Active Immunosurveillance by $CD8^+$ T Lymphocytes during Acute and Latent Herpes Simplex Virus-1 Infection

by

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2. Liu T, **Khanna K.M**., Carriere B.N., and Hendricks R.L. 2001. Gamma Interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. *J. Virol.* **75** (22): 11178-11184.

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Active Immunosurveillance by $CD8^+$ T Lymphocytes during Acute and Latent Herpes Simplex Virus-1 Infection

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Herpes simplex virus type-1 (HSV-1) infection results in the establishment of a latent infection in sensory ganglia of the peripheral nervous system (PNS). In humans the virus can sporadically reactivate and gain access to the primary sites of infection and cause considerable tissue damage. The mechanisms involved in establishment and maintenance of latency and the sporadic reactivation of HSV-1 infected neurons in sensory ganglia is poorly defined. Elucidating the mechanisms involved in maintenance of latency of HSV-1 would be extremely valuable in controlling the high prevalence of disease in communities around the world. We have shown that $CD8^+$ T lymphocytes can block HSV-1 reactivation from latency in sensory neurons of the trigeminal ganglion ex vivo.

The mechanism that the $CD8^+$ T cells employ to prevent reactivation includes release of the antiviral cytokine IFNγ, but other mechanisms, such as the lytic effect of perforin and granzymes appear to be important as well. In order to further understand the cellular immune response against the latent HSV infection we have attempted to dissect the $CD8^+$ T cell immune response in the TG during acute and latent infection. Specifically, we determined the antigen specificity and TCR expression of the $CD8^+$ T cells that accumulate in the TG at various times post infection. We have investigated the state of $CD8^+$ T cell memory after HSV-1 infection and clarified the mechanisms used by $CD8^+$ T cells to prevent reactivation from latency

Acknowledgements

"Brief is the noise of fame, that passing guest. They all must die – the hero and the knave. The greatest king goes to eternal rest, and every dog comes pissing on his grave"

-Johann Wolfgang von Goethe

I must begin my acknowledgments by thanking my dear parents. They have given me my life, their love has been the reason for my existence, their integrity and support has been a compelling force in my growth as a human being and as a scientist. My respect for them is unending and my comparison for them is with the Divine.

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1. INTRODUCTION

1.1. Herpes Simplex Virus

Herpes Simplex viruses (HSV) are one of the most widely studied viruses in the world. The name herpes comes from the Greek word 'herpein', which means to creep. Like other herpesviruses, they have very unique biological properties, particularly their ability to form latent infections and reactivate periodically to cause herpetic lesions. Herpes Simplex Virus 1 (HSV-1) is an alphaherpes virus, which is part of a large family of viruses collectively called the herpesviridae. This thesis will primarily explore the dynamic equilibrium of HSV-1 infection involving a tripartite interaction between the virus, the host neuron and the local immune components.

1.2. Viral Structure

The HSV virion (Figure 1) is approximately 200 nm in diameter and is made up of four basic elements: (i) a core that contains a spool around which the double stranded DNA (dsDNA) is wrapped; (ii) an icosahedral capsid made up of 162 hexagonal capsomeres and five viral proteins surrounding the core and is 100-110 nm in diameter; (iii) A tegument surrounds the capsid and contains viral proteins that are critical for initiating viral gene expression; and (iv) an outer envelope, which is a lipid bilayer and contains numerous embedded glycoproteins (1).

1.3. Viral genome

The viral genome, when in the virion, comprises of a linear double stranded DNA approximately 153,000 base pairs (bp) in length. The genome is organized into two main segments of long (L) and short (S) domains (Figure. 2). The genome contains two unique

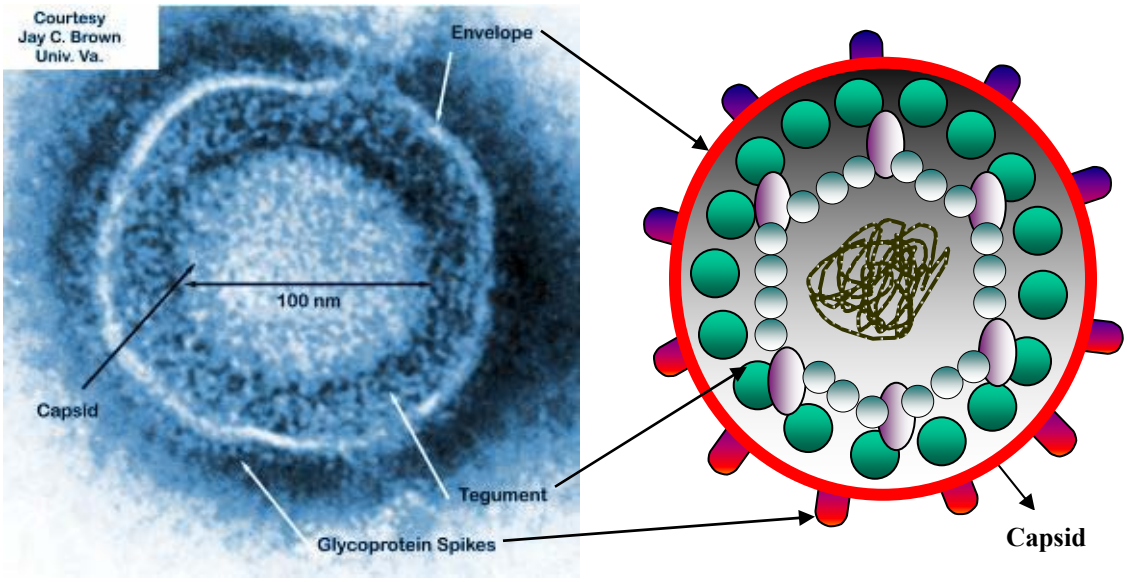

Figure 1: Electron micrograph (left) and a schematic (right) of HSV-1 structure showing the four basic components of the virion.

regions unique long (U_L) and unique short (U_S). There are short inverted repeats between and flanking the unique sequences L and S denoted as LTRa, LTRb, and LTRc. The genome is represented by a particular scheme in which the first nucleotide of the shortest terminal repeat is LTRa, adjacent to LTRb followed by the U_L region, LTRa',b' and LTRc' and ending with LTRc and LTRa, as shown in Figure. 2. The viral genome may contain variable number of LTRa copies (2,1).

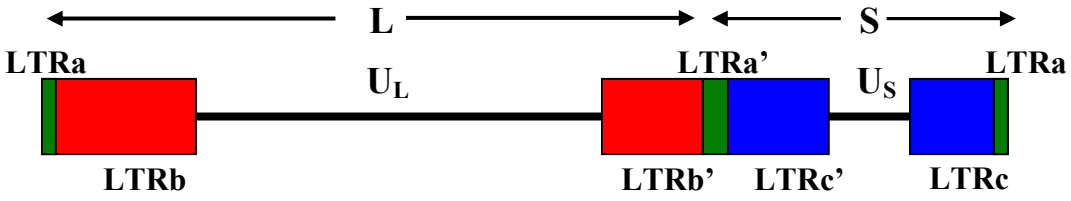

Figure 2: Schematic illustration of the arrangement of DNA in the HSV-1 genome*. (Figure not to scale)*

1.4. HSV-1 infection and gene expression

The HSV-1 genome encodes more than 80 unique proteins that are expressed in a regulated and sequential manner (Figure. 4). There are three main classes of gene transcripts encoded by HSV-1 DNA; immediate early genes (α proteins), early genes (β proteins) and late genes that are further divided into γ1 and γ2 genes.

1.4.1. Viral life cycle

One of the unique properties of HSV-1 is its ability to virtually infect all types of mammalian cells. The infection begins (Figure. 3) when the virus attaches to the myriad of extracellular matrix proteins such as heparan sulfate on the cell surface. Viral proteins involved in the initial attachment process are primarily gB and gC. The second step in the attachment process involves the binding of viral protein gD with many coreceptors on the cell, including members of the tumor necrosis factor (TNF) receptor family, and immunoglobulin (Ig) superfamily. After the interaction of gD with cellular coreceptors the viral envelope fuses with the plasma membrane, an event mediated by glycoproteins gB, gD, and gH. After the fusion of the viral envelope with the plasma membrane, the capsid with the tegument proteins along with the viral DNA is released in the cytoplasm and the viral glycoproteins remain embedded at the surface of the cell. The viral nucleocapsid and certain tegument proteins (VP16) are transported to the nucleus. The virus docks at the nuclear pores and releases the viral DNA inside the nucleus. Upon release into the nucleus VP16 interacts with host transcription factors to initiate the expression of immediate early (α) genes (without new viral protein synthesis), which in turn activate early (β) genes that mediate DNA replication of HSV genome. Expression of β and γ1 genes require α protein expression.

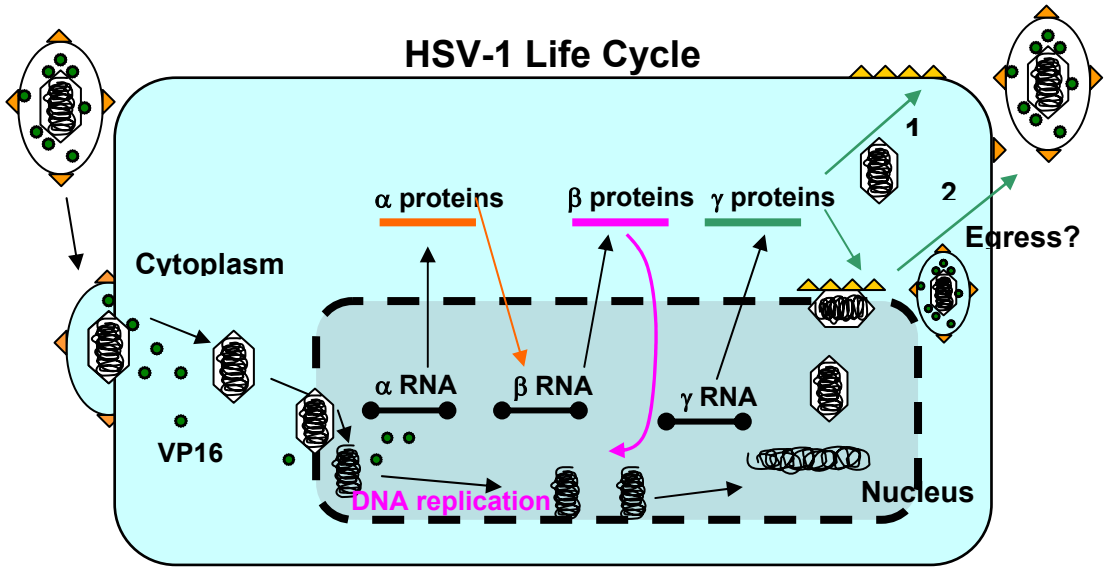

Figure 3: Schematic representation of the HSV-1 life cycle.

Following DNA synthesis γ1 gene transcription is augmented and γ2 gene expression commences (γ2 gene expression requires the onset of DNA synthesis). After late proteins are translated in the cytoplasm the premature glycoproteins are localized to the inner and outer membrane of the cell's nuclear membrane. These proteins are further modified in the Golgi apparatus and the mature glycoproteins are then transported to the plasma cell membrane. There are two proposed pathways by which the virus egresses from the infected cell (Figure 3). The first pathway shows (Pathway 1 in Figure 3) the virus buds out of the inner nuclear membrane and fuses with the outer nuclear membrane and in the process is de-enveloped and transported through the Trans Golgi apparatus, where it acquires its envelope and egresses out through the plasma membrane. In the second pathway (Pathway 2 in Figure 3) the virus buds off the outer nuclear membrane with an intact envelope and is transported to the plasma membrane of the cell

where it egresses out of the cell. A recent study by Grunewald et.al (3) now favors the first pathway.

1.4.2. HSV-1 lytic infection and gene expression

Immediate Early (α) Gene Expression: HSV-1 infection of non-neuronal cells usually leads to the death of the host cell. In most cells, HSV-1 produces a lytic infection in which it expresses its entire genome with the exception of latency associated transcripts (LAT). During lytic infection viral gene expression is remarkably regulated (Figure. 4). Viral tegument protein VP16 augments α gene expression by interacting with two other cellular proteins by binding to a target sequence TAATGARAT. This results in the expression of five α gene products ICP4, ICP0, ICP27, ICP47 and ICP22 (4). α Gene expression peaks between two to four hours after infection.

ICP4 is an essential viral α protein that serves as a transcription factor to activate early and late proteins (5). ICP4 can also serve as a repressor, inhibiting the expression of immediate-early genes (6). ICP0 is a non-essential protein but can activate viral gene expression at low multiplicity of infection (MOI). ICP0 is essential for efficient reactivation of HSV-1 from latency (7). ICP0 localizes to the nucleus where it can influence chromatin structure by breaking down histone proteins and other nuclear proteins such as nuclear domain 10 (ND10) bodies (8,9,10). The ability of ICP0 to influence viral and host cell chromatin structure and accessibility of transcription factors to viral and host cell DNA makes it a good candidate for regulating reactivation (11). ICP0 has also been shown to inactivate interferon associated gene cascade, thus implicating it in immune evasion (12,13).

ICP27 is an essential α protein required for HSV-1 lytic infection and essential for early and especially late gene expression. ICP27 has myriad of functions; (14,1) it regulates splicing,

translation and post-translational modification of viral gene expression. ICP27 localizes to the nucleus and regulates the nuclear transport of viral mRNA transcripts. ICP27 also controls host cell gene expression by interacting with small nuclear ribonucleoprotein particles (snRNPs) and inhibiting host cell mRNA splicing (15).

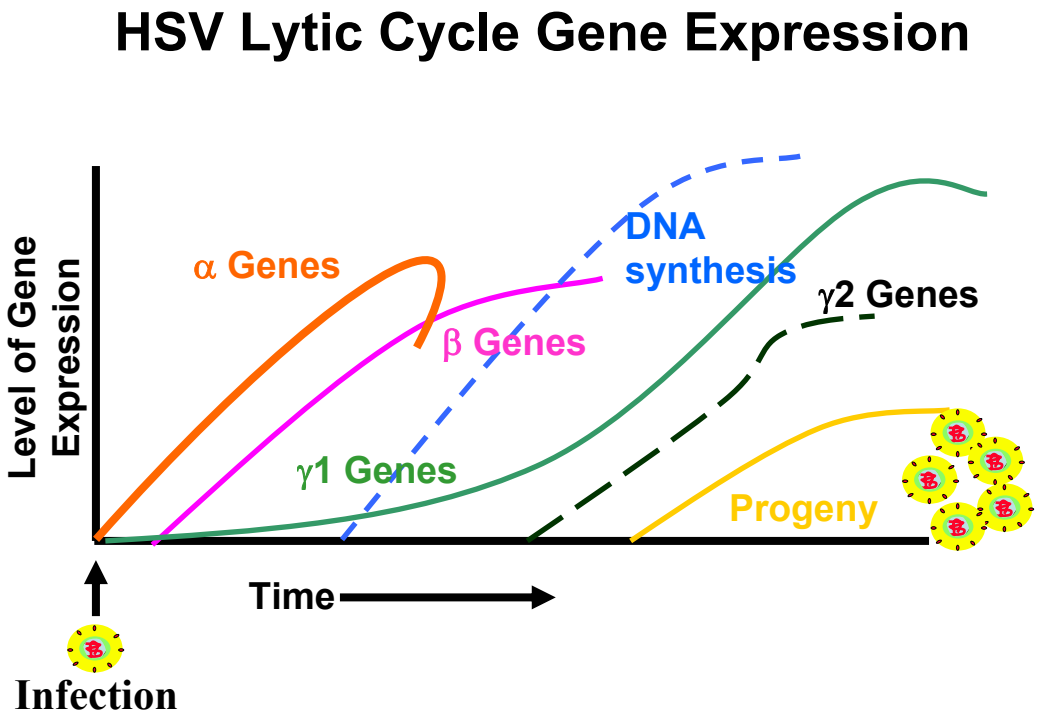

Figure 4: HSV-1 gene expression during lytic infection. *The x-axis denotes time after infection of a cell by HSV-1. The y-axis represents level of α, β, and γ gene expression. The dotted blue line represents DNA synthesis. Note that γ1 genes can be expressed in the absence of DNA synthesis but are elevated substantially after the onset of DNA replication. Note that this figure only denotes general chronology of events.*

ICP47 is not an essential protein for viral lytic infection but plays a very important role in immune evasion. ICP47 is a soluble 12-kDa protein that effectively blocks antigen presentation of viral peptides by blocking the transporter associated with antigen processing (TAP) complex. TAP1 and 2 are encoded by the major histocompatibility complex (MHC) cluster and play a crucial role in the endogenous antigen presentation pathway by which viral antigens are presented to $CD8^+$ T cells in the context of MHC class I molecules (16). Viral proteins are processed into small peptides by the proteasomes in the cytosol and subsequently transported into the lumen of the endoplasmic reticulum (ER), where they bind to the MHC class I molecules

to be presented at the surface of the infected cell. TAP1 and TAP2 form a non-covalent complex at the surface of the ER and facilitate the shuttling of viral peptides from the cytosol to the ER (17). Residues 2-35 of ICP47 prevent the transport of viral peptides by binding to the TAP1 and TAP2 molecules and weakening the interaction between the two molecules, thus precluding efficient activation of HSV specific cytotoxic T lymphocytes (CTL) (18). ICP47 is especially effective in blocking human TAP, but a recent study showed that it can also inhibit murine TAP function (19).

The fifth IE gene product ICP22 is not essential for viral replication but is important in the expression of ICP0 and some late genes (14).

Early (β) Gene Expression: Soon after the synthesis of α proteins, β genes are expressed. Early genes are primarily involved in DNA replication (20). β Gene expression begins as early as three hours after infection and reaches its peak between four and seven hours post infection (PI). DNA replication begins upon β protein expression and continues for up to fifteen hours PI.

One of the β gene products thymidine kinase (TK) is not required for viral DNA synthesis in non-neuronal cells because the virus is able to employ cellular TK; however it is essential for viral replication in neuronal cells because neurons do not contain their own TK. For this reason, virus lacking TK is unable to reactivate once it establishes latency in ganglionic neurons (21,22). HSV TK can phosphorylate purine nucleosides and deoxyribonucleosides in addition to thymidine. Guanosine analogs acyclovir and gancyclovir are prodrugs that have been effectively used for many years to combat herpetic infections. Acyclovir is only effective in HSV infected cells because it requires a triple kinase activity to be converted into a triphosphate derivative, which can then serve as a substrate for HSV-1 DNA polymerase. Once the

triphosphate acyclovir derivative is incorporated into the HSV DNA, the growing DNA chain terminates since acyclovir lacks the 3' OH sugar group(20).

Late (γ) Gene Expression: The viral late genes are subdivided into γ1 and γ2 genes. γ1, also called leaky genes, are expressed at low levels before viral DNA synthesis (Figure. 4), but at much higher levels after viral DNA synthesis is initiated. Expression of γ2 genes is completely dependent on initiation of viral DNA synthesis. Some of the γ1 genes include structural proteins such as glycoprotein B (gB) and glycoprotein D (gD). γ2 Genes encode for proteins such as gC, gH, U_S11, and U_S9. The exact mechanisms that direct late gene expression are not well understood, however α and β genes such as ICP4, ICP27 and ICP8 are necessary for late gene expression (6, 1,14). After the onset of DNA synthesis late genes that encode structural proteins are expressed in considerably high levels to ensure the generation of high numbers of infectious viral progenies.

1.4.3. Effect on the Host Cell after HSV-1 Productive Infection

HSV-1 is an exceptionally cytopathic virus; cells productively infected with HSV undergo substantial changes, which ultimately lead to their death. The molecular, chemical and structural changes that occur in the productively infected epithelial cell are extensive therefore, only an overview will be provided. For a broader and excellent discussion please see reference 1. Upon infection of an epithelial cell by HSV-1, viral glycoproteins are embeded in the cellular membrane. Once HSV-1 productive infection proceeds, the microtubular structure of the host cell is disrupted and the Golgi apparatus is fragmented leading to the rounding up and adherence of infected cells. One of the most detrimental effect of HSV infection is the shut off of host DNA, RNA and protein synthesis. Host mRNA and proteins are degraded and transcriptional and translational processes are completely inhibited. Viral tegument protein called the virion

host shut-off protein (vhs) is largely responsible for initiating and carrying out the host shut off processes (14). Another profound effect of HSV-1 infection is on the host cell cycle machinery (1). Since the virus shuts off host DNA synthesis there is an indirect block in the cell cycle at the G1 phase. HSV infection also leads to inhibition of cyclin dependent kinases (CDK) such as cdk2 and cyclin D3 proteins. However recent studies have implicated certain CDKs in viral reactivation from latency in sensory ganglia (23)

1.4.4. Latent Viral Infection

The most intriguing property of HSV infection is its ability to establish a latent infection in neurons of sensory ganglia. Understanding the mechanisms that regulate the establishment and maintenance of latency as well as reactivation of virus from latency continues to be the most challenging problem in biomedical research.

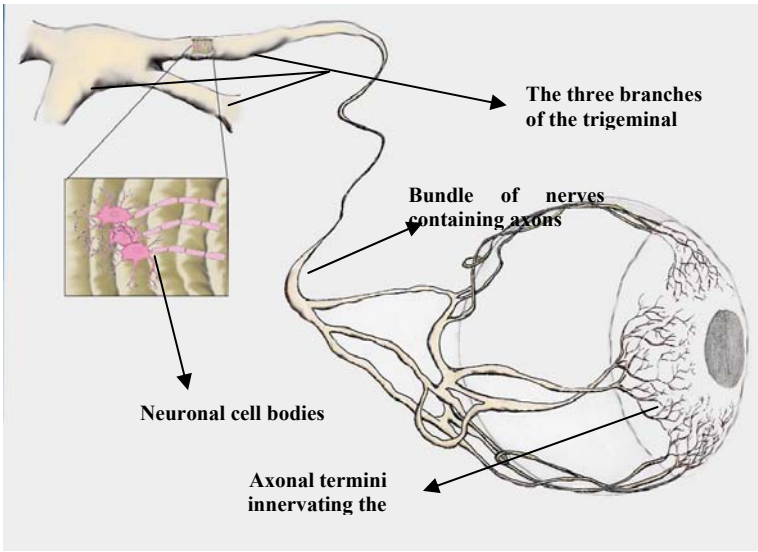

Figure 5: Graphic representation of the anatomy of the peripheral nervous system, *where the eye may serve as the peripheral sight of infection. The eye is innervated heavily with axonal termini of the sensory neurons contained in the trigeminal ganglion. The trigeminal ganglion is made up of three branches. The inset shows the neuronal cell bodies present in the ophthalmic branch of the trigeminal ganglion.*

After HSV-1 infection at a peripheral site (i.e. eye, mouth, etc.) the virus briefly replicates in the epithelial cells (where the productive infection follows the temporal pattern of gene expression described above) followed by retrograde axonal transport to the body of the neuronal cell in sensory ganglia that innervate the primary site of infection where it establishes latency for the life on the individual (Figure. 5). During stable latency the virus remains in a non-replicating episomal state. In humans and rabbits the virus can sporadically reactivate as result of poorly defined stimuli, such as immunosuppression, stress and exposure to UV-B irradiation (1,4). Upon reactivation the virus travels via the anterograde axonal transport and gains access to the primary site of the infection and cause recurrent herpatic disease.

Although there is a consensus that latently infected neurons harbor a functional viral genome but fail to produce virions, the definition of latency at the molecular level remains controversial. Establishment of latency appears to be a rather promiscuous property of HSV. Recombinant viruses lacking essential proteins such as ICP4, ICP8 and TK are capable of establishing latency, but fail to reactivate (24,1). During latency the most abundant viral transcripts produced are the 2 and 1.5 kb latency-associated transcripts (LATs). LATs are stable intronic RNA sequences that are anti-sense to ICP0 (25). To date no viral proteins that are uniquely expressed during HSV-1 latency have been found given that, no LAT translation product has been discovered (4). $LAT^{-/-}$ HSV-1, however, is capable of establishing a latent infection. Recent studies have demonstrated anti apoptotic role of LATs; neurons that were infected with LAT deletion viruses were more susceptible to apoptosis *in vivo* and *ex vivo* (26,27), although the mechanism is yet to be defined.

For many years it was believed that HSV latency represented a quiescent state in which transcription of the viral genome was limited to LATs. As LATs do not encode any proteins, expression of any lytic genes was considered reactivation from latency (28,25). However, many recent studies, including ours, support a more dynamic view of latency, which suggests that limited viral gene expression continues in the absence of virion formation and viral DNA replication (29,30,31,32,33). Low-level expression of α gene ICP4, and β gene TK were detected *in vivo* by nested RT-PCR (30) as well as in *ex vivo* latent TG cultures (32). In both studies, γ2 gene (gC) expression was not detected confirming the lack of viral reactivation. A recent histological study of latently infected mouse TG revealed expression of transcripts for ICP4, TK, and in rare cases gC expression was also detected (29).

Therefore, the factors that contribute to the maintenance of HSV latency still remain poorly defined. Evidence emerging from our laboratory (reviewed later) suggests the involvement of the immune system in regulating HSV-1 latency.

1.4.5. Ocular Murine Model of Latency

In humans and rabbits, HSV-1 reactivates spontaneously and can be readily detected in teardrops of the eyes; however there are obvious disadvantages to studying viral latency in humans and rabbits. The mouse model of latency has been the most widely used experimental system for investigating HSV latency and reactivation. Just as in humans and rabbits, HSV-1 corneal infection of the murine eye results in brief viral replication at the corneal epithelial cells followed by transport of the virus to the ophthalmic branch of the trigeminal ganglion where it briefly replicates and establishes a latent infection by 8 days PI (Figure. 6). There are no known differences in the state of the latent viral genome in animals and humans. The murine experimental system is particularly attractive because (i) spontaneous reactivation in mice is

extremely rare and can be induced experimentally at a time of our choosing (34,35,36); (ii) availability of highly inbred, knockout and transgenic strains of mice is extremely valuable for investigating the role of the immune system in regulating viral latency and reactivation, and finally; (iii) the overabundance of available murine immunological reagents greatly exceeds any other animal experimental model. Since MHC-H2k^{b} restricted viral epitopes recognized by CD8^{+} T cells are defined, we used the C57BL/6 mice for majority of the studies presented here.

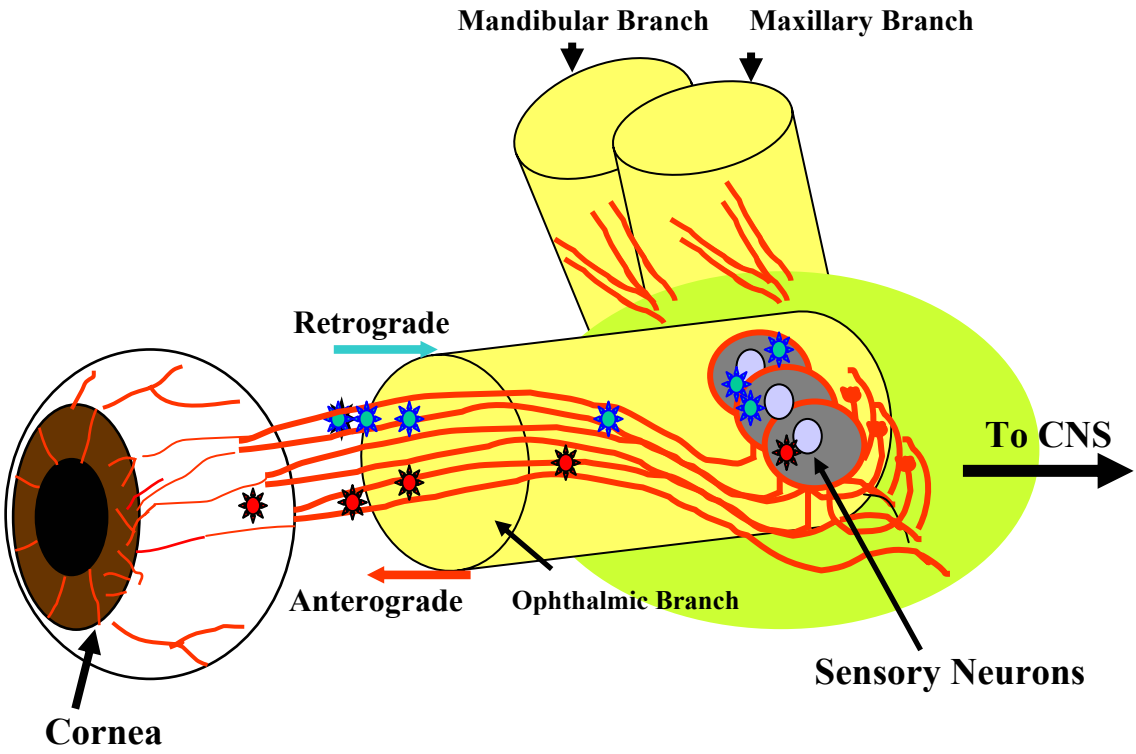

Figure 6: The murine experimental model of HSV-1 latency. *The cornea serves as the primary site of infection. Once the virus gains access to axonal termini innervating the cornea it travels retrograde (blue) and establishes latency in the neuronal cell bodies in the ophthalmic branch of the TG. Upon induced reactivation the virus emerges from latency and travels anterograde (red) and can cause herpetic disease at the primary site of the infection (cornea). In very few cases the virus can access the central nervous system (CNS) and cause encephalitis. Note that the TG is made up of three branches; mandibular, maxillary and ophthalmic. The mandibular and maxillary branches contain neurons that innervate the mouth and the face.*

1.5. HSV Infections

Documentation of herpetic diseases was first done as far back as in the Greek and Roman eras. But it was not until the early 1900s when the true infectious nature of the virus was discovered (37). In the 1930s investigators were baffled by a paradoxical finding, only those individuals who had neutralizing antibodies to HSV were the ones who showed herpetic disease. In the next few decades advances in medicine resolved some of the anomalous observations and by the 1970s HSV was reclassified into HSV-1, which was found to cause infections "above the waist" and HSV-2, which primarily caused infections "below the waist".

Infections caused by herpesviruses have become a considerable public health problem in the world. There are eight known human herpesviruses that share many infectious properties and have many genetic similarities. These include varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpsesviruses 6, 7 and 8. The two most related and genetically conserved herpesviruses are the HSV-1 and HSV2. This thesis will focus mainly on HSV-1 with emphasis on the primary and memory immune responses to HSV-1 infection during latency.

1.5.1. HSV Pathology and Pathogenesis

HSV predominantly infects mucosal sites such as the eye, oropharyngeal areas, and genital areas. HSV-1 is the primary cause of ocular and oropharyngeal infections and recent epidemiological data suggest that both HSV-1 and HSV-2 can cause genital herpes, although HSV-2 remains the major culprit (37,38). Given that HSV is an especially cytopathic virus, the results of a productive infection at mucosal and mucocutaneous sites are clinically unpleasant. HSV infection induces painful skin lesions as a result of cellular death and inflammation; there is

degradation of cellular plasma membrane leading to the formation of giant multinucleated cells, pustule and scabbing. In rare instances HSV can infect the CNS, leading to death or permanent brain

damage (39,40,37). The pathology after a recurrent infection as a result of a reactivation is milder presumably because of an enhanced immune response. HSV-1 infection of the eye leads to the destruction of corneal epithelium causing dendritic lesions. The lesions can be detected by topical treatment of the eye with fluorescien. The ocular dendritic lesions in the mouse cornea are strikingly similar to human corneal lesions (Figure 7)

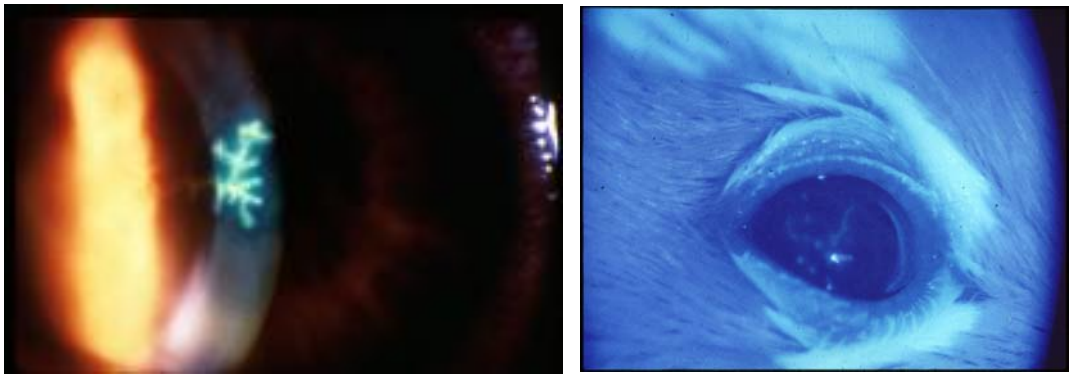

Figure 7: Dendritic lesions caused by HSV-1. *Topical treatment of fluoroscene on the eye reveals the dendritic lesions caused by HSV-1 infection of the eye as a result of epithelial cell damage. Notice the similarities in the lesion caused by the virus in humans (left) and mouse (right).*

The virus forms a latent infection in the ophthalmic branch of the trigeminal ganglion. In humans, the virus sporadically reactivates and can reinfect the eye, a condition called recurrent herpetic disease that can lead to herpes stromal keratitis (HSK). Repeated infection of the eye can lead to permanent corneal damage and in some cases total loss of vision.

HSV is a remarkably successful pathogen because it can hide in post mitotic neuronal cells where antiviral drugs pose no danger. Fortunately, HSV cannot survive out side living

cells; therefore the virus can only spread through close contact with mucosal surfaces or abraded skin. In most cases the virus spreads only to the ganglionic neurons that innervate the primary site of infection, but in special cases such as immuno-compromised and neonatal individuals, virus can spread to other branches of sensory ganglia, causing a disseminated infection (37,38).

1.5.2. HSV Epidemiology

There are many factors that contribute the spectrum of diseases caused by HSV such as age, geographical settings and socioeconomic status of individuals. Studies with cadavers have shown that about 18% of people up to the age of 20 have latent HSV-1 in the TG, and virtually 100% of cadavers aged 60 or older tested positive for latent HSV-1. In the United States, at least 50% of the population is currently infected with HSV-1, however the rates are declining (38). HSV-1 remains the leading cause of infectious blindness with at least 50,000 new or recurrent cases reported each year (40). It is estimated that 8.4/100,000 new cases of ocular HSV occur each year and 149/10,000 ocular HSV cases are prevalent in the population of United States (38). Ocular HSV infections include acute conjunctivitis, where 21% of all conjunctivitis cases in the US are caused by HSV infections. About 20-25% of all HSV ocular infections lead to stromal keratitis. In adults ocular HSV infection rarely leads to encephalitis or death, however in newborn and young children, ocular or oropharangeal HSV infection can be a cause of considerable morbidity and permanent brain impairment (38,37).

