p.i. The data are combined from two independent experiments. The mean numbers of total $CD8^+$ T cells and the percentages of gB-CD8 between Rescue and gCp-gB is not significantly different as assessed by Students' *t*-test ($p>0.05$). **B)** The left panel represents the total number of $CD8^+$ T cells and the right panel represents the percentage gB-$CD8^+$ T cells per TG from mice infected with 1 x 10^5 PFU/eye HSV. The mean for the percentage of gB-$CD8^+$ T cells is shown within each bar graph. The data are combined from three independent experiments. The mean percentages of total and $gB_{498-505}$-specific CD8 T cells between Rescue and gCp-gB is significantly different at all time points tested as assessed by Students' *t*-test ($p<0.05$).

5.4.5 gCp-gB fails to activate gB-CD8 during latency.

Non-HSV-specific $CD8^+$ T cells (OT-1) are gradually lost from the latently infected TG over time, while a stable population of HSV-specific $CD8^+$ T cells is maintained, suggesting that antigenic exposure is required to maintain the TG resident $CD8^+$ T cell population (145). Therefore, we hypothesized that the low frequency of gB- $CD8^+$ T cells in gCp-gB infected TG during latency might reflect reduced antigenic exposure. We assessed gB-CD8 activation based on intracellular granzyme B expression in TG harboring equal ganglionic loads of gCp-gB and rescuant virus. We measured the absolute number (Fig. 5-6 left) and frequency (Fig. 5-6 right) of granzyme B positive gB-CD8 during the acute phase at 8 d.p.i, when there is high level of antigen and during latency at 34 d.p.i where antigenic exposure is minimal. While the absolute number of gB-CD8 at 8 d.p.i was significantly different between gCp-gB and Rescue, the frequency of gB-CD8 retained in the TG for each infection were almost equal (although significantly different). However at 34 d.p.i, the frequency of gB-CD8 was greatly reduced in gCp-gB infected TG, consistent with reduced antigenic exposure.

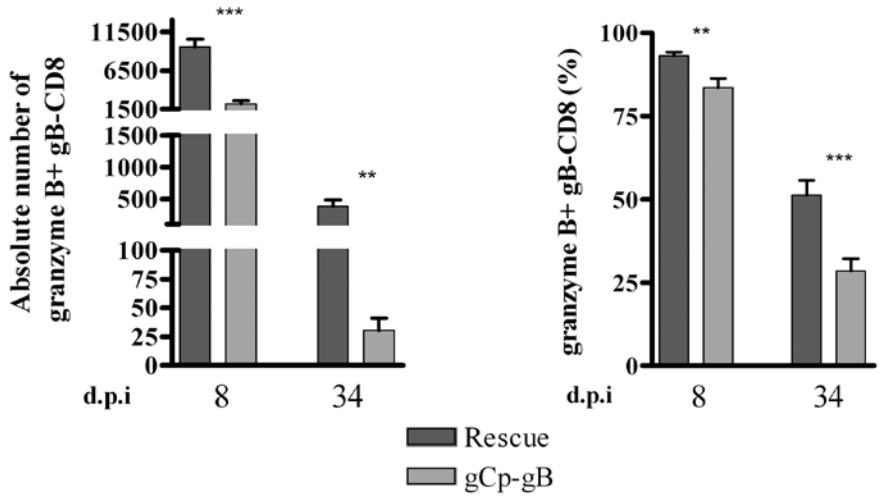

Figure 5-6. Fewer gB-CD8 in the TG of gCp-gB mice are activated during latency.

The graphs represent the absolute number of gB-CD8$^+$ T cells (left) and percentage of gB-CD8 (right) in infected TG expressing intracellular granzyme-B at 8 or 34 d.p.i. from mice infected with 3 x 10^4 PFU/eye Rescue and 1 x 10^5 PFU/eye gCp-gB to yield equal genome loads. The data are combined from two independent experiments. The differences between the absolute numbers and percentages are significantly different at all time points as assessed by a Students' *t*-test ($p < 0.01$).

5.4.6 The diminished gB-CD8 population in gCp-gB latently infected TG is not due to growth impairment.

To determine if the altered gB-CD8 frequency in gCp-gB infected TG during latency resulted from impaired virus replication during acute infection we compared the absolute number and frequency of gB-CD8 in TG that were latently infected with gCp-gB or a second recombinant HSV-1 in which replication was similarly impaired by disruption of the U_S3 kinase gene (U_S3KO; Fig. 5-1 iv). In U_S3KO, gB is expressed from its native promoter and thus made at low levels prior to viral DNA synthesis. The U_S3KO and gCp-gB viruses induce a similar load of latent virus in the TG at 34 d.p.i. (Fig. 5-7A), but the TG harboring latent U_S3KO virus exhibited a significantly larger population of gB-CD8 (Fig. 5-7B), indicating that late gB

expression, rather than a low copy number, leads to the reduced frequency of gB-CD8 in TG harboring latent gCp-gB.

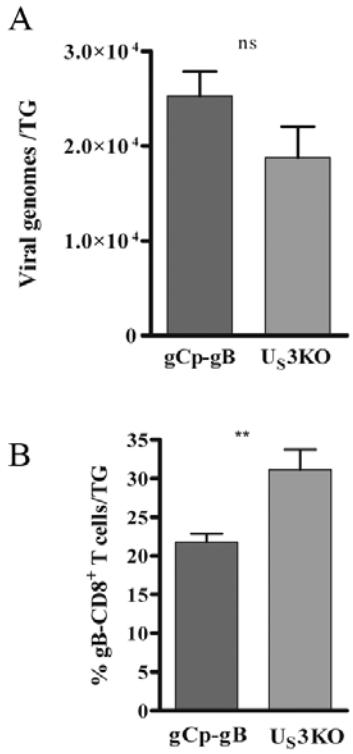

Figure 5-7. Delaying gB contributes to the diminished gB-CD8 response in the TG.

Mice were infected at 1 x 10^5 PFU/eye with gCp-gB or another replication impaired HSV-1 lacking the U_S3 kinase (U_S3KO). The data shown are combined from two independent experiments. **A)** Viral genome copies were determined by real time PCR at 34 d.p.i. The data is represented as the mean +/- SEM of the mean. The differences between gCp-gB and U_S3KO genome copy numbers are not significantly different as assessed by Students' *t*-test ($p>0.05$). **B)** Single cell suspensions of TG infected for 34 days were stained for CD45, CD8 and $gB_{498-505}$ TCR expression and analyzed by flow cytometry. The data is presented as the mean +/- SEM of the mean. The percentage of $gB_{498-505}$-specific CD8 T cells (B) is significantly different between Rescue and gCp-gB at all time points tested as assessed by Students' t-test ($p=0.0034$).

5.4.7 gB-CD8 are capable of blocking reactivation of gCp-gB.

The early expression of gB before viral DNA synthesis might provide a requisite window of opportunity for gB-CD8$^+$T cells to prevent full reactivation and virion formation. To test this hypothesis, cultures of dispersed TG harboring similar loads of latent gCp-gB or rescuant virus were depleted of endogenous CD8$^+$ T cells, reconstituted or not with purified gB-CD8, and monitored for HSV-1 reactivation based on recovery of infectious virus from culture supernatants. Figure 5-8A verifies that the TG cell suspensions were effectively depleted of endogenous CD8$^+$ T cells. In the absence of added gB-CD8$^+$ T cells, reactivation was observed in neurons harboring both gCp-gB and the rescuant, although the reactivation frequency was slightly lower in neurons harboring gCp-gB (Fig. 5-8B). Notably, late gB expression did not impair the ability of the gB-CD8$^+$ T cells to block reactivation. Thus, gB-CD8 can act very late in the viral life cycle to block full reactivation and formation of infectious virus.

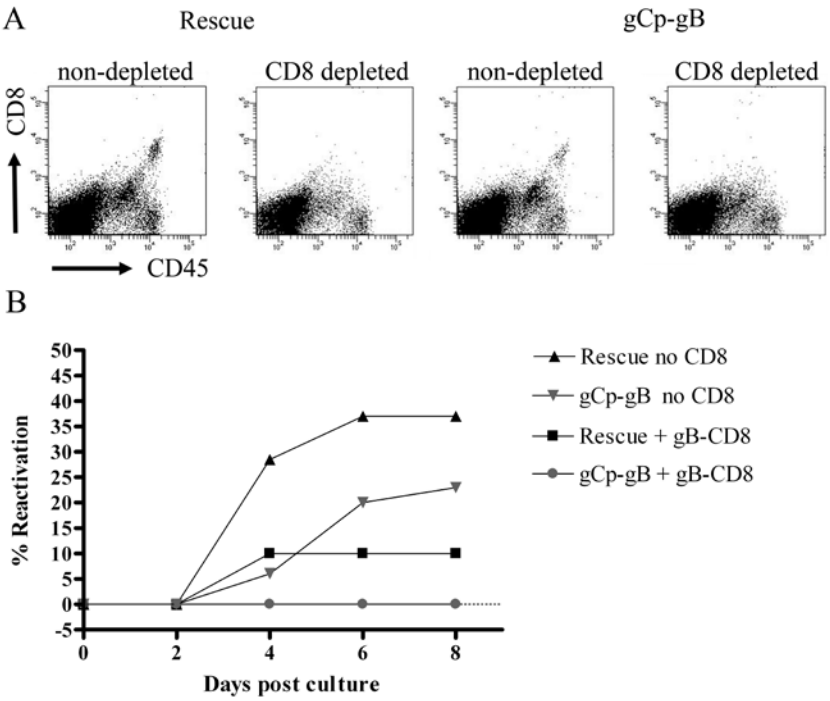

Figure 5-8. gB-CD8 can block gCp-gB reactivation.

Mice were infected with 3 x 10^4 PFU/eye Rescue and 1 x 10^5 PFU/eye gCp-gB to establish equal genome copies during latency. TG were excised at 34 d.p.i. and single cell suspensions were depleted of CD8 as described in the materials and methods section. Depleted TG were plated as $1/5^{th}$ TG cultures and half the cultures received add-back of $gB_{498-505}$-specific CD8 T cells (gB-CD8 T cells) and the other half did not. Reactivation was monitored by sampling supernatants for infectious virus via plaque assay. **A)** Representative dot plots before and after CD8 depletion, showing depletion efficacy. **B)** Representative graph showing reactivation frequency of Rescue and gCp-gB virus with or without gB-CD8 add-back. The experiment was repeated three independent times with similar results.

5.4.8 Growth impairment in primary cells involves the eIF2α pathway.

The impaired growth of gCpgB virus in the TG was intriguing, since the majority of gB is made following viral DNA replication in HSV-1 RE infected cells. In the course of these studies, it was reported that gB also acted at a level to counteract the cell induced stress response involving

an ER-resident kinase, PERK (113). ER stress results in PERK activation by auto-phosphorylation which subsequently phosphorylates eIF2α, a eukaryotic translation initiation factor required for translation. Mulvey *et al* showed HSV-1 gB binds to PERK to block phosphorylating eIF2α. Impaired gCp-gB growth in the TG may be partly caused by the inability of gB to affect the PERK stress response pathway early on in the lytic cycle. We therefore determined if eIF2α phosphorylation was higher in gCp-gB infected cells. For this work, a primary cell type derived from the cornea was used, since we rationalized that subtle differences in viral growth might be more apparent in cell lines closes to the natural host. When a multistep growth curve was assessed on primary corneal fibroblast (PCF) cells following a low mulitplicity infection of 0.01, we found at 24 h.p.i that gCp-gB shows a significant reduction in viral titer within the infected cell compared to the Rescue (Fig. 5-9A). This is in contrast to the lack of growth difference observed in Vero cells (Fig. 5-3A). We then assessed the levels of phosphorylated eIF2α in PCF cells infected with gCp-gB or Rescue in a single step infection.. In each case of viral infection, thapsagargin, a chemical inducer of the ER stress response, induced signficantly large amounts of phosphorylated eIF2α, indicating that eIF2α could be potentially phosphorylated in infected cells. In rescue virus infected cells, relatively low levels of eIF2α were detected at all time points. However, there was overall a detectable increase in eIF2α phosphorylation in gCp-gB infected cells compared to Rescue at all time points. Furthermore, there was a noticeable reduction of phosphorylated eIF2α at late times post infection in Rescue-infected cells coinciding with a peak in gB-accumulation. In gCp-gB-infected cells, there was a more modest reduction in phosphorylated eIF2α indicating that this virus was impaired in its ability to overcome this axis of the stress response. In both viruses, when gB expression was minimized by performing infections in the presence of PAA, eIF2α

remained phosphorylated demonstrating that gB expression was required for the dephosphorylation of eIF2α (Fig. 5-9B)

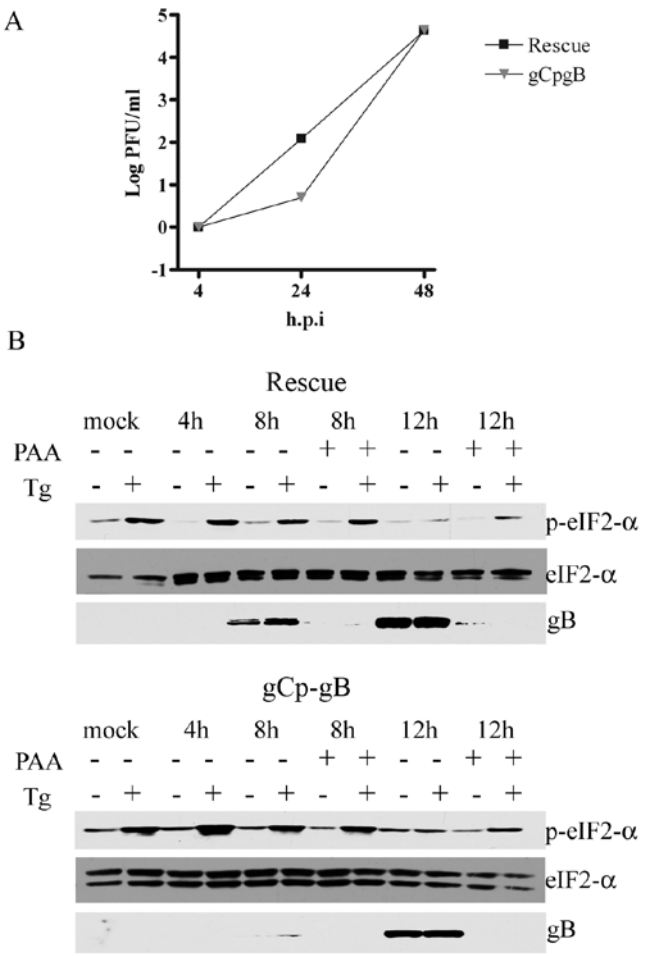

Figure 5-9. Growth impairment of gCp-gB in primary cells is in part due to host control through eIF2α.

