# Contributions of Innate and Adaptive Immunity in the Development of Herpes Stromal Keratitis After Corneal HSV-1 Infection

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Acute herpes simplex virus type 1 (HSV-1) infection is readily cleared by the host's immune system; however, HSV-1 also establishes a lifelong latent infection within neurons. HSV-1 can remain latent indefinitely but can also reactivate and cause recurrent peripheral disease. The cornea is one potential site of HSV-1 infection and viral reactivation can lead to the development of herpes stromal keratitis (HSK). HSK is characterized by progressive corneal opacity, neovascularization, and hypoesthesia that can lead to blindness. Although most people contract HSV-1 by adulthood, only a fraction develop HSK, despite many showing evidence of viral shedding. This disparity raises the questions of what triggers HSK development in only some individuals and how we can prevent reactivation events that contribute to HSK development. It has previously been shown that HSV-1-specific $CD8^+$ T cells are crucial in preventing reactivation in the neurons of the trigeminal ganglion (TG); however, a portion of these cells undergo functional impairment. To better understand how function is regulated in these cells, we assessed the expression of checkpoint molecules and unexpectedly found that T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), which has previously been associated with functional impairment, is found on highly functional cells within the TG. Tim-3 expression on $CD8^+$ T cells was influenced by viral gene expression and marked cells that produced effector molecules during both acute and latent infection, suggesting that Tim-3 expression indicates recent stimulation, rather than exhaustion. These results suggest that T cell impairment in HSV-1 infection may be

phenotypically distinct from exhaustion in chronic infections. To address what triggers HSK development, we utilized pathogenic and nonpathogenic strains of HSV-1 to assess the immune response during infection. We found that mice infected with the pathogenic strain had lower viral titers in the cornea but a more robust immune response, which included increased numbers of inflammatory innate immune cells and upregulation of proinflammatory mediators. This suggests that early interventions that blunt the innate response to HSV-1 may result in more favorable visual outcomes. Collectively, this work further defines HSV-1-specific $CD8^+$ T cell impairment in the TG and identifies early innate responses as orchestrating HSK development.

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## Preface

First and foremost, I would like to thank my mentors. My PhD journey began with Dr. Robert Hendricks and I am grateful that I was able to start my foray into immunology under the tutelage of a mentor with such a wealth of knowledge, eternal optimism, and sense of humor. As my PhD studies conclude, I am thankful for the guidance of Dr. Anthony St. Leger, who not only took over my training without complaint when Bob retired, but has also given me the freedom to steer my projects and encouraged me to embrace new advances in the field to further my work. I also greatly appreciate the support and feedback from my committee members Drs. Paul Kinchington, John Williams, Louise D'Cruz, and Lawrence Kane.

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Finally, I would like to thank my family and friends for always being there for me. I would especially like to thank my husband Jason for always believing in me and my mom, who has always been my inspiration and my number one cheerleader from the moment I declared that I wanted to be a scientist.

## List of Abbreviations

Bat3 – HLA-B associated transcript 3

BTLA – B- and T-lymphocyte attenuator

CEACAM1 – Carcinoembryonic antigen-related cell adhesion molecule-1

CTLA-4 – Cytotoxic T-lymphocyte protein 4

dpi – Days post-infection

DC – Dendritic cell

DLN – Draining lymph node

dsDNA – Double-stranded DNA

EAE – Experimental autoimmune encephalomyelitis

FGL1 – Fibrinogen-like protein 1

GAG – Glycosaminoglycans

gB – Glycoprotein B

gB-CD8$^+$ T cells – CD8$^+$ T cells specific for the immunodominant gB epitope

gC – Glycoprotein C

gD – Glycoprotein D

gH – Glycoprotein H

gL – Glycoprotein L

GzmB – Granzyme B

HBV – Hepatitis B virus

HCF-1 – Host cell factor-1

HCV – Hepatitis C virus

HIV – Human immunodeficiency virus

HMGB1 – High mobility group protein-1

HSV-1 – Herpes simplex virus 1

HSV-2 – Herpes simplex virus 2

HVEM – Herpesvirus entry mediator

IFN – Interferon

IgV – immunoglobulin variable

IL – Interleukin

iNOS – inducible nitric oxide synthase

ISGs – Interferon stimulated genes

ITIM – Immunoreceptor tyrosine-based inhibitory motif

ITSM – Immunoreceptor tyrosine-based switch motif

JAK1 – Janus kinase 1

KO – Knockout

LAG3 – Lymphocyte activation gene 3

LAT – Latency-associated transcript

LCMV – Lymphocytic choriomeningitis virus

MAVS – Mitochondrial antiviral signaling protein

MDA5 – Melanoma differentiation-associated protein 5

miRNA – MicroRNA

NF-κB – Nuclear factor kappa-light chain enhancer of activated B cells

NK – Natural killer

OAS – 2′,5′-oligoadenylate synthetase

Oct-1 – Octamer binding protein-1

PACSIN1 – protein kinase C and casein kinase substrate in neurons 1

PAT – proline-alanine-threonine

PD-1 – Programmed death-1

PD-L1 – Programmed death-ligand 1

PD-L2 – Programmed death-ligand 2

PI3K – Phosphoinositide 3-kinase

PKR – Protein kinase R

PP1 – Protein phosphatase 1

PtdSer – Phosphatidylserine

PVR – Poliovirus receptor

RAGE – Receptor for advanced glycation end products

RIG-I – Retinoic acid-inducible gene I

ROS – Reactive oxygen species

RR1-$CD8^+$ T cells – $CD8^+$ T cells specific for the subdominant RR1 epitopes

SHIP1 – SH2 domain-containing inositol-5-phosphatase 1

STAT1 – Signal transducer and activator of transcription 1

Subdom-$CD8^+$ T cells – $CD8^+$ T cells specific for the eighteen subdominant epitopes

TAM – Tyro3, Axl, and Mer

TAP – Transporter associated with antigen processing

TCR – T cell receptor

TGF-β – Transforming growth factor-beta

TIGIT – T-cell immunoreceptor with Ig and ITIM domains

Tim-3 – T-cell immunoglobulin and mucin-domain containing-3

TLR – Toll like receptor

TNF – Tumor necrosis factor

$U_L$ – Unique long

$U_S$ – Unique short

VEGF – Vascular endothelial growth factor

WT – Wild-type

VZV – Varicella zoster virus

# 1.0 Introduction

## 1.1 HSV-1 Infection

There are hundreds of viruses known to infect humans, ranging from innocuous ones that go unnoticed by the host, to those that cause symptoms terrifying enough to inspire movies to be made about them (1). Herpes simplex virus type 1 (HSV-1) falls somewhere in the middle, with most pathologies caused by HSV-1 being fairly mild and more of a nuisance than a life-threatening event. This relatively benign infection and its ability to transition from a lytic to a lifelong latent infection has likely contributed to how HSV-1 has successfully permeated the human population and become one of the most common viral infections worldwide.

### 1.1.1 Epidemiology

HSV-1 is one of nine viruses in the *Herpesviridae* family that infects humans and is easily contracted through oral-to-oral contact. Other well-known family members include varicella zoster virus (VZV), which causes chickenpox and herpes zoster (shingles), and herpes simplex virus type 2 (HSV-2), which is the primary cause of genital herpes lesions but can also cause oral lesions similar to those seen in HSV-1 infection. Although the prevalence of HSV-1 varies from country to country, a study that assessed infection rates in individuals under the age of 50 found that the worldwide incidence of HSV-1 is estimated to be almost 70% within this population, with the Americas having the lowest overall average incidence and Africa and the Mediterranean having the highest (2).

In the United States, a little over 50% of people between the ages of 14 and 49 are seropositive for HSV-1; however, this number has been slowly decreasing over time, with the decline being attributed to better access to sanitation and improved living conditions (3). Within this population, seroprevalence of HSV-1 increases with age, with 30% of individuals aged 14-19 having HSV-1 as compared to almost 64% of individuals aged 40-49 (3). When cadavers were assayed for the presence of HSV-1 using PCR-based methods, 100% of people over the age of 60 were found to be positive for HSV-1, suggesting that it is merely a question of when, not if, a person will contract HSV-1 during their lifetime (4). When demographic factors are considered, being female or having an income that falls below the poverty line puts one at a higher risk for contracting HSV-1. The likelihood of becoming infected with HSV-1 is also increased for individuals who are sexually active, especially if they have had three or more partners (3). Although the incidence of HSV-1 may be slowly declining within the United States, a significant proportion of the population worldwide will contract HSV-1 during their lifetime, representing a large healthcare burden and necessitating further study into how to prevent and treat HSV-1 infections.

### 1.1.2 HSV-1 Mediated Disease States and Treatments

As a virus that can readily infect epithelial cells, HSV-1 has a multitude of sites that it is able to target for its initial infection. Of particular importance are the oral, genital, and ocular mucosal surfaces. The vast majority of HSV-1 infections affect the oral mucosa, with initial infection and reactivation events resulting in the formation of painful lesions on the lips and gums. Although HSV-2 is the primary cause of herpes-associated genital lesions worldwide, HSV-1 can also result in the development of these lesions and is, in many developed countries, overtaking

HSV-2 as the primary cause of herpes-associated genital lesions (2, 5-7). Although less common than oral or genital infections, HSV-1 infection of the cornea can cause severe inflammation and result in significant visual and quality of life impairment.

With a substantial portion of the world being infected with HSV-1, an effective vaccine could have a significant impact on reducing the health care burden created by these diseases. Unfortunately, despite a considerable amount of effort, none of the current vaccine candidates have resulted in meaningful protection against HSV-1 infection. Thus, the current treatments for HSV-1 infection are primarily based on limiting the course of lytic infection, preventing reactivation events that result in new rounds of productive infection, and controlling the immune response to the infection to prevent extensive tissue damage. The development of antivirals has been key in limiting lytic infections. Most patients who are undergoing treatment receive acyclovir (or one of its derivatives), a nucleoside analog that selectively targets infected cells (due to interactions with the viral thymidine kinase and viral DNA polymerase) and inhibits viral replication by terminating DNA synthesis and inactivating the viral polymerase (8). Additional antivirals such as foscarnet and cidofovir can also be used to control HSV-1 infection and function by inhibiting the viral DNA polymerase (9, 10). However, foscarnet and cidofovir are more toxic than acyclovir and thus are usually only used in patients with acyclovir resistant strains of HSV-1. When HSV-1 infects the cornea, additional treatments including corticosteroids are sometimes administered alongside antivirals to temper inflammation and prevent subsequent damage to the tissues of the eye (11).

### 1.1.3 HSV-1 Virion and Genome Organization

HSV-1 is a large dsDNA virus with a genome contained within an icosahedral capsid that is surrounded by tegument proteins before being enclosed by a bilayered envelope that is derived

from the host cell during the production of infectious virus (Figure 1). This envelope contains an average of 650 glycoprotein spikes that facilitate viral entry into host cells and extend the diameter of the virion to ~225nm (12). The tegument that is contained within the virion is comprised of over a dozen viral proteins with functions that range from transactivation of viral gene expression to immune evasion, allowing for rapid viral takeover of the host cell.

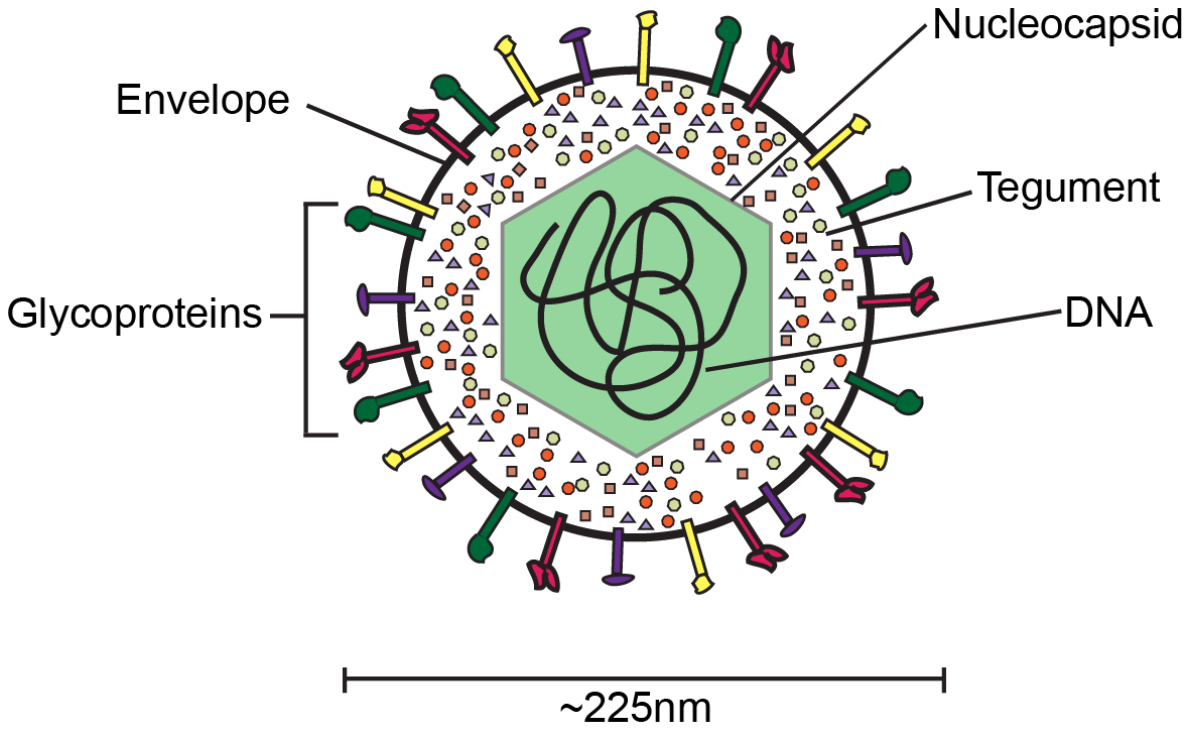



**Figure 1. HSV-1 Virion Structure.**
The dsDNA genome of HSV-1 is contained within an icosahedral capsid that is enclosed within a lipid envelope studded with viral glycoproteins. Tegument proteins are found within the space between the capsid and the envelope and play critical roles in initiating viral transcription and shut down of host cell processes.

The ~152kb HSV-1 genome is linear and consists of connected unique long ($U_L$) and unique short ($U_S$) regions that are each flanked by repeat regions (Figure 2) (13-17). These repeat regions vary in length between strains, and the repeats on one end of each unique region are inverted on the other end (17, 18). Although the HSV-1 genome is made up of a single linear piece

of dsDNA, four isomers of the genome exist due to the short and long segments having two possible orientations (19). Although these four isomers were originally thought to be present in equal amounts in infected cells, more recent work has shown the ratios to be strain and cell/tissue type specific (19, 20).

