NONCYTOTOXIC LYTIC GRANULE-MEDIATED MAINTENANCE OF HSV-1 NEURONAL LATENCY

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Reactivation of herpes simplex virus (HSV) from neuronal latency is a common and potentially devastating cause of disease worldwide. CD8 T cells can completely inhibit HSV reactivation, and IFN-γ affords a portion of this protection. We now show that CD8 T cell lytic granules are also required for the maintenance of HSV type 1 (HSV-1) neuronal latency both *in vivo* and in *ex vivo* cultures, and that their directed release into junctions with neurons in latently infected ganglia does not induce neuronal apoptosis. Our findings support a non-lethal mechanism of viral inactivation by demonstrating that the lytic granule component, granzyme B, degrades the HSV-1 immediate early protein, ICP4, which is essential for further viral gene expression. These findings reveal a novel non-apoptotic function of granzyme B in the context of inhibiting viral reactivation from latency.

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1.0 INTRODUCTION

1.1 HSV-1 CLASSIFICATION AND VIRION STRUCTURE

Herpes simplex virus type 1 (HSV-1), also known as human herpesvirus 1, is a member of the *Herpesviridae* family of viruses. Members of the *Herpesviridae* family generally share a particular virion architecture, including a linear double-stranded DNA core, an icosahedral capsid, an amorphous tegument, and a glycoprotein-rich envelope. *Herpesviridae* family members also have four distinct biological characteristics in common, namely *i)* expression of various enzymes involved in nucleic acid metabolism (*e.g.* thymidine kinase) and protein processing (*e.g.* protein kinases); *ii)* nuclear synthesis of viral DNA and capsids with cytoplasmic processing of the final virion; *iii)* production of infectious progeny that eventually destroy the infected cell; and *iv)* the ability to establish latency (1). Latency is defined as the retention of the viral genomes within an infected cell for a prolonged period without production of infectious virions. During latency, viral genomes exist as closed circular molecules known as episomes (2). Importantly, viral gene expression is dramatically reduced during latency, with only the latency associated transcripts (LATs) being abundantly transcribed (3-5). However, as discussed below, this does not mean that no other lytic transcripts are produced. Latent genomes are capable of replicating upon reactivation from latency; however, the mechanisms involved in HSV-1 reactivation remain incompletely understood.

Based on distinct biological properties, the *Herpesviridae* family of viruses is classified into three subfamilies, the *Alpha-, Beta-, and Gamma-herpesvirinae*. HSV-1 is a member of the *Alphaherpesvirinae* subfamily, which is characterized by a variable host range (*e.g.* mammals, birds, and reptiles), a short reproductive cycle, rapid cell-to-cell spread in culture, efficient destruction of infected cells, and the ability to establish latency primarily within neurons of sensory ganglia (1). Indeed, HSV-1 establishes latency solely within ganglionic neurons of humans and animal models (6, 7). HSV type 2 (HSV-2) and varicella zoster virus (VZV) are other examples of *Alphaherpesvirinae* subfamily viruses.

1.2 HSV-1 GENE EXPRESSION

During primary lytic infection with HSV-1, more than 80 viral genes are sequentially expressed in a highly regulated fashion. HSV-1 genes can be categorized into three main kinetic classes, namely immediate early (α), early (β), and late (γ). At high multiplicities of infection (MOI), such as 10 plaque-forming units of virus per cell, the immediate early gene products are synthesized within two to four hours post-infection. There are six α proteins, including ICP0, ICP4, ICP22, ICP27, ICP47, and $U_s1.5$, with all but ICP47 acting to transactivate β gene expression. Early gene expression requires at least the presence of ICP4 and peaks at approximately four to eight hours after infection. Several early proteins function to promote viral DNA replication, which is required for expression of most late genes. HSV-1 late gene products are subdivided into two groups differing in their dependence on viral DNA replication for expression. These two groups are the γ_1, or leaky late, and γ_2, or true late, genes. Glycoprotein B (gB) is an example of a γ_1 gene that is expressed relatively early during infection and whose

expression is stimulated mildly by viral DNA synthesis, while γ_2 genes, such as gC, are expressed later during infection and absolutely require DNA replication for expression (1). As discussed below, an epitope within gB is dominant in the CD8 T cell response to HSV-1 infection in the C57BL/6 mouse. Therefore, the timing of viral gene expression becomes important in determining when in the viral life cycle CD8 T cells are able to recognize infected cells expressing gB. Indeed, CD8 T cells have been shown to recognize gB on the surface of infected cells within two hours of infection (8). It must also be noted that, although the exact sequence of viral gene expression following reactivation from latency remains unknown, several studies suggest that the cascade of HSV-1 gene expression follows a similar pattern to that observed during primary lytic infection (9-13).

1.3 HSV-1 EPIDEMIOLOGY AND PATHOGENESIS

HSV-1 is a common cause of infection worldwide. Clinical history is not an accurate determinant of the prevalence of HSV-1 infection due to the high rate of asymptomatic carriers. Therefore, other methods must be used to determine the number of individuals who have been exposed to HSV-1. Serology for HSV-1-specific antibodies is a common method used to estimate the prevalence of HSV-1 infection. A recent study conducted in the United States discovered that the overall seroprevalence of HSV-1 has declined from 62% to 57.7% over the past two decades (14). Studies from Europe and Australia reveal a slightly higher prevalence of 74.5-98.8% depending on age (15, 16). In the majority of studies, HSV-1 seroprevalence increases with age (17). Perhaps a more sensitive method, PCR analysis has revealed that 89.1-100% of trigeminal ganglia from post-mortem adults contained HSV-1 DNA (18, 19).

Viral latency has been defined as the retention of functional viral genomes without production of transmissible virions (20). By this definition, reactivation from latency occurs at the point in which functional virus particles are produced and shed at the periphery. Reactivation of latent HSV, commonly associated with stress or immune compromise, can lead to a variety of pathological conditions, including herpes labialis, genital lesions, HSV encephalitis, and HSV stromal keratitis. While herpes labialis, also known as the common cold sore, is often painful and unsightly, HSV encephalitis and HSV stromal keratitis are life- and sight-threatening pathologies, respectively. HSV encephalitis is the most common cause of sporadically fatal viral encephalitis in the United States affecting between 1 in 250,000 to 1 in 500,000 individuals per year (21, 22). Moreover, HSV stromal keratitis is the most common cause of blindness of infectious etiology in developed nations (23) and the third-leading indication for penetrating keratoplasty (24). The significant morbidity and mortality associated with HSV-1 reactivation underscore the importance of understanding the mechanisms used by both the virus and the host during the maintenance of latency to aid the development of effective therapeutic vaccines that prevent viral reactivation.

1.3.1 Ocular HSV-1 infection

Of particular importance to our lab in the Department of Ophthalmology are the ocular pathologies related to HSV-1 infection. HSV-1 remains a leading cause of unilateral infectious corneal blindness worldwide (25). The global prevalence of HSV ocular infection has been estimated to be nearly 10 million people, including approximately one-half million cases in the United States (23, 26, 27). A further 21 to 31 individuals per 100,000 are newly diagnosed annually in developed nations (23, 26, 28). However, as mentioned above, it is thought that

clinical surveys grossly underestimate the true prevalence and incidence of HSV infections because approximately two-thirds of primary HSV infections are asymptomatic or not clinically recognized (29). Excluding cases of neonatal infections, estimates suggest that greater than 90-95% of ocular HSV infections are caused by HSV-1 (30, 31).

HSV stromal keratitis is a potentially blinding consequence of ocular HSV-1 infection. Even though HSV stromal keratitis accounts for only 2% of initial HSV-1 ocular presentations, it is the cause of 20-61% of recurrent disease (26, 32, 33). A history of previous HSV stromal keratitis episodes significantly increases the probability of subsequent stromal disease (34). HSV stromal keratitis can manifest in multiple forms including necrotizing and non-necrotizing disease. One study of 104 patients with HSV stromal keratitis revealed that disease was of the necrotizing type in 7%, non-necrotizing in 88%, and a mix of the two in 5% (35).

In necrotizing HSV stromal keratitis, necrosis, ulceration, and dense leukocytic infiltration of the corneal stroma are present and often accompanied by an overlying epithelial defect.(36, 37) Without timely and effective therapies, necrotizing HSV stromal keratitis can quickly lead to devastating corneal perforation. HSV-1 DNA, antigen, and even intact virions have been recovered from corneas with necrotizing HSV stromal keratitis (38-40), and active viral replication is implicated in the disease pathogenesis (36, 37). Thus, both immune and viral mechanisms can contribute to the pathogenesis of necrotizing HSV stromal keratitis.

The other type of HSV stromal keratitis is referred to as non-necrotizing or immune stromal keratitis, and the pathogenesis of this type of disease is less clear. Stromal inflammation is the hallmark of non-necrotizing HSV stromal keratitis (36, 37). This inflammation is mediated largely by lymphocytes, with CD4 T cells typically playing a more prominent role in mouse models (41-44). Inflammation may be focal, multifocal, or diffuse leading to thinning, scarring,

and neovascularization of the normally avascular cornea (45). It is hypothesized that stromal inflammation is driven by either HSV-1-specific $CD4^+$ T cells stimulated directly by viral antigens, bystander cytokine activation of CD4 T cells, autoantigens unmasked and mimicked by HSV-1 corneal infection, or a combination of these processes (46). It must also be noted that HSV-1 antigen and DNA have occasionally been detected in quiescent corneal buttons from patients with a history of HSV-1 stromal keratitis (40, 47-50). However, in these studies, it is not clear which type of stromal disease previously afflicted the cornea, and many included corneas with disciform keratitis in the non-necrotizing category further confounding interpretation of the results. Thus, roles for viral DNA, antigen, or replication in the pathogenesis of non-necrotizing stromal disease cannot be ruled out, and it is possible that non-necrotizing and necrotizing HSV-1 stromal keratitis represent a spectrum of disease rather than separate pathological processes.

1.4 HSV-1 LATENCY

A hallmark of the herpes family of viruses is their propensity to cause recurrent disease. Interestingly, in most cases, recurrent HSV disease is not the result of reinfection from an external source, but is rather due to reactivation of virus that has established a latent infection within the host. Upon infection of mucosal or abraded epithelial surfaces, HSV fuses to and enters innervating peripheral sensory nerve termini, and the viral nucleoplasmid is shuttled via retrograde axonal transport to the neuronal nucleus in the cell body within the connecting sensory ganglion (51). Thus, following corneal infection, HSV establishes latency within neurons of the ophthalmic branch of the trigeminal ganglion. HSV replicates briefly within infected sensory neurons, and then establishes a latent infection in a portion of these neurons.

Latency is operationally defined as the retention of the viral genome within a cell for a prolonged period without production of infectious virions. Indeed, latent HSV persists in sensory ganglia for the life of the individual. In some infected people, latency is periodically interrupted in one or a few neurons leading to virion formation, anterograde transport to the peripheral site innervated by the neuron, and viral shedding with or without recurrent clinical lesions.

In recent years, technological advances have allowed a characterization of latently infected ganglia in terms of the number of neurons harboring latent viral DNA and the number of viral genomes contained within individual latently infected neurons. This detailed level of analysis is critical in determining the relationship between latent viral burden and the frequency and magnitude of reactivation. Early studies investigating the amount of latent HSV-1 DNA within infected ganglia indicated that each ganglion-derived cell contained between 0.1 and one viral genome equivalents (52-54). Since neurons are estimated to make up only 10% of total sensory ganglion cells, it was hypothesized that each latently infected neuron contained multiple copies of HSV-1 DNA (55). Indeed, using sensitive PCR methods, it was determined that individual latently infected murine neurons contained <10 to >1,000 HSV genomes (56, 57), while laser-capture microscopy of latently infected human ganglia revealed and average of 11 HSV DNA copies per neuron (58). Furthermore, the number of HSV-1 genome copies per latently infected neuron has been shown to be directly proportional to the ability of virus to reactivate (59). Additionally, the titer of the viral inoculum directly correlates with the number of neurons that become latently infected, which also determines the frequency of viral reactivation (60). Therefore, both the number of latently infected neurons and the latent viral burden within individual neurons correlate with the ability of HSV-1 to reactivate within infected ganglia.

1.4.1 Immune control of acute HSV-1 ganglionic infection

The mechanisms underlying this unique capacity of HSV-1 to establish, maintain, and reactivate from a latent state has been the focus of extensive research over the past several decades. Both the innate and adaptive immune systems have been implicated in controlling viral replication within sensory ganglia during acute HSV-1 infection. Following corneal HSV-1 infection of mice, type 1 interferons (IFN) are produced within infected ganglia, and overexpression of IFN-α1 within infected ganglia significantly inhibited viral replication (61). Additionally, macrophages and γδ T cells infiltrate HSV-infected trigeminal ganglia and control the majority of viral replication at this site (62-64). Macrophages were found to secrete nitric oxide (NO) and tumor necrosis factor alpha (TNF-α), both of which possess potent antiviral activity, within infected ganglia (62), while depletion of γδ T cells during acute infection significantly increased HSV-1 replication and eliminated the majority of IFN-γ within the ganglia (65). Therefore, the innate cells that infiltrate sensory ganglia acutely infected with HSV-1 appear to control viral replication and spread through secretion of various cytokines.

A functional adaptive immune response is also required for complete elimination of replicating HSV-1 from infected ganglia. Mice lacking an adaptive immune system (severe combined immune deficiency; SCID) succumb to fatal viral encephalitis within about two weeks of infection; however, HSV-1 neuronal latency was still established equally compared to wild-type mice possessing an intact adaptive immune system (66, 67). Adoptive transfer of splenocytes or purified αβ T cells from immunized mice fully protected HSV-infected SCID

mice from death, while transfer of immune serum prolonged survival without affecting overall mortality (68, 69). Thus, αβ T cells of the adaptive immune system appear to be required for survival following infection with HSV-1, but they are not required for the establishment of neuronal latency.

1.4.2 The latency-associated transcripts (LATs)

A potential breakthrough in HSV biology occurred in the mid-1980s when a series of viral transcripts referred to as latency associated transcripts (LATs) were found to be prominently expressed in latently infected neurons. These transcripts are first detectable during the lytic phase of HSV-1 infection; however, much higher levels of LATs are detected within infected trigeminal ganglia during latency (70, 71). The possibility that this family of viral transcripts that appeared to be uniquely expressed during latency might represent a mechanism for establishing or maintaining HSV latency spurred extensive research into the properties of LATs. Unfortunately, the enthusiasm for LATs as the holy grail of HSV latency was somewhat diminished by early studies demonstrating that the LAT region is not absolutely required for HSV-1 establishment and maintenance of latent infections or for reactivation from latency (72-74). Nonetheless, recent findings do support a role for LATs in determining the propensity of HSV-1 and HSV-2 to establish latency in different subtypes of sensory neurons and in protecting latently infected neurons from undergoing apoptosis (75-78).

1.4.3 Host cell and viral mechanisms of maintaining HSV-1 neuronal latency

Upon establishment of latency, the HSV-1 genome circularizes into episomes (2, 53, 54), which assemble into nucleosomes (79). The transition from latent to lytic infection during HSV-1 reactivation may be associated with a conversion from this circular genome to a linear form regulated by both host and viral factors. Cross-regulation between host-derived IFN-γ and the HSV-1 immediate early gene product, ICP0, a promiscuous transactivator of HSV-1 lytic genes, appears to influence this transition. ICP0 has been shown to degrade proteins associated with nuclear domain 10 bodies (80), which are discrete nuclear substructures that HSV-1 genomes associate with early during infection, thereby allowing gene transcription and subsequent viral reactivation to occur. Importantly, IFN-γ is able to block HSV-1 reactivation in *ex vivo* cultures of latently infected trigeminal ganglia in part by inhibiting ICP0 promoter activity (81).

Epigenetic modifications, particularly histone methylation and acetylation, of the latent HSV-1 genome have also been shown to play a role in the transition from latency to lytic infection during viral reactivation (82). During latency, portions of the LAT-encoding gene, including the promoter and enhancer regions, are associated with acetylated histones, indicative of active chromatin or euchromatin, consistent with the high levels of LAT mRNA produced during latency (83, 84). Alternatively, most lytic genes promoters are associated with methylated histones, indicative of inactive or heterochromatin, during latency (85). Upon reactivation in ganglia explant cultures, the LAT enhancer region is rapidly deacetylated, while the ICP0 promoter becomes acetylated, followed by an increase in the production of ICP0 mRNA (86).

Consistent with these findings, histone deacetylase inhibitors, such as sodium butyrate, have been shown to induce reactivation of latent HSV-1 in certain mouse strains by modifying viral DNA-associated histones in a similar manner to that seen following *ex vivo* culture of latently infected ganglia (19, 87).

More recently, HSV-derived microRNAs have been implicated in maintaining HSV-1 in a latent state via post-transcriptional regulation of viral gene expression (88, 89). Several different species of microRNAs expressed during latency were shown to inhibit production of HSV-1 immediate early proteins, including ICP0 and ICP4 (89), both of which can promote efficient reactivation from latency (90). Thus, HSV-1 expresses at least two primary microRNAs in latently infected neurons that may facilitate the establishment and maintenance of viral latency.

1.5 IMMUNE CONTROL OF HSV-1 LATENCY

Although the above studies identified mechanisms that might contribute to the maintenance of HSV latency and reactivation from the latent state, there is no obvious way to exploit this knowledge in developing a strategy for preventing recurrent herpetic disease. Alternatively, exciting observations began to emerge in the mid 1990s implicating the host immune system in maintaining HSV-1 latency in mice. Mice harbor latent HSV-1 in sensory ganglia following infection at peripheral sites, and the virus can be induced to reactivate from latency *in vivo* by exposure of latently infected mice to stressful stimuli such as hyperthermia, ultraviolet irradiation, and psychological stressors (91-94), and *in vitro* by explanting and culturing dispersed latently infected sensory ganglia (95, 96). However, HSV-1 does not spontaneously

reactivate from latency in murine sensory ganglia *in vivo* as is observed in latently infected sensory ganglia of humans, rabbits, and guinea pigs, suggesting that the mechanisms responsible for maintaining HSV-1 latency are well-developed in mice.

Contrasting with the apparent stability of HSV-1 latency in mouse sensory ganglia is the growing evidence demonstrating a maintained adaptive immune response to HSV-1 within latently infected ganglia. CD69-expressing CD4 and CD8 T cells, inflammatory cytokines including IFN-γ, TNF-α, and interleukin (IL)-6, as well as transcripts for molecules involved in inflammatory cell chemoattraction, such as RANTES, CCR5, and CXCR3, are present throughout latency in ganglia of HSV-1-infected mice (63, 97-103). Although no complete infectious virions are produced during stable latency, a limited array of lytic genes, such as ICP4, and even proteins are expressed by some latently infected neurons in mice (103-105). Combined, these findings gave rise to a proposed dynamic model of HSV-1 latency in which HSV-specific CD8 T cells maintained within latently infected ganglia are continually scanning the microenvironment in an attempt to curtail reactivation and prevent formation of intact virions and shedding in the periphery. Early studies also suggest an important role of CD4 T cells in the progression of acute HSV-1 infection into stable latency (106, 107). However, little is known about the mechanism that CD4 T cells employ in the clearance of virus and establishment of the latent state. Understanding the factors that lead to viral reactivation, the immune mechanisms involved in preventing reactivation, and the fate of latently infected neurons will assist in the design of better therapeutic modalities to treat recurrent herpetic disease.

CD8 T cells appear to be one critical component of the adaptive immune response to HSV-1 within infected ganglia. In general, CD8 T cells exert their effector function either by exocytosis of lytic granules, which contain cytolytic molecules including perforin and

granzymes, or by secretion of the antiviral cytokines IFN-γ and TNF-α (108). Following release of CD8 T cell lytic granules toward virally infected cells, perforin facilitates entry of other granule components, such as granzymes, into the infected cell generally leading to caspase activation and apoptosis (109). Mice deficient in CD8 T cells due to *in vivo* antibody depletion are unable to control HSV-1 replication and are significantly impaired in their ability to clear the virus from infected ganglionic neurons after primary infection (110). Additionally, granzyme A is required to restrict the spread of HSV-1 within infected ganglia (111). Furthermore, a significant proportion of neurons undergo cell death in the absence of CD8 T cells (110). These data taken together suggest a nonlytic, protective role for CD8 T cells within infected ganglia.