HSV-2 infections, which result in genital herpes, are also widespread. Unlike HSV-1, incidence of HSV-2 infections is steadily rising in developed countries. 22% of adults in the US are HSV-2 seropositive, with incidence considerably higher in African Americans (~45%) than in Caucasians (17.6%). Genital herpes is principally transmitted sexually in genital secretions,

and contact with infected mucosal surfaces (37,38). There are 500,000 new cases each year and prevalence of latent HSV-2 infection is estimated to be in the range of 40 to 60 million people in the US.

The high prevalence of HSV-1 and 2 infections in the world can be attributed to the chronic nature of the virus. An individual can be infectious even though he/she may be asymptomatic; viral shedding occurs in almost all latently infected individuals. Clinics have reported viral shedding in saliva, eye, and in genital areas in the absence of any herpetic lesion or disease, which makes preventing the transmission of HSV even harder.

Therefore, it is clear that HSV infections are increasing and becoming a menace to the public heath systems around the world. The problem is expected to get worse because of the increasing number of individuals that are immunocompromised as a result of human immunodeficiency virus (HIV) infections, transplant patients and patients suffering form autoimmune disorders who are on immunosuppressive drugs. Moreover, reactivation of HSV-1 increases with age and since the life expectancy of population in developed countries continues to increase the problem of HSV mediated diseases will worsen.

Management of primary and recurrent HSV infections is limited to the use of antiviral drugs such as acyclovir and gancyclovir. These drugs are effective only during productive infection, and are incapable of eliminating latent virus or preventing the virus from establishing latency (38,37,41). All attempts to devise an effective vaccine have been largely unsuccessful.

1.6. T Lymphocytes and Adaptive Immunity

During an invasion of a pathogen, T lymphocytes are an invaluable weapon employed by our immune system to encumber the ensuing assault to the body. There are two main classes of

T lymphocytes; T helper cells (Th or $CD4^+$ cells) that express the CD4 protein, and cytotoxic T lymphocytes (CTL or $CD8^+$ cells) that express the CD8 coreceptor.

$CD4^+$ T cells recognize foreign antigens presented by antigen presenting cells (APC) in the context of major histocompatibilty complex II (MHC II) molecules. $CD4^+$ T cells are principally helper cells that are important in augmenting the function of $CD8^+$ T cell mediated and humoral immunity, moreover recent studies have confirmed the importance of $CD4^+$ T cells in maintaining $CD8^+$ T cell memory (42-44) . There are two types of Th cells: Th1 and Th2. Generation of Th1 cells requires the presence of interleukin 12 (IL-12), which is mainly secreted by dendritic cells (DC) and natural killer cells (NK cells). Th1 cells are a major source of antiviral cytokines such as interferon γ (IFNγ) and tumor necrosis factor α (TNFα). Th1 cells assist in the generation of an effective cell mediated immunity by secreting growth factors such as IL-2, that are necessary for clonal expansion of $CD8^+$ T cells as well as enhancing the cytotoxic functions of $CD8^+$ T cells. $CD4^+$ T cells can activate DCs by providing costimulation by way of CD40-CD40 ligand (CD40L) interaction; this "licensing" makes DCs better antigen presenting cells for T lymphocytes (45). Th2 cells are generated in response to IL-4 secreted by DCs. Th2 cells activate B cells by secreting cytokines such as IL-4 and IL-5, as well as providing the CD40L to stimulate the CD40-CD40L pathway. IL-4 and CD40-CD40L interaction between B cells and Th2 cells is extremely important in inducing isotype switching and clonal expansion of B cells, which, leads to the differentiation of B cells into plasma cells. Plasma cells produce copious amounts of antibodies to combat extracellular pathogens. Many studies have demonstrated the importance of CD4 T cell responses in immunity to HSV infections (46,47,48) but for the sake of relevance and brevity this thesis will concentrate primarily on $CD8^+$ T cells.

1.6.1. Antigen Recognition and Processing

Major Histocompatibility Complex Class I:

Intracellular pathogens such as viruses and some bacteria, once inside a cell, remain hidden from the innate and humoral immune responses; therefore our body has evolved to devise a very efficient system of alerting $CD8^+$ T cells to the presence of intracellular pathogens. MHC class I molecules are expressed on virtually all cell types including neurons. MHC I molecules are made up of a 43-kDa heavy chain (α), and a 12-kDa light chain (β). The heavy chain consists of three immunoglobulin like domains: α1, α2 and α3, which non-covalently associate

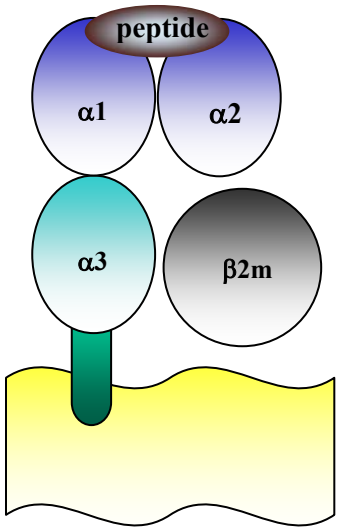

Figure 8: A structural schematic of the MHC I molecule. *The heavy chain contains the three α domains and spans the plasma membrane. β2m is the light chain and associates extensively with the three domains of the heavy chain. The peptide binds in the groove contained with in the α1 and α2 domains.*

with the light chain β2-microglobulin (β2m). The α3 domain spans the plasma membrane and has a binding site for the CD8 coreceptor (Figure 8). The heavy chain amino terminal domains

α1 and α2 form the peptide-binding groove, which accommodates peptides 8-9 residues in length (49).

Antigen Processing and Presentation:

MHC class I antigen processing and presentation (outlined in Figure 9) constitutes a complex series of events that lead to the presentation of foreign and endogenous peptides in the context of MHC I molecules to T cell receptors (TCRs). MHC class I light chains are synthesized in the endoplasmic reticulum (ER) where they remain in a partially folded state bound to chaperone protein calnexin (50). β2m binds to the α light chain further stabilizing the complex. Other chaperone proteins such as calreticulin and tapasin bind to the α-β2m complex bringing it in close proximity to the TAP subunits. Viral and other cytoplasmic proteins are processed and

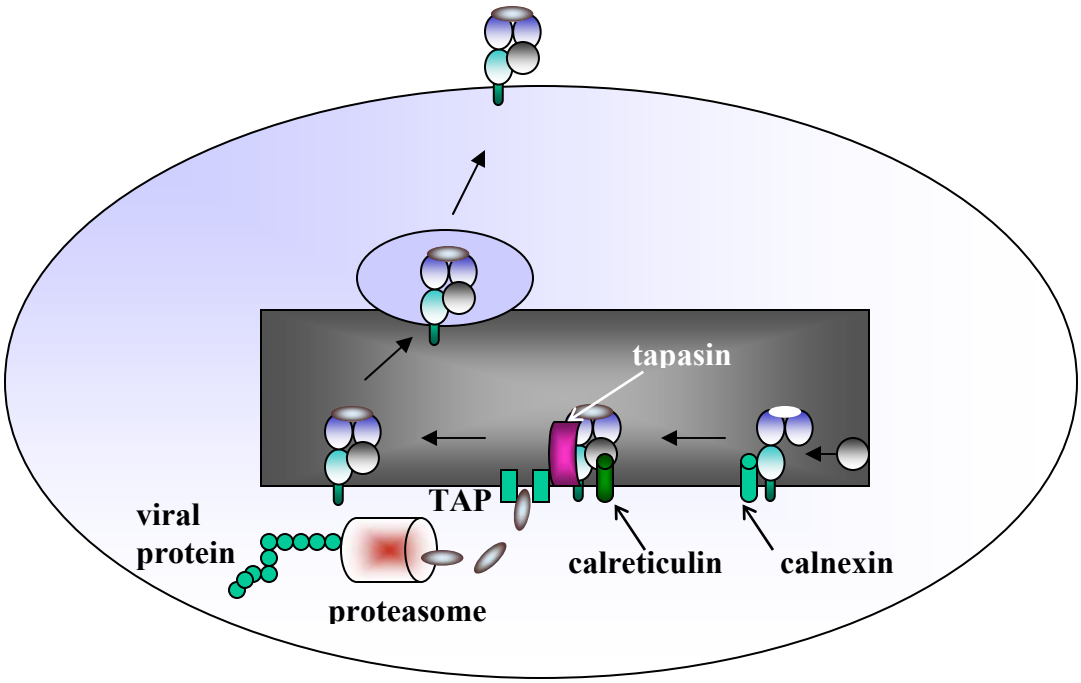

Figure 9: MHC class I antigen presentation pathway. *Steps in the antigen processing and presentation pathway are outlined in the text.*

degraded into small peptides by the action of several processing enzymes and proteosomes. Small 8-10mer peptides are transported inside the ER by the ATP dependent TAP transporters where they associate with the empty MHC I complexes. The properly folded MHC-peptide complexes are transported to the surface of the cell by the Golgi transport system (49,50). Extracellular proteins can also be taken up by antigen presenting cells and shuttled into the endogenous MHC class I pathway of antigen presentation, a process that is referred to as cross priming (51). Cross priming is particularly important in eliminating highly cytopathic viruses that can cause extensive cellular necrosis, thus releasing viral proteins into the extracellular space (52).

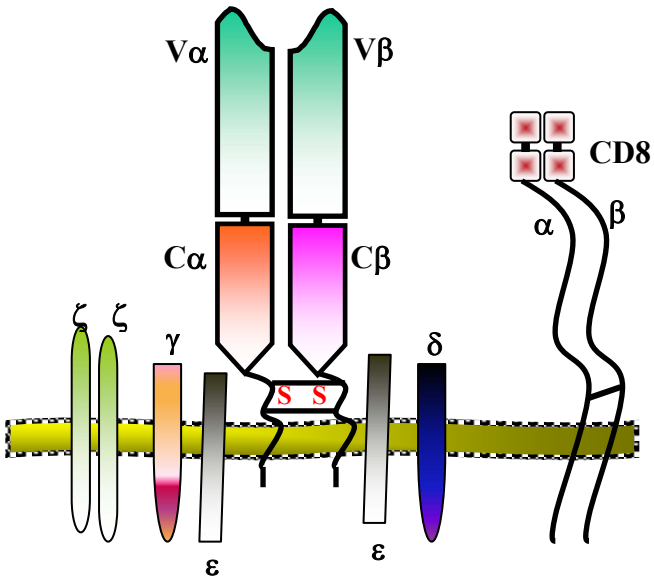

Figure 10: T Cell Receptor Complex. *The TCR and accessory molecules involved in intracellular signaling are shown. The CD8 coreceptor is a homodimer that binds to invariant chain of the MHC molecule stabilizing the TCR MHC-peptide interactions.*

T Cell Receptors:

Adaptive immune response to intracellular infectious agents is initiated by the recognition of antigen by T cell receptors expressed on $CD8^+$ and $CD4^+$ cells. The structure of the TCR (Figure10) is similar to the antigen-binding portion of an antibody molecule. The TCR is comprised of an α and a β chain, which are linked by a disulfide bond. Each chain consists of variable (Vα and Vβ) as well as constant (Cα and Cβ) regions. The membrane proximal constant regions have cytoplasmic extensions called the hinge regions that contain the disulfide bonds linking the two chains together. The variable regions of the α and β domains form the complementarity-determining regions (CDRs). These hypervariable regions confer to the TCR an astonishing ability to recognize numerous different antigens. There are three CDR loops that form the MHC-peptide binding sites on the TCR α and β variable regions. The CDR3 loop is a highly hypervariable region that contributes the most to the binding of peptide, while the CDR1 and CDR2 loops interact predominantly with the MHC (50,53). After the T cell recognizes the antigen being presented by MHC the TCRs on the surface of the T cells cluster together along with other adhesion molecules to form an organized complex between the antigen-presenting cell forming an immunological synapse (54). The central region of the organized synapse is called the supramolecular activation cluster or cSMAC, which contains the TCRs as well as costimulatory molecules (55). Astonishingly, even if a single TCR engages with a peptide-MHC molecule the events that lead to the formation of the cSMAC of T cells are initiated (56). The functional activation of T cells is carried out by intracellular signaling molecules and accessory proteins in the CD3 complex that include the δ, γ and ζ chains. The proteins of the CD3 complex contain immunoreceptor tyrosine-based activation motifs (ITAMs), which when

phosphorylated induce a cascade of signaling events leading to the expression of certain genes like c-fos, c-jun and NFκB activating the T cells to become effector cells (57).

1.6.2. $CD8^+$ T Cells

Effector Mechanisms:

$CD8^+$ T cells are an important arsenal in the fight against viral and bacterial infections. Recent advances in technology such as MHC tetramers, intracellular cytokine staining as well as improvement in imaging have underscored the important role(s) $CD8^+$ T cells play in immunosurveillance and subsequent eradication or control of viruses, bacteria and tumors (58,59). The two main effector mechanisms employed by $CD8^+$ T cells are: (a) perforin/granule and Fas mediated destruction of infected cells and (b) secretion of antiviral cytokines (60).

Cytolytic mechanisms of $CD8^+$ *T cells:*

There are two major pathways by which $CD8^+$ T cells kill their target cells; perforin/granule release and Fas-FasL. Several studies have shown the importance of perforin/granzyme mediated killing in eliminating or controlling viral infections (61-64). However, in some infection models lack of perforin did not hinder the adequate clearance of the pathogen (65). CTLs harbor lytic granules in their cytoplasm that store perforin and other proteases called granzymes. Granzyme A (GrA) and GrB are the most important molecules that are critical in inducing apoptosis in target cells. GrB is a potent apoptotic protease and can induce death in a target cell within a few minutes (66). However, GrA is less potent and can cause delayed apoptosis of virally infected cells (67,68,69,70). After a CTL interacts with an infected target cell the TCRs polarize towards the interface with the target cell forming an immunological synapse. Upon CTL activation the granules also polarize towards the target cell and are released within the synapse in an organized secretory domain (71,72).

The other mechanism by which CTLs cause target cell apoptosis is by the interaction of FasL with Fas expressing target cells. This interaction initiates a cascade of signaling events mediated by fas-associating protein with a death domain (FADD) that can lead to caspase activation and eventually the death of the target cell. This pathway, however, is more promiscuous and considerably slower than perforin mediated apoptosis (65,60).

Noncytolytic mechanisms:

In the tug of war for survival between the virus and its host, recent studies have demonstrated that noncytolytic mechanisms utilized by lymphocytes and other cells of the immune system are critical in order to tip the balance in favor of the host (32,73,74).

Cytokines secreted by myriad of cell types during innate as well as adaptive immune responses play a pivotal role in controlling or eliminating viral infections.

$CD8^+$ T cells primarily secrete interferon γ (IFNγ), tumor necrosis factor α (TNFα) and interleukin 2 (IL-2). IFNγ and TNFα have direct and indirect antiviral activities. IFNγ can positively regulate antigen processing and presentation by augmenting proteasome function and MHC gene expression. Apart from having direct aniviral activities both cytokines have many immunoregulatory functions by which they activate other immune cells such as NK cells, macrophages and T cells to become better effector cells. They can also enhance the production of chemokines and other cytokines. IFNγ is critical in skewing the immune response to a Th1 phenotype, which is necessary to combat intracellular pathogens (75,60,76).

1.6.3. $CD8^+$ T cell Memory

Formation of immunological memory is the inimitable feature of the adaptive immune response and is simply defined as a more efficient and vigorous immune response upon re-exposure to the same antigen. Despite recent progress, the mechanisms underlying the

formation, maintenance and impairment of T cell memory remain poorly understood. Unresolved issues relevant to T cell memory include: (a) the role of antigen, especially in persistent viral infections in memory formation and maintenance; (b) what are the factors that contribute to tissue-specific homing and retention of effector/memory cells; and (c) the mechanisms responsible for homeostasis of memory T cell pools in lymphoid and nonlymphoid tissues.

After a viral infection the $CD8^+$ T cell immune response in lymphoid organs follows a pattern characterized by: (i) initiation of the immune response; (ii) clonal expansion of viral specific $CD8^+$ T cells; (iii) a substantial reduction of effector $CD8^+$ T cells and finally; (iv) formation of a stable memory pool (77-80). There is a substantial increase in lymphoid antigen specific $CD8^+$ T cells during the expansion phase of an antiviral immune response. Seven days after lymphocytic choriomeningitis virus (LCMV), infection viral specific $CD8^+$ T cells account for more than 80% of all $CD8^+$ T cells present in the draining lymph nodes (78). Thirty days after LCMV infection the magnitude of memory $CD8^+$ T cell pool that formed depended on the initial burst size of $CD8^+$ T cell clonal expansion. Recent studies have shown that in response to a viral infection, memory T cells can be found in lymphoid organs as well as in the peripheral non-lymphoid tissues where they may reside permanently or recirculate throughout the body. A surprising observation made from these studies was that the trafficking of antigen specific effector T cells to non-lymphoid tissues was not antigen dependent. These findings challenge the existing concept that activated T cells only migrate to primary sites of infection and reside mainly in the lymphoid organs (81,82,83). However, the capacity of the $CD8^+$ T cells retained in non-lymphoid tissues to protect upon secondary challenge with pathogen is yet to be determined. These and other findings (84) have provided the basis for a premise that memory T cells can be

subdivided based on their function, anatomical location as well as surface expression of chemokine receptors and cell adhesion molecules. The "effector memory" cells primarily migrate to and reside in non-lymphoid tissues. Functionally these cells produce IFNγ and are cytotoxic directly ex-vivo even months after infection (81). Phenotypically, effector memory cells are $CCR7^{lo}$, $CD62^{lo}$, $CD44^{hi}$ (84,85). "Central memory" cells are both phenotypically and functionally distinct from effector memory cells; they are largely retained in lymphoid organs, and produce copious amounts of IL-2 but little IFNγ. Lymphoid memory cells lack granzyme B expression and, therefore, do not retain direct *ex vivo* cytotoxic activity (84,77,81). However, in persistent infections the concept of the existence of mutually exclusive central and effector memory T cells may be an over simplification (86).

Recent studies have clarified mechanisms that regulate formation and maintenance of memory but many questions remain unanswered. The majority of the models used to study memory utilize non-cytopathic viral infections that are readily cleared from body. However, our understanding of T cell memory in persistent and latent viral infections remains at a rudimentary level. In mice, LCMV specific memory $CD8^+$ T cells can survive without antigen persistence or interaction with cognate MHC molecules (87). This suggests that maintenance of memory T cells is antigen independent. However, some investigators argue that mere survival of memory T cells does not connote their functionality. These investigators make a distinction between memory, and "functional" memory. They postulate that efficient functional T cell memory is maintained only when low-level antigen persists in the body (88,89). This concept is supported by clinical data obtained from HIV patients that suggest a direct correlation between viral loads and the size of the memory T cell pool in the blood. A recent study showed that memory $CD4^+$ T cells can

be maintained in presence or absence of MHC interaction; however, the former were functionally defective (90).

Members of cytokine family that use the common cytokine receptor γ chain (γc) are extremely important in mediating the progress of $CD8^+$ T cell responses to pathogens. Of particular interest are IL-2, IL-15 and IL-7. IL-2R and IL-15R use the same β chain but unique α chains. All three cytokines use the common γ chain. There is evidence of differential expression of cytokine receptors during an anti-viral immune response; naïve T cells express IL-7Rα, IL-15Rα, and IL-2Rβ, but not IL-2Rα. Memory $CD8^+$ T cells express the β chain of the IL-2/IL-15R as well as IL-7Rα (91-93). Thus, it appears each cytokine may have a distinct function at different stages of a $CD8^+$ T cell response to viral infections. Several recent studies have suggested that IL-2 and IL-15 have contrasting roles in $CD8^+$ T cell memory formation and maintenance (94,95, 96, 97). There is conflicting data on the role of IL-2 at different phases of the $CD8^+$ T cell immune response. In vitro data indicate that IL-2 exposed $CD8^+$ T cells become efficient effector memory T cells that migrate primarily to the non-lymphoid organs, while IL-15 exposed $CD8^+$ T cells become central memory cells (98). Adoptive transfer experiments with IL-$2^{-/-}$ and IL-$2R^{-/-}$ CD8 T cells have shown the importance of IL-2 in the sustained expansion of $CD8^+$ T cells during the acute phase of VSV infection (97,99). There is considerable evidence that IL-15 is very important in the maintenance and formation of an adequate memory $CD8^+$ T cell pool (100,101,102,103). IL-15 is not only important in homeostatic proliferation of memory $CD44^{hi}$ $CD8^+$ T cells but also in survival of memory cells by inducing anti-apoptotic molecules such as BCL-2 and BCL-X (104). In spite of these recent advances, the role of IL-2 and IL-15 in modulating $CD8^+$ T cell responses to persistent or latent viral infection is not defined. Whether

or not the generation and maintenance of extra-lymphoid memory $CD8^+$ T cell population requires IL-15 and/or IL-2 has yet to be delineated.

1.7. Immune Responses to HSV-1 Infection

The nature of the immune response at primary sites (such as skin and cornea) and lymphoid organs during acute HSV-1 infection has been extensively investigated, yet little is known about the immunological events that occur in sensory ganglia (the site of latent infection). After HSV-1 infection the immune response in the cornea is characterized by a complex series of events. Although the cornea is considered an immune privilege site, HSV-1 infection in mice leads to an elevated leukocyte infiltration dominated by polymorphonuclear leukocytes (PMN), followed largely by $CD4^+$ T cells, which mediate the continued infiltration of PMNs by secreting Th1 cytokines IFNγ and IL-2. $CD4^+$ T cell mediated inflammation leads to severe corneal scarring and loss of vision by 21 days post infection (105-109,110,111). For the sake of relevance and brevity the subsequent discussion will primarily deal with acute and latent immune responses in lymphoid organs and the TG after HSV-1 corneal infection.

1.7.1. Immune Response to Acute HSV-1 Infection

Unlike LCMV and VSV, HSV infection does not represent a systemic, but rather a localized infection. Since HSV is notorious for its ability to block the MHC-I antigen processing pathway, it was believed that $CD4^+$ T cells alone were critical in clearing primary HSV infection in humans and in mice (112,113,114,115). However recent human studies by Lawrence Corey have demonstrated that $CD8^+$ T cells are equally important in clearance of HSV-1 from primary sites of infection (116-119).

A number of studies using a murine model of HSV-1 corneal and cutaneous infection have established the importance of T lymphocytes during acute infection (46,120). The immune

response to HSV-1 in the draining lymph nodes is characterized by a relatively meek $CD8^+$ T cell activation. 5 days after a cutaneous HSV infection, 5% of the lymphoid $CD8^+$ T cells are HSV specific, of those 90% recognize an epitope contained in the γ1 gene product gB ($gB_{498-505}$) and a smaller fraction recognize an epitope contained in early protein, ribonucleotide reductase ($RR1_{822-829}$) (121-123,124). The TCR usage of gB specific $CD8^+$ T cells is dominated by Vβ10 and a subdominant Vβ8 elements (125). Since HSV-1 causes a localized infection it does not infect the draining lymph nodes but studies with foot pad infection show a rapid transport of viral antigens presumably by langerhan cells and dendritic cells to the draining lymph nodes. gB specific $CD8^+$ T cells were activated with in hours of infection indicating rapid processing and presentation of gB epitope from the primary sites of infection to the LN by APCs (126,127). Furthermore, using fibroblasts as targets and gB specific $CD8^+$ T cells, Mueller et.al. demonstrated the rapid rate by which gB can be presented to activate $CD8^+$ T cells (128).

After corneal or foot pad infection HSV-1 is transported to the sensory ganglia within 24 hours, where it briefly replicates and establishes latency in neurons by 8 days PI. Histological studies indicate the initial leukocyte infiltrate in the TG includes macrophages, NK cells and γδ T cells, followed by $CD8^+$ and $CD4^+$ T cells, just before replicating virus is cleared (5-7 days PI) (129). Macrophages and γδ T cells are the dominant leukocytic population during the lytic phase of the infection (3-7 Days PI), and control early HSV-1 replication in the TG. Depletion of γδ T cells lead to a decrease in IFNγ as well as a dramatic increase in HSV-1 titers in the TG (130). Macrophage depletion significantly reduced the expression of IL-12, iNOS and TNFα, which resulted in increased HSV-1 replication and spread in the ophthalmic branch of the TG (131). Although depletion of γδ T cells or macrophage did not lead to an increase in duration of HSV-1 replication or affect its ability to establish latency , $CD8^+$ and $\alpha\beta^+$ T cell depletion

significantly augmented HSV-1 neurovirulence resulting in lethal viral encephalitis (131,130,132). The mechanisms by which $CD8^+$ T cells provide protection from encephalitis are not well understood. A study by Simmons' group demonstrated the importance of GrA in preventing viral spread in the TG during acute HSV-1 infection (133) suggesting a role of the cytolytic machinery. Other antiviral cytokines may also play a crucial role in preventing viral spread into the CNS.

1.7.2. Immune Response to Latent HSV-1 Infection

There is evidence for a constant host immune surveillance in latently infected TG. Extensive histological analysis by our laboratory of mouse TG after HSV-1 corneal infection revealed that $CD8^+$ T cells continued to accumulate until 14 days PI which is long after the establishment of latency. The $CD8^+$ T cells were predominantly in close apposition to the neuron cell bodies. These cells seemingly remained in the ganglion beyond 90 days PI (129). Support for the role of the immune system during latency was further strengthened by studies that demonstrated persistent cytokine and chemokine production in latently infected murine TGs. Expression of chemokines that are essential for attracting T cells such as regulated on activation, normal T cell expressed and secreted (RANTES), and T cell-derived cytokines (i.e. IFNγ and TNFα) can be readily detected in latently infected TGs as far beyond as 90 days PI (129,134,135,136,22).

2. STATEMENT OF THE PROBLEM

HSV infections around the world are increasing, providing a formidable challenge for public health systems. Apart from alarming rise in the incidence of herpetic disease because of immunosuppression and sexual transmission, herpes stromal keratitis continues to be a leading

cause of blindness by an infectious agent (38,137). Understanding the mechanisms involved in latency and reactivation has been a goal of many researchers for over half a century, but progress has been slow. Until recently, the role of the immune system in controlling HSV-1 latency was largely ignored due to the prevalent concept that HSV-1 latent infection in sensory ganglia represents a quiescent state wherein a lack of viral gene and MHC-I expression conceals the virus from the host immune system. As outlined in the previous section, findings in our and other laboratories challenge this concept. In addition, work by Simmons' group has clearly confirmed the expression of MHC-I molecules on neurons by histological and cytometric analysis (138,139). Eradicating latent HSV-1 from sensory neurons is not currently feasible and current vaccine strategies have been unsuccessful in preventing complete establishment of latency (140,141,142). Recent findings implicating the possible role of the host immune system during latent infection provide promise for immunologic approaches to intervention in recurrent herpetic disease. Therefore, the purpose of this study was to completely understand the adaptive immune response that occurs at the site of the latent infection, and to test the hypothesis that $CD8^+$ T cells are intricately involved in regulating viral latency and gene expression, with the goal of facilitating better vaccination strategies to combat herpetic infections. To this end we have attempted to dissect the $CD8^+$ T cell immune response in the TG during acute and latent infection by determining the antigenic specificity and their phenotypic and functional properties. We have investigated the ability of $CD8^+$ T cell subsets to prevent reactivation and regulate viral gene expression, as well as the CTL mechanisms essential for maintaining latency. We have also characterized the mechanisms that govern the retention of HSV-specific memory/effector $CD8^+$ T cells in latently infected TG.

3. MATERIALS AND METHODS

The contents of section 3 has been modified from articles (32,33,73). Copyright permission is on file with Kamal Khanna.

3.1. HSV-1 Infection

6–8-wk-old female C57BL/6, perforin KO (C57BL/6JPfp<tm15dz) (Jackson Laboratory, Bar Harbor, ME), BALB/C (Frederick Cancer Research Center, Frederick, MD), and IL-15 KO (Taconic Farms, Germantown, NY) mice were anesthetized by intramuscular injection of 2.0 mg of ketamine hydrochloride and 0.04 mg of xylazine (Pheonix Scientific; St Joseph, MO) in 0.2 ml of HBSS (Biowhittaker, Walkersville, MD). The RE strain of HSV-1 used in these studies was grown in Vero cells, and intact virions were purified on Percoll (Pharmacia LKB Biotechnology, Inc.Piscataway, NJ) gradients. Corneas of anesthetized mice were scarified 10 times in a crisscross fashion with a sterile 30- gauge needle, and the eyes were infected topically with 3 μl of RPMI (Biowhittaker) containing 10^5 PFU of HSV-1.

3.2. Single cell suspensions of TG

At various times after HSV-1 corneal infection, mice were perfused with PBS, and TGs were excised and treated with collagenase type I (800 units/ml; Sigma Chemical Co., St Louis, MO) for 1.5 h at 37°C. The TGs were dissociated into single cell suspension by trituration with a P200 pipet.

3.3. Reagents

Peptides $gB_{498-505}$ (SSIEFARL) and $RR1_{822-829}$ (QTFDFGRL) were purchased from Research Genetics (Invitrogen Corporation, Carlsbad, California). Peptide purity was confirmed to be > 95% by reverse phase HPLC analysis. Phycoerythrin (PE)-conjugated H-$2K^b$ tetramers complexed with $gB_{498-505}$ or $RR1_{822-829}$ were generated by the NIAID Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA).

PE-conjugated and purified H-$2K^b$:Ig dimer, PE-conjugated anti CD8α (clone 53-6.7); fluorescein isothiocyanate-conjugated anti CD44 (IM7), anti CD69 (H1.2F3), and anti IFNγ (XMG1.2); and Cy-Chrome™-conjugated anti CD45 (30-F11), were purchased from BD Pharmingen, San Diego, CA. Isotype control antibodies were purchased from BD Pharmingen; Caltag, Burlingame, CA; and Jackson ImmunoResearch (West Grove, PA).

3.4. Immunohistochemistry

Following perfusion with 1 X PBS, mice were sacrificed and TG were excised from latently infected mice and processed for frozen sectioning or whole organ staining. For frozen sectioning the TG were embedded in optimal cryogenic temperature (OCT) and immediately frozen in liquid nitrogen, and 10 μm serial sections were cut using a microtone apparatus at -20°C. Frozen TG sections were incubated with rat anti mouse CD8α (53-6.7, 1μg/ml, Pharmingen) overnight at 4°C. The sections were then washed 3 times with 1 X PBS for 5 min each, fixed with 2% paraformaldehyde for 30 min at 4°C, followed by the addition of Alexa Fluor® 546 goat anti rat IgG (2 μg/ml, Molecular Probes, Eugene,OR) diluted in 2% normal goat serum and PBS. The sections were incubated at 4°C for 3 h, washed, mounted with Immu-

Mount, Thermo Shandon, Pittsburgh, PA, and analyzed using a Bio-Rad 2000 confocal microscope (Bio-Rad, Richmond, VA)

Alternatively, whole TGs were stained using a modification of a previously described *in situ* tetramer staining procedure (143,144). Briefly, excised TG were washed in PBS and incubated overnight at 4°C in round bottom 96-well plates with PE conjugated MHC class I tetramers (2μg/ml) and rat anti mouse CD8α (53-6.7, 1μg/ml, Pharmingen) diluted in 2% normal goat serum and PBS. Next day tissues were washed 5 times for 15 min each with 1 X PBS, and fixed with 2% paraformaldehyde for 30 min at 4°C, and incubated overnight at 4°C with Alexa Fluor® 546 goat anti rat IgG (2 μg/ml, Molecular Probes) and rabbit anti-PE (1μg/ml, Biomeda, Hayword, CA) diluted in 2% normal goat serum and PBS. The tissues were then washed as described above and incubated for 6 h at 4°C with Alexa Fluor® 488 goat anti rabbit IgG (2 μg/ml, Molecular Probes). The tissues were washed similarly and mounted on slides using Immu-Mount. The stained whole mount tissues were analyzed by confocal microscopy. The image acquisition was done using Bio-Rad Laser Sharp® and image analysis was performed using Metamorph® software (Universal Imaging Corp, Downingtown, PA).

3.5. Flow Cytometry

Single cell suspensions of TG were pooled and passed through a 40-micron filter. Aliquots of TG cells (2 TG equivalents) were added to 5 ml polystyrene round bottom tubes (Becton Dickinson, Franklin Lakes, NJ). FC receptors on the cells were blocked by incubating the single cell suspension with anti-FC antibody for 15 min at 4°C and stained for the cell surface markers (0.5μg/ml) for 30 min at 4°C. The cells were then washed twice and fixed in 1% paraformaldehyde (PFA, Electron Microscopy Sciences, Fort Washington, PA) and stored at 4°C

until analyzed. The cells were acquired on a FACSCalibur or LSR II (Becton Dickinson) and analyzed using WinMDI data analysis software (J. Totter, The Scripps Clinic, La Jolla, CA). Single cell suspensions of TG were first stained with the anti CD8α antibody for 30 min. at 4°C followed by $gB_{498-505}$ or $RR1_{822-829}$ tetramers for 20 min. at room temperature. Cells were fixed with 1% PFA, and analyzed immediately by flow cytometry.

3.6. Intracellular IFNγ detection

Intracellular IFNγ stains were carried out using Cytofix/Cytoperm kit with Golgiplug™ (BD Pharmingen) in accordance with the manufacturer's instructions. Briefly, aliquots of pooled TG cells (2 TG equivalents) were incubated with 1 x 10^6 stimulator cells and Golgiplug™ for 6 hours at 37°C in flow tubes. Stimulator cells were the B6WT3 fibroblast cell line that were uninfected, infected with HSV-1 at an MOI of 5 for 6 hours, transfected to produce the $gB_{498-505}$ peptide (B6/T-350gB) (145), or transfected to produce the $RR1_{822-829}$ peptide (B6/T-350RR1) (123). After the 6 hrs incubation, the cells were stained for cell surface molecules and intracellular cytokines. Fc receptors were blocked by incubating the TG single cell suspension with 1μg FC block™ (BD Pharmingen) for 15 min at 4°C. The cells were then washed twice with flow wash buffer (1% FCS, 0.1% NaN3 in 1XPBS) and stained with fluorochrome-conjugated antibody for the cell surface proteins CD45, and CD8α for 30 min at 4°C. The cells were washed twice with flow wash buffer and resuspended in Cytofix/Cytoperm™ solution for 20 min at 4°C to fix and permeabilize. The cells were washed twice with 1X Perm/Wash™

buffer followed by incubation with 0.5µg/stain of flourochrome-conjugated anti-IFNγ diluted in 1X Perm/Wash™ buffer for 30 min at 4°C. The cells were washed twice with 1X Perm/Wash™ buffer followed immediately by flow cytometric analysis.