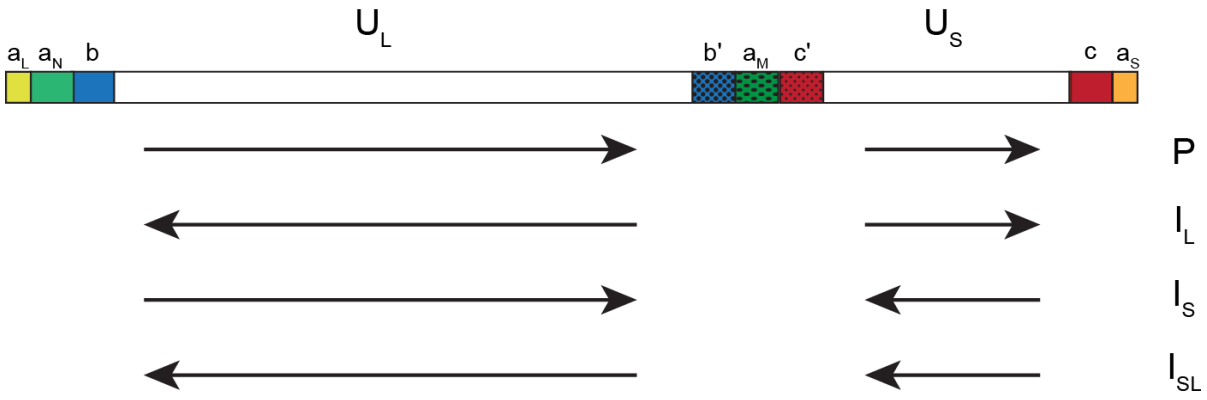



**Figure 2. HSV-1 Genome Structure.**
The HSV-1 genome is comprised of unique long ($U_L$) and unique short ($U_S$) regions framed by terminal repeats. $a_L$ and $a_S$ are found on the termini of the $U_L$ and $U_S$ regions, respectively. $a_N$ and $a_M$ indicate sequences with variable lengths. Due to inversions of both the $U_L$ and $U_S$ regions, four isomers of the HSV-1 genome can occur as indicated by the directional arrows under the unique segments.

The HSV-1 genome encodes at least 80 proteins and additionally produces a number of transcripts called latency associated transcripts (LATs) that do not encode proteins but may play a role in establishment and maintenance of viral latency (21). The genes themselves are divided into three classes, immediate-early (α), early (β), and late (γ), and these genes are expressed at different times during the viral lifecycle.

### 1.1.4 HSV-1 Life Cycle

HSV-1 has a two-phase life cycle consisting of a short lytic infection followed by a latent infection in neurons that can persist indefinitely. In response to certain stimuli, the virus can reactivate from this latent state. Viral reactivation is defined as expression of lytic gene products followed by the production of infectious viral particles. The severity of disease resulting from reactivation events can vary from person to person, with some people experiencing recurrent pathologies while others shed the virus asymptomatically.

#### 1.1.4.1 Lytic Infection

Initial HSV-1 infection can occur in a variety of tissue types, with epithelial cells in mucosal tissues being a common target. Five of the viral glycoproteins found embedded in the viral envelope are known to play important roles in facilitating viral attachment and entry into the host cell. Glycoprotein B (gB) first tethers the virus particle to the host cell through interactions with a variety of glycosaminoglycans (GAGs), with heparan sulfate being the preferred target (22). Glycoprotein C (gC) can also bind to GAGs and contribute to binding to the host cell, although studies using gC-null viruses have shown that it is not required for infection in many cell types (23). Following virion binding to heparan sulfate, glycoprotein D (gD) binds to one of (at least) three receptors, herpesvirus entry mediator (HVEM), nectin-1, or heparan sulfate that has been modified by 3-O-sulfotransferases (24-28). The fact that HSV-1 is able to utilize multiple receptors for cell entry likely contributes to its broad tissue tropism and overall success in infecting the majority of the population.

Once gD has bound its receptor, viral entry is initiated. This can occur through fusion of the viral envelope with the host cell membrane or fusion with the vesicle membrane after

endocytosis (Figure 3-1). There have also been several reports suggesting that HSV-1 can enter some cell types through phagocytosis (29, 30). While there are still many parts of the membrane fusion process that are not well understood, the general consensus in the field is that after binding one of its receptors, gD undergoes a conformation change that activates the fusion complex, consisting of gB, glycoprotein H (gH), and glycoprotein L (gL). gH and gL form a heterodimer on the viral envelope and the conformational change in gD presumably induces subsequent conformational changes in gH/gL that activate gB and position it close enough to the cell membrane to trigger fusion with the viral envelope.

Following fusion at the host cell or vesicle membrane, the viral capsid is transported into the cell nucleus via microtubules (31). Once there, the viral capsid docks at a nuclear pore (Figure 3-2) and the viral genome is released into the nucleus where it can subsequently circularize, associate with histones, and form a type of disordered chromatin, which helps regulate viral gene expression (Figure 3-3) (32, 33). Viral replication begins with the transcription of the immediate early (α) genes (Figure 3-4). This transcription requires no *de novo* protein synthesis, as the tegument proteins that are packaged with the virus, in combination with host factors, form a fully competent transcriptional unit. VP16, one of the viral tegument proteins, plays a critical role in this process by recruiting a number of host factors including octamer binding protein-1 (Oct-1) and host cell factor-1 (HCF-1) (which associate with VP16 and bind to TAATGARAT sequences in viral promotors), demethylases, transcription factors, and RNA polymerase II to immediate early gene promotors (34-39). Once the immediate early genes are transcribed, their mRNAs are exported to the cytoplasm and translated by host machinery. There are six proteins produced from the immediate early gene transcripts: ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$. These proteins are required for the efficient expression of early (β) and late (γ) genes and can also function to

suppress the immune response to infection and prevent shutdown of viral replication by the host cell.

Following immediate early gene expression, early and late genes are transcribed (Figure 3-5 and 3-7) and genome replication is initiated (Figure 3-6). Genome replication requires the synthesis of the early gene proteins, as at least seven of them directly contribute to replication by providing necessary components of the replication complex, such as the DNA polymerase that is encoded by $U_L30$ and $U_L42$ (40-42). Once translated, these proteins are transported into the nucleus where they associate with the viral genome and form replication compartments. These compartments enlarge and merge as the infection proceeds, eventually filling the nucleus (43). Although the exact mode of genome replication has yet to be fully defined for all stages of infection and cell types, it is generally accepted that the viral genome is ultimately produced in long concatemers that are comprised of many repeats of the genome fused together (44).

After genome replication begins, there is an increase in the expression of late genes, many of which encode the tegument and capsid proteins required for new virion assembly (Figure 3-7) (45, 46). Once these late gene products are produced in sufficient quantities, the capsid proteins are transported into the nucleus where the procapsid is assembled around the scaffolding proteins encoded by $U_L26$ and $U_L26.5$ (47-49). Following assembly of the procapsid, the scaffolding proteins are cleaved by the protease function of $U_L26$ and the capsid undergoes a conformational change (50, 51). This allows for the insertion of the viral genome in which the free end of a genome concatemer is fed into the empty capsid via a portal, followed by cleavage of the genome at the packaging sites *pac1* and *pac2* once one full length genome has been inserted into the capsid (Figure 3-8) (52-54).

With viral genome packaging completed, the last step in productive viral infection is the procurement of the viral tegument proteins and envelope. To leave the nucleus, the virus exits via an intermediate envelopment step in which the virion acquires an envelope at the inner nuclear membrane that it then loses at the outer nuclear membrane (55). From there the viral capsid localizes to either the trans-Golgi network or endosomes that contain the required tegument and glycoproteins and merges with it to form a completed virion that is encapsulated by a secondary membrane (Figure 3-9) (56-58). The encapsulated virus is then trafficked to the cell surface, where the secondary membrane can undergo fusion with the cell membrane and release the virion into the extracellular space (Figure 3-10).

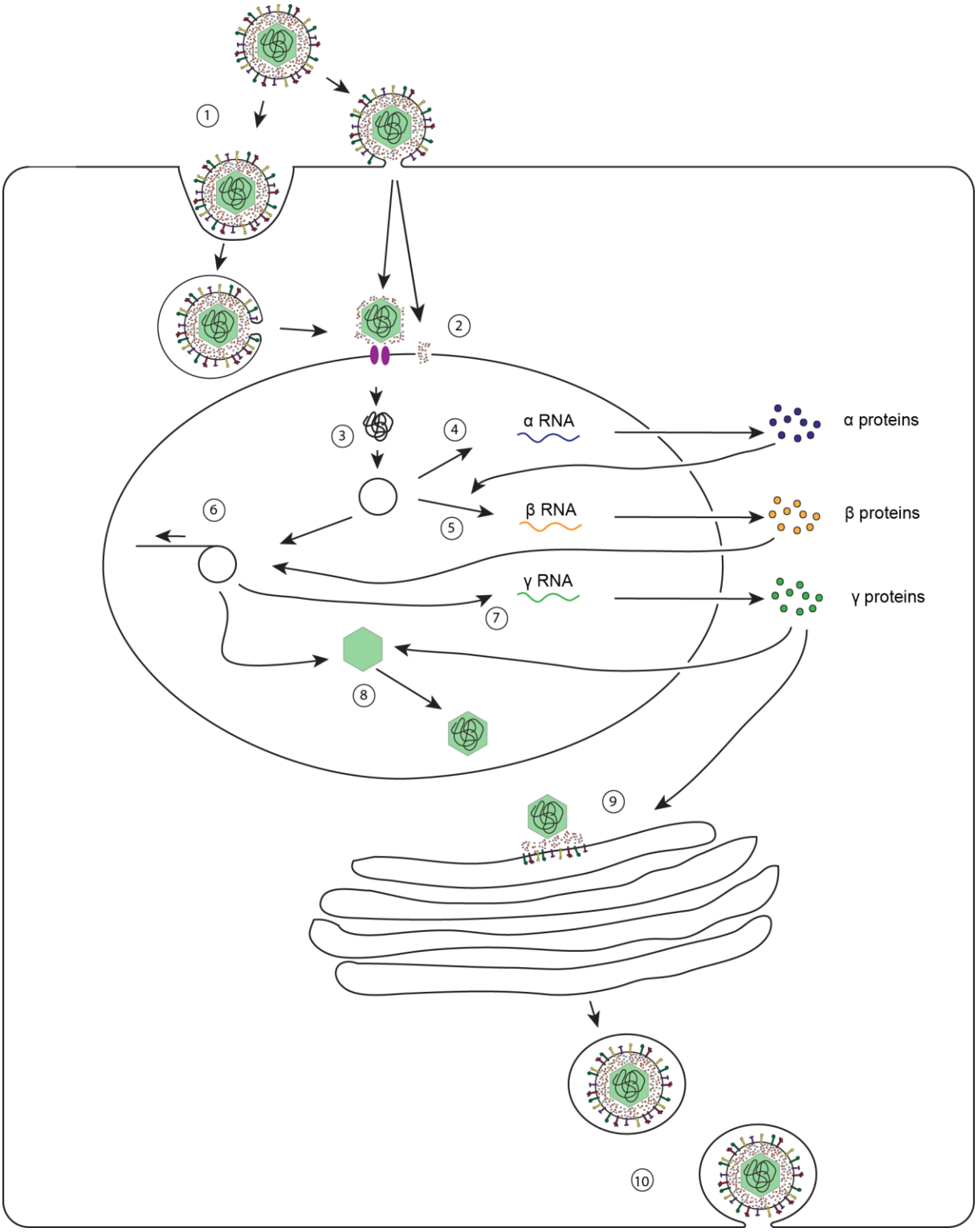



**Figure 3. HSV-1 Replication.**
(1) After binding of the HSV-1 glycoproteins to their receptor(s), the virus can enter the host cell by either fusing with the plasma membrane or by undergoing endocytosis. (2) The viral capsid is trafficked to a nuclear pore where the viral DNA is inserted into the nucleus. Essential tegument proteins such as VP16 also enter the nucleus at this time via interactions with host proteins such as HCF. (3) Once in the nucleus, the viral DNA can circularize. (4) The

immediate early (α) genes are the first genes to be transcribed, with the resulting mRNA transported into the cytoplasm and translated by host machinery. (5) The newly translated α gene products transactivate early (β) genes. (6) The β gene products initiate viral DNA replication. (7) Viral DNA replication enhances late (γ) gene expression. (8) Viral capsids are assembled within the host nucleus and viral DNA is inserted. (9) The viral capsid exits the nucleus and associates with viral tegument proteins and glycoproteins at the trans-Golgi network and acquires a double membrane. (10) The virus fuses with the host cell membrane, losing its outer membrane and expelling a complete viral particle.

#### 1.1.4.2 Latent Infection and Reactivation

HSV-1 can readily infect a wide assortment of cell types and rapidly replicate itself through the lytic infection cycle. However, much of HSV-1's success as a pathogen is due to its ability to infect neurons and switch into a dormant state called latency, allowing it to evade the host immune system. HSV-1 latency can be defined as a lack of infectious viral particle production with little or no viral gene expression. Although reactivation events can restart the lytic infection cycle, the virus is able to be maintained in this latent state for extended periods of time.

During primary lytic infection, HSV-1 can infect the neurons that innervate the tissues where the initial infection occurs through fusion with the cell membrane at the axon termini. From there, the viral capsid is transported via retrograde axonal transport to the neuronal nucleus where the viral genome circularizes into an episome. Although the virus can undergo lytic replication at this point, lytic gene expression is suppressed within a few days of initial infection (59). This suppression of productive viral infection is likely due to a number of factors including, inefficient transport of the tegument protein VP16 into the nucleus (as the nucleus can be a considerable distance from the axon termini in neurons), insufficient access to required host factors, repression by host and viral gene products, and packaging of most of the viral genome into heterochromatin (60-65). While lytic gene expression tapers off during neuronal infection and is only sporadically detected during latent infection, expression of the LATs increases during the first few days of infection in neurons and is stably expressed throughout latency (59, 66, 67).

As LATs are the primary viral gene transcripts found within latently infected neurons, understanding the role of these transcripts in the control of viral replication has long been a goal of the field. As a group, the LATs consist of a number of splice variants (from a parent transcript of about 9 kb) that do not seem to encode proteins. The LAT gene is flanked by chromatin insulating elements that likely contribute to the preferential transcription of the LATs as compared to the rest of the HSV-1 genome, which is transcriptionally repressed during latency by heterochromatin packaging (68). Although the LAT parent transcript itself is found within latently infected neurons, it appears that the smaller LATs that are produced through splicing are more stable and are thus present in higher amounts (69, 70). It was originally thought that the LATs were required to initiate viral latency; however, multiple studies using LAT knockout viruses have provided evidence that argues against this (71-73). While LATs may not be necessary for the establishment of latency, they do play an important role in suppressing lytic viral gene expression through miRNA production, by enriching the formation of heterochromatin on lytic genes, and by contributing to the prevention of neuronal death (21, 74-79).

Through the combined contributions of the LATs, host miRNAs, and the host immune system (described in detail in Section 1.1.7.2), viral latency can be efficiently maintained for the life of the host. However, certain perturbations such as trauma or UV exposure can upset this tenuous stalemate between the host and virus and reinitiate the lytic viral lifecycle (80, 81). Like the establishment and maintenance of latency, viral reactivation is a complex process that has not been linked to one single event or expression of a single viral protein, despite many studies that have investigated the role of HSV-1 proteins such as ICP0 and VP16 in initiation of reactivation (82-85). Instead, it has been proposed by several groups that viral reactivation occurs as a two-step process (86, 87). In the first phase of reactivation, or “animation” phase, low levels of viral gene

expression occurs in a disordered fashion that does not appear to require VP16, which is unique from the orderly cascade that is observed during primary infection (88, 89). This low level of gene expression can be detected in a variety of ways, including direct detection by sensitive PCR techniques and indirectly through using reporter mice and by assessment of activation of HSV-1-specific T cells within infected TGs (67, 90-93). The gene expression observed during phase I appears to occur despite repressive heterochromatic marks on the HSV-1 genome (94).