The majority of CD8 T cells found in HSV-1 latently infected ganglia of C57BL/6 mice are specific for an epitope within the viral envelope molecule, glycoprotein B ($gB_{498-505}$) (97). Nearly all of the CD8 T cells within latently infected ganglia persistently express the early activation marker, CD69, suggesting recent stimulation (97). Furthermore, a significantly higher proportion of gB-specific CD8 T cells than gB-nonspecific CD8 T cells also express the lytic granule component, granzyme B (112), suggesting persistent antigenic stimulation of gB-specific CD8 T cells within latently infected ganglia. Importantly, ganglionic gB-specific CD8 T cells polarize their T cell receptors (TCRs) toward neurons within HSV-1 latently infected murine TGs forming an apparent immunologic synapse (97). Such TCR polarization implies the presence of major histocompatibility complex (MHC) class 1/gB peptide complexes on the surface of latently infected neurons. Consistent with this observation, persistent activation of gB-specific CD8 T cells within latently infected sensory ganglia depends on antigen presentation by ganglia parenchymal cells (*i.e.* neurons and their support cells) and not on bone marrow-derived antigen-presenting cells (113). This concept is further supported by the observations that CD8 T

cells are unable to prevent reactivation in HSV-1-infected neurons that are MHC class 1-incompatible (97) or in neurons infected with recombinant HSV-1 strains expressing cytomegalovirus (CMV) immune evasion molecules that inhibit MHC class 1 presentation (114). These data, combined with the fact that HSV-1 establishes latency only in neurons (115, 116), has lead to the hypothesis that infected neurons directly present viral antigen to gB-specific CD8 T cells during attempted reactivation.

Latent HSV-1 can be induced to reactivate following excision and culture of dispersed ganglia cells (103). CD8 T cells from lymph nodes of mice acutely infected with HSV-1 are able to completely protect (103), and a gB-specific CD8 T cell line (2D5) can nearly completely protect (97) HSV-1 latently infected neurons from viral reactivation in *ex vivo* cultures. Further, HSV-specific CD8 T cell-mediated protection is antigen-specific, dose-dependent, and MHC-restricted (97). Recombinant IFN-γ has been shown inhibit reactivation in a portion of neurons within *ex vivo* cultures of HSV-1 latently infected trigeminal ganglia (81, 117), and CD8 T cells appear to be a significant source of INF-γ in *ex vivo* ganglia cultures (117). Interestingly, CD8 T cells protect *ex vivo* cultures of latently infected ganglionic neurons without depleting the pool of latently infected neurons (103). Combined with the observations demonstrating significant neuronal death in the absence of CD8 T cells (110), these data strongly suggest that HSV-1-specific CD8 T cells utilize nonlytic mechanisms to protect HSV-1 latently infected neurons from viral reactivation.

Consistent with the notion of noncytolytic CD8 T cell immunosurveillance of HSV-1 latently infected ganglia is the observation that many HSV-1-specific CD8 T cells also express the CD94/NKG2A inhibitory heterodimer (112, 118). Expression of this receptor on virus-specific CD8 T cells downregulates antigen-specific cytotoxic activity (119) without affecting

secretion of the antiviral cytokine, IFN-γ (118). Furthermore, a portion of neurons within HSV-1 latently infected trigeminal ganglia were shown to express Qa-1, the major ligand of the CD94/NKG2A heterodimer, and blocking this interaction in *ex vivo* ganglia cultures led to increased neuronal cell lysis (112). Therefore, it appears that specific neurons can dictate which effector mechanism(s) are employed by CD8 T cells to block HSV-1 reactivation from latency.

Humans are the only natural host for HSV-1, and the virus appears to have adapted well to its host. The virus reactivates from latency and proliferates at mucosal surfaces in many infected individuals, permitting spread to new hosts. In contrast, HSV-1 spontaneously reactivates to a much lesser extent in mouse sensory ganglia (120). The enhanced reactivation in human ganglia may reflect at least in part the heightened efficiency with which the HSV-1 immediate early gene product, ICP47, inhibits the human transporter associated with antigen processing (TAP) relative to the mouse TAP (121). Since TAPs have an essential role in the loading of viral peptides on MHC class 1 for presentation to CD8 T cells, this effect of ICP47 might preferentially reduce the efficiency of human CD8 T cell immunosurveillance, particularly in neurons with low level production of both viral proteins and MHC class 1. Indeed, ICP47 was shown to enhance HSV neurovirulence in mice by blocking the CD8 T cell response (122).

Importantly, many of the immunologic phenomena associated with latent HSV-1 infection in mice have been reproduced in human studies examining cadaveric ganglia. Expression of HSV-1 immediate early genes, such as ICP0 and ICP4, has been detected in latently infected human ganglia, and activated CD8 T cells have been found surrounding HSV-1 LAT^+ neurons (123-126). Also, trigeminal ganglia in which HSV-1 LAT^+ neurons were identified contained significantly more CD8, IFN-γ, TNF-α, and the T cell chemoattractant RANTES mRNA transcripts compared to ganglia whose neurons tested negative for LAT

expression (123, 124). It has recently been demonstrated that CD8 T cells resident in human cadaveric ganglia express an effector memory phenotype and cluster around HSV-1 latently infected but not varicella zoster virus latently infected neurons (125). Consistent with studies in the murine model, neuronal damage was not observed in latently infected human trigeminal ganglia despite granzyme B expression by many of the CD8 T cells surrounding HSV-1 latently infected neurons (125, 126). These findings in human ganglia, which replicate previous findings in mice, support the relevance of using the murine model of HSV-1 infection to identify mechanisms of HSV-1 latency that may be applicable to humans.

2.0 STATEMENT OF THE PROBLEM

CD8 T cells, many of which contain the cytotoxic molecule granzyme B within lytic granules, surround apparently healthy neurons within HSV-1 latently infected ganglia of humans and mice. Furthermore, CD8 T cells are required to maintain HSV-1 in a latent state preventing reactivation and recurrent disease. CD8 T cells produce IFN-γ, which blocks HSV-1 reactivation in some, but not all latently infected neurons. Therefore, CD8 T cells must employ additional effector mechanisms during the maintenance of HSV-1 neuronal latency. Release of lytic granules represents another important CD8 T cell effector mechanism. However, lytic granule release is generally thought to be lethal to target cells, an effect that would be detrimental to nonregenerating neurons within sensory ganglia. Using a murine model of HSV-1 corneal infection, my dissertation research tested the overarching hypothesis that CD8 T cells use lytic granules in a noncytotoxic manner to block HSV-1 reactivation from neuronal latency by directly degrading HSV-1 protein(s) required for further viral propagation.

3.0 SPECIFIC AIMS

The specific aims that were initially set forth for my dissertation research project as well as the progress made toward these aims are outline below.

Specific Aim 1: To investigate whether HSV-specific CD8 T cells secrete lytic granules through immunological synapses with neurons from latently infected trigeminal ganglia, and whether lytic granule release results in neuronal apoptosis.

Hypothesis: HSV-specific CD8 T cells form immunological synapses with and are directly activated by HSV-1 latently infected neurons to release lytic granules, but that directed lytic granule exocytosis does not cause neuronal caspase activation or apoptosis.

Imaging revealed that HSV-specific CD8 T cells formed stable conjugates containing lytic granule secretory domains directed toward neurons in *ex vivo* cultures of dispersed trigeminal ganglia latently infected with HSV-1. While HSV-specific CD8 T cells induced caspase activation and apoptosis of susceptible fibroblasts, neither caspase activation nor morphologic signs of apoptosis were evident in neurons targeted by CD8 T cell lytic granules. We also demonstrated CD8 T cell polarization of granzyme B toward neurons *in situ* by staining and imaging intact HSV-1 latently infected trigeminal ganglia tissue suggesting continued use of lytic granules during CD8 T cell immunosurveillance of HSV-1 neuronal latency *in vivo*.

Specific Aim 2: To investigate whether the lytic granule components perforin and granzyme B are necessary for the establishment and/or maintenance of HSV-1 neuronal latency.

Hypothesis: Perforin and granzyme B are not required for the establishment but are necessary for the maintenance of HSV-1 neuronal latency.

Previous reports have indicated that natural killer and CD8 T cells, the two major lymphocyte classes containing lytic granules, are not required for the establishment of HSV-1 neuronal latency. In support of these reports, our data demonstrate that perforin- and granzyme B-deficient mice clear replicating virus from infected corneas and trigeminal ganglia with similar kinetics to wild-type mice. However, we now show that HSV-1 latency within perforin- and granzyme B-deficient ganglia is unstable with significant elevations in viral genome copies between 10 and 14 days post-infection. Furthermore, perforin- and granzyme B-deficient CD8 T cells are compromised in their ability to inhibit HSV-1 reactivation from latency in *ex vivo* ganglia cultures compared to wild-type CD8 T cells.

Specific Aim 3: To investigate whether granzyme B inhibits HSV-1 reactivation by cleaving essential HSV-1 proteins thereby inactivating the viral life cycle prior to reactivation.

Hypothesis: Granzyme B cleaves an HSV-1 immediate early protein that is required for further viral gene transcription to inactivate the viral life cycle prior to reactivation.

Using the GraBCas bioinformatics tool, we identified putative granzyme B cleavage sites in certain HSV-1 proteins, including the immediate early protein, ICP4, that is absolutely required for further viral gene transcription. We then investigated ICP4 within HSV-infected fibroblasts as a potential target of CD8 T cell granzyme B. Indeed, wild-type but not perforin- or granzyme

B-deficient CD8 T cells significantly reduced the level of ICP4 within HSV-1-infected fibroblasts. Further, we demonstrated that recombinant human granzyme B directly cleaved ICP4 in lysates of cells either transfected with an ICP4-expressing plasmid or infected with HSV-1 as well as ICP4 purified by immunoprecipitation from HSV-1-infected cells.

4.0 MATERIALS AND METHODS

4.1 MICE

Six- to 10-week-old female C57BL/6J (WT; Jackson Laboratory), C57BL/6-Prf1^{tm1Sdz}/J (perforin$^{-/-}$; Jackson Laboratory), or C57BL/6J mice deficient in granzyme B (granzyme B$^{-/-}$; a kind gift from Dr. Timothy Ley, Washington University School of Medicine) were used in this research.

4.2 VIRUS AND OCULAR INFECTIONS

The wild-type RE strain of HSV-1 (HSV-1 RE) as well as a recombinant HSV-1 RE that expresses EGFP from the ICP0 promoter (pICP0-EGFP HSV-1 RE) were grown in Vero cells, and intact virions were purified using OptiPrep gradient columns (Accurate Chemical and Scientific Corporation) according to the manufacturer's instructions. Plaque-forming units (pfu) of HSV-1 were determined by standard viral plaque assays on Vero cells. Prior to corneal infection with HSV-1, mice were anesthetized by intraperitoneal injection of 2.0 mg ketamine hydrochloride and 0.04 mg xylazine (Phoenix Scientific) in 0.2 mL HBSS (BioWhittaker). The corneas of anesthetized mice were then scarified with a sterile 30-gauge needle in a crisscross fashion. $1x10^5$ pfu of purified HSV-1 in 3 μl RPMI (BioWhittaker) were applied to the scarified

corneas. Notably, the number of times a cornea is scratched prior to application is a critical determinant of the magnitude of infection and subsequent immune response, with a higher number of scratches leading to increased viral loads and immune cell infiltrates into HSV-infected ganglia (See Appendix A, Figure 24). All animal studies were approved by and conducted in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

4.3 GENERATION OF SINGLE-CELL TRIGEMINAL GANGLIA SUSPENSIONS

At the indicated times after infection, mice were euthanized by exsanguination. Trigeminal ganglia were excised, digested in DMEM containing 10% FBS and 400 U/ml collagenase type 1 (Sigma-Aldrich) per trigeminal ganglia for 1 hr at 37° C. Finally, trigeminal ganglia were dissociated into single-cell suspensions by repeated trituration through 200 μl pipette tips.

4.4 FLOW CYTOMETRY

Single-cell suspensions of trigeminal ganglia were prepared as above. The cells were passed through a 40 μm filter to remove debris and incubated with anti-CD16/CD32 antibody to block nonspecific antibody binding to $F_c\gamma$ receptors. Cells were then surface stained with fluorochrome-conjugated antibodies at 4° C. For intracellular staining, surface-stained cells were washed and then fixed and permeabilized in Cytofix/Cytoperm (BD), washed in Perm/Wash (BD), and then stained with fluorochrome-conjugated antibodies diluted in Perm/Wash. Finally,

the cells were washed and analyzed on a FACSAria flow cytometer (BD). Fluorochrome-conjugated antibodies against the following surface molecules were used: α-CD45 (clone 30-F11), α-CD3ε (clone 145-2C11), α-CD8α (clone 53-6.7), CD107a (LAMP-1; clone 1D4B), and appropriate isotype controls (all from BD Biosciences). Fluorochrome-conjugated antibodies against the following intracellular molecules were used: α-granzyme B (clone GB11) and isotype control (both from Caltag) as well as α-IFN-γ (clone XMG1.2) and isotype control (both from BD Biosciences). PE-conjugated H-2K^b/gB$_{498–505}$ (SSIEFARL) tetramers or biotinylated H-2K^b/gB$_{498–505}$ monomers (Tetramer Core Facility, NIAID, NIH) conjugated to streptavidin-bound Qdots (Invitrogen) at a molar ratio of 1:1 were used to identify the H-2K^b-restricted HSV-1 gB$_{498–505}$-specific CD8 T cell population.

4.5 ISOLATION AND EXPANSION OF GLYCOPROTEIN B-SPECIFIC CD8 T CELLS FROM HSV-1 LATENTLY INFECTED TRIGMEINAL GANGLIA

Trigeminal ganglia from mice latently infected with HSV-1 were excised and dissociated into single-cell suspension as described above. Suspensions were then cultured with B6WT3 fibroblasts (127) transfected to stably express glycoprotein B for 10 days in RPMI containing 10% FBS, 10% Rat T-Cell Culture Supplement with ConA (BD Biosciences), and 5% methyl α-D-mannopyranoside (Sigma-Aldrich). CD8 T cells were then positively selected by MACS bead separation according to the manufacturer's instructions. Flow analysis revealed that >95% of the resulting cells stained positively for gB$_{498–505}$/H-2K^b tetramer (see Figure 2A). Nearly all of the gB-specific CD8 T cells from WT and perforin$^{-/-}$ trigeminal ganglia expressed granzyme B, while the granzyme B$^{-/-}$ gB-specific CD8 T cells were devoid of granzyme B (see Figure 14A).

Moreover, after a 6-hr stimulation with targets pulsed with 10^{-6} M $gB_{498-505}$ peptide (SSIEFARL; Invitrogen Life Technologies), gB-specific CD8 T cells from all three strains of mice expressed similar levels of CD107a on their surface and contained similar levels of intracellular IFN-γ (See Figure 14B).

4.6 LIVE-CELL IMAGING OF GLYCOPROTEIN B-SPECIFIC CD8 T CELL INTERACTIONS WITH B6WT3 FIBROBLASTS

Real-time epifluorescence microscopy was used to image interactions between gB-specific CD8 T cells and B6WT3 fibroblast targets. To image the movement of lytic granules within gB-specific CD8 T cells, these cells were labeled with 60 nM LysoTracker Red (Invitrogen) for 1 hr, washed, and added to $gB_{498-505}$ peptide-pulsed B6WT3 fibroblasts plated in either Bioptics Delta T or MatTek coverslip-bottom dishes. Time-lapse fluorescence and differential interference contrast (DIC) images were acquired every 30 seconds on either a Nikon TE 2000E inverted microscope with a 1.4 numerical aperture (NA) 60X oil objective and a Cascade 1K camera or an Olympus IX81 inverted microscope with a 1.4 NA 60X oil objective and QImaging Retiga EXi Fast CCD camera (QImaging). Metamorph software (Molecular Devices) was used to acquire all images and to drive the microscopes. Cells were maintained at 37° C using heated stage inserts during imaging, and pH was maintained using HEPES-buffered media.

To image apoptosis of B6WT3 fibroblasts exposed to gB-specific CD8 T cells, $gB_{498-505}$ peptide-pulsed B6WT3 fibroblasts were labeled with the membrane dye, DiI (Invitrogen), according to the manufacturer's protocol and plated in either Bioptics Delta T or MatTek coverslip-bottom dishes. Unlabeled gB-specific CD8 T cells were added to the dishes and fluorescence and DIC images were acquired as above.

4.7 FIXED-CELL IMAGING OF GLYCOPROTEIN B-SPECIFIC CD8 T CELL INTERACTIONS WITH EITHER B6WT3 FIBROBLASTS OR CELLS IN TRIGEMINAL GANGLIA CULTURES

Fixed-cell confocal or wide-field microscopy was used to visualize the polarization of CD8 T cell effector molecules toward neuronal and fibroblast targets and to visualize caspase activation within target cells. HSV-1-infected B6WT3 fibroblasts or *ex vivo* trigeminal ganglia cultures were incubated with previously isolated and expanded gB-specific CD8 T cells for 1-48 hrs prior to staining for active caspases with Red-VAD-FMK (Calbiochem) according to the manufacturer's protocol. Where indicated, gB-specific CD8 T cells were labeled with DiI (Invitrogen) according to the manufacturer's protocol prior to addition to cultures. For Ab staining, cultures were fixed in 2% paraformaldehyde, fluorescently stained for surface CD8α, CD107a, and/or gB-specific TCR with $gB_{498–505}$/H-$2K^b$ tetramers, permeabilized in PBS containing 0.1% Triton X-100, washed in PBS, and then fluorescently stained for intracellular NeuN and/or granzyme B. Images were acquired on either a Nikon TE-2000 wide-field inverted microscope with either a 1.4 NA 60X oil or 1.3 NA 40X oil objective and a Cascade 1K camera, a Nikon C1 Digital Eclipse confocal microscope with a 1.4 NA 60X oil objective, or an Olympus

Fluoview 1000X confocal microscope with either a 1.4 NA 60X oil or 1.3 NA 40X oil objective. Images were acquired by sequential scanning to avoid fluorescence crossover, and, where indicated, Z stacks were acquired at Nyquist sampling frequency through the full thickness of the cells. All image reconstructions were made using Metamorph.

4.8 IMMUNOFLUORESCENCE STAINING OF INTACT TRIGEMINAL GANGLIA TISSUE

Intact trigeminal ganglia were excised, washed in PBS with 4% FBS, and incubated with directly a conjugated anti-CD8α antibody diluted in PBS with 4% FBS overnight. The tissue was then extensively washed in PBS with 4% FBS. For intracellur NeuN and granzyme B staining, surface-stained tissue was fixed and permeabilized in Cytofix/Cytoperm (BD), washed in Perm/Wash (BD), and then stained with anti-NeuN and anti-granzyme B antibodies diluted in Perm/Wash. Images were acquired on an Olympus Fluoview 1000X confocal microscope with a 1.4 NA 60X oil objective. Images were acquired by sequential scanning to avoid fluorescence crossover, and, where indicated, Z stacks were acquired at Nyquist sampling frequency through the tissue. All image reconstructions were made using Metamorph.

4.9 QUANTITATIVE REAL-TIME PCR

DNA was isolated from dispersed trigeminal ganglia using DNeasy columns (Qiagen) and diluted to 1 ng/µl in nuclease-free dH_2O. DNA (25 ng) or water control was mixed in duplicate with an equal volume TaqMan Universal PCR Master Mix (Roche) and an HSV-1 glycoprotein H (gH)-specific primer-probe set, custom designed and synthesized by ABI Assays-by-Design service (Applied Biosystems). Samples 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. Sequences of the gH forward primer: 5'-GACCACCAGAAAACCCTCTTT-3', reverse primer: 5'ACGCTCTCGTCTAGATCAAAGC-3', and probe: 5'-(FAM)TCCGGACCATTTTC(NFQ)-3'. Since the HSV-1 genome contains a single copy of the gH gen, viral genome copy number could 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.

4.10 DETECTING HSV-1 REACTIVATION IN DISPERSED TRIGEMINAL GANGLIA CULTURES

Single-cell trigeminal ganglia suspensions were prepared as above. Dissociated cells were cultured in DMEM (Biowhittaker) containing 10% FBS (Atlanta Biologicals), 10 mM HEPES buffer (GIBCO), 10 U/ml recombinant murine IL-2 (R&D Systems), and 50 µM 2-Mercaptoethanol (Fisher Scientific). Individual culture wells were monitored for reactivation by serially testing culture supernatant fluid for live virus by standard viral plaque assays on Vero

cell monolayers. Where indicated, cultures were supplemented with monoclonal antibodies against IFN-γ (20 μg/ml; clone R4-6A2) or CD8α (100 μg/ml, clone 2.43), supplemented with recombinant IFN-γ (1,000 U/ml; R&D Systems), or depleted of endogenous CD8 T cells by magnetic Dynal bead (Invitrogen) depletion of $CD8\alpha^+$ cells or by antibody/complement-mediated lysis (Low-Tox-M Rabbit Complement; Cedarlane) of $CD8\alpha^+$ cells prior to culture initiation. The efficiency of $CD8^+$ T cell depletion was routinely greater than 95% as assessed by flow cytometric analysis.