A) Representative graph of growth curves in primary corneal fibroblasts (PCF) cells. Confluent cells were infected at an MOI = 0.01 with either Rescue of gCp-gB. At indicated times post infection cells and supernatants were harvested and infectious virus was assayed via plaque assay. The difference between viral titers of Rescue and gCp-gB is significantly different at 24 h.p.i ($p<0.05$). **B)** eIF2α phosphorylation pattern in PCF cells. Confluent cells were infected at an MOI = 5 with or without 350ug/ml phosphonoacetic acid (PAA). Cells were treated with 0.5uM thapsigargin (Tg) 30 minutes prior to harvest or left untreated. Samples were analyzed by western blotting for eIF2α, phosphorylated eIF2α (p-eIF2α) and gB.

5.5 DISCUSSION

To our knowledge this is the first study to assess the influence of viral gene expression kinetics on host immunity. We employed an infection model in which latent virus influences the functional characteristics of the memory $CD8^+$ T cell population (146). The sequential expression of HSV-1 genes during lytic infection has been appreciated for some time, but little is known about the importance of expression kinetics for viral virulence or for targeting of viral proteins by host immunity. Using classical molecular approaches gB was shown to be produced early in the viral life cycle before viral DNA replication, leading to its classification as a leaky-late γ1 gene (122, 130). Very low levels of protein synthesis are required to sensitize cells for recognition by $CD8^+$ T cells (163). Accordingly, while gB is hardly detectable by immunoblotting at very early stages of infection, Carbone and colleagues showed *de novo* gB synthesis can be detected by gB-CD8 as early as 2 hours post infection (112). Moreover, $gB_{498-505}$ represents a strongly immunodominant epitope that is recognized by up to 70% of HSV-specific $CD8^+$ T cells in lymphoid organs of C57BL/6 mice. Collectively, these findings suggest that early expression of gB might have important implications for both replication and host immunity.

We observed that gCp-gB exhibited a significant loss of neurovirulence in infected mice. HSV-1 gB is required for HSV-1 entry into cells by mediating fusion of the viral envelope to the host cell membrane (52, 172). However, several recent studies have demonstrated other functions for gB during the viral life cycle. Importantly, gB is able to counteract the host-mediated shut down of protein translation thus allowing viral replication to continue. gB is able to do this by binding to PERK, a kinase able to detect cell stress, and consequentially preventing the phosphorylation of eIF2α, thus allowing protein translation to continue (113). This appears to

be a redundant mechanism to allow viral replication to continue, since eIF2α phosphorylation is also reversed by γ34.5, also a γ1 gene (18, 176). In our studies we observed some growth impairment of gCp-gB virus in the cornea and a more severe impairment in the TG *in vivo*, but a normal growth curve was observed when gCp-gB virus was grown in Vero cells in vitro. This is in agreement with our observation of gCp-gB virus growth impairment when grown in primary corneal fibroblasts that was correlated with a slight increase in eIF2α phosphorylation. Thus the growth impairment of gCp-gB seen in the TG of infected mice could be in part due to the virus' inability to overcome the host response. Other studies have also shown that phosphorylation of gB is essential for the egress of nucleocapsids from the inner nuclear membrane (185). So there also exists the possibility that transport of gCp-gB capsids from the cornea to the nerve body is compromised which could also contribute to its poor growth in the TG. Our findings emphasize the selective importance of the kinetics of gB expression for viral growth in certain cell types. The mechanisms underlying the growth impairment of gCp-gB are currently under investigation.

HSV-1 latency differs from chronic infections in that viral DNA replication does not take place and viral protein expression is largely silenced. However, HSV-1 latency is no longer considered an entirely antigenically silent state. Recognition of latent virus by $CD8^+$ T cells was first suggested by the observation that activated $CD8^+$ T cells surround infected neurons in murine latently infected TG, where spontaneous reactivation and virion formation was not observed (87). Subsequent studies confirmed this notion by demonstrating that gB-CD8 release lytic granules into an immunologic synapse *in situ* (67, 70), and that the endogenous $CD8^+$ T cells in dispersed latently infected TG can block HSV-1 reactivation from latency in *ex vivo* TG cultures (67, 88). gB-CD8 employ both IFN-γ and lytic granules to prevent HSV-1 reactivation in neurons with preservation of neuronal viability (31, 70). Moreover, recent findings suggest

that ganglionic $CD8^+$ T cells that are not specific for $gB_{498-505}$ are nevertheless HSV-specific and capable of blocking HSV-1 reactivation from latency in *ex vivo* TG cultures (145).

We hypothesized that detection of viral protein early in the reactivation process is essential for the ability of $CD8^+$ T cells to block full HSV-1 reactivation prior to virion formation. Moreover, the maintenance of a constant level of latent viral genomes in the TG over long periods of latency in mice (our unpublished observations) suggested that inhibition of reactivation likely occurs prior to viral DNA synthesis. Indeed gC transcripts are not detectable when latently infected TG are cultured in the presence of $CD8^+$ T cells capable of blocking reactivation (88). To test these theories we created a recombinant virus (gCp-gB) that expresses gB as a late protein from the γ2 regulated gC promoter, thus abrogating expression of gB protein prior to DNA synthesis. This should not influence the level of gB expression since gB and gC are expressed at similar levels at late times post infection (154). The expansion of naïve HSV-specific $CD8^+$ T cells in the lymphoid organs appears to result from cross-presentation of viral antigens rather than by direct presentation by infected antigen presenting cells (7). Accordingly, any of the kinetic classes of HSV-1 proteins could theoretically be presented, and delayed expression of gB should not greatly influence the initial expansion of gB-$CD8^+$ T cells in mice infected with gCp-gB. This was in fact observed as quantitatively similar expansion of gB-CD8 was observed in draining lymph nodes of mice infected with gCp-gB and rescuant virus. Moreover, we observed only a modest (though statistically significant) reduction in the magnitude of the gB-$CD8^+$ T cell population in gCp-gB acutely infected TG (8 d.p.i) when compared those infected with the rescue virus. One of the most striking observations was that during latency the frequency of gB-CD8 was diminished over time in TG harboring latent gCp-gB. Combined with our observation that activated Ova-specific $CD8^+$ T cells that enter the TG

during acute infection are lost over time during latency (145), these findings suggest a lack of antigenic exposure to gB-CD8 when gB is expressed as a true late gene in gCp-gB.

Persistent antigenic exposure of gB-CD8 was also suggested by previous studies that demonstrated that gB-CD8 in latently infected TG exhibit a more activated phenotype and are less reliant on homeostatic proliferation signals than their counterparts in non-infected tissues (145). Here we show that significantly fewer gB- CD8 are positive for intracellular granzyme B in TG harboring latent gCp-gB compared to rescuant, even when the load of latent virus was equalized. Since granzyme B represents an activation marker for $CD8^+$ T cells (145, 162), this data provides further support for the concept that gB-CD8 receive less antigenic exposure in latently infected TG when gB is expressed as a true late gene after viral DNA synthesis. Furthermore this data is also consistent with the idea that in wild type infections, sporadic expression of gB by reactivating neurons is quickly recognized by gB-CD8 which are then able to block reactivation prior to DNA replication.

We have previously shown that gene expression kinetics are similar during reactivation and lytic replication, at least with respect to γ1 (gB) and γ2 (gC) genes (refer Chapter 4). In a wild type virus, gB is expressed relatively early so it seems obvious to surmise that gB-CD8 can recognize their viral antigen early and shut down reactivation before DNA replication. We hypothesized that once DNA replication takes place it is too late in the viral life cycle for $CD8^+$ T cells to block reactivation, as the virus has already committed to assembly and egress. However, our findings establish that gB-CD8 can indeed block reactivation even after viral DNA replication. This is consistent with the ability of the $CD8^+$ T cell effector molecule IFNγ to block reactivation even after late gene expression (31). $CD8^+$ T cells can employ lytic granules and IFN-γ to block reactivation of wild type HSV-1 from latency without neuronal destruction (70). It remains to be determined if the effector mechanism(s) employed by gB-CD8 to block

reactivation when gB is expressed only after viral DNA synthesis are compatible with neuronal preservation.

The data from this study can be used to design better therapeutic vaccines for HSV-1. While $CD8^+$ T cells responding to late antigens are still able to block reactivation, they are not retained in high numbers in the TG during latency. This makes late viral antigens a poor choice as the sole immunogen. There is an obvious utility to targeting viral proteins expressed before DNA replication. A block in reactivation after viral DNA synthesis would permit an accumulation of viral genomes in neurons, which is associated with a greater likelihood of reactivation (60). A study by Straus and colleagues demonstrated that the rate of HSV-1 reactivation is proportional to the number of latent genomes and inversely proportional to the number of $CD8^+$ T cells retained in the TG. They therefore suggest that vaccines should be evaluated for their ability to induce and maintain a virus-specific $CD8^+$ T cell response as well as a low genome load in the ganglia (60). Our findings suggest that immunizing against viral α, β, and γ1 proteins that are expressed before DNA replication would optimize the capacity of the immune system to maintain HSV-1 in a latent state and prevent recurrent disease.

6.0 ANALYSIS OF THE gB-CD8$^+$ T CELL RESPONSE IN C57BL/6 MICE THROUGH OVEREXPRESSION OF AND MUTATIONS IN THE IMMUNODOMINANT $gB_{498-505}$ EPITOPE USING RECOMBINANT HSV-1

6.1 ABSTRACT

In HSV-1 latently infected trigeminal ganglia (TG), a small but stable number of $CD8^+$ T cells surround latently infected neurons and actively block reactivation. Periodically, external stress stimuli can result in loss and dysfunction of some of these TG-resident $CD8^+$ T cells, leading to reactivation and recurrent disease. In the absence of an existing vaccine to prevent reactivation, we addressed whether it would be possible to augment the antigen-specific $CD8^+$ T cell response in the TG to larger numbers. To test this, we exploited the C57Bl/6 mouse model in which approximately half of the HSV-specific $CD8^+$ T cells retained in the TG are specific to one epitope on glycoprotein B ($gB_{498\text{-}505;}$ gB-CD8). We created a recombinant HSV-1 (13LAT) which expresses a multimer of thirteen tandem repeats of the $gB_{498\text{-}505}$ epitope from a promoter that is highly active during latency (the LAT promoter), thus providing consistent exposure of antigen to surrounding $CD8^+$ T cells. Compared to a wild type behaving control virus, 13LAT replicated efficiently in the TG and also established latency with similar genome loads. Even though 13LAT induced a 2-fold greater total $CD8^+$ T cell response in the TG, the frequency of gB-CD8 was not altered by overexpression of these peptides. Furthermore, the greater number of $CD8^+$ T cells did not appear to prevent reactivation *ex vivo*. Since we were not able to induce a greater gB-CD8 response or differentiate the gB-CD8 response induced by endogenous gB protein and our ectopic peptides present in the 13LAT virus, we engineered new recombinant HSV-1 in which the endogenous gB epitope is mutated. We tested four mutant viruses and one wild type derivant. While the wild type derivant still induced a gB-CD8 response, all mutant viruses were successful in abrogating a gB-CD8 response in the TG. Based on these studies, we will rederive the multimer peptide viruses into the background of one of these viruses. This will allow us to test the efficacy of our vaccine strategy in the absence of an endogenous gB peptide.

6.2 INTRODUCTION

HSV-1 infections in humans can reactivate frequently and lead to recurrent disease. Primary HSV-1 infection of the ocular or orofacial region results in the establishment of latency in the trigeminal ganglia (TG). Recurrent reactivation of the virus from the TG results in amplification of virus in the periphery where it can cause several complications. Viral replication in the eye can lead to a severe immunopathology known as herpes stromal keratitis (HSK). Virus in the eye initiates an immune infiltrate into the cornea which can cause disorganization and destruction of the collagen matrix of the stroma and the formation of scar tissue (9, 82, 110). Corneal scarring resulting from repeated HSK events can ultimately lead to blindness. HSV-1 is the leading cause of infectious blindness in the developed world (86).

Following an HSV-1 infection, $CD8^+$ T cells infiltrate the TG and a maximum infiltrate is observed concurrent with the establishment of latency at around 8 d.p.i. The $CD8^+$ T cell population then undergoes a massive contraction phase from 8 d.p.i until 34 d.p.i where a small but steady population is retained in the TG. In the latently infected TG, $CD8^+$ T cells polarize their TCR toward these neurons and release cytokines and lytic granules into the neurons (67, 70). TG-resident $CD8^+$ T cells also remain in an activated state during latency suggesting exposure to antigen (67). Further proof of antigen exposure comes from studies demonstrating the presence of several viral transcripts within latently infected TG in the absence of infectious virus (39, 99) . These $CD8^+$ T cells are responsible for maintaining the virus in a latent state and their depletion results in reactivation (41). Furthermore, $CD8^+$ T cells maintain HSV-1 latency without causing neuronal apoptosis (70). In *ex vivo* reactivation assays, when TG from 14 d.p.i are cultured in the presence of endogenous $CD8^+$ T cells, reactivation is not observed. However, when TG from 34 d.p.i are cultured in a similar assay, full reactivation is observed (88). The

difference between these two time points is that there are far fewer numbers of $CD8^+$ T cells present at 34 d.p.i TG compared to 14 d.p.i. Addition of exogenous $CD8^+$ T cells to 34 d.p.i *ex vivo* cultures successfully prevents reactivation (70). A recent study corroborating this data demonstrates that the rate of reactivation is inversely proportional to the number of $CD8^+$ T cells retained in the TG and directly proportional to the number of latent genomes (60). *In vivo*, compromising either $CD8^+$ T cell number or function through *in vivo* depletion or stress treatment leads to reactivation (41). Combined, these data demonstrate that during latency, there is a delicate balance between viral latency and the number of $CD8^+$ T cells maintained in the TG and that functional or numerical impairment of $CD8^+$ T cells or both can lead to reactivation.