In phase II of viral reactivation, there is a transition from disordered gene expression to the normal α, β, γ cascade that occurs during productive infection. This transition is likely due to an accumulation of necessary viral gene products, such as VP16, reaching a threshold that allows the virus to overcome the suppressive measures that normally maintain it in a latent state. Although it is not entirely clear what events need to occur to achieve full reactivation, is has been hypothesized that VP16's ability to recruit histone demethylases and other chromatin remodeling enzymes to the repressed HSV-1 genome contributes to the re-initiation of viral gene expression (95). As the HSV-1 genome transitions into a more transcriptionally conducive state as repressive heterochromatic marks are removed, VP16 can once again transactivate viral gene expression of immediate early genes. Once the immediate early genes are translated, additional viral proteins such as ICP0 likely contribute (via its E3 ubiquitin ligase activities) to the continued removal of repressive heterochromatin, allowing for the expression of the full HSV-1 genome and production of infectious viral particles.

### 1.1.5 Herpes Stromal Keratitis

Herpes stromal keratitis (HSK) is a debilitating disease of the cornea that can develop after HSV-1 infection. Unlike many of the HSV-1 induced disease states, much of the pathology that

defines HSK appears to be due to the immune response to the virus rather than viral replication itself. Corneal HSV-1 infection starts in the epithelial cells of the cornea where viral replication can cause lesions. These lesions usually resolve within several days of initial infection and primary infection does not usually result in any lasting pathology (96, 97). While the virus is replicating within the corneal epithelium, it is also able to infect and establish latency in the neurons of the trigeminal ganglion (TG), which provide sensation to the eye. The virus persists in the TG in its latent state until reactivation is initiated, which results in productive viral infection first in the TG neurons and subsequently in the cornea after anterograde transport of the virus down the axons of the TG neurons (98). Although many infected individuals asymptomatically shed virus at the tear film, only a subset go on to develop HSK (99, 100).

HSK is defined as the progressive development of opacity, edema, hypoesthesia, and neovascularization of the normally avascular cornea. HSK is classified into two types, necrotizing and non-necrotizing. Necrotizing HSK consists of inflammation in the corneal stroma, coupled with defects in the epithelial cells that are presumably due in part to active viral replication while non-necrotizing HSK is characterized as stromal inflammation without obvious epithelial involvement (101, 102). HSK can develop after a single reactivation event; however, in most individuals, HSK develops after multiple reactivation events. Although the symptoms of HSK can be managed by the administration of antivirals and topical corticosteroids, repeated reactivation events can lead to a progressive accumulation of scar tissue in the cornea and ultimately an impairment of vision (103, 104). Once excessive scarring has occurred within the cornea, treatment to restore vision is currently limited to corneal transplants.

### 1.1.6 Animal Models of Corneal HSV-1 Infection

The primary host of HSV-1 is the human, although for both ethical and practical reasons most of the research on HSV-1 has made use of tissue culture and animal models. The two most prominent animal models used in corneal HSV-1 research are the rabbit and the mouse. Both of these animals can be readily infected with HSV-1 and maintain latent infections. The rabbit can spontaneously reactivate and shed virus, making it a useful model for studying recurrent disease (105, 106). The mouse exhibits a more stable latent infection that is difficult to experimentally reactivate and is thus well-suited to studying the maintenance of latency (80, 107, 108). In terms of corneal HSV-1 infection, both the mouse and the rabbit develop a primary epithelial disease that mimics human infection. Both animals are also able to develop immune-mediated HSK, although murine corneal HSV-1 infection induces the development of HSK shortly after primary infection (Figure 4) as opposed to development after reactivation. The mouse model benefits from a wide variety of available transgenic strains and immunological reagents, which are somewhat limited in the rabbit model. As such, the experiments in the following chapters use the mouse model, which has proved to be of great utility in dissecting the complicated immune response to HSV-1 infection.

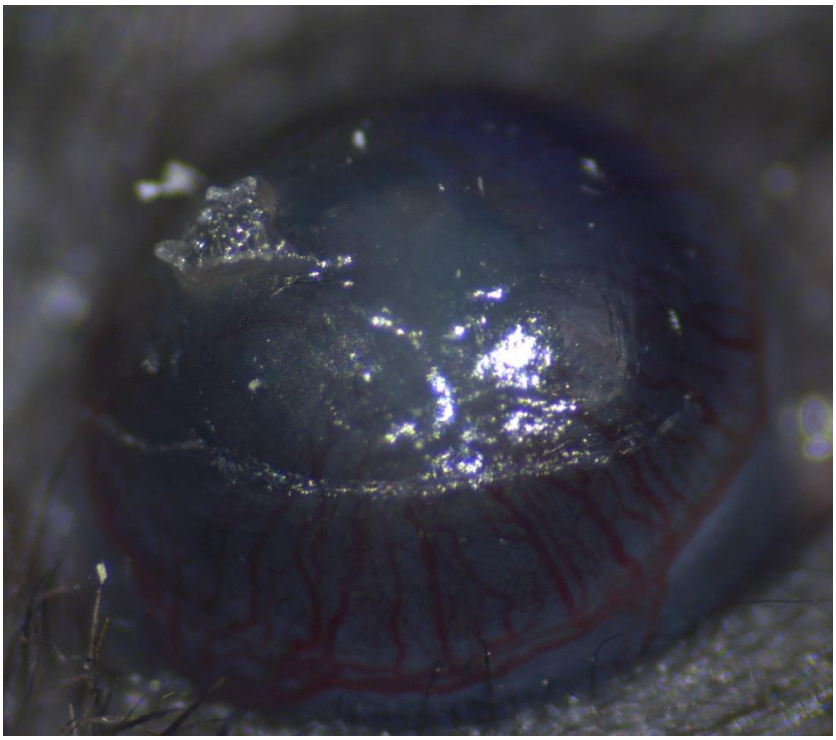


**Figure 4. HSK in the C57BL/6 mouse.**
C57BL/6 mice infected with the HSV-1 strain RE develop a severe HSK characterized by neovascularization, edema, opacity, and loss of corneal sensitivity. Image shown is of a mouse cornea 21 dpi with HSV-1 RE.

### 1.1.7 Immune Response to HSV-1 Infection

With its two-phase lifecycle, HSV-1 presents a challenging problem for the host's immune system. Not only does the host need to rapidly respond to the initial infection and control it, it also needs to help maintain viral latency without damaging the neurons that the virus hides in. An efficient innate immune response serves to quickly rein in viral replication while the adaptive immune response develops a complex relationship with neurons that allows for suppression of viral replication without irreparable damage to the neurons themselves. However, the virus is not defenseless in this battle with the host's immune system and has developed a considerable arsenal of immune evasion techniques that actively seek to limit the host's ability to clear the infection.

The following two sections will focus on the host's immune response during acute and latent HSV-1 infection respectively, while section 1.1.7.3 will briefly address some of HSV-1's most well characterized immune evasion techniques.

#### 1.1.7.1 Acute Infection

##### *Innate Response*

As the initial step in host defense against HSV-1 infection, the innate immune response employs a wide range of sensing mechanisms to inhibit viral takeover. These sensors are widely expressed across many different tissue types, including both the cells at the barrier sites where the virus enters the host and the infiltrating immune cells that help control and clear the infection. The virus first encounters these sensors on the surface of the epithelial cells that it targets in its primary infection in the form of Toll-like receptors (TLRs). HSV-1 infection has been shown to activate innate responses at the cell surface through TLR2, due to interactions with gB and gH/gL (109-111). Once inside the host cell, the virus can induce signaling through TLR3, which recognizes dsRNA, and TLR9, which detects unmethylated CpG motifs (112, 113). Signaling through these receptors results in the synthesis of proinflammatory cytokines like interleukin (IL)-6 and tumor necrosis factor (TNF)-α and initiates the production of the type I interferons (IFN)-α and -β, which are essential for control of HSV-1 infection (114-116). In addition to TLR3, HSV-1 dsRNA can also be sensed by protein kinase R (PKR), which phosphorylates eIF2α and stops protein translation, and 2′,5′-oligoadenylate synthetase (OAS), which activates RNase L and leads to RNA degradation (117-119). Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) also recognize HSV-1 RNA in the cytoplasm and their signaling cascade, which shares the adapter mitochondrial antiviral signaling protein (MAVS), is similar to

that of the TLRs, resulting in cytokine and type I interferon production (120, 121). HSV-1 DNA can be detected by a number of sensors including IFN-γ-inducible protein (IFI16), DNA-dependent activator of interferon regulatory factors (DAI), absent in melanoma 2 (AIM2), and cyclic GMP-AMP synthase (cGAS) (122-126). Detection of viral DNA by most of these sensors also results in cytokine and interferon production. Additionally, AIM2 and IFI16 have been shown to trigger inflammasome formation, which activates caspase 1 and results in the cleavage of pro-IL-1β and pro-IL-18 to their active, proinflammatory forms.

With the abundance of sensors that can detect HSV-1, the host is able to quickly raise the alarm and rapidly recruit various immune effectors after infection first occurs. This recruitment is mediated by chemokines such as CXCL1, CXCL10, CCL5, and CCL7, which are expressed as part of the downstream signaling cascades initiated by the triggering of the aforementioned sensors. Within the first few days of infection, these chemokines attract neutrophils, dendritic cells (DCs), macrophages, natural killer (NK) cells, and γδ T cells to the site of infection, where several of these cell types play a crucial role in limiting viral replication.

Neutrophils are some of the first responders to HSV-1 infection and infiltration of infected tissues has been demonstrated within hours of initial infection (127). While neutrophils presumably contribute to control of infection by the production of reactive oxygen species (ROS) and proinflammatory chemokines and cytokines, they appear to be dispensable for limiting HSV-1 replication and can actually contribute to the immunopathology observed during corneal HSV-1 infection (128, 129). A population of DCs reside within noninfected murine corneas and additional DCs infiltrate within the first few days of HSV-1 infection (130, 131). These DCs play a role in HSV-1 control by activating other infiltrating immune cell types, through production of type I interferons, and by bridging the innate and adaptive immune responses by functioning as antigen

presenting cells (APCs) that prime T cell responses within both the draining lymph nodes (DLNs) and the infected tissues themselves (121, 132).

DCs have also been shown to create a chemokine gradient in the cornea that is important for the recruitment of inflammatory monocytes and NK cells (133). NK cells function by patrolling tissues to look for aberrant expression of proteins on the cell surface that indicate that the cell is infected or otherwise compromised. Once such a cell is identified via the collective feedback from receptors that provide either inhibitory or activation signals to the NK cell, cytotoxic granules and IFN-γ are released. These effector molecules can directly inhibit viral replication in the infected cell or activate other immune cells such as macrophages. Removal of NK cells during corneal HSV-1 infection results in slower clearance of the virus, increased mortality, and an increase in lesion severity, indicating that NK cells have a significant role in controlling early infection in this tissue (133-135).

Inflammatory monocytes and macrophages are also important for control of HSV-1 infection (136, 137). These cells are readily activated by TLR signaling and produce type I interferons and, in the context of infection, proinflammatory cytokines like TNF-α and IL-6. These cells can also produce IL-12, which serves to increase production of IFN-γ by NK cells (138, 139). Increased production of IFN-γ by NK cells can work in a feedback loop to reciprocally induce macrophage production of ROS and nitric oxide (NO), which is important in controlling viral replication (137). γδ T cells also may contribute to the control of viral infection in the TG and the brain, since mice that lack γδ T cells display increased viral burden (140). In the TG it appears that γδ T cells, not NK cells, are the primary source of IFN-γ required for macrophage activation and control of viral replication at early times post-infection (137).

### *Adaptive Response*

As a rapidly replicating virus, HSV-1 is, by necessity, mostly controlled by the innate immune response both at the site of primary infection and in the neurons that house the latent virus. However, the adaptive immune response is also essential in controlling viral infection as RAG knockout mice, which lack B and T cells, are unable to clear the virus from the cornea or skin and eventually succumb to encephalitis or limb paralysis (141, 142). Further work into the role of the adaptive response showed that despite priming the production of antibodies by activated B cells, the humoral response to HSV-1 is not sufficient to prevent infection and control viral replication, although it can help limit viral spread through infected tissues (143, 144). HSV-1 infection also primes robust $CD4^+$ and $CD8^+$ T cell responses and, unlike the humoral response, T cells have important functions in controlling viral replication both in the cornea and the TG.

Unlike innate immune responses, adaptive immune responses are specific to the encountered pathogen and as such, require additional steps in their development. T cell responses are initiated by the processing of viral antigens by professional APCs located within the DLNs. In these tissues, DCs are the predominant APCs and can take up antigen or free virus directly from the lymphatics or from the site of viral infection. These cells then proteolytically cleave viral proteins and load the resulting peptide fragments on either MHCI (recognized by $CD8^+$ T cells) or MHCII (recognized by $CD4^+$ T cells) complexes which are then transported to the cell surface for presentation. Naïve T cells found within the DLNs sample the surface of the DCs and once the correct combination of MHC complex and antigen for their unique T cell receptor (TCR) is encountered, signaling through the TCR, costimulatory receptors, and cytokine receptors results in T cell activation. After being activated, T cells proliferate and mature into effector T cells that patrol infected tissues. When these T cells recognize their cognate antigen, they can release

cytokines and/or degranulate to either help control viral infection or eliminate infected cells. Once the immunological threat has been contained, the population of responding T cells contracts and select members of the activated population are maintained, both in the lymphatic tissues and in the tissues of the original site of infection as memory T cells. These cells are long-lived and can rapidly respond to stimulation should they reencounter their antigen.

Activated $CD4^+$ and $CD8^+$ T cells can be detected within infected tissues by around five days post HSV-1 infection (145). $CD4^+$ T cells have been shown to help control viral replication as CD4 knockout mice experience a delayed clearance of the virus from infected corneas (146, 147). Although $CD4^+$ T cells contribute to viral clearance, they can also contribute to immunopathology, as is seen in the development of HSK (148-150). Highlighting the role of Th1 and Th17 $CD4^+$ T cells in corneal tissue damage, studies have shown that neutralization of the effector cytokines IL-2, IFN-γ, or IL-17 significantly reduces the severity of HSK (151-153). While $CD4^+$ T cells appear to be a regulator of corneal inflammation at later times in infection, it appears that neutrophils, not the $CD4^+$ T cell themselves, may have a primary role in mediating the immune damage to the cornea that occurs during the progression of HSK. Prolonged production of IFN-γ and IL-2 by $CD4^+$ T cells permits neutrophils to infiltrate and survive for extended periods of time in the cornea. These neutrophils contribute to HSK through the production of metalloproteinases, which cause extracellular matrix breakdown and promote neovascularization in the cornea by indirectly increasing the amount of available vascular endothelial growth factor (VEGF) (154).

Recent work from our laboratory has also implicated $CD4^+$ T cells in the development of the exposure keratitis (damage due to desiccation) that can sometimes occur during murine HSK development and greatly exacerbate its severity (155). This exposure keratitis occurs due to the

loss of corneal sensory nerves in infected corneas, leading to a decrease in blinking which is necessary to redistribute the tear film over the surface of the cornea and protect the integrity of the epithelial layers. When the tear film is disrupted, corneal desiccation and inflammation occur, further damaging the cornea. While the exact function and antigenic specificity of $CD4^+$ T cells in this loss of corneal sensation are still under investigation, it is clear that these cells play a significant role in the damage that occurs in the cornea during HSV-1 infection. Of note, humans possess consensual blink reflex (both eyes blink even with stimulus in only one) while mice do not (155). This likely contributes to the milder HSK normally seen in human patients compared to mouse models of HSK. However, some humans do develop corneal hypoesthesia and severe keratitis, which suggests a similar immune progression in humans as in mice.