4.11 ICP4 CLEAVAGE BY GRANZYME B IN LYSATES OF TRANSFECTED/INFECTED CELLS

293T cells were transfected with a plasmid encoding full-length ICP4 from its native promoter (P1-2; kindly provided by Dr. Neal Deluca, University of Pittsburgh) or B6WT3 fibroblasts were infected with HSV-1 at a multiplicity of infection 10 for 6 hrs. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and soluble lysate was obtained after centrifugation. Soluble lysate was added with the indicated concentrations of recombinant human granzyme B (Calbiochem) to ICE buffer (10 mM HEPES/KOH, 2 mM EDTA, and 1% NP-40; pH 7.4) and incubated for the indicated times at 37° C. Samples were electrophoresed on an 8% SDS-polyacrylamide gel prior to Western blotting with a rabbit anti-ICP4 polyclonal antibody (kindly provided by Dr. Neal Deluca, University of Pittsburgh).

4.12 IMMUNOPRECIPITATION OF ICP4 FROM HSV-1-INFECTED CELL LYSATES

B6WT3 fibroblasts were infected at a multiplicity of infection of 10 with pICP0-EGFP HSV-1 (128) for 6 hrs. The infected cells were lysed in RIPA buffer, and the soluble fraction was incubated with rabbit anti-ICP4 polyclonal antibody for 4-16 hrs at 37°C. A 30% (V/V) solution of Protein A-Sepharose CL-4B beads (Sigma-Aldrich) was then added and incubated for 1 hr at 4° C. The beads were washed extensively in RIPA buffer. Bead-bound ICP4 was then exposed to recombinant granzyme B and analyzed by immunoblot with a mouse anti-ICP4 monoclonal antibody (clone: 10F1; Virusys Corp.).

4.13 IN VITRO CLEAVAGE OF ICP4 BY GLYCOPROTEIN B-SPECFIC CD8 T CELL GRANZYME B

B6WT3 fibroblasts were infected with pICP0-EGFP HSV-1 RE (128) for 1 hr. Cells were then washed and incubated with WT, perforin$^{-/-}$, or granzyme B$^{-/-}$ gB-specific CD8 T cells (see section 3.5 above) at an effector-to-target ratio of 6:1 for 5 hrs. Cells were then harvested by scraping and washed in PBS. Half of the cells were stained for CD8α and analyzed by flow cytometry for EGFP expression on CD8α^{-} cells, while the other half of the cells was lysed and analyzed for ICP4 expression by Western blotting. Optical density (O.D.) measurements were made on scanned films with ImageJ software (NIH).

4.14 STATISTICAL ANALYSIS

All statistical analyses were computed with GraphPad Prism software. p values <0.05 were considered statistically significant.

5.0 RESULTS

5.1 LYTIC CAPACITY OF CD8 T CELLS WITHIN HSV-1 LATENTLY INFECTED TRIGEMINAL GANGLIA

To initially investigate the lytic capacity of CD8 T cells within HSV-1 latently infected ganglia, we determined their level of granzyme B expression. A significant population of gB-specific CD8 T cells within HSV-1 latently infected trigeminal ganglia expressed granzyme B directly *ex vivo* (Figure 1A&B). Lytic granule membranes contain CD107a and CD107b (LAMP-1 and -2, respectively) that insert into the plasma membrane of the CD8 T cell at the site of lytic granule release and identify CD8 T cells that have recently released lytic granules (129). Consistent with the finding that nearly half of the trigeminal ganglia-resident CD8 T cells are specific for the HSV-1 $gB_{498-505}$ epitope (Figure 1A), approximately half of these cells mobilized CD107a to the cell surface (Figure 1C), indicating release of lytic granules upon stimulation with gB peptide-pulsed fibroblasts.

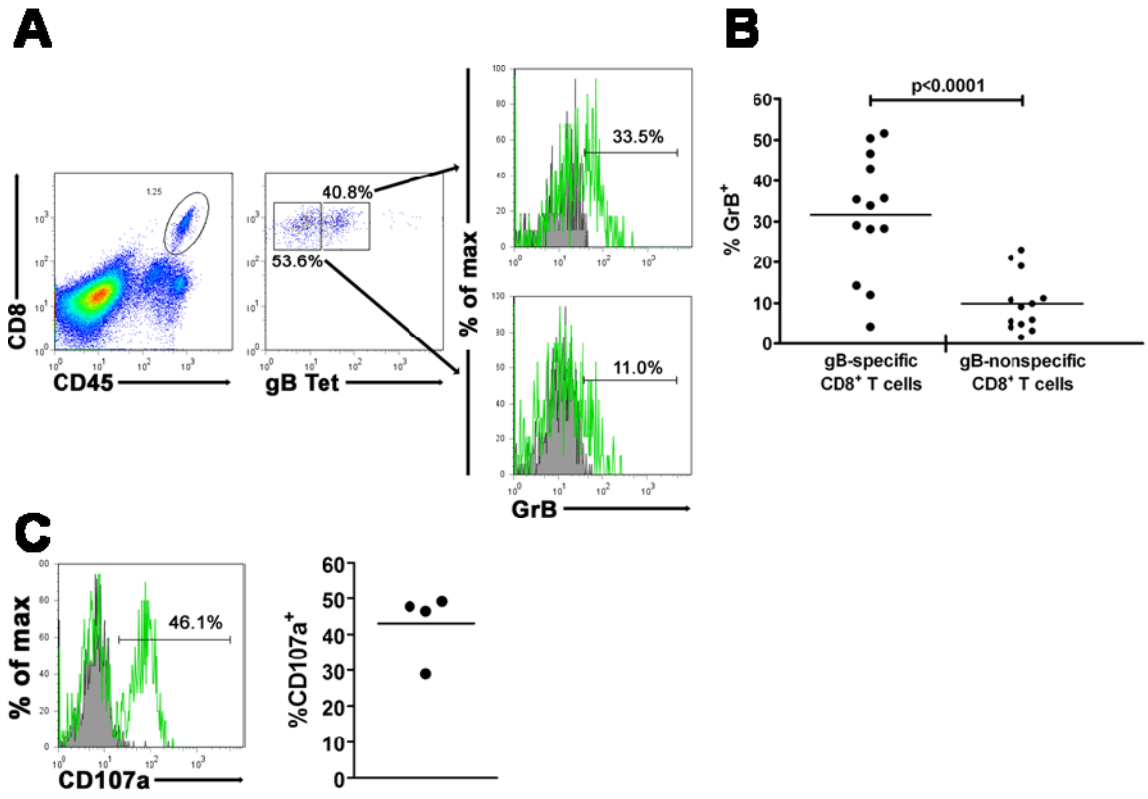


Figure 1. gB-specific CD8 T cells from HSV-1 latently infected trigeminal ganglia express the lytic granule component granzyme B and are capable of lytic granule exocytosis.
Trigeminal ganglia were removed from wild-type (WT) mice at 35 day post-infection and dispersed with collagenase. Cells were then stained for CD45, CD8α, gB-specific TCR (gB Tet) and granzyme B (GrB) prior to flow cytometric analysis. (A) Representative dot plots showing gating on gB Tet^+ $CD8^+$ T cells and histograms (grey plot: isotype control) showing GrB expression (green line) by gB Tet^+ $CD8^+$ T cells (top) and gB Tet^- $CD8^+$ T cells (bottom). (B) Scatter plot of data from three separate experiments (horizontal bar = mean). p value calculated by Student's T test. (C) Trigeminal ganglia cells were stimulated for 6 hrs with $gB_{498\text{-}505}$ peptide-pulsed targets in the presence of GolgiPlug and anti-CD107a mAb prior to flow cytometric analysis. Left: Histogram is gated on $CD45^+$ $CD8\alpha^+$ cells. Right: Scatter plot of all data (horizontal bar = mean).

To aid in further characterizing the lytic potential of CD8 T cells that reside within HSV-1 latently infected ganglia, we isolated and expanded gB-specific CD8 T cell lines from HSV-1 latently infected trigeminal ganglia. After multiple rounds of stimulation with B6WT3 fibroblasts stably transfected to express glycoprotein B, greater than 95% of the isolated and expanded CD8 T cells stained positively with H-2K^{b}/gB$_{498\text{-}505}$ tetramer (Figure 2A). Nearly 100% of the gB-specific CD8 T cells grown from wild-type mice expressed granzyme B (see Figure 14). Consistent with the T cell receptor (TCR) variable (V) β chain usage by gB-specific CD8 T cells from HSV latently infected ganglia directly *ex vivo* (130), isolated and expanded gB-specific CD8 T cells predominantly used Vβ10b and Vβ8 (Figure 2B). Thus, the gB-specific CD8 T cells used in the studies below were all armed with granzyme B and representative of the endogenous CD8 T cell population within latently infected ganglia in terms of TCR Vβ usage as opposed to being clonally derived from a single cell.

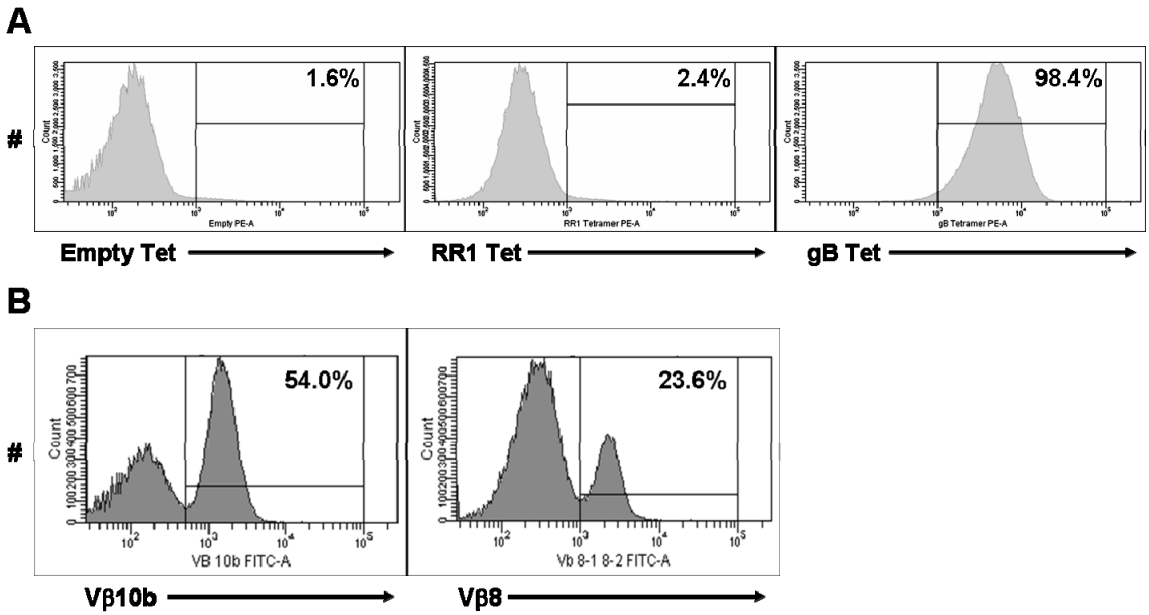


Figure 2. Phenotype of gB-specific CD8 T cells isolated and expanded from HSV-1 latently infected ganglia.

(A) gB-specific CD8 T cells isolated and expanded form HSV-1 latently infected (>30 days post-infection) trigeminal ganglia were stained for CD8α and either ribonucleotide reductase 1-specific (RR1 Tet; negative control) or glycoprotein B-specific (gB Tet) T cell receptors or left unstained (Empty Tet) and analyzed by flow cytometry. (B) The same gB-specific CD8 T cells were stained for CD8α and either the T cell receptor variable (V) β10 or Vβ8 chains and analyzed by flow cytometry.

Live-cell imaging was used to track the movement of lytic granules within previously isolated and expanded gB-specific CD8 T cells (see Figure 2) during interaction with gB peptide-pulsed fibroblasts. Over the course of 9.5 minutes, the lytic granules of gB-specific CD8 T cells polarized to the junction with gB peptide-pulsed (Figure 3), but not non-pulsed (data not shown), fibroblast targets. A similar time-scale for CD8 T cell lytic granule polarization was previously reported (131).

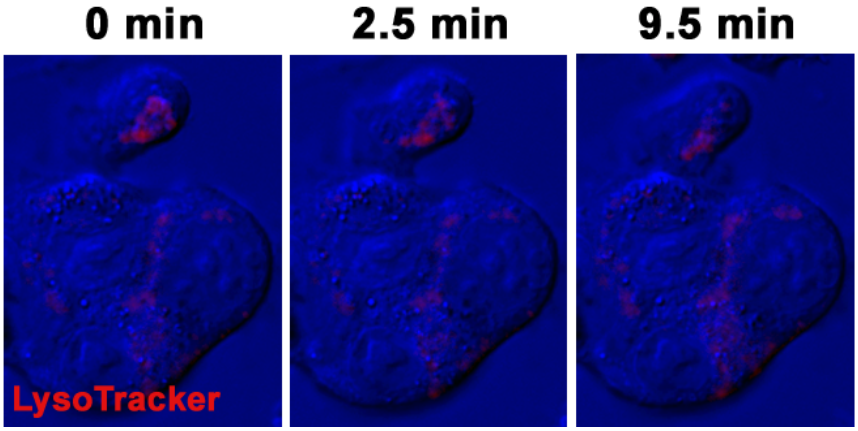


Figure 3. gB-specific CD8 T cells from HSV-1 latently infected trigeminal ganglia polarize their lytic granules toward gB peptide-pulsed fibroblasts.
gB-specific CD8 T cells previously isolated and expanded from HSV-1 latently infected trigeminal ganglia were labeled with LysoTracker Red (top cell) and added to cultures of $gB_{498-505}$ peptide-pulsed fibroblast targets (bottom cell) prior to live-cell imaging. DIC shown in blue.

Imaging also revealed CD107a expression on the surface of gB-specific CD8 T cells at the contact site with target fibroblasts (Figure 4A), consistent with a secretory domain of an immunological synapse (131). Importantly, CD107a was only observed on the surface of CD8 T cells productively interacting with pulsed targets and not on CD8 T cells that were not contacting any target cells (Figure 4B).

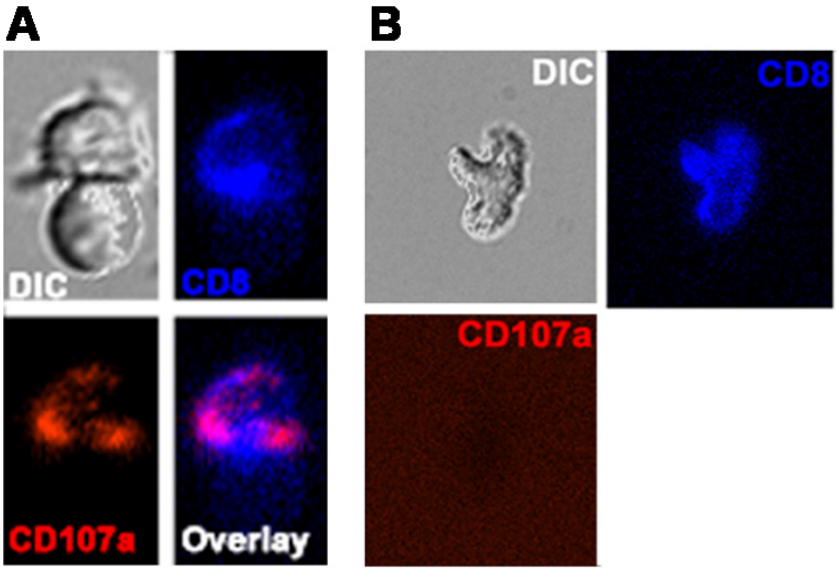


Figure 4. gB-specific CD8 T cells release lytic granules in a directed manner toward gB peptide-pulsed fibroblast targets.
gB-specific CD8 T cells previously isolated and expanded from HSV-1 latently infected trigeminal ganglia were incubated with $gB_{498\text{-}505}$ peptide-pulsed fibroblast targets for 1 hr prior to staining for CD8α and CD107a. (A) Polarization of CD107a on a gB-specific CD8 T cell toward a target cell. (B) Absence of surface CD107a expression on gB-specific CD8 T cells not interacting with any target cells.

The functionality of the released lytic granules was established by signs of apoptosis including cellular condensation with membrane blebbing in targeted gB peptide-pulsed fibroblasts within 30-45 minutes of adding the gB-specific CD8 T cells (Figure 5). Thus, gB-specific CD8 T cells from latently infected trigeminal ganglia possess lytic granules containing granzyme B that are capable of inducing apoptosis of peptide-pulsed non-neuronal targets.

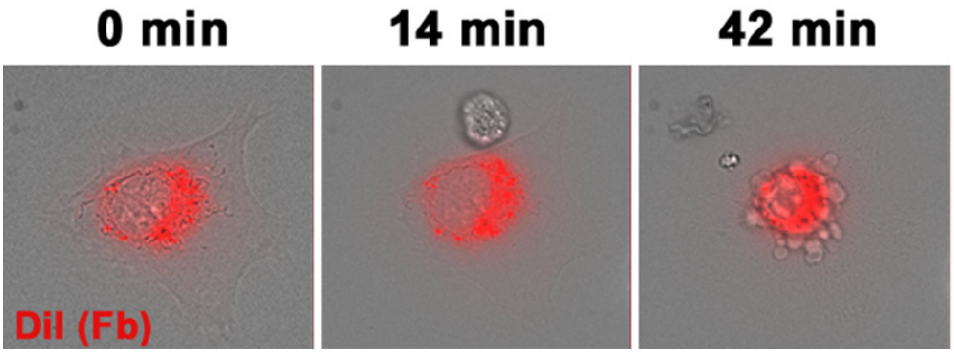
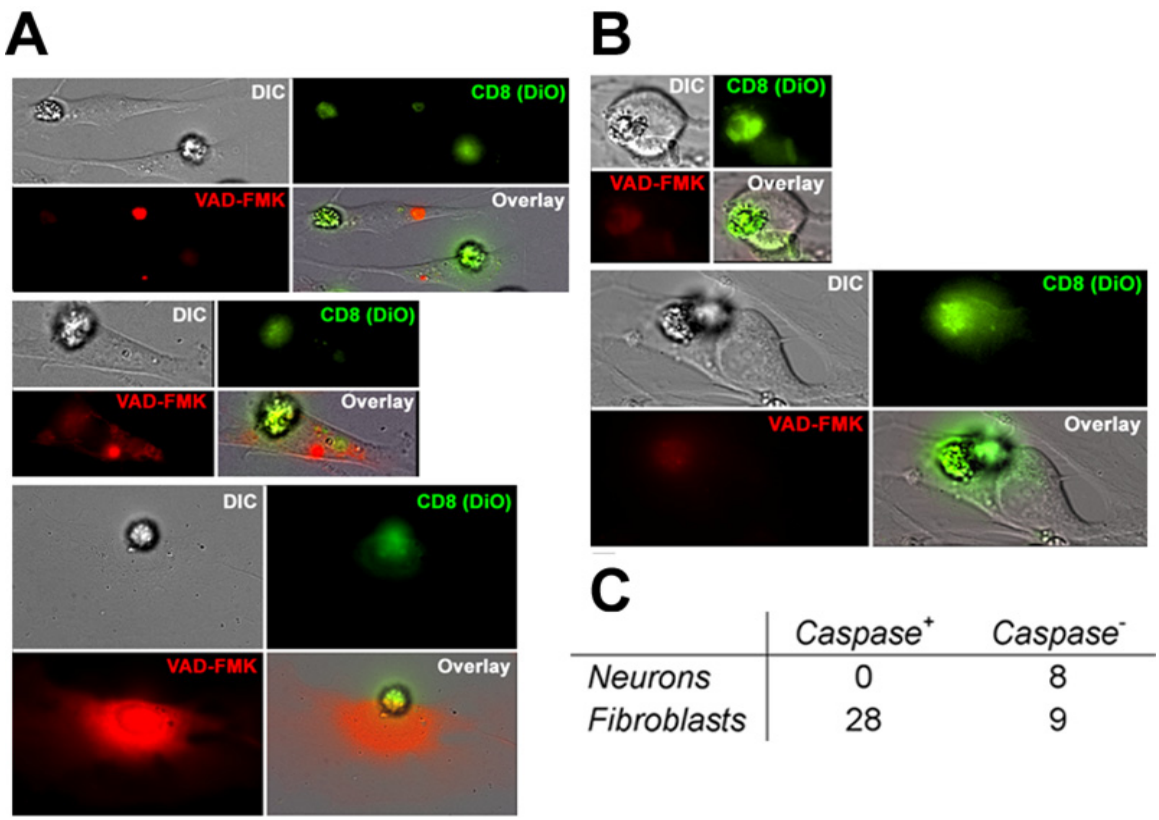


Figure 5. gB-specific CD8 T cells induce apoptosis of gB peptide-pulsed fibroblast targets. Unlabeled gB-specific CD8 T cells isolated and expanded from HSV-1 latently infected trigeminal ganglia were added to DiI-labeled $gB_{498\text{-}505}$ peptide-pulsed fibroblast targets. Morphological changes associated with apoptosis were visualized by live-cell imaging in the target cells at the indicated times after adding the gB-specific CD8 T cells. No morphological changes associated with apoptosis were observed in nonpulsed fibroblast targets incubated with the same gB-specific CD8 T cells (data not shown).