3.7. Preparation of TG cultures

3.7.1. TG cultures supplemented with gB-specific $CD8^+$ T cell clone

Single cell suspensions of pooled TGs were added to each well (1/2 TG/well) of a 48-well tissue culture plate, and the cells were cultured with DMEM (Biowittaker) containing 10% FCS (HyClone, Logan, UT), and 10 U/ml recombinant murine IL-2 (R&D Systems, Inc., Minneapolis, MN). Where indicated, the TG cells were depleted of $CD8^+$ T cells by immunomagnetic separation using anti-CD8-coated dynabeads® (Dynal ASA, Oslo, Norway).

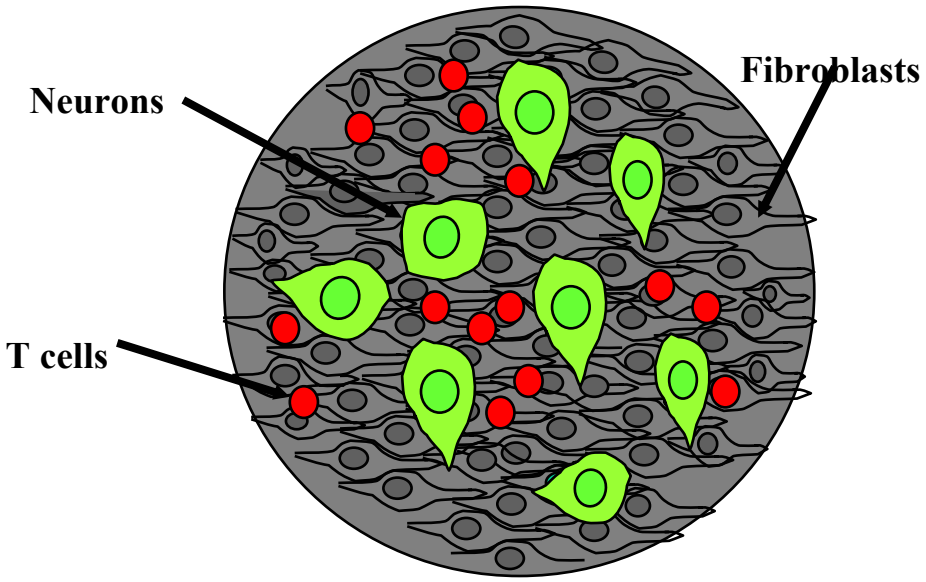

Figure 11: Schematic of a TG culture. *4 days after culture initiation a monolayer of fibroblasts forms (grey cells) and neurons (green) and T cells (red) can be found on top of the monolayer of fibroblasts. After a viral reactivation event, plaques can be observed when fibroblasts around the reactivation foci are destroyed forming an area of cellular clearance.*

Single cell suspension of pooled TGs were treated with anti-CD8α MAb-coated magnetic beads for 30 min at 4°C followed by seven rounds of magnetic separation using a high power magnet. The efficiency of $CD8^+$ T cell depletion was routinely greater than 98% as determined by flow cytometry. The $CD8^+$ T cell clone (2D5) specific for the HSV-1 $gB_{498-505}$ epitope was maintained as previously described (146). Briefly, 2D5 cells were maintained in complete Iscove's Modified Dulbecco's Medium (IMDM) (Biowhittaker) containing 10% FCS (HyClone) and $5x10^{-5}$ Molar beta-2-mercaptoethanol (β2-ME). Complete IMDM was further supplemented with 10% rat T-cell-Stim (Becton Dickinson). The 2D5 cells were maintained by 3 day stimulation and 3 day rest cycle, B6/T-350gB fibroblast cells were used as stimlators. For TG cultures 2D5 cells used after a 3-day rest period in which no stimulant was added. On the day of the experiment the 2D5 cells were removed from the wells using versene, washed and added to the TG cultures at various doses. Naïve $CD8^+$ T cells were obtained from the spleens of uninfected mice by magnetic activated cell sorting (MACS®, Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The naïve $CD8^+$ T cells were washed and added to the TG cultures.

3.7.2. TG cultures to determine IFNγ mediated protection

At 35 days after HSV-1 corneal infection, the ipsilateral TG was excised and treated with collagenase type 1 (800 U/ml; Sigma, St. Louis, Mo.) for 1.5 h at 37C and dispersed into single cells by triturating as previously described (32). The cells from multiple TG were pooled, and the neurons were counted. The equivalent number of cells from one TG (approximately 10,000 neurons) was added to each well of a 24-well tissue culture plate (catalog. no. 353047; Falcon), and the cells were incubated in culture medium consisting of Dulbecco's modified Eagle's medium, 10% fetal calf serum (HyClone, Logan, Utah), and recombinant murine IL-2 (10 U/ml;

R & D Systems, Inc., Minneapolis, Minn.). Some cultures received ACV (50 μg/ml; Glaxo Wellcome, Inc., Research Triangle Park, N.C.) during the first 4 days of incubation. This treatment was shown to be optimal in preliminary experiments. After 4 days, the ACV-containing medium was removed and the cultures were rinsed with culture medium twice, and then they were incubated for an additional 10 days in culture medium alone or culture medium supplemented with monoclonal antibody (mAb) to IFNγ (20 μg/ml, clone R4-6A2) or CD8α (100 pg/ml, clone 2.43) or with mouse recombinant IFNγ (rIFNγ) (1,000 World Health Organization units/ml; R & D Systems, Inc.). Where indicated, TG cell suspensions were depleted of $CD8^+$ T cells or $CD45^+$ cells by treatment with anti CD8α MAb or anti-CD45 MAb-coated magnetic beads (6 beads/cell, Dyna- beads; Dynal) followed by seven rounds of magnetic separation. The resulting TG cell suspensions contained less than 1% of the depleted cell population as assessed by immunofluorescent staining with PE-conjugated anti-CD8α mMb (clone 53-6.7; PharMingen) or biotinylated anti-CD45 mAb (clone m1/9.3.4.HL2) and FITC-conjugated streptavidin followed by flow cytometric analysis. Some neurons and supporting cells were lost from the TG cell suspensions during immunomagnetic depletion, but adjustments were made so that the depleted and nondepleted TG cultures contained comparable numbers of neurons. Preliminary studies established that mock depletion with uncoated beads did not influence assay results, so this control was not included in the studies described herein.

3.8. IFNγ titration in TG cultures

At various times after culture initiation 50μl of medium were removed from each culture and tested for IFNγ content using a standard-enzyme linked immunosorbent assay (ELISA). Plates were coated with coating buffer (0.1M Na_2HPO_4; dissolve 1.42 gram of sodium phosphate

(Sigma) into 80 ml H_2O, pH 9.0) containing anti-IFNγ antibody (clone R4-6A2) at 2 μg/ml and kept overnight at 4°C. The next day plates are washed 0.05%Tween-20/PBS four times and blocked with 1%BSA/PBS for 1hr at room temperature. The plates are washed as before and the diluted samples are added in duplicates into each well and incubated for 2 hr at room temperature. The supernatant was decanted and plates were washed 6 times before the addition of biotin-conjugated anti-IFNγ detection antibody (R&D; 1μg/ml) for 1hr at room temperature. The plates were washed 6 times and incubated with Avidin-horseradish peroxidase (Pharmingen, SA-HRP; 1:1000) for 30 min at room temperature. The plates were again washed 8 times and the TMB developing solution was added to each well and allowed to stand for 15 min at room temperature, the reaction was stopped by the addition of 100μl per well of 1N H_2SO_4. The optical density of the wells was detected by a standard ELISA plate reader. The concentration of the protein was measured by comparing OD readings to the standard curve generated by serial dilution of recombinant IFNγ standards**.** The sensitivity of the assay was 15.6 pg/ml.

3.9. Monitoring of TG cultures for HSV-1 reactivation from latency

Within 3 days of TG culture initiation, the neurons could be observed on a monolayer of fibroblast-like cells. The cultures were monitored using three criteria to define HSV-1 reactivation from latency. A culture was considered positive for HSV-1 reactivation if microscopic examination revealed the presence of viral cytopathic effects (CPE), or if infectious virus was detected in serial samples of culture supernatant (50μl/culture) using a standard viral plaque assay on monolayers of Vero cells. After each sampling, the medium was replaced with an equal volume of fresh medium of the same composition (32). To further confirm monitoring accuracy, selected cultures that were negative for reactivation based on the above criteria were

tested for the presence of HSV-1 γ2 gene (glycoprotein H) transcripts by RT-PCR. Cultures that lacked viral cytopathic effect and infectious virus were uniformly negative for gH transcripts.

3.10. Reverse transcription PCR

Ten days after initiation of TG cultures, cells were scraped off the surface of the wells and total RNA was extracted from the cells using RNeasy™ columns (Quiagen, Valencia, CA) according to manufacturer's instructions. Total RNA was treated with DNase-1 using the DNA-free™ kit according to manufacturer's instructions (Ambion INC, Austin, TX). RT-PCR was performed using the GeneAmp® Gold RNA PCR core kit (Applied Biosystems, Foster City, CA). The cDNA encoding glycoprotein H and house keeping gene hypoxanthineguanine phosphoribosyl transferase (HPRT) were amplified and by using the following primer pairs through 40 cycles:

The following primers were used: gH, left (TTT ATG GTT CGT GGG GGT TA); right (GGT CTT CGG GAT GTA AAG CA) and HPRT, left (CTG GTG AAA AGG ACC TCT CG), and right (TGA AGT ACT CAT TAT AGT CAA GGG CA). The reaction conditions for the RT were: 25°C for 10 min followed by 42°C for 12 min and for the PCR reaction: (i) Initial activation of AmpliTaq gold 95°C for 4min, (ii) annealing and extension at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min and (iii) final extension at 72°C for 10 mins.

3.11. In vivo Treatment of anti-IL2

At various times post infection IL-15 $^{-/-}$ or C57BL/6 mice were treated IP with 1mg of anti-IL-2 mAb (clone S4B6-1) diluted in 500 µl of endotoxin free PBS (Biowhittaker). Anti-IL-2 antibody was checked for endotoxin levels before use in in vivo studies.

3.12. BrdU Stain

WT and IL15-KO mice were administered 1mg of anti-IL2 or PBS intraperitoneally (IP) at D6 PI. All mice either received 1mg of BrdU (BD, Pharmingen) solution IP at D6 and at D7 PI or equal volume of PBS (negative control). The mice were sacrificed at D8 PI, the TG and the draining submandibular lymph nodes were extracted and a single cell suspension was obtained for each individual LN and TG for each mouse in the four experimental groups. Each TG single cell suspension and 2 x 10^6 LN cells was washed and the Fc receptors were blocked. Cell surface proteins CD8α, CD4, CD45, and gB specific TCR was stained by incubating the single cell suspensions of TG and LNs with fluorochrome-conjugated antibodies and gB-MHC I dimer for 1 hr at 4°C. The cells were washed twice with flow wash buffer and resuspended in Cytofix/Cytoperm™ solution (BD Pharmingen) for 20 min at 4°C to fix and permeabilize. The cells were washed once with 1X Perm/Wash™ buffer (BD Pharmingen) and incubated with Cytoperm Plus™ buffer for 10 min at 4°C. The cells were washed once with 1X Perm/Wash™ and refixed for 5 min at 4°C with Cytofix/Cytoperm™ solution. The cells were washed and resuspended in 100µl of DNase solution (300µg/ml) and incubated for 1hr at 37°C followed by incubation with 50µl of 1X Perm/Wash™ buffer containing fluorescent anti-BrdU and isotype control for 20 min at room temperature and analyzed immediately using a flow cytometer (BD, BSR II)

3.13. Annexin-V Apoptosis Detection

WT and IL15-KO mice were administered 1mg of anti-IL2 or PBS intraperitoneally (IP) at D6 PI. The mice were sacrificed at D8 PI, the TG and the draining submandibular lymph nodes were extracted and a single cell suspension was obtained for each individual LN and TG

for each mouse in the four experimental groups. Each TG single cell suspension and 2 x 10^6 LN cells was washed and the Fc receptors were blocked. Cell surface proteins CD8α, CD4, CD45, and gB specific TCR was stained by incubating the single cell suspensions of TG and LNs with fluorochrome-conjugated antibodies and gB-MHC I dimer for 1 hr at 4°C were washed twice with cold PBS and then resuspended in 100µl of 1X binding buffer (BD Pharmingen) containing FITC-Annexin-V (BD Pharmingen) at a 1:20 dilution for 15 min at room temperature. 200µl of 1X binding buffer was added to each tube before immediate flow cytometric analysis.

4. RESULTS

The contents of sections 4.1 - 4.5.2 have been modified from articles (33,73). Copyright (2004), with permission from "Elevier" and "The American Society of Microbilogy". Copyright permission is on file with Kamal Khanna.

4.1. Immune Infiltration into the Trigeminal Ganglion

Background and Rationale:

Early studies with rabbit ocular infection models (147) first demonstrated the infiltration of T lymphocytes in the TG after corneal infection. Moreover the presence of cytokine transcripts (136) in the TG long after the establishment of latency indicated a continued immune response in the peripheral nervous system even though the virus was allegedly veiled from the immune system. These observations lead our laboratory to perform an in-depth histologic study of the TG after HSV-1 corneal infection in AJ mice. $CD8^+$ T cells invade the TG around 7 days after HSV-1 corneal infection, reach maximum density around 14 days post infection (p.i.), and are then retained in significant numbers seemingly for the life of the animal (36,22), therefore we employed flow cytometry to determine the kinetics and the phenotype of the $CD8^+$ T cell immune response in the TG during acute and latent phases after HSV-1 corneal infection.

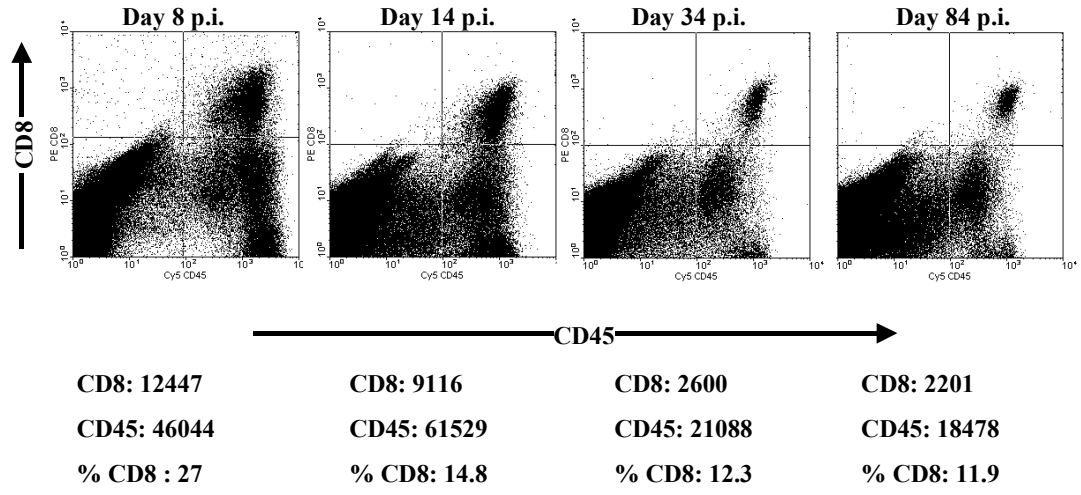

Figure 12: Infiltration of CD8$^+$ T cells in the TG after HSV-1 corneal infection*. Single-cell suspensions of TG obtained from mice 8, 14, 34, and 84 days after HSV-1 corneal infection were simultaneously stained for CD45 and CD8. For each reaction the equivalent number of cells from 2 TGs were stained and a total of 5 x 10^5 events were collected. Forward and side scatter gates were set to encompass the CD45 population, and the frequency of CD8$^+$ T cells was expressed as a percentage of CD45$^+$ cells (% CD8). These data are representative of 2 - 4 independent experiments.*

Using flow cytometric analysis, we quantified CD8$^+$ T cells and CD45$^+$ cells (bone marrow-derived infiltrating cells) in ipsilateral (infected) and contralateral (uninfected) TG single-cell suspensions obtained at various times after uniocular HSV-1 corneal infection of C57BL/6 mice. The highest density of CD8$^+$ T cells was present in the ipsilateral ganglion 8 days p.i., the population declined through day 34 p.i., and then a constant pool of CD8$^+$ T cells was maintained at least through day 84 p.i (Figure 12). In contrast, fewer than 100 CD8$^+$ T cells were detected in the contralateral (uninfected) TG (not shown). Recent reports have indicated that T lymphocytes migrate to many nonlymphoid tissues in response to a viral infection irrespective of the presence of antigen therefore we performed histological analysis of mouse TG after corneal infection to determine the distribution of CD8$^+$ T cells within the three branches of

the ganglion.Within the ipsilateral TG, $CD8^+$ T cells were concentrated within the ophthalmic branch containing neurons that innervate the cornea (Figure 13). The $CD8^+$ T cells appear to surround the neurons and are in close apposition to the neuronal cell bodies only in the ophthalmic branch of the ganglion, which contains neurons that innervate the cornea. HSV-1 uniformly established a latent infection in the TG neurons by 14 days p.i. as demonstrated by a lack of replicating virus in extracts of freshly isolated TG obtained 14 and 34 days p.i. (data not shown). Thus, $CD8^+$ T cells are selectively retained in infected regions of the TG long after viral latency is established.

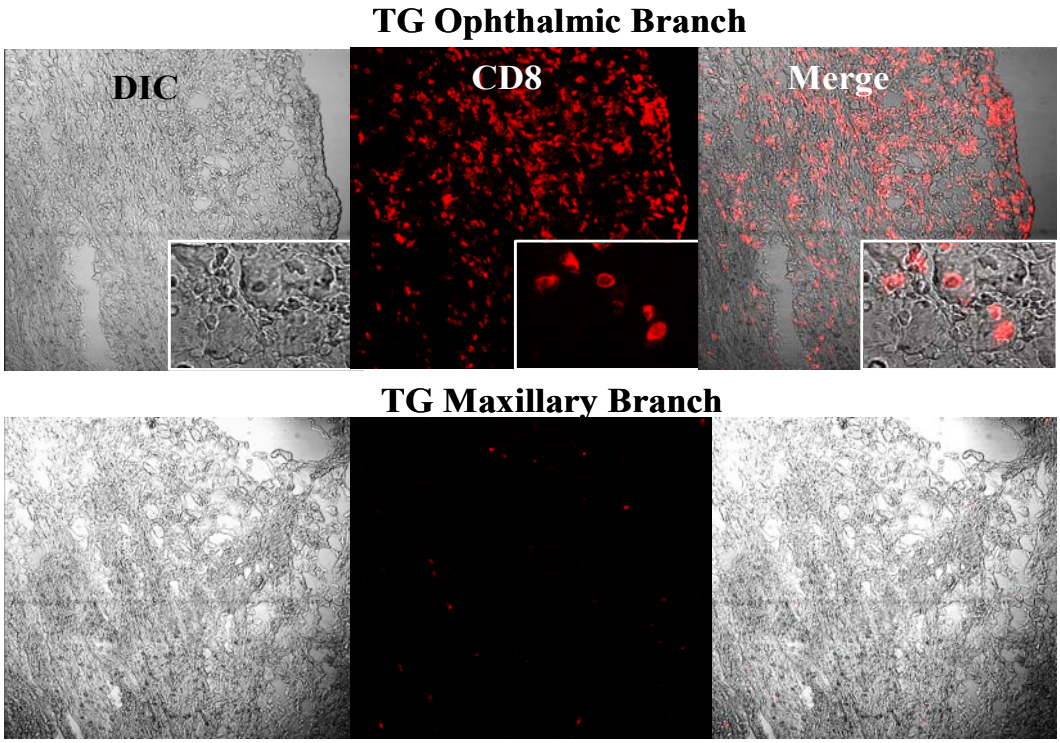

Figure 13: Selective retention of $CD8^+$ T cells in latently infectecd tissues. *TG were excised 34 days after HSV-1 corneal infection, and frozen sections were stained for CD8. Representative fields from the ophthalmic (A) and maxillary (B) branches of the TG are shown. Superimposed fluorescence and DIC images show preferential accumulation of $CD8^+$ T cells (red) among the neuronal cell bodies in the ophthalmic branch of the latently infected TG.*

4.1.1. Activation Phenotype of $CD8^+$ T cells in the TG

Single-cell suspensions of TG obtained from C57BL/6 mice at various times after uniocular HSV-1 corneal infection were simultaneously stained for CD45, CD8, and for either the CD69 (early activation) or CD44 (activation/memory) markers (Figure 14). In the ipsilateral TG, CD44 was consistently expressed on $CD8^+$ T cells from day 8 through day 84 p.i. (Figure 14A). In contrast, the percentage of $CD8^+$ T cells that expressed CD69 was very low at 8 days p.i., increased through day 34 p.i., and then remained constant through day 84 p.i. (Figure 14B). The $CD8^+$ T cells in the TG also showed a shift to elevated expression of CD8 between day 8 and day 34 PI (Figure 14C). These findings demonstrate that $CD8^+$ T cells were activated in the TG after viral latency was established and the TG $CD8^+$ T cell population exhibits a shift to a more activated phenotype during the course of latency.

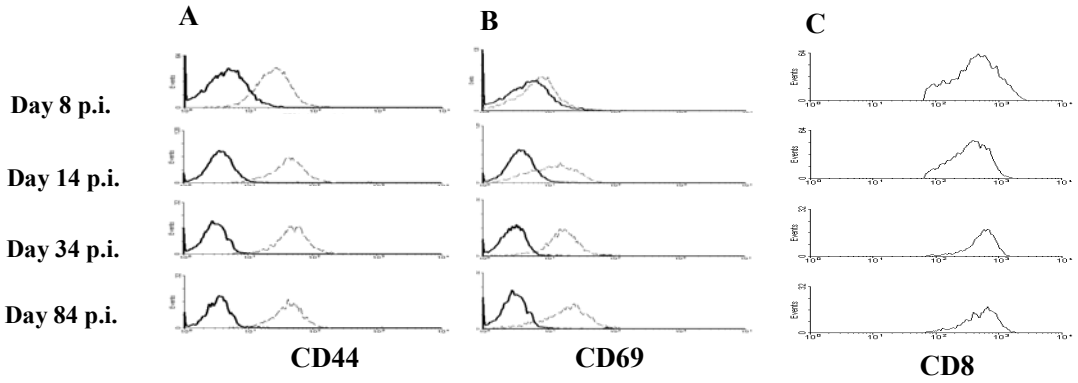

Figure 14: Activation phenotype of $CD8^+$ T cells present in the TG. *Single cell suspensions of TG were analyzed for expression of: (A) CD44, (B) CD69, (C) CD8 (dashed lines), or isotype control (dark line). Following flow cytometric analysis, the forward angle and side scatter gates were set on the $CD45^+$ population. Backgating on the $CD8^+$ population determined the proportion of $CD8^+$ T cells that expressed the activation markers. These data are representative of 2 - 4 independent experiments.*

4.2. Antigen Specificity of $CD8^+$ T cells in the TG

Backgound and Rationale:

Previous studies have shown that the CTL response to HSV-1 infection in the lymph nodes consists of T cells specific for the immunodominant gB epitope (glycoprotein B) and for the subdominant epitope RR1 (ribonucleotide reductase) (121,123,141,146). However, we have shown that in the TG IE protein ICP4 and early protein ICP8 are expressed in a fraction of the neurons that harbor latent virus *in vitro* (32), and other studies have demonstrated the presence of IE transcripts in latently infected TGs (30). Therefore we hypothesize that the differences in antigen presentation and the state of viral replication between the primary sites and the peripheral nervous system will influence the phenotype of the $CD8^+$ T cells that infiltrate and remain in the TG. In addition, the antigen specificity and immunodominant determinants of the $CD8^+$ T cells may vary at different times PI. The state of latency at early time points like day 14 may be different from later time points such as day 30 or 60 because of factors like differential MHC class I expression on neurons early vs. late PI (138,139)**.** Data from our laboratory shows that at 14 days PI the endogenous $CD8^+$ T cells can protect sensory neurons from reactivation in vitro but at day 34 exogenous $CD8^+$ T cells are required (32). Other studies have provided evidence for epitope shifting in a neurotropic persistent viral infection. They showed that the majority of the $CD8^+$ T cells that infiltrate the CNS upon infection are cytolytic and are specific for a certain epitope but at later time points after infection there is a shift in the epitope specificity of the T cells along with the loss of cytolytic function (148). We therefore hypothesize that the nature of the $CD8^+$ T cell response may be quite dissimilar during the lytic (day 5-7), early latent infection (day 14) and at later time points during latency (day 30 and 60) in the trigeminal ganglion. Using tetramers and intracellular cytokine stain we identified the antigen specificity of the $CD8^+$ T cells and the nature of immunodominance at various times after corneal infection in the TG.

Results:

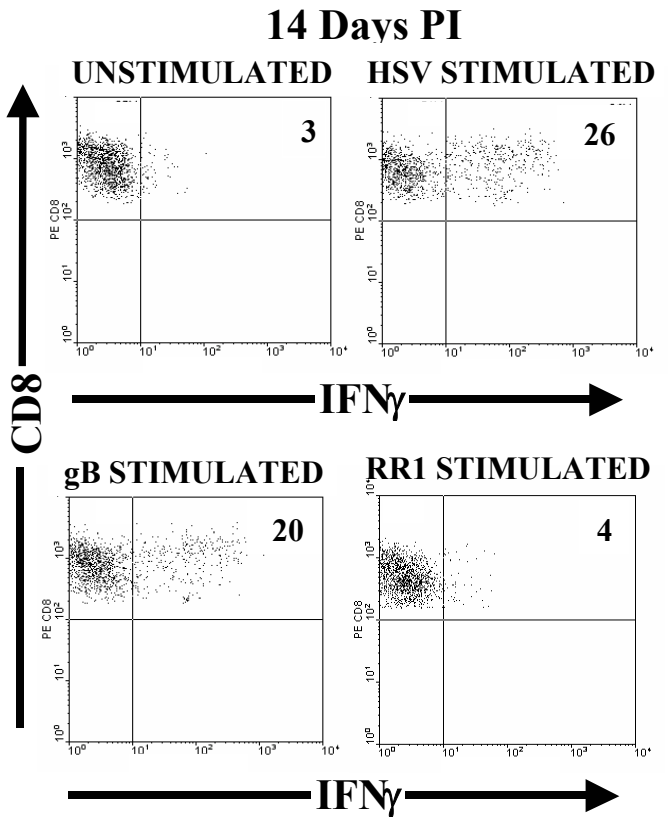

Figure 15: CD8$^+$ T cells retained in the TG during latency are specific for HSV-1 protein gB and produce IFNγ directly ex vivo. *Single cell suspensions of TG obtained 14 days after corneal infection were incubated with the indicated stimulator cells for 6 hours in the presence of GolgiPlug™ and stained for intracellular IFNγ. The stimulator cells were HSV infected (HSV stimulated), $gB_{498\text{-}505}$ peptide pulsed (gB stimulated), RR1 pulsed (RR1 stimulated) or uninfected (Unstimulated).*

The antigen specificity and the ability of the CD8$^+$ T cells to secrete IFNγ in the HSV-1 latently infected C57BL/6 TG was determined by stimulating the TG-derived lymphocytes for 6 hours with HSV-1 infected or $gB_{498\text{-}505}$ transfected stimulator cells, and then staining simultaneously for CD45, CD8, and intracellular IFN-γ. In TG obtained 14 days PI, a similar percentage of CD8$^+$ T cells produced IFN-γ when stimulated with HSV-1 infected (21.5 ± 3.3%) or $gB_{498\text{-}505}$ peptide-pulsed (20.0 ± 0%) stimulator cells (Figure 15). Thus, it appears that most

HSV-specific $CD8^+$ T cells in the TG 14 days p.i. are specific for the immunodominant $gB_{498-505}$ peptide, but the majority of these are incapable of producing IFN-γ when stimulated.

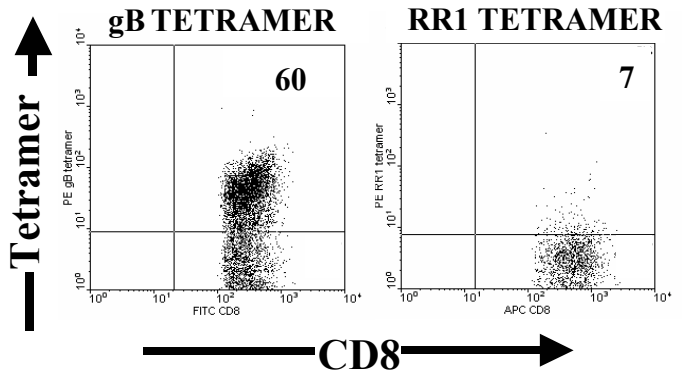

Figure 16: Antigen specificity of $CD8^+$ T cells in the TG. *Single cell suspensions of TG obtained at 14 days PI were stained with an anti CD8 mAb, anti CD45 mAb, and either $gB_{498-505}/K^b$ or $RR1_{822-829}/K^b$ tetramers. A total of 5 x 10^5 events were collected. The dot plots represent the CD8 gated population. These data are representative of 2 - 4 experiments.*

Alternatively, antigen specificity of $CD8^+$ T cells was also determined by staining TG-derived lymphocytes with tetramers containing the immunodominant $gB_{498-505}$ or subdominant ribonucleotide reductase 1 ($RR1_{822-829}$) epitopes. Surprisingly, a much higher percentage of $CD8^+$ T cells reacted with the gB tetramer (58.0 ± 2.0%) compared to the ability of these cells to secrete IFNγ directly ex vivo (comapare figure 16 and 15). $CD8^+$ T cells reactive to a subdominant epitope on RR1 were low to undetectable by both tetramer staining and IFN-γ production (Figure 16 and 15). Therefore there is a strong skewing towards one immunodominant viral determinant, gB, the majority of the $CD8^+$ T cells in the TG by 14 days PI are specific for the late viral protein glycoprotein B.

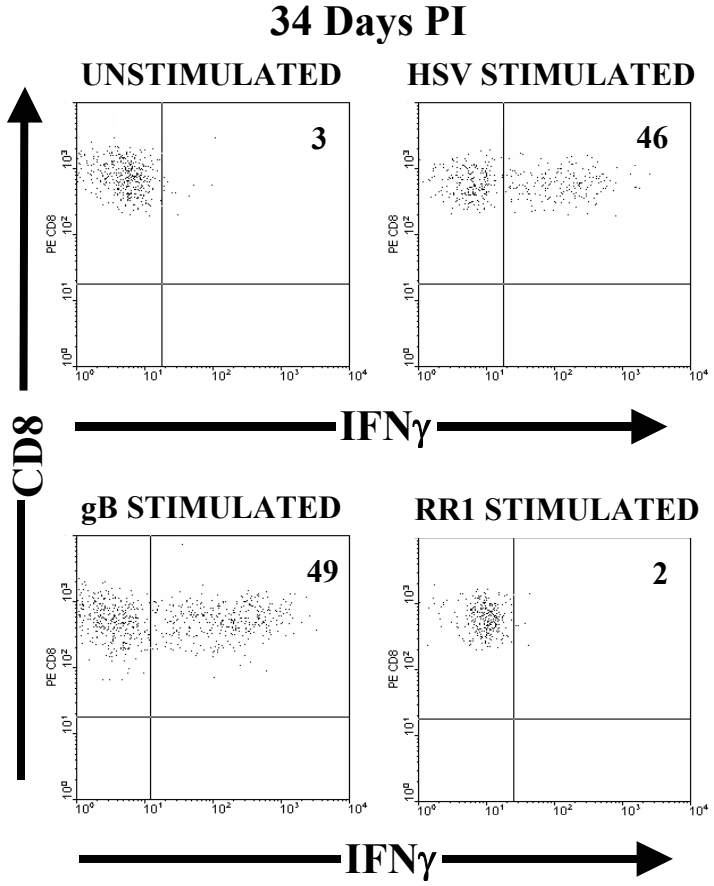

Figure 17: CD8$^+$ T cells retained in the TG at 34 days PI during latency can secrete IFNγ directly ex vivo. *Single cell suspensions of TG obtained 34 days after corneal infection were incubated with the indicated stimulator cells for 6 hours in the presence of GolgiPlug™ and stained for intracellular IFNγ. The stimulator cells were HSV infected (HSV stimulated), $gB_{498-505}$ peptide pulsed (gB stimulated), RR1 pulsed (RR1 stimulated) or uninfected (Unstimulated).*

At 34 days p.i. (Figure 17) there was a similar proportion of CD8$^+$ T cells in the TG that produced IFN-γ in response to HSV-1 infected (43.0 ± 3.5%) and $gB_{498-505}$ peptide-pulsed (47.0 ± 3.0%) stimulator cells. However, the proportion of CD8$^+$ T cells that produced IFN-γ in response to either stimulation was significantly higher at 34 days than at 14 days p.i. (compare Figure. 17 and 15).