Like $CD4^+$ T cells, $CD8^+$ T cells are found within most HSV-1-infected tissues within a week of initial infection. In the cornea, the overall numbers of $CD8^+$ T cells are much lower than $CD4^+$ T cell numbers and in mice that lack $CD4^+$ T cells, a more mild and somewhat transient HSK occurs, suggesting that $CD8^+$ T cells lack the capacity to cause HSK (146). While $CD8^+$ T cells do not appear to cause chronic HSK development, they are capable of controlling viral replication when transferred into HSV-1-infected RAG knockout mice (141). Within the TG, $CD8^+$ T cells primarily control viral replication starting around six dpi, preventing spread of the virus into the CNS (156, 157).

#### 1.1.7.2 Latent Infection

The host immune system efficiently clears HSV-1 from the epithelial cells that support primary infection, but the virus is nonetheless able to infect the nerves that innervate the initial site of infection. Unlike epithelial cells, neurons cannot regularly regenerate, so the host has developed

mechanisms to protect neurons from excessive immune responses. These protective measures allow the establishment of a latent HSV-1 infection that can persist indefinitely.

After corneal HSV-1 infection, the virus enters latency within the TG. Latency is maintained not only by host and viral factors (reviewed in Section 1.1.4.2), but also by activated T cells. $CD8^+$ T cells appear to be primarily responsible for the immune control of viral latency, although $CD4^+$ T cells indirectly contribute by facilitating the development of fully functional $CD8^+$ T cells during activation (157, 158). $CD8^+$ T cells have been shown in both mice and humans to surround neurons within the latently infected TG and form immune synapses, suggesting that these cells can see antigen, directly interact with neurons, and prevent viral reactivation (92, 159, 160). Indeed, work from our laboratory has shown that IFN-γ and granzyme B (GzmB) produced by $CD8^+$ T cells can prevent viral reactivation (161, 162). Interestingly, these interactions between $CD8^+$ T cells and neurons via IFN-γ and GzmB do not result in neuron cell death. It appears that these effector molecules can instead function through cleavage of the essential HSV-1 protein ICP4 (by GzmB) or by blocking ICP0 promotor activity (by IFN-γ), thus preventing the subsequent downstream gene expression that is required for production of infectious virus (162, 163). IFN-γ signaling also likely helps prevent HSV-1 reactivation by inducing an antiviral state within the neurons by upregulating various interferon stimulated genes (ISGs) such as 2′-5′OAS and PKR and the expression of MHC molecules, although some known downstream effects of IFN-γ signaling such as apoptosis are presumably suppressed in these neurons as a protective measure.

### *$CD8^+$ T cell Immunodominance During HSV-1 Infection*

The ability of $CD8^+$ T cells to interact with neurons within the HSV-1-infected TG and prevent viral reactivation through the production of effector molecules indicates both that there are low levels of viral protein expression present within the latently infected TG and that these $CD8^+$

T cells are specific to HSV-1. Work from our laboratory and others has established that this is the case. It was previously demonstrated that the majority of virus-specific $CD8^+$ T cells primed by HSV-1 infection in the C57BL/6 (B6) mouse are specific for a single, unusually prominent, immunodominant epitope from gB ($gB_{498-505}$, SSIEFARL) (164-166). Our laboratory has shown that in the HSV-1-infected TG, ~50% of the $CD8^+$ T cells are gB specific and that the remaining $CD8^+$ T cells are specific to an additional eighteen subdominant epitopes (145, 166, 167). Collectively, these data establish that essentially all the $CD8^+$ T cells found within the latent HSV-1-infected TG are specific to HSV-1. This conclusion is further supported by work showing that $CD8^+$ T cells must receive antigenic stimulation to be maintained within the HSV-1-infected TG, with activated, non-HSV-1-specific $CD8^+$ T cells being able to infiltrate the TG but being unable to be maintained within the TG over time (145, 168).

HSV-1 specific $CD8^+$ T cells are detected within the infected TG starting at around five dpi. Using a tetramer specific to the gB epitope, the virus-specific $CD8^+$ T cell response can be separated into two relatively equally sized populations, the gB-$CD8^+$ T cells (tetramer positive) and the Subdom-$CD8^+$ T cells (tetramer negative, collectively made up of $CD8^+$ T cells responding to the eighteen subdominant epitopes). When these populations are followed throughout the course of infection, it is evident that the immunodominance hierarchy is maintained from early times post-infection into latency (Figure 5) (145). Interestingly, TG gB-$CD8^+$ T cells peak one day later than Subdom-$CD8^+$ T cells during acute infection, although both populations contract at a similar rate and maintain a consistent ratio well into latent infection (145).

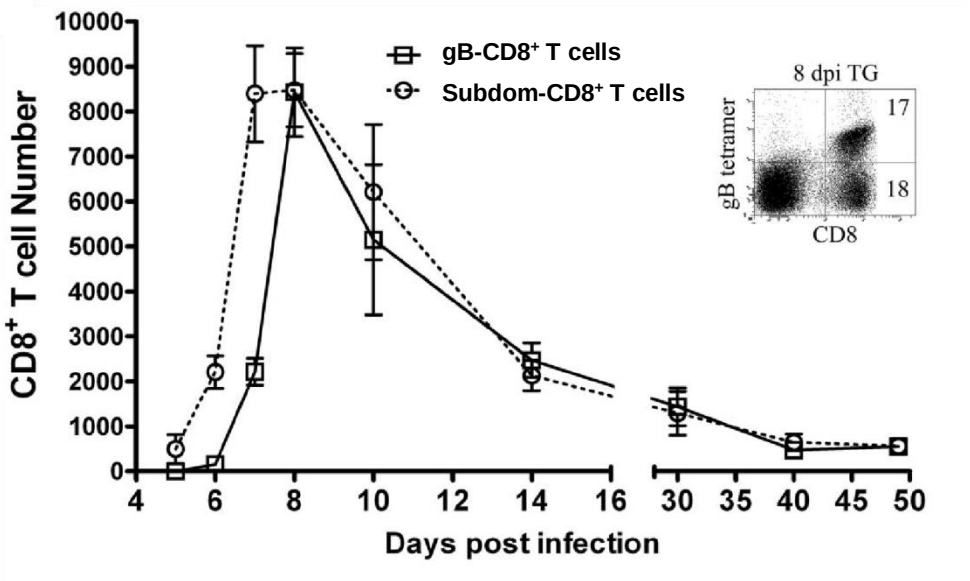



**Figure 5. Expansion and contraction of virus-specifc gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells in the HSV-1-infected TG.**
TGs from the indicated time points post-infection were processed and stained for CD8 and gB tetramer to allow for the quantification of the total number of gB-CD8$^+$ T cells (solid line) and Subdom-CD8$^+$ T cells (dashed line) during HSV-1 infection. Numbers shown are the mean ± SEM. The inset flow panel represents staining for CD8 and gB tetramer in an 8 dpi TG. Figure adapted from Sheridan, et.al., 2009 (145).

### *CD8$^+$ T Cell Functionality During HSV-1 Infection*

While these two virus-specific CD8$^+$ T cell populations enter and are maintained in equal numbers within the HSV-1-infected TG for extended periods of time, there are some substantial differences in their functionality during both acute and latent infection. During acute infection, gB-CD8$^+$ T cells express significantly higher percentages of activation markers such as CD69 and GzmB, than do Subdom-CD8$^+$ T cells (145). The gB-CD8$^+$ T cells also undergo significantly more proliferation within the infected TG than the Subdom-CD8$^+$ T cells at early times post-infection (145). When these populations were assessed for their capacity to produce IFN-γ and TNF-α during acute and latent infection, gB-CD8$^+$ T cells maintained their multifunctionality into latency, while Subdom-CD8$^+$ T cells experienced a significant decrease in their ability to respond to cognate antigen at latent timepoints (169). Staining of Subdom-CD8$^+$ T cells with tetramers revealed that

these cells are not lost from the TG but instead persist in a state of suboptimal function (169). A gradual loss of T cell function over time is a process known as T cell exhaustion. T cell exhaustion is generally seen in chronic infections or in cancer, when antigen persists in the afflicted tissues for an extended period of time. The development, consequences, and treatments for T cell exhaustion as well as the implications of functional impairment of HSV-1-specific $CD8^+$ T cells in preventing viral reactivation will be discussed in further detail in Section 1.2.

#### 1.1.7.3 HSV-1 Immune Evasion

In most viral infections, the immune system is able to clear the virus within a week or so of initial infection. HSV-1 manages to evade this complete clearance by establishing residence in neurons, which are protected from the full extent of the immune response. While this evasion technique is a key component of the virus's strategy to persist within the host, HSV-1 also encodes several proteins with functions that can actively inhibit innate or adaptive immune responses. As HSV-1 encodes over 80 proteins, the ways in which it can inhibit the immune response are numerous and varied. Although a detailed accounting of all of these strategies is beyond the scope of this dissertation, a few that impact key regulatory points in the immune response are summarized in the subsequent paragraphs.

As a tegument protein that is transported into the host cell along with the viral capsid, the virion host shutoff (vhs) protein is well positioned to help the virus combat the host immune response. An RNase, vhs plays a key role in limiting host protein translation via the degradation of mRNAs (170-172). This degradation of mRNA limits not only regular host cell processes but also innate immune responses that require the synthesis of new gene products. However, the role of vhs in HSV-1 immune evasion is not limited to just destabilization of RNA transcripts. Although the method of action has not yet been completely defined, infections with vhs null viruses result

in a substantial increase in both PKR activation and the formation of stress granules, suggesting that vhs is normally capable of subverting these processes (173-176).

Many viral infections are equipped with strategies to prevent PKR activation and stress granule formation as activation of PKR limits translation initiation and stress granules effectively sequester host cell translational machinery, which many viruses require for translation of their own gene products. Inhibition of PKR appears to be an important checkpoint in the immune response to HSV-1, as the virus encodes several proteins in addition to vhs that can impede PKR signaling. ICP34.5 interferes with the downstream signaling of PKR by recruiting protein phosphatase 1 (PP1) to phosphorylated eIF2α (177, 178). eIF2α is essential for translation initiation and is inactivated by phosphorylation via PKR. The recruitment of PP1 by ICP34.5 to eIF2α results in the dephosphorylation of eIF2α and restores translation.

Another HSV-1 protein, US11, can also interfere with PKR signaling, but unlike ICP34.5, US11 can directly interfere with PKR's activity both by binding directly to PKR and preventing phosphorylation of eIF2α and by binding RNA to prevent PKR activation (179-181). US11 has additional immune evasion roles through its ability to inhibit other RNA sensors such as RIG-I and MDA5, by preventing their association with the adaptor molecule MAVS, and OAS, likely via its RNA binding capacity that allows for the sequestration of RNA to avoid OAS activation (119, 182).

Many of the innate signaling molecules that recognize HSV-1 result in activation of nuclear factor kappa-light chain enhancer of activated B cells (NF-κB). Activation of NF-κB can have many downstream effects, including inducing the production of cytokines and chemokines, contributing to the activation and function of T cells, and regulating the inflammasome. As NF-κB is an important component in innate immune responses, it is not surprising that several HSV-1

proteins are able to interfere with its function. The HSV-1 proteins US3, ICP0, VP16, and UL24 have all been shown to interfere with NF-κB signaling, with a general mechanism of preventing relocation of NF-κB into the nucleus (183-186). Also crucial in the innate immune response to HSV-1 infection is autocrine or paracrine interferon signaling. The HSV-1 protein UL36 inhibits interferon signaling by binding to the type I interferon receptor and preventing its interaction with Janus kinase 1 (JAK1). Additionally, ICP27 has been shown to inhibit interferon signaling by preventing the phosphorylation of signal transducer and activator of transcription 1 (STAT1) (187, 188). Interference by HSV-1 at either of these steps prevents the transcription of downstream ISGs.

HSV-1 can also interfere with adaptive immune responses by preventing the processing and presentation of antigens on MHCI. ICP47 has been shown to bind to transporter associated with antigen processing (TAP) and inhibit its function of transporting viral peptides from the cytoplasm into the ER, which partly prevents antigen presentation on MHCI molecules to $CD8^+$ T cells (189). Interestingly, the ability of ICP47 to inhibit TAP differs between humans and mice, with ICP47 binding to human TAP with a much higher affinity than to murine TAP (190). This disparity between binding in humans and mice is one potential explanation why humans spontaneously reactivate the virus while mice do not, as diminished antigen presentation in humans could inhibit the effectiveness of $CD8^+$ T cell control over the latent virus.

### 1.2 T cell Exhaustion

Between its innate and adaptive components, the mammalian immune system is well armed to mount both a rapid response and long-term immunity after an infectious insult. However, this robust response must also be tempered so that the immune system itself does not cause tissue

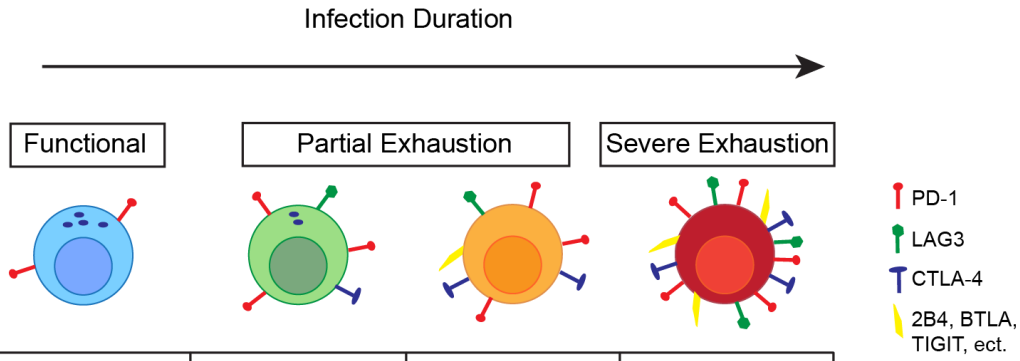
damage. In general, this system works well, but when the insult is not cleared before these checks are activated, immune impairment can occur.

### 1.2.1 Chronic Viral Infections

Most viral infections are short-lived, with the host immune system quickly responding to and eliminating the threat. However, viruses such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) can persist within the host as chronic infections, despite the induction of a strong immune response. This paradox suggests that the induced immune response is not completely functional or is "exhausted" and thus is unable to clear the infection. This hypothesis was first proven to be true in a murine lymphocytic choriomeningitis virus (LCMV) infection model. In mice, some strains of LCMV result in an acute infection while others can cause a chronic infection. When the virus-specific $CD8^+$ T cell response was followed over time in mice chronically infected with LCMV, it became apparent that a population of cells underwent a loss of function and was eventually deleted at later times in infection (191, 192). Since these initial studies, T cell exhaustion has been shown to occur in many human viral infections, including HCV, HIV, and hepatitis B virus (HBV) infections (193-196).