5.2 NEURONS WITHIN HSV-1-INFECTED TRIGEMINAL GANGLIA ARE RESISTANT TO APOPTOSIS INDUCED BY CD8 T CELLS

Caspases are cysteine proteases that are major effectors of the apoptotic process mediated by granzyme B (132). To initially investigate whether HSV-specific CD8 T cells induce apoptosis of targeted neurons in HSV-1 latently infected trigeminal ganglia, we assessed for differential activation of caspases in neurons and fibroblasts targeted by CD8 T cells within *ex vivo* trigeminal ganglia cultures allowed to reactivate permitting spread of virus from neurons to surrounding fibroblasts. Trigeminal ganglia from wild-type mice infected greater than 30 days previously were excised, dispersed into single-cell suspensions, and cultured in the absence of exogenous gB-specific CD8 T cells. After 60 hours of culture, a time that we routinely see HSV-1 reactivation but before cultures succumb to excessive viral cytopathic effect, we added exogenous gB-specific CD8 T cells, incubated the cultures for another hour to allow conjugate formation, and then imaged either neuron/CD8 T cell or fibroblast/CD8 T cell interactions for caspase activation. In the two trigeminal ganglia cultures imaged in this manner, we witnessed a total of 8 neuron/CD8 T cell interactions. We also imaged a total of 37 fibroblast/CD8 T cell interactions, although many more of these interactions were present in the cultures than were imaged. Of the 37 fibroblast/CD8 T cell interactions imaged, 28 of the fibroblasts targeted by CD8 T cells stained positively for active caspases (Figure 6A&C). Conversely, zero of the 8 neurons targeted by CD8 T cells contained active caspases (Figure 6B&C). This differential caspase activation in fibroblasts compared to neurons was statistically significant as assessed by Fisher's Exact Test. ($p=0.0005$). While not definitive, this observation suggested either that CD8 T cells do not release their lytic granules into neurons or that lytic granule release does not activate the caspase system of neurons within HSV-1-infected ganglia.



	Caspase$^+$	Caspase$^-$
Neurons	0	8
Fibroblasts	28	9

Figure 6. gB-specific CD8 T cells activate caspases in HSV-1-infected fibroblasts but not neurons within dispersed trigeminal ganglia cultures.
HSV-1 latently infected trigeminal ganglia were excised from WT mice, dispersed into single cells, and cultured for 3 days to allow viral reactivation from neurons and spread to surrounding fibroblasts. Isolated and expanded gB-specific CD8 T cells were labeled with DiO and then added to the reactivated cultures for 1-3 hours prior to staining with a fluorescently labeled pan-caspase inhibitor (VAD-FMK). (A) Representative images of gB-specific CD8 T cells interacting with fibroblasts demonstrating intracellular areas of early punctate (top), multifocal (middle), and diffuse late (bottom) caspase activation within the targeted fibroblasts. (B) Representative images of gB-specific CD8 T cells contacting neurons (identified by size and morphology), which lack active caspase staining. (C) Contingency table showing a significant difference in the number of fibroblasts compared to the number of neurons contacted by gB-specific CD8 T cells and containing active caspases ($p=0.0001$ as calculated by Fisher's Exact Test).

5.3 POLARIZATION OF CD8 T CELL GRANZYME B TOWARD NEURONS WITHIN HSV-1 LATENTLY INFECTED GANGLIA

To address whether or not CD8 T cells release their lytic granules toward neurons, we first documented CD8 T cell polarization of granzyme B toward junctions with neurons within latently infected ganglia *in situ* (Figure 7A). This extended our previous observation of gB-specific CD8 T cell TCR polarization toward neurons *in situ* (97), and suggested ongoing use of directed lytic granule release by CD8 T cells during immunosurveillance of latently infected trigeminal ganglia. Co-localization of gB-specific TCR and granzyme B at CD8 T cell/neuron junctions was further demonstrated in *ex vivo* ganglia cultures (Figure 7B). Moreover, none of the targeted neurons exhibited morphologic signs of apoptosis, adding further support to the notion that neurons are selectively resistant to apoptosis induction by lytic granules.

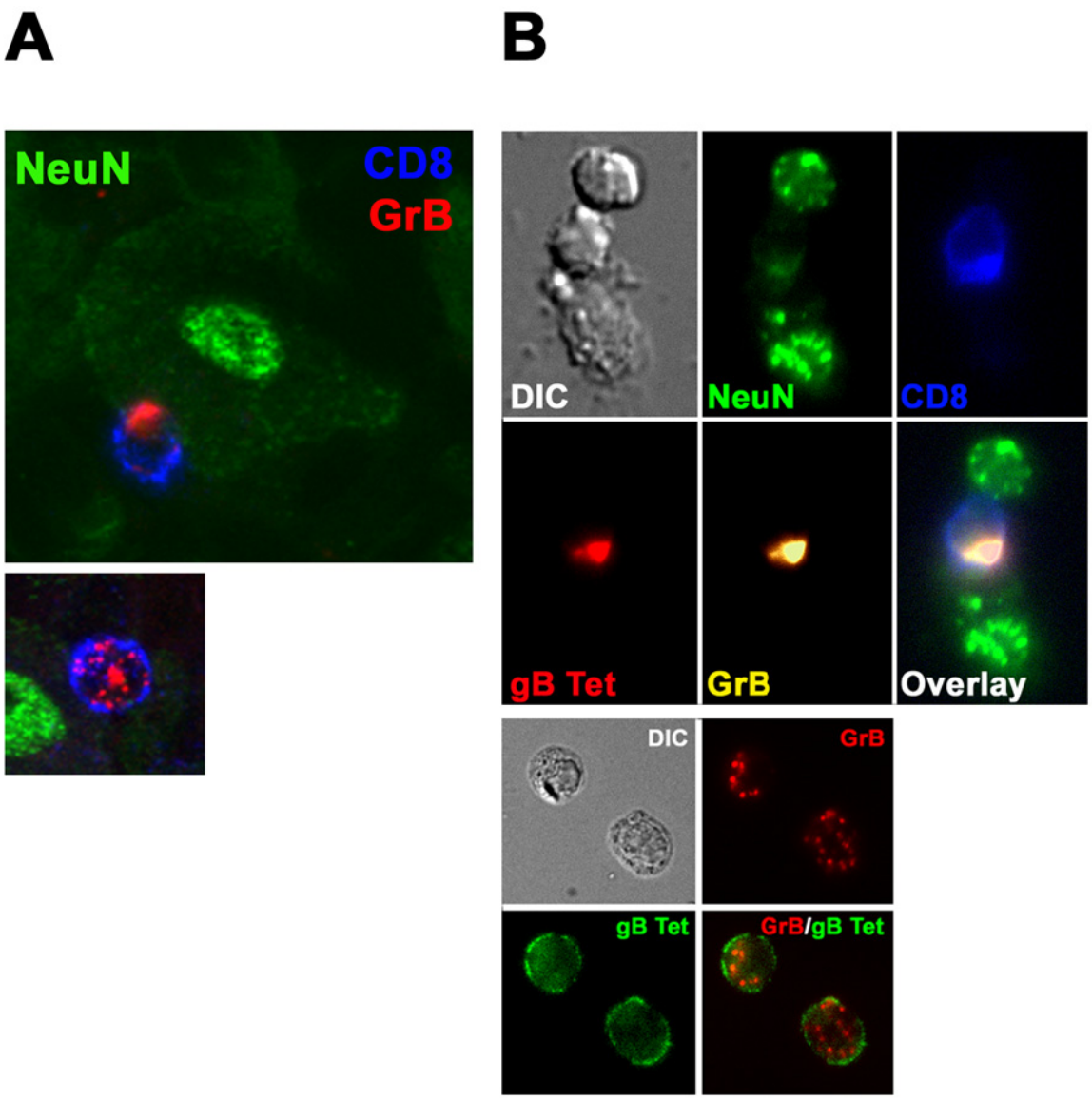


Figure 7. CD8 T cells polarize lytic granule components toward neurons within HSV-1 latently infected trigeminal ganglia.

(A) Intact trigeminal ganglia were excised 15 days post-infection (dpi) and stained for CD8α, NeuN (neuronal nucleus), and granzyme B (GrB). Top: GrB polarization within a CD8 T cell toward the junction with a $NeuN^{+}$ neuron. Bottom: Representative image of the majority of CD8 T cells within latently infected ganglia where GrB is dispersed throughout the cell. (B) Trigeminal ganglia excised at 16 dpi were dispersed, plated on cover slips, incubated for 1 hr, and then stained for CD8α, the neuron marker NeuN, gB-specific TCR (gB Tet) and GrB. Top: A CD8 T cell in contact with two neurons polarizes its TCR and lytic granules toward the bottom neuron only. Bottom: CD8 T cells not contacting targets show no TCR polarization and diffuse punctate intracellular GrB staining.

5.4 CD8 T CELLS WITHIN HSV-1 LATENTLY INFECTED GANGLIA RELEASE LYTIC GRANULES TOWARD NEURONS WITHOUT INDUCING APOPTOSIS

Histologic studies of HSV-1 latently infected human (133) and murine (134) ganglia have failed to detect morphologic signs of apoptosis in neurons that are in direct contact with activated CD8 T cells. Furthermore, we previously reported that CD8 T cell inhibition of HSV-1 reactivation in *ex vivo* trigeminal ganglia cultures is reversible when CD8 T cell function is compromised, consistent with a non-lytic block of reactivation (103). We now extend those findings by directly demonstrating that lytic granule release by CD8 T cells does not induce apoptosis of HSV-1 latently infected neurons. Single-cell suspensions of latently infected trigeminal ganglia were prepared and incubated with wild-type (WT) gB-specific CD8 T cells, which completely inhibit viral reactivation from latency (see Table 2). The cultures were then fluorescently stained for surface CD8α and CD107a (marker of lytic granule release), and for intracellular expression of NeuN (pan-neuronal nucleus marker) and activated caspases. Of 13 documented CD8 T cell/neuron interactions characterized by CD107a inclusion in an apparent immunological synapse, none of the targeted neurons contained active caspases (illustrated in Figure 8A). CD8 T cells contacting non-neuronal cells or not contacting any cell showed no surface CD107a expression (Figure 8B). Although not activated by CD8 T cell lytic granules, neuronal caspases were present and could be activated by ethanol treatment (Figure 8C). Therefore, we conclude that HSV-1 latently infected neurons are preferentially protected from apoptosis mediated by gB-specific CD8 T cells lytic granules in *ex vivo* trigeminal ganglia cultures.

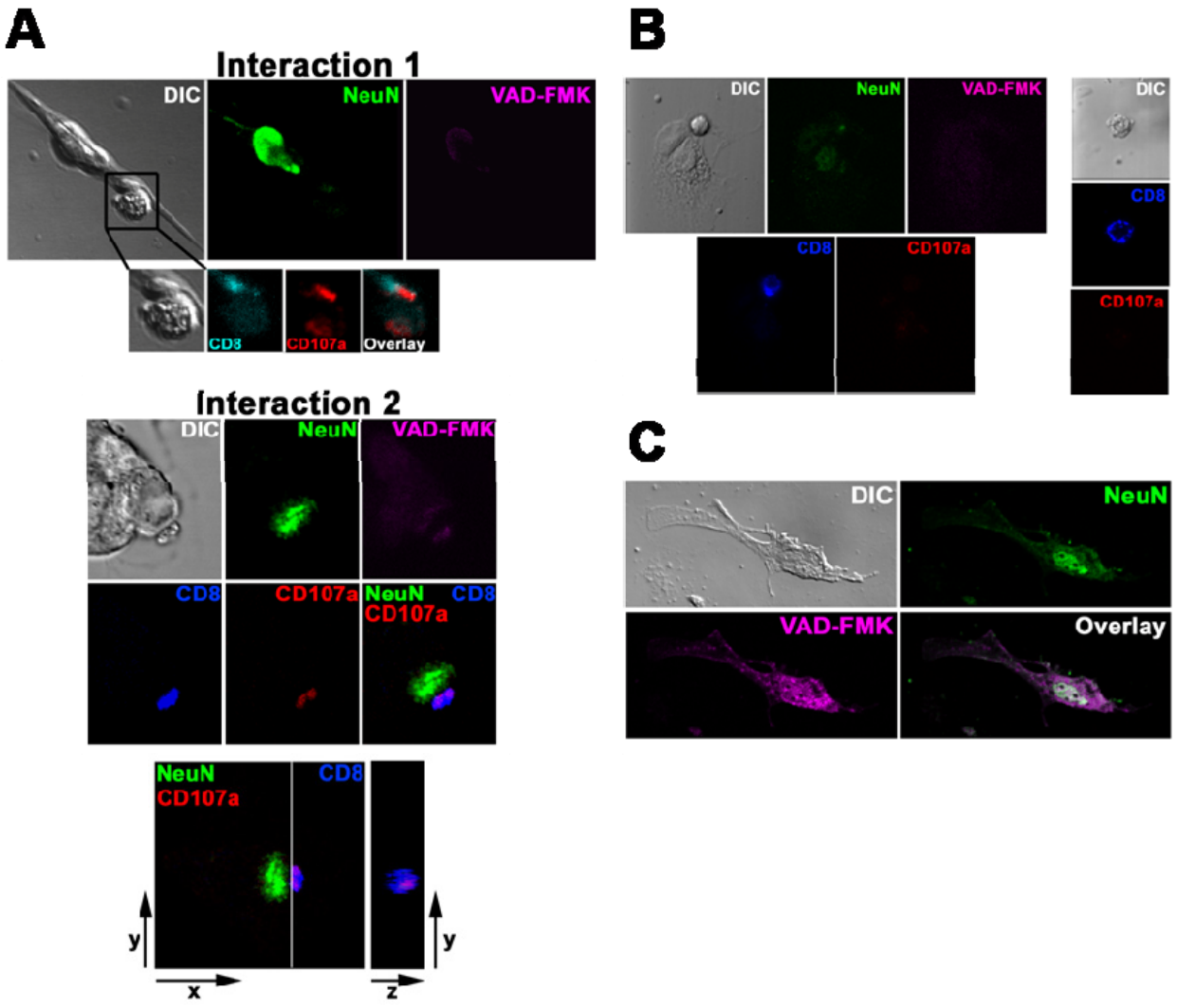


Figure 8. CD8 T cells release lytic granules toward neurons within HSV-1 latently infected trigeminal ganglia without activating neuronal caspases.
Latently infected trigeminal ganglia were dispersed, plated on cover slips, and cultured with wild-type gB-specific CD8 T cells from culture initiation to prevent reactivation from latency. (A&B) After 24-48 hrs, cultures were stained for surface CD8α and CD107a as well as intracellular active caspases (VAD-FMK) and NeuN (neuronal nuclear stain) prior to confocal imaging. All fluorescence images are single-plane 3D reconstructions of sequential Z sections through the entire thickness of the cells. (A) Two representative interactions between $NeuN^+$ neurons and CD8 T cells with CD107a polarized toward the junction with the neuron. In the bottom panel of Interaction 2, the plane demarcated by the line between the cells (left) is shown *en face* (right) demonstrating an apparent secretory domain of an immunological synapse between the neuron and CD8 T cell. (B) Left: Representative interaction between a $NeuN^-$ cell (i.e. fibroblast that is not latently infected) and a CD8 T cell lacking surface CD107a expression. Right: Representative image of a CD8 T cell not in contact with a target cell lacking CD107a surface expression. (C) The same cultures treated with 10% ethanol to induce apoptosis. Note positive staining of $NeuN^+$ neuron for active caspases.

5.5 LYTIC GRANULE COMPONENTS ARE NOT REQUIRED FOR THE ESTABLISHMENT OF HSV-1 NEURONAL LATENCY IN VIVO

Having demonstrated that CD8 T cells do in fact release lytic granules toward neurons within HSV-1 latently infected ganglia with apparent neuronal survival, we next assessed the requirement for lytic granule components in establishing and maintaining HSV-1 neuronal latency. Studies employing wild-type mice or mice deficient in the lytic granule components perforin (perforin$^{-/-}$) or granzyme B (granzyme B$^{-/-}$) assessed CD8 T cell use of lytic granules in establishing and maintaining HSV-1 latency. First, all three strains cleared virus from infected corneas with similar kinetics (Figure 9). Also, HSV-1 latency is consistently established by 8-10 days post-infection in wild-type mice as evidenced by a lack of detectable live virus in directly explanted trigeminal ganglia (Table 1) with a mean retention of ~50,000 viral genome copies per trigeminal ganglia (Figure 10). Perforin$^{-/-}$ and granzyme B$^{-/-}$ trigeminal ganglia contained no detectable live virus by 10 days post-infection (Table 1) and established a similar latent viral load by 8-10 days post-infection (Figure 10). Therefore, the lytic granule components perforin and granzyme B are not required for the initial establishment of HSV-1 latency.

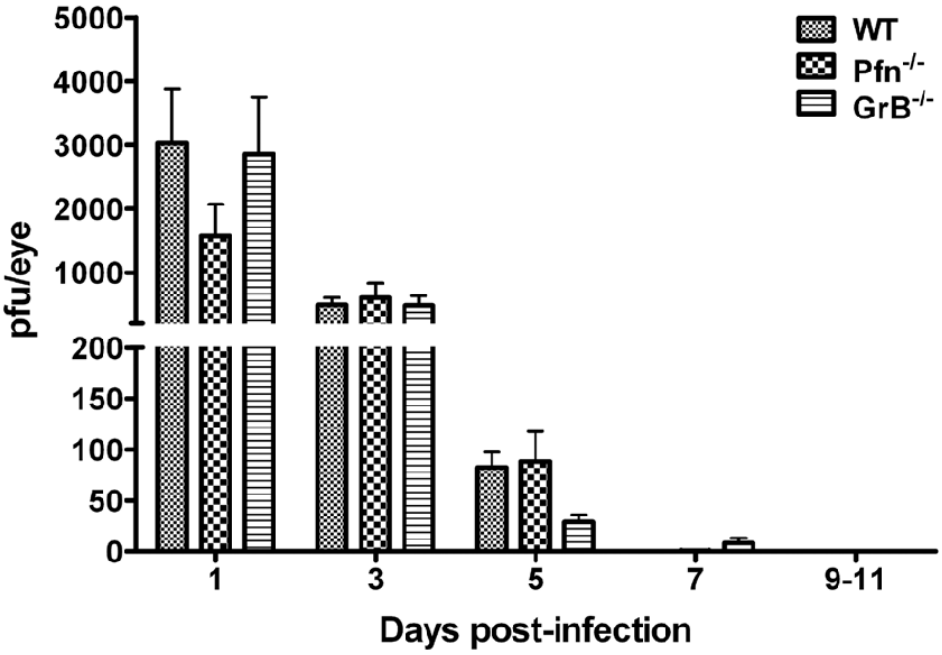


Figure 9. Perforin and granzyme B are not required to clear replicating HSV-1 from infected corneas.
Eye swabs from the indicated mice at the indicated times were analyzed by plaque assay for replicating virus. Pooled data from two independent experiments (n=6-10 per condition) presented as mean ± SEM. ANOVA revealed no statistical differences between groups at any time point.

Table 1. Perforin and granzyme B are not required to clear replicating HSV-1 from infected trigeminal ganglia.
Trigeminal ganglia (TG) from wild-type (WT) mice and mice deficient in perforin ($Pfn^{-/-}$) or granzyme B ($GrB^{-/-}$) were excised at the indicated days post-infection (dpi), homogenized, and analyzed by plaque assay for replicating HSV-1.