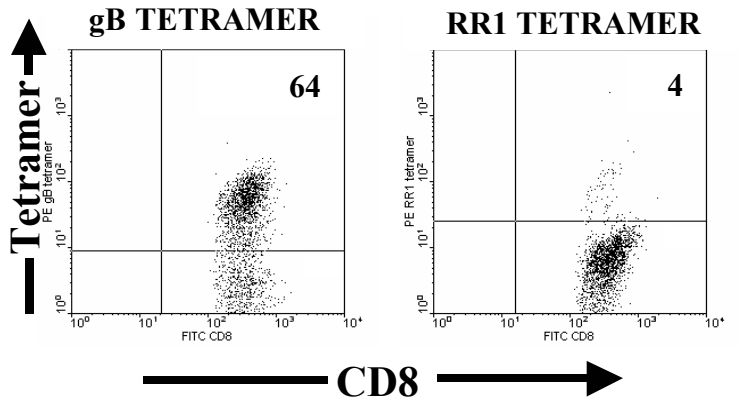

Figure 18: Antigen specificity of CD8$^+$ T cells in the TG at 34 days PI. *Single cell suspensions of TG obtained at 34 days PI were stained with an anti CD8 mAb, anti CD45 mAb, and either $gB_{498\text{-}505}/K^b$ or $RR1_{822\text{-}829}/K^b$ tetramers. A total of 5 x 10^5 events were collected. The dot plots represent the CD8 gated population. These data are representative of 2 - 4 experiments.*

The proportion of CD8$^+$ T cells in the day 34 TG (Figure 18) that recognized the $gB_{498\text{-}505}$ tetramer (62.0 ± 2.0%) was similar to that observed in day 14 TG. Thus, in the latently infected TG, there is selective enrichment of gB-specific CD8$^+$ T cells capable of producing IFN-γ, however there is no indication of an epitope shift between early vs. late time points during latency

Simultaneous in situ tetramer and immunofluorescence staining of whole TG obtained 34 days after HSV-1 corneal infection demonstrated selective localization of $gB_{498\text{-}505}$ tetramer positive CD8$^+$ T cells within the ophthalmic branch of the TG (Figure 19)

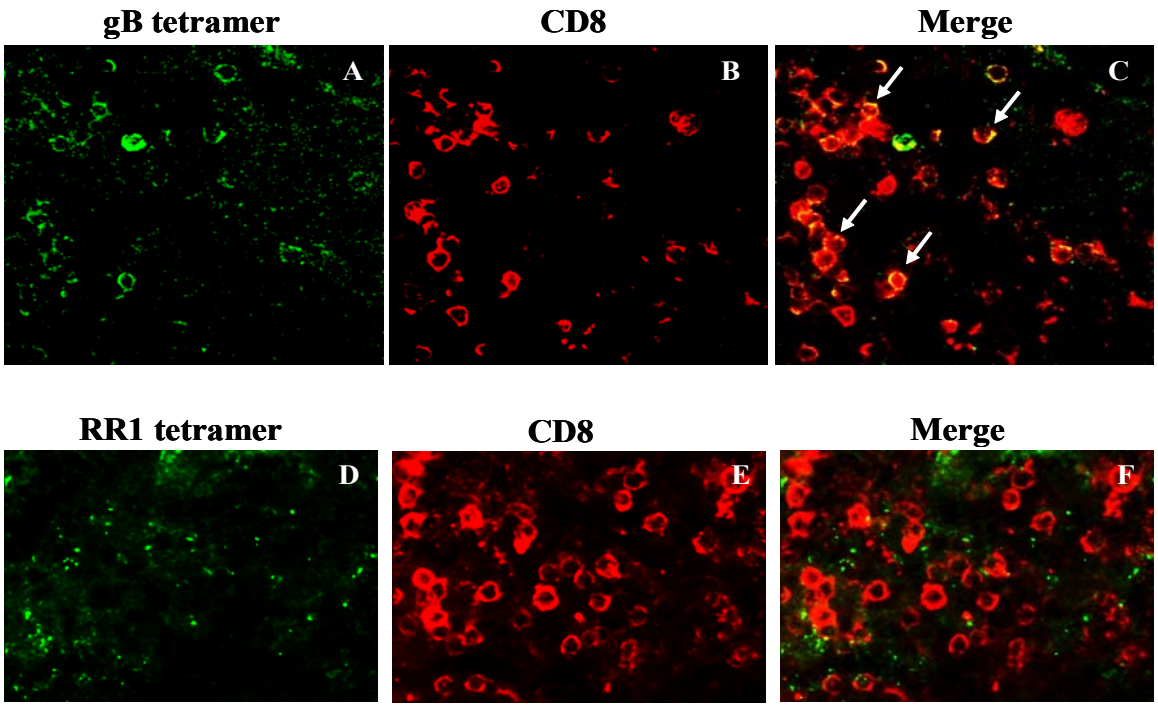

Figure 19: In situ tetramer stain of latently infected TG. *TG were excised and $gB_{498-505}$-specific $CD8^+$ T cells were identified by simultaneously staining the whole tissue with MHC class I tetramers containing the $gB_{498-505}$ epitope (green) and CD8 (red). The tissues were examined by confocal microscopy, and presented as a merged image of a Z-series. Areas of overlap between the CD8 and tetramer-bound TCR appear yellow. (A-C) The majority of $CD8^+$ T cells that localized to the area of neuron cell bodies in the ophthalmic branch of the TG were tetramer positive. RR1 tetramer was used as negative control. (D-F) Note the lack of RR1 tetramer staining on $CD8^+$ T cells in the ganglion.*

The majority of the $CD8^+$ T cells were observed to be $gB_{498-505}$ tetramer positive, and these cells were localized to the area of the ophthalmic branch containing neuron cell bodies. No RR1 tetramer positive cells were observed (Figure 19), which agreed with the flow data, and served as a negative control for tetramer staining. In most $gB_{498-505}$ tetramer positive cells, tetramer staining was polarized and appeared patchy or ring-shaped, suggestive of immunologic synapse formation. The ring-shaped staining pattern might reflect an immature synapse, or the fact that the tetramer can only bind to TCR that are not engaged by the epitope on the target cell

or that can be competed off the cell-bound epitope by the tetramer. Thus, tetramer competition for TCR binding in the central region of the synapse might be more demanding than that at the periphery of the synapse, resulting in a ring of staining around the synapse.

In many of the $gB_{498\text{-}505}$ tetramer positive $CD8^+$ T cells, the TCR was polarized to the area of the cell that appeared to be in direct contact with a neuron (Figure 20), consistent with the notion that $gB_{498\text{-}505}$ tetramer positive $CD8^+$ T cells can directly monitor viral gene activity in latently infected neurons.

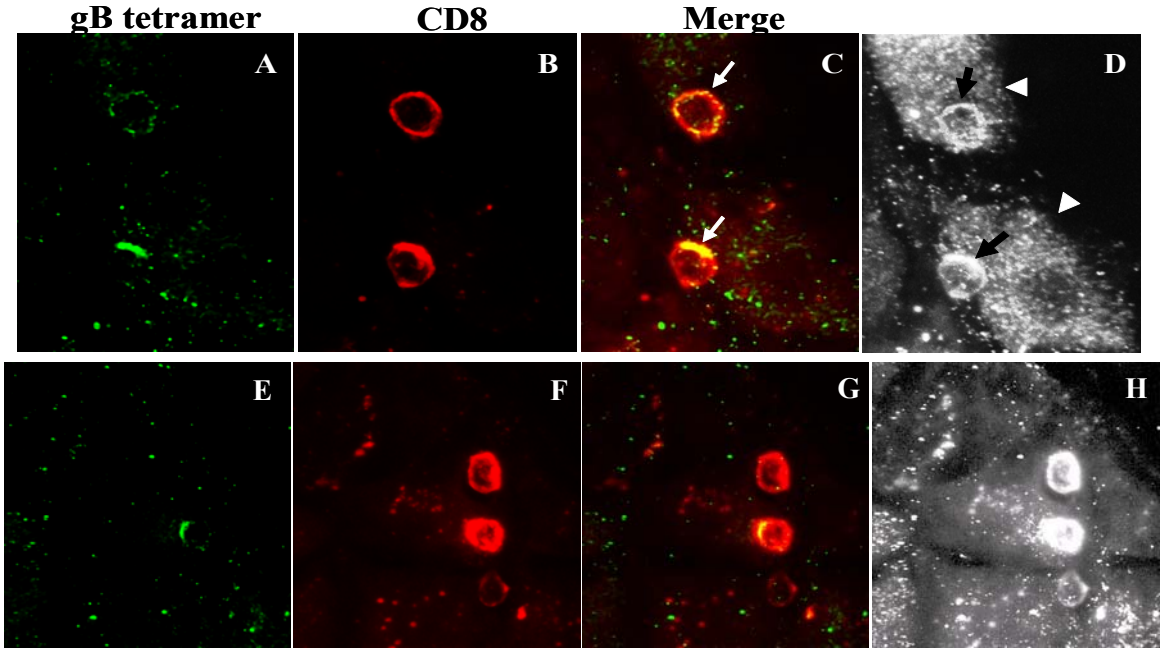

Figure 20: T cell receptor polarization towards neuronal cell bodies. *(A-H) Most of the $CD8^+$ T cells exhibited ring- or patchy-foci of TCR polarization. (D and H) Grayscale image showing $CD8^+$ T cells (arrows) in direct apposition to neurons (arrowheads); taking advantage of the intrinsic autofluorescence of neurons when excited with an argon laser and collected with a 500 longpass filter. Simultaneous staining of whole TG tissue with $gB_{498\text{-}505}$ MHC class I tetramers (green) and CD8 (red). Colocalization of TCR and CD8 appears as green.*

Figure 20 (A-D and E-H) shows two such examples exhibiting the possibility of $CD8^+$ T cells directly interacting with neuronal cell bodies presumably harboring latent virus. The presence of a distinct TCR polarization suggests formation of an organized immunological synapse with the neurons of the peripheral nervous system.

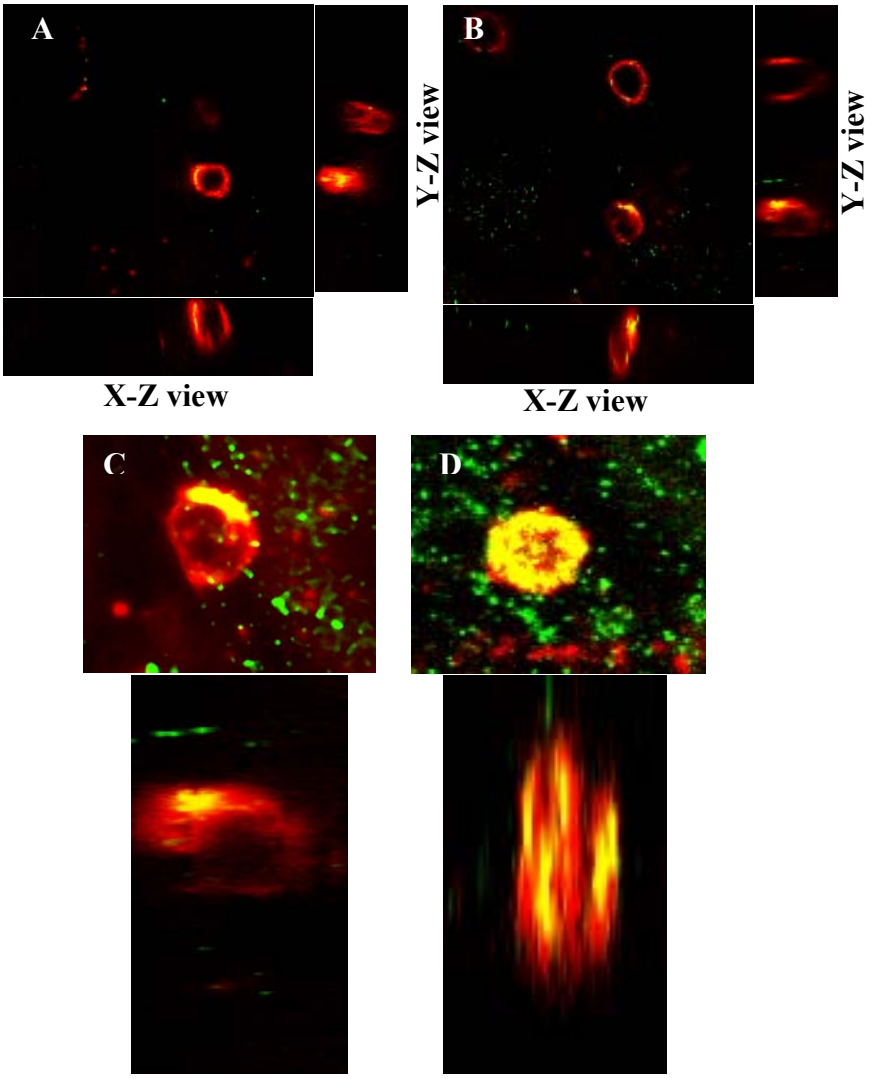

Figure 21: Orthogonal views of $CD8^+$ T cells in the TG. *Using Metamorph™ software orthogonal views showing an immunological synapse on the two $CD8^+$ T cell shown in figure 20 were constructed (A&B). Orthogonal views of a $CD8^+$ T cell exhibiting TCR polarization towards a neuronal cell body (C), compared a CD8 T^+ cell in the TG showing dispersed TCR arrangement (D). Simultaneous staining of whole tissue with MHC class I tetramers containing the $gB_{498\text{-}505}$ epitope (green) and CD8 (red).*

To further investigate the likelihood of gB specific $CD8^+$ T cells directly interacting with neurons in the latently infected TG we took advantage of the Metamorph software and derived orthogonal *zy* and *xz* views of the $CD8^+$ T cells that show or do not show gB specific TCR polarization (Figure 21). The orthogonal *yz* views in Figure 21A-C clearly show gB specific TCR aggregation in an organized immunological synapse exhibiting the formation of a cSMAC on those $CD8^+$ T cells that were in close apposition to neurons in the TG. Note the dispersed TCR (Figure 21D) on the surface of a $CD8^+$ T cell that did not exhibit receptor polarization and was not in close proximity to a neuron (compare Figure 21C and D). To our knowledge this is the first example of a demonstration of the formation of an immunological synapse in vivo and a direct interaction of $CD8^+$ T cells with neurons.

4.3. Expression of Glycoprotein B During Latency

Background and Rationale:

$CD8^+$ T cells retained in the TG during latency appear to be activated as demonstrated by their activation phenotype, antigen directed retention only in the infected ipsilateral TG and the appearance of direct interaction with neurons as well as formation of an organized immunological synapse with neuronal cell bodies. These data suggest that a late (γ1) viral gene product may be presented to $CD8^+$ T cells even during latency and may explain the state of $CD8^+$ T cells in the latent TG. To investigate this possibility we performed RT-PCR on whole TG tissues that were latently infected with HSV-1 using gB specific primers, but we obtained variable results. We had difficulties in obtaining consistent clear results with the RT-PCR approach because the gB gene is an unspliced gene, which prevented us from using intron spanning primers. To avoid these complexities we constructed a recombinant virus that

expresses enhanced green fluorescent protein (EGFP) under the control of the gB promoter (gBp-EGFP HSV-1), therefore we were able to detect gB promoter activity in vivo.

Results:

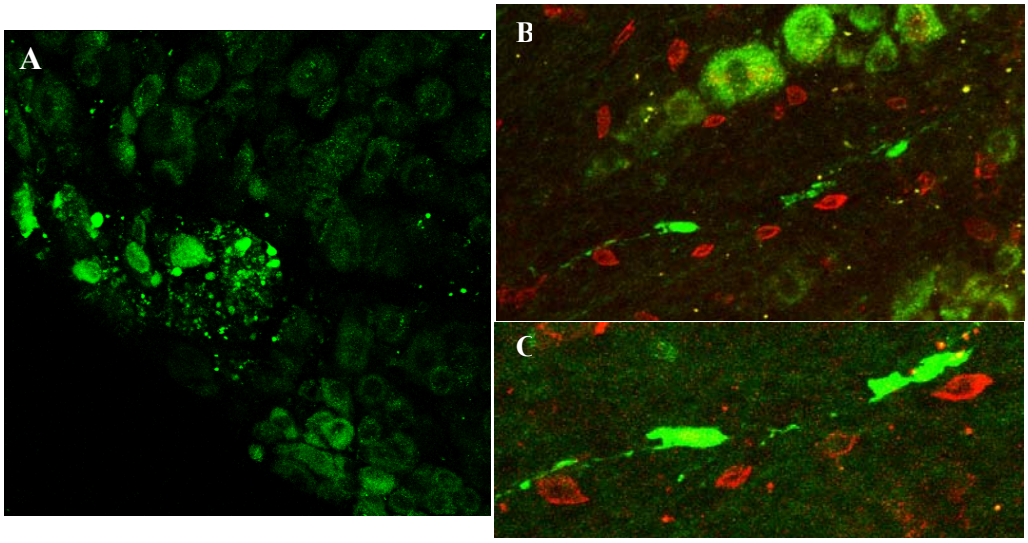

Figure 22: ***Confocal images of whole TG 5 days after corneal infection with gBp-EGFP HSV-1.*** *Whole TGs were excised 5 days PI, and stained for CD8α (red) and gB promoter activity is exhibited by green fluorescent protein (green). At 5 days PI replicating virus can be seen in neurons (green) of the ophthalmic branch of the TG (A). $CD8^+$ T cells (red) are beginning the infiltration in the TG and can be seen in close apposition to green neurons (B), and also axons exhibiting gB promoter activity (C).*

Using whole tissue staining and confocal microscopy we were able to detect expression of the gB gene in vivo during acute HSV-1 infection (D5 PI; Figure 22). $CD8^+$ T cells appear to interact with neurons and axons showing evidence of gB promoter activity suggesting antigen presentation by neuronal cells to $CD8^+$ T cells.

To determine if gB, a γ1 gene product, is expressed during latency mice were infected with the gBp-EGFP HSV-1 and sacrificed at 34 days PI, TGs were excised and whole tissues were analysed by confocal microscopy to detect expression of EGFP as a function of gB promoter activity.

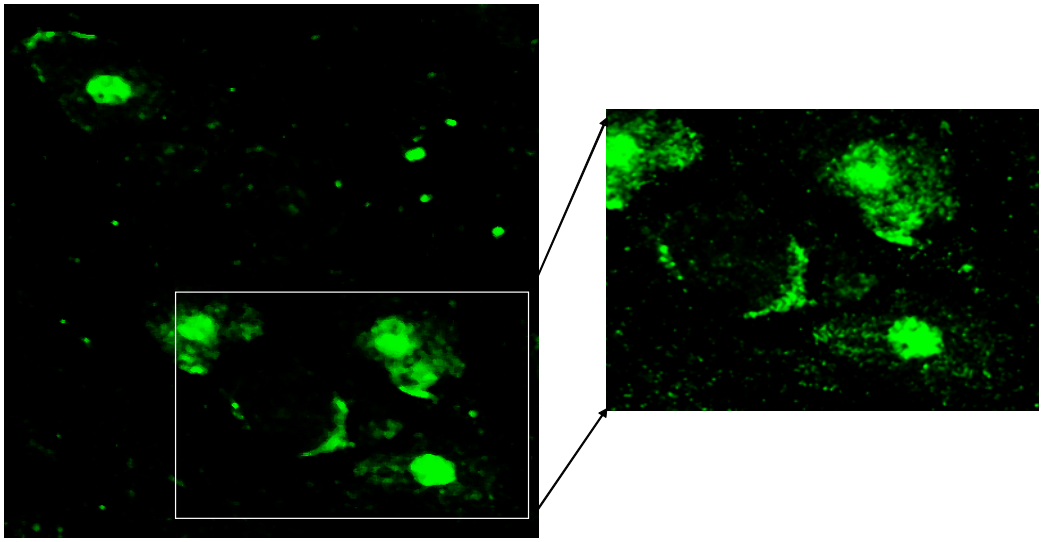

Figure 23: Whole mounts of TG infected with gBp-GFP HSV-1 34 days PI. *Mice were infected with gBp-GFP HSV-1 and sacrificed at 34 day after corneal infection. TGs were excised and green fluorescence was detected by confocal microscopy.*

As shown in Figure 23 three to four neurons exhibited GFP expression indicating gB promoter activity confirming the possibility of γ1 gene expression during latency and continued antigen presentation to $CD8^+$ T cells retained in the TG after the establishment of latency. Interestingly in some neurons GFP was detected localized in the nucleus (Figure 23).

4.4. Role of $CD8^+$ T Cells in Preventing Reactivation and Control of Viral Gene Expression during Latency

Background and Rationale:

The data presented above indicate that $CD8^+$ T cells infiltrate the TG at a time when HSV-1 replication has nearly ceased and latency is beginning to be established. The $CD8^+$ T cells remain in the TG seemingly for the life of the animal, moreover the $CD8^+$ T cell population exhibits a shift to a more activated phenotype during the course of latency. Earlier work by Simmons' group has established the role of $CD8^+$ T cells in controlling viral replication during lytic infection in the sensory ganglion (132). Furthermore studies with of αβ T cell-deficient mice have revealed that after HSV-1 corneal infection the virus was largely controlled early, but the majority of the mice died as a result of encephalitis presumably as a result of poor maintenance of viral latency (130). HSV-1 does not appear to spontaneously reactivate from latency in the sensory ganglia of mice in vivo. However, the virus can reactivate from mouse ganglion in vitro; we hypothesize that the capacity of HSV-1 to reactivate from latency in explant cultures of mouse ganglia might reflect the separation of the ganglionic neurons from HSV specific $CD8^+$ T cells. Our histologic studies indicate that $CD8^+$ T cells are not only retained in the TG long after the establishment of latency, but they appear to interact directly with neurons and form an organized immunological synapse, which suggests continued antigen presentation to gB specific $CD8^+$ T cells by latently infected neurons. The possibility of antigen presentation to $CD8^+$ T cells in the ganglion by neurons is further strengthened by the results obtained by infection of mice with gBp-EGFP HSV-1 (Figure 23) demonstrating the expression of gB in latently infected neurons.

Results:

The above findings invited the hypothesis that $CD8^+$ T cells specific for a single immunodominant epitope of the HSV-1 γ_1 gene product gB can block HSV-1 reactivation from latency.

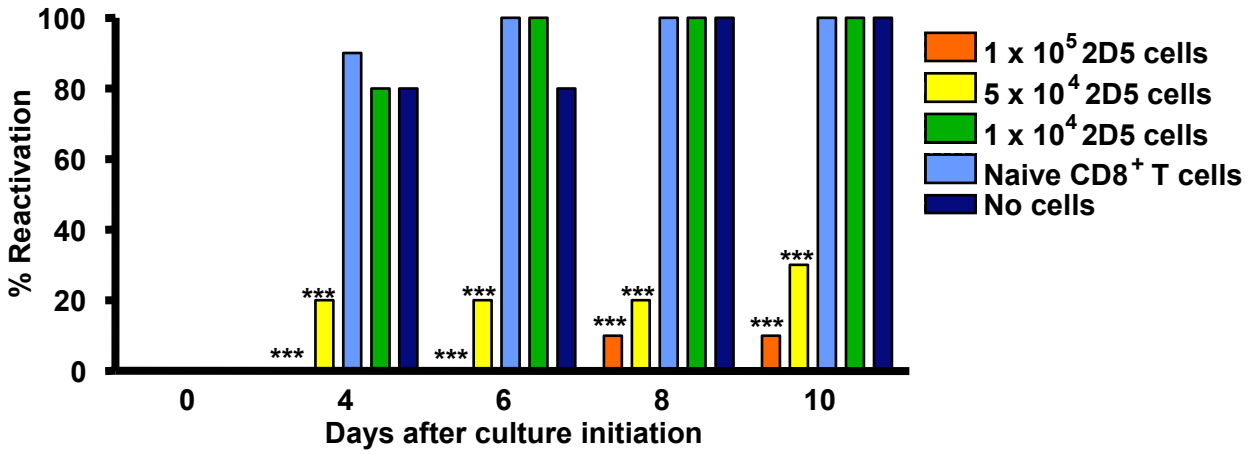

Figure 24: A $CD8^+$ T cell clone specific for the $gB_{498-505}$ epitope (2D5) can block HSV-1 reactivation from latency in TG cultures. *TG were excised from C57BL/6 mice 34 days after corneal infection and TG cells (0.5 TG equivalent/culture) were cultured with the indicated $CD8^+$ T cells, $1x10^5$, $5x10^4$, or $1x10^4$ 2D5 cells (n=10) and naïve cells (n=5). At the indicated times cultures were examined and HSV-1 reactivation was monitored as described in Methods. A Survival Curve Analysis determined the significance of differences in reactivation frequency (compared to cultures receiving naïve $CD8^+$ T cells). (*** $p< 0.0001$). The reactivation frequency in cultures that received naïve $CD8^+$ T cells was not significantly different ($p = 0.5485$) from that in cultures to which no $CD8^+$ T cells were added.*

To investigate this point, cultures were prepared with single cell suspensions of C57BL/6 TG that were excised 34 days after HSV-1 corneal infection. To these cultures were added: 1) varying numbers of 2D5 cells, an HSV $gB_{498-505}$ peptide-specific $CD8^+$ T cell clone derived from C57BL/6 mice; 2) naïve $CD8^+$ T cells isolated from the lymph nodes of uninfected C57BL/6 mice; or 3) no $CD8^+$ T cells. The cultures were then monitored for HSV-1 reactivation as described in Methods. All cultures that received 1 x 10^5 naïve $CD8^+$ T cells exhibited HSV-1

reactivation with similar kinetics to that observed in cultures to which no $CD8^+$ T cells were added. The addition of 2D5 cells to MHC compatible C57BL/6 TG cultures suppressed HSV-1 reactivation in a dose-dependent manner (Figure 24). At inputs of 1 x 10^5 and 5 x 10^4, 2D5 cells significantly ($p< 0.0001$ and $p < 0.001$, respectively) suppressed HSV-1 reactivation from latency in C57BL/6 TG cultures throughout the 10-day culture period. In contrast those cultures that received 1 x 10^4 2D5 cells, naïve CD8 T cells or no cells reactivated promptly and by day 10 after culture initiation 100% of them had replicating virus.

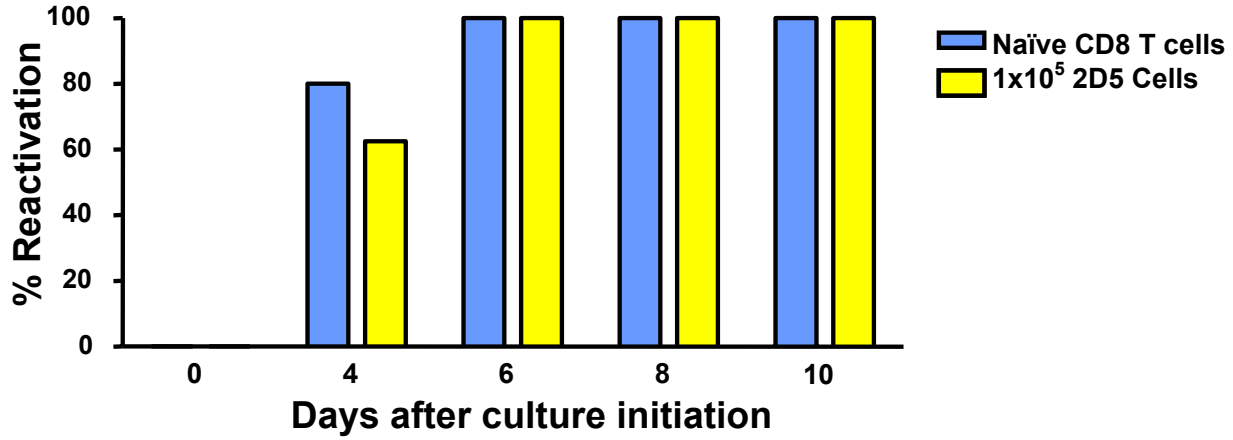

Figure 25: 2D5 cells cannot block HSV-1 reactivation in allogeneic D34 Balb/c TG cultures. *TG were excised from Balb/c mice 34 days after corneal infection and TG cells (0.5 TG equivalent/culture) were cultured with the indicated $CD8^+$ T cells, 1 x 10^5 2D5 cells (n=8) and naïve $CD8^+$ T cells (n=5). At the indicated times cultures were examined and HSV-1 reactivation was monitored as described in Methods.*

$CD8^+$ T cells can produce many antiviral factors such as cytokines and the proposed $CD8^+$ T cell antiviral factor (CAF) that does not require cell-cell interaction or MHC restriction (149). To determine if the $CD8^+$ T cell protection from reactivation in the ex vivo cultures is MHC restricted, single cell suspensions of Balb/c TG that were excised 34 days and cultured as mentioned above. In contrast to C57BL/6 cultures (Figure 24) at the highest input (1 x 10^5

cells/culture) 2D5 cells did not significantly ($p = 0.45$) alter the course of HSV-1 reactivation from latency in the MHC incompatible BALB/c TG cultures (Figure 25), suggesting that the protection rendered by $CD8^+$ T cells in the TG ex vivo cultures is indeed MHC restricted and presumable requires cell-cell contact.

Since the TG cultures in the above experiment contained host $CD8^+$ T cells that were present in the TG at the time of excision, the relative contribution of the endogenous $CD8^+$ T cells and added 2D5 cells could not be determined. To address this issue, TG were excised 34 days after HSV-1 corneal infection of C57BL/6, and depleted of $CD8^+$ T cells by immunomagnetic separation. Either 2D5 cells or naïve syngeneic $CD8^+$ T cells were then added to the CD8-depleted TG cultures, and the cultures were monitored for HSV-1 reactivation. Again, naïve $CD8^+$ T cells failed to influence HSV-1 reactivation from latency, while 2D5 cells inhibited HSV-1 reactivation from latency in a dose-dependent manner (Figure 26).

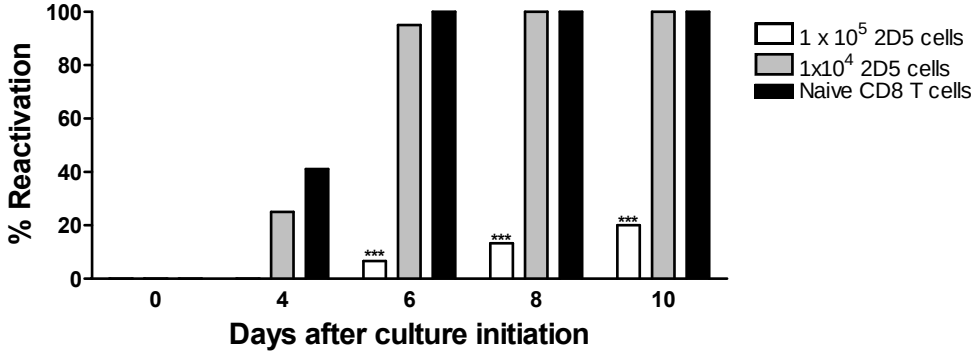

Figure 26: 2D5 Cells Can Block HSV Reactivation in CD8-Depleted Day 34 C57BL/6 TG cultures. *TG were excised from C57BL/6 mice, depleted of endogenous $CD8^+$ T cells, and cultured with $1x10^5$ (n=15) or $1x10^4$ (n=20) 2D5 cells, or with $1x10^5$ naïve $CD8^+$ T cells (n=16). At the indicated times cultures were examined and HSV-1 reactivation was monitored as described in Methods. A Survival Curve Analysis determined the significance of differences in reactivation frequency (compared to cultures receiving naïve $CD8^+$ T cells (*** $p< 0.0001$).*

The 2D5 cells are cytotoxic and produce IFN-γ when stimulated with the $gB_{498-505}$ epitope. However, treating TG cultures with anti-CD8 mAb 6 days after culture initiation

blocked the 2D5 protective function and allowed the previously latent virus to reactivate as indicated by the cytopathic effect (CPE) of the virus (not shown). The prompt reactivation after the addition of anti-CD8 mAb suggests that 2D5 cells could prevent HSV-1 reactivation in TG cultures without eliminating the reservoir of latenly infected neurons. In addition protected TG cultures did not show any expression of the γ_2 gene, gH (Figure 27). However γ_2 gene expression was readily detected in cultures that reactivated as a result of the addition of anti-CD8 mAb. Thus, 2D5 cells were able to block HSV-1 reactivation in at least some of the neurons by a non-cytolytic mechanism and by regulating viral gene expression.

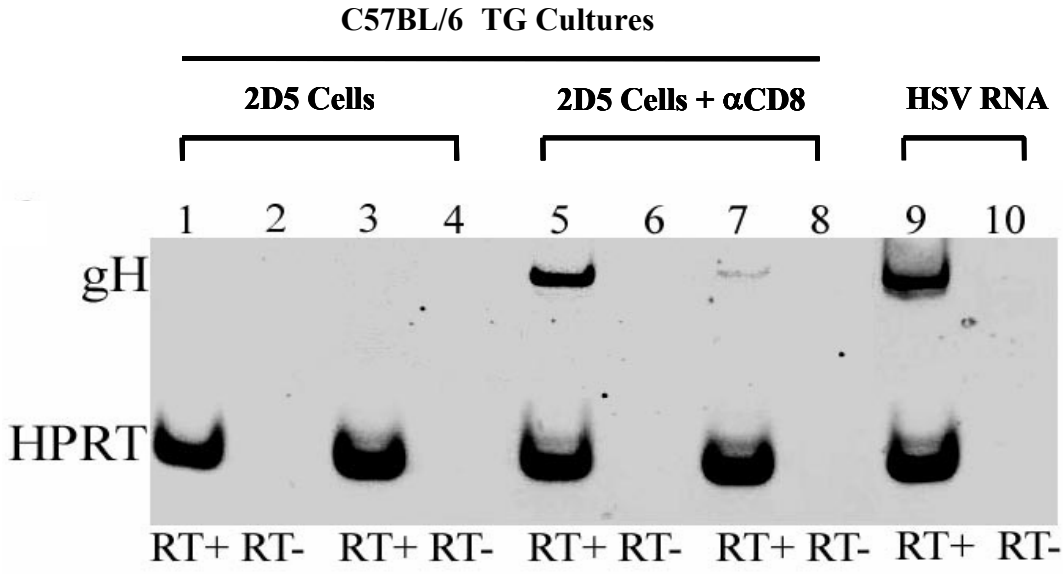

Figure 27: gB specific CD8$^+$ T cell clone can block HSV-1 γ2 gene expression in D34 TG cultures. *Ten days after culture initiation, total RNA was extracted from CD8$^+$ T cell-depleted TG cultures that received 1x10^5 2D5 cells, and was analyzed for HSV-1 gH transcripts by RT-PCR as described in Methods. Each sample is comprised of pooled RNA from two cultures. Lanes 1-4 represent cultures that did not receive anti-CD8 mAb and showed no viral CPE or infectious virus. Lanes 5-8 represent cultures that received anti-CD8 mAb on day 6 of culture and were positive for viral CPE and infectious virus. Note that the cultures represented in columns 7 & 8 were nearly destroyed by the virus, resulting in greatly reduced total RNA and gH transcripts. RNA extracted from a freshly excised TG obtained 5 days after corneal infection (during lytic virus infection) served as a positive control (lanes 9 and 10). Transcripts for the housekeeping gene HPRT were similarly amplified to demonstrate equal loading of RNA from each sample.*

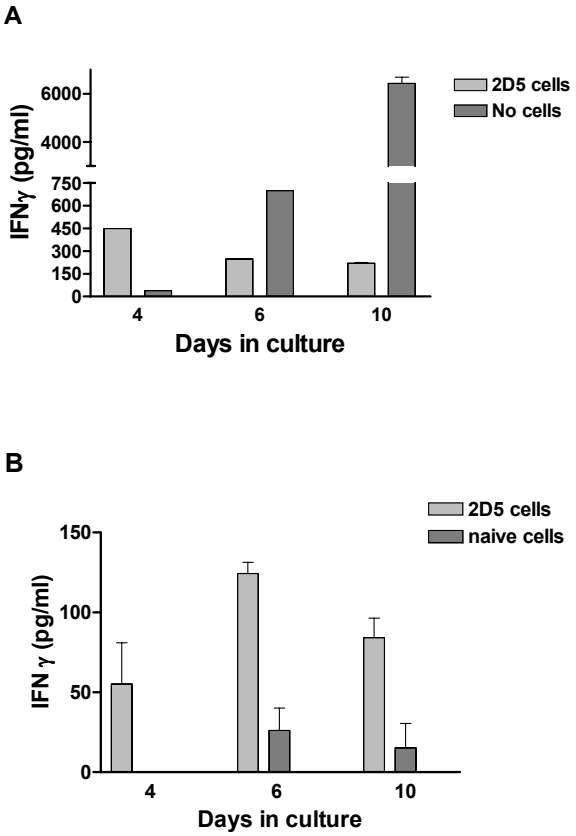

Figure 28: IFN-γ production in TG cultures. *At the indicated times, supernatant fluids from the cultures described in Figure. 24 and 26 and were tested for IFN-γ content by ELISA. Data are presented for undepleted (A) and $CD8^+$ T cell depleted (B) C57BL/6 TG cultures that received $1x10^5$ 2D5 cells, or $1x10^5$ naïve $CD8^+$ T cells.*

We have demonstrated that IFN-γ can block HSV-1 reactivation (see next result section) from latency in *ex vivo* TG cultures (73). Therefore, supernatant fluids from the cultures shown in Figure 24 and 26 were tested for IFN-γ by ELISA (Figure 28). C57BL/6 TG cultures containing endogenous $CD8^+$ T cells, but no added $CD8^+$ T cells produced significant amounts of

IFN-γ, but only after HSV-1 reactivated from latency (Figure 28A and 24, respectively). C57BL/6 TG cultures that were depleted of endogenous $CD8^+$ T cells and did not receive 2D5 cells, or received naïve $CD8^+$ T cells produced low to undetectable levels of IFN-γ even after HSV-1 reactivation from latency (Figure 28B and 26, respectively) demonstrating that HSV-specific $CD8^+$ T cells are responsible for most or all IFN-γ production in these cultures.