Currently, there is no therapeutic vaccine available for preventing HSV-1 reactivation. The concept behind therapeutic vaccines to boost the existing local immunity to HSV-1 such that even in times of stress (a common trigger for reactivation), the immune system may prevent full viral reactivation or at least limit disease at the periphery. Therapeutic vaccine designs have centered on the use of subunit and attenuated live virus vaccines to prevent HSV reactivation. While they have demonstrated some efficacy in animal models, they have not been successful in human trials (114-115, 134, 160). Subunit vaccine trials in humans were successful in boosting neutralizing antibody responses in seropositive individuals but did not reduce shedding rates (161). A trial with an attenuated live virus vaccine proved to be safe to use in humans but provided no benefit to vaccinated individuals as their shedding rates did not differ from unvaccinated individuals. Furthermore, this vaccine did not prevent asymptomatic shedding as 82% of vaccinated individuals were PCR positive for virus in the periphery (29).

The use of viral immunodominant peptide epitopes that are recognized by $CD8^+$ T cells have been used as immunogens in prophylactic vaccine studies. Tandem repeats of HSV-1 $CD8^+$ T cell peptides that were delivered via adenovirus or bacterial vectors were enough to confer

protective immunity in challenged mice or minimize viral load at the site of infection (16, 189). Transgenic mice or rabbits expressing human MHC molecules have been used recently to study the $CD8^+$ T cell response as well as vaccine efficacy in a humanized model (19, 21, 188). In such a model, immunization rabbits with known human $CD8^+$ T cell epitopes from seropositive individuals induced a strong HSV-1 specific $CD8^+$ T cell response and also reduced HSV-1 ocular replication and corneal disease after challenge (20). Various adjuvants have been tested to maximize effectiveness of vaccine delivery including ligand epitope antigen presentation (LEAPS) consisting of a T cell epitope of an HSV-1 protein conjugated to a peptide sequence of β-2-microglobulin; localized delivery of epitopes mixed with known immunostimulatory molecules such as oligodeoxy-nucleotides (ODNs) induced a strong protective peptide specific response and antibody response in animals (46, 114, 136).

While the studies mentioned above have shown some success as prophylactic vaccines, similar strategies have yet to be investigated in the form of therapeutic vaccines. The main struggle faced by scientists is to design a vaccine that prevents full reactivation of the virus. A vaccine that is able to stop symptomatic shedding, but not able to prevent asymptomatic shedding could be detrimental since it would still allow transmission of the virus from person to person. Given the strong role for $CD8^+$ T cells to block reactivation, vaccine strategies should be aimed at augmenting the existing $CD8^+$ T cell response to HSV-1 in the TG. While boosting the overall cellular response to HSV is necessary, more importantly, the local $CD8^+$ T cell response at the site of latency has to be augmented. This would be the optimal approach such that in situations where function of some of the $CD8^+$ T cells in the TG becomes compromised due to stress or other reactivation stimuli, there are enough functional resident $CD8^+$ T cells to overcome this dysfunction and still prevent reactivation. In this study, we employ a method of overexpressing the immunodominant HSV-1 peptide recognized by C57Bl/6 mice ($gB_{498\text{-}505}$)

from recombinant HSV-1 and we assess whether this results in greater antigen-specific $CD8^+$ T cell response that can prevent reactivation.

6.3 MATERIALS AND METHODS

6.3.1 Construction and generation of recombinant multimer HSV-1 containing $gB_{498-505}$ epitope repeats.

The recombinant viruses containing $gB_{498-505}$ epitope repeats were generated on the HSV-1 RE background (Fig. 6-1). The primers used to generate the epitope sequence also included the 6 residues before and 4 residues after the actual epitope region (amino acids: RIKKTTSSIEFARLNFTY). The two primers (complementary to each other) used were: gBF 5' GATCCCATGGCGCATAAGACCACCTCCTCCATCGAGTTCGCCAGGCTGCAGTTTACG TACAACCACAAA 3' and gBR 5' GAT CTT TGT GGT TGT ACG TAA ACT GCA GCC TGG CGA ACT CGA TGG AGG AGG TGG TCT TAT GCG CCA TGG TGG 3'. The contain overlaps of GATC on each end and which represents either a BamHI or a BglII site. The oligos were phosphorylated, combined together and heated to 70^{o} C to anneal them to each other. The annealed product was then ligated and cut with BamHI and BglII at 37^{o}C for 3 hours to eliminate head to head and tail to tail dimmers. The ligated products were run on an agarose gel and the bigger DNA bands were cut out (which represent bands containing compatible BamHI ends). The multimer fragments were then cloned into a previously described vector, gC-EGFP in which EGFP is driven from the gC promoter (31). gC-EGFP was cut with BamHI and the fragments containing BamHI ends were ligated into this vector generating a new vector in which the gC promoter drove expression of the peptide multimer fragments fused to EGFP. Isolated colonies were then tested for inserts that had BamHI at the back end of the insert. Fragments containing 4 and 13 repeats were sequenced for their integrity (Fig. 6-1B). Into these were insered the thymidine kinase (*tk)* (from Lst-1 plasmid, a kind gift of Neal Deluca), the LAT LAP2 promoter

(LAP2 plasmid, a kind gift of Bill Goins) the gB promoter (from our gB-EGFP plasmid detailed in Chapter 3), all generated from HinDIII-BamHI cuts from their respective vectors (Fig. 6-1C). The resulting plasmids were then cut with HinDIII and NheI and RFP was inserted into them generating a final plasmid in which the gC promoter drives expression of RFP and either the tk, LAP2 or gB promoters drive the expression of either 4 or 13 repeats of the gB epitope fused to EGFP (Fig. 6-1D). The final plasmids were linearized with SspI and cotransfected along with HSV-1 RE DNA to generate recombinant plaques. Recombinant viruses were screened by their gain of RFP fluorescence and subjected to several rounds of purification. The purified viruses were checked for purity by Southern blotting analysis. The multimer repeats were also cloned into EGFP-C3 or EGFP-N1 vectors to generate 4 or 13 repeats of the peptide fused to either the C-terminus (6C3 or 13C3) or the N-terminus (6N1 or 13N1) of EGFP and expressed under the CMV IE promoter. We generated these constructs to test peptide expression *in vitro* assays. The CMV constructs generated are listed in Table 1 and the multimer viruses created are listed in Table 2. The above mentioned constructs and viruses were generated by Christina Ferko.

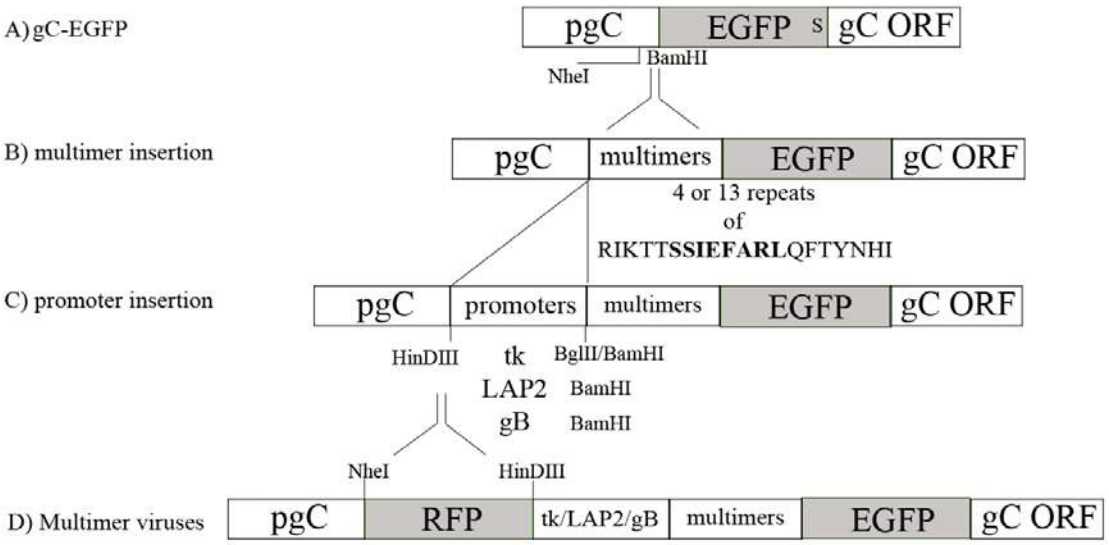

Figure 6-1. Construction of $gB_{498-505}$ epitope multimer viruses.

A) Representation of the gC-EGFP construct in which EGFP expression is controlled by the gC promoter. **B)** Multimer insertion involved restriction digest of gC-EGFP construct with BamHI and insertion of 4 or 13 repeats of the multimer into this site. **C)** Insertion of various promoters. The construct containing the multimers was digested with HinDIII and BgllII and BamHI (*tk* promoter) or BamHI alone (LAP2 and gB promoters). The respective promoters were digested with the same enzymes from their parent constructs listed in the materials and methods and ligated to create constructs in which the multimer expression was under the control of the *tk*, LAP2 or gB promoters. **D)** The constructs were then each digested with NheI and HindIII and RFP was inserted into the construct. The final construct was such that the gC promoter drove the expression of RFP followed by one of the tk, LAP2 or gB promoters driving the expression of 4 or 13 repeats of the gB multimer fused to EGFP. The constructs were recombined into the gC locus of HSV-1 RE and the recombinant viruses are null for gC protein. The resultant viruses are listed in table 2.

Table 1. List of multimer constructs under the CMV IE promoter

Construct	*EGFP terminus*	*Number of repeats*
4N1	N-terminus	4
13N1	N-terminus	13
4C3	C-terminus	4
13C3	C-terminus	13

Table 2. List of multimer viruses

Virus	*Promoter*	*Number of repeats*
4LAT	LAP2	4
13LAT	LAP2	13
4TK	TK	4
13TK	TK	13
4gB	gB	4
13gB	gB	13

6.3.2 Construction and generation of gB null (gBKO) HSV-1

We generated gB null viruses that do not express gB Protein. To do this the back end of gB (excluding the epitope region) was amplified using the following primers: gBBackF 5' GCGCCTAGGCTCGGATCCCAGTTTACGTACAAC 3' and gBBackR 5' GAGCGGAATTCATTTACAACAAACCCCCCATCA 3'.

This fragment was cloned into a previously existing plasmid used to make the gC-Rescue in Chapter 5. Thus the resulting plasmid contained gB promoter driving the expression of gB and had unique BamHI and AvrII sites within the back end of the gB sequence (plasmid 1973) (Fig. 6-2 i). EGFP was then amplified from EGFP-C3 using the following primers: gBF-EGFP 5' CCC TAG GCT ACC TGA CGG CGG GCA CGA CGG 3' and 5' TTG TAC GTA GGA TCC TTA CTT GTA CAG CTC GTC 3'. The previous construct was cut with BamHI and AvrII and the EGFP amplicon was cloned into that site resulting in a plasmid containing the gB promoter driving the expression of EGFP fused to the back end of gB (plasmid 1976) (Fig. 6-2 i). This

plasmid effectively does not express functional gB and is missing the epitope region. The plasmid was then linearized with an sspI cut and cotransfected into gB-Veros (Veros expressing gB from the native gB promoter) along with WT HSV-1 RE genomic DNA. Selection of plaques was based on gain of EGFP fluorescence and plaque picks were subjected to several rounds of purification (Fig. 6-2 iii). Transfection of gBKO genomic DNA into Veros confirmed the absence of functional gB as no plaques were formed in this cell type due to the lack of complementing gB to allow viral replication. The gBKO viruses were generated by Kip Kinchington and Srividya Ramachandran.

6.3.3 Generation of DNA constructs and recombinant HSV-1 containing mutations in the $gB_{498-505}$ epitope region.

The gB epitope mutants that were created for this study are listed in Table 3 along with their names and respective mutations (in bold and red) that were inserted into the $gB_{498-505}$ sequence. The forward primer used for all constructs was gBFrontF:

5’ GCCCTAGGCTACCTGACGGGGGGCACGACGGGCCCCCGTAG 3’.

The reverse primers (gBFrontR) used to generate each of the mutations are listed for each mutant in Table 4. The gB front sequence was amplified from HSV-1 RE viral DNA and cloned into a pGEM vector for easy selection. Following selection of positive clones and sequencing confirmation, each of the mutant constructs were digested with SnaBI and AvrII and ligated into the 1976 construct mentioned previously also digested with the same enzymes (1976 construct expresses EGFP fused to the back end of gB). The resulting construct now contains gB promoter driving the expression of full length gB with the respective mutations in the $gB_{498-505}$ region (gB-

plasmids) (Fig. 6-2 iv). The plasmids were also engineered to be expressed under the CMV IE promoter for high expression *in vitro* assays. To do this, the constructs were digested with AvrII and EcoRI and ligated into the EGFP-C3 backbone which was cut with NheI and EcoRI (dropping out the EGFP sequence). The resulting plasmids contained the gB epitope mutants expressed from the CMV promoter (CMV-plasmids).