Although T cell exhaustion has been demonstrated in both $CD4^+$ and $CD8^+$ T cells, the majority of studies have focused on $CD8^+$ T cells. T cell exhaustion is generally defined as the gradual loss of effector functions, including IL-2, IFN-γ, and TNF-α production, and a loss of proliferative ability in response to antigen (Figure 6) (197-199). This diminished function seems to occur in a stepwise fashion, with proliferative capacity and IL-2 production the first things to be lost, followed by TNF-α and lastly IFN-γ production (198, 199). This progressive loss of function is accompanied by a concurrent increase in expression of inhibitory checkpoint molecules

(further described in Section 1.2.2), which have important roles in preventing immunopathology. Exhausted cells also downregulate the expression of the IL-7 and IL-15 receptors and instead rely on frequent exposure to their antigen to signal their continued survival (200-202). In the most extreme forms of T cell exhaustion, cells can undergo apoptosis and be eliminated. While T cells that undergo exhaustion do lose functionality over time, not all exhausted T cells become completely nonfunctional and instead can typically still respond to stimuli and perform their effector functions in a limited capacity. This leads to a tenuous balance in which the immune system can limit further viral or tumor spread but at the same time is unable to complete clear it.




| | | | | |
|---|---|---|---|---|
| IFN-γ | +++ | ++ | + | +/- |
| TNF-α | ++ | + | +/- | - |
| Cytotoxicity | ++ | + | +/- | - |
| Proliferation | ++ | + | +/- | - |
| IL-2 | +/- | - | - | - |
| Apoptosis | - | - | +/- | ++ |

**Figure 6. T cell exhaustion.**
After activation, T cells are highly polyfunctional and can proliferate after stimulation. If virus-specific T cells are unable to clear the virus and are persistently stimulated, they eventually become exhausted and progressively loose effector functions and their ability to proliferate. This loss of function and proliferation is accompanied by an upregulation of inhibitory checkpoint molecules and eventually leads to cell death.

While early investigations into T cell exhaustion focused on the progressive loss of effector functionality, more recent studies have demonstrated that exhausted T cells also exhibit altered

patterns of transcription factor expression. In normal T cell development, expression of the transcription factor T-box expressed in T cells (T-bet) is generally high in effector T cells and contributes to the production of effector molecules such as IFN-γ and can prevent the expression of inhibitory checkpoint molecules like programed death 1 (PD-1) (203, 204). In exhausted T cells found in chronic viral infections, T-bet expression is eventually reduced and the transcription factor eomesodermin (EOMES) is upregulated along with PD-1 (205, 206). The distinct expression of T-bet vs. EOMES, along with PD-1 expression, partitions these exhausted T cells into separate populations that appear to have differing capacities for functional rescue, with T-bet$^{hi}$ cells expressing moderate levels of PD-1 and responding to blockade of PD-1 signaling, while EOMES$^{hi}$ PD-1$^{hi}$ cells are refractory to this treatment (207).

Another facet of the T cell response that is atypical during exhaustion is the development of T cell memory. During an acute viral infection, antigenic stimulation of the responding T cells is limited by efficient viral clearance. Once antigenic stimulation ceases, the majority of the antigen-specific T cells undergo apoptosis, except for a subset that upregulate expression of the IL-7 receptor (208). These memory T cells do not need to respond to their antigen to receive pro-survival signaling and instead, homeostatically proliferate in response to IL-7 and IL-15 (209, 210). During exhaustion, sustained exposure to antigen precludes the development of T cell memory by the selective differentiation of memory T cell precursors into terminally differentiated exhausted T cells (211). Although this transition from memory precursors to exhausted T cells can be prevented early in infection by removal of the antigen, at later times in chronic infection these cells become committed to exhaustion and cannot be rescued, thereby diminishing the pool of available memory precursors and impairing memory development (211).

### 1.2.2 Checkpoint Molecules

After T cell activation, numerous “checkpoint” molecules are transiently upregulated. Checkpoint molecules are broadly classified into stimulatory and inhibitory molecules that can tune the immune response so that it is able to both efficiently respond to threats while also avoiding excessive activation that leads to immunopathology. During chronic viral infections and cancer, repeated antigenic stimulation of T cells can cause some of these inhibitory checkpoint molecules to once again be upregulated (Figure 7). When these molecules encounter their ligands, suppression of T cell signaling can occur, blunting T cell functionality.

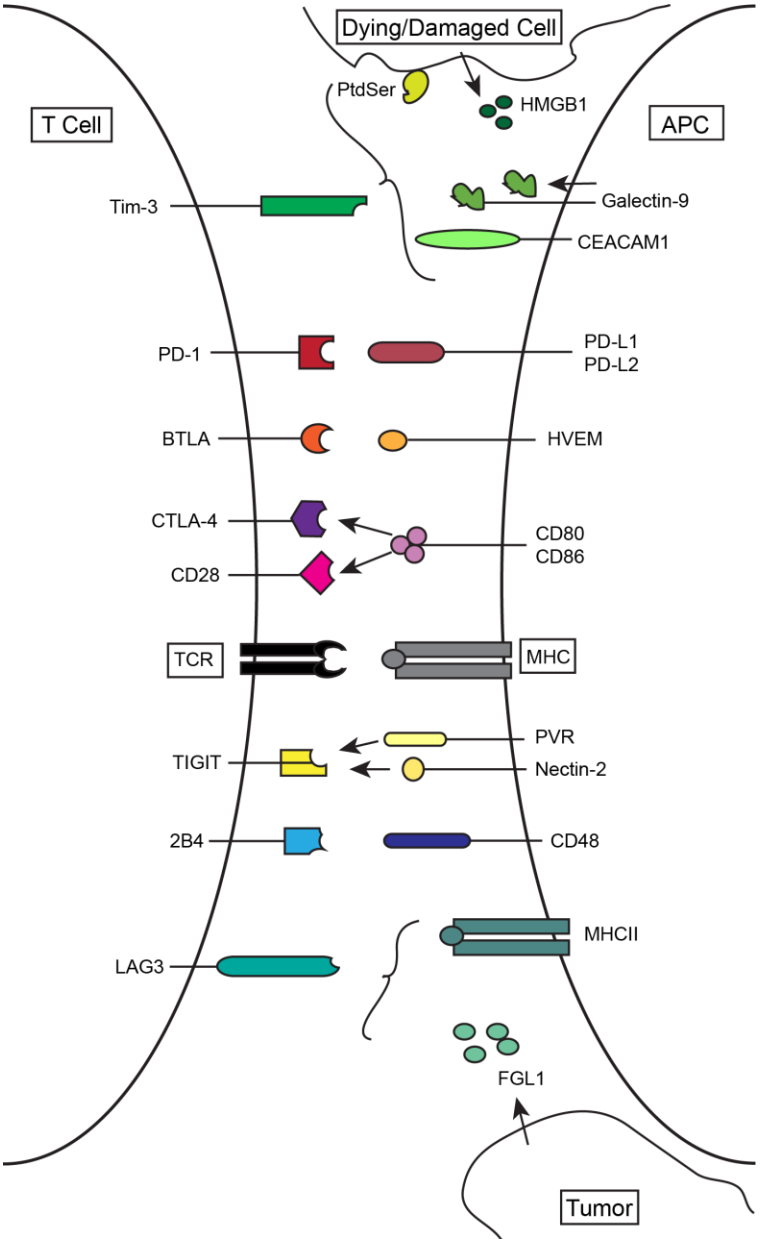



**Figure 7. Checkpoint molecules.**
Checkpoint molecules are transiently upregulated after T cell activation and serve to temper T cell responses. These molecules can be re-upregulated in situations where an immunological threat fails to be cleared and T cells are repeatedly stimulated. Upregulation of these molecules in this scenario can limit T cell function and prevent viral clearance.

#### 1.2.2.1 PD-1, BTLA, and CTLA-4

Members of the CD28 family of receptors, PD-1, B- and T-lymphocyte attenuator (BTLA), and Cytotoxic T-lymphocyte protein 4 (CTLA-4) all have roles in limiting T cell responses. PD-1 was first identified in a screen of cell lines after induction of programmed cell death and has two ligands; programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2) (212). PD-L1 is expressed on many different immune and non-immune cell types, while PD-L2 appears to be expressed primarily on immune cell types such as DCs. When PD-1 encounters its ligands, signaling occurs through its cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) domains that recruit SHP-1 and SHP-2 phosphatases to the TCR (213). These phosphatases interfere with signaling molecules such as Zap70 and phosphoinositide 3-kinase (PI3K), which down-regulates signaling though the TCR and costimulatory molecules (214, 215). Interfering with PD-1 signaling has demonstrated that it has important roles in both regulating T cell function during persistent antigen stimulation and in modulating T cell tolerance (216, 217). Similar to PD-1, signaling though BTLA occurs via two ITIM domains after binding its ligand HVEM, resulting in decreased IL-2 production and T cell proliferation (218-220).

One of the first inhibitory checkpoint molecules to be studied, CTLA-4 can bind to the same ligands (CD80 and CD86, expressed on APCs) as the costimulatory receptor CD28. CTLA-4 binds to its ligands with a higher affinity than CD28 and may function, in part, by binding CD80 and CD86 and preventing their association with CD28. Although part of the same family as PD-1 and BLTA, CTLA-4 does not appear to contain ITIM or ITSM domains, and it is not yet truly understood how CTLA-4 inhibits T cells responses. Despite this, there is clear evidence that CTLA-4 signaling functions to inhibit T cell cells, as mice lacking CTLA-4 succumb to organ

failure three to four weeks after birth due to uncontrolled T cell activation and proliferation (221, 222).

#### 1.2.2.2 LAG3

Located near CD4 in the human genome, lymphocyte activation gene 3 (LAG3) is structurally similar to CD4 and also binds MCHII molecules (223-225). LAG3 binds to MHCII with a higher affinity than CD4 and like the interaction between CTLA-4 and CD28, may compete for binding. Recent work has identified fibrinogen-like protein 1 (FGL1) as an additional LAG-3 ligand that is abundantly produced in cancer cells and contributes to downregulation of T cell function in tumors (226). Inhibitory LAG3 signaling appears to be related to a KIEELE motif in the cytoplasmic tail and causes the downregulation of proliferation and effector cytokines in T cells, although the exact mechanism of how this occurs is not yet known (227, 228).

#### 1.2.2.3 TIGIT

In 2009, T-cell immunoreceptor with Ig and ITIM domains (TIGIT) was added to the list of inhibitory checkpoint molecules (229, 230). TIGIT binds the poliovirus receptor (PVR) with high affinity as well as less strongly to Nectin-2 (229-231). TIGIT is expressed not only on T cells but also on NK cells and much of TIGIT's signaling has been determined from work in NK cells. TIGIT contains an ITIM domain and an ITT-like motif, and phosphorylation of a tyrosine in either of these domains is sufficient to enable signaling in mice (232). It has been shown in NK cells that phosphorylation at these tyrosine residues recruits SH2 domain-containing inositol-5-phosphatase 1 (SHIP1), which can subsequently inhibit PI3K and NF-κB signaling (233, 234). In both NK cells and T cells, signaling through TIGIT results in loss of both cytokine production and effector function.

#### 1.2.2.4 2B4

Like TIGIT, 2B4 is found on both T cell and NK cells, as well as several other immune cells types, and most of what is known about 2B4 signaling has been determined from NK cell studies. After binding CD48, 2B4 signaling is mediated through four ITSM domains that can recruit a number of different adapter proteins that can exert either stimulatory or inhibitory signaling (235). This choice of binding partners appears to be dependent on the ratio of 2B4 to the adaptor protein SAP, with high levels of SAP leading to activation of the cell while low levels of SAP allow for the recruitment of alternative molecules such as the phosphatases SHP-1 and SHP-2, which abrogate TCR signaling and inhibit T cell functionality (236, 237).

#### 1.2.2.5 Tim-3

First described in the early 2000s, T-cell immunoglobulin and mucin domain 3 (Tim-3) was originally identified as a cell surface marker that distinguishes Th1 $CD4^+$ T cells from Th2 $CD4^+$ T cells in mice (238). Tim-3 is one of several identified Tim family members and is conserved between mice and humans. Although first identified on T cells, Tim-3 has since been shown to also be expressed on other immune cells types such as NK cells, macrophages, and DCs. Tim-3 was initially identified as having inhibitory effects on T cell function, as early studies showed that blocking Tim-3 worsens experimental autoimmune encephalomyelitis (EAE, a murine model of multiple sclerosis) and hastens the onset of diabetes in nonobese diabetic mice (238, 239). However, more recent studies have shown that Tim-3 signaling can also have stimulatory effects in some cases, suggesting that the role of Tim-3 in immune cell function is highly context dependent (240, 241).

### *Ligands*

Tim-3 is currently known to have four ligands: galectin-9, carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1), phosphatidylserine (PtdSer), and high mobility group protein-1 (HMGB1). Galectin-9, the first discovered Tim-3 ligand, was identified by using mouse Tim-3–Ig fusion proteins incubated with lysates from a lymphoma cell line to affinity purify candidates that were then subjected to mass spec (242). Galectin-9 is expressed as both a secreted and membrane bound form by many different cell types, including immune cells like NK cells, macrophages, and T cells (243). As a family, galectins are known to bind to β-galactoside sugars and galectin-9 has previously been shown to bind to lactose (243). Galectin-9 appears to bind to Tim-3 though similar interactions with carbohydrate groups in the Tim-3 immunoglobulin variable (IgV) domain (242). Administration of galectin-9 to *in vitro* Th1 T cell cultures induced apoptotic and necrotic cell death, while *in vivo* administration resulted in a decrease in the production of IFN-γ (242). Additional studies that have assayed the ability of galectin-9 to signal though Tim-3 have demonstrated that galectin-9 can also bind to T cells independently of Tim-3 and that human Tim-3 does not appear to bind galectin-9 at all. Together, these findings indicate that the role of galectin-9 in T cell function and survival is more complex than simply signaling through Tim-3 (244, 245).

CEACAM1 has a structure similar to Tim-3, with an IgV domain and a cytoplasmic tail, and is expressed on epithelial cells, endothelial cells, and many immune cell types including NK cells, DCs, and T cells (246, 247). CEACAM1 is able to dimerize with other CEACAM1 molecules or other CEACAM family members, as well as bind to additional nonrelated ligands such as galectin-3 (248, 249). Signaling though CEACAM1 has been demonstrated to inhibit T cell proliferation and cytokine production through its two ITIMS, which can recruit SHP-1 (250).

CEACAM1 is able to bind to Tim-3 via interactions between their respective IgV domains and this interaction can occur in either *cis* or *trans* (251). Deletion of CEACAM1 results in a decrease in Tim-3 expression on T cells, suggesting that CEACAM1 is necessary for stable Tim-3 expression at the cell surface. When antibodies to Tim-3 and CEACAM1 were co-administered in a model of colorectal cancer, $CD8^+$ T cells expressed higher levels of IFN-γ, and the tumor burden was decreased as compared to control mice or mice treated with antibodies to only Tim-3 or CEACAM1 (251).

PtdSer is a phospholipid that is found within membranes of virtually all cells and is normally confined to the cytoplasmic face of the membranes. When cells undergo apoptosis, membranes become disrupted and some PtdSer is displayed on the extracellular side. This aberrant expression of PtdSer is a signal for phagocytic cells to engulf the apoptotic cell. This process is normally mediated by the Tyro3, Axl, and Mer (TAM) family receptors, but Tim-3 has also been shown to bind to PtdSer through its IgV domain (252, 253). Interactions between Tim-3 expressed on phagocytic cells and PtdSer results in phagocytosis of the apoptotic cell (253). Nonphagocytic cells that express Tim-3, such as T cells, can also bind to PtdSer, although it is not currently known what effect this binding has on T cell function (252).

As a DNA binding protein, HMGB1 is normally found within the cell nucleus. However, after cell stress or damage occurs, this protein can be transported out of the nucleus and secreted into the extracellular space. Once secreted, HMGB1 can bind to receptor for advanced glycation end products (RAGE) on the surface of macrophages and DCs and trigger TLR9 signaling, which initiates a proinflammatory response (254). Tim-3 expressed at high levels on DCs that infiltrate tumors can alternatively bind to HMGB1 with an affinity that is similar to the affinity of HMGB1 for RAGE (255). Binding of Tim-3 to HMGB1 inhibits nucleic acid signaling within DCs and

appears to impair antitumor responses, as blockade of Tim-3 enhanced proinflammatory antitumor DC responses during treatment with DNA vaccines and cytotoxic cancer therapies (255).