Interestingly, the cultures that received a protective dose of 2D5 cells ($1x10^5$/culture) showed early production of IFN-γ on days 4 and 6 of culture, and the IFN-γ levels declined thereafter. These data suggest that the addition of exogenous gB-specific $CD8^+$ T cells to the TG cultures allows more rapid interaction with reactivating neurons, thus permitting a protective $CD8^+$ T cell response within the timeframe necessary to block HSV-1 reactivation from latency. Together these findings demonstrate that $CD8^+$ T cells can block HSV-1 reactivation from latency in TG cultures by a gB-specific, dose-dependent, MHC-restricted, non-cytolytic mechanism that is at least partially IFN-γ-dependent.

4.5. Mechanisms Employed by $CD8^+$ T cell to Prevent HSV-1 Reactivation

Background and Rationale:

Our studies have provided compelling evidence that $CD8^+$ T cells can prevent reactivation of latent HSV-1 in ex vivo TG cultures, but the mechanisms by which the $CD8^+$ T cells confer such protection are yet to be fully understood. Two of the most common mechanisms utilized by $CD8^+$ T cells to eliminate invading pathogens are secretion of antiviral cytokines and destruction of virus infected cells through induction of apoptosis through the release of lytic granules containing perforin, GrA and GrB.

HSV-1 infection of mice results in a latent infection in sensory ganglia, but the virus does not normally reactivate from latency. There is a substantial infiltration of CD4 and $CD8^+$ T cells, which are retained in the TG seemingly for the life of the animal. Moreover leukocytic infiltration of the latently infected mouse TG is associated with the presence of T cells secreted cytokines IFNγ, TNFα and IL-6 suggesting persistent activation of the infiltrating cells (136,129,22). Cytokine production in the TG was inhibited by treatment of latently infected mice with the antiviral drug acyclovir (ACV), consistent with the involvement of viral proteins in T cell activation. Results from studies in our and other laboratories are consistent with the notion that individual latently infected neurons may periodically lose the capacity to inhibit expression of HSV-1 genes, resulting in low-level and perhaps intermittent production of viral proteins. These viral proteins (such as γ1 gene product gB; see sections 4.2 and 4.3) might stimulate T cells in the ganglion to produce cytokines and perhaps degranulation of cytolytic granules that prevent further progression through the viral life cycle to the stage of virion production. Therefore in the next set of studies we determined the role of IFNγ and the lytic machinery (perforin and Fas-FasL mechanisms) in preventing reactivation of latent HSV-1 in ex vivo TG cultures.

One of the most potent mechanisms utilized by $CD8^+$ T cells is the Fas (CD95) and perforin mediated destruction of viral infected cells through apoptosis. Fas mediated destruction of target cell operates through the binding of Fas receptor on the infected cell with the Fas ligand (FasL) on the $CD8^+$ T cells. This Fas-FasL binding triggers the release of cysteine proteases called caspases, that lead to the death of the infected target cell. The second mechanism by which target cells are destroyed by $CD8^+$ T cells is the release of a pore forming molecule perforin (68kDa) from granules. Upon recognition of foreign antigens presented by class I major

histocompatibility complex (MHC-I) on infected cells the granules present in the $CD8^+$ T cells are activated and perforin is subsequently released. T cell mediated cytolysis involves granule components such as; perforin, serine protease GrB and GrA (60,150). Perforin polymerization in target cell membranes facilitates entry of granzymes. However recent studies have shown that perforin also plays an important role in redistribution of granzymes once these molecules enter the cytoplasm. Perforin may be essential for nuclear translocation of granzymes as well as their release from endosomal vesicles (66,151,152). Once in the cytoplasm of the target cell, GrA and GrB are believed to function independently initiating separate pathways. GrB (32 kDa) is proteinase with aspase activity and cleaves at aspartate residues (66), it is believed to be the more potent of the two granzymes and induces apoptosis rapidly. GzmB can activate cysteinyl aspartate-specific proteinases (caspases) by directly cleaving them at specific aspartate residues. Once activated caspases can activate each other. Activated caspases translocate to the nucleus and begin the destruction of DNA repair enzymes such as poly (ADP-ribose) polymerase (PARP) and DNA dependent protein Kinase (DNA-PKcs). In addition, GrB has been reported to directly cleave PARP and DNA-PKc increasing the efficiency and rate of apoptosis. The ultimate result of such events is lack of DNA repair and eventual fragmentation of cellular DNA (66,153). The exact mechanism employed by GrA in apoptosis or viral clearance has not yet been completely elucidated, however it appears to function independently of GrB (69,154,70). GrA (65kDa) is a tryptase with specificity for basic residues such as lysine or arginine and is thought to activate a caspase independent apoptotic pathway. The contribution of GrA in target cell apoptosis is a subject of controversy, a recent study identified a GrA activated DNase called NM23-H1. NM23-H1 is a tumor suppressor protein and is responsible for inducing cell-death events such as DNA damage. An inhibitor protein SET keeps NM23-H1 inactivated in the

cytoplasm, GrA cleaves SET allowing NM23-H1 to translocate to the nucleus and cause DNA damage (155). Shresta et al, propose a model for the pathways of GrA and GrB function. They suggested that GrB is the primary inducer of apoptosis but in certain instances, such as GrB resistant tumors and infections with viruses that encode proteins to inhibit GrB (CrmA encoded by cowpox virus) GrA can be very valuable (70). There are limited data on the role of granzymes in HSV-1 infection; a recent study implicated GrA in preventing HSV-1 neuronal spread in the TG at 5 days after HSV-1 corneal infection. At 5 days PI TGs isolated from $GrA^{-/-}$ mice contained considerably higher PFU of HSV-1, in addition many more neurons (10 fold higher) were infected with HSV-1 in TG of $GrA^{-/-}$ mice compared to WT mice. Apart from its ability to cause target cell death, there are many noncytolytic functions that have been proposed for GrA, such as facilitating T cell migration and extravasation into subendothelial spaces, augmenting an inflammatory response mediated by IL-1 and IL-6 and perhaps acting as a cytokine itself (151,156). GrA activates pourokinase, initiating a proteolytic cascade leading to the production of plasmin, which in turns can inactivate enveloped viruses (Ectromelia virus) (157,67). Interestingly GrA can cleave extracellular matrix proteins and thrombin receptors on neurons. (158). Simmons et al, recently demonstrated the important role of GrA in restricting HSV-1 spread in mouse TG during the lytic phase of infection (133), however the role of the lytic machinery during latency is yet to be defined.

Results:

4.5.1. IFNγ Can Prevent HSV-1 Reactivation from Latency in Sensory Neurons

In our previous study using Balb/c mice (32), when TG were obtained 34 days PI, the endogenous $CD8^+$ T cells could delay, but could not prevent, reactivation, unless supplemented with exogenous $CD8^+$ T cells obtained from the lymph nodes of HSV-1 infected, but not

uninfected, mice. This failure of the endogenous $CD8^+$ T cells to block reactivation in day 34 TG cultures might reflect their reduced numbers (compared to those of day 14 TG cultures), but it might also reflect functional change in the $CD8^+$ T cells that remain in the TG between 14 and 34 days after HSV-1 infection. To determine if IFNγ can prevent HSV-1 reactivation in ex vivo TG cultures balb/c TG were excised 35 days after corneal infection, dissociated into single cell suspension and cultured (1TG/well) in 24 well plates. The cultures were incubated in medium alone or with recombinant IFNγ (rIFNγ; 1000 U/ml). Reactivation was monitored by assaying the supernatant for infectious virus by plaque assay. 100% of the cultures reactivated in the presence or absence of rIFNγ (Figure 29). The rate of reactivation was slightly delayed, the viral titers were slightly reduced (not shown), and reversion to undetectable virus titers occurred 2 days earlier in rIFNγ-treated cultures. However, none of these differences were statistically significant at a *P* value of <0.05.

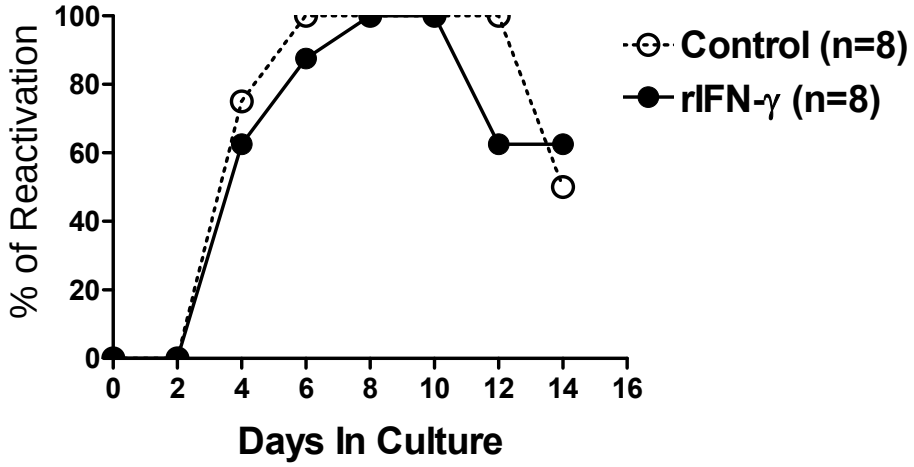

Figure 29: rIFNγ alone cannot block HSV-1 reactivation. *TG were excised 35 days after HSV-1 corneal infection. Single cell suspensions were prepared, and replicate cultures were incubated in culture medium alone (control) or medium containing rIFNγ. The supernatant fluids were sampled every day and assayed for infectious virus. The data are expressed as percentage of cultures with detectable virus.*

The findings described above (Figure 29) led us to propose that latent HSV-1 began to reactivate early after TG excision, and by the time of culture initiation, it had progressed too far into the viral life cycle to be controlled by IFNγ. Therefore, we reasoned that 4 days of exposure to the antiherpetic drug ACV would reestablish latency in all neurons and enhance the effectiveness of IFNγ and $CD8^+$ T cells in blocking HSV-1 reactivation from latency. To test this possibility, day 35 TG cultures were incubated for 4 days with ACV. The cultures were then incubated in culture medium alone or culture medium that was supplemented with rIFNγ at 0, 24, 48, or 72 hrs after removal of ACV. Addition of rIFNγ within 24 hrs after removal of ACV dramatically reduces HSV-1 reactivation from latency in TG cultures ($P < 0.001$; Figure 30). However, rIFNγ had no effect on HSV-1 reactivation on HSV-1 reactivation from latency when treatment was delayed until 48 hrs after removal of ACV.

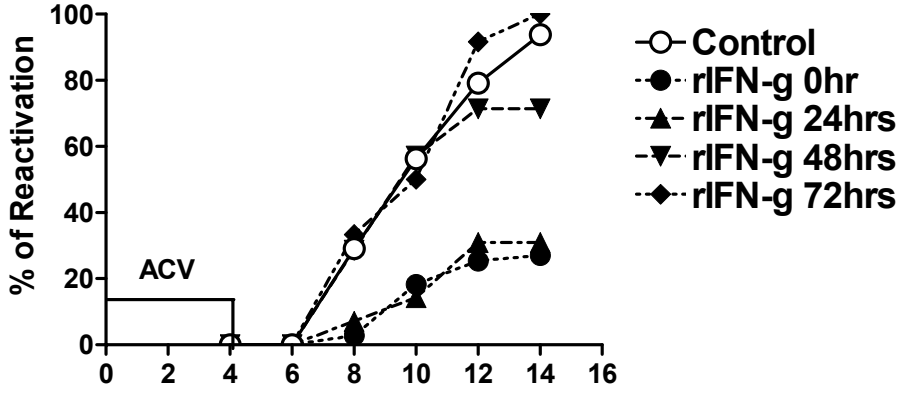

Figure 30: IFNγ can block HSV-1 reactivation from latency in ACV treated TG cultures. *Day 35 TG cell cultures were treated with ACV (50 μg/ml) for 4 days, rinsed and then incubated for an additional 10 days in medium alone (n=26) or culture medium supplemented with rIFNγ at 0 hr (n=25), 24 hrs (n=10), or 72 hrs (n=10) after removal of ACV. Culture supernatant were sampled every other day and assayed for infectious HSV-1 by plaque assay. Reactivation frequency was significantly reduced ($P < 0.001$) relative to medium only controls by IFNγ treatment at 0 and 24 hrs only.*

Interestingly, IFNγ production was not detectable during ACV treatment, and following ACV removal, it was produced with similar kinetics to that seen in cultures that were not treated with ACV (data not shown). Depletion of $CD8^+$ T cells from the TG cell suspension eliminated IFNγ production. Thus, $CD8^+$ T cells were the main source of IFNγ in cultures of latently infected TG, and ACV delayed IFNγ production. ACV at concentrations of up to 200μg/ml did not significantly affect IFNγ production by 2D5 cells in response to stimulator cells that express the appropriate viral epitopes. Thus, a direct ACV block of IFNγ production by $CD8^+$ T cells cannot account for the delay in IFNγ production observed in ACV-treated TG cultures.

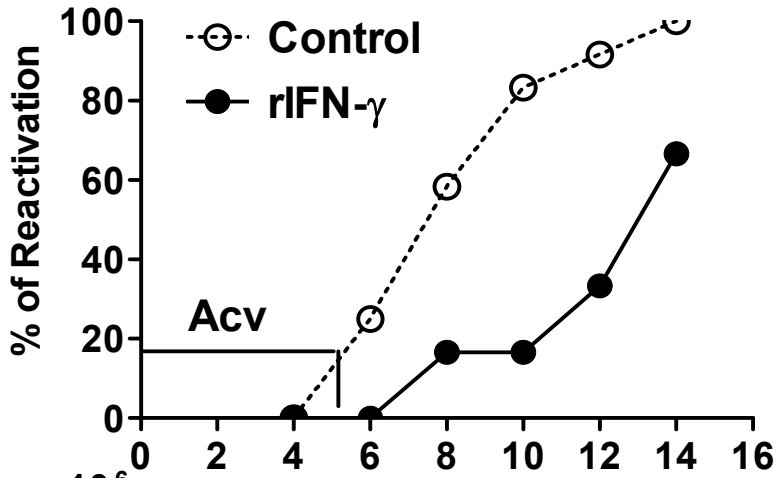

Figure 31: IFNγ can directly and indirectly inhibit HSV-1 reactivation from latency. *TG were excised 35 days after HSV-1 corneal infection and single cell suspensions were pooled, depleted $CD45^+$ cells (n = 12), and incubated with culture medium containing ACV for 4 days. After removal of ACV, cultures were incubated with medium alone or medium supplemented with rIFNγ for an additional 10 days. Culture supernatant fluids were sampled every other day and assayed for infectious HSV-1 (indicating HSV-1 reactivation from latency) in a plaque assay. IFNγ significantly reduced the reactivation frequency ($P < 0.001$) in $CD45^+$ cell-depleted TG cultures.*

Data in Figure 30 clearly established that IFNγ could prevent HSV-1 reactivation from latency when present early in the reactivation process. However these cultures contained $CD8^+$ T cells and other inflammatory cells that were present in the TG at the time of excision. Thus, it was not clear if IFNγ directly blocked HSV-1 reactivation from latency in neurons or acted indirectly by enhancing a protective response of $CD8^+$ T cells or other inflammatory cells.

To address this issue, day 35 TG cell suspensions were depleted of $CD8^+$ T cells and incubated with ACV for 4 days. After removal of ACV, medium alone or medium supplemented with rIFNγ was added, and HSV-1 titers in culture supernatants were measured on alternate days. HSV-1 reactivation was delayed and significantly reduced in cultures that were treated with rIFNγ (data not shown) ($P < 0.001$). It is noteworthy that rIFNγ did appear to be somewhat less effective at blocking HSV-1 reactivation in the absence of endogenous $CD8^+$ T cells (60% versus 20%, data not shown). Moreover, rIFNγ treated CD8 T-cell-depleted TG cultures that reactivated were ultimately destroyed by the virus. As noted above, the virus did not destroy TG cultures that contained $CD8^+$ T cells. These data demonstrate that IFNγ can block HSV-1 reactivation from latency by a mechanism that is at least partially $CD8^+$ T cell independent but is ineffective at blocking the spread of HSV-1 following a reactivation event. To further support the concept that IFNγ can directly block HSV-1 reactivation from latency in neurons, day 35 TG cells were prepared and depleted of $CD45^+$ (bone marrow-derived) cells. Cultures were prepared and treated with ACV for 4 days followed by addition of medium alone or medium supplemented with rIFNγ. As shown in Figure 31, rIFNγ significantly delayed and reduced HSV-1 reactivation from latency in TG cultures lacking any detectable $CD45^+$ cells ($P < 0.001$). Taken together, these data suggest that IFNγ can act directly on latently infected neurons to

inhibit HSV-1 reactivation from latency, but it can also augment a protective response that appears to be mediated primarily by $CD8^+$ T cells.

4.5.2. Role of Perforin in Preventing HSV-1 Reactivation from Latency

We clearly established the important role of IFNγ in blocking HSV-1 reactivation in ex vivo TG cultures (Section. 4.5.1), next we wished to determine what role the lytic machinery of $CD8^+$ T cells plays in preventing HSV-1 reactivation. Our studies have demonstrated that $CD8^+$ T cells can prevent reactivation without eliminating the reservoir of all the neurons harboring latent HSV-1. However we cannot rule out the possibility that certain neurons harboring several copies of latent HSV-1 could be destroyed by perforin mediated mechanisms. Moreover studies in our laboratory have confirmed that $CD8^+$ T cells are more efficient than IFNγ alone at blocking HSV reactivation, suggesting an IFNγ independent mechanism. In addition two recent studies have implicated the importance of perforin and granzymes in controlling early HSV-1 replication in neurons (159,133).

Thus, to determine a role of perforin in maintaining HSV-1 latency, TGs from C57BL/6 and perforin knock out mice 14 days after corneal infection were excised and single cell suspensions were cultured in medium alone. 100% of the PfP cultures reactivated compared to 25% of the WT cultures (Figure 32). To determine if $CD8^+$ T cells were responsible for this protection, TGs were excised from C57BL/6 and perforin knock out (PfP) mice 14 days after corneal infection and single cell suspensions were cultured in medium alone or medium containing anti-CD8 mAb.

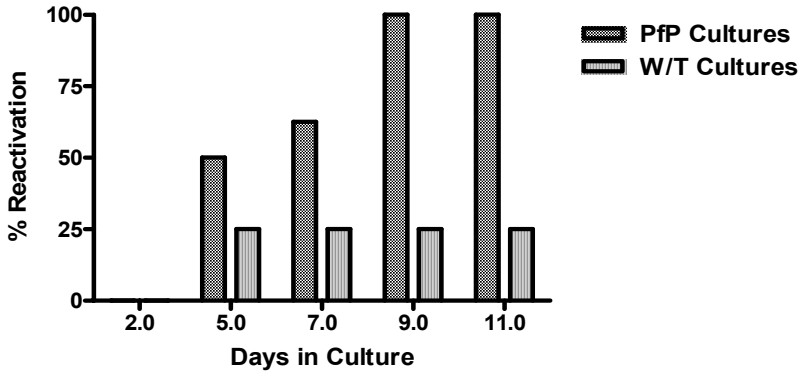

Figure 32: Perforin plays an important role in preventing HSV-1 reactivation. *TGs were excised 14 days after corneal infection from C57BL/6 (WT; n=8) and perforin KO (PfP; n=8) mice. Single cell suspensions were prepared and cultured (1TG/well) in medium alone. Culture supernatant were sampled every other day and assayed for infectious HSV-1 by plaque assay.*

100% of the PfP cultures reactivated compared to 25% of the WT cultures (Figure 33). The protection in WT cultures was mediated by the endogenous $CD8^+$ T cells present in the TG at the time of excision since addition of anti-CD8 mAb resulted in loss of protection. These data suggest that early after the establishment of latency (Day 14 PI) $CD8^+$ T cells need perforin to protect ex vivo TG cultures from HSV-1 reactivation. Interestingly PfP cultures that received anti-CD8 mAb showed accelerated reactivation compared to PfP cultures in medium alone. Since PfP mice secrete copious amounts of IFNγ, the delayed reactivation in PfP cultures that did not receive anti-CD8 mAb suggests lytic and non-lytic (IFNγ secretion) mechanisms are important in blocking HSV-1 reactivation early during latency.

Addition of anti-FasL mAb to latently infected TG cultures did not significantly affect reactivation frequency (data not shown). These data suggest that of the two cytolytic mechanisms

used by $CD8^+$ T cells to fight viral infections, only perforin mediated mechanism(s) is important in maintaining HSV-1 in a latent state.

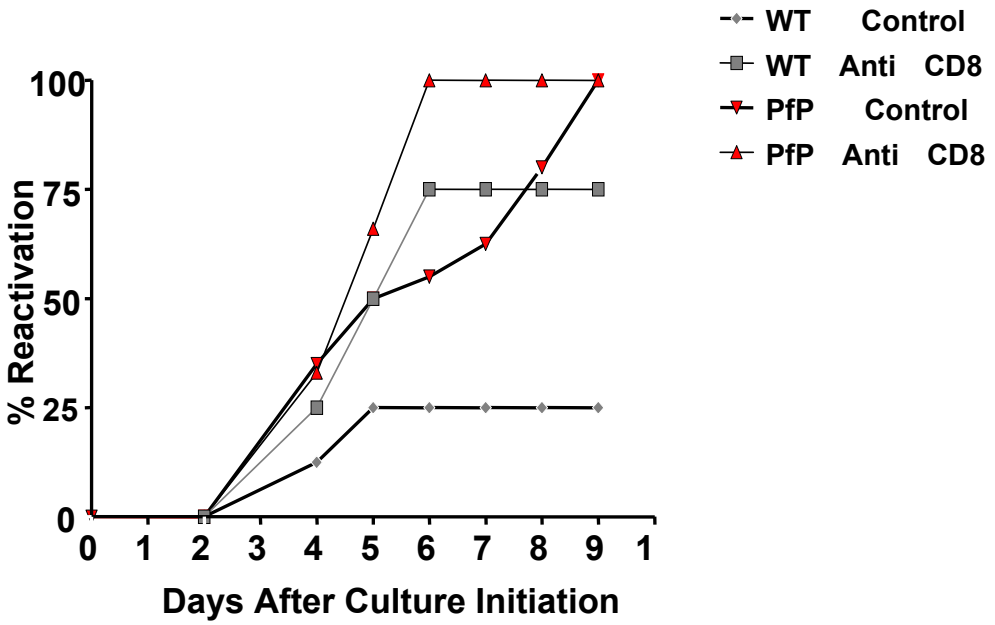

Figure 33: $CD8^+$ T cells in Day 14 TG from perforin KO mice cannot block HSV-1 reactivation from latency. *TGs were excised 14 days after corneal infection from C57BL/6 (WT) and perforin KO (PfP) mice. Single cell suspensions were prepared and cultured (1TG/well) as indicated, for each group n=5. Both WT and PfP cultures received 100 µg/ml of anti-mouse CD8α or control mAb on the day of culture initiation (d 0). Culture supernatant were sampled every other day and assayed for infectious HSV-1 by plaque assay.*

In the experiments with C57BL/6 mice we observed spontaneous reactivation in a small number of TG cultures. In order to ensure the establishment of stable latency in all neurons after excision and derivation of single cell suspensions of TG we used the anti-herpetic drug ACV as in the previous section. TGs from C57BL/6 and perforin knock out mice 14 days after corneal infection were excised and single cell suspensions were cultured as indicated in Figure 34. Even after 4 day treatment with ACV, 100% of PfP cultures reactivated compared to 20% of WT cultures at day 10 after culture initiation. The addition of anti-IFNγ mAb in PfP cultures resulted in accelerated reactivation frequency, moreover addition of rIFNγ reduced reactivation rate even

in PfP culutures, again suggesting the importance of IFNγ along with perforin in protecting TG cultures from HSV-1 reactivation at 14 days PI.

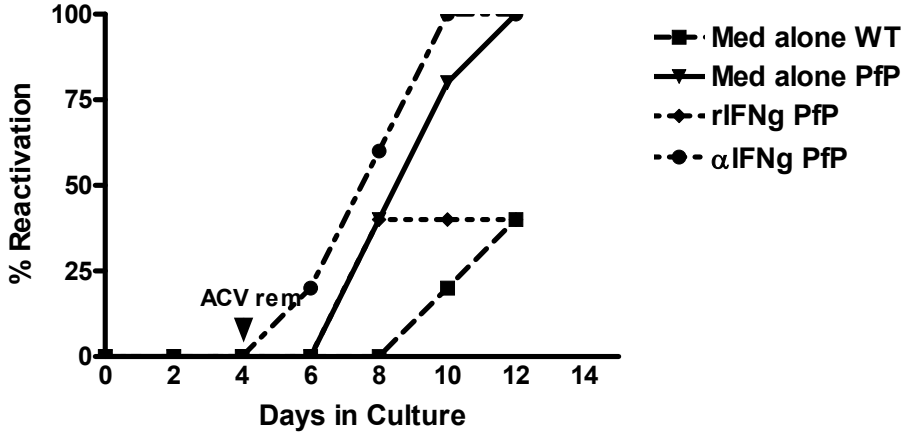

Figure 34: Perforin is required for efficient protection from HSV-1 reactivation in latent ex vivo TG cultures. *TGs were excised 14 days after corneal infection from C57BL/6 (WT) and perforin KO (PfP) mice. Single cell suspensions were prepared and cultured (1TG/well) as indicated. TG cell cultures were treated with ACV (50 μg/ml) for 4 days, rinsed and then incubated for an additional 10 days in medium alone or rIFNγ (1000 U/ml) or anti-IFNγ (20 μg/ml). For all groups n=8. Culture supernatant were sampled every other day and assayed for infectious HSV-1 by plaque assay.*

The data in the above 3 figures clearly established that perforin mediated mechanisms are important in protecting ex vivo TG cultures from HSV-1 reactivation early (Day 14 PI) during the latent stage of infection, although, the possibility of a non-cytotoxic mechanism involving selective utilization of GrA rather than GrB cannot be ruled out. To investigate the role of perforin in protecting latently infected TG cultures at 34 days PI, TG single cell suspension were prepared and cultured as described above. Interestingly PfP cultures reactivated sooner and with higher reactivation frequency compared to WT cultures, however the difference between the two groups at 34 days PI was not as significant as at day 14 PI (Figure 35). In addition supplementing

cultures with rIFNγ greatly reduced the reactivation frequency of both PfP and WT cultures, suggesting that at 34 days PI the requirement of perforin to prevent HSV-1 reactivation is reduced compared to 14 days PI. At 34 days after HSV-1 corneal infection non-cytolytic mechanisms such as IFNγ secretion appear to be essential for maintaining latency. It should be noted that in this particular experiment over all reactivation frequency of TG cultures was lower (~60% maximum reactivation percentage) compared to previous experiments where maximum reactivation percentage was close to 100%.

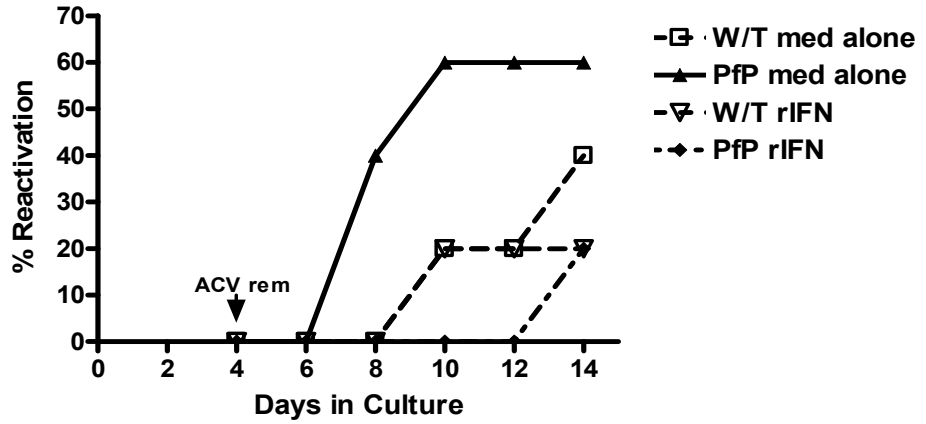

Figure 35: At 34 Days PI perforin mediated mechanisms are not as important in blocking HSV-1 reactivation. *TGs were excised 34 days after corneal infection from C57BL/6 (WT) and perforin KO (PfP) mice. Single cell suspensions were prepared and cultured (1TG/well) as indicated. TG cell cultures were treated with ACV (50 μg/ml) for 4 days, rinsed and then incubated for an additional 10 days in medium alone or rIFNγ (1000 U/ml). For all groups n=10. Culture supernatant were sampled every other day and assayed for infectious HSV-1 by plaque assay.*

At 34 days PI experiments with C57BL/6 and PfP mice (Figure 35) show that IFNγ secretion presumably by $CD8^+$ T cells in ex vivo latent TG cultures was necessary to prevent reactivation. We have shown that IFNγ can block HSV-1 reactivation in ex vivo TG cultures derived from Balb/C mice (Section 4.5.1). We were concerned about the compensatory mechanisms that may exist in PfP mice, since there is evidence that PfP mice secrete more IFNγ

that WT mice after viral infections (160) Therefore supernatant fluids from WT and PfP cultures were tested for IFNγ by ELISA (Figure 36). IFNγ production in protected PfP and WT TG cultures (medium alone) was identical, however in cultures that reactivated, IFNγ content in PfP cultures was greater compared to WT (data not shown). IFNγ production peaked around 8 days after culture initiation but declined steadily thereafter. Although IFNγ production in WT and PfP cultures was identical at 8 days, the reactivation frequency was higher in PfP compared to WT cultures at the same time (Compare Figure 36 and 35).

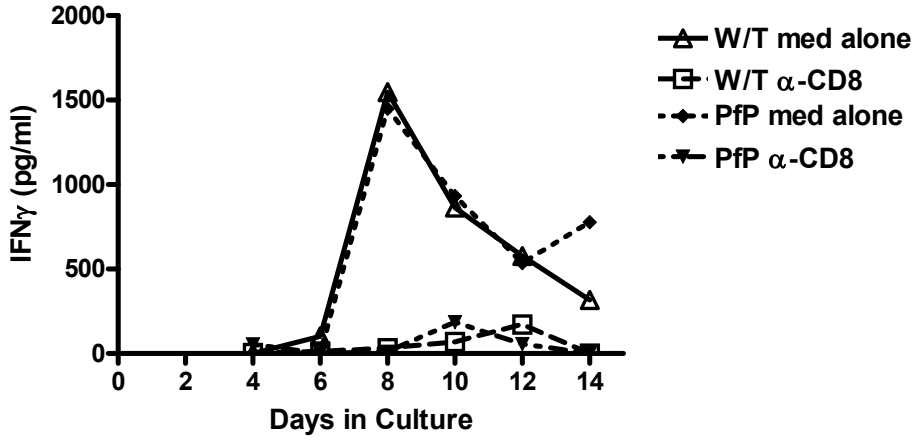

Figure 36: IFNγ production in PfP and WT cultures day 34 PI. *At the indicated times, supernatant fluids from the protected cultures (WT med alone and PfP med alone) described in Figure 35 at the indicated time points after culture initiation were combined and tested for IFNγ content by ELISA. TG cell cultures were treated with ACV (50 μg/ml) for 4 days, rinsed and then incubated for an additional 10 days in medium alone or anti-CD8 mAb (100 μg/ml).*

Data in Figure 36 also confirms that the main source of IFNγ in the WT and PfP cultures is $CD8^+$ T cells given that addition of anti-CD8 mAb completely abrogated the production of IFNγ in both types of cultures.