In attempting to generate recombinant viruses from the gB-plasmid constructs, we found that none of the plasmids yielded viable recombinant plaques. Therefore, to repair the back end of these gB constructs, HSV-gCRescue or RE genomic DNA was digested with EcoRI $^{+}$ SnaBI. The 6282bp fragment was excised and eluted and then ligated to the 5kb fragment generated from a digest of the gB-plasmid DNAs with the same restriction enzymes. The resulting constructs were sequentially digested with EcoRI and then HindIII. The 8kb fragment was excised, gel-purified, and co-transfected with HSV-gBKO1 infectious DNA in Vero cells. Any plaques that formed were pooled and screened by PCR amplification of 500 bp spanning the gB promoter and front end of gB ORF containing the unique AvrII site. The PCR products were then digested with AvrII to confirm that recombination of the gB-epitope mutant constructs and the gBKO DNA had taken place. The epitope mutant constructs were generated by Drew Comrey and Michael Yee.

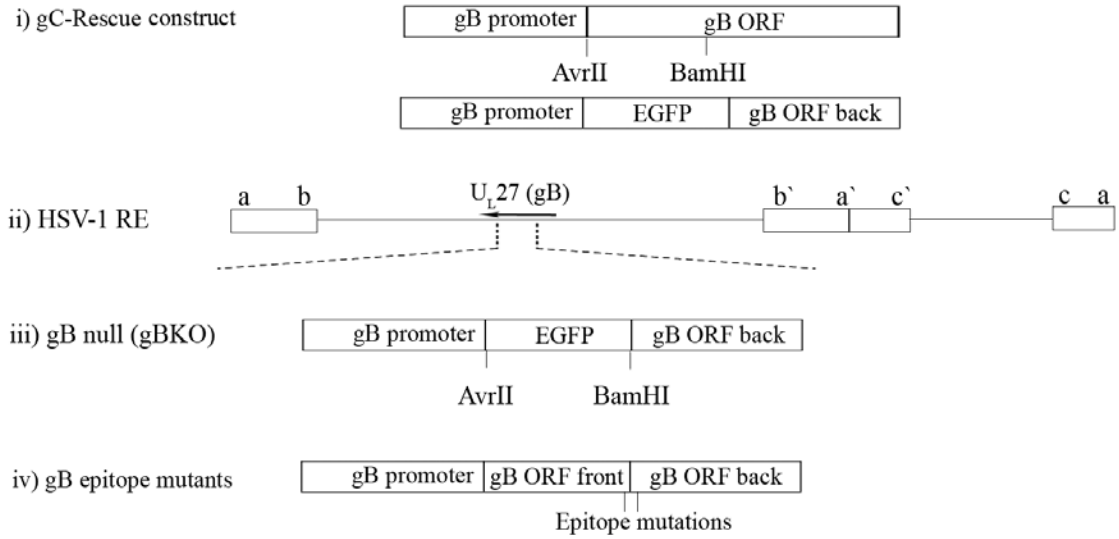

Figure 6-2. Construction of gBKO viruses and $gB_{498-505}$ epitope mutant viruses.

i) Representation of the gC-Rescue construct designed in Chapter 5. The construct was digested with AvrII and BamHI and EGFP that was also digested from construct EGFP-C3 with the same enzymes was inserted which effectively disrupts the gB ORF. **ii)** Representation of the HSV-1 RE genome highlighting the gB. **iii)** Representation of the gBnull (gBKO) virus within the gB locus of the virus. **iv)** Representation of the epitope mutant viruses showing the region the mutations were inserted. The mutants were generated via recombination into gBKO DNA. The mutants are listed in Table 3.

Table 3. List of $gB_{498-505}$ mutations.

Number	*Name*	*Mutation*
1	WT	SSIEFARL (none)
2	L8A	SSIEFAR**A**
3	F5L	SSIE**L**ARL
4	S1G	**G**SIEFARL
5	S1L	**L**SIEFARL
6	S1G/L8A	**G**SIEFAR**A**
7	S1G/I3A	**G**S**A**EFARL
8	L8A/R7K	SSIEFA**KA**
9	S1G/I3N/F5L/E4S (SIFE)	**G**S**NSL**ARL

Table 4. List of reverse primers used to generate $gB_{498-505}$ epitope mutants.

Name	*Reverse primers used*
WT	5' GTT GTA CGT AAA CTG CAG CCT GGC GAA CTC GAT GGA GGA GGT GGT CTT GAT GCG CTC CA 3'
L8A	5 ' GTT GTA CGT AAA CTG AGC CCT GGC GAA CTC GAT GGA GGA GGT GGT CTT GAT GCG CTC CA 3'
F5L	5' GTT GTA CGT AAA CTG CAG CCT GGC CAA CTC GAT GGA GGT GGT CTT GAT GCG CTC CA 3'
S1G	5' GTT GTA CGT AAA CTG CAG CCT GGC GAA CTC GAT GGA GGT GGT CTT GAT GCG CTC CA 3'
S1L	5' GTT GTA CGT AAA CTG CAG CCT GGC GAA CTC GAT GGA CAA GGT GGT CTT GAT GCG CTC CA 3'
S1G/L8A	5' GTT GTA CGT AAA CGT AGC CCT GGC GAA CTC GAT GGA CCC GGT GGT CTT GAT GCG CTC CA 3'
S1G/I3A	5' GTT GTA CGT AAA CTG CAG CCT GGC GAA CTC GGC GGA CCC GGT GGT CTT GAT GCG CTC CA 3'
L8A/R7K	5' GTT GTA CGT AAA CGT AGC CTT GGC GAA CTC GAT GGA GGA GGT GGT CTT GAT GCG CTC CA 3'
S1G/I3N/F5L/E4S	5' GTT GTA CGT AAA CTG CAG CCT GGC CAA GCT GTT GGA CAA GGT GGT CTT GAT GCG CTC CA 3'

6.3.4 Complementation assay

Confluent monolayers of Vero cells were transfected with 4μg of plasmids expressing one of the epitope knockout gB proteins or wild type gB using the Fugene transfection reagent (Roche Diagnostics). After 18 hours, cells were infected with gBKO1 virus at an MOI of 5 for 1 hour. Following media replacement, cells were incubated at 37^{o}C for 24 hours prior to harvest. Cells and supernatants were harvested and subjected to three freeze-thaw cycles and titrated onto both Vero cells and gB-vero cells and counted for plaque forming units.

6.4 RESULTS

6.4.1 Expression of multimers by CMV constructs and recombinant viruses.

We first tested whether the CMV promoter expressed constructs (Table 1) and the recombinant viruses expressed multimers of the gB peptide (Table 2). We expected a high rate of misfolding of these fusion proteins and thus to be targeted to the proteosome for degradation. To block proteosome degradation and allow us to detect the fusion peptides via western blots, the transfections and infections were allowed to proceed either in the in the presence or absence of MG132, a known inhibitor of the proteosome pathway (28, 147). The expected size of the expressed peptide fused to EGFP was 35 kDa for 4 repeats of the multimer and and 55kDa for 13 repeats of the multimer. When fusion peptides were expressed from the CMV IE promoter either as C- or N-terminal fusions, we did not observe fragment sizes of the expected sizes. Both in the presence or absence of MG132, only a band corresponding to the size of EGFP was observed (Fig. 6-3). Even at 48 hours post transfection, there was no accumulation of larger size bands (Fig. 6-3 lower panel). Thus it did not appear that the CMV-constructs expressed detectable levels of the peptide-EGFP fusion protein or that the amounts of MG132 were not sufficient to block proteosome mediated degradation. When the same experiment was performed with the recombinant viruses, interestingly we observed a band corresponding to approximately 35 kDa in all samples in which MG132 was added (Fig. 6-4). We did not observe the expected 55kDa band in cells infected with viruses 13gB and 13TK suggesting that the 35kDa might be a more stable form of the fusion peptide. Thus from these assays, we could not conclusively determine whether the peptide-EGFP fusion proteins were being expressed correctly.

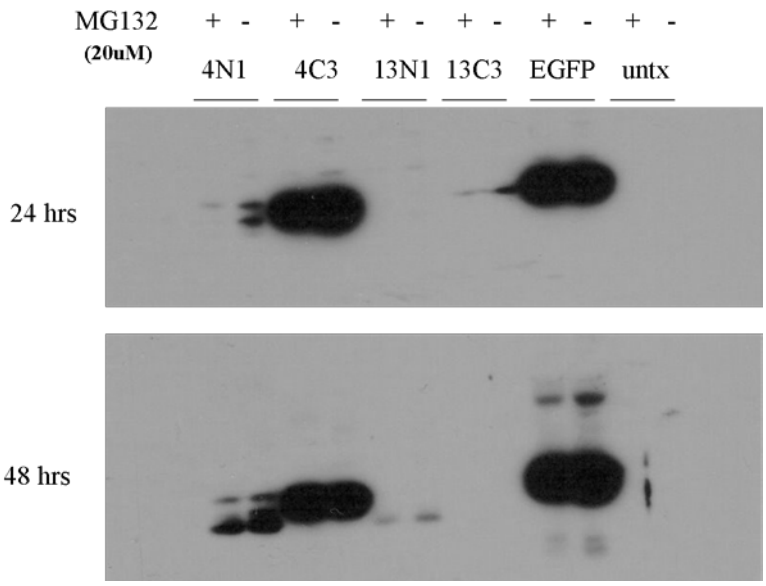

Figure 6-3. Expression of multimer peptides by CMV constructs.

Confluent Vero cells were transfected with plasmids 4N1, 4C3, 13N1, 13C3, EGFP or left untransfected. Media was replaced 4 hours post transfection with or without 20μM of MG132, a proteosome inhibitor drug. Cells were harvested for protein 24 or 48 hours later and analyzed by immunoblotting for EGFP. The times and drug treatment are shown in the panels above the blots. The upper blot represents samples harvested at 24 hours after transfection and the lower blot represents samples harvested at 48 hours after transfection.

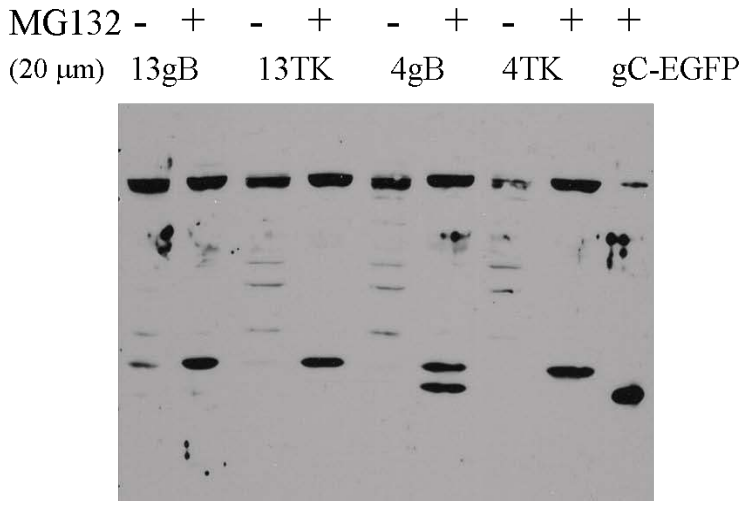

Figure 6-4. Expression of multimer peptides by recombinant viruses.

Vero cells were infected at an MOI of 5 with 13gB, 13TK, 4gB, 4TK or gC-EGFP for 1 hour at room temperature. Media was replaced after one hour with or without 20μM of MG132. Cells were harvested at 24 h.p.i and analzyed by immunoblotting for EGFP.

6.4.2 Peptides expressed from the CMV promoter are able to stimulate gB-CD8 to produce IFNγ.

Since we could not confirm expression of the peptides by western blot, we performed an *in vitro* assay to determine if the peptides expressed from the CMV IE promoter could stimulate gB-CD8 from infected TG of C57BL/6 to produce IFNγ (Fig 6-5). We transfected B6WT3 cells (syngeneic fibroblasts from C57BL/6 mice) with the plasmids we created (Table 1) along with EGFP as a negative control. We also infected B6WT3 cells with HSV-1 RE (WT) or our control virus gC-EGFP and used 18 hour infected cell lysates for this experiment. TG infected for 8 days with WT virus were dispersed and combined with transfected or infected cells in the presence of golgiplug to allow IFNγ to accumulate within gB-CD8. Both HSV-1 RE and gC-EGFP infected cells induced a high IFNγ production by gB-CD8. IFNγ production by cells transfected with 4N1, 13N1 and 13C3 were comparable to infected cell controls (Fig. 6-3). In contrast, 4C3 did not induce an IFNγ response and this response was similar to cells transfected with EGFP which served as the negative control. Interestingly, the plasmids containing more number of repeat peptides (13N1 and 13C3) did not induce greater IFNγ production by gB-CD8 compared to the one containing fewer numbers of repeats (4N1). It is possible that overexpression of these plasmids in this transfection assay saturates the amount of peptide synthesized such that plasmids expressing more copies of the peptide do not have a greater effect. However, from this experiment it can be concluded that while plasmids, 4N1, 13N1 and 13C3 seem to express the epitope in frame and induce a strong IFNγ response, plasmid 4C3 was not functional in doing so (Fig. 6-5). This demonstrates that plasmids 4N1, 13N1 and 13C3 express the peptides in frame which gave us the impetus to continue to characterize the recombinant viruses we created.

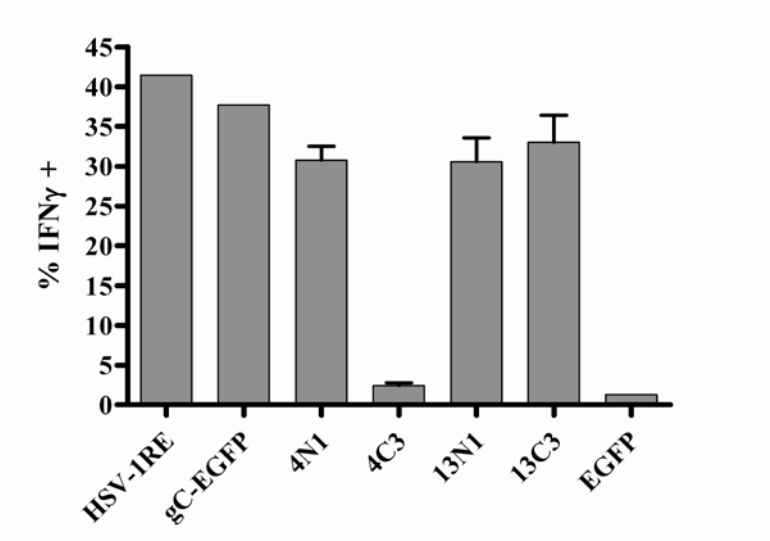

Figure 6-5. Peptide constructs are able to stimulate gB-CD8 from infected TG to produce IFNγ.