Type of mouse	*HSV-1$^+$ TG/Total TG*			
	5 dpi	*10 dpi*	*14 dpi*	*20 dpi*
WT	5/5	0/8	0/8	0/7
$Pfn^{-/-}$	6/6	0/6	0/8	0/6
$GrB^{-/-}$	6/6	0/6	0/8	0/5

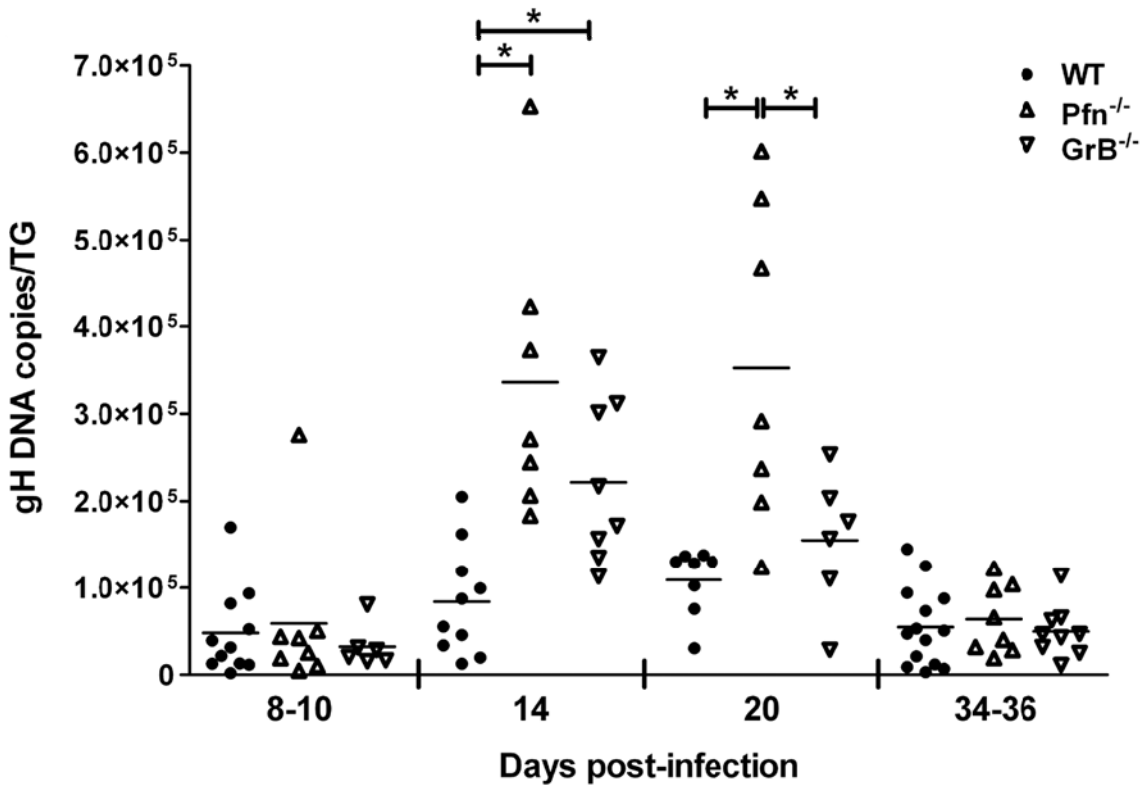


Figure 10. HSV-1 genome copy number within infected trigeminal ganglia of wild-type, perforin$^{-/-}$, and granzyme B$^{-/-}$ mice.
At the indicated times after infection, DNA was extracted from trigeminal ganglia of wild-type (WT), perforin-deficient (Pfn$^{-/-}$), and granzyme B-deficient (GrB$^{-/-}$) mice, and the number of HSV-1 genome copies in individual ganglia was determined by quantitative real-time PCR (horizontal bar = mean). * $p<0.05$ as calculated by ANOVA with Bonferroni post-test.

5.6 LYTIC GRANULE COMPONENTS ARE REQUIRED FOR THE MAINTENANCE OF HSV-1 NEURONAL LATENCY IN VIVO

Despite establishing an equivalent viral load by 10 days following infection, latency was unstable in HSV-1-infected perforin-deficient and granzyme B-deficient compared to wild-type trigeminal ganglia as indicated by a significant increase in the number of viral genome copies between 10 and 14 days post-infection (Figure 10). This rise in viral genome copies in perforin-deficient trigeminal ganglia continued through 20 days post-infection, while the viral load in granzyme B-deficient trigeminal ganglia was only slightly elevated compared to wild-type ganglia at this time. However, the viral load in both perforin- and granzyme B-deficient trigeminal ganglia declined back to wild-type levels by 30 days post-infection (Fig 10). Interestingly, no live virus could be detected in perforin- or granzyme B-deficient trigeminal ganglia by standard viral plaque assays of homogenized tissue at either 14 or 20 days post-infection possibly suggesting an additional lytic granule-independent block in the HSV-1 reactivation process following DNA replication. Thus, perforin and granzyme B are required to inhibit the early steps in the HSV-1 reactivation process *in vivo*, at least during the early stages of latency.

The histone deacetylase inhibitor, sodium butyrate, has been shown to induce reactivation of latent HSV-1 in Balb/c mice with viral shedding at the cornea (135). As an initial investigation into whether sodium butyrate treatment also induced reactivation of latent HSV-1 in B6 mice, which are more resistant to HSV-1 infection than Balb/c mice, latently infected wild-type B6 mice were treated with titrated doses of sodium butyrate via intraperitoneal injection and assayed for replicating virus in eye swabs each of three days following treatment. Additionally, trigeminal ganglia were harvested three days following treatment and assayed for late viral gene

expression, presence of live virus, and viral genome copy number. A single dose of 1200 mg/kg sodium butyrate induced reactivation defined as the presence of live virus in either eye swabs or trigeminal ganglia in 100% of Balb/c mice (135). Although treatment of HSV-1 latently infected B6 mice with 1200 mg/kg sodium butyrate did induce promoter activity from both the HSV-1 glycoprotein B and glycoprotein C genes (136), doses of sodium butyrate ranging from 1200-2400 mg/kg did not induce reactivation defined as the presence of live virus in eye swabs or trigeminal ganglia in any B6 mice (data not shown). Additionally, the latent viral load was not significantly increased within ganglia from treated mice compared to PBS-treated controls (Figure 11A).

To determine if mice deficient in critical lytic granule components were more susceptible to sodium butyrate-induced HSV-1 reactivation, latently infected wild-type B6 mice or B6 mice deficient in perforin or granzyme B were treated with a single dose of 3000 mg/kg sodium butyrate (greater than two times the LD_{50} for Balb/c mice). Interestingly, all of the treated B6 mice survived without detectable reactivation measured by live virus in eye swabs or trigeminal ganglia (data not shown) or by an increase in viral genome copy number in trigeminal ganglia (Figure 11B). Thus, B6 mice appear to be more resistant to sodium butyrate-induced HSV-1 reactivation than Balb/c mice, consistent with previous reports using other methods to induce HSV-1 reactivation from latency in these strains of mice (137).

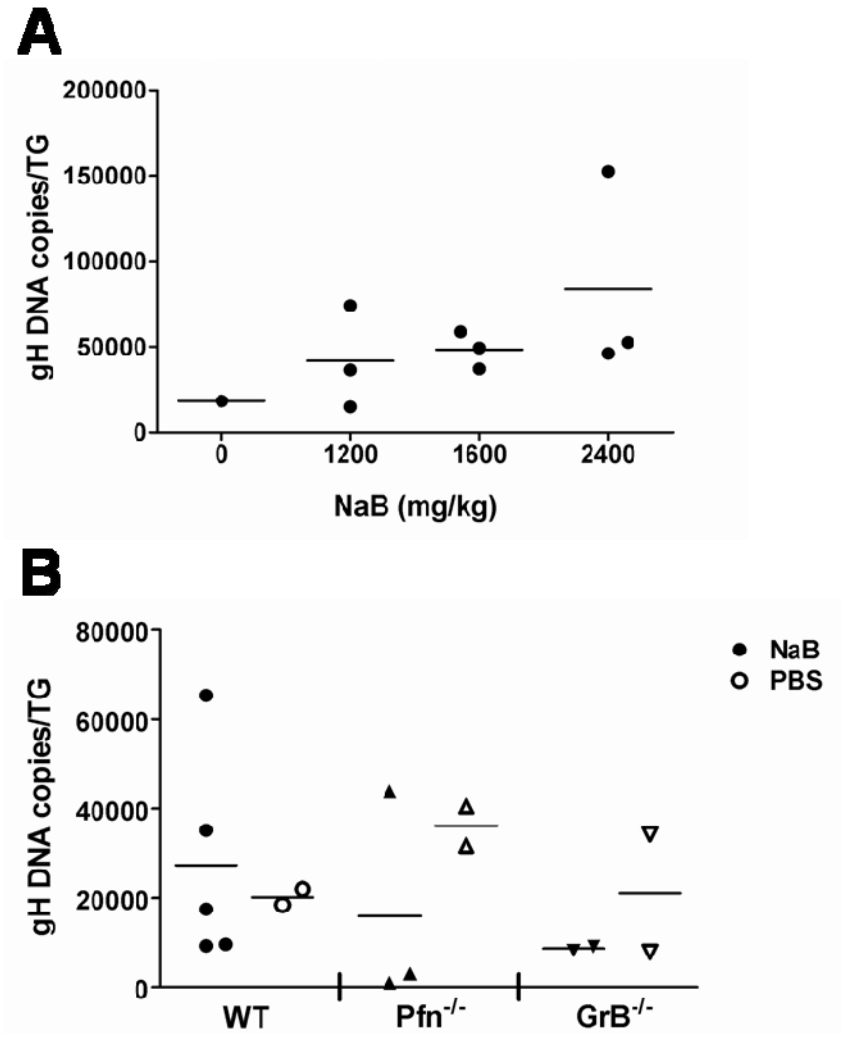


Figure 11. Sodium butyrate treatment does not induce reactivation of HSV-1 in latently infected ganglia of wild-type, perforin$^{-/-}$, or granzyme B$^{-/-}$ B6 mice.
(A) Wild-type B6 mice were given a single intraperitoneal injection of the indicated doses of sodium butyrate (NaB) 70 days following HSV-1 corneal infection. Three days later, trigeminal ganglia were excised and the number of HSV-1 glycoprotein H (gH) DNA copies was determined by real-time PCR. (B) Wild-type (WT) B6 mice or B6 mice deficient in perforin (Pfn$^{-/-}$) or granzyme B (GrB$^{-/-}$) received a single intraperitoneal injection of either 3000 mg/kg NaB or PBS 57 days following infection. Three days later, the number of gH DNA copies was determined as in (A). No statistical differences between NaB- and PBS-treated groups were revealed for WT, Pfn$^{-/-}$, or GrB$^{-/-}$ mice by Student's T test.

5.7 LYTIC GRANULE COMPONENTS ARE REQUIRED FOR THE MAINTENANCE OF HSV-1 LATENCY IN EX VIVO GANGLIA CULTURES

A recent report established that the rate of HSV reactivation from latency in *ex vivo* trigeminal ganglia cultures correlated directly with the viral load and indirectly with the number of CD8 T cells within the ganglia (138). Latently infected trigeminal ganglia from wild-type, perforin-deficient, and granzyme B-deficient mice infected for >30 days contained identical numbers of HSV-1 genome copies (Figure 10) as well as total and gB-specific CD8 T cells (Figure 12). Thus, any differences in reactivation frequency in latently infected trigeminal ganglia of these mice should be directly attributable to the composition of the lytic granules of trigeminal ganglia-resident cells.

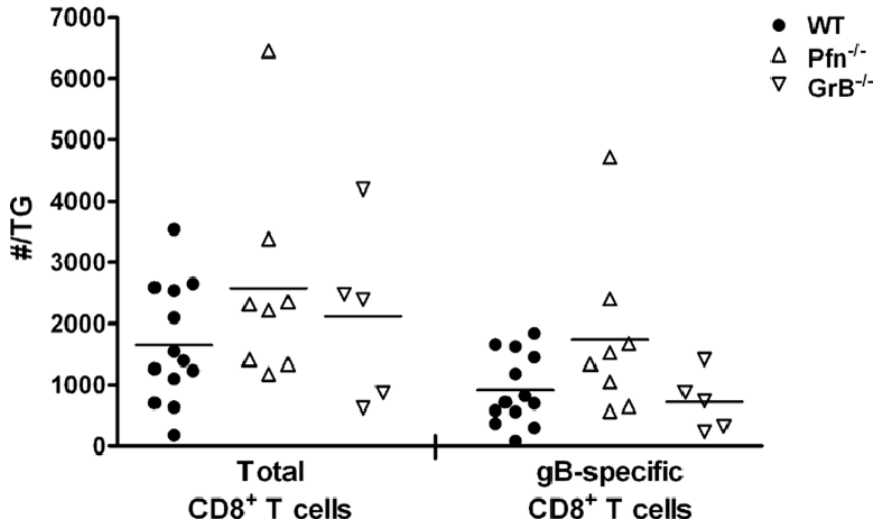


Figure 12. Wild type, perforin$^{-/-}$, and granzyme B$^{-/-}$ HSV-1 latently infected ganglia contain similar numbers of total and gB-specific CD8 T cells.
Trigeminal ganglia (TG) from wild-type (WT) mice or mice deficient in perforin (Pfn$^{-/-}$) or granzyme B (GrB$^{-/-}$) were excised 35-36 days post-infection, dispersed into single cells, and stained for CD45, CD8α, and with H-2K^{d}/gB$_{498\text{-}505}$ tetramers for gB-specific T cell receptors prior to flow cytometric analysis. Scatter plot shows the absolute number of total CD8 T cells and gB-specific CD8 T cells per TG. Data pooled from two independent experiments. ANOVA analysis revealed no significant differences between groups.

Trigeminal ganglia were obtained from perforin-deficient, granzyme B-deficient, and wild-type mice infected for greater than 30 days, dispersed into single cells, and cultured at the equivalent of one-fifth trigeminal ganglia per well of 48-well tissue culture plates. HSV-1 reactivation from latency was assessed by serially testing culture supernatant fluids for infectious virus using standard virus plaque assays every 48 hours. HSV-1 latently infected trigeminal ganglia from perforin-deficient and granzyme B-deficient mice reactivated to a significantly greater extent than did trigeminal ganglia from wild-type mice (Figure 13), suggesting that perforin and granzyme B are necessary for optimal inhibition of HSV-1 reactivation within latently infected neurons in *ex vivo* trigeminal ganglia cultures.

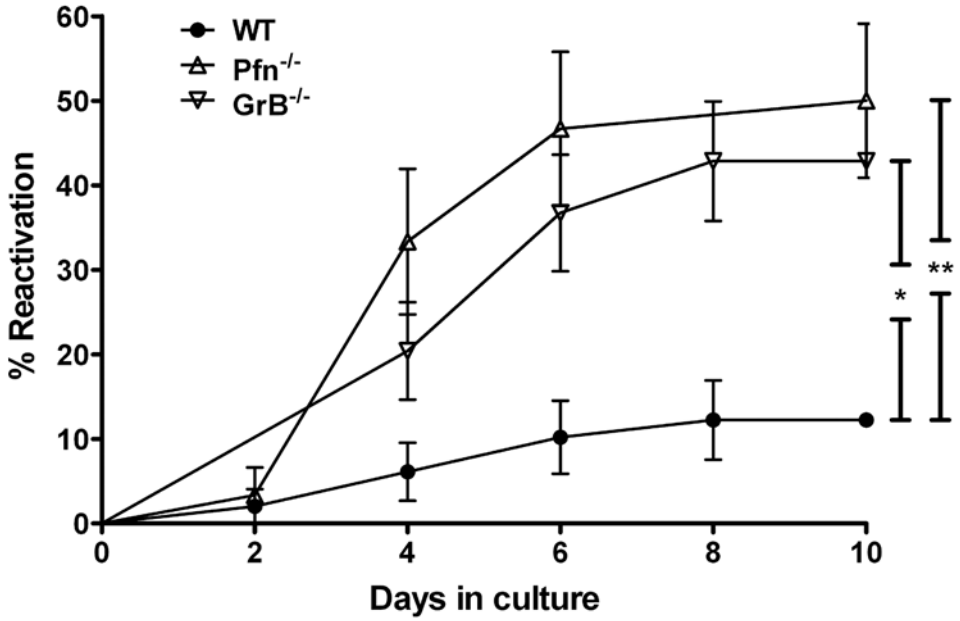


Figure 13. HSV-1 reactivation within *ex vivo* cultures of latently infected wild-type, perforin$^{-/-}$, and granzyme B$^{-/-}$ trigeminal ganglia.
Latently infected (>30 days) trigeminal ganglia obtained from wild-type (WT) mice or those deficient in perforin (Pfn$^{-/-}$) or granzyme B (GrB$^{-/-}$) were dispersed and cultured at one-fifth TG per well of 48-well culture plates. HSV-1 reactivation was indicated by the presence of infectious virus in serially sampled supernatants. Pooled data from two experiments are presented as mean and standard error. * p=0.0009; ** p=0.0002 calculated by survival curve analysis (Log-rank Test).

5.8 PERFORIN$^{-/-}$ AND GRANZYME B$^{-/-}$ CD 8 T CELLS ARE DEFICIENT IN THEIR ABILITY TO BLOCK HSV-1 REACTIVATION

Interpretation of the above findings was compromised by uncertainties about the relative susceptibility of neurons from wild-type, perforin-deficient, and granzyme B-deficient mice to reactivation, and the possible contribution of lytic granules from cells other than CD8 T cells to blocking HSV-1 reactivation. To directly address these issues, trigeminal ganglia were obtained from wild-type mice, depleted of >95% of the endogenous CD8 T cells, and cultured with varying numbers of gB-specific CD8 T cells that were previously isolated and expanded from latently infected trigeminal ganglia of wild-type, perforin-deficient, or granzyme B-deficient mice. Greater than 95% of the isolated and expanded CD8 T cells from all three strains of mice stained positively for $gB_{498\text{-}505}$/H2-K^b MHC class I tetramer (see Figure 2 for wild-type data). Nearly all of the gB-specific CD8 T cells from wild-type and perforin-deficient trigeminal ganglia expressed granzyme B, while granzyme B-deficient gB-specific CD8 T cells were devoid of granzyme B (Figure 14A). Moreover, gB-specific CD8 T cells from all three strains of mice expressed similar levels of CD107a on their surface and contained similar levels of intracellular IFN-γ after a six-hour stimulation with gB peptide-pulsed targets (Figure 14B).

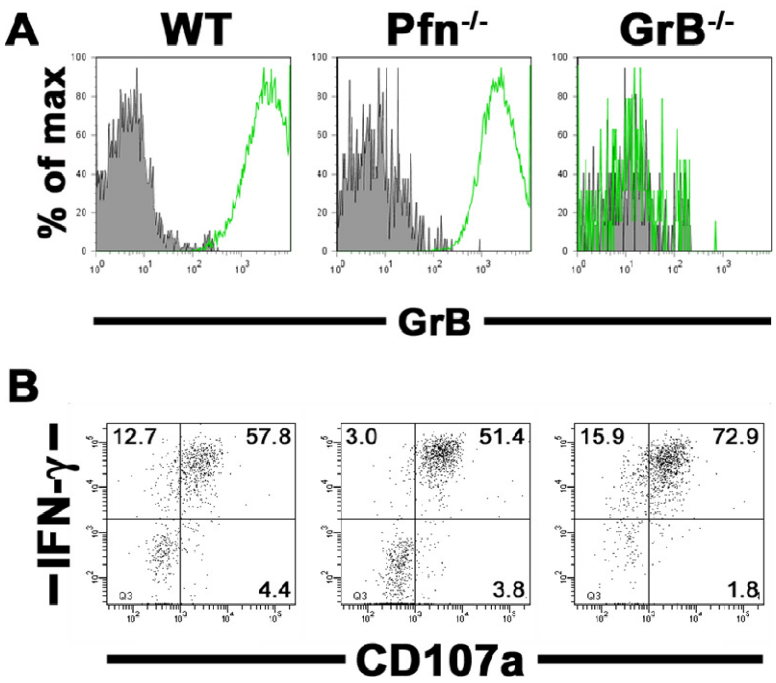


Figure 14. gB-specific CD8 T cells isolated from latently infected wild-type, perforin$^{-/-}$, and granzyme B$^{-/-}$ mice exhibit similar effector functions.
gB-specific CD8 T cells isolated and expanded from HSV-1 latently infected trigeminal ganglia of wild-type (WT) mice or those deficient in perforin (Pfn$^{-/-}$) or granzyme B (GrB$^{-/-}$) were (A) stained for intracellular granzyme B (GrB) or (B) stimulated for 6 hrs with gB peptide-pulsed targets in the presence of FITC-labeled anti-CD107a monoclonal antibody and GolgiPlug, surface stained for CD8α^{+}, permeabilized and stained with APC-labeled anti-IFN-γ, and analyzed by flow cytometry. Dot plots are gated on CD8α^{+} cells.

As illustrated in Table 2, HSV-1 reactivation from latency in *ex vivo* cultures of CD8 T cell-depleted wild-type trigeminal ganglia was completely abrogated by the addition of as few as $2x10^4$ wild-type gB-specific CD8 T cells. In contrast, inhibition of reactivation required $5x10^4$ granzyme B-deficient gB-specific CD8 T cells, and reactivation was not inhibited by as many as $1x10^5$ perforin-deficient gB-specific CD8 T cells (highest dose tested). Thus, perforin- and granzyme B-deficient CD8 T cells are less efficient than wild-type CD8 T cells in blocking HSV-1 reactivation from latency in an identical pool of latently infected neurons.

Table 2. gB-specific CD8 T cells deficient in perforin or granzyme B are impaired in their ability to block HSV-1 reactivation in a common pool of latently infected neurons.
HSV-1 reactivation detected by plaque assay of supernatants of dispersed latently infected wild-type trigeminal ganglia depleted of endogenous CD8 T cells and cultured at one-fifth ganglia per well with the indicated type and number of gB-specific CD8 T cells from culture initiation. +, reactivation detected within 10 days of culture. -, no reactivation detected within 10 days of culture. X, condition not tested. Data pooled from two independent experiments (n=15-35 cultures per condition).