### *Structure and Signaling*

Like all Tim family members, the extracellular portion of Tim-3 is composed of an IgV domain containing a group of highly conserved cysteines and a mucin domain (256, 257). The mucin domain is secured at the cell membrane by a transmembrane domain which links it to its cytoplasmic tail. Unlike most inhibitory molecules, the cytoplasmic tail of Tim-3 does not contain any common inhibitory motifs such as an ITIM or ITSM. While Tim-3 may not contain canonical inhibitory motifs, the cytoplasmic domain does contain several tyrosines that have been demonstrated to be phosphorylated (258, 259). The phosphorylation of these residues has been shown to be mediated by the kinases Lck, Fyn, or Itk (258, 259).

Phosphorylation of tyrosine residues generally facilitates the recruitment of SH2 domain-containing adaptor molecules. A screen of several of these adaptor molecules showed binding of the kinases Fyn and Lck and the PI3K adaptor protein p85 to phosphorylated tyrosines on Tim-3 (258). While the intermediate steps in Tim-3 signaling are not yet clear, it has been shown *in vitro* that Tim-3 signaling is associated with increased activation of PLC-γ1 and an increase in S6 phosphorylation, which correlates with higher levels of NF-κB and activating protein-1 (AP-1) activation in cells expressing Tim-3 (258). Stimulation of T cells *in vitro* also showed that Tim-3 expression correlates with increased production of IFN-γ and IL-2, suggesting that Tim-3 expression is, at least in some instances, stimulatory (258). Similar observations have been made *in vivo*, with $CD8^+$ T cells from human patients infected with *Mycobacterium tuberculosis* and $CD4^+$ T cells from LCMV-infected mice exhibiting increased cytokine expression in cells expressing Tim-3 compared to cells that do not (241, 260). A recent study has additionally shown

that Tim-3 can have a stimulatory role even in $CD8^+$ T cells in mice infected with LCMV, with $Tim\text{-}3^+$ $CD8^+$ T cells being more activated during acute infection (261).

The cytoplasmic tail of Tim-3 can additionally be bound by the protein HLA-B associated transcript 3 (Bat3) (262). This association with Bat3 appears to occur in the absence of phosphorylation, and phosphorylation of the cytoplasmic tail of Tim-3 after galectin-9 binding displaces Bat3. Similar to Tim-3, Bat3 can also bind activated Lck, though this interaction is disrupted after antibody binding of Tim-3. When Bat3 is ablated from cells, functionality decreases and expression of checkpoint molecules, including Tim-3, increases (262). Although the exact role of Bat3 in Tim-3 signaling is not clear, it appears that its binding to Tim-3 can modulate Tim-3 signaling and may help to reconcile why Tim-3 has been shown to have both inhibitory and stimulatory roles.

### *Functions*

Despite being most commonly associated with T cell exhaustion, Tim-3 was first shown to have inhibitory effects in autoimmunity. In the study that first identified Tim-3 on T cells, blocking Tim-3 resulted in worsening EAE and an increase in mortality (238). Since this inaugural study, Tim-3 has been suggested to have inhibitory effects during a variety of viral and bacterial infections and in cancer. During chronic LCMV infection of mice, Tim-3 is co-expressed on exhausted T cells with other markers such as PD-1, and contributes to a more severe exhaustion phenotype (263). When antibodies to Tim-3 and PD-1 were administered simultaneously *in vivo*, T cell function and viral control was enhanced, suggesting that Tim-3 contributes to exhaustion in this model (263). Similar to LCMV infection, Tim-3 has been shown to be upregulated on $CD8^+$ T cells in humans with HIV and chronic HCV infections, and treatment with anti-Tim-3 antibodies resulted in restoration of T cell functions (264-266). Tim-3 has also been postulated to have an

inhibitory role in T cell function in chronic *Mycobacterium tuberculosis* infection in mice, with Tim-3 expression accumulating on $CD4^+$ and $CD8^+$ T cells over the course of infection and where administration of anti-Tim-3 antibodies restores T cell function (267). Interestingly, this report also showed that only T cells that expressed Tim-3 in combination with other markers of exhaustion exhibited impaired function, while T cells that expressed Tim-3 without co-expression of PD-1 were largely functional, suggesting that Tim-3 can have dual roles even within the context of a chronic infection (267).

High levels of Tim-3 expression have also been observed in various cancers, including renal cell carcinoma, melanoma, gastric cancer, and non-Hodgkin lymphoma. Tim-3 appears to be preferentially expressed on T cells found at the site of the tumor, suggesting that antigen expression within the tumor is responsible for Tim-3 upregulation (268-271). When Tim-3 expression was tracked over time in several different types of cancer, high expression of Tim-3 correlated with a less-favorable prognosis (270, 271). In several studies, use of anti-Tim-3 antibodies increased T cell function and reduced tumor burden, which was taken as evidence of an inhibitory role for Tim-3 within tumors (268, 269, 271). Like in chronic viral infections, Tim-3 is often co-expressed with PD-1 in cancer and the function of these cells is highly attenuated. With the current popularity of checkpoint molecule therapies in cancer treatment, there is hope that combination anti-PD-1 and -Tim-3 treatments will prove to be effective in reinvigorating immune responses in cancers.

While most of the early literature about the function of Tim-was focused on the role of Tim-3 as a negative regulator of immune function, a growing number of studies have shown that Tim-3 can also function in a stimulatory manner. In LCMV infection, $CD8^+$ and $CD4^+$ T cells expressing Tim-3 have both been shown to exhibit enhanced functionality during acute infection compared to cells that do not express Tim-3 (260, 261). In *Mycobacterium tuberculosis* infection

in humans, Tim-3-expressing $CD4^+$ and $CD8^+$ T cells from both active and latent infections expressed increased levels of IFN-γ and perforin and were more effective at controlling infection (241). In mice infected with *Mycobacterium tuberculosis*, macrophage activation and bacterial killing mediated by IL-1β was enhanced via an interaction between Tim-3 on T cells and galectin-9 on macrophages (272). In acute bacterial infection with *Listeria monocytogenes*, mice lacking Tim-3 expression experienced a decrease in $CD8^+$ T cell numbers compared to wild-type mice, in addition to diminished primary and recall responses (240). With evidence of a stimulatory function for Tim-3 now available from several different infections, it is clear that the role of Tim-3 during infection can no longer be assumed to be inhibitory and must be carefully assessed in each unique environment.

With four known ligands, expression on varied cell types, and a complicated and incompletely understood signaling pathway, describing the functions of Tim-3 is not a straightforward task. Although Tim-3 was originally described as functioning in an inhibitory manner in T cells, over 15 years of research has shown us that describing Tim-3's role as solely inhibitory is inaccurate, as there are now numerous studies that show that in some cases, Tim-3 can enhance function in several immune cell types, including T cells. Further complicating matters is the use, in many studies, of antibodies to Tim-3 whose ascribed blocking functions have not yet been fully characterized, and indeed, a recent paper showed that several Tim-3 antibodies prevent binding of some, but not all, Tim-3 ligands (273). Use of global Tim-3 knockout mice may also be somewhat problematic, as we now know that Tim-3 is expressed on many cell types and it is difficult to predict how the lack of Tim-3 on all of these cells impacts the experimental endpoints. Collectively, the diverse studies that have assessed the role that Tim-3 plays in regulating immune

responses ultimately suggest that Tim-3's function is highly context dependent and based on what type of cell it is expressed on and what ligands are available for binding.

#### 1.2.2.6 Checkpoint Molecule Immunotherapies

The identification of cell-surface checkpoint molecules as mediators of T cell exhaustion has prompted the development of antibody-based immunotherapies that can be used to reinvigorate T cell responses. Although there is hope that these immunotherapies will be able to be used in chronic infectious diseases such as HIV and HBV, the bulk of clinical trials for checkpoint molecule inhibitors have thus far focused on their use in various types of cancers, where T cell exhaustion can also occur. Accordingly, the first FDA approved immunotherapy developed to CTLA-4 (ipilimumab) was initially tested for use in metastatic melanoma (274). When survival data were pooled from patients who received ipilimumab in various clinical trials, there was an overall 20% survival rate at ten years post-treatment, which was an improvement on the 10% survival rate historically seen in advanced melanoma patients that had received alternative treatments (275, 276). Also improved in comparison to other treatments was the durability of the response to ipilimumab, with the survival curve leveling out at around 3 years post-treatment, indicating that the effects of anti-CTLA-4 are long-lasting (275).

With the success of anti-CTLA-4 therapy, it is not surprising there has been a race to develop blocking antibodies to other checkpoint molecules. Since the development of ipilimumab, there have been numerous blocking antibodies developed to PD-1 and its ligand PD-L1 that are currently FDA approved for treatment of several types of cancer. Although the long-term outcomes for PD-1/PD-L1 blockade therapies are just now starting to be assessed, it appears that like ipilimumab, anti-PD-1 and -PD-L1 treatments lead to a better prognosis in the long term as compared to traditional cancer treatments (277). With the development of PD-1 and PD-L1

inhibitors, combination checkpoint blockade therapy became possible. Indeed, initial studies combining CTLA-4 and PD-1 blockade have shown a notable reduction in cancer progression and tumor burden compared to monotherapies (278, 279). Inhibitors to other checkpoint molecules like Tim-3 and LAG-3 are currently in clinical trials. It will be interesting to see how these therapies perform alone and in combination with the existing CTLA-4 and PD-1/PD-L1 therapies.

Checkpoint molecule inhibitors are now valuable tools for treating cancer. With the initial clinical trials for use of checkpoint molecule therapies in chronic infectious diseases currently underway, it is likely that their use in human medicine will only continue to increase. However, there are several aspects of checkpoint molecule therapy that merit careful consideration when it is being chosen for treatment. Like any immunotherapy, checkpoint molecule blockade can lead to increased adverse effects associated with excessive immune activation and in some cases these events can be quite severe (280). Although many of these effects can be managed, some have ultimately resulted in premature death, although the percentage of fatalities in checkpoint molecule therapy in cancer is on par with or better than other types of treatments (280). Additionally, only a subset of patients is responsive to checkpoint molecule blockade, highlighting the need for both a better understanding of how these molecules work and the identification of biomarkers that allow for effecting screening of candidates before treatments are started.

### 1.2.3 T Cell Exhaustion/Impairment During HSV-1 Infection

Work from our laboratory has shown that functional impairment of $CD8^+$ T cells can occur after corneal HSV-1 infection (169). As discussed above, a similar loss in functionality has been observed in several chronic infections and has been shown to contribute to pathogen persistence. Although HSV-1 infection is, by definition, not a chronic viral infection as it undergoes periods

without production of infectious virus, some aspects of latent HSV-1 infections can mimic a chronic infection in regard to their effects on suppressing the immune system. With prevention of viral reactivation being a target for controlling HSV-1 infection, the ability to enhance the functionality of HSV-1 specific $CD8^+$ T cells is of considerable interest, especially considering the recent advances in checkpoint molecule immunotherapies. Understanding how T cell impairment in HSV-1 develops and persists, and how this compares to T cell exhaustion in chronic viral infections and in cancers, will be important in determining the suitability of current and future T cell reinvigoration therapies for use in HSV-1 infections.

It is commonly accepted that the development of T cell exhaustion can be triggered by the chronic exposure of $CD4^+$ and $CD8^+$ T cells to high levels of their cognate antigen. Although HSV-1 certainly has abundant gene expression and subsequent antigen production during acute infection and reactivation events as it forms new viral particles, detection of viral gene expression during latency has been challenging. Accordingly, it has been a long-held opinion that LAT, which does not encode a protein product, is the only gene expressed during latency. Despite this, more recent work using sensitive detection techniques has shown that HSV-1 transcripts, and even proteins driven by HSV-1 promoters, are produced in a disordered manner during latency as part of the first phase of viral reactivation (67, 86, 90, 91). Viral protein expression during latency has also been indirectly shown by the observation that HSV-1-specific $CD8^+$ T cells associate and form immune synapses with neurons (92, 162). Additionally, TGs infected with a virus lacking the gB epitope fail to retain gB-$CD8^+$ T cells, suggesting that antigen must be present during latency to maintain the ganglion T cell resident population (168). Despite the levels of viral gene expression being so low as to be undetectable by most common molecular biology methods, prior observations suggest that viral gene expression, potentially due to abortive attempts to reactive, is still sufficient to

impart functional impairment of HSV-1-specific $CD8^+$ T cells, similar to that seen in chronic viral infections (169).

As the prototypical inhibitory checkpoint molecule, PD-1 was one of the first such molecules to be studied during HSV-1 infection. Like in typical T cell exhaustion, PD-1 expression is upregulated during latent HSV-1 infection (281, 282). When gB-tetramer was used to distinguish between gB-$CD8^+$ T cells and Subdom-$CD8^+$ T cells, it became evident that PD-1 was preferentially expressed on the Subdom-$CD8^+$ T cells (281, 282). Using tetramers specific to a subset of the subdominant epitopes, it was further demonstrated that both the percentage of cells that express PD-1 and the expression level of PD-1 per cell are variable between subdominant epitopes, perhaps pointing to differences in specific subdominant epitope expression during latency (282). When expression of one of the ligands for PD-1, PD-L1, was assessed within the HSV-1-infected TG over time, increasing numbers of neurons were found to express PD-L1 in response to IFN-γ production. As virus-specific $CD8^+$ T cells interact with neurons within the infected TG during viral infection, interactions between neurons expressing PD-L1 and $CD8^+$ T cells expressing PD-1 have the potential to limit the viral-specific $CD8^+$ T cell response and contribute to the observed decline in Subdom-$CD8^+$ T cell function during latency (169, 282). This hypothesis was tested using PD-L1 knockout mice and despite there being an increase in Subdom-$CD8^+$ T cells numbers in these mice, there was a significant decrease in the overall percentage of cells that were able to produce either IFN-γ or GzmB. This suggests that disruption of PD-1: PD-L1 signaling promotes survival of, but does not restore function to, impaired cells in HSV-1 infection (282).

Although much of the work in understanding the mechanisms of T cell exhaustion has focused on the role of checkpoint molecules, soluble mediators such as IL-10 and TGF-β also play

a role in development of exhaustion (283, 284). Within the HSV-1 latently infected TG, around 15% of $CD4^+$ T cells express IL-10 (169). When IL-10 signaling was blocked *in vivo* using an antibody against IL-10, Subdom-$CD8^+$ T cells preferentially expanded within the TG. *In vitro* stimulations of $CD8^+$ T cells from anti-IL-10 antibody-treated mice showed that increased numbers of Subdom-$CD8^+$ T cells were able to produce IFN-γ. However, the frequency of Subdom-$CD8^+$ T cells that were IFN-$\gamma^+$ was similar between untreated and treated mice. This indicates that blockade of IL-10 signaling increases proliferation of both functional and nonfunctional cells but does not rescue the function of impaired Subdom-$CD8^+$ T cells. (169).

Despite the similarities between decreased T cell function in HSV-1, cancer, and other infections after persistent exposure to antigen, T cell impairment/exhaustion during HSV-1 infection seems to exhibit some phenotypic differences. Although PD-1 expression appears to mark impaired cells during HSV-1 infection and IL-10 appears to limit proliferation, blockade of either one is not sufficient to reverse the loss of function, despite similar blockades reinvigorating exhausted populations in other systems. These differences demonstrate a need for further understanding of how T cell impairment is controlled during HSV-1 infection.