TG were obtained 8 days after corneal infection with HSV-1 RE (WT), dispersed with collagenase, and stimulated for 6 h with syngeneic B6WT3 cells that were mock transfected, infected with HSV-1 RE, gC-EGFP or transfected with plasmids 4N1, 4C3, 13N1, 13C3 or EGFP. Cells were surface stained for CD45 and CD8, and stained for intracellular IFN-γ. The graph depicts the mean percent of IFN-γ positive cells (n = 2/group) and standard error of the mean for each stimulation. The IFN-γ response of gB stimulated $CD8^+$ T cells from 4C3 and EGFP transfections is significantly ($p < 0.05$) different from the response HSV-RE and gC-EGFP as assessed by Students' t test.

6.4.3 Replication of HSV-13LAT in vivo in the TG is similar compared to gC-EGFP

For studies in mice, we chose to characterize the 13LAT virus which expresses 13 repeats of the peptide fused to the N-terminus of EGFP under the LAT promoter. In our *in vitro* assay, both constructs containing the peptides fused to the N-terminus of EGFP induced an IFNγ response in gB-CD8 (Fig. 6-5). Furthermore, during latency, the LATs are the only abundant transcripts to be found within infected neurons indicating that this promoter is highly active during latency (77). By using this promoter we can predict that there might be persistent expression of the

peptides and thus constant antigenic exposure to surrounding $CD8^+$ T cells. Also, by using the virus expressing 13 repeats of the epitope, we can maximize antigen presentation to $CD8^+$ T cells. We also used a previously defined recombinant HSV-1, gC-EGFP (31) as the control virus. gC-EGFP has been shown in previous studies to behave exactly like WT virus in terms of viral replication and induction of an immune response. Furthemore, both 13LAT and gC-EGFP express fluorescent markers (RFP and EGFP respectively) from the gC promoter allowing us to monitor reactivation *ex vivo* through fluorescent microscopy.

We first determined the replication kinetics of both 13LAT and gC-EGFP during acute phase replication in the TG. At 4 d.p.i which is the peak of viral replication in the TG, both 13LAT and gC-EGFP viruses grew to equal titers, demonstrating that the additional expression of repeat gB epitopes did not impair the ability of 13LAT to access and replicate in the TG (Fig. 6-6).

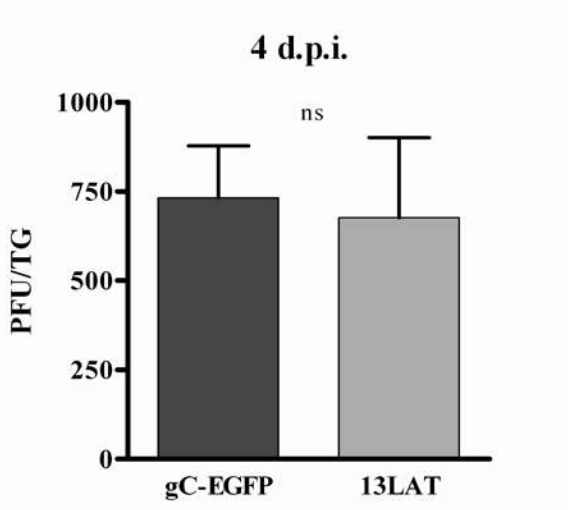

Figure 6-6. HSV-13LAT replication in the TG is similar to gC-EGFP.

Mice were infected with gC-EGFP or 13LAT at 1×10^5 PFU/eye. At 4 d.p.i., TG were excised, homogenized and subjected to three freeze–thaw cycles prior to determination of viral titers on Vero cells. The graph represents the mean viral titers ± SEM of the mean. Titers of both the

viruses in the TG were not significantly different ($p > 0.05$) at this time point as assessed by a Student's *t* test (n=10).

6.4.4 HSV-13LAT establishes latency with similar genome copies compared to gC-EGFP

We next examined the ability of 13LAT to establish latency compared to gC-EGFP. We used real-time quantitative PCR to measure the latent genome copies retained in the TG at 34 d.p.i. While 13LAT established latency with slightly higher genome copies compared to gC-EGFP, this was not statistically significant. Therefore, 13LAT is able to establish latency at similar genome copies compared to gC-EGFP (Fig. 6-7).

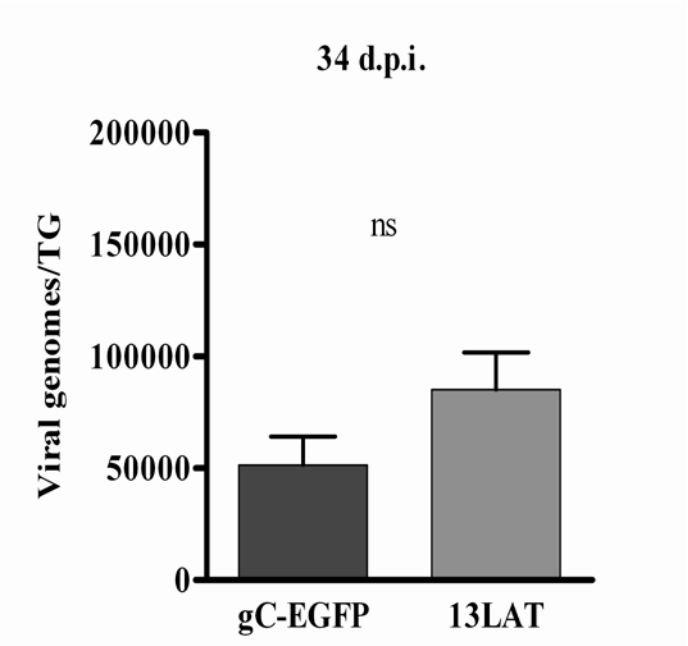

Figure 6-7. HSV-13LAT establish latency with similar copies compared to gC-EGFP.

Mice were infected with gC-EGFP or 13LAT at an infectious dose of $1x10^5$ or $3x10^4$ PFU/eye. TG were excised at, 34 d.p.i and genome copies were determined by real time PCR. The latent viral genome copies retained in TG infected with gC-EGFP and 13LAT are not significantly different ($p \geq 0.0.5$) as analyzed by a Student' *t*-test (n=5).

6.4.5 HSV-13LAT induces a greater overall $CD8^+$ T cell response compared to gC-EGFP but not a gB-CD8 response in the TG.

We next assessed the $CD8^+$ T cell response generated in the TG of mice infected with either gC-EGFP or 13LAT. At 14 d.p.i, both viruses induced a similar total $CD8^+$ T cell infiltrate within the infected TG (Fig. 6-8 A). However, by 34 d.p.i, the total number of $CD8^+$ T cells retained in the TG of 13LAT infected mice was approximately 2-fold greater than gC-EGFP. This corresponds with the slightly greater (although not statistically significant) number of latent genomes retained in TG of 13LAT infected mice compared to those infected with gC-EGFP (Fig. 6-7). However, an analysis of the corresponding gB-CD8 response revealed no difference in both the total numbers (Fig. 6-8 B) and the frequency of gB-CD8 (Fig. 6-8 C) between the two viruses at either 14 or 34 d.p.i. So while 13LAT is able to induce a greater overall $CD8^+$ T cell response in the TG, overexpression of the peptide does not appear to alter the gB-CD8 response in the latently infected TG.

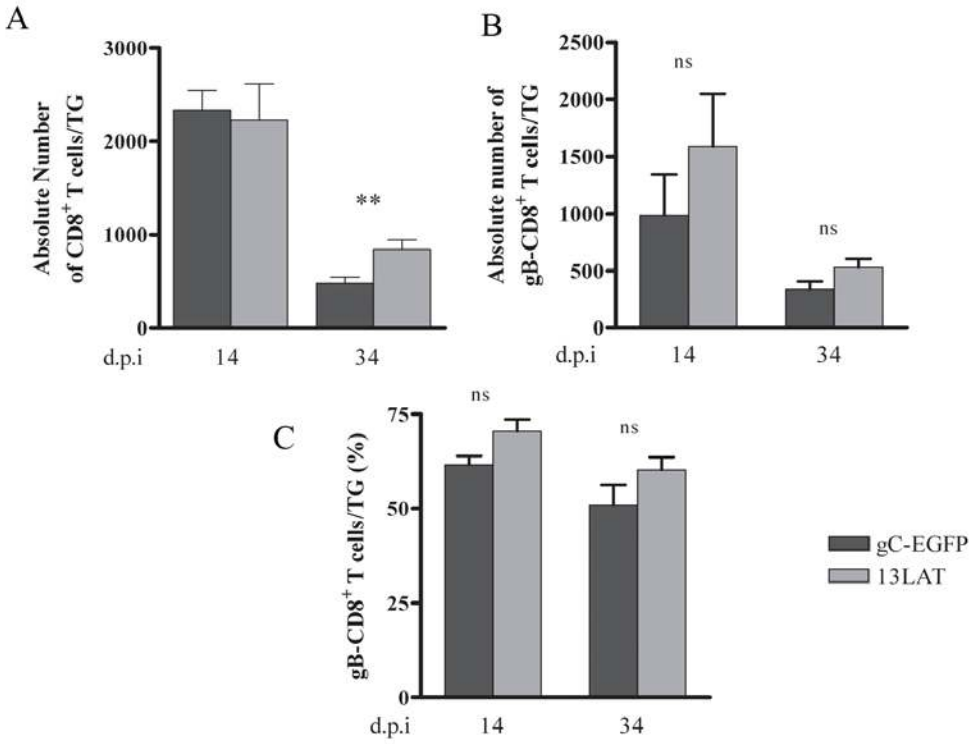

Figure 6-8. Comparison of the CD8$^+$ T cell response within TG infected with gC-EGFP and 13LAT.

Mice were were infected with 1 x 10^5 PFU/eye with gC-EGFP or 13LAT. TG were excised at 14 and 34 d.p.i., dissociated into single cell suspensions and surface stained for expression of CD45, CD8 and $gB_{498\text{-}505}$-T cell receptor and analyzed by flow cytometry. The data are represented as the mean $^+$/- SEM of the mean. The data are combined from two independent experiments. **A)** Total number of CD8$^+$ T cells. The total number of CD8$^+$ T cells at 34 d.p.i is significantly different between gC-EGFP and 13LAT infected TG ($p<0.05$) as assessed by a Students' *t* test. **B)** The total number of gB-CD8 per TG. **C)** The percentage gB-CD8 cells per TG. For B) and C), the means are not significantly different between gC-EGFP and 13LAT at either of the time points tested as assessed by a Students' *t* test ($p\geq0.05$).

6.4.6 The frequency of circulating CD8$^+$ T cells in 13LAT infected mice is similar those infected with gC-EGFP.

Since we observed no difference in the gB-CD8 numbers in the TG of 13LAT infected mice, we investigated whether this may be due to the inability of gB-CD8 to infiltrate the TG or there were

fewer gB-CD8 in the periphery. We did this by measuring the frequency of circulating gB-CD8 in the blood of infected mice. Both total numbers of $CD8^+$ T cells and gB-CD8 as well as the frequency of gB-CD8 were similar in blood of mice infected with either gC-EGFP or 13LAT (Fig. 6-9) . Thus 13LAT does not seem to induce a greater gB-CD8 response in the periphery of infected mice compared to gC-EGFP.

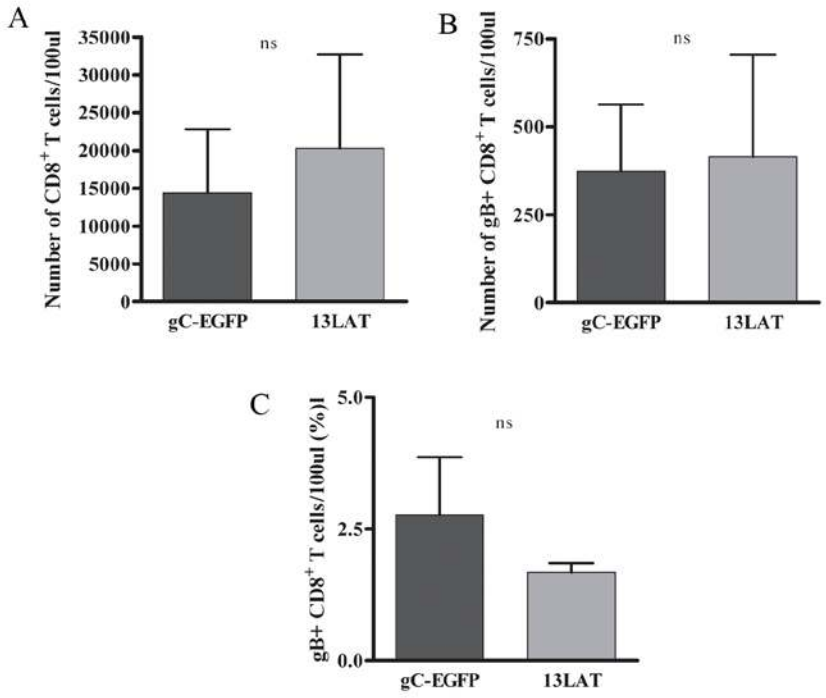

Figure 6-9. Comparison of the $CD8^+$ T cell response within blood of mice infected with gC-EGFP and 13LAT.

Mice were were infected with 1 x 10^5 PFU/eye with gC-EGFP or 13LAT. At 34 d.p.i, 100 ul blood was collected from infected mice and surface stained for expression of CD45, CD8 and $gB_{498-505}$-T cell receptor and analyzed by flow cytometry. The data are represented as the mean $^+$/- SEM of the mean. The data are combined from two independent experiments. **A)** Total number of $CD8^+$ T cells. **B)** The total number of gB-CD8 per TG. **C)** The percentage gB-CD8 cells per TG. The means are not significantly different between gC-EGFP and 13LAT for A, B or C as assessed by a Students' *t* test ($p \geq 0.05$).