# gB-CD8 per well	Type of gB-CD8		
	WT	*Pfn*$^{-/-}$	*GrB*$^{-/-}$
$2x10^3$	+	x	x
$2x10^4$	-	+	+
$4x10^4$	-	+	+
$5x10^4$	-	+	-
$1x10^5$	-	+	-

5.9 INFILTRATION OF NATURAL KILLER CELLS INTO HSV-1-INFECTED GANGLIA

The data presented above, particularly Table 2, clearly demonstrate a role for CD8 T cell lytic granules in maintaining HSV-1 neuronal latency. However, it is conceivable that other cell types expressing perforin and granzyme B may also play a role in blocking HSV-1 reactivation. In addition to CD8 T cells, natural killer (NK) cells are another type of lymphocyte that use lytic granule exocytosis as an effector mechanism. Thus, we investigated the infiltration of NK cells, compared to CD8 T cells, into trigeminal ganglia following HSV-1 corneal infection. The well-documented expansion that peaks at eight days post-infection, contraction, and maintenance of the CD8 T cell pool within HSV-infected trigeminal ganglia is shown in figure 15A&C. NK cells accumulate in acutely infected ganglia with numbers peaking at 5 days post-infection (Figure 15B&C). However, NK cells then contract to naïve levels by 14 days post-infection. Thus, CD8 T cells, but not NK cells, are selectively retained in HSV-1 latently infected ganglia.

Although not definitive, the observation that NK cell numbers diminished back to naïve levels during HSV-1 latency suggested that these cells were likely not involved in the long-term maintenance of viral latency. To more directly address this issue, we depleted $NK1.1^+$ cells from dispersed latently infected trigeminal ganglia prior to *ex vivo* culture. Culture supernatants were then serially tested for live virus via plaque assays. Results from a single experiment revealed that depleting $NK1.1^+$ cells did not increase the rate or frequency of reactivation compared to non-depleted cultures (data not shown). Obviously, this experiment requires repetition, but the data thus far indicate that NK cells are likely not playing a prominent role in maintaining HSV-1 neuronal latency.

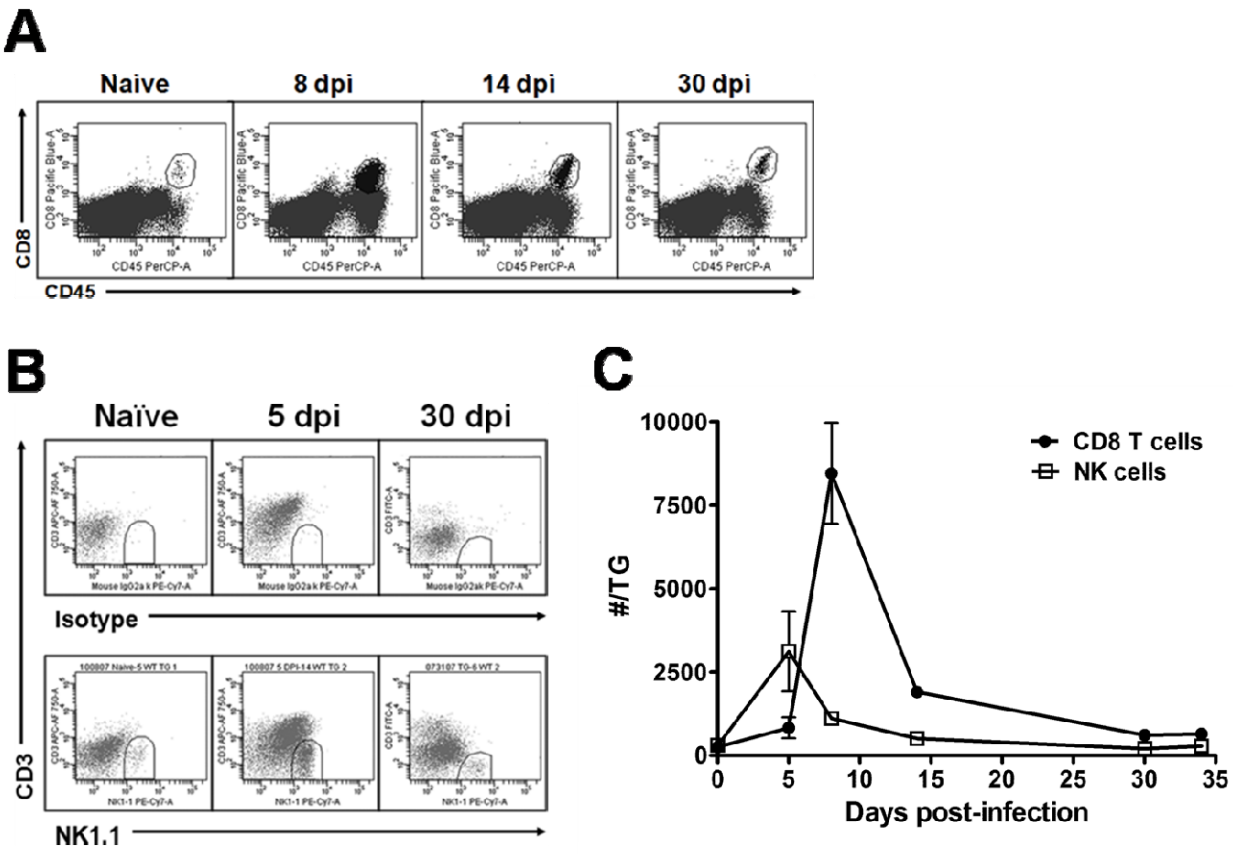


Figure 15. CD8 T cell and NK cell infiltration into HSV-infected ganglia.
Trigeminal ganglia were removed from wild-type mice at the indicated days post-infection (dpi) and dispersed with collagenase. Cells were then stained for CD45, CD3, CD8α, or NK1.1 prior to flow cytometric analysis. (A) Representative dot plots showing gating on $CD8^+$ T cells. (B) Representative dot plots gated on $CD45^+$ cells showing gating on $CD3^-NK1.1^+$ (NK) cells. (C) Plot of the number of CD8 T cells and NK cells at various times post-infection. Data represented as mean ± SEM. (n = 4-8 per condition).

5.10 EXPRESSION OF NATURAL KILLER CELL RECEPTORS ON NK CELLS AND CD8 T CELLS FOLLOWING HSV-1 INFECTION

A family of receptors, collectively known as NK cell receptors, has been shown to regulate effector functions of both NK cells as well as some CD8 T cells. Different members of this family of receptors act to either augment (*e.g.* NKG2D) or inhibit (*e.g.* NKG2A) CD8 T cell and NK cell effector functions, especially lytic granule-mediated cytotoxicity (166). Given the noncytotoxic lytic granule-mediated maintenance of HSV-1 neuronal latency described above, it was of interest to investigate the expression of these receptors on ganglia-resident CD8 T cells and NK cells.

In naïve trigeminal ganglia, a higher percentage of NK cells express the inhibitory NKG2A receptor than the stimulatory NKG2D receptor (Figure 16), suggesting that under normal conditions, the NK cell population is biased toward inhibiting undue effector responses, which would be expected in sensory ganglia made up of nonregenerating neurons. Interestingly, five days following HSV-1 infection, this trend reversed with the majority of NK cells expressing the stimulatory NKG2D receptor and fewer expressing the inhibitory NKG2A receptor (Figure 16). This increase in NKG2D expression coincides with the peak of acute HSV-1 infection in HSV-infected ganglia suggesting increased NK cells effector function during this time. Therefore, NK cells may play an important role in curbing acute ganglionic infection.

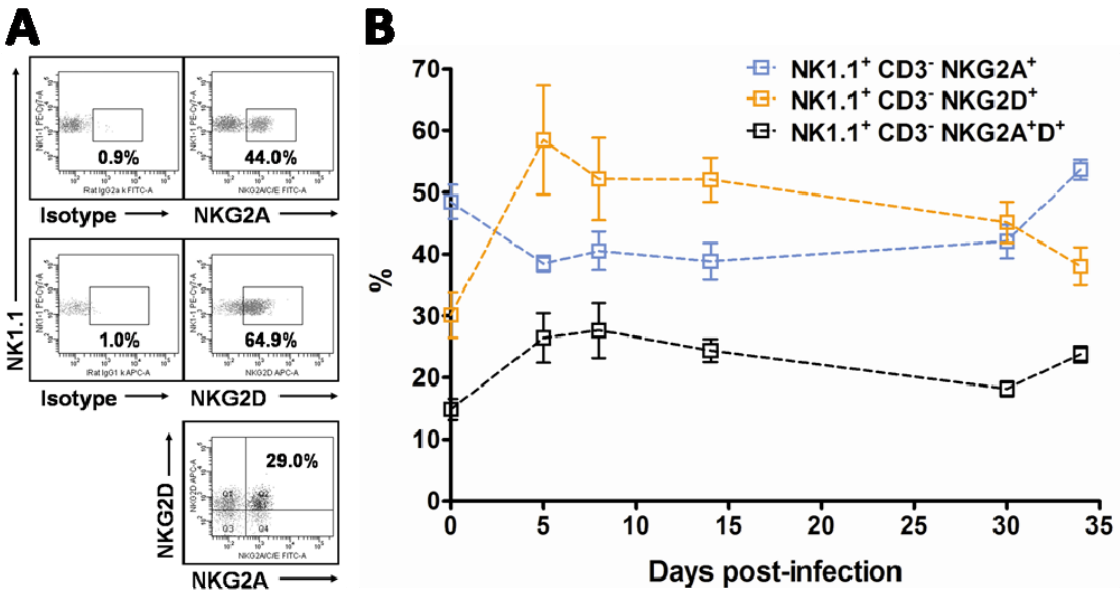


Figure 16. NK cell receptor expression on NK cells within HSV-infected ganglia.
Trigeminal ganglia were removed from wild-type mice at the indicated days post-infection (dpi) and dispersed with collagenase. Cells were then stained for CD45, CD3, NK1.1, NKG2A/C/E, and NKG2D prior to flow cytometric analysis. (A) Representative dot plots from 5 dpi trigeminal ganglia gated on $CD3^{-}NK1.1^{+}$ cells showing $NKG2A^{+}$, $NKG2D^{+}$, and $NKG2A^{+}D^{+}$ populations. (B) Plot of the percentage of NK cells expressing NKG2A, NKG2D, or both at various times post-infection. Data represented as mean ± SEM. (n = 4-8 per condition).

Indeed, the viral load within acutely infected trigeminal ganglia five days after infection, prior to significant CD8 T cell infiltration, was significantly increased in perforin- and granzyme B-deficient mice compared to wild-type controls (Figure 17). By eight days post-infection, the percent of NK cells expressing NKG2D had declined, and by 35 days after infection the percent of NK cells expressing NKG2A and NKG2D was nearly identical to that seen in a naïve ganglion (Figure 16). Thus, NK cells within trigeminal ganglia upregulate stimulatory NK cell receptors early during HSV-1 infection but downregulate these receptors to naïve levels following the establishment of viral latency.

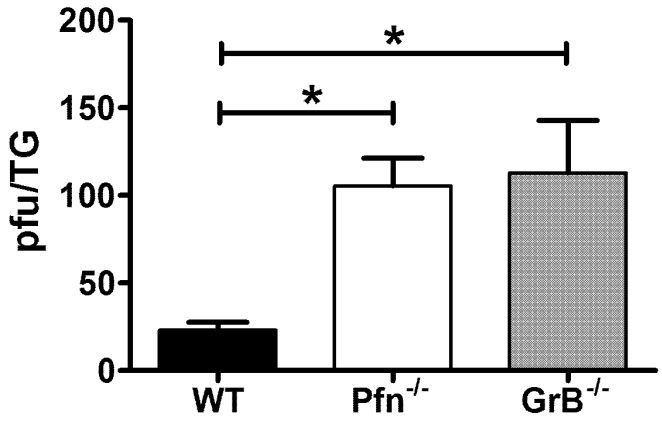


Figure 17. Perforin- and granzyme B-deficient ganglia contain higher viral titers during acute HSV-1 infection.
Trigeminal ganglia (TG) were excised from wild-type (WT) mice or mice deficient in perforin ($Pfn^{-/-}$) or granzyme B ($GrB^{-/-}$) five days following HSV-1 corneal infection. Supernatants from homogenized TG were assayed for replicating virus by plaque assay and the number of plaque-forming units (pfu) was quantified per TG. *, ANOVA revealed significant differences ($p<0.05$) between WT and both $Pfn^{-/-}$ and $GrB^{-/-}$ TG (n=5-6 TG/group).

In contrast to NK cells, CD8 T cells in HSV-infected trigeminal ganglia did not upregulate NK cell receptors until 14 days following infection. At this time, the percentage of CD8 T cells expressing NKG2A, NKG2D, or both was increased approximately three-fold over naïve levels (Figure 18). The percentage of CD8 T cells expressing NKG2A, NKG2D, or both remained elevated at least 35 days after infection. Interestingly, the majority of CD8 T cells expressing NKG2A were gB-specific, while NKG2D was expressed nearly equally on gB-specific and gB-nonspecific CD8 T cells (Figure 19). It should be noted that the antibody used to detect NKG2A in these studies also recognizes NKG2C and NKG2E; however, it was previously shown that only NKG2A is expressed by CD8 T within HSV-1-infected ganglia (167).

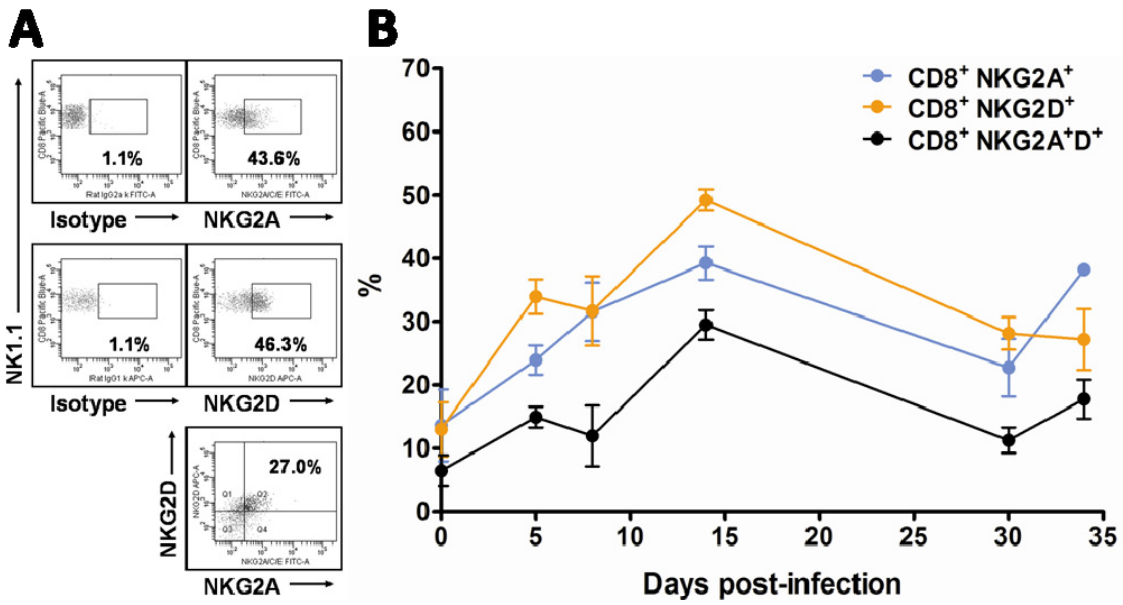


Figure 18. NK cell receptor expression on CD8 T cells within HSV-infected ganglia. Trigeminal ganglia were removed from wild-type mice at the indicated days post-infection (dpi) and dispersed with collagenase. Cells were then stained for CD45, CD8α, NKG2A/C/E, and NKG2D prior to flow cytometric analysis. (A) Representative dot plots from 14 dpi trigeminal ganglia gated on $CD8^+$ cells showing $NKG2A^+$, $NKG2D^+$, and $NKG2A^+D^+$ populations. (B) Plot of the percentage of CD8 T cells expressing NKG2A, NKG2D, or both at various times post-infection. Data represented as mean ± SEM. (n = 4-8 per condition).

When multiple NK cell receptors are expressed by a single cell, it is thought that the balance of cumulative signals from activating versus inhibitory receptors determines whether effector functions are augmented or suppressed. Since the percentage of CD8 T cells expressing both NKG2A and NKG2D peaked at 14 days post-infection and remained elevated compared to naïve levels, the regulation of CD8 T cell effector functions by NK cell receptors may be determined by the type and density of NK cell receptor ligands expressed by latently infected neurons. Although very little is known about the expression of NK cell receptor ligands on neurons within HSV-infected ganglia, Qa-1, a non-classical MHC class 1 molecule that binds to NKG2A/CD94 heterodimers, has been shown to be upregulated on neurons following HSV-1 infection (145). However, preliminary characterization of Qa-1-deficient B6 mice has revealed no significant differences from wild-type mice in terms of mortality (i.e. no mice succumb to infection consistent with a noncytotoxic mechanism of controlling HSV-1 neuronal infection), rate of viral clearance from infected corneas, viral titers in acutely infected (five days post-infection) ganglia, viral genome copy number in latently infected (>30 days post-infection) ganglia, rate or magnitude of viral reactivation in *ex vivo* cultures of latently infected (>30 days post-infection) ganglia, or the number of total or gB-specific CD8 T cells within latently infected (>30 days post-infection) ganglia (data not shown). The functional role of NK cell receptors and their ligands in controlling ganglionic HSV-1 infection requires further study.

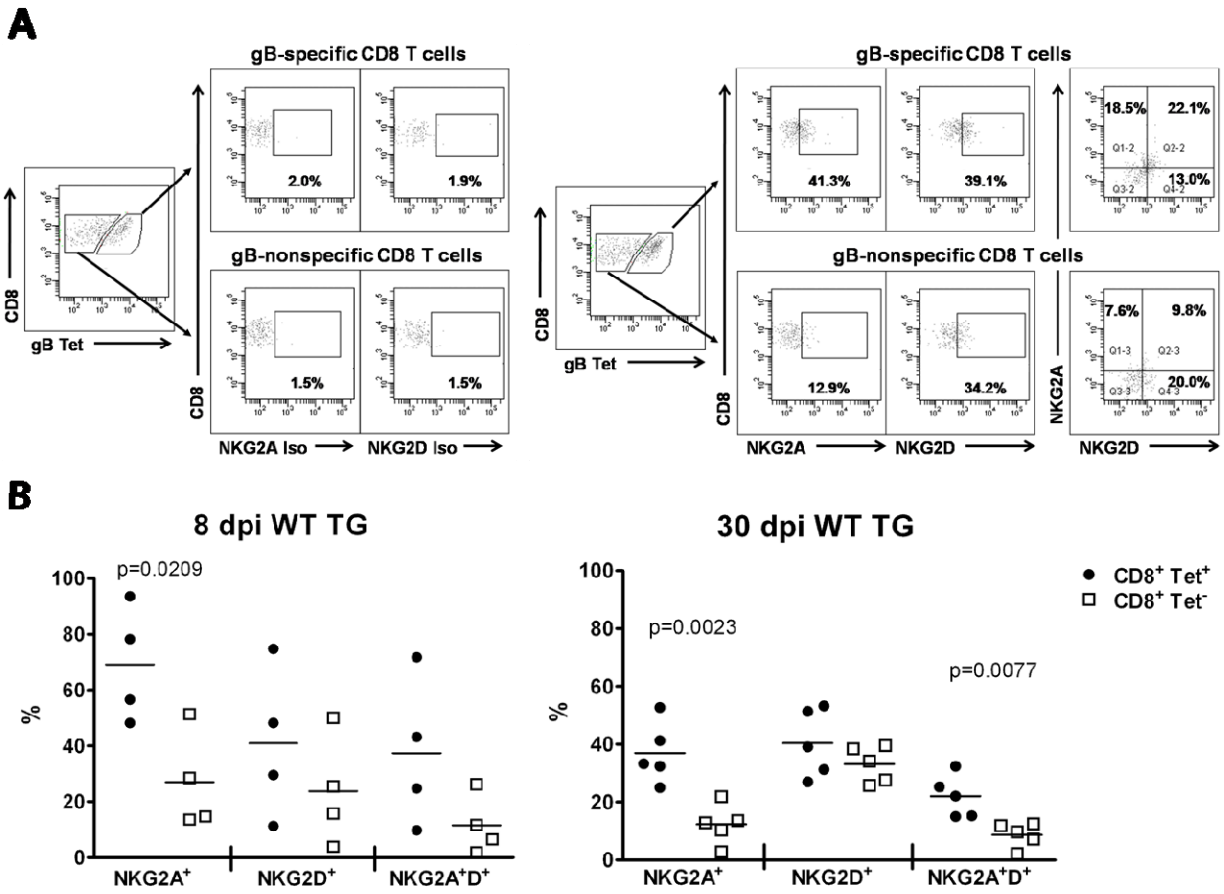


Figure 19. NK cell receptor expression on gB-specific and gB-nonspecific CD8 T cells within HSV-infected ganglia.