## 2.0 Specific Aims

Without an effective vaccine to prevent HSV-1 infection and no way for the host to clear the virus completely due to latency in protected neurons, treatments for managing the development of HSK after corneal HSV-1 infection are limited to preventing viral reactivation in the TG and regulating the inflammation in the cornea when a reactivation event does occur. The following studies have investigated the contributions of the immune response to both aspects of this control.

**Aim 1- Determine the expression pattern of the checkpoint molecule Tim-3 and ascertain if it is a marker of functional impairment in the HSV-1 latently infected TG.**

$CD8^+$ T cells help maintain vial latency within the TG, however, functional impairment of Subdom-$CD8^+$ T cells compromises this control. Thus, ways to enhance T cell functionality within the TG would be useful in bolstering the immune control of viral reactivation. In chapter 3, we have assessed the expression of checkpoint molecules during HSV-1 infection and investigated how the expression of Tim-3 is associated with function, rather than inhibition, during infection.

**Aim 2- Define the contributions of the immune response to the development of HSK after corneal HSV-1 infection.**

HSK is known to be immune-mediated and development can occur rapidly after viral replication in the cornea. However, not everyone with a corneal HSV-1 infection develops HSK, suggesting that the immune response can be manipulated to prevent HSK development in susceptible individuals. In chapter 4, we compare immune responses to a pathogenic and nonpathogenic stain of HSV-1 to identify how HSK is triggered after viral infection.

## 3.0 Differential Expression of Immune Checkpoint Molecules on CD8$^+$ T Cells Specific for Immunodominant and Subdominant Herpes Simplex Virus 1 Epitopes

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### 3.1 Summary

Herpes simplex virus 1 (HSV-1) causes a lifelong infection of neurons that innervate barrier sites like the skin and mucosal surfaces like the eye. After primary infection of the cornea, the virus enters latency within the trigeminal ganglion (TG), from which it can reactivate throughout the life of the host. Viral latency is maintained, in part, by virus-specific CD8$^+$ T cells that nonlethally interact with infected neurons. When CD8$^+$ T cell responses are inhibited, HSV-1 can reactivate, and these recurrent reactivation events can lead to blinding scarring of the cornea. In the C57BL/6 mouse, CD8$^+$ T cells specific to the immunodominant epitope from glycoprotein B maintain functionality throughout latency while CD8$^+$ T cells specific for subdominant epitopes undergo functional impairment that is associated with the expression of the inhibitory checkpoint molecule programmed death 1 (PD-1). Here, we investigate the checkpoint molecule, T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), which has traditionally been associated with CD8$^+$ T cell exhaustion. Unexpectedly, we found that Tim-3 was preferentially expressed on

highly functional ganglionic $CD8^+$ T cells during acute and latent HSV-1 infection. This, paired with data that show that Tim-3 expression on $CD8^+$ T cells in the latently infected TG is influenced by viral gene expression, suggests that Tim-3 is an indicator of recent T cell stimulation, rather than functional compromise, in this model. We conclude that Tim-3 expression is not sufficient to define functional compromise during latency; however, it may be useful in identifying activated cells within the TG during HSV-1 infection.

## 3.2 Importance

Without an effective means of eliminating HSV-1 from latently infected neurons, efforts to control the virus have centered on preventing viral reactivation from latency. Virus-specific $CD8^+$ T cells within the infected TG have been shown to play a crucial role in inhibiting viral reactivation and with a portion of these cells exhibiting functional impairment, checkpoint molecule immunotherapies have presented a potential solution to enhancing the anti-viral response of these cells. In pursuing this potential treatment strategy, we found that Tim-3 (often associated with $CD8^+$ T cell functional exhaustion) is not upregulated on impaired cells but instead is upregulated on highly functional cells that have recently received antigenic stimulation. These findings support a role for Tim-3 as a marker of activation rather than exhaustion in this model, and we provide additional evidence for the hypothesis that there is persistent viral gene expression in the HSV-1 latently infected TG.

## 3.3 Introduction

Herpes simplex virus 1 (HSV-1) is a prevalent human pathogen, with over 50% of adults in the United States testing positive by late adulthood (3). HSV-1 infection can result in a number of pathologies that depend on both the mucosal site of primary infection, as well as the neurons that ultimately harbor the latent virus and are the site of sporadic reactivation events that lead to recurrent peripheral disease. One potential site of viral infection, the cornea, plays a crucial role in vision by transmitting and focusing light so that images can be processed by the retina and brain. Without an optically clear cornea, visual acuity and quality of life are severely reduced.

During acute infection, HSV-1 replicates in the corneal epithelium and gains access to the axons of sensory neurons before being cleared from the cornea by innate immune cells like macrophages and NK cells (133, 135, 285, 286). Using retrograde axonal transport, HSV-1 travels to neuronal cell bodies in the trigeminal ganglion (TG) where it establishes latency. Although the virus is able to be maintained in a latent state indefinitely, various stressors such as UV light exposure, hormone fluctuation, or trauma, can cause viral reactivation, which results in active viral replication within infected neurons and anterograde transport of infectious virus down axons back to the periphery (80, 81, 287). These reactivation events can, in some individuals, result in the development of recurrent herpes stromal keratitis (HSK), which is characterized by a progressive development of corneal opacity, neovascularization, edema, and hypoesthesia, which can ultimately lead to loss of vision (288). As there is no effective and approved vaccine for HSV-1, therapeutic efforts have primarily focused on controlling viral replication, restricting inflammation, and preventing reactivation from viral latency.

Viral latency is maintained by several factors including host miRNAs, viral miRNAs derived from the Latency-Associated Transcript (LAT), and immune cells such as virus-specific

$CD8^+$ T cells (21, 61, 157). Virus-specific $CD8^+$ T cells have been shown to infiltrate the infected TG (159) and interact with infected neurons to prevent viral reactivation (92). In the C57BL/6 mouse, 50% of TG-associated $CD8^+$ T cells are specific for an immunodominant epitope found on glycoprotein B (gB) (164). Previous work from our laboratory identified the remaining epitope specificities of the $CD8^+$ T cell repertoire in this model, which consists of eighteen subdominant HSV-1 epitopes from viral proteins including ribonucleotide reductase 1 (RR1), glycoprotein C, infected cell protein (ICP) 8 and others (166). While the mechanism governing the immunodominance hierarchy remains incompletely understood, 80% of the epitopes recognized by HSV-1-specific $CD8^+$ T cells are from proteins expressed before DNA synthesis. These data, together with our previous studies, support the notion that $CD8^+$ T cell activity is required to prevent viral reactivation from latency (162). When functionality between the gB-specific (gB-$CD8^+$ T cells) and subdominant epitope-specific (Subdom-$CD8^+$ T cells) $CD8^+$ T cell groups was assessed at latency, a higher frequency of gB-$CD8^+$ T cells produced granzyme B (GzmB) directly *ex vivo* and IFN-γ and TNF-α after *in vitro* peptide stimulation than Subdom-$CD8^+$ T cells (169). Since $CD8^+$ T cell functionality plays an important role in suppressing viral gene expression and preventing reactivation, improving the function of TG-resident Subdom-$CD8^+$ T cells provides a potentially useful strategy for preventing reactivation in the TG.

Loss of functionality in T cells after prolonged exposure to their cognate antigen is a phenomenon that has received considerable attention in recent years in both chronic viral infection and tumor models. In these models, $CD8^+$ T cells progressively lose their capacity to respond to their antigen after repeated stimulations over an extended period of time, with the affected cells being considered “exhausted” (191, 197, 289, 290). This development of exhausted cells allows the perpetuation of viral infection or tumor growth. As such, there has been substantial enthusiasm

for the development of immunotherapies to reverse this loss in functionality. The major targets of these therapies have centered on checkpoint molecules such as programed death-1 (PD-1) and cytotoxic T-lymphocyte protein 4 (CTLA-4), although numerous others are in development (274, 291). The specific contributions of individual checkpoint molecules are not yet fully understood; however, it is generally accepted that increased expression of single and/or co-expression of multiple checkpoint molecules results in functional compromise (292). Therapies blocking these molecules have successfully reinvigorated exhausted $CD8^+$ T cells in animals and the clinic, resulting in more efficient viral/tumor clearance and increased patient survival (263, 274, 292-294).

Here, we have defined the expression of several classical checkpoint molecules during HSV-1 latency. We show that while the expression levels of the majority of assessed molecules are low in ganglionic $CD8^+$ T cell populations during HSV-1 latency, T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) is preferentially upregulated on functional gB-$CD8^+$ T cells rather than impaired Subdom-$CD8^+$ T cells. Although other laboratories have reported similar expression levels of Tim-3 on these populations (281, 295), our study is the first to correlate the expression pattern of Tim-3 with functionality in this model. We found that Tim-$3^+$ cells can readily respond to peptide stimulation and are in fact, highly multifunctional. Furthermore, during latency, we were able to modulate Tim-3 expression on TG-resident $CD8^+$ T cells by using strains of the virus with altered expression patterns of viral $CD8^+$ T cell epitopes, suggesting that Tim-3 may serve as a T cell activation marker in this model. Our data also suggest that functionally compromised cells may acquire this phenotype during acute infection rather than gradually throughout latency. Collectively our results indicate that despite the traditional classification of Tim-3 as an inhibitory checkpoint molecule, its expression should not automatically imply

functional impairment. Instead, Tim-3 expression in the TG may serve as a marker of recent T cell activation and may be helpful in determining the levels of expression of viral genes during latency.

## 3.4 Materials and Methods

### 3.4.1 Mice

Six-week-old C57BL/6 female mice were purchased from Jackson Laboratories and infected at 7-8 weeks of age via administration of $1x10^5$ PFU of purified HSV-1 in 3 µl of PBS onto a cornea that was scarified in a crosshatch pattern using a 30G needle, with the viral inoculant massaged into the cornea with the eyelids. Prior to infection, mice were anesthetized with 2.5 mg of ketamine and 0.25 mg of xylazine i.p. After infection, 6.25 µg of antisedan was administered i.p. Mice were infected bilaterally with one eye receiving HSV-1 strain RE and one eye receiving HSV-1 strain KOS for experiments shown in all figures except for Figure 12. In Figure 12, mice were bilaterally infected with the viruses indicated in the figure legend. For clarity, data in all figures except Figure 12 are from RE-infected TGs as no notable differences were found between the RE- and KOS- infected TGs. All experimental animal procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and the animals were handled in accordance with guidelines established by the Institutional Animal Care and Use Committee.

### 3.4.2 Virus

Viruses used were wild-type HSV-1 KOS and RE strains and genetically modified HSV-1 strains S1L, gC, and ICP0, which were made on the KOS background. The S1L virus lacks the immunodominant $gB_{498\text{-}505}$ epitope through the mutation of residue 498 (SSIEFARL to LSIEFARL). Characterization of the T cell infiltrates to S1L and its construction were recently described (168). The S1L virus was then used to generate the recombinant gC and ICP0 HSV-1 strains. These viruses contain four copies of the gB epitope and flanking sequences (residues 494-509) linked to EGFP that is expressed from the indicated viral promoters in the gC locus. The construction and characterization of these viruses have been described in detail elsewhere (296). Briefly, the following pUC19-based recombinant HSV-1 KOS gC-locus targeting plasmids were constructed: 1) p.gCp-pep4-EGFP, which contains a $4XgB_{494\text{-}509}$-EGFP expression cassette immediately downstream of the native gC promoter and 2) p.gC-ICP0p-pep4-EGFP, which has an interrupted gC promoter and an ectopically placed ICP0 promoter driving $4XgB_{494\text{-}509}$-EGFP expression. Clean virus was then generated by classical homologous DNA recombination with the above-described linearized gC targeting plasmids and S1L infectious viral DNA, followed by three rounds of plaque purification based on gain of fluorescence and confirmation of recombinant promoter viruses by southern blotting. Virus strains were prepared as follows: flasks with Vero cell monolayers were infected with a MOI of 0.01 and monitored until cytopathic effect was observed in more than 90% of cells. 5 M NaCl was added to the existing culture media in each flask to a final concentration of 0.45 M, flasks were rocked for 1 h at room temperature to release cells into the media, and cells were collected into 50 ml conical tubes and centrifuged at 6,000 g for 10 min at 4ºC. Supernatants were then filtered through a 0.8 µm filter, overlaid onto a 50% sucrose cushion (0.22 µm filtered) in 38.5 ml polypropylene tubes (Beckman Coulter

Cat#326823), and pelleted at 142,000 g for 1 h. After carefully removing most of the supernatant, the virus pellet was resuspended in the remaining sucrose cushion and media, aliquoted, and stored at -80ºC. Before use, viral stocks were titered on Vero cells. Promoter kinetics and epitope expression in the gC and ICP0 HSV-1 strains were confirmed by *in vitro* gB-$CD8^+$ T cell stimulations with infected B6 fibroblasts at 4, 8, and 24 hours post-infection. Strong ICP0 promoter driven epitope is detected at 4 hours while no significant gC promoter driven epitope is detectible until 24 hours, as measured by gB-$CD8^+$ T cell activation.

### 3.4.3 Reagents

Ketamine, xylazine, and antisedan were purchased from Henry Schein. BrdU (Cat#B5002), DNaseI (Cat#D5025), and Liberase (Cat#5401119001) were purchased from Millipore-Sigma. Brefeldin A (Cat#B7450), anti-CD45-PerCP (Clone 30-F11), anti-CD8- Allophycocyanin-H7 or Allophycocyanin-Cy7 (Clone 53-6.7), anti-IFN-γ-Allophycocyanin (Clone XMG1.2), anti-TNFα-PE-Cy7 (Clone MP6-XT22), anti-CD107a-FITC (Clone 1D4B), anti-Granzyme B- Brilliant Violet (BV)421 (Clone GB11), and FITC anti-BrdU Sets containing FITC anti-BrdU (Clone 3D4) and FITC Mouse IgG1,κ Isotype (Clone MOPC-21) were purchased from BD Biosciences. Anti-Tim-3-PE (Clone #215008) was purchased from R&D Systems. Anti-PD-1-PE-Cy7 (Clone RMP1-30) was purchased from Biolegend. Anti-PD-1-FITC (Clone RMP1-30), anti-TIGIT-FITC (Clone GIGD7), and anti-LAG3-FITC (Clone eBioC9B7W) were purchased from eBioscience. Anti-CLTA-4-PE-Cy7 (Clone UC10-4F10-11) was purchased from Tonbo Biosciences. Anti-BTLA-PE-Vio770 (Clone REA224) and Anti-2B4-VioBright FITC or Allophycocyanin-Vio770 (Clone REA388) were purchased from Miltenyi Biotec. Aqua Live Dead (Cat# L34957) and Anti-Granzyme B-Allophycocyanin (Clone GB11) were purchased from Invitrogen. iScript (Cat#170-

8898) was purchased from Bio-Rad. The NIH Tetramer Core Facility provided the H2-$K^b$ tetramers containing the immunodominant $gB_{498-505}$ peptide conjugated to BV421 and the subdominant $RR1_{982-989}$ and $RR1_{822-829}$ peptides conjugated to Allophycocyanin. All peptides were purchased from Invitrogen. The BD Biosciences Cytofix/Cytoperm kit (Cat#554714) was used for all intracellular staining and the Cytofix/Cytoperm Plus reagent (Cat#561651) was additionally used for BrdU staining experiments. B6WT3 fibroblasts were maintained in DMEM containing 5% FBS, 1% Penicillin and Streptomycin, and 1% Gluta-MAX (297). Vero cells were maintained in DMEM containing 10% FBS, 1% Penicillin and Streptomycin, and 1% Gluta-MAX.