6.4.7 HSV-13LAT reactivation is similar to gC-EGFP

The use of fluorescent viruses has enabled us to rapidly detect reactivation *in vitro* by visualizing the spread of fluorescence within latently infected TG cultures (31). Both gC-EGFP and 13LAT viruses have fluorescence tags that enable us to monitor reactivation through fluorescence microscopy. Since 13LAT latently infected TG harbored a larger number of $CD8^+$ T cells compared to gC-EGFP, we wanted to investigate if this would result in a lower reactivation frequency of 13LAT infected cultures. Examination of *ex vivo* cultures of TG latently infected with gC-EGFP or 13LAT revealed fluorescence plaques that expressed EGFP or RFP respectively. We observed that the percentage of 13LAT cultures that reactivated were slightly lower at earlier times post culture, however by 8 days post culture, similar number of cultures reactivated for both viruses (Fig. 6-10). Thus the increased number of total $CD8^+$ T cells within 13LAT infected TG might offer protection from reactivation early, it does not appear to block reactivation at later times post culture initiation compared to gC-EGFP infected TG cultures.

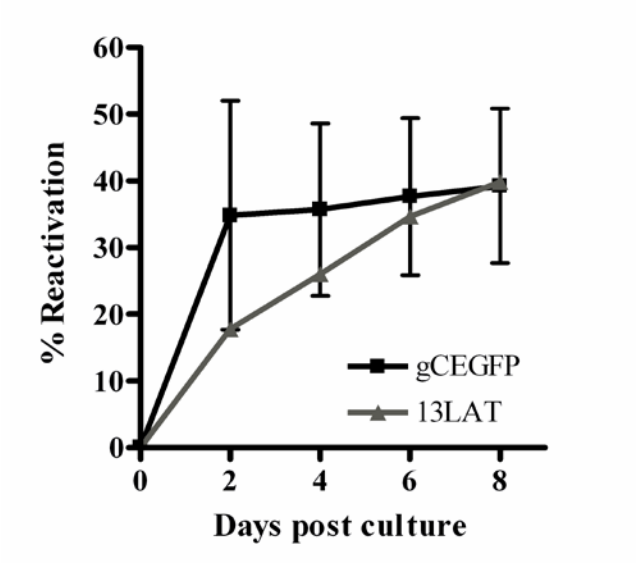

Figure 6-10. Reactivation of HSV-13LAT is similar to gC-EGFP.

TG were excised 34 d.p.i. after corneal infection with gC-EGFP or 13LAT, dispersed with collagenase, and cultured (0.2 TG/well) at 37 °C. At the indicated times following culture initiation, HSV-1 reactivation from latency was detected based on spread of EGFP fluorescence from neurons to surrounding fibroblasts. The mean percent of cultures exhibiting HSV-1 reactivation from latency and standard error of the mean is shown at each time point.

6.4.8 Alternate strategy for developing recombinant viruses expressing gB multimers.

The studies described in this chapter so far were not completely successful in identifying whether peptide expression could be detected both *in vitro* and within infected neurons during latency. However, the ability of the CMV-constructs to stimulate gB-CD8 indicates that at least *in vitro*, the peptides are expressed in frame, undergo the proteosome pathway and are presented on MHC-I molecules for presentation to $CD8^+$ T cells. We also did not observe a difference in the gB-CD8 response generated by gC-EGFP or 13LAT infections and therefore could not determine whether the peptides were being expressed during latency. Since the 13LAT virus also contains an existing endogenous gB epitope present within the native locus, we were unable to differentiate the gB-CD8 response generated from this epitope from the ectopically expressed peptides. Thus we had to formulate an alternative strategy to address this question. This required the engineering of several additional recombinant viruses. First we generated a virus in which the gB gene is abrogated such that it does not express native gB protein. Using this virus as a backbone, we then engineered several viruses containing mutations in the epitope region which would restore fully functional gB protein into the virus but abrogate recognition of the $gB_{498\text{-}505}$ peptide by gB-CD8 (gB-epitope mutant viruses). The following studies describe the generation and characterization of these viruses both *in vitro* and *in vivo*. Future studies will involve the re-engineering of the multimer viruses into the gB-epitope mutants, thus allowing us to address

whether overexpression of $CD8^+$ T cell peptides can induce an antigen-specific $CD8^+$ T cell response within latently infected ganglia.

6.4.9 HSV-gBKO viruses do not express glycoprotein B.

We independently isolated two HSV-gBKO viruses. Our strategy involved recombination of EGFP into the front end of gB such that the resultant viruses were fluorescent but did not express functional gB. Since gB is an essential viral protein required for virus entry and replication, selection for recombinants had to be performed in a complementing cell line containing a plasmid expressing gB from the native gB promoter (gB-Veros) (Fig. 6-2). To confirm that our gBKO viruses were null for gB protein and that recombination between gBKO DNA and the gB plasmid from the gB-Veros did not occur, we analysed gBKO infected lysates for gB expression by western blot. Vero cells infected with an MOI of 10 with WT-RE, gBKO1 or gBKO2 were harvested at 1, 12 and 24 h.p.i and analysed for gB expression. At both 12 and 24 h.p.i, WT RE expressed high levels of gB but this expression was not observed in either gBKO1 or gBKO2 infected cell lysates indicating that both these viruses do not express gB (Fig. 6-11 top panel). A western blot analysis using anti-HSV antibody revealed a similar pattern of protein expression indicating that the gBKO viruses were not defective in synthesizing other viral proteins (Fig. 6-11 bottom panel).

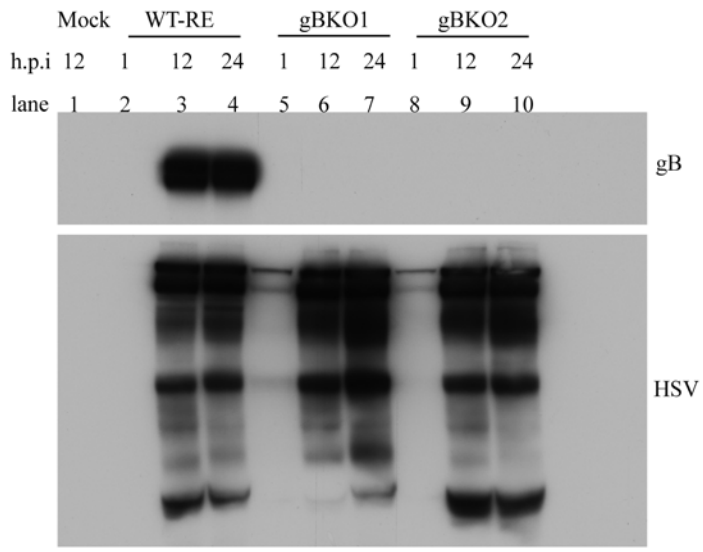

Figure 6-11. HSV-gBKO1 and HSV-gBKO2 viruses do not express any gB protein.

Confluent monolayers of Vero cells were infected with an MOI = 10 with HSV-1 RE, gBKO1 and gBKO2 or mock infected. Total SDS PAGE separated proteins were analyzed by immunoblotting for using polyclonal antibodies to gB and HSV. The times of harvest are indicated above each lane. The top panel represents an immunoblot for gB and the bottom panel is for total HSV proteins.

6.4.10 The $gB_{498-505}$ epitope mutant DNA constructs are unable to stimulate gB-CD8 to produce IFNγ.

The $gB_{498-505}$ epitope mutant DNA constructs were tested for their ability to stimulate WT gB-CD8 to produce IFNγ. For this assay, we used gB-CD8 that were expanded *in vitro* from TG latently infected with WT virus. The positive control (gB) in which gB is expressed in high amounts from a CMV promoter stimulated gB-CD8 to high levels. In contrast, none of the epitope mutant constructs were able to stimulate gB-CD8 and the levels of IFNγ production were similar to mock transfected cells (Fig. 6-12 top panel). gB expression was confirmed for all constructs via western blotting (Fig. 6-12 bottom panel). The WT condition represents a construct which was made simultaneously with the epitope knockout constructs but in which the

epitope was not disrupted. Stimulation of gB-CD8 with the WT construct was similar to the positive gB control demonstrating that the strategy used to generate these constructs maintained the integrity of the protein and allowed it to be expressed in frame (Fig. 6-12 top panel).

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Figure 6-12. $gB_{498-505}$ epitope mutant constructs are unable to stimulate gB-CD8 to produce IFNγ.

B6WT3 (1 x 10^5 cells) were transfected with 5μg of plasmids expressing one of the gB epitope mutant proteins or wild type gB. 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. 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.

6.4.11 The $gB_{498-505}$ epitope mutant DNA constructs are able to complement gBKO virus growth

Prior to generating recombinant viruses using each of the epitope mutant constructs, we performed complementation assays to determine whether these mutations generated within the gB protein had any effect on its function. Vero cells were transfected with each of the plasmids and 18 hours later infected with the gBKO1 virus at an MOI of 5. Cells were harvested 48 hours later and titrated on both Veros and gB-Veros. None of the conditions produced plaques on Veros (not shown) demonstrating that recombination between the transfected plasmids and gBKO1 virus did not occur and also that gBKO1 virus is truly null for gB expression. Both untransfected and EGFP transfected controls produced no plaques when titrated on gB-Veros demonstrating that the gBKO1 virus was unable to replicate and amplify itself within infected Vero cells (Fig. 6-13 top panel). Complementation with the positive control gB plasmid resulted

in high virus titers up to 10^7 PFU/ml (Fig. 6-13 top panel). Compared to this, all the epitope mutant constructs were able to complement the gBKO1 virus but to varying levels. Both S1G/I3A and S1G/L8A mutants were able to complement the gBKO1 virus similar to levels seen with the positive control. The F5L and L8A/R7K mutants were also able to complement the gBKO1 virus, they yielded virus titers that were approximately 2 log lower than the positive control. The remaining plasmids were able to complement the gBKO1 virus to about 1 log lower than the positive control (Fig. 6-13 top panel). Expression of gB from all transfections was confirmed via western blotting (Fig. 6-13 bottom panel).

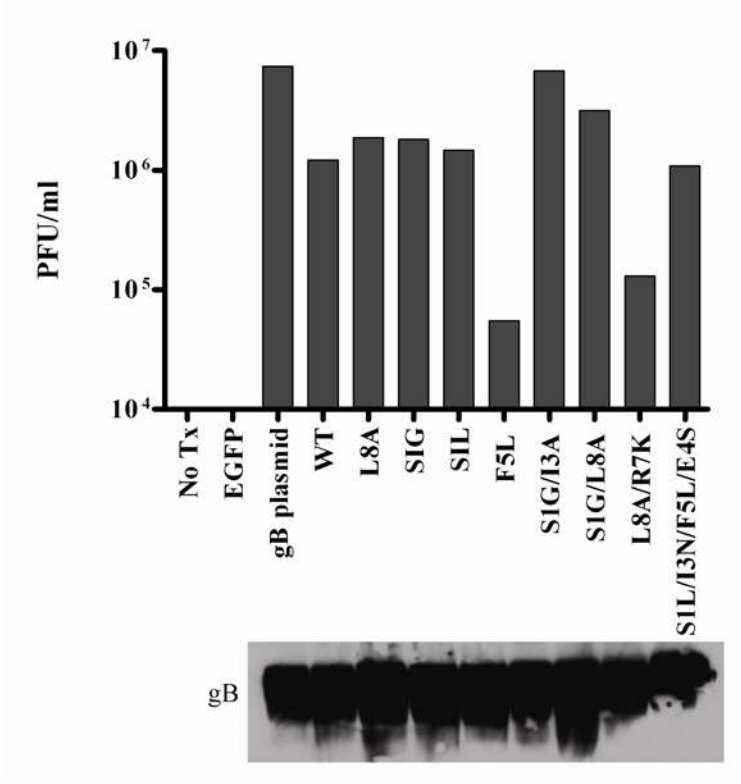

Figure 6-13. $gB_{498\text{-}505}$ epitope mutant constructs are able to complement the gBKO virus.

Confluent Vero cells were transfected with one of the above plasmids or left untransfected. 18 hours later, cells were infected with gBKO1 virus at an MOI of 5. 24 hours post infection, cells and supernatants were harvested, subjected to three freeze-thaw cycles and titrated on to Vero cells. The graph represents the plaque forming units (PFU)/ml calculated for each virus after titration onto Vero cells. The bottom panel represents an immunoblot of gB confirming protein expression from each transfection.

6.4.12 Replication of $gB_{498-505}$ epitope mutant viruses in the TG.

We next wanted to text viral pathogenicity and replication of some of the epitope mutant viruses in C57BL/6. For this chapter, only WT, F5L, L8A, S1L and SIFE mutants were tested in C57BL/6 mice as those were the viruses available at the time the experiment was performed. We measured the viral titers in the TG at 4 d.p.i which is normally the peak of viral replication in the TG (refer to Chapters 4 and 5). Surprisingly, the WT control did not replicate as efficiently in the TG as expected (Fig.6-14). This might indicate that during the isolation process, an unknown mutation was introduced into the WT recombinant which affects its pathogenicity. Interestingly, the F5L mutant grew to high titers in the TG, similar to levels observed with wild-type or rescue viruses in our previous studies (refer to Chapters 4 and 5). Both the L8A and S1L mutants were similarly impaired compared to the WT and the SIFE mutant produced no plaques at all. This shows that the F5L mutation in gB does not compromise the ability of this virus to replicate in the TG (Fig. 6-14).