Trigeminal ganglia (TG) were removed from wild-type mice at the indicated days post-infection (dpi) and dispersed with collagenase. Cells were then stained for CD45, CD8α, gB-specific T cell receptor (gB-Tet), NKG2A/C/E, or NKG2D prior to flow cytometric analysis. (A) Dot plots from 30 dpi TG gated on $CD8^+$ cells showing isotype controls (left) or representative samples (right) stained for NKG2A and NKG2D. (B) Plots of the percentage of either gB-specific or gB-nonspecific CD8 T cells expressing NKG2A, NKG2D, or both at 8 dpi (left) and 10 dpi (right). Horizontal bars denote mean values. p values were calculated by Student's t test.

5.11 GRANZYME B CLEAVES THE ESSENTIAL HSV-1 IMMEDIATE EARLY PROTEIN ICP4

We hypothesized that granzyme B might inhibit the viral life cycle without killing the host neuron by directly cleaving an HSV-1 protein important for viral propagation. To initially investigate whether any HSV-1 proteins contained putative granzyme B cleavages sites, we used the GraBCas bioinformatics program, which predicts putative granzyme B cleavage sites within protein sequences (139). Using position specific scoring matrices based on experimentally determined substrate specificities, the GraBCas program predicts potential granzyme B cleavage sites within entered protein sequences. The tool was validated by testing known substrates and has a sensitivity of 80% and a specificity of 82% at a cutoff value of 1.2 (139), which was used in all analyses below. As shown in Table 3, four of ten HSV-1 proteins tested contained putative granzyme B cleavage sites predicted by the GraBCas program.

Table 3. Putative granzyme B cleavage sites within HSV-1 proteins.
The GraBCas bioinformatics program was used to predict granzyme B cleavage sites within sequences of the listed HSV-1 proteins. TK, thymidine kinase; gB, glycoprotein B, gC, glycoprotein C.

HSV-1 protein	HSV-1 gene class	# of putative granzyme B cleavage sites
ICP0	α	0
ICP4	α	2
ICP22	α	2
ICP27	α	0
ICP47	α	2
ICP8	β	0
TK	β	0
VP16	γ	0
gB	γ_1	1
gC	γ_2	0

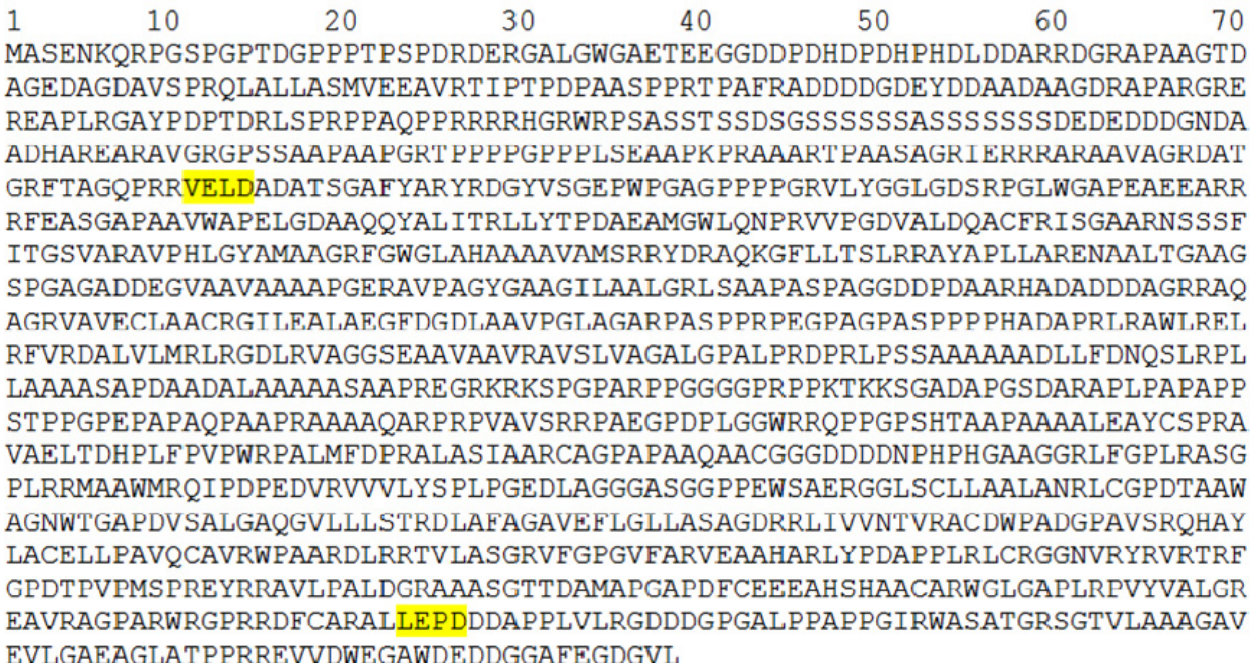


Figure 20. Predicted granzyme B cleavage sites within HSV-1 ICP4.
Protein sequence of ICP4 (Entrez #: NP_044676) with the granzyme B cleavage sites predicted by the GraBCas program highlighted in yellow.

Interestingly, two distinct granzyme B cleavage sites were identified in ICP4 (Figure 20), an HSV-1 immediate early (α) protein that is absolutely required for viral transcription beyond the α genes (140). Because ICP4 is essential for further HSV-1 gene expression (140) and because of available reagents, we chose to first investigate whether ICP4 was cleaved by granzyme B in biochemical assays. To determine if the granzyme B within CD8 T cell lytic granules could cleave ICP4 within HSV-1-infected cells, syngeneic fibroblasts were infected for 1 hr with a recombinant HSV-1 that expresses EGFP from the promoter for the α gene, ICP0, and incubated for a further 5 hrs with either wild-type, perforin$^{-/-}$, or granzyme B$^{-/-}$ gB-specific CD8 T cells at ratios of 6 T cells to 1 fibroblast. Half of the cells from each culture were analyzed by flow cytometry for the level of infection based on EGFP expression from the ICP0 promoter (Figure 21A), while the other half were assessed for ICP4 expression by Western blot (Figure 21B). ICP4 protein levels were dramatically reduced in lysates of fibroblasts that were exposed to wild-type gB-specific CD8 T cells relative to those exposed to no gB-specific CD8 T cells, perforin$^{-/-}$ gB-specific CD8 T cells, or granzyme B$^{-/-}$ gB-specific CD8 T cells, even when densitometry readings were adjusted for the number of infected (EGFP^{+}) cells remaining in the cultures (Figure 21B). Thus, the granzyme B released by CD8 T cell lytic granules is able to degrade ICP4 in infected targets.

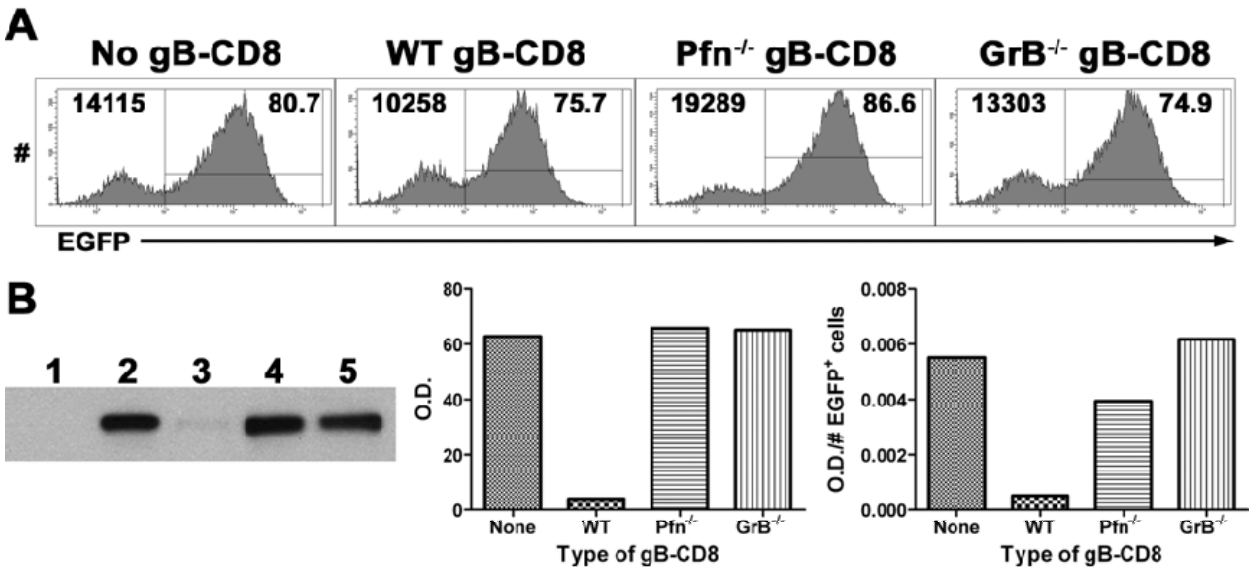


Figure 21. Granzyme B released by wild-type gB-specific CD8 T cells degrades ICP4 within HSV-1-infected fibroblasts.

B6WT3 fibroblasts were infected with a recombinant HSV-1 that expresses EGFP from the promoter of the α gene, ICP0, for 1 hour and then exposed to either wild-type (WT) gB-specific CD8 T cells (gB-CD8) or those deficient in perforin ($Pfn^{-/-}$) or granzyme B ($GrB^{-/-}$) for 5 hrs prior to flow cytometric analysis for EGFP (A) and Western blotting for ICP4 protein (B).(A) Histograms showing the total number of fibroblasts recovered from each culture (top left of each histogram) and the percentage of recovered fibroblasts that are infected ($EGFP^{+}$; top right of each histogram). (B) Western blot (left; Lane 1: Noninfected; 2: Infected/No gB-CD8; 3; Infected/WT gB-CD8; 4: Infected/$Pfn^{-/-}$ gB-CD8; 5: Infected/$GrB^{-/-}$ gB-CD8) and optical density (O.D.) readings that were not adjusted (middle) or adjusted for the number of infected fibroblasts recovered from each culture (right). Data representative of two experiments with similar results.

To formally demonstrate degradation of ICP4 by granzyme B, lysates of 293T cells transfected with a plasmid expressing ICP4 or fibroblasts infected with HSV-1 for 6 hours were exposed to recombinant human granzyme B. Granzyme B degraded ICP4 in a concentration- and time-dependent manner (Figure 22A-C). However, the possibility that granzyme B was activating other cellular proteases in the lysates that were then degrading ICP4 needed to be ruled out. Direct cleavage of ICP4 by granzyme B was established by granzyme B degradation of ICP4 purified by immunoprecipitation from HSV-1-infected fibroblasts (Figure 22D).

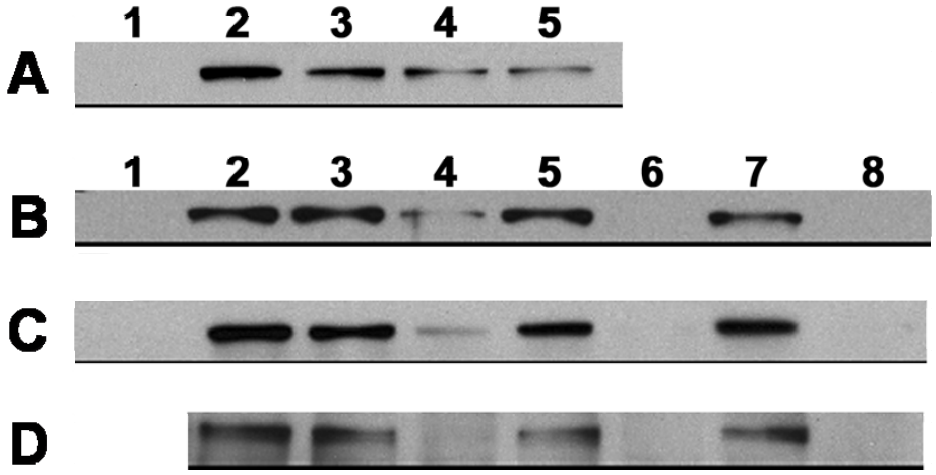


Figure 22. Granzyme B directly cleaves the essential HSV-1 IE protein ICP4.
(A) Lysates from 293T cells that were either non-transfected (Lane 1) or transfected with an ICP4-expressing plasmid (Lanes 2-5) were exposed to the indicated concentrations of recombinant granzyme B for 1 hour (hr) at 37° C (Lane 1: 0 nM, 2: 0 nM, 3: 25 nM,4: 50 nM, 5: 100 nM). (B) Lysates from 293T cells that were either non-transfected (Lane 1) or transfected with an ICP4-expressing plasmid (Lanes 2-8) were exposed to 100 nM granzyme B (Lanes 4, 6, 8) or 0 nM granzyme B (Lanes 1-3, 5, 7) for the indicated times at 37° C (Lanes 1&2: 0 hr, 3&4: 1 hr, 5&6: 2 hrs, 7&8: 3 hrs). (C) Lysates from B6WT3 fibroblasts that were noninfected (Lane 1) or infected with HSV-1 for 6 hrs (Lanes 2-8) were exposed to 100 nM granzyme B (Lanes 4, 6, 8) or not (Lanes 1-3, 5, 7) for the indicated times at 37° C (Lanes 1&2: 0 hr, 3&4: 1 hr, 5&6: 2 hrs, 7&8: 3 hrs). (D) ICP4 was immunoprecipitated from HSV-1-infected fibroblast cell lysates before exposure to 100 nM granzyme B (Lanes 4, 6, 8) or 0 nM granzyme B (Lanes 2-3, 5, 7) for the indicated times at 37° C (Lane 2: 0 hr, 3&4: 1 hr, 5&6: 2 hrs, 7&8: 3 hrs).

6.0 DISCUSSION

Most HSV-1-induced pathology is associated with recurrent disease, which is the result of HSV-1 reactivation from latency in sensory ganglia and transport of the virus to peripheral tissues or to the brain stem. Thus, understanding the mechanisms that control HSV-1 latency is of paramount importance. A growing literature supports a dynamic form of HSV-1 latency in which persistent attempts at reactivation in a small number of neurons at any given time is thwarted by HSV-specific CD8 T cells that colonize the ganglion following primary infection (reviewed in (141)). The mechanisms employed by CD8 T cells in controlling HSV-1 latency remain incompletely defined. The fact that activated CD8 T cells surround multiple apparently healthy latently infected neurons in sensory ganglia of both mice (63, 134) and humans (133, 142), and in many cases focus their T cell receptor to the neuronal junction (97), suggests the use of non-lytic mechanisms by CD8 T cells in controlling HSV-1 latency. Consistent with this theory is published evidence that CD8 T cells can employ IFN-γ to block HSV-1 reactivation from latency in some neurons (117, 128). However, the fact that recombinant IFN-γ can only prevent reactivation in a portion of neurons suggested a role for additional CD8 T cell effector mechanisms in controlling HSV-1 latency.

Expression of granzyme B by CD8 T cells is associated with activation (143), and is seen mainly in effector cells and effector memory cells (144). The HSV-specific CD8 T cells that are retained for prolonged periods in HSV-1 latently infected trigeminal ganglia of mice and humans

express an effector memory phenotype, and many express granzyme B (97, 126, 133, 145). Our current findings establish that CD8 T cells from latently infected trigeminal ganglia not only express granzyme B but are also capable of directed release of lytic granules toward susceptible non-neuronal targets leading to caspase activation and obvious signs of apoptosis.

The observations that gB-specific CD8 T cells within latently infected trigeminal ganglia are armed and capable of killing susceptible non-neuronal targets and that they form apparent immunologic synapses with multiple neurons with no obvious signs of neuronal destruction suggested at least two possibilities: either gB-specific CD8 T cells do not release their lytic granules toward infected neurons or latently infected neurons are selectively resistant to apoptosis induction by lytic granule components. Our results establish that gB-specific CD8 T cells do polarize and release their lytic granules into the junction with latently infected neurons in *ex vivo* trigeminal ganglia cultures. However, lytic granule exocytosis does not lead to either activation of the caspase system or observable signs of apoptosis in neurons within latently infected ganglia. Importantly, we observed susceptible fibroblasts undergoing various stages of caspase activation ranging from small localized areas of caspase activity, consistent with early stages of apoptosis, to more diffuse caspase staining patterns indicative of later stages of the apoptotic process (146). None of the neurons targeted by CD8 T cell lytic granules possessed even punctate staining for active caspases. Therefore, we are confident that the time-course used in these experiments was sufficient to detect activation of caspases, and that HSV-1 latently infected neurons are preferentially protected from caspase activation mediated by virus-specific CD8 T cells. The possibility that neurons targeted by CD8 T cells undergo a caspase-independent form of apoptosis is unlikely as granzyme B can directly activate caspase-3 as well as induce mitochondrial membrane disruption causing release of proteins, such as cytochrome c,

that also activate caspases (132). Thus, regardless of the apoptotic pathway undertaken, the pan-caspase inhibitor used in our studies should have detected any activation of caspases. Further, we did not observe any overt morphologic signs of apoptosis in these neurons.

The mechanism(s) employed by targeted neurons to protect themselves from CD8 T cell-mediated apoptosis remains to be elucidated. Many neurons within HSV-1 latently infected trigeminal ganglia express Qa-1 (145), a non-classical MHC class I molecule that regulates the effector functions of NKG2A/CD94-expressing CD8 T cells (147). Blocking this interaction in *ex vivo* cultures of HSV-1 latently infected trigeminal ganglia increased neuronal destruction by CD8 T cells (145). Thus, neuronal Qa-1 expression may render some neurons resistant to CD8 T cell cytotoxicity; however, the mechanism of this regulation requires further study.

Expression of latency-associated transcripts (LATs) by HSV-1 latently infected neurons is another potential explanation for the lack of caspase activation detected within these cells upon attack by CD8 T cells. LATs possess anti-apoptotic activity that is mediated at least in part by inhibition of caspase activation (148-150). The role of LATs in neuronal resistance to CD8 T cell lytic attack has not been determined but offers a promising avenue for future studies.

The fact that lytic granules released from HSV-specific CD8 T cells do not induce apoptosis in latently infected neurons suggested the alternative possibilities that lytic granule release toward neurons is inconsequential or that lytic granules function in a non-lytic manner to control viral latency. These possibilities were distinguished by testing the involvement of the essential lytic granule components perforin and granzyme B in control of HSV-1 latency *in vivo* and in *ex vivo* trigeminal ganglia cultures. Our data showing undetectable live virus and nearly identical latent viral loads in the trigeminal ganglia of perforin- and granzyme B-deficient compared to wild-type mice 8-10 days after infection suggest that these lytic granule components

are not required for the establishment of HSV-1 neuronal latency. This finding is in agreement with a previous study demonstrating that T lymphocytes and natural killer (NK) cells, the two major immune cells capable of expressing perforin and granzyme B, play little, if any, role in the establishment of HSV-1 neuronal latency (66). However, we now show that latency is not stable in perforin- or granzyme B-deficient mice as indicated by the dramatic increase in viral load between 10 and 14 days post-infection, an elevation that is maintained through at least 20 days post-infection in perforin-deficient mice. The viral load in perforin- and granzyme B-deficient trigeminal ganglia returned to wild-type levels by 30 days post-infection, possibly suggesting greater dependence on perforin and granzyme B for preventing HSV-1 reactivation early in latency. This could be due to HSV latency becoming more stable (less prone to reactivation) over time as suggested by findings that it is easier to induce HSV-1 reactivation in mice early in latency (151) and that recent acquisition is a predictor for human shedding of HSV-2 (152). These results are also consistent with the recent findings of Orr et al. showing that infection with HSV-1 recombinants expressing genes that inhibit MHC class I-restricted antigen presentation to CD8 T cells did not alter the establishment of HSV-1 latency or latent viral load but did result in increased reactivation frequencies both *in vitro* and *in vivo* (153).

Alternatively, the finding that HSV-1 genome copy number in latently infected perforin- and granzyme B-deficient ganglia returns to wild-type levels by 30 days post-infection despite apparent reactivation between 10 and 14 days post-infection might indicate that viral reactivation leads to neuronal destruction. However, neuronal destruction would imply production of infectious virus, which was not detectable in extracts of trigeminal ganglia obtained from perforin- or granzyme B-deficient mice at 14 or 20 days post-infection when viral genome copy number was elevated. It is also possible that reactivation might be halted after viral DNA

replication *in vivo* by a perforin- and granzyme B-independent mechanism with subsequent viral DNA degradation. This latter possibility would suggest that HSV-1 reactivation is regulated at several points by different mechanisms and would be consistent with our previous observation that IFN-γ can block reactivation at a late stage in the viral life cycle that occurs after late gene expression (128).