### 3.4.4 Flow Staining

TGs were excised and digested in 100 µl of DMEM containing 0.2 U/ml liberase for 50 minutes at 37ºC before being triturated into a single cell suspension. TG suspensions were filtered through 35 µm filter top flow tubes and were stained for surface markers, gB tetramer, and viable cells with Aqua Live Dead for 1 h at room temperature in the dark in PBS or PBS containing 10% FBS. For experiments that included intracellular staining, cells were fixed in Cytofix/Cytoperm for 20 min at 4ºC, washed with perm wash, stained with intracellular antibodies in perm wash for 30 min at 4ºC, washed with perm wash, and resuspended in FACS buffer (1% FBS and 0.1% sodium azide in PBS). For BrdU staining, cells were fixed in Cytofix/Cytoperm as described, washed in perm wash, incubated in Cytofix/Cytoperm Plus reagent for 10 min at 4ºC, washed in perm wash, and refixed in Cytofix/Cytoperm for 5 min at 4ºC before being incubated with 0.3 mg/ml DNaseI in PBS for 1 h at 37ºC. After DNaseI treatment, cells were washed in perm wash, incubated with anti-BrdU antibody for 20 min at room temperature, washed with perm wash, and washed and resuspended in FACS buffer. For experiments that did not involve intracellular

staining, cells were fixed in 1% Paraformaldehyde for 20 min at 4ºC, washed in FACS buffer, and resuspended in FACS buffer before being run.

### 3.4.5 Stimulations

For stimulation experiments, $1x10^6$ B6WT3 cells/ml were pulsed with 0.9 µg/ml gB peptide in stim media (RPMI containing 10% FBS, 1% Penicillin and Streptomycin, and 1% Gluta-MAX) for 60 min at 37ºC and 5% $CO_2$, with shaking every 10 mins. B6WT3s were then washed with stim media, resuspended in stim media containing 5 µg/ml brefeldin A, 50 µM 2-Mercaptoethanol, and CD107a-FITC antibody, and $5x10^5$ B6WT3s were added to each dissociated TG. Stimulations were incubated for 6 h at 37ºC and 5% $CO_2$ and then stained for surface markers and intracellular cytokines as described above.

### 3.4.6 Nanostring

TGs excised from 10-12, 8 dpi mice were dissociated and stained as described above for viable cells, CD45, CD8, gB Tetramer, Tim-3 and PD-1. Since essentially all $CD8^+$ T cells in the HSV-1-infected TG are HSV-1 specific (166), gB Tetramer staining was used to gate the $CD8^+$ T cell population into gB-$CD8^+$ T cells or Subdom-$CD8^+$ T cells and Tim-$3^-$PD-$1^-$, Tim-$3^+$PD-$1^-$, Tim-$3^+$PD-$1^+$, and Tim-$3^-$PD-$1^+$ cells from each population were sorted into PBS. Cells were pelleted and resuspended in iScript at a concentration of 250 cells/µl followed by vortexing for 30 sec to lyse cells. The lysate was then pelleted to clear cell debris and stored at -80ºC until use. RNA from 1 µl of this cell lysate was amplified by using the nCounter® Low RNA Input Kit (Nanostring) with 10 cycles of amplification. This amplified sample was then run with the

nCounter® PanCancer Immune Profiling Panel. After normalization to housekeeping genes, the indicated populations were assessed for fold change differences and a threshold of a 1.5-fold change averaged over the three experimental replicates (each replicate consisted of cells from the pooled TGs of 10-12 mice for a total of 20-24 TGs/replicate) was used to identify genes of interest in the gB-CD8$^+$ T cell population. These genes were further used to assess the Subdom-CD8$^+$ T cell population as well as compare between the gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells.

### 3.4.7 Data Analysis

All flow samples were run on a BD FACSAria. Total cell numbers were determined using counting beads. Gates were set on fluorescence minus one controls. All flow analysis was done in FlowJo and graphs and statistical analysis were done in Prism (GraphPad). Statistical tests used and n for each experiment are indicated in the figure legends.

### 3.4.8 Data Availability

The Nanostring data has been deposited in the NCBI's Gene Expression Omnibus, GEO Series accession #GSE137973.

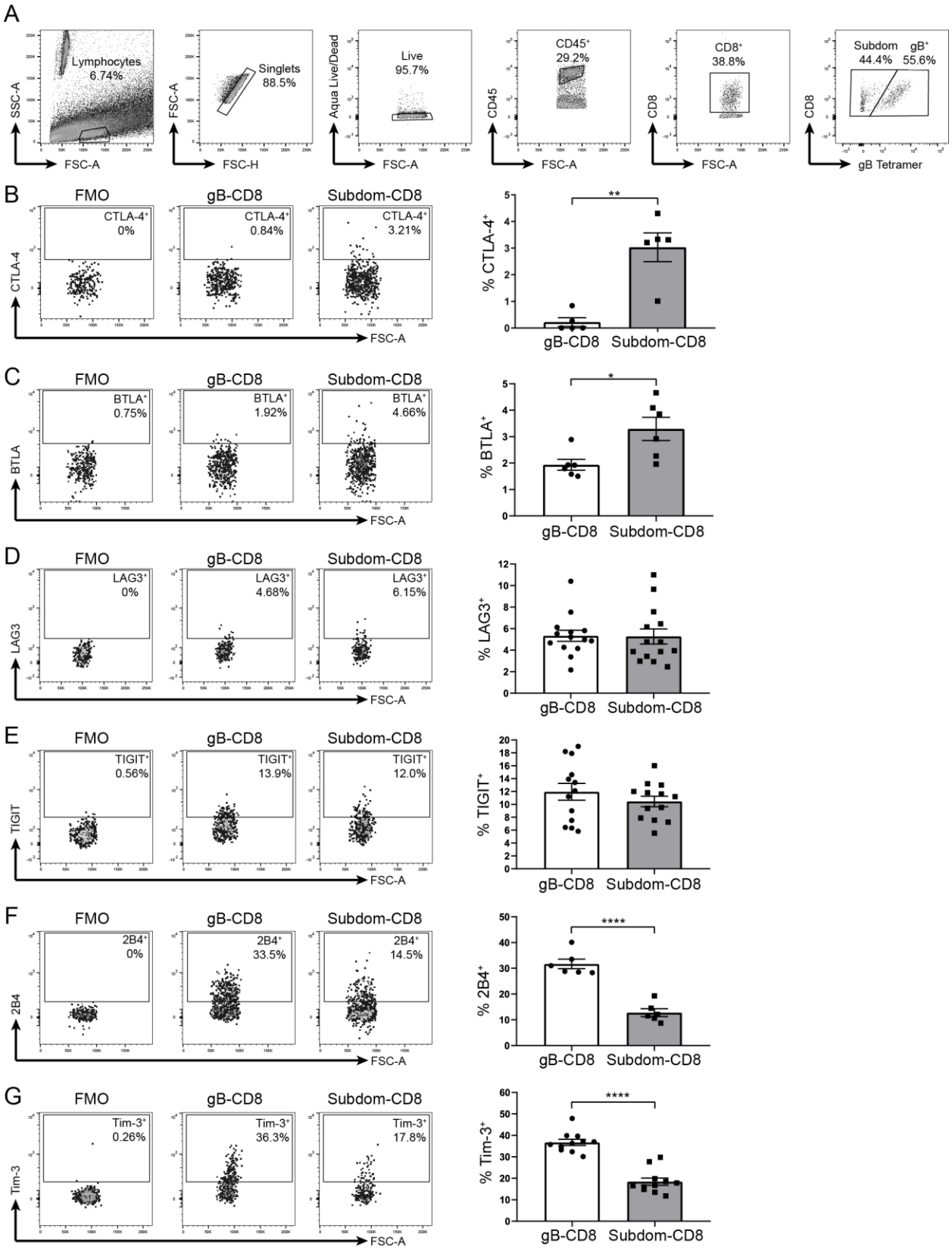
## 3.5 Results

### 3.5.1 Tim-3 and 2B4 are preferentially expressed on gB-CD8$^+$ T cells during latent HSV-1 infection

The expression of a single checkpoint molecule does not necessarily indicate functional impairment; however, the simultaneous expression of multiple checkpoint molecules is a hallmark of CD8$^+$ T cell exhaustion (292). Our laboratory has recently shown that during latent HSV-1 infection within the TG, CD8$^+$ T cells recognizing 18 subdominant epitopes from HSV-1 viral proteins (Subdom-CD8$^+$ T cells) collectively express high levels of the inhibitory checkpoint molecule PD-1. In contrast, CD8$^+$ T cells specific for the immunodominant epitope, $gB_{498\text{-}505}$ (gB-CD8$^+$ T cells), found in the same TGs express low levels of PD-1 (282). The expression of other checkpoint molecules on TG-CD8$^+$ T cells remained largely unknown, so we assessed the expression of six additional checkpoint molecules on gB-CD8$^+$ T cells (gB tetramer positive cells) and Subdom-CD8$^+$ T cells (gB tetramer negative cells) at latency (33-43 days post-infection (dpi)) (Figure 8A).

Similar to the expression pattern of PD-1, Cytotoxic T-lymphocyte protein 4 (CTLA-4) and B- and T-lymphocyte attenuator (BTLA) were expressed on a higher percentage of Subdom-CD8$^+$ T cells than gB-CD8$^+$ T cells, although overall levels of both checkpoint molecules were relatively low in both groups (Figure 8B-C). In addition, gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells had equivalent expression levels of lymphocyte-activation gene 3 (LAG3) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (Figure 8D-E). In contrast to PD-1, CTLA-4, and BTLA expression, a higher percentage of gB-CD8$^+$ T cells expressed 2B4 and Tim-3 than Subdom-CD8$^+$ T cells (Figure 8F-G). Therefore, despite the canonical expression pattern of

checkpoint molecules such as PD-1, BTLA, and CTLA-4 on Subdom-CD8$^+$ T cells, which exhibit functional impairment, we showed that Tim-3 and 2B4 are enriched on gB-CD8$^+$ T cells, which retain functionality throughout latent infection. These data call into question the association of these molecules with functional exhaustion in this model.


A
Lymphocytes
6.74%
SSC-A
FSC-A
Singlets
88.5%
FSC-A
FSC-H
Live
95.7%
Aqua Live/Dead
FSC-A
CD45+
29.2%
CD45
FSC-A
CD8+
38.8%
CD8
FSC-A
Subdom gB+
44.4% 55.6%
CD8
gB Tetramer
B
FMO
gB-CD8
Subdom-CD8
CTLA-4+
0%
CTLA-4+
0.84%
CTLA-4+
3.21%
CTLA-4
FSC-A
% CTLA-4+
**
gB-CD8
Subdom-CD8
C
FMO
gB-CD8
Subdom-CD8
BTLA+
0.75%
BTLA+
1.92%
BTLA+
4.66%
BTLA
FSC-A
% BTLA+
*
gB-CD8
Subdom-CD8
D
FMO
gB-CD8
Subdom-CD8
LAG3+
0%
LAG3+
4.68%
LAG3+
6.15%
LAG3
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gB-CD8
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FMO
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TIGIT+
0.56%
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13.9%
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gB-CD8
Subdom-CD8
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gB-CD8
Subdom-CD8
2B4+
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2B4+
33.5%
2B4+
14.5%
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FSC-A
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gB-CD8
Subdom-CD8
G
FMO
gB-CD8
Subdom-CD8
Tim-3+
0.26%
Tim-3+
36.3%
Tim-3+
17.8%
Tim-3
FSC-A
% Tim-3+
****
gB-CD8
Subdom-CD8

**Figure 8. Tim-3 and 2B4 are preferentially expressed on gB-CD8$^+$ T cells during latent HSV-1 infection.**
TGs from latently infected mice (33-43 dpi) were removed, processed into single cell suspensions, and stained for viability, CD45, CD8, CTLA-4, BTLA, LAG3, TIGIT, 2B4, or Tim-3, and gB tetramer to distinguish the gB-CD8$^+$ T cells from the Subdom-CD8$^+$ T cells and analyzed by flow cytometry. Representative dot plots from one mouse showing the expression of the indicated checkpoint molecules on the gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells are shown along with the FMO gating control with a graph from one representative experiment shown on the right (B-G) In the graphs, bars represent mean ± SEM. Differences between groups were assessed by unpaired t tests, *P ≤ 0.05, **P ≤ 0.01, **** P ≤ 0.0001. (A) Gating strategy is as follows: lymphocytes were first gated by size, doublets were excluded, dead cells were excluded, CD45$^+$ cells were gated, CD8$^+$ cells were gated, and CD8$^+$ cells were split into gB-CD8$^+$ T cell or Subdom-CD8$^+$ T cell groups by using the gB tetramer before looking at the various checkpoint molecules. (B) Expression of CTLA-4 (stained both extracellularly and intracellularly), n=5, representative of two independent experiments. (C) Expression of BTLA, n=6, representative of two independent experiments. (D) Expression of LAG3, n=14, representative of two independent experiments. (E) Expression of TIGIT, n=13, representative of two independent experiments. (F) Expression of 2B4, n=6, representative of four independent experiments. (G) Expression of Tim-3, n=11, representative of ten independent experiments.

### 3.5.2 Tim-3 is primarily expressed on PD-1$^-$-CD8$^+$ T cells in the latently infected TG

Tim-3 has been shown to mark functionally exhausted CD8$^+$ T cells during infection with LCMV, HIV, and HCV, as well as in tumors (263, 265, 266, 298). In several of these models the severity of exhaustion in Tim-3 expressing cells is dependent on the co-expression of PD-1, with cells expressing both checkpoint molecules being notably less functional than cells that express either PD-1 or Tim-3 alone (263, 298). This is consistent with the idea that functional exhaustion is a result of accumulating expression of multiple inhibitory checkpoint molecules. It was previously reported that Tim-3 marks exhausted CD8$^+$ T cells in the latent TG during HSV-1 infection (281, 295); however, we wanted to assess Tim-3 expression on TG-CD8$^+$ T cells that expressed or did not express the canonical exhaustion marker PD-1. When we assessed the expression of these two checkpoint molecules on CD8$^+$ T cells in the latently infected TG (Figure 9A), it was apparent that there was little overlap between the two markers, with fewer than 50 total cells/TG or less than 10% of the CD8$^+$ T cells being Tim-3$^+$PD-1$^+$ in both the gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups (Figure 9B-C). This lack of strong co-expression between Tim-3 and PD-1, and the preferential expression of Tim-3 on gB-CD8$^+$ T cells and PD-1 on Subdom-CD8$^+$ T

cells, indicated that Tim-3 may not be functioning in the typically inhibitory manner that is seen in most chronic diseases.

As our Tim-3 and PD-1 co-expression data implied that Tim-3 might not be inhibitory in the latently infected TG, we next wanted to determine if Tim-3 plays a role in $CD8^+$ T cell proliferation, which is often diminished during functional exhaustion (197). After a two-day *in vivo* BrdU pulse, we found that levels of proliferation in $Tim\text{-}3^+$ cells were not significantly different than in $Tim\text{-}3^-$ cells in both the gB-$CD8^+$ T cells and Subdom-$CD8^+$ T cells, implying that these cells are not exhausted (Figure 9D). Collectively, these results show that Tim-3 expression is largely independent of PD-1 expression and further suggest that Tim-3 may not mark cells that have lost functionality in the latently infected TG.