4.6. Role of IL-2 and IL-15 in modulating effector and memory $CD8^+$ T cell responses in a latent viral infection

Background and Rationale:

In response to viral infection, antigen specific effector $CD8^+$ T cells migrate to non-lymphoid tissues irrespective of the presence of antigen, where they are subsequently retained as effector memory cells (81,161). There are myriad of factors that can contribute to the migration and maintenance of memory T cells in non-lymphoid tissues including: (i) antigen persistence; (ii) chemokines and cell adhesion molecules; and (iii) cytokines.

We have shown that after HSV-1 corneal infection of mice there is a substantial infiltration of $CD8^+$ T cells in the TG. The highest density of $CD8^+$ T cells was present in the ipsilateral ganglion 8 days p.i., the population declined through day 34 p.i., and then a constant pool of $CD8^+$ T cells was maintained for the life of the animal (33). Both in situ and ex vivo tetramer staining revealed that 60% of the $CD8^+$ T cells that were retained in the ganglion were specific for the immunodominant epitope ($gB_{498-505}$) on the γ1 gene product gB. All $CD8^+$ T cells were $CD44^{hi}$ and surprisingly 80% expressed CD69. CD69 expression was greater among gB specific $CD8^+$ T cells. These data indicate that the HSV-1 specific $CD8^+$ T cells present in the TG 30+ days post infection are memory cells belonging to the effector memory T cell population.

The $CD8^+$ T cell response observed in lymphoid organs of mice infected with LCMV and vesicular stomatitis virus (VSV) (77,78,81) is characterized by: (i) initiation of the immune response; (ii) clonal expansion of viral specific $CD8^+$ T cells; (iii) a substantial reduction of effector $CD8^+$ T cells; and (iv) formation of a stable memory pool. The $CD8^+$ T cell response that develops in the TG after HSV-1 corneal infection follows a similar course, with the following exception: (i) virus can be readily detected in the TG, albeit in a latent state; and (ii)

virtually all the memory $CD8^+$ T cells in the TG retain CD69 expression, and exhibit TCR polarization to junctions with neuron cell bodies. Together these findings suggest preferential TCR stimulation through direct contact with infected neurons. These observations beg the question: what are the mechanisms that mediate the infiltration, expansion and retention of effector and memory $CD8^+$ T cells in the TG and lymphoid organs? Cytokines such as IL-2 and IL-15 participate in the differentiation of naïve $CD8^+$ T cells into effector and subsequently memory $CD8^+$ T cells. IL-2 and IL-15 belong to family of cytokines that use the common cytokine receptor γ chain (γc-chain). IL-15 and IL-2 receptors are comprised of three components, the high affinity receptor (α-chain), the relatively low affinity receptor CD122 (β-chain) and the γc chain. The α-chain chain for each cytokine is unique where as the β and γc-chains are shared (93). While the binding of each cytokine is controlled by the α and β-chain, signal transduction is mediated by β and γc chains.

Based primarily on in vitro studies, IL-2 thought to be an essential growth factor for T lymphocytes following antigenic stimulation (162). However, cell expansion appears to be normal in $IL\text{-}2^{-/-}$ and $IL\text{-}2R^{-/-}$ mice following antigenic challenge, and these mice actually develop severe lymphoproliferative diseases (163,164,165). There are conflicting findings regarding the role of IL-2 in $CD8^+$ T cell expansion and in the generation and maintenance of $CD8^+$ T cell memory. In some studies IL-2 was essential for initial $CD8^+$ T cell expansion (166,97), where as other studies have dismissed the role of IL-2 in $CD8^+$ T cell proliferation (163, 167). Using adoptive transfer of $IL\text{-}2^{-/-}$ and $IL\text{-}2R^{-/-}$ $CD8^+$ T cells, D'Souza et al. demonstrated that IL-2 was required for sustained expansion but not for the initiation of $CD8^+$ T cell cycling (99). Therapeutic use of IL-2 was recently tested at different stages of $CD8^+$ T cell response after LCMV infection (168). Interestingly, rIL-2 administration during the expansion

phase (1-7 days PI) accelerated the contraction of antigen specific $CD8^+$ T cells but did not influence the extent of initial T cell expansion or the size of the T cell memory pool. In contrast, IL-2 increased the size of the memory pool when administered during the contraction and memory phases. In contrast, in vivo treatment with anti-IL2 antibody was beneficial to memory $CD8^+$ T cells, demonstrating negative effects of IL-2 on memory cells (95).

Another member of the γc family of cytokines is IL-15, which has many important immunological properties. Studies with IL-$15^{-/-}$ and IL-$15R^{-/-}$ mice have clearly implicated IL-15 as essential for memory $CD8^+$ T cell maintenance and survival (100,169,170). IL-15 mediates low-level memory $CD8^+$ T cell division; thus, preventing the attrition of the memory T cell pool long after virus is cleared from the body. The primary sources of IL-15 are macrophages, monocytes and dendritic cells, in addition IL-15 mRNA is found in many non-lymphoid tissues. Besides being a T cell growth factor recent human studies have implicated IL-15 as a potent chemoattractant for T cells as well as an effective inducer of antibody production by B cells (171,172). The beneficial properties of IL-15 on antigen specific memory $CD8^+$ T cells is well established, however its role in $CD8^+$ T cell expansion early after a viral infection is not well understood. The role of IL-15 in primary $CD8^+$ T cell expansion has been dismissed, given that mice lacking IL-15-Rα or IL15 were able to mount efficient primary $CD8^+$ T cell response to acute viral infections (173,169,100), although, another study implicated IL-15 in early expansion of alloreactive $CD8^+$ T cells in vivo (174).

Each of these cytokines may have a distinct function at different stages of a $CD8^+$ T cell response to viral infections. At some stage IL-2 and IL-15 may work in concert while in others their roles may contrast (174,95). The role of IL-2 and IL-15 in modulating $CD8^+$ T cell responses to persistent and latent viral infection in non-lymphoid tissues in vivo is poorly

understood. Unlike the majority of the viral mouse models that use acute systemic viral infections to study IL-2 and IL-15 functions, HSV-1 is a localized infection, where the virus establishes latency in sensory ganglia and is never cleared from the body. In the next set of studies we wished to determine how IL-2 and IL-15 contribute in shaping $CD8^+$ T cell response at different stages of HSV-1 infection and whether the presence of latent virus influences the functions of these cytokines.

Results:

4.6.1. IL-15 but not IL-2 is essential for initial expansion of viral specific and antigen independent $CD8^+$ T cells in the LN

Foot pad infection with HSV-1 has revealed that the magnitude of $CD8^+$ T cells response in draining LN is highest at 5 days PI (124). To examine the effect of IL-15 on T cell expansion in LN after HSV-1 corneal infection, WT and IL-$15^{-/-}$ mice were infected with HSV-1 and sacrificed at day 5. Draining submandibular LN were extracted and single cell suspensions were stained for CD8, CD4 and gB specific TCR. $CD8^+$ T cell expansion in LN was dramatically reduced in IL-$15^{-/-}$ mice, however $CD4^+$ T cell expansion was not affected by the lack of IL-15 (Figure 37A). Similarly, gB specific $CD8^+$ T cells were also reduced in IL-$15^{-/-}$ mice (Figure 37B). These data suggest that IL-15 is required for early expansion of antigen specific and non specific $CD8^+$ T cells, suggesting a role of IL-15 in bystander activation of naïve or memory $CD8^+$ T cells.

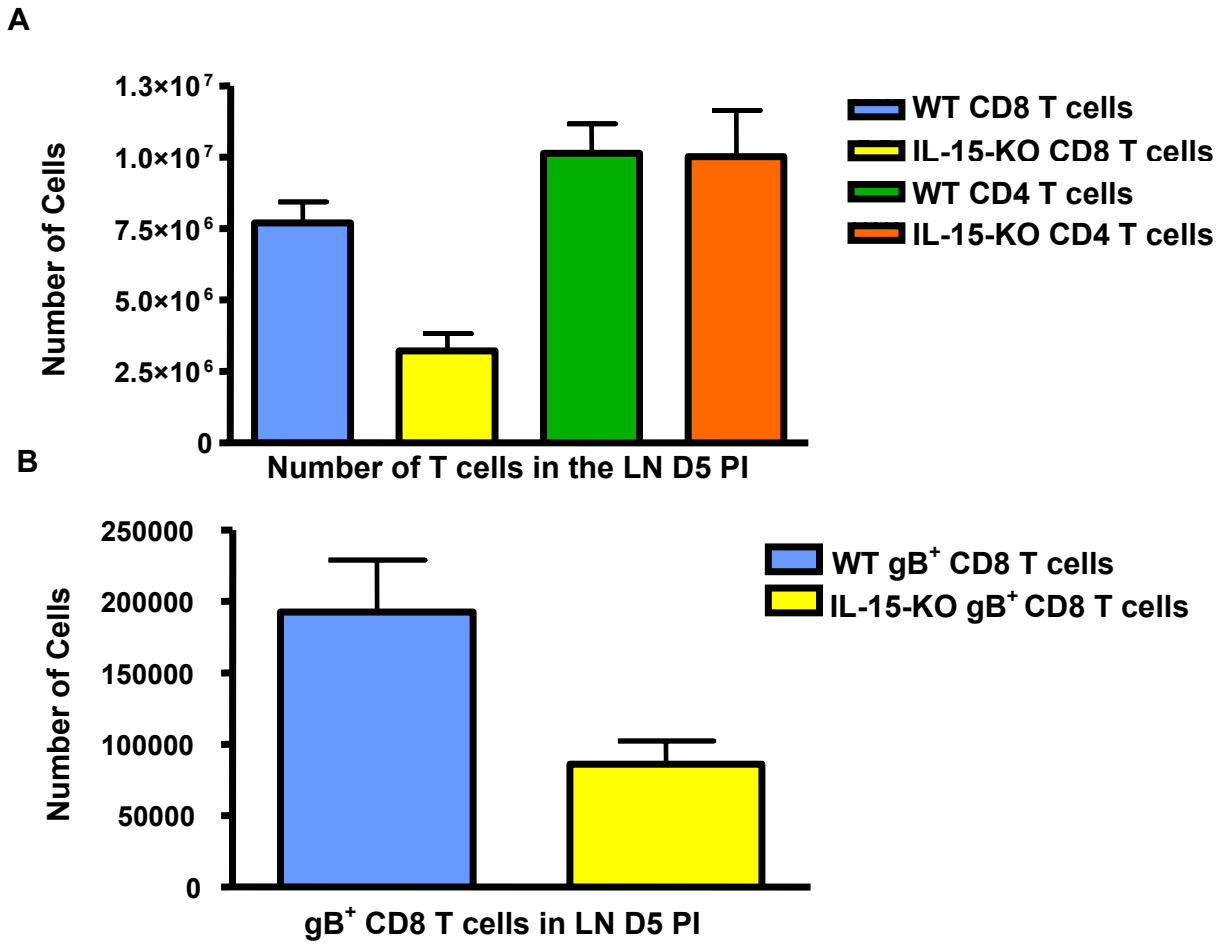

Figure 37: Primary expansion of lymphoid CD8$^+$ T cells 5 days after HSV-1 corneal infection is dependent on IL-15. *LN cells from WT or IL-15$^{-/-}$ mice 5 days PI were stained for CD8, CD4 (A) or gB specific TCR (B). Mean percent ± SEM (n=3) **P=0.0097. A non parametric Student t test was performed to determine the statistical significance.*

4.6.2. IL-15 and IL-2 are both essential for regulating primary T cell responses in the TG:

Our previous studies have shown that CD8$^+$ T cells reach their peak density at 8 days after corneal infection (Figure 12), therefore to determine the period of greatest CD8$^+$ T cell expansion we compared the magnitude of CD8$^+$ T cell proliferation in the TG between 6 and 8 days PI. Figure 38 shows that number of CD8$^+$ T cells in WT mice increase more than 7 fold between day 6 and 8 after corneal infection. CD8$^+$ T cell

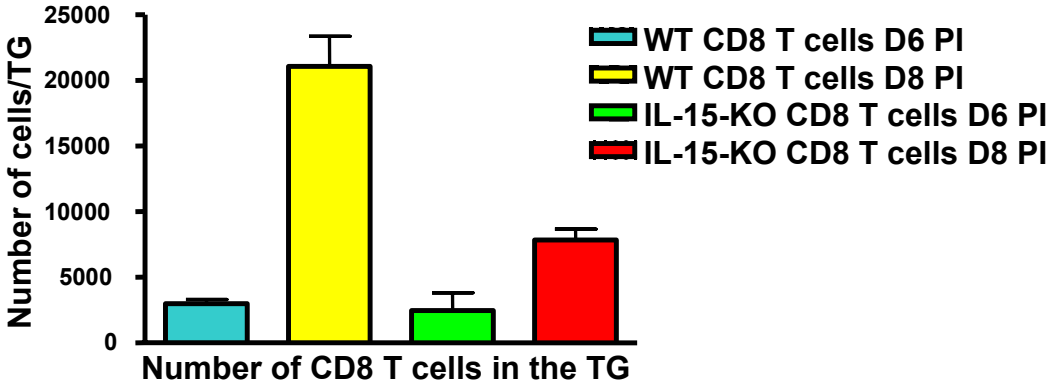

Figure 38: Number of ganglionic CD8$^+$ T cells increase by 7 fold between 6 and 8 days PI. *Single cell suspensions of TG obtained from C57BL/6 (WT; n=3) and IL-15$^{-/-}$ (IL-15-KO; n=3) mice 6 and 8 days (WT; n=6, and IL-15-KO; n=9) after corneal infection were stained for CD8. Total number of CD8$^+$ T cells in each TG was determined by running the entire single cell suspension of each TG through the flow cytometer. Error bars indicate mean percent ± SEM.*

infiltrate at 6 days after corneal infection in WT and IL-15$^{-/-}$ mice was similar; however the increase in CD8$^+$ T cell number between day 6 and 8 was severely compromised in IL-15$^{-/-}$ mice.

To determine if IL-2 and IL-15 regulate ganglionic primary T cell responses *in vivo*, WT and IL-15$^{-/-}$ mice were either untreated or treated with 1mg anti-IL-2 mAb at day 6 after corneal infection and sacrificed at 8 days PI. TGs from each mouse in the indicated groups were extracted and single cell suspensions were stained for CD8, CD4, CD45 and MHC class-I dimers were used to identify HSV-1 gB-specific CD8$^+$ T cells. Compared to WT untreated mice the magnitude of CD8$^+$ and CD4$^+$ T cell responses in the TG 8 days after corneal infection was significantly compromised in IL-15$^{-/-}$ mice as well as anit-IL-2 treated mice (Figure 39A). Untreated mice and mice treated with isotype matched control antibody (for anti-IL-2 mAb) showed no difference in CD8$^+$ or CD4$^+$ T cell sponses in the TG or LN (data not shown). Therefore as a control, a subset of mice in all subsequent experiments were left untreated.

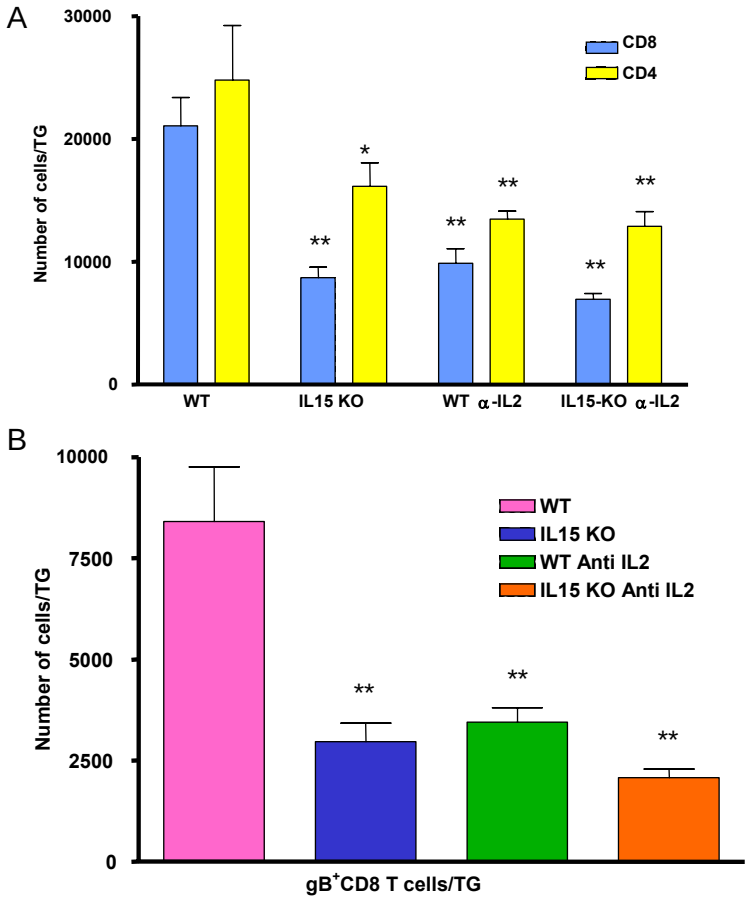

Figure 39: IL-15 and IL-2 are both required for adequate T cell responses in the TG at 8 days PI. *C57BL/6 or IL-15$^{-/-}$ mice mice were either untreated (WT; n=6, IL15 KO; n=9) or treated with anti-IL-2 mAb (1mg) IP (WT Anti IL2; n=9, IL15 Anti-IL2; n=9), at day 6 PI. Single-cell suspensions of TG obtained from mice 8 days after HSV-1 corneal infection were simultaneously stained for CD45, CD4 and CD8 (A) or CD45, CD8 and gB-specific TCR (B). For each reaction single cell suspensions from every individual TG was stained and all the events from each TG were collected. Forward and side scatter gates were set to encompass the CD45 population (A), or CD8^{+} population (B). Significance of differences between WT and all indicated groups were determined by one way ANOVA (non-parametric) analysis with Bonferroni post test comparing all groups together. *P<0.01 and **P<0.001, error bars indicate mean percent ± SEM. The above graph represents combined data of 3 independent experiments.*

MHC class-I dimers loaded with HSV-1 gB$_{498\text{-}505}$ peptide were used to determine the effect of IL-15 and IL-2 on antigen specific CD8^{+} T cells. As shown in Figure 39B gB specific

$CD8^+$ T cells were reduced by more than 70% in IL-$15^{-/-}$ and in anti-IL-2 treated mice. Interestingly, the diminution in the magnitude of viral specific $CD8^+$ cell response was consistently greater in IL-$15^{-/-}$ mice treated with anti-IL-2 antibody (Figure 39B; compare IL-15-KO Anti-IL-2 group with WT Anti-IL-2 and IL-15-KO groups), suggesting an additive effect of blocking both cytokines together as opposed to individually *in vivo*. Thus, data in Figure 39 indicate that IL-2 and IL-15 are important in mediating primary viral specific as well as antigen independent $CD8^+$ T cell responses in latently infected non-lymphoid organs such as the sensory ganglion.

4.6.3. IL15 but not IL2 is required for primary $CD8^+$ T cell response in LN at 8 days after HSV-1 corneal infection

To determine if IL-2 and IL-15 influence primary lymphoid $CD8^+$ T cell response *in vivo*, WT and IL-$15^{-/-}$ mice were either left untreated or treated with 1mg anti-IL-2 mAb at day 6 after corneal infection and sacrificed at 8 days PI. Single cell suspension of LN were stained for CD8 and gB specific TCR. Number of $CD8^+$ T cells in the lymph nodes was dramatically reduced in IL-$15^{-/-}$ mice but the number of $CD8^+$ T cells in anti IL-2 treated mice was similar to the control (Figure 40A). The number of antigen specific $CD8^+$ T cells in the lymph nodes was also negatively affected by the lack of IL-15 (Figure 40B); however the reduction was not as impressive as the reduction in total number of antigen independent $CD8^+$ T cells. The data in Figure 40 and 37 suggest the requirement of IL-15 in the bystander activation of antigen independent naïve or memory lymphoid $CD8^+$ T cells as well as the early expansion of antigen specific lymphoid $CD8^+$ T cells after HSV-1 corneal infection. In all three experimental groups of mice the expansion of $CD4^+$ T cells in the LN was not affected (data not shown), suggesting

neither IL-15 nor IL-2 regulate early lymphoid $CD4^+$ T cell responses of mice infected with HSV.

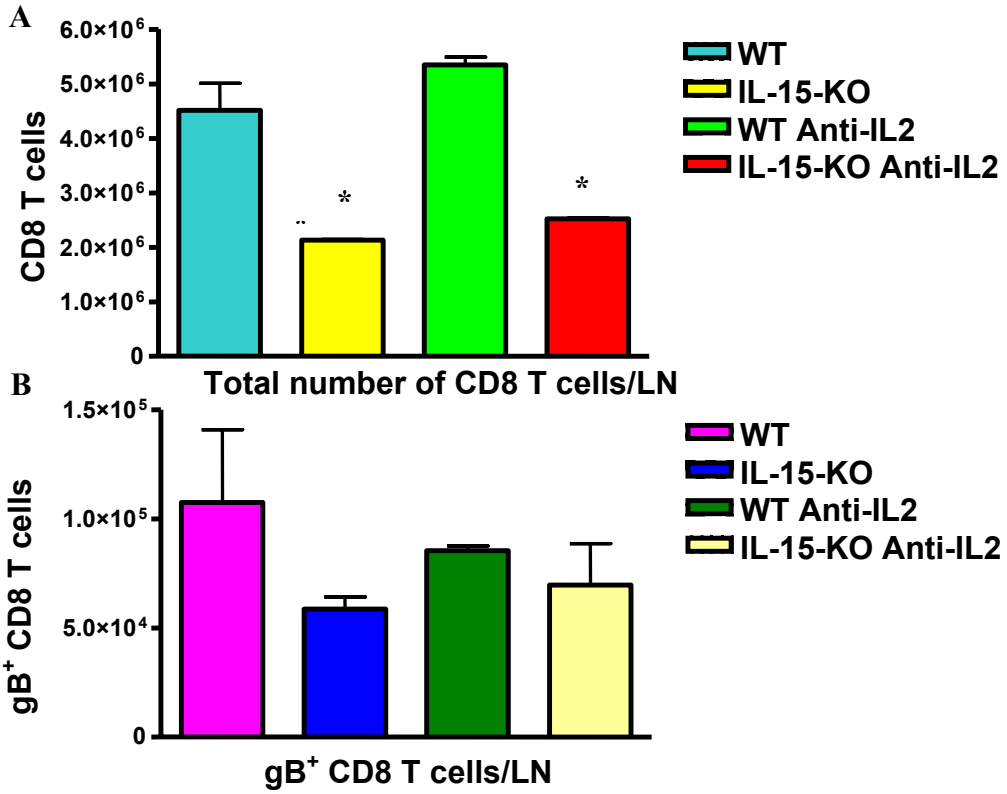

Figure 40: IL15 but not IL2 is required for primary $CD8^+$ T cell response in the LN 8 days after HSV-1 corneal infection. *C57BL/6 or IL-$15^{-/-}$ mice were either untreated (WT; n=3, IL15 KO; n=3) or treated with anti-IL-2 mAb (1mg) IP (WT Anti IL2; n=3, IL15 Anti-IL2; n=3), at day 6 PI. Single-cell suspensions of LN obtained from mice 8 days after HSV-1 corneal infection were simultaneously stained for CD8 (A) or CD8 and gB-specific TCR (B) Significance of differences between WT and all indicated groups were determined by one way ANOVA (non-parametric) analysis with Bonferroni post test comparing all groups together. *P<0.01, error bars indicate mean percent ± SEM. The above graph is a representative of 2 independent experiments.*

Judging from the data acquired from experiments done with LN and TG, it appears that IL-15 and IL-2 can exert their effects on T cells differentially depending on the location of T cells in the body.

4.6.4. IL-15 drives optimal proliferation of antiviral and antigen independent $CD8^+$ T cells in the LN

To confirm that the reduction in $CD8^+$ T cell numbers in the lymph nodes at D8 PI was the result of a lack of proliferation, mice in each group were treated with BrdU at 6 and 7 Days PI. Flow cytometric analysis was performed at 8 days PI (Figure 41A). Incorporation of BrdU in $CD8^+$ T cells between day 6 and 8 PI was considered an indication of cell division during the short 48 hour period. In IL-$15^{-/-}$ mice only 50% of the $CD8^+$ T cells incorporated BrdU between day 6 and 8 after HSV-1 corneal infection, which correlated well with the reduction in absolute number of $CD8^+$ T cells in the LNs (Figure 41A.) As expected blocking IL-2 in WT mice showed no effect as the percentage of $CD8^+$ T cells that were BrdU positive was similar to WT untreated mice. The percentage of viral specific $CD8^+$ T cells that incorporated BrdU between day 6 and 8 PI was also reduced significantly in IL-$15^{-/-}$ mice (Figure 41B)**.** As anticipated from data in Figure 40 we did not observe any greater reduction in BrdU positive $CD8^+$ T cells in IL-$15^{-/-}$ mice treated with anti-IL-2 mAb compared to untreated IL-$15^{-/-}$ mice, confirming that IL-15 and not IL-2 drives the turnover of both viral specific and antigen independent $CD8^+$ T cells in LN early after HSV-1 corneal infection.

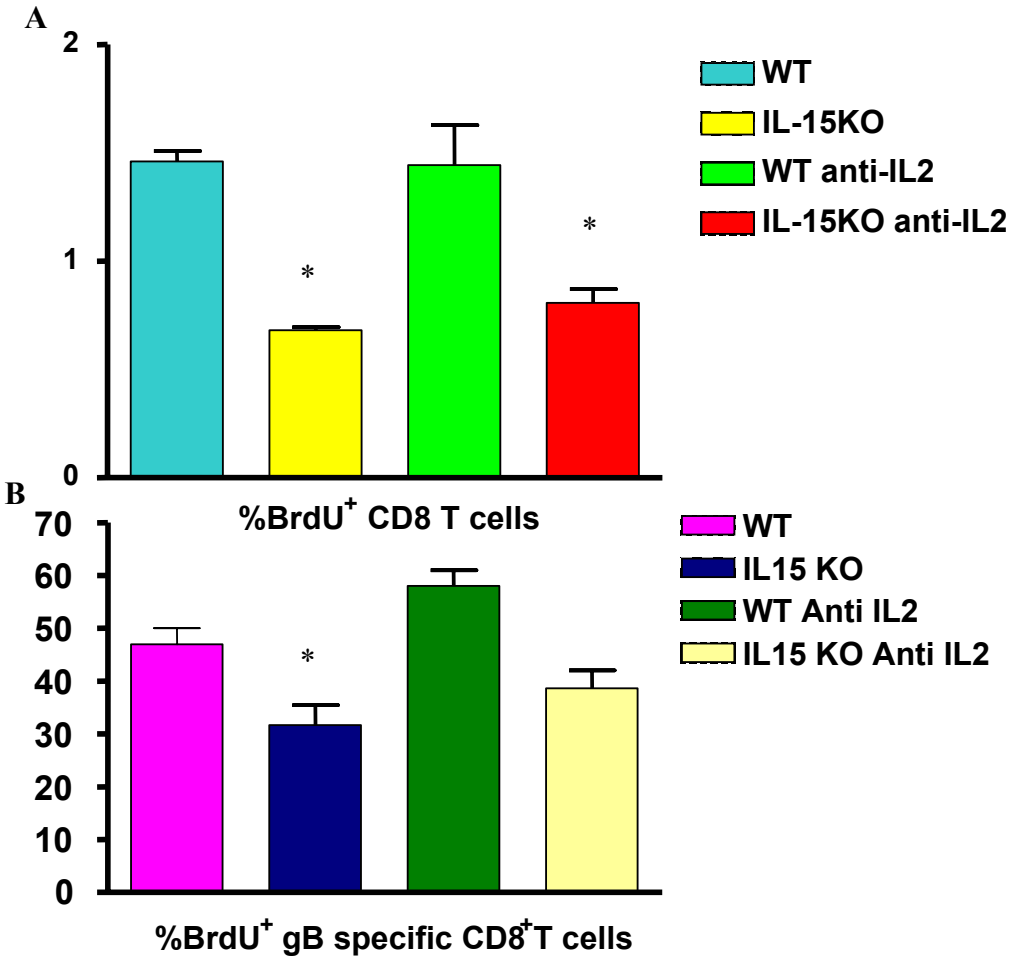

Figure 41: The reduction in number of lymphoid CD8$^+$ T cells in IL15-KO mice at 8 days PI is a result of poor proliferation. *Mice in each indicated groups were administered 1mg of BrdU IP at day 6 and 7 after corneal infection. C57BL/6 and IL-15$^{-/-}$ mice were either left untreated or treated with 1mg anti-IL-2 mAb at day 6 PI. Single-cell suspensions of LN obtained from mice 8 days after HSV-1 corneal infection were simultaneously stained for CD8, gB-specific TCR and incorporated BrdU. Percentage of total number of CD8$^+$ T cells that incorporated BrdU between day 6 and 8 PI (A), and percentage of gB-specific CD8$^+$ T cells that incorporated BrdU between day 6 and 8 PI (B). Significance of differences between WT and all indicated groups were determined by one way ANOVA (non-parametric) analysis with Bonferroni post test comparing all groups together. *P<0.01, error bars indicate mean percent ± SEM. For all indicated groups n=3.*

4.6.5. Neither IL-15 nor IL-2 mediates primary $CD8^+$ T cell proliferation in TG

To determine if the dysregulation in the level of $CD8^+$ T cells response in the TG of IL-$15^{-/-}$ mice, and mice treated with anti-IL2 mAb was due to a decrease in proliferation (as seen in the LN); mice in each experimental group were given BrdU interperitoneally at day 6 and 7 PI and sacrificed at 8 days after HSV-1 corneal infection. Ganglionic $CD8^+$ T cells that incorporated BrdU were considered to have recently divided between days 6 and 8 PI; however the possibility that some $CD8^+$ T cells may have divided outside of the TG (i.e. LN) during the 48 hr treatment with BrdU and migrated to the TG cannot be completely eliminated.

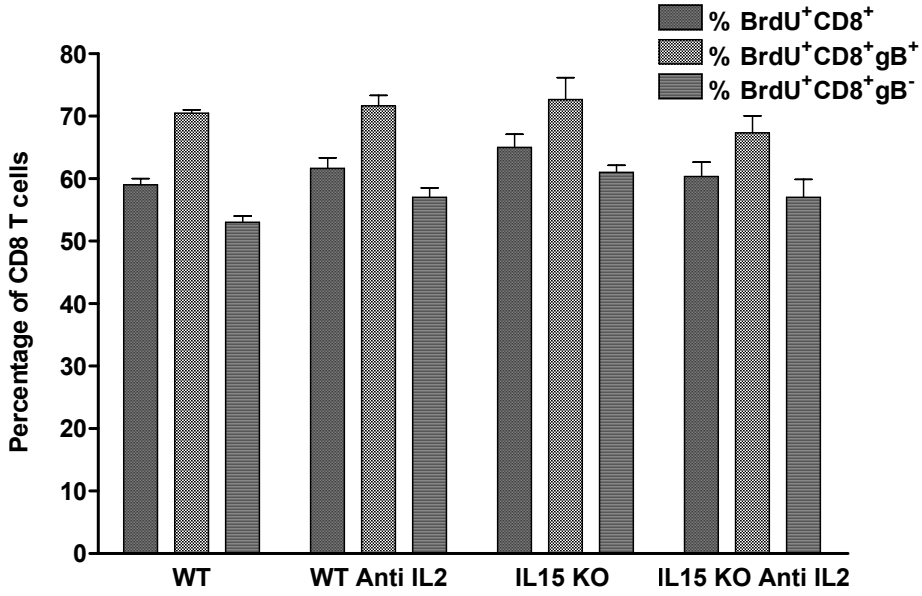

Figure 42: The dysregulation in the level of $CD8^+$ T cells response in the TG was not due to a decrease in proliferation. *BrdU incorporation in ganglionic $CD8^+$ T cells was determined in the same mice in Figure 41. Single-cell suspensions of TG obtained from mice (the same mice as in figure 42) 8 days after HSV-1 corneal infection were simultaneously stained for CD8, gB-specific TCR and incorporated BrdU. Total number of $CD8^+$ T cells that were BrdU positive ($BrdU^+CD8^+$), gB-dimer positive CD8 T cells that were BrdU positive ($BrdU^+CD8^+gB^+$), and gB-dimer negative CD8 T cells that were BrdU positive ($BrdU^+CD8^+gB^-$) are shown.*

As shown in Figure 42 the lack of IL-2 or IL-15 did not affect the proliferation of gB-dimer^{+} or gB-dimer^{-} CD8^{+} T cells suggesting neither of the cytokines drive CD8^{+} T cell turnover during the lytic phase of HSV-1 infection in sensory ganglion. The reduction in CD8^{+} T cell expansion between day 6 and 8 PI in the TG of virally infected IL-15$^{-/-}$ mice could be at the level of priming in the LN as CD8^{+} T cell proliferation in the LN of IL-15$^{-/-}$ mice was compromised. However, the reduction in CD8^{+} T cell expansion in anti-IL2 treated mice still remains inexplicable. Notably the highest percentage of BrdU incorporation was seen in gB-specific CD8^{+} T cells indicating preferential proliferation of viral specific cells but surprisingly more than 50% of gB-dimer^{-} CD8^{+} T cells incorporated BrdU, suggesting an unusually high turnover of apparently antigen independent CD8^{+} T cells in the TG. We did not observe proliferation of subdominant population of RR1 specific CD8^{+} T cell (data not shown) in any groups of mice suggesting the likelihood of substantial bystander activation of CD8^{+} T cell in the TG.

4.6.6. Do IL-15 and IL-2 mediate lymphoid or non-lymphoid CD8^{+} T cell survival early after HSV-1 corneal infection?

Since IL-15 and IL-2 can promote T cell survival, we wished to determine if the reduction in primary CD8^{+} T cell response in the TG of IL-15$^{-/-}$ mice and mice treated with anti-IL-2 mAb was a result of increased cell death. Therefore we tested the apoptotic potential of ganglionic CD8^{+} T cells at 8 days PI in vivo by using the annexin-V assay. One of the earliest events in a cell undergoing apoptosis is the exposure of phosphatidylserine (PS) on the surface of the cell, annexin-V binds to PS with high affinity and can be used to identify early apoptotic cells (175). More than 40% of ganglionic CD8^{+} T cells in all groups of mice were annexin-V positive indicating no increase in cell death in mice lacking IL-15 or IL-2 (Figure 43). Cell death in viral specific CD8^{+} T cells was also similar in all groups of mice tested (data not shown). However, at

8 days PI it may already be too late to detect early apoptotic cells in the TG, hence the assay will have to be repeated earlier i.e. 6 or 7 days after HSV-1 corneal infection. These data show that blocking IL-2 neither compromised primary $CD8^+$ T cell proliferation nor increased cell death in the TG of mice infected with HSV-1.