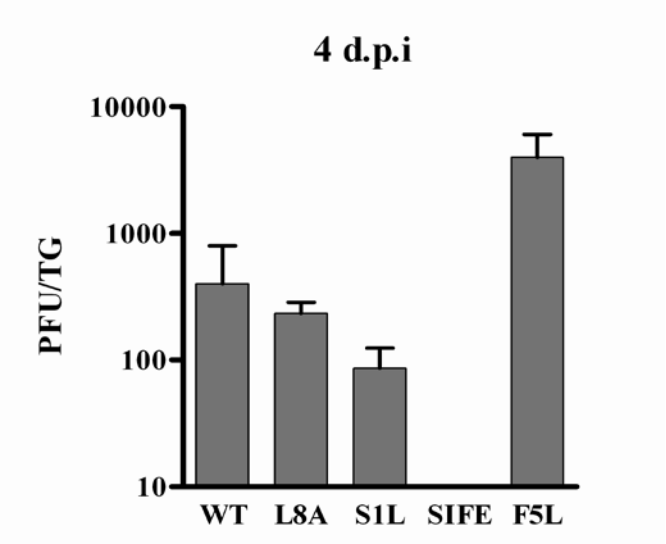

Figure 6-14. Replication of $gB_{498-505}$ epitope mutant viruses in the TG.

Mice were infected with 1 x 10^5 PFU/eye with HSV-1 WT, HSV-1 L8A, HSV-1 S1L, HSV-1 F5L or HSV-1 SIFE. At 4 d.p.i, TG were harvested and subjected to three freeze thaw cycles and infectious virus released into the supernatant was titrated on to Vero cells. The graph represents the mean virus titer for each virus ± SEM of the mean (n = 5 mice).

6.4.13 The $gB_{498-505}$ epitope mutant viruses do not induce a gB-CD8 response in the TG

The main goal in generating the epitope knockout viruses was to abolish their ability to induce a gB-CD8 response in the TG of C57BL/6 mice. Thus we measured the $CD8^+$ T cell response generated in mice infected with WT, L8A, F5L, S1L and SIFE mutants at 14 d.pi (Fig. 6-15). Concordant with their poor ability to replicate in the TG, the WT, L8A, S1L and SIFE mutants all generated a very poor total $CD8^+$ T cell response in the TG (Fig. 6-15 A). In contrast, the total $CD8^+$ T cell infiltrate in the TG of F5L infected mice was greater, similar to numbers observed previously using wild type viruses (refer Chapters 4 and 5). However, when we measured the total and frequency of gB-CD8 in the TG of infected mice, we observed a significant number of

gB-CD8 in TG of mice infected with WT but very few gB-CD8 in TG of mice infected with the L8A, S1L, SIFE or F5L mutants (Fig. 6-15 B). When this was translated to the frequency of gB-CD8 in the TG, as expected, approximately 40-45% of the total $CD8^+$ T cells in WT infected TG were gB-CD8. In contrast to this, the percentage of gB-CD8 in the L8A, S1L, SIFE or F5L infected TG represented around or less than 5% of the total $CD8^+$ T cells (Fig. 6-15 C). This demonstrates that the L8A, S1L, SIFE and F5L mutants did not generate a significant gB-CD8 infiltrate within infected TG while the WT virus does (Fig. 6-15).

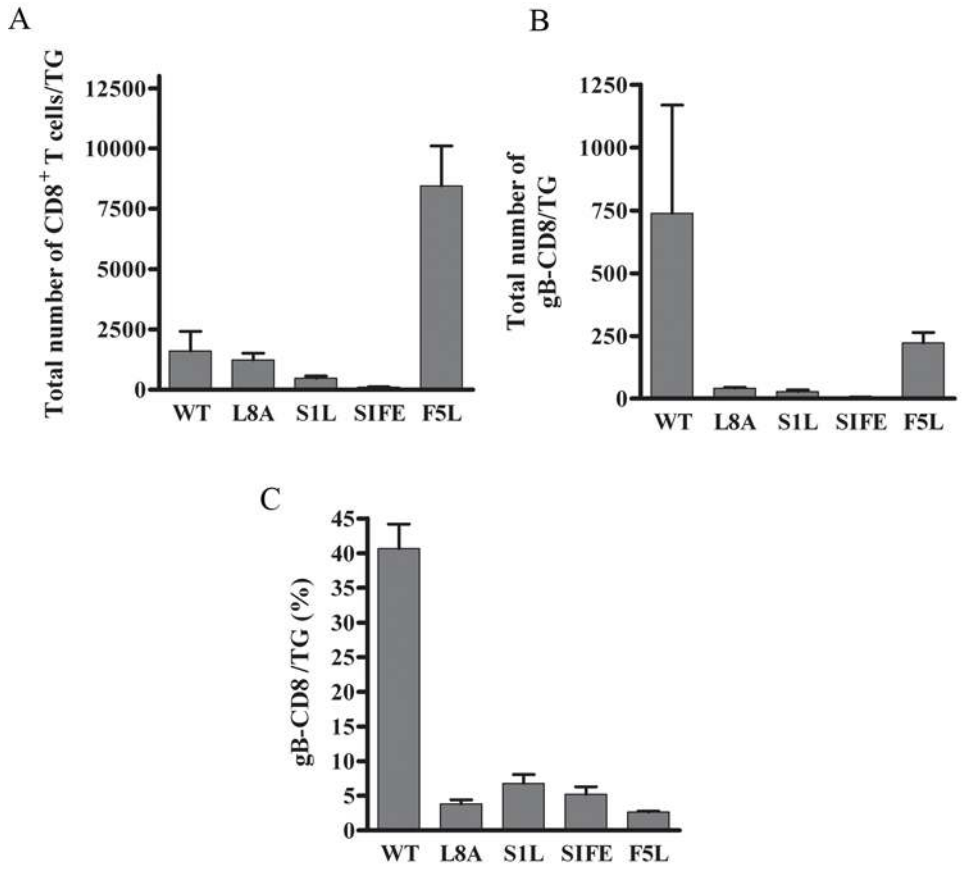

Figure 6-15. Comparison of the $CD8^+$ T cell and the gB-CD8 response within TG infected with $gB_{498-505}$ epitope mutant viruses.

Mice were were infected with 1 x 10^5 PFU/eye with HSV-1 WT, HSV-1 L8A, HSV-1 S1L, HSV-1 F5L or HSV-1 SIFE. TG were excised at 14 d.p.i., dissociated into single cell suspensions and surface stained for expression of CD45, CD8 and $gB_{498-505}$-T cell receptor and analyzed by

flow cytometry. The data are represented as the mean $^{+}$/- SEM of the mean. **A)** Total number of $CD8^{+}$ T cells.. **B)** The total number of gB-CD8 per TG. **C)** The percentage gB-CD8 cells per TG.

6.5 DISCUSSION

Currently, there is no available vaccine to prevent HSV-1 reactivation. Therapeutic vaccine trials in both animals and humans have been successful in boosting the cellular and antibody responses in the periphery but have not been effective in augmenting the existing $CD8^{+}$ T cell response at the site of latency. While an increased immune response in the periphery can act to minimize viral shedding and transmission, it is not able to prevent reactivation of the virus entirely and thus cannot prevent recurrent disease. Many prophylactic vaccine trials in animal models have been successful in the use of $CD8^{+}$ T cell epitope peptides as immunogens to prevent or reduce the severity of an initial HSV-1 infection. In the absence of an available vaccine to prevent HSV-1 reactivation, we sought to use a similar approach to design a novel vaccine strategy in which we could augment the existing $CD8^{+}$ T cell immunosurveillance at the site of latency. In this study, we exploited the C57BL/6 mouse model in which, the $gB_{498\text{-}505}$ peptide represents the immunodominant epitope recognized by virus-specific $CD8^{+}$ T cells following infection. In the C57Bl/6 mouse, approximately a thousand $CD8^{+}$ T cells are retained within the latently infected TG at 34 d.p.i and beyond. Out of these, approximately half represent gB-CD8. We thus assessed whether a recombinant HSV-1 which expresses tandem repeats of the viral $gB_{498\text{-}505}$ epitope

could induce a larger gB-CD8 response in the TG during latency, which would be able to prevent reactivation.

Our strategy was to augment the $CD8^+$ T cell response in the ganglia by overexpressing the $gB_{498-505}$ epitope within latently infected neurons. We first generated several constructs containing either 4 or 13 tandem repeats of the $gB_{498-505}$ peptide fused to EGFP. In our initial studies characterizing peptide expression *in vitro*, we did not observe the expected size fragment of the fusion peptides in a western blot assay (Fig. 6-3 and Fig. 6-4) but constructs expressing the peptides were able to stimulate gB-CD8 to produce IFNγ confirming that the peptides were being expressed in frame (Fig. 6-5). We note that the constructs expressing more numbers of peptide repeats did not induce a greater IFNγ response compared to ones expressing fewer numbers of repeats. This assay required expression of peptides via a transfection assay which often results in overexpression of proteins. It is possible that the high amount of peptide expressed within each transfection, saturated the assay to a level that made it impossible to determine differences in the level of stimulation by cells expressing 4 or 13 repeats of the epitope.

Following the above mentioned studies, we generated several viruses in which tandem repeats (4 or 13) of the epitope were expressed from the the LAT (LAP2), gB or tk promoters (Table 2). However, we only characterized the virus expressing 13 repeats of the epitope from the LAP2 promoter (HSV-13LAT) *in vivo*. During latency, the only transcripts to be expressed in high amounts are the LATs and the LAT promoters are highly hyperacetylated, demonstrating active transcription (77). Thus we chose to evaluate this virus so that there is persistent exposure of viral antigen to surrounding $CD8^+$ T cells. The 13LAT virus was able to replicate efficiently in the TG to the control (gC-EGFP) demonstrating that the addition of exogenous peptides had no detrimental effects on viral pathogenesis. Real time PCR analysis of the latent genomes revealed a slightly greater (although not significantly different) number of latent genomes

retained in TG of mice infected with the 13LAT virus compared to gC-EGFP. However, since the assay was only limited to five mice, it is not possible to conclude from this data set whether this small increase in genome copy number could be significant in future experiments. A greater overall $CD8^+$ T cell response (2-fold increase) was observed in TG infected with 13LAT compared to gC-EGFP which could have been contributed by the greater number of genomes retained in 13LAT infected mice. However, we did not observe a greater gB-CD8 response in 13LAT infected mice. It is possible that during the recombination process, an unknown mutation could have been introduced into these viruses thus shifting them out of frame making them non-functional. Studies in chronic viral infection models have demonstrated $CD8^+$ T cell exhaustion resulting from high levels of antigen exposure and in some extreme cases, even deletion of antigen-specific $CD8^+$ T cells (42, 111, 183). It is possible in our study that by expressing the peptides from the LAP2 promoter, there is overexposure of this antigen to gB-CD8 and instead of inducing a greater gB-CD8 response it leads to their exhaustion or depletion from the TG. In future studies, we will explore the use of the viruses in which the peptides are expressed from the gB and *tk* promoters. Since these promoters will be active only periodically when the virus is attempting to reactivate, antigen expression from these promoters may not overwhelm the immune response.

Our overall goal with using the peptide viruses is to determine if they are useful as vaccine strategy to block reactivation. A disadvantage in performing these studies in C57BL/6 mice is that it is nearly impossible to induce reactivation *in vivo* in this mice strain. While a reactivation stimuli or administration of anti-CD8 antibody results in an increase in copy number within latently infected TG, we have never been able to detect infectious virus either in the TG or in the cornea. Reactivation studies must be performed using *ex vivo* assays which require far greater numbers of $CD8^+$ T cells to prevent reactivation compared to that needed *in vivo*. For

these reasons, it is difficult to assess vaccine efficacy as we cannot evaluate whether vaccinated mice can be protected from induced reactivation *in vivo*. Our future vaccines studies might be performed in BALB/b mice which mount a $CD8^+$ T cell immune response identical to C57BL/6 mice. Since these mice have a BALB/c background, inducing reactivation *in vivo* may also be possible (117). This will allow us to induce reactivation *in vivo* and test vaccine efficacy by measuring both increases in copy number and infectious virus production. Furthermore these mice develop HSK similar to BALB/c mice and any vaccine strategy can also be assessed by its ability to reduce HSK in these mice.

Since we did not observe a difference in the gB-CD8 infiltrate in TG of mice infected with HSV-13LAT compared to the gC-EGFP control, we could not determine whether overexpression of the epitope region was functional in this virus. For this reason, we chose an alternative strategy in which the native gB epitope had been mutated such that induction of a gB-CD8 response was abrogated. We tested four mutant viruses HSV-L8A, HSV-S1L, HSV-F5L and HSV-S1G/I3N/F5L/E4S (HSV-SIFE) and one wild type (WT) derivant that were available at the time the experiments were performed. Interestingly, even though gB protein expressed from all the epitope mutant DNA constructs were able to complement gBKO virus in an *in vitro* complementation assay, different growth properties of the corresponding recombinant viruses were observed *in vitro*. Interestingly, the WT virus showed some growth deficiency in the TG at 4 d.p.i., indicating that an unknown mutation could have been introduced into the virus that compromised its pathogenicity. Out of the four other mutants tested, HSV-F5L virus replicated the best in the TG and demonstrated titers in the TG similar to those seen with wild type viruses from our previous studies (refer to Chapter 4 and 5). It is possible that the growth impairment seen for HSV-L8A, HSV-S1L and HSV-SIFE could also be due to an unknown mutation introduced into the viruses during the recombination process. However, despite the growth

impairment of the WT derivant, viral infection still induced a large frequency of gB-CD8 in the TG (~45%) which is typical of wild type viruses (67). In contrast, TG infected with the four mutant viruses did not demonstrate a similarly great gB-CD8 response. Interestingly, in mice infected with the mutant viruses, there were still a small percentage of $CD8^+$ T cells that could be detected by $gB_{498-505}$-tetramer staining. These cells could reflect $CD8^+$ T cells of an alternative specificity (not wild type $gB_{498-505}$) in which the epitope is close enough to $gB_{498-505}$ that the tetramer staining is still able to bind and detect some of these $CD8^+$ T cells. Alternatively, the mutations made in each of the viruses were not sufficient in completely ablating the activation and expansion of gB-CD8. Since unexpectedly, the WT control virus did not show the robust growth properties we expected *in vivo*, it is difficult to fully and comprehensively interpret the data from the above mentioned experiments. However, since HSV-F5L was not compromised in its replication in the TG and did not induce a significant gB-CD8 response, a rescue virus will be derived from the F5L background which will be used as our control. This will help us understand whether this viral backbone contains some other mutation that makes it more pathogenic and will ultimately serve as the true control for the HSV-F5L virus. We expect the rescuant to be fully replication competent *in vivo* comparable to the parental virus, HSV-1 RE. Once the replication kinetics of HSV-F5L and its corresponding rescuant have been established, we will derive new recombinant viruses containing the peptide repeats in the absence of an endogenous $gB_{498-505}$ response. This will allow us to determine whether expression of the gB epitope from an ectopic site will induce a gB-CD8 response in the TG and also allow us to determine whether the number of repeats (4 or 13) has any effect on the magnitude of this response.