To investigate the mechanism as to how lytic granules may inhibit HSV-1 reactivation without lysing HSV-1 latently infected neurons, we tested the hypothesis that granzyme B directly cleaves an HSV-1 protein required for viral propagation. An initial bioinformatics analysis of HSV-1 protein sequences revealed that the HSV-1 immediate early (α) protein ICP4 contained two consensus granzyme B cleavage sites, and subsequent biochemical assays demonstrated that ICP-4 is, in fact, degraded by granzyme B. As we did not detect any stable ICP4 cleavage fragments when blotting with either polyclonal or monoclonal anti-ICP4 antibodies, it is possible that ICP4 is further degraded at sites other than those predicted by the GraBCas program. Importantly, ICP4 is absolutely required for progression of viral transcription beyond the α genes (140). Furthermore, ICP4 is one of only a few HSV-1 lytic genes that has been shown to be expressed in latently infected ganglia (104, 105). Combined with our finding that lytic granules are required to inhibit HSV-1 reactivation but do not lyse latently infected neurons, granzyme B cleavage of ICP4 likely acts to inactivate full viral reactivation.

Functioning as a transcription factor, most of the ICP4 protein within an HSV-1-infected cell resides in the nucleus (154). Therefore, this is the site where granzyme B-mediated cleavage of ICP4 would likely be most effective at inactivating the virus. Indeed, granzyme B has been shown to cleave several protein substrates in the nucleus of target cells during lytic granule-mediated apoptosis (155-158). The flux of proteins into the nucleus through nuclear pore

complexes in the nuclear envelope is tightly regulated, with many proteins requiring a nuclear localization signal (NLS) for entry (159). Both human and murine granzyme B contain sequences resembling known nuclear localization signals, and nuclear import of granzyme B requires cytosolic factors, such as importins, which can mediate granzyme B nuclear entry (160, 161). In addition, transport of granzyme B into the nucleus of intact target cells is dependent on perforin (156, 158, 161, 162). Thus, granzyme B, with the aid of perforin, has the ability to localize to the nucleus of target cells, the site where most IPC4 protein resides and functions within HSV-1-infected cells.

Of the ten HSV-1 protein sequences examined, three others also contained predicted GrB cleavage sites, including the α proteins, ICP22 and ICP47, and the γ_1 protein, gB. We concentrated on ICP4 because of its critical role in regulating expression of β and γ genes, but degradation of ICP22, which is a nonessential transactivator of HSV-1 genes, and gB, which is required for HSV-1 cellular penetration, might also aid in blocking full reactivation and infectious virion formation. ICP47, which functions to block CD8 T cell recognition of infected cells by inhibiting the transporter associated with antigen presentation (121), also contains two putative granzyme B cleavage sites. It is conceivable that granzyme B-mediated cleavage of ICP47 could neutralize this viral defense mechanism thereby enhancing the overall CD8 T cell response to HSV-1 infection. Moreover, a lytic granule-mediated protective mechanism might be employed by CD8 T cells in dealing with other members of the herpes virus family, since the varicella zoster virus IE62 protein, which is analogous to ICP4 of HSV-1, also contains two predicted granzyme B cleavage sites.

Our data clearly demonstrate an important role for lytic granules in maintaining HSV-1 latency in *ex vivo* trigeminal ganglia cultures. Although granzyme B appears to represent an important mechanism by which CD8 T cell lytic granules inhibit HSV-1 reactivation from latency, we note that CD8 T cells deficient in perforin are more compromised than those deficient in granzyme B in maintaining HSV-1 latency *in vivo* and in a common pool of neurons *ex vivo*. These observations suggest the involvement of other lytic granule components in the maintenance of viral latency. Granzyme A is a likely candidate as this granzyme has been implicated in the initial control of viral replication in acutely infected trigeminal ganglia (111) with apparent preservation of neuronal viability (134).

Understanding the mechanisms that control HSV-1 latency is of paramount importance because HSV-induced pathology is associated with viral shedding following reactivation from latency in sensory ganglia. A recent report demonstrated that viral microRNAs expressed during latency can inhibit production of multiple HSV-1 immediate early proteins, including ICP4 (163). Such mechanisms, in addition to epigenetic modifications of the viral genome (82), likely contribute to the stable latency that appears to exist in the vast majority of latently infected neurons. We propose that at any given time, some neurons escape these control mechanisms and require protection from HSV-specific CD8 T cells (reviewed in (141) and illustrated in Figure 23). It is these neurons that might represent those most likely to reactivate *in vivo*.

Our findings resolve the apparent paradox that potentially cytotoxic CD8 T cells surround apparently healthy latently infected neurons in HSV-infected ganglia of both mice and humans. We show that CD8 T cell lytic granules can block the HSV-1 life cycle through a non-lytic mechanism and that granzyme B can directly cleave ICP4, a viral protein required for expression of early and late viral genes. The fact that gB, a γ_1 gene product, is detectable by gB-specific

CD8 T cells within 2 hours of infection (8) would permit ICP4 degradation very early in the viral life cycle during both lytic infection and reactivation from latency. This mechanism might be particularly efficient during attempted HSV-1 reactivation events where ICP4 expression has escaped microRNA-mediated and epigenetic repression. We propose a tripartite relationship in which HSV-1 latency is maintained through the activity of the virus, the host neuron, and the contiguous CD8 T cells permitting viral persistence with neuronal survival. Preservation of granzyme B cleavage sites in a critical HSV-1 regulatory protein might represent a compromise by the virus designed to avoid destruction of the host cells that harbor it in a latent state.

Although many individuals acquire HSV-1 very early in life, the incidence of seroconversion increases into adulthood. Thus, a prophylactic vaccine might protect a portion of the population from acquiring latent HSV-1 infections. However, universal protection from HSV by a prophylactic vaccine appears unlikely. Therefore, a critical challenge facing herpes virologists and viral immunologists is to exploit the available knowledge of HSV-1 latency to develop new therapeutic strategies for maintaining HSV-1 in a latent state. It is our belief that currently the best hope for accomplishing this goal lies in our evolving understanding of the interaction between HSV-specific CD8 T cells and HSV-1 latently infected neurons. Development of a therapeutic vaccine targeting the viral proteins recognized by CD8 T cells that are retained within latently infected ganglia would seem to be a useful approach. In B6 mice, most HSV-specific CD8 T cells that are generated following an acute infection recognize a single epitope on the viral leaky late gene product, gB. Moreover, HSV-specific CD8 T cells of this specificity are further enriched in the memory CD8 T cell population that is retained in latently infected ganglia. This might suggest that CD8 T cells of this specificity are especially efficacious in preventing HSV-1 reactivation from latency. Indeed, the fact that gB-specific CD8

T cells are effective in blocking HSV-1 reactivation from latency suggests that this viral protein is a good target for CD8 T cell intervention in the reactivation process in mice. In humans, the CD8 T cell response to HSV-1 appears to be more heterogeneous. Whether all specificities of human HSV-specific CD8 T cells are equally effective at blocking HSV-1 reactivation from latency remains to be determined. Investigating the antigen specificity of CD8 T cells that are retained within latently infected human trigeminal ganglia might aid in the design of therapeutic vaccines for humans. Recent studies in which T cells were expanded from latently infected human trigeminal ganglia suggest the feasibility of such an approach (125). Although unsuccessful human vaccine studies have included gB as an immunogen, these studies have largely targeted antibody responses (164). Our findings would suggest that vaccine formulations designed to additionally activate CD8 T cells would be more efficacious at preventing reactivation of latent virus and shedding at the periphery.

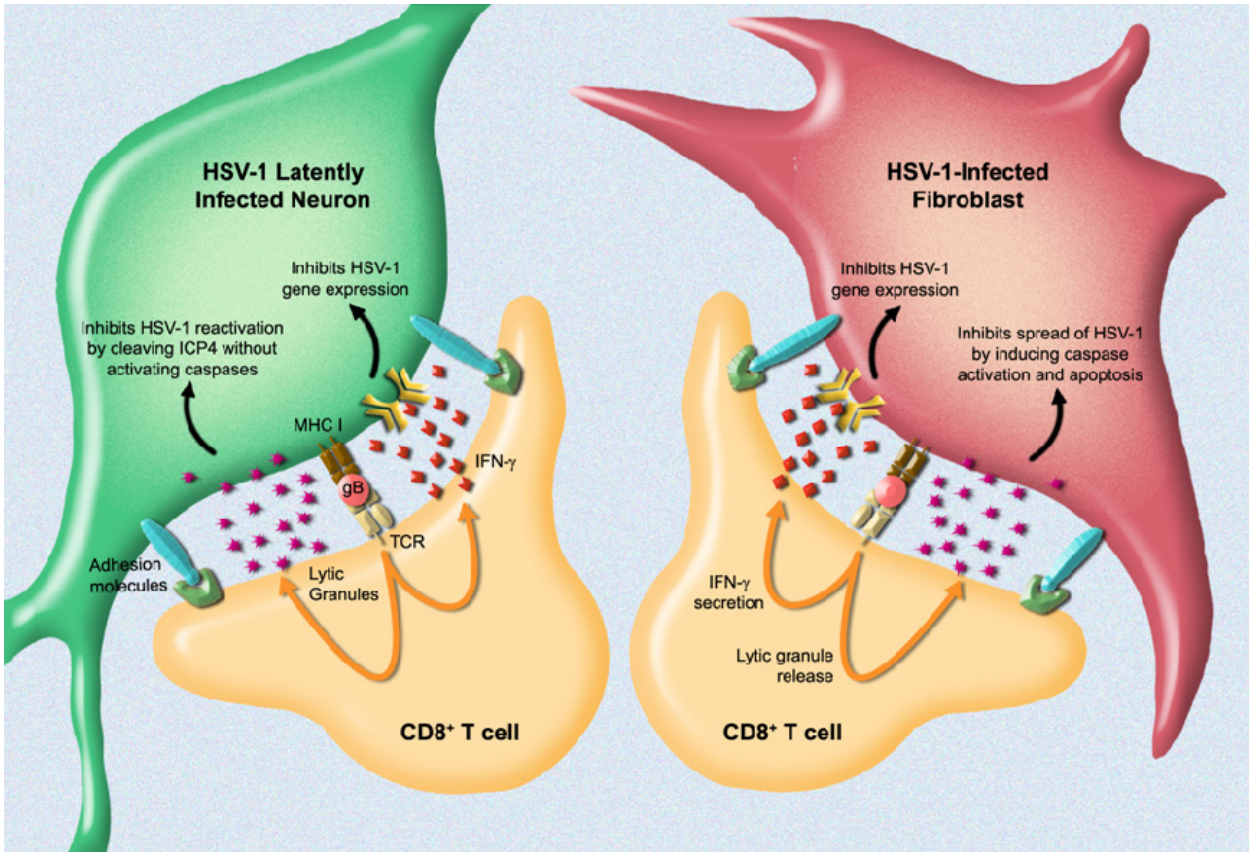


Figure 23. Schematic model of HSV-specific CD8 T cell interactions with either HSV-1-infected fibroblasts or neurons.
HSV gB-specific CD8 T cells form immunologic synapses with HSV-1 latently infected neurons or lytically infected fibroblasts. In both cases, IFN-γ and lytic granules are secreted into the CD8 T cell/target cell junction. Although CD8 T cells can block HSV-1 reactivation from latency in all neurons, IFN-γ can only block HSV-1 reactivation approximately half of the neurons that require CD8 T cell protection, presumably because only about half of trigeminal ganglia neurons express IFN-γ receptors (our unpublished observation). gB-specific CD8 T cells can also block HSV-1 reactivation from latency in neurons through a non-lytic mechanism involving granzyme B cleavage of HSV-1 ICP4, a protein that is critical for transactivation of HSV-1 early and late genes. Neurons are protected from granzyme B-induced caspase activation and the resulting apoptosis by an as yet undefined mechanism. Lytic granule release into lytically infected fibroblasts also results in granzyme B cleavage of ICP4, but the caspase system of these cells is activated leading to apoptosis. We hypothesize that the retention of granzyme B cleavage sites in a critical HSV-1 regulatory protein represents a compromise that permits HSV-1 to be harbored for prolonged periods in sensory neurons, emerging only when T cell function is compromised.

7.0 SUMMARY

Herpes simplex virus type 1 (HSV-1) infection is generally acquired early in life and affects the majority of the world's population by middle age. Following primary infection, HSV-1 establishes lifelong latency in sensory neurons. Reactivation of latent HSV-1 in trigeminal ganglia neurons can lead to retrograde viral transport to the brain causing HSV encephalitis, the leading cause of sporadically fatal viral encephalitis in the U.S. Anterograde transport of reactivated HSV-1 can lead to orofacial lesions, such common cold sores, as well as ocular pathology, with recurrent corneal infections representing a leading cause of infectious blindness worldwide. Prophylactic antiviral therapy can reduce the rate of recurrent herpetic disease, but this therapy is not curative, requires daily dosings, and only acts once the virus has reactivated from latency. Thus, understanding and exploiting the mechanisms responsible for maintaining HSV-1 in a latent state represents a promising approach to preventing these devastating diseases. Emerging evidence demonstrates a role for CD8 T cells in maintaining HSV-1 latency within infected ganglia. However, prudence dictates that exploitation of a mechanism with the potential cellular lethality of CD8 T cells should be approached with caution, especially when directed toward nonregenerating sensory neurons. Therefore, a major goal of our lab is to understand the effector mechanisms employed by CD8 T cells in preventing HSV-1 reactivation from neuronal latency.

Using a mouse model of ocular HSV-1 infection, our lab previously established that CD8 T cells can completely block HSV-1 reactivation from neuronal latency. We also showed that CD8 T cells can secrete the antiviral cytokine IFN-γ to inhibit reactivation in some, but not all, latently infected neurons, suggesting involvement of additional CD8 T cell effector functions. Release of lytic granules represents another important CD8 T cell effector mechanism. However, lytic granule release is generally thought to be lethal to target cells, an effect that would be detrimental to nonregenerating neurons. My dissertation research tested the hypothesis that CD8 T cells used lytic granules in a noncytotoxic manner to block HSV-1 reactivation from neuronal latency by directly degrading HSV-1 protein(s) required for further viral propagation. The results of this research demonstrated that CD8 T cells do, in fact, use lytic granules in a nonlytic manner to inhibit HSV-1 reactivation within nonregenerating sensory neurons. Mechanistically, we demonstrated that perforin and granzyme B, two key lytic granule components, are required for this inhibition and that granzyme B directly cleaves the essential HSV-1 transactivating protein, ICP4, thereby shutting down the viral life cycle early in the reactivation process. The most exciting aspects of this work are the demonstration that lytic granules can function in a noncytotoxic manner and the discovery of a novel granzyme B substrate that is not a part of the cellular apoptotic pathway but rather a viral protein that is required for further viral gene expression. Thus, a therapeutic vaccine designed to induce a potent CD8 T cell response against latent HSV-1 may provide protection from reactivation without eliminating non-regenerating sensory neurons.

8.0 FUTURE DIRECTIONS

8.1 THE ROLE OF HSV-1 LATENCY ASSOCIATED TRANSCRIPTS IN PROTECTING HSV-1 LATENTLY INFECTED NEURONS FROM LYTIC GRANULE-MEDIATED APOPTOSIS

As noted above, the HSV-1 latency associated transcripts (LATs) function to block apoptosis at least in part through inhibition of caspase activation (148-150). In a previous report, significantly more neurons within ganglia infected with a LAT-deficient HSV-1 underwent apoptosis compared to those infected with a wild-type LAT-competent virus (150). Here, we describe a CD8 T cell lytic granule-mediated, yet noncytotoxic, mechanism of maintaining HSV-1 neuronal latency. It is of great interest to determine if those neurons susceptible to apoptosis when infected with LAT-deficient HSV-1 are those targeted by CD8 T cell lytic granules.

8.2 THE ROLE OF GRANZYME-MEIDATED CLEAVAGE OF HSV-1 PROTEINS OTHER THAN ICP4

We established that granzyme B directly cleaves the essential HSV-1 immediate early protein ICP4. Several other important HSV-1 proteins (see Table 3), as well as the varicella zoster virus protein IE62, which is analogous to HSV-1 ICP4, also contain putative granzyme B cleavage

sites. Additionally, granzyme H has been shown to cleave the adenovirus DNA-binding protein (165). Therefore, granzyme-mediated degradation of viral proteins may be a more pervasive mechanism of viral control than currently recognized. Investigation into whether granzyme B can cleave other HSV-1 proteins and the functional outcomes of such cleavage remain to be determined.

8.3 THE ROLE OF GRANZYMES OTHER THAN GRANZYME B IN MAINTAINING HSV-1 NEURONAL LATENCY

We define here a role for granzyme B in blocking HSV-1 reactivation from neuronal latency. As mentioned above, perforin-deficient CD8 T cells appeared to be less efficient than granzyme B-deficient CD8 T cells in the maintenance HSV-1 neuronal latency suggesting use of other granzymes in this process. Granzyme A has been implicated in blocking the interneuronal spread of HSV-1 within acutely infected ganglia (111); however, the role this granzyme plays in maintaining HSV-1 neuronal latency has yet to be investigated. Therefore, further research is required to determine whether any other granzymes function to maintain HSV-1 neuronal latency along with granzyme B.

8.4 THE ROLE OF NATURAL KILLER (NK) CELLS AND NK CELL RECEPTORS IN CONTROLING NEURONAL HSV-1 INFECTION

The results presented above define the kinetics of NK cell infiltration into HSV-1-infected ganglia as well as the kinetics of both stimulatory and inhibitory NK cell receptor expression on NK cells and CD8 T cells following HSV-1 infection. Further, our data suggest that NK cells may be more important during acute rather than latent ganglionic HSV-1 infection. Additional studies are required to elucidate the exact roles NK cells and NK cell receptors play in modulating neuronal HSV-1 infection.

APPENDIX A

SUPPLEMENTAL DATA

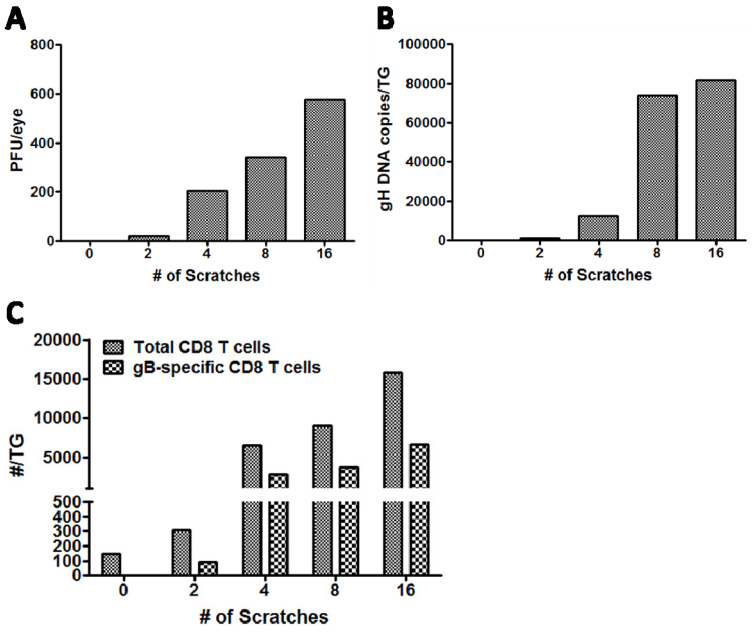


Figure 24. Effect of corneal scarification on HSV-1 infection and immune response. Corneas of wild-type B6 mice were scratched with a sterile 30-guage needle the indicated number of times prior to topical application of $1x10^5$ pfu of HSV-1. Scratches were defined as full-length scarifications from limbus to limbus in a criss-cross fashion over the entire surface of the cornea. For example, 16 scratches refers to eight scratches across the width of the cornea in one direction and eight scratches across the width of the cornea in the perpendicular direction. (A) Viral titers from eye swabs taken two days after infection assessed by plaque assay. (B) HSV-1 glycoprotein H (gH) DNA copies per trigeminal ganglia (TG) at eight days post-infection assessed by real-time PCR. (C) The number of total and gB-specific CD8 T cells per TG at eight days post-infection assessed by flow cytometry. All other experiments presented in this dissertation used 8-16 scratches.

APPENDIX B

PUBLICATIONS

1. Knickelbein, J.E., K.M. Khanna, M.B. Yee, C.J. Baty, P.R. Kinchington, R.L. Hendricks. Noncytotoxic lytic granule-mediated $CD8^+$ T cell inhibition of HSV-1 reactivation from neuronal latency. Submitted.
2. Knickelbein, J.E., S.C. Watkins, R.L. Hendricks. Stratification of antigen-presenting cells within the normal cornea. In preparation.
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