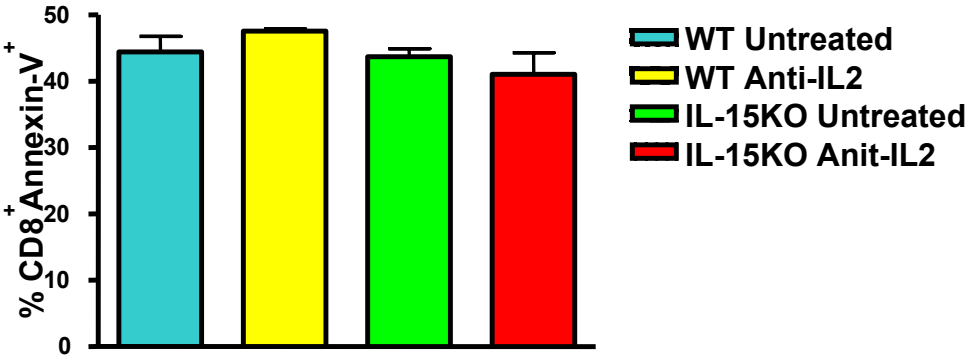

Figure 43: Apoptotic potential of $CD8^+$ T cells in the TG at 8 days after HSV-1 corneal infection. *Apoptosis of ganglionic $CD8^+$ T cells from the indicated groups of mice was determined by annexin-V binding.*

We next examined annexin-V binding on $CD8^+$ T cells in the LNs of mice at 8 days PI. To our surprise percentage of $CD8^+$ T cells that were annexin-V positive was significantly higher in IL-$15^{-/-}$ mice that received anti-IL2 antibody compared to all the other 3 groups of mice (Figure 44B). A similar pattern of apoptosis was detected in gB specific $CD8^+$ T cells (data not shown). Lymphoid $CD8^+$ T cell apoptosis was elevated in all groups of mice compared to WT untreated mice. The apoptosis data in Figure 44 do not however correlate well with the absolute number of $CD8^+$ T cells present in the LN at 8 days PI, for instance we did not observe any difference in lymphoid $CD8^+$ T cell numbers between anti-IL2 treated and untreated IL-$15^{-/-}$ mice (Figure 41)

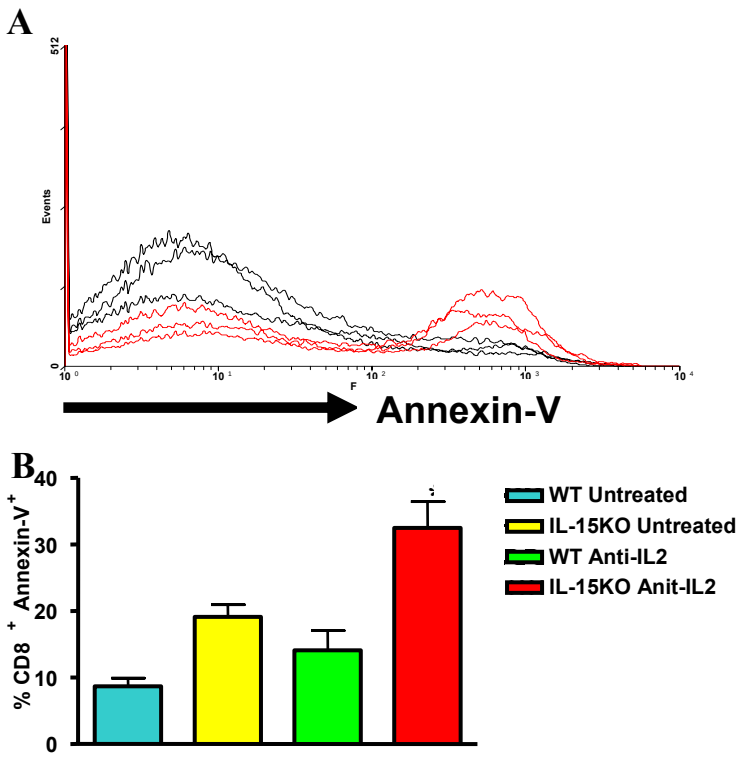

Figure 44: Apoptotic potential of CD8$^+$ T cells in the LN at 8 days after HSV-1 corneal infection. *Apoptosis of Lymphoid CD8$^+$ T cells from the indicated groups of mice was determined by annexin-V binding. Histogram demonstrating the intensity of annexin-V binding on lymphoid CD8$^+$ T cells isolated from anti-IL-2 treated (1mg at day 6PI) IL-15$^{-/-}$ (red lines) and WT untreated (black lines) at 8 days PI (A). Each line in the histogram represents 1 mouse with n=3 for each group. Percentage of annexin-V positive CD8$^+$ T cells in the LN of the indicated groups of mice at 8 days PI (B). Significance of differences between anti-IL-2 treated IL-15$^{-/-}$ mice and all indicated groups were determined by one way ANOVA (non-parametric) analysis with Bonferroni post test comparing all groups together. *P<0.01, error bars indicate mean percent ± SEM. For all indicated groups n=3.*

Since annexin-V binding is a sign of early apoptosis, the higher level of CD8$^+$ T cell apoptosis observed in anti-IL-2 treated IL-15$^{-/-}$ mice indicates that a lack of IL-2 and IL-15 together can accelerate the contraction phase of CD8$^+$ T cell response in the LN of mice infected with HSV-1.

4.6.7. IL-15 is Essential for the Formation of Effector and Central Memory $CD8^+$ T cell Pool after HSV-1 Corneal Infection

IL-15 has been shown to regulate homeostatic proliferation of memory $CD8^+$ T cells in lymphoid organs (169,100), on the other hand IL-2 has been determined to be a negative (95) as well as a positive regulator of lymphoid memory $CD8^+$ T cells (168). HSV-1 infection provides us with a unique model to study how cytokines influence lymphoid and extra-lymphoid memory T cells. It would be interesting to determine how would the presence of latent virus as well as the possibility of infrequent antigen presentation in the TG (29,33) affect the ability of IL-2 and IL-15 to regulate central and effector HSV-1 specific memory $CD8^+$ T cells. To this end, we repeated the above experiments at 65 days after HSV-1 corneal infection. WT and IL-$15^{-/-}$ mice were either treated with control or anti-IL-2 antibody at 6 days and sacrificed at 65 days after HSV-1 corneal infection. The effector/memory $CD8^+$ T cell pool in the TG was considerably smaller in control as well as anti-IL-2 antibody treated IL-$15^{-/-}$ mice compared to both groups of WT mice (Figure 45). Notably, the memory $CD8^+$ T cell pool in the TG of anti-IL-2 treated WT mice was similar to control WT mice suggesting that effector memory $CD8^+$ T cell pool was not affected even though primary $CD8^+$ T cell response (Day 8 PI; Figure 39) in the TG of anti-IL-2 treated WT mice was markedly compromised. Viral specific splenic memory $CD8^+$ T cell pool (Central memory cells) was also noticeably reduced in IL-$15^{-/-}$ mice.

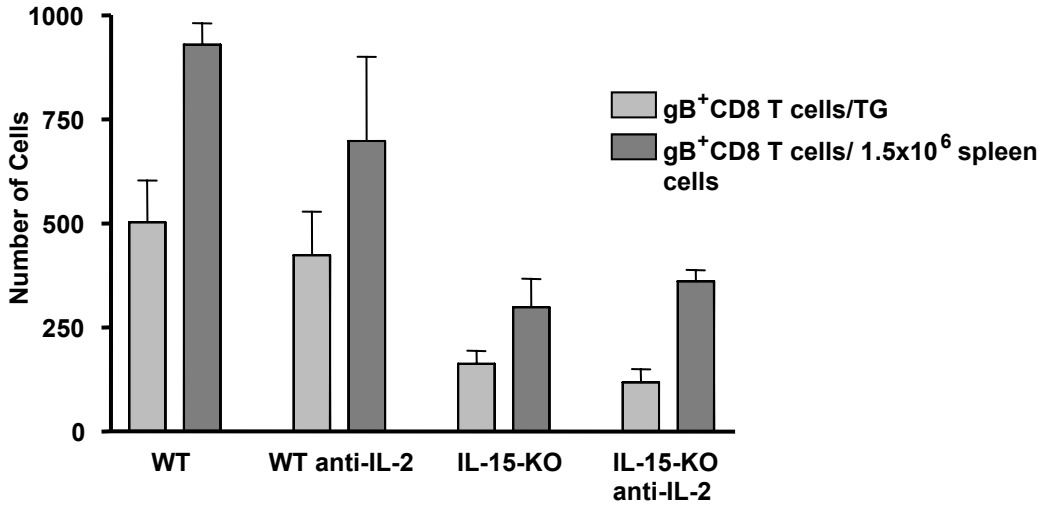

Figure 45: IL-15 is required for the maintenance of viral specific memory CD8$^+$ T cells. *C57BL6 mice were treated with 1mg control (WT) or 1mg anti-IL-2 antibody (WT anti-IL-2). IL-15$^{-/-}$ mice were were treated with 1mg control (IL-15-KO) or 1mg anti-IL-2 antibody (IL-15-KO anti-IL-2) at 6 days PI. Mice were sacrificed at 65 days PI, Single-cell suspensions of TG and spleen were stained for CD8, and gB-specific TCR. The data shows total of number of gB-specific CD8$^+$ T cells cells per TG or 1.5 x 10^6 spleen cells. Error bars indicate mean percent ± SEM. For all indicated groups n=3.*

In Figure 14 we demonstrated that more than 80% of the CD8$^+$ T cells retained in the TG as late as 84 days PI expressed the early activation marker CD69. There have been several recent reports implicating the role of IL-15 in upregulating CD69 on T cells and B cells (176-180), yet we did not observe any reduction in the percentage of CD69$^+$ CD8 T cells in the TG of IL-15$^{-/-}$ mice or anti-IL-2 treated mice at any time points after the establishment of latency (data not shown).

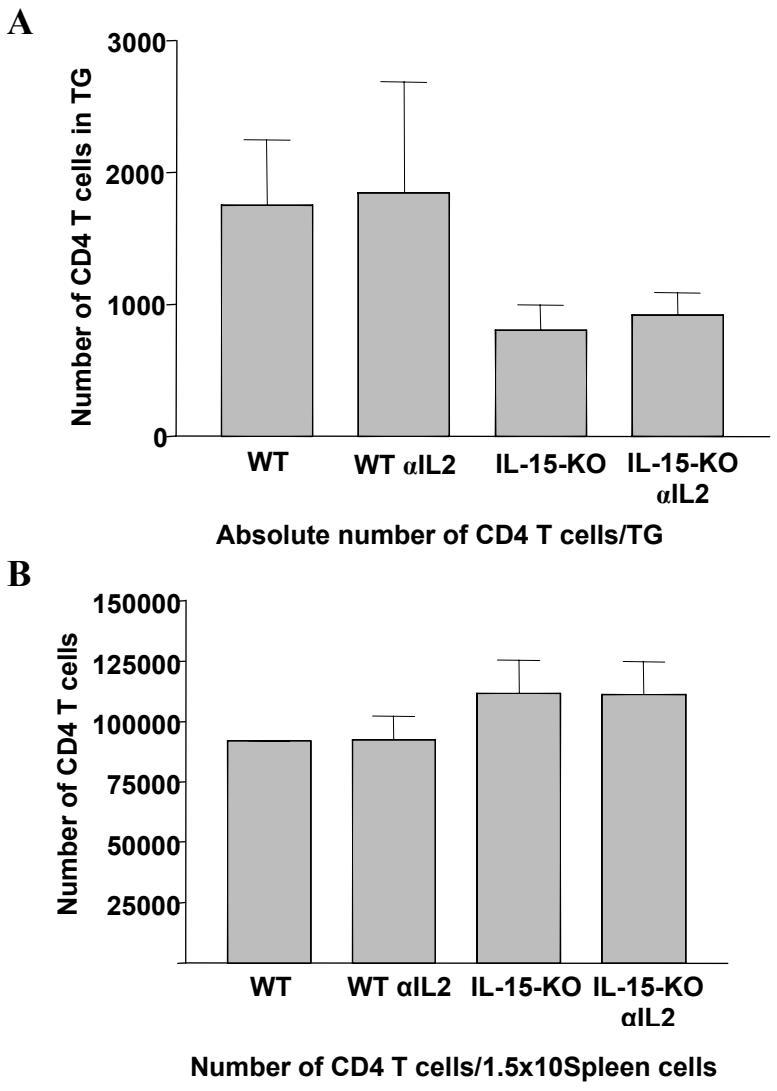

Figure 46: IL-15 is required for the maintenance of non-lymphoid memory CD4$^+$ T cells. *C57BL6 mice were treated with 1mg control (WT) or 1mg anti-IL-2 antibody (WT anti-IL-2). IL-15$^{-/-}$ mice were were treated with 1mg control (IL-15-KO) or 1mg anti-IL-2 antibody (IL-15-KO anti-IL-2) at 6 days PI. Mice were sacrificed at 65 days PI, Single-cell suspensions of TG and spleen were stained for CD4, and CD45. The data shows total of number of CD4$^+$ T cells cells per TG (A) or 1.5 x 10^6 spleen cells (B). Error bars indicate mean percent ± SEM. For all indicated groups n=3.*

We also determined if $CD4^+$ T cell responses in the TG as well as in secondary lymphoid organs were affected by the lack of IL-15 or (and) IL-2 at 65 days after HSV-1 corneal infection. Number of $CD4^+$ T cells retained in the TG at 65 days PI was markedly reduced in IL-$15^{-/-}$ mice (Figure 46A) compare to WT controls, where as number of $CD4^+$ T cells in the spleen was unaffected by the lack of IL-2 or IL-15 (Figure. 46B). This result is contrary to other studies that have found no evidence that proliferation or survival of $CD4^+$ T cells is influenced by IL-15 (102,170,181). However these studies only examined the effect of IL-15 on lymphoid and splenic $CD4^+$ T cells. Therefore contrary to lymphoid $CD4^+$ T cells, effector/memory $CD4^+$ T cells (those retained in the TG) depend on IL-15 for their proliferation and (or) survival after HSV-1 infection.

5. DISCUSSION

The contents of sections 5.1 and 5.2 have been modified from articles (33,73). Copyright permission is on file.

5.1. HSV-1 Specific Memory $CD8^+$ T cells are selectively Retained in Latently Infected TG and can Regulate Viral Gene Expression.

Our current findings challenge the notion that HSV-1 latent infection in sensory ganglia represents a quiescent state wherein a lack of viral gene expression conceals the virus from the host immune system. Instead, we establish that a dynamic HSV-specific $CD8^+$ T cell response is maintained in the TG for at least 75 days after HSV-1 latency is uniformly established. Several lines of evidence support the notion that HSV-specific $CD8^+$ T cells are persistently stimulated within the latently infected ganglion. First, at least 60% of the $CD8^+$ T cells in the latently infected TG were HSV-specific as demonstrated by binding to the $gB_{498-505}$ tetramer *ex vivo*. Second, during the early stages of $CD8^+$ T cell infiltration into the infected TG, a time when virus replication was subsiding and latency was being established in sensory neurons, few $CD8^+$ T cells expressed the early activation marker CD69. However, CD69 expression on ganglion-derived $CD8^+$ T cells gradually increased through day 34 p.i., long after latency was uniformly established in the TG. Moreover, CD69 expression was maintained for at least 84 days p.i. Third, the variable size and level of CD8 expression on the $CD8^+$ T cell population that initially infiltrated the TG (day 8 –14 p.i.) gradually gave way to a more homogeneous, large, $CD8^{hi}$ population during latency (day 34 – 84 pi), indicative of a shift to a more uniformly activated $CD8^+$ T cell population. Finally, *in situ* staining of latently infected TG with the $gB_{498-505}$ tetramer revealed TCR polarization on many $CD8^+$ T cells towards the T cell-neuron junction. This latter observation is suggestive of TCR aggregation in lipid rafts, as occurs during T cell

activation (182). Although there have been a few previous reports of *in situ* tetramer staining (143,144), we believe this is the first report of such staining following infection in a non-transgenic model. Together, these findings suggest that HSV gB-specific memory $CD8^+$ T cells are maintained in an activated state through a direct interaction with latently infected neurons that persistently or episodically express a portion of the viral genome including the γ_1 gene gB.

We also noted a significant increase from 14 – 34 days p.i. in the proportion of ganglion-derived $gB_{498\text{-}505}$ tetramer positive $CD8^+$ T cells that produced IFN-γ in response to $gB_{498\text{-}505}$ peptide-pulsed target cells. Their levels then remained constant through day 84 p.i. However, it should be noted that the total number of $CD8^+$ T cells in the TG dropped during the period of 14-34 days p.i., so that the actual number of IFN-γ^+, $gB_{498\text{-}505}$ peptide-specific $CD8^+$ T cells remained relatively constant. Thus, the increased proportion of IFN-γ-secreting cells could reflect either selective retention of a small population of cells capable of producing IFN-γ in response to gB peptide at day 14, or expansion of IFN-γ^+ $CD8^+$ T cells in the TG through cell division or a functional change. We tend to favor the theory that the number of IFN-γ-producing cells in the TG increases during latency, because we routinely detect significantly more IFN-γ production by one TG equivalent of day 34 TG than by one TG equivalent of day 14 TG in *ex vivo* cultures (data not shown). Other viral infection models have demonstrated a similar enrichment for IFN-γ producing $CD8^+$ T cells at sites of infection (148,183). Together these findings suggest that $CD8^+$ T cells that are retained at a peripheral site of infection undergo an antigen-driven maturation process resulting in enhanced IFN-γ production. Since IFN-γ can block HSV-1 reactivation from latency in sensory neurons, this functional shift would appear to produce a $CD8^+$ T cell population that is ideally suited to the function of long-term protection with minimal tissue destruction.

It was recently reported that IFN-γ and TNF-α expression persisted in TG that were latently infected with an HSV-1 mutant lacking viral thymidine kinase (Tk^- HSV-1) (22). Since Tk^- HSV-1 mutants are unable to replicate their DNA in neurons, these findings suggest that maintenance of cytokine production occurred in the absence of viral DNA synthesis within the ganglion. DNA synthesis is not only required for production of infectious virions, but is also necessary for expression of the γ_2 class of HSV-1 genes during a lytic infection. In contrast, the α, β, and γ_1 genes are expressed in the absence of viral DNA synthesis. Thus, it is reasonable to speculate that HSV α, β, and γ_1 genes may be expressed in latently infected neurons in the absence of viral DNA synthesis and γ_2 gene expression. Our current studies clearly establish that a $gB_{498-505}$ epitope-specific $CD8^+$ T cell clone (2D5) can block HSV-1 reactivation from latency in *ex vivo* TG cultures derived from syngeneic C57BL/6 mice, but not from allogeneic Balb/c mice. This result established that the protective response is MHC restricted, and thus involves TCR engagement by an MHC I/ $gB_{498-505}$ peptide complex. Although sensory neurons do not normally express detectable MHC, they do express MHC I during the lytic phase of HSV-1 infection (139). Thus a reactivation stimulus that overcomes repression of viral gene expression might result in concordant expression of MHC I, allowing rapid presentation of viral epitopes to $CD8^+$ T cells.

We show that virtually 100% of HSV-1–specific $CD8^+$ T cells in the latently infected TG at 34 days p.i. recognized the $gB_{498-505}$ epitope, and that all $gB_{498-505}$ tetramer positive cells were $CD69^+$. A similar immunodominance of the $gB_{498-505}$ epitope is seen during the inductive phase of the immune response to HSV-1 (124).It is somewhat surprising that a viral late gene product would be targeted by $CD8^+$ T cells, and that these T cells could block HSV-1 reactivation from latency. One might predict that a viral late gene product would be expressed too late in the viral

life cycle to elicit a $CD8^+$ T cell response capable of blocking virion formation. It is noteworthy, however, that although gB is a γ_1 gene, $CD8^+$ T cells specific for gB are activated very early after infection (127), and the $gB_{498\text{-}505}$ epitope appears to be expressed on cells within hours of infection (128).

These observations suggest a "just in time" mechanism in which $CD8^+$ T cells within the ganglion may only respond when the virus reaches the point in its life cycle where γ_1 genes are expressed, immediately prior to replication of its DNA. Such a mechanism would require a rapid response by the $CD8^+$ T cells capable of shutting down further progression of the viral life cycle, possibly by inhibiting viral DNA synthesis. Indeed, we show in *ex vivo* TG cultures that $CD8^+$ T cells reactive to the γ1 gene product gB block expression of the γ2 gene gH. The capacity of $CD8^+$ T cells to provide such exquisite regulation of HSV-1 gene expression *in vivo* is suggested by their juxtaposition to neuron cell bodies in the ophthalmic branch of the latently infected TG, and their persistent stimulation by neurons as indicated by CD69 expression and polarization of their TCR towards the T cell-neuron junction.

Based on this and our previous studies, we propose that $CD8^+$ T cells employ multiple mechanisms to block HSV-1 reactivation from latency. The addition of $CD8^+$ T cells from the lymph nodes of HSV-1 infected mice (32), or the addition of the $gB_{498\text{-}505}$ epitope-specific $CD8^+$ T cell clone, 2D5 at the initiation of day 34 TG cultures completely blocked HSV-1 reactivation from latency. In contrast, the addition of IFNγ (1000 U/ml) to day 34 TG cultures had no effect on reactivation frequency, unless the cultures were first treated for four days with the antiherpetic drug acyclovir (73). The addition of IFNγ to cultures within 24 hours of acyclovir removal effectively prevented HSV-1 reactivation from latency. These findings suggest that IFN-γ can block HSV-1 reactivation only when present early in the reactivation process.

Apparently during TG culture preparation some neurons exceed the window of opportunity for IFNγ protection, and require some other $CD8^+$ T cell mechanism such as perforin/granzymes to block reactivation (Section. 4.5.2).

There is growing evidence that memory $CD8^+$ T cells may reside in non-lymphoid tissues following viral infections (81,161). In one study, $CD8^+$ T cells were shown to reside in multiple tissues that did not appear to harbor viral genome or proteins, leading to the suggestion that memory $CD8^+$ T cell retention might not be an antigen-driven process (81). However, in that study the virus was administered intravenously, so the presence of very low levels of viral protein in multiple tissues could not be formally excluded. Following HSV-1 corneal infection, $CD8^+$ T cells were selectively retained in the ophthalmic branch of the ipisilateral TG, where the viral genome is readily detectable. Moreover, while high levels of CD44 was consistently expressed on $CD8^+$ T cells in the latently infected TG, CD69 expression actually increased during the first 34 days p.i., and was then maintained on 90% of $CD8^+$ T cells through 84 days p.i. Our findings are consistent with the notion that HSV $gB_{498\text{-}505}$ peptide -specific $CD8^+$ T cells are retained in the latently infected TG in an activated, memory phenotype due to persistent low-level antigenic stimulation. The concept that functional T cell memory requires persistent antigen has been proposed and supported by experimental data (184,185,186,90). Although CD69 expression has been observed on memory $CD8^+$ T cells in tissues that appear to lack antigen (161,187), in one study CD69 expression diminished over time (161). Combined with the observation that $gB_{498\text{-}505}$ peptide -specific $CD8^+$ memory T cells appear to primarily reside outside of the lymphoid organs following primary HSV-1 infection (188), our findings suggest that the latently infected TG could be a site of retention of HSV-specific $CD8^+$ effector memory T cells.

There appears to be a dynamic balance between HSV-1 latency and reactivation involving a tripartite interaction among the virus, the host neuron, and the local immune components. We propose that the capacity of latently infected neurons to repress viral and MHC gene expression may be frequently perturbed, permitting rapid processing and presentation of some viral gene products to surrounding $CD8^+$ T cells. The more frequent reactivation of HSV-1 in human TG may reflect a less efficient $CD8^+$ T cell response. This would be consistent with the fact that the HSV-1 ICP47 protein binds more efficiently to human than to mouse TAPs, thus inhibiting the loading of antigenic peptides on human MHC I (189). In neurons that express very little MHC I, ICP47 might delay the activation of resident $CD8^+$ T cells long enough to permit some virion formation. We propose that an appropriate vaccine targeting $CD8^+$ T cells specific for viral proteins that are expressed during latency might prove highly efficacious in preventing recurrent herpetic disease.

5.2. How do $CD8^+$ T cells Prevent HSV-1 Reactivation?

The nervous system is a preferred site of viral persistence. The susceptibility of neurons to persistent viral infections has been attributed to the fact that neurons are post-mitotic and poor targets for T-cell surveillance due to low expression of MHC molecules. On the other hand, there is growing evidence that T cells might play an important role in maintaining certain viruses in a persistent or latent state in the central or peripheral nervous system (136,190,148,183). A recent study involving persistent mouse hepatitis virus infection of the central nervous system demonstrated that antigen-specific $CD8^+$ T cells were retained for prolonged periods in the central nervous system (148). The $CD8^+$ T cells that were retained in the brain after clearance of infectious virus were functionally distinct from those present during the acute phase of infection, in that the former exhibited dramatically reduced lytic function but maintained IFNγ production.

This shift in $CD8^+$ T cell function might represent an adaptation designed to accommodate the changing needs of the tissue as the infection progresses from the acute to the latent or persistent stage. During the acute stage of infection both lytic and nonlytic mechanisms might be required to eliminate replicating virus in the infected tissue. In contrast, non- lytic, cytokine-mediated mechanisms might be sufficient to maintain the virus in a latent or persistent state, while avoiding unnecessary tissue destruction. Using two different strains of mice we have provided clear evidence that $CD8^+$ T cells are capable of preventing HSV-1 reactivation in sensory neurons (32,33). Using ex vivo TG cultures derived from Balb/C mice we previously demonstrated that $CD8^+$ T cells that were present in the TG 14 days after HSV-1 corneal infection could completely block HSV-1 reactivation from latency, however, the endogenous $CD8^+$ T cells present in TG obtained 34 days PI infection delayed, but could not prevent, HSV-1 reactivation. HSV-1 reactivation was completely blocked in day 34 TG cultures when supplemented with exogenous $CD8^+$ T cells obtained from draining lymph nodes 7 days after HSV-1 corneal infection, moreover addition of 2D5 cells (gB-specific $CD8^+$ T cell clone) to day 35 ex vivo cultures derived from C57BL/6 mice prevented HSV-1 reactivation. It is not clear if the exogenous cells merely provided a critical density of HSV-reactive $CD8^+$ T cells in the culture, or if the $CD8^+$ T cells that are generated during acute infection possess a different functional program than those retained in the ganglion during latency. We have demonstrated that $CD8^+$ T cells retained in the TG at 35 days after HSV-1 corneal infection show enhanced ability to secrete IFNγ when compared to $CD8^+$ T cells obtained from day 14 infected mice (33). Thus, in the latently infected TG, there is indeed a selective enrichment of gB-specific $CD8^+$ T cells capable of producing IFN-γ, however unlike with the murine hepatitis virus (148) there is

no indication of an epitope shift. It should also be noted that upon antigenic stimulation 2D5 cells can kill as well as secrete IFNγ.

Therefore in the present study we attempted to answer the following important questions; whether or not IFNγ can block HSV-1 reactivation from latency and if perforin mediated cytolytic mechanisms are necessary for $CD8^+$ T cells to confer such exquisite protection. Our findings (Section 4.5.1) demonstrated that rIFNγ cannot block HSV-1 reactivation from latency when added at the initiation of day 35 TG cultures. (Recent data in our lab indicates that some neurons are susceptible to IFNγ control while others are not). Clearly, the TG cultures differ in many ways from the in vivo TG. For instance, the neurons are undoubtedly stressed during excision, which might compromise their intrinsic ability to inhibit HSV-1 gene expression. In addition, the $CD8^+$ T cells are separated from the latently infected neurons until contact can be reestablished in culture. Moreover, the cytokines that are produced by the $CD8^+$ T cells may be diluted and washed away more rapidly in ex vivo cultures than they are in the TG in situ. We believe the inability of endogenous $CD8^+$ T tells in day 35 TG or rIFNγ to block HSV-1 reactivation might reflect some of these changes that occur in the TG upon excision. We proposed that a brief treatment of TG cultures with ACV might alleviate these problems by pushing the reactivating HSV-1 genomes back into the latent state that existed prior to TG excision and providing the $CD8^+$ T cells time to reestablish contact with infected neurons and to be activated. ACV treatment did render latently infected neurons susceptible to IFNγ inhibition of HSV-1 reactivation. Inhibition of HSV-1 reactivation was only observed when latently infected neurons were exposed to IFNγ within 24 h after ACV removal from cultures. This observation suggests that IFNγ blocks an early step in HSV-1 reactivation from latency. The rIFNγ was less effective at blocking HSV-1 reactivation when endogenous $CD8^+$ T cells were

depleted from the day 35 TG cells. Furthermore, addition of 2D5 cells to day 35 C57BL/6 cultures provided protection from reactivation without the addition of ACV (Figure 24 and 26) indicating the likelihood that $CD8^+$ T cells utilize more than one mechanism to block HSV-1 reactivation. However, the effectiveness of IFNγ was not further compromised by depletion of all bone marrow-derived cells from the TG cultures. Thus, IFNγ appears to inhibit HSV-1 reactivation in part through augmentation of a $CD8^+$ T cell response.

IFNγ delayed and reduced HSV-1 reactivation in day 35 TG cultures that were depleted of all detectable $CD45^+$ cells. The latter observation suggested that IFNγ can also directly inhibit HSV-1 reactivation in neurons. To our knowledge, this is the first direct demonstration that IFNγ can block HSV-1 reactivation from latency in neurons. In vivo studies comparing wild-type and IFNγ knockout (GKO) mice on a BALB/c background demonstrated more rapid reactivation of HSV-1 from latency following induction by hyperthermic stress (190) or UV-B corneal irradiation (191) in GKO mice. In the former study the overall incidence of reactivation was also increased in IFNγ deficient mice, whereas this difference was not observed in the latter study. Another recent study using an in vivo reactivation model with GKO mice on C57BL/6 background demonstrated that IFNγ was critical in preventing reactivation and subsequently death of HSV-1 infected mice (192). Our findings with the ex vivo model of HSV-1 reactivation from latency in sensory neurons are in good general agreement with in vivo studies using mice with targeted disruption of the IFNγ gene. Moreover, our model avoids the complication of differences in control of the acute infection, establishment of latency, and possible compensatory mechanisms in GKO mice, and should facilitate studies of latency at the molecular level.

The mechanism by which IFNγ prevents HSV-1 reactivation is not yet known. During lytic infections, IFNγ has been shown to inhibit expression of ICP4, a potent transactivator of

HSV gene expression, destabilize HSV mRNA, and stabilize HSV-1 association with nuclear protein aggregates called ND10 bodies, which inhibit HSV-1 gene expression (193,194). Any of these mechanisms could contribute to the effect of IFNγ on HSV-1 reactivation in neurons, although ND10 bodies have been difficult to detect in neurons (195). Recent studies have identified an immediate early viral protein ICP0, which can block IFNγ function by inhibiting interferon induced gene expression (12,13). Using in vitro assays the inhibition by ICP0 was dependent on its level of expression in infected cells. Thus, it is reasonable to suggest that in ex vivo TG cultures (and perhaps in vivo), the effectiveness of IFNγ would likely depend on the level as well as the timing of the cytokine expression. During a reactivation event the level of viral gene expression may reach a critical point where elevated concentration of ICP0 may render IFNγ ineffective at maintaining viral latency (especially if timely expression of IFNγ is not achieved). This would explain the need for IFNγ early in reactivation. In such an event, IFNγ independent mechanism(s) (i.e. perforin) are likely to play a critical role in blocking HSV-1 reactivation. However, whether or not ICP0 functions with the same efficiency in neurons was not tested.

To determine if lytic granule-mediated mechanisms are important in blocking HSV-1 reactivaion from latency, we evaluated the ability of PfP $CD8^+$ T cells to protect HSV-1 reactivation in ex vivo TG cultures. To this end we first compared reactivation frequencies in PfP and WT TG cultures at 14 days PI. $CD8^+$ T cells present in the TG at the time of excision in PfP mice were incapable of inhibiting HSV-1 reactivation from latency, where as those present in WT TG blocked reactivation with greater efficiency. This suggested that a perforin-mediated mechanism was important in preventing reactivation early during latency. As mentioned above, the stress induced by explanting TGs can trigger events that can lead to HSV-1 reactivation in

certain neurons, which may artificially increase the need for cytolytic mechanisms to prevent full reactivation. For this reason we treated day 14 cultures with ACV for 4 days in order to truly evaluate the requirement of perforin for maintaining viral latency. Treatment of PfP and WT cultures with ACV yielded similar results confirming the need for perforin in maintaining latency at 14 days after HSV-1 corneal infection. However, the need for perforin in day 34 TG cultures was not as dramatic. Although, reactivation was delayed in WT TG cultures , by 14 days in culture, reactivation frequencies of PfP and WT mice were similar, although reactivation was delayed in WT TG cultures. Moreover, supplementing WT and PfP cultures with rIFNγ completely alleviated the need for perforin. Interestingly in day 34 TG cultures that lacked viral reactivation IFNγ levels in supernatants reached their peak at 8 days after culture initiation but began to decline soon thereafter. At the time when IFNγ levels began to drop WT cultures started reactivating suggesting the importance of IFNγ in preventing reactivation at 34 days PI. These results confirm our hypothesis that granule-mediated mechanisms are indeed essential in preventing HSV reactivation early during latency when a greater array of viral proteins are expressed, but non-lytic mechanisms such as IFNγ secretion are more important at 34 days PI when a more stable viral latency is established. Moreover, attempts to detect MHC class I proteins on TG neurons at 34 days PI have failed, however at 14 days PI MHC class I molecules were readily detectable on neurons suggesting the possibility of elevated viral gene expression at 14 days PI which may coincide with MHC class I expression.