The studies characterized in Chapter 5 demonstrate that while $CD8^+$ T cells responding to late antigens are able to block reactivation, they are not retained in high numbers in the TG during latency. Previous studies have demonstrated the non-$gB_{498-505}$ population of $CD8^+$ T cells

is also HSV-specific and can block reactivation. However the identity of these $CD8^+$ T cells is currently unknown. The F5L mutant virus will prove useful in determining the epitope specificity of the other $CD8^+$ T cells. By comparing the identity of the non-$gB_{498-505}$ $CD8^+$ T cell repertoire generated in a wild type infection to that generated in the F5L mutant, we will be able to determine whether the repertoire is altered in the absence of the immunodominant $gB_{498-505}$ epitope. This will help us further our understanding on how $CD8^+$ T cells specific to immunodominant epitopes influence the infiltration and retention of those specific to non-immunodominant epitopes. Furthemore, 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.

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 our studies in mice, our aim is to show a proof of principle that by expressing multiple copies of a particular $CD8^+$ T cell epitope, we can augment the immune response to that antigen and subsequently prevent reactivation. However, since humans do not react to the same $CD8^+$ T cell epitopes, the challenge facing scientists is to be able to predict viral epitopes that are reactive in humans. The dynamics of how pre-existing memory $CD8^+$ T cells react to reactivating virus is also not fully understood. Knowledge on how the existing $CD8^+$ T cell memory population is boosted in response to reactivation is needed to design better vaccines. Also, since therapeutic vaccines would need to be delivered locally to the site of latency, an effective and safe method of delivery needs to be generated. 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 seems to play a critical and important part in the efficacy of a vaccine. While vaccination with peptide vaccines along 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.

7.0 CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this thesis has significantly improved our knowledge on HSV-1 latency and how $CD8^+$ T cells responds to viral infection. Previous to this work, a strong role for $CD8^+$ T cells in controlling of viral latency had been established (66, 87-88); however it was not clear how HSV-1 antigen expression during latency and reactivation influenced the way $CD8^+$ T cells responded to virus in latently infected ganglia. Using HSV-1 gB and gC as representative early and late viral antigens, we show that during reactivation events, gB expression occurs much sooner than gC expression, indicating lytic replication kinetics are maintained during reactivation. We also show that $CD8^+$ T cells responding to late antigens are not retained in high numbers in latently infected TG, but are still capable of blocking reactivation indicating they can act very quickly even after DNA replication. Finally, by mutational analysis of a HSV-specific $CD8^+$ T cell peptide, we show that $CD8^+$ T cells responding to the original peptide do not infiltrate the TG. These studies all demonstrate that during an HSV-1 infection, only antigen-specific $CD8^+$ T cells infiltrate the TG and antigen exposure during latency is responsible for their retention in the TG. This work has great implications toward designing better immuogens for therapeutic vaccines to prevent HSV-1 reactivation.

The studies performed in Chapter 4 describe the development and use of a novel recombinant HSV-1 which expresses two fluorescent proteins from two different lytic promoters. We chose the gB and gC promoters as typical $\gamma 1$ and $\gamma 2$ promoters to drive EGFP and RFP

expression respectively. The recombinant virus pgB/pgC replicated efficiently in culture and *in vivo* and induced an immune response similar to wild type virus. This virus also reactivated to similar levels as wild type HSV-1, expressing high levels of EGFP and RFP. Previous to this study, it was not clear whether lytic gene expression kinetics were maintained during latency. We show that EGFP expression (from γ1 gB promoter) occurs much sooner than RFP expression (from γ2 gC promoter) in *ex vivo* cultures demonstrating kinetic activity comparable to lytic replication. The highly activated phenotype of gB-CD8 in latently infected TG of C57BL/6 mice also led us to investigate if we could visualize antigen expression during latency. We wanted to determine if we could capture EGFP expression from the gB promoter in the absence of RFP from the late gC promoter. However, whole mount analysis of latently infected TG did not reveal either EGFP or RFP expression. It is possible that extremely low level EGFP expression was below our level of detection via confocal imaging. For ease of recombination, we had chosen to insert the gB-EGFP-gC-RFP cassette into the gC locus since gC is not essential for replication (52). Since DNA replication does not occur during latency and lytic genes are hypermethylated to maintain latency (179), it is also possible that gene expression from this locus was silenced in our recombinant virus. Therefore, during latency the gB promoter may be periodically active from its native locus, this activity could have been repressed within the gC locus due to histone modifications or due to the presence of repressors. This silencing was de-repressed when we treated latently infected mice with sodium butyrate, a histone deacetylase inhibitor and a known inducer of HSV-1 reactivation. Whole mounts of TG from treated mice demonstrated high levels of both EGFP and RFP expression. Therefore, while this virus remains a powerful tool to study gene expression during lytic replication and reactivation, our strategy did not allow us to observe gene expression during latency. A previous study showed the ability of IFNγ to block reactivation both before and after DNA replication in separate cultures using single fluorescent

viruses (31). By co-culturing latently infected TG with HSV-specific $CD8^+$ T cells, we can determine the frequency of cultures in which reactivation is stopped prior to DNA replication (gB-EGFP expression only) and in which reactivation is stopped following DNA replication (gB-EGFP and gC-RFP expression). This will further our understanding of how quickly $CD8^+$ T cells respond to reactivating virus and the mechanisms involved in blocking reactivation.

Following the previous study, we used an alternative approach to determine whether antigen expression occurred during latency and more importantly, how important this was to maintain an antigen-specific $CD8^+$ T cell response in the TG. Our observation that a stable number of HSV-1 genomes are maintained during latency led us to hypothesize that DNA replication did not occur during latency and consequently, γ2 proteins should not be expressed during this time. Using gB as our model antigen, we altered its expression from a γ1 protein that is expressed early during infection to one that is dependent on DNA replication. Surprisingly, this recombinant virus (gCp-gB) grew very poorly *in vivo* in infected TG. Several roles for gB have been implicated including viral entry, counteracting host response and viral egress (52, 113, 185). Therefore, altering gB expression seems to severely diminish viral growth, underscoring the importance of early gB expression for viral replication. The gB-CD8 infiltrate into the TG during latency was substantially diminished compared to the rescue virus. This phenomenon was not due to the inability of this virus to generate a gB-CD8 response in the lymph nodes or due to its poor growth. During the acute stages of infection, the infiltrating gB-CD8 in gCp-gB infected TG showed activation levels similar to the gB-CD8 in Rescue infected TG further demonstrating they were once exposed to high levels of antigen during acute infection. However, these gB-CD8 in gCp-gB infected TG fail to maintain this activated phenotype during latency (34 d.p.i) indicating the loss of antigen exposure during this time. These experiments show that periodic anrtigen exposure during latency is responsible for maintaining an activated, antigen-specific

$CD8^+$ T cell infiltrate in the TG that is capable of blocking reactivation. Future studies will involve a further analysis of the phenotype of the infiltrating gB-CD8. One of the more interesting aspects of this study was that viral latency is still maintained in the absence of a predominant gB-CD8 response. A small percentage of gB-CD8 are still retained in the TG and the proportion of gB-CD8 T cells reduces over time indicating that some other population of HSV-specific CD8 T cells may be expanding to take up the space created from the loss of the gB-CD8. A previous study has shown that the non-gB-CD8 population is also HSV-specific and able to block reactivation (145). It will be important to determine which viral antigens the non-gB-CD8 population responds to in gCp-gB infected TG and how the range specificity is affected when immunodominance is altered. In these studies we have not thoroughly explored the phenotype of the existing gB-CD8 in gCp-gB infected TG and whether they are functional *in ex vivo* assays. Sorting gB-CD8 from non-gB CD8 and performing *ex vivo* proliferation assays in response to antigen can give us a better idea of whether these cells are still functionally competent even though they are not exposed to high levels of antigen *in vivo*. The inflammatory environment of the TG latently infected with gCp-gB may be different from Rescue infected TG with respect to chemokine expression and dependence on cytokines such as IL-15 for attraction and survival of $CD8^+$ T cells in the TG. Future studies will involve characterizing these specific differences to further understand whether they are a contributing factor to the diminished gB-CD8 response seen in gCp-gB infected mice.

Interestingly, in *ex vivo* reactivation assays, gB-CD8 maintained their ability to block reactivation of gCp-gB infected cultures indicating that $CD8^+$ T cells can act very quickly, even after DNA replication. They may be able to act at a late stage by blocking viral assembly or even egress of the virus from infected cells. Future studies could involve understanding which viral protein functions are altered in the presence of $CD8^+$ T cells responding to reactivation.

Immunofluorescence imaging could reveal a differential localization of proteins involved in assembly or egress. It will be important to understand which stage of viral replication $CD8^+$ T cells act to block reactivation. Using chamber systems in which the soma and axons of neurons are separated, it will be important to determine whether assembly or trafficking of virus is affected in the presence of $CD8^+$ T cells. It is also possible that some late proteins are degraded by granzymes thus affecting the structural integrity of replicating virus. The importance of perforin and granzymes in blocking reactivation of also needs to be explored by using gB-CD8 missing one or both of these components. Performing *ex vivo* reactivation assays with TG cultures latently infected with gCp-gB or the dual fluorescent virus in the presence of granzyme or perforin null gB-CD8 will give us a better idea of which stage in reactivation these components are able to act on.

In order to design a novel vaccine strategy to prevent HSV-1 reactivation, we generated several recombinant viruses that expressed multiple copies of the immunodominant $gB_{498\text{-}505}$ $CD8^+$ T cell epitope from various HSV-1 promoters. Our strategy was to overexpress the epitope such that a greater gB-CD8 infiltrate was retained in the TG, capable of blocking reactivation upon stress stimuli. We tested one of the recombinant viruses *in vivo* that expressed thirteen repeats of the peptide from the LAT promoter (HSV-13LAT). While this virus demonstrated replication kinetics similar to the control, the gB-CD8 response was not greater in HSV-13LAT infected TG compared to the control. We then speculated that the endogenous gB protein was expressed to such high levels that the gB-CD8 response generated from the protein masked any gB-CD8 infiltrate induced by peptide expression. We then adopted a new strategy in which we generated a panel of mutant viruses in which the $gB_{498\text{-}505}$ epitope was abrograted such that it did not induce an appreciable gB-CD8 response within infected TG. We tested a subset of our recombinants in mice and observed that one mutant (gB-F5L) replicated to similar levels as a

wild type virus indicating that this mutation did not affect gB function or viral growth. In contrast to wild type viruses, the gB-CD8 infiltrate within TG of mice infected with gB-F5L was less than 2% of the total $CD8^+$ T cells. This gives us confidence that this virus does not induce a gB-CD8 infiltrate into the TG and we can now use this virus to re-derive the multimer viruses into this background. This will allow us to test these viruses in mice and observe whether we generate a gB-CD8 response simply by expressing $CD8^+$ T cell epitopes. It might also be worthwhile to move our studies into Balb/B mice which mount an immune response similar to C57BL/6 mice but unlike C57BL/6 mice, reactivation can be induced in Balb/B mice. Following that we can determine whether the number of epitopes has an affect on the magnitude of the gB-CD8 response. Finally, in Balb/B mice, we can ask the question whether a greater gB-CD8 response in the TG is capable of blocking reactivation *in vivo*, as a therapeutic vaccine strategy. Using the epitope mutant viruses (especially HSV-F5L) we can also try and determine the identity of other antigen-specific $CD8^+$ T cells retained in the TG. Following wild-type infection, all the $CD8^+$ T cells retained in the TG are HSV-1 specific and can block reactivation. It will be interesting to investigate how the range of specificities of the infiltrate will be altered in the absence of the immunodominant gB epitope. It is possible that other epitopes that were previously sub-dominant will now become predominant and occupy a larger proportion of the repertoire of the TG-resident $CD8^+$ T cells. The identity of these previously unknown epitopes will further our understanding on how antigen expression affects the development and retention of antigen-specific $CD8^+$ T cells in the TG.

The work presented here has given us significant insight on HSV-1 replication and on how HSV-1 influences the development and retention of antigen-specific $CD8^+$ T cells in latently infected TG. The ultimate goal of this research was to understand which immunogens would be the best choice to use in therapeutic vaccines. Our studies show that proteins expressed

early in infection and reactivation are more potent inducers of a $CD8^+$ T cell response and therefore make better candidates as therapeutic vaccine targets. While translating this work to human studies will require significant amount of work in terms of the best vaccination approach, it gives us a strong foundation towards developing an effective vaccine against HSV-1.

APPENDIX

PUBLICATIONS

1) **Ramachandran S**, Davoli, KA, Yee, MB, Hendricks, RL and Kinchington, PR. 2010. Delaying the expression of herpes simplex virus type 1 glycoprotein B to a true late gene alters neurovirulence and inhibits the gB-$CD8^+$ T cell response in the trigeminal ganglion. Submitted to *Journal of Virology*.

2) **Ramachandran S**, Knickelbein JE, Ferko C, Hendricks RL, Kinchington PR. 2008. Development and pathogenic evaluation of recombinant herpes simplex virus type 1 expressing two fluorescent reporter genes from different lytic promoters. *Virology.* 378(2):254-64.

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