These data raise interesting questions about how $CD8^+$ T cells might deal with virus infections in sensory neurons that cannot be regenerated, and what factors determine which aspects of the $CD8^+$ T cell functional program are exerted. There is evidence in humans and mice that HSV-1 corneal infection is associated with partial loss of corneal sensation, suggesting

that some neurons are killed either by the virus or by immune mechanisms (196). However, we have not noticed immunopathology in latently infected TG, and our previous studies clearly established that $CD8^+$ T cells can block HSV-1 reactivation from latency in TG cultures by a non-lytic mechanism(s) (32,33). In fact, a $gB_{498-505}$ specific $CD8^+$ T cell clone that is both cytotoxic and produces IFN-γ when stimulated was able to block HSV-1 reactivation from latency in TG cultures without eliminating the latently infected neurons.

Epitope density is one factor that has been proposed as a differential signal for $CD8^+$ T cell expression of non-cytotoxic (i.e., IFN-γ) or cytotoxic (i.e., lytic granule release) effector mechanisms (197-200). The apparent immunologic synapse formation between $CD8^+$ T cells and neurons within latently infected TG suggests that the neurons are able to express the $gB_{498-505}$ epitope that is recognized by the T cell receptor on these cells. Neurons up-regulate MHC class I expression during HSV-1 lytic infection 138,139, but MHC class I expression diminishes to an undetectable level when the virus enters a latent state. This suggests concordant regulation of MHC class I and viral gene expression in neurons. We propose that under conditions that permit the viral genome to emerge from a quiescent state MHC class I gene expression is concomitantly initiated. Thus viral proteins that are expressed early in reactivation are processed and presented with MHC class I at an initial low density on the surface of the neuron. The epitope density would increase as reactivation progressed. A recent study demonstrated that the $gB_{498-505}$ epitope is expressed on cells very early (within 2 hours) after a lytic infection is initiated 128, and may also be expressed early during reactivation. We propose that exposure of $gB_{498-505}$ specific $CD8^+$ T cells to neurons early in reactivation, when the $gB_{498-505}$ epitope density would presumably be low, would induce IFN-γ production but not lytic granule release by the $CD8^+$ T cells (Figure 48). In contrast, encounter of $CD8^+$ T cells with a neuron late in the reactivation

process, when $gB_{498-505}$ epitope density would be high might result in induction of the entire functional program of the $CD8^+$ T cells, including lytic granule release. The susceptibility of the neurons to apoptosis induction by the lytic granules might be determined by the level of accumulation of viral LATs or proteins such as the US3 and US5 that inhibit apoptosis (201,202).

An alternative explanation for the differential use by $CD8^+$ T cells of lytic and non-lytic mechanisms to control HSV-1 reactivation from latency might lie in the observation that memory $CD8^+$ T cells lose granzyme B expression, but maintain expression of IFN-γ (77). Memory $CD8^+$ T cells regain granzyme B expression upon stimulation. Thus, lytic granule release by $CD8^+$ T cells might only be possible if IFN-γ fails to rapidly shut down HSV-1 gene expression during reactivation.

Regulation either by epitope density or through loss of granzyme B would accommodate the use of non-lytic mechanisms early in reactivation and lytic mechanisms late in reactivation. A recent report demonstrated that only very rare neurons (1 in every 10 latently infected mouse TG) express viral γ2 genes (29). These rare neurons were surrounded by leukocytes and appeared to be dying. In contrast, our studies demonstrated that most HSV-1 specific $CD8^+$ T cells in latently infected TG express an activation phenotype and exhibit TCR polarization toward junctions with multiple neurons (33), and the neurons appeared healthy. These findings are consistent with the notion that $CD8^+$ T cells normally block HSV-1 reactivation in neurons through a non-lytic mechanism, but can kill those rare neurons that reach a late stage of HSV-1 reactivation and are refractory to non-lytic mechanisms.

5.3. How do IL-2 and IL-15 Regulate T cell responses in the TG after HSV-1 Infection

In the present study, we examined the requirement of IL-15 and IL-2 during the expansion and memory phases of T cell responses in non-lymphoid (sensory ganglion) and secondary lymphoid tissues after HSV-1 corneal infection. HSV-1 infection provides us with a unique model to study how cytokines influence lymphoid and extra-lymphoid T cell responses. After HSV-1 corneal infection in mice the virus briefly replicates in the corneal epithelium followed by retrograde axonal transport to the body of the neuronal cell in the trigeminal ganglion (TG) where it establishes a latent infection. Recent data from our lab demonstrated that HSV-1 latent infection is a dynamic process characterized by a robust T cell response in the sensory ganglion and sustained immunosurveillance by $CD8^+$ T cells long after the establishment of latency. There is considerable evidence to suggest persistent viral gene expression and antigen presentation during HSV-1 latency (33,29,22). Although, recent studies have investigated the role of IL-15 and IL-2 in the expansion, and memory phases of T cell responses following acute viral infections, the understanding of how these cytokines function in an environment of constant viral antigen presentation and infrequent reactivation in tertiary tissues is limited. Two recent studies elegantly showed that the immune response to an antigenic challenge is not limited to the secondary lymphoid organs; in actuality a large percentage of antigen-specific T cells migrate to tertiary tissues (81,82). The current study adds new dimensions to our understanding of how IL-15 and IL-2 regulate T cell responses in lymphoid organs as well as non-lymphoid sites during the lytic and latent phase of a viral infection.

In vitro studies have determined IL-2 as essential for T cell proliferation (162), however, its requirement in vivo for T cell expansion and memory, after viral infections is still unclear.

Experiments with IL-$2^{-/-}$ and $CD25^{-/-}$ (IL-$2R\alpha^{-/-}$) mice have yielded puzzling results that are difficult to interpret since these mice rapidly develop severe autoimmune diseases (203,204,165). In a recent study, the role of IL-2 during primary $CD8^+$ T cell expansion was examined by adoptive transfer of IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ OT-1 TCR transgenic $CD8^+$ T cells (specific for OVA) into normal hosts. The mice were then infected with recombinant VSV that expressed soluble OVA. IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ OT-1 cells expanded normally in secondary lymphoid organs, but failed to expand adequately in the intestinal lamina propria (LP). The reduced $CD8^+$ T cell expansion in the LP was attributed to a lack of sustained cell division during primary responses to VSV-OVA (97,99). However, another study reached a different conclusion (173). They used $CD122^{-/-}$ (IL-$2R\beta^{-/-}$) transgenic mice ($Tg^{-/-}$) that are resistant to autoimmune disorders normally associated with non-transgenic IL$2R\beta^{-/-}$ mice. $Tg^{-/-}$ mice were immunized with OVA in complete Freund's adjuvant (CFA) and primary and secondary $CD8^+$ T cell responses were evaluated. $CD8^+$ T cell responses were normal in $Tg^{-/-}$ mice after immunization with OVA, suggesting that IL-2 or IL-15 were not essential for $CD8^+$ T cell expansion in lymphoid tissues. Both of these studies used transgenic mouse models that may render interpretation of results difficult. Adoptive transfer of a large number of naïve OT-1-TCR transgenic $CD8^+$ T cells can dramatically increase the precursor frequency and may alter the true requirements of cytokines in vivo. Moreover, TCR transgenic $CD8^+$ T cells were isolated from four week old IL-$2^{-/-}$ and IL-$R\alpha^{-/-}$ OT-1 mice that develop serious lymphoproliferative diseases, although later than in non transgenic IL-$2^{-/-}$ mice. Primary $CD8^+$ T cell responses in $Tg^{-/-}$ mice were measured after administration of soluble OVA in CFA and not after a natural viral infection. Additionaly, $Tg^{-/-}$ mice have $CD8^+$ T cells that express functional high affinity IL-15Rα and IL-2Rα. We avoided the complexities associated with using such mouse models by employing a simpler approach. To

assess IL-2 function we used a monoclonal antibody (95) to block IL-2 during the expansion phase of $CD8^+$ T cell response after HSV-1 corneal infection. In addition IL-$15^{-/-}$ mice were used to examine the role of IL-15 during primary and memory phases of T cell responses to HSV-1 infection. Since we were particularly interested in determining the requirement of IL-2 during the primary T cell response in the TG, we delayed the administration of anti-IL-2 Ab until day 6 PI; this is after the $CD8^+$ T cell response peaks in the lymph nodes (124), but just prior to the maximum $CD8^+$ T cell expansion in the TG (between day 6 and 8 PI). Both $CD8^+$ and $CD4^+$ T cell numbers were dramatically reduced in IL-$15^{-/-}$ mice and in WT mice that received only one injection of anti-IL-2 antibody. Intriguingly, both viral specific and antigen independent $CD8^+$ T cells were negatively affected by the lack of IL-15 or IL-2. The effect however, was more pronounced in viral specific $CD8^+$ T cells. This suggested that both IL-2 and IL-15 are essential for T cell expansion in tertiary tissues like the sensory ganglion. We further show that there are notable differences in the effect of IL-15 and IL-2 on lymphoid vs. non-lymphoid T cells. In contrast to the TG, $CD4^+$ and $CD8^+$ T cell expansion in the lymph nodes between 6 and 8 days PI was not affected by the treatment of anti-IL2 antibody. However, as in the TG, $CD8^+$ T cell expansion in the lymph node was significantly reduced in IL-$15^{-/-}$ mice at 5 and 8 days after corneal infection. Interestingly $CD4^+$ T cell expansion in the LN of IL-$15^{-/-}$ mice was normal. These data are in agreement with D'Souza et.al who showed that lack of IL-2 function lead to a reduction in $CD8^+$ T cell response only in tertiary tissues but not in secondary lymphoid organs (97,99).

Our results clearly show that IL-15 is vital for primary ganglionic $CD8^+$ and $CD4^+$ T cell responses as well as lymphoid $CD8^+$ T cell expansion. Antigen specific $CD8^+$ T cell response in the TG was severely compromised in IL-$15^{-/-}$ mice as well as mice that received anti-IL2

antibody; there was an 80% drop in the number of antigen specific $CD8^+$ T cells retained in the TG at 8 days after corneal infection. These findings suggest an even greater dependence on IL-15 and IL-2 by $CD8^+$ T cells in non-lymphoid tissues. Conversely, IL-15 was not required for $CD4^+$ T cell response in the lymph nodes. Contrary to our findings in the TG, other researchers have found that IL-15 does not play any role in $CD4^+$ T cell responses (102). However, none of the studies thus far have examined the effect of IL-15 on non-lymphoid $CD4^+$ T cells after viral infections.

Our conclusions about the positive regulation of IL-15 in initial proliferation of lymphoid $CD8^+$ T cells are at odds with recent reports that did not find any correlation with IL-15 and primary $CD8^+$ T cell expansion (169,100). There are important differences between the infection models used in the above studies that may explain the conflicting results. Mice in the aforementioned studies were infected systemically (intravenous injections) with LCMV (169) or VSV (100). Because LCMV directly infects secondary lymphoid organs a systemic infection with it results in an unusually large $CD8^+$ T cell burst size; where more than 80% of the lymphoid $CD8^+$ T cells are viral specific (78). It is likely that such an extraordinary inflammatory response leads to elevated expression of other cytokines and chemokines that can alter the requirements of IL-15. HSV-1 corneal infection is a localized mucosal infection that does not lead to viral replication in the lymphoid organs (126). Furthermore, at the peak of the immune response in the LNs only 2% of the $CD8^+$ T cells are viral specific at 5 days PI in the draining lymph nodes.

IL-2 and IL-15 share the same β and γ chain cytokine receptors and both cytokines can exert positive effects on T cell expansion. To determine if deficiency in both cytokines would further weaken primary T cell responses, we blocked IL-2 function between day 6 and 8 PI in IL-

$15^{-/-}$ mice. We consistently observed a more pronounced reduction in antigen specific $CD8^+$ T cell numbers in anti-IL-2 treated IL-$15^{-/-}$ mice compared to IL-$15^{-/-}$ mice alone, however, the reduction was not statistically significant. Although, simultaneously blocking IL-2 and IL-15 did not result in a significant drop in T cell responses in LN or TG, we hypothesize that IL-15 and IL-2 may function sequentially. There is evidence that IL-15 can enhance IL-2Rα (CD25) expression on $CD8^+$ T cells (205), however the effect of IL-2 on CD25 expresion is controversial (205,168).

A possible explanation for the lack of optimal T cell response during the expansion phase of the immune response in the TG and LN of IL-$15^{-/-}$ and anti-IL-2 treated mice could be decreased proliferation, an increase in apoptosis or a combination of the two. In WT mice there is an 8 fold increase in ganglionic $CD8^+$ T cell numbers between day 6 and 8 after HSV-1 corneal infection. To assess the level of T cell proliferation in the TG and LNs, we treated mice with BrdU at day 6 and 7 PI. Lymphoid $CD8^+$ T cell proliferation was indeed compromised in IL-$15^{-/-}$ mice but was normal in anti-IL-2 treated WT mice, suggesting that IL-15 contributes to the proliferation of anti viral $CD8^+$ T cells in the LNs during the expansion phase of an immune response. On the contrary, the percentage of $CD8^+$ T cells that incorporated BrdU in the TG between 6 and 8 days PI was normal in all three groups of mice. Thus, the dysregulation in the level of $CD8^+$ T cell response in the TG was not due to impaired proliferation. An alternative explanation for the weak T cell expansion phase could be a reduction in sustained turnover of T cells in the absence of IL-2 and IL-15. A similar conclusion was reached by a recent study in which IL-$2R^{-/-}$ $CD8^+$ T cells underwent fewer divisions after antigenic challenge compared to those from WT mice (99).

Why should there be notable differences in the way cytokines regulate T cell responses in lymphoid vs. non lymphoid tissues? We believe that antigen dose may dictate the level of requirement for IL-15. Early during a viral infection the level of antigen presentation is high and this initial antigen encounter by T cells can trigger early rounds of cell division but as the virus is cleared from the body, sustained T cell proliferation may become increasingly dependent on IL-15 and (or) IL-2. After an HSV-1 infection there is no evidence of replicating virus in the LN, however the virus does travel to the TG where it briefly replicates and by day 8 majority of the replicating virus is cleared. It is conceivable that a lack of replicating virus in the LN leads to a milieu of limited antigen dose where $CD8^+$ T cell proliferation is highly dependent on IL-15. In the TG at 6 days PI replicating virus is readily available to initiate T cell division but in the subsequent 24 to 48 hours the presence of cytokines becomes increasingly important for sustaining cellular division. There are other important differences between lymphoid and non-lymphoid tissues that can also contribute to the observed differences in the T cell responses after HSV-1 infection. (i) The cytokine milieu in the LN may be quite different compared to the sensory ganglia. (ii) The context in which the cytokines are presented to receptors on T cells may be different in non-lymphoid tissues; there is evidence that cytokines such as IL-2 can bind to the extracellular matrix in extra-lymphoid tissues (206,207). (iii) $CD8^+$ T cells in the TG compared to the LN may differ phenotypically and (or) functionally. We are currently examining the IL-2 and IL-15 receptor expression on lymphoid and ganglionic $CD8^+$ T cells in order to discern tissue specific phenotypic differences. Recent reports have also implicated IL-15 and IL-2 in regulating T cell migration and homing to sites of infection (208,209,210), likewise it is possible that migration of $CD8^+$ and $CD4^+$ T cells to the TG is adversely affected by the lack of IL-2 and IL-15. However, at day 6 PI we did not observe any significant

differences in the number of T cells present in the TG. To address some of the issues mentioned above, an adoptive transfer study with CFSE labeled $CD8^+$ T cells will be done.

In our present study we made the additional important observation: an unusually high degree of bystander $CD8^+$ T cell activation in the TG. At 8 days after corneal infection more than 50% of the gB-dimer negative $CD8^+$ T cells in the TG incorporated BrdU, and in the lymph nodes a majority of the dividing $CD8^+$ T cells were antigen-independent. This putative bystander activation of $CD8^+$ T cells was severely diminished by the lack of IL-15. While IL-15 has been shown to promote $CD44^{hi}$ memory $CD8^+$ T cell proliferation in an antigen-independent manner (103,170,211,212), a role for IL-15 in regulating bystander activation-induced proliferation of naïve and memory $CD8^+$ T cells during the course of infection has not been demonstrated. Bystander activation appears to be more prominent after a localized infection with HSV-1 compared to a systemic infection with LCMV. After an LCMV infection more than 80% of the responding $CD8^+$ T cells are viral specific. However, 5 days after HSV-1 corneal infection, viral specific $CD8^+$ T cells account for only 2% of all $CD8^+$ T cells present in the LN. A recent in vitro study with human T cells favors such a possibility (213).

IL-2 and IL-15 can negatively regulate T cell death; however we did not observe any preferential increase in apoptotic potential of $CD8^+$ T cells in the TG at 8 days PI. However, detecting apoptotic cells in extra-lymphoid tissues is difficult, given that apoptotic cells are cleared immediately by phagocytes even before the exposure of PS on the cell surface. Moreover, collagenase treatment and the harsh physical breakup of TG in to single cell suspensions can result in non-specific exposure of PS in otherwise non apoptotic (Annexin-V^-) T cells. We also examined the level of $CD8^+$ T cell death in the lymph nodes. There was elevated $CD8^+$ T cell apoptosis in IL-$15^{-/-}$ mice, but surprisingly the highest percentage of apoptotic $CD8^+$

T cells was found in anti-IL-2 treated IL-15$^{-/-}$ mice (>30% of CD8^{+} T cells). We conclude that IL-15 contributes to the survival of antigen specific and bystander CD8^{+} T cells in LNs. In addition, blocking IL-2 and IL-15 simultaneously increases effector CD8^{+} T cell death and thereby accelerates the contraction phase of lymphoid CD8^{+} T cell response after HSV-1 corneal infection.

Our data are consistent with recent findings that IL-15 is essential for the maintenance of memory CD8^{+} T cells retained in secondary lymphoid organs (169,100,102,170). The present study adds another dimension to the understanding of the role of IL-15 in regulating memory T cells during a latent/persistent infection. We show that IL-15 is necessary to prevent attrition of viral specific memory CD8^{+} T cells in non-lymphoid sites such as the TG. Interestingly, CD4^{+} T cell numbers were also notably reduced in the TGs of IL-15$^{-/-}$ mice at 65 days PI but splenic CD4^{+} T cell numbers were normal. To our knowledge this is the first report that has examined the effect of IL-15 on CD4^{+} and CD8^{+} T cells in extra-lymphoid tissues during the primary and memory phases of an anti-viral immune response.

The current paradigm that initial clonal burst size determines the size of virus-specific memory CD8^{+} T cell population is largely based on studies performed with lymphoid T cell populations (78,214,215). Our findings are consistent with this paradigm with one important exception. As noted earlier, anti-IL-2 treated mice showed a dramatic decrease (20% of normal) in the number of effector CD8^{+} T cells at the peak of the immune response in the TG after HSV-1 corneal infection. Yet, this reduction in effector CTL expansion did not result in a smaller memory CD8^{+} T cell pool. Thus, at least in the TG it appears that the magnitude of HSV-specific memory CD8^{+} T cell pool is not dependent on the initial burst size. In addition, the

$CD8^+$ T cells that became memory cells do not require the presence of IL-2 during the expansion phase of an anti-viral response.

How does our current study impact our understanding of the immune response during an HSV infection? We have previously shown that there is a constant T cell monitoring of HSV-1 gene expression during latency. After HSV-1 corneal infection T cells are recruited to the TG in great numbers and are subsequently retained in the tissue for the life of the animal. $CD8^+$ T cells exhibit an activation phenotype, and combined with the juxtaposition of $CD8^+$ T cells with neuron cell bodies in latently infected TG suggests a condition of constant antigen presentation which can lead to T cell exhaustion or anergy as seen in other chronic/persistent infections (160,216,217,218,219). Human studies with HIV patients have revealed that one explanation for the impairment of the $CD8^+$ T cell adaptive immune response during the course of HIV infection is the dysregulated production of IL-15 as well as IL-2. Therefore, we believe that IL-2 and particularly IL-15 play a pivotal role in the expansion of effector cell in tertiary tissues, and in preventing ganglionic T cell exhaustion and functional impairment normally associated with chronic infections. Recently we demonstrated that IFNγ appears to inhibit HSV-1 reactivation in part through augmentation of a $CD8^+$ T cell response, furthermore, several laboratories have observed a uniform expression of IFN-γ and TNF-α in latently infected TG (22,136,129,135). Upregulation of IL-15 expression by IFNγ (103,211) in the TG may very well be one of the means IFNγ augments $CD8^+$ T cell responses during latency.

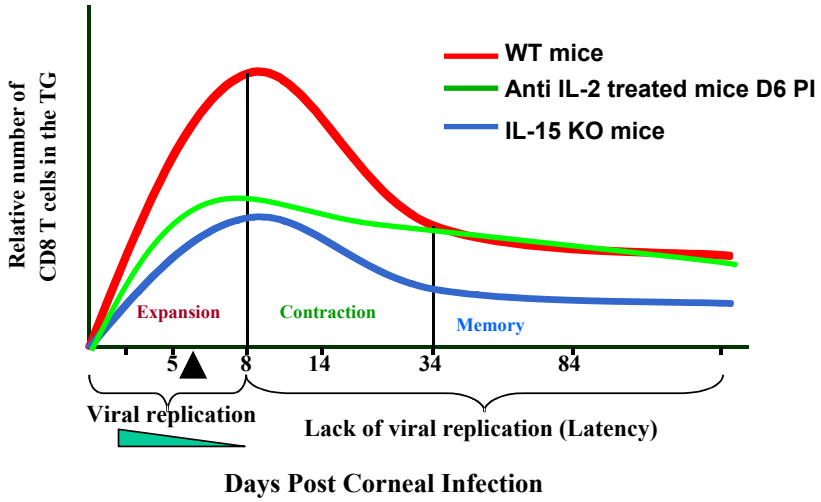

Figure 47: Summary of events in the TG. *After HSV-1 infection, $CD8^+$ T cells go through the typical expansion and contraction phase in the TG. By day 34 PI a stable pool of memory $CD8^+$ T cell is established. Anti IL-2 treated mice (at day 6 PI) have a reduced expansion phase, but form a normal memory $CD8^+$ T cell pool. IL-$15^{-/-}$ mice have a reduced expansion phase and fail to form a normal memory $CD8^+$ T cell population.*

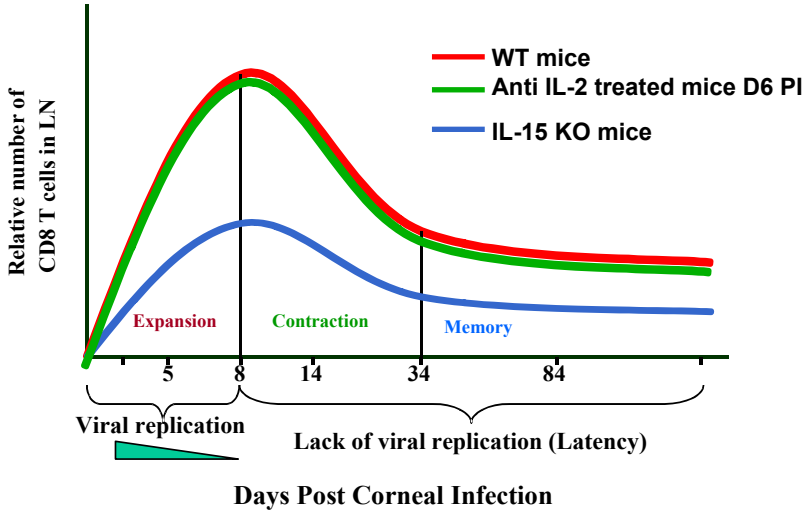

Figure 48: Summary of events in the lymphoid organs. *After HSV-1 corneal infection, $CD8^+$ T cells in the LN go through the typical expansion and contraction phase. By day 34 PI a stable pool of memory $CD8^+$ T cell is established. Anti IL-2 treated mice (at day 6 PI) go through a normal expansion phase, as well as form a normal memory $CD8^+$ T cell pool. IL-$15^{-/-}$ mice have a reduced expansion phase, and fail to form a normal memory $CD8^+$ T cell population in the spleen.*

Considering the data presented in this study, we propose that after HSV-1 corneal infection, corneal langerhan and dentritic cells interact with T cells in secondary lymphoid organs leading to the primary T cell expansion, which is in part dependent on IL-15 (mainly for $CD8^+$ T cells) but IL-2 independent. Activated T cells migrate to the TG (Figure 47 and 48) where they continue to divide (as result of replicating virus), and this subsequent expansion/survival is mediated by IL-2 and (or) IL-15. Next follows the contraction phase where a majority of the $CD8^+$ T cells die and a fraction of them survive and are retained in an activated state for the life of the animal. It is possible that effector cells expressing high levels of IL-7 receptor but not IL-2R (220) survive to bemome memory $CD8^+$ T cells. The maintenance of memory T cells is highly dependent on IL-15 even in the face of continual antigen presentation.

In conclusion, our findings have confirmed and added to the understanding of how effector and memory T cells responses are controlled by IL-2 and IL-15 in secondary lymphoid and non-lymphoid tertiary tissues. In addition we challenge the current tenets underlying the role of IL-15, which are based on observations of events occurring in lymphoid organs after acute viral infections that are readily cleared from the body. We demonstrate a novel role of IL-15 in regulating primary and memory $CD4^+$ T cell responses in non-lymphoid tissues. We also demonstrate that IL-15 is necessary for $CD8^+$ T cell expansion in the lymph nodes as well as in the TG after HSV-1 corneal infection. And finally we confirm that IL-15 prevents attrition of memory $CD8^+$ T cells in lymphoid in addition to non-lymphoid sites.

6. SUMMARY

Parts of Section 6 are modified from the article titled "**Immunity to latent viral infection: many skirmishes but few fatalities"** submitted to Trends in Immunology.

The body of work presented in this thesis has greatly advanced our understanding of HSV infections. Our findings are leading to a paradigm shift in our conception of HSV-1 latency from a virus/neuron relationship to a tripartite interaction involving the virus, neuron, and host immunity. In this study we have provided clear evidence that HSV-1 latency does not represent a silent infection that is ignored by the host immune system. To the contrary, we demonstrate that after HSV-1 corneal infection there is an antigen-directed retention of memory $CD8^+$ T cells in the latently infected branch of the TG, where they provide active surveillance of HSV-1 gene expression. The expansion and retention of T cells in the TG after HSV-1 infection is in part regulated by IL-2 and IL-15. Finally we also show that $CD8^+$ T cells can prevent viral reactivation from latency in sensory neurons by IFNγ and perforin mediated mechanisms. Based on the data presented here we propose a model of immune surveillance during HSV-1 latency and reactivation (Figure 49).

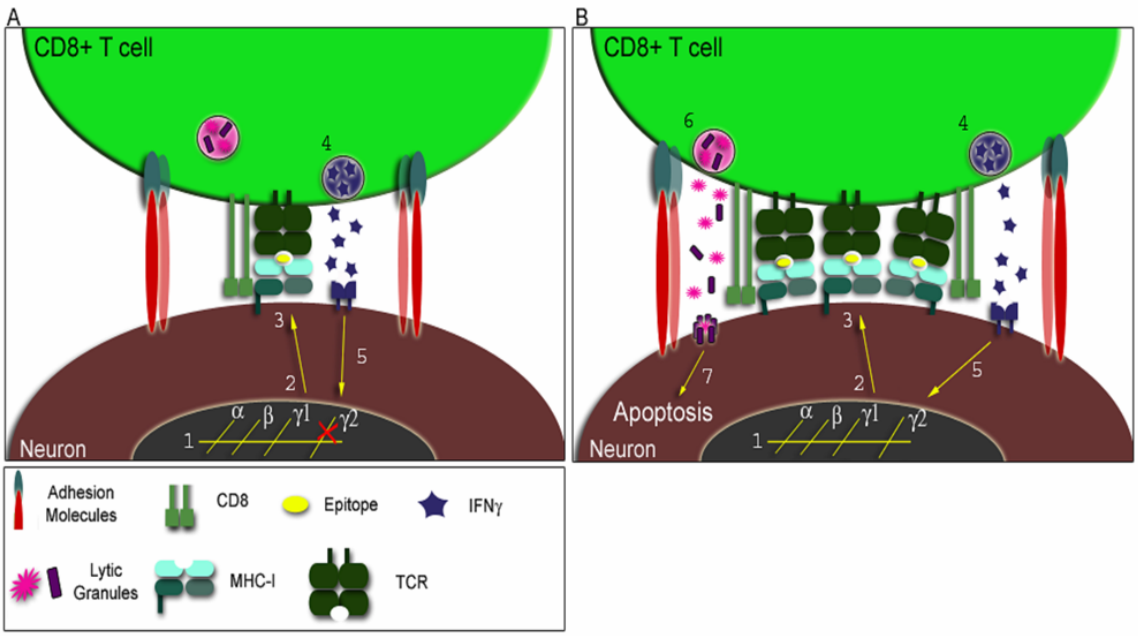

Figure 49: ***Model of immune surveillance during HSV-1 latency and reactivation.***

(A) Although HSV-1 can replicate efficiently in mouse sensory neurons, spontaneous reactivation from latency with virion formation as seen in other species (i.e., humans, rabbits, etc) does not occur in mouse sensory ganglia. However, we propose that at any given time in an HSV-1 latently infected mouse trigeminal ganglion, multiple neurons express viral α, β, and even γ1 gene products, which are rapidly processed to peptides and presented in the context of MHC I on the neuron surface (1). In C57BL/6 mice, HSV-1 α, and β gene products are ignored by $CD8^+$ T cells, but an immunodominant epitope on the HSV-1 γ1 gene product, gB binds to the $CD8^+$ T cell receptor (2). Since gB and MHC I are likely to be expressed at a low level early in reactivation, a low density of these gB/MHC class I complexes might selectively induce IFN-γ production by $CD8^+$ T cells (3). At this stage in reactivation, IFN-γ can block progression to expression of γ2 genes and virion formation (4, 5). The neuron is not destroyed, but the virus is pushed back into a more stable state of latency.

(B) We propose that encounter with a neuron at a late stage of reactivation will activate the full functional program of $CD8^+$ T cells for two reasons. From a teleological standpoint, the inability of IFN-γ to regulate viral gene expression late in the viral life cycle would require $CD8^+$ T cells to employ an alternative mechanism to prevent virion formation (1,2). From a mechanistic standpoint we propose that as the reactivation process progresses, the density of gB/MHC I complexes increases on the surface of the neuron (3). Thus, a $CD8^+$ T cell that encounters a neuron late in the reactivation process might receive strong TCR stimulation from a high epitope density, leading to activation of both IFN-γ production (4,5) and lytic granule release (6), potentially inducing apoptosis (7). The capacity of the neuron to survive this encounter might depend on the level of accumulation of anti-apoptotic viral proteins such as US3 and US5.

Since recurrent HSV-1 disease results from reactivation of latent virus as opposed to exogenous re-infection, these findings have important implications for vaccine development. We propose that a vaccine that targets the $CD8^+$ T cells that are retained in the latently infected sensory ganglia might be efficacious in preventing recurrent herpetic disease. A viral α gene product, ICP47 inhibits antigen presentation to $CD8^+$ T cells more efficiently in humans than in mice (189). We propose that a more efficient $CD8^+$ T cell response to HSV-1 might account for the lack of spontaneous HSV-1 reactivation in mice. By extension, increasing the efficiency of the $CD8^+$ T cell response might have a similar effect in humans. Clearly the HSV vaccines tested to date have had disappointing efficacy in humans. However, a therapeutic vaccine containing epitopes that are expressed early in HSV-1 reactivation might augment $CD8^+$ T cell protection from reactivation, while minimizing destruction of sensory neurons. An interesting caveat is that periodic reactivation in neurons and shedding of virus might enhance immunity at peripheral sites, preventing exogenous re-infection. Thus, an effective vaccine might have to target both reactivation of virus from latency in sensory ganglia and lytic infection at peripheral sites.

We are elated by emerging data from human studies in our and other labs that confirm our findings in the mouse model (Figure 50) (118,119,221). A recent study published in the American Journal of Pathology found a similar infiltration of $CD8^+$ T cells in human TGs latently infected with HSV-1 (222). The $CD8^+$ T cells were in close apposition to neurons that expressed viral LATs, in contrast uninfected or VZV infected TGs shown little or no T cell infiltrate. In addition, quantitative RT-PCR of infected TGs revealed elevated levels of inflammation characterized by expression of IFNγ, TNFα and chemokines such as IP-10 and RANTES.

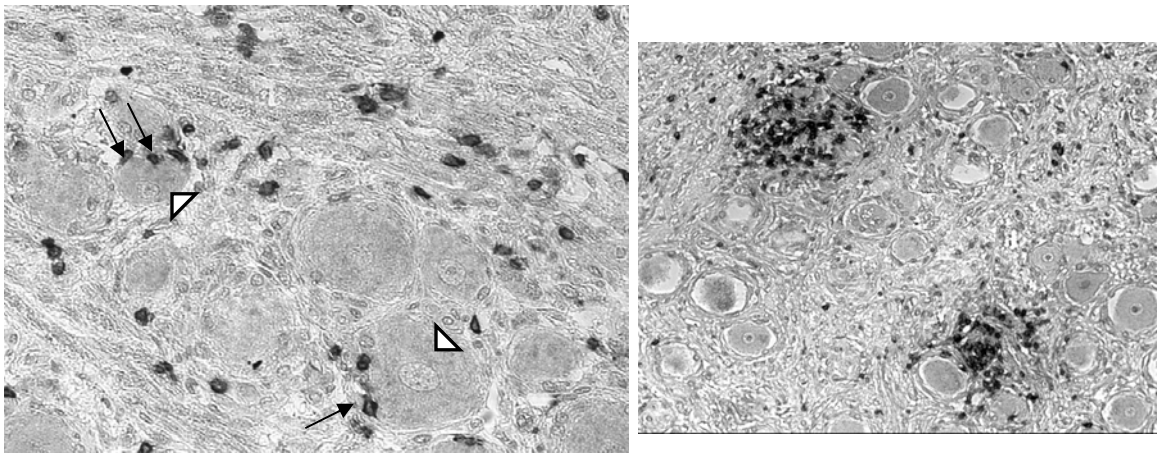

Figure 50: CD8$^+$ T cell infiltrate found in human TGs of individuals infected with HSV-1. *Paraffin embedded human TGs (kindly provided by Dr. Y. Jerold Gordon) were stained with purified anti-human CD8, followed by biotin-conjugated secondary antibody. Horse raddish peroxidase ABC kit was used to detect the secondary antibody. Note that many CD8$^+$ T cells (arrows) are in close apposition to neuronal cell bodies (white arrow heads). We also noticed clusters of T cells surrounding neurons in a few field of views (right pictogram); perhaps indicating a reactivation event.*

APPENDIX A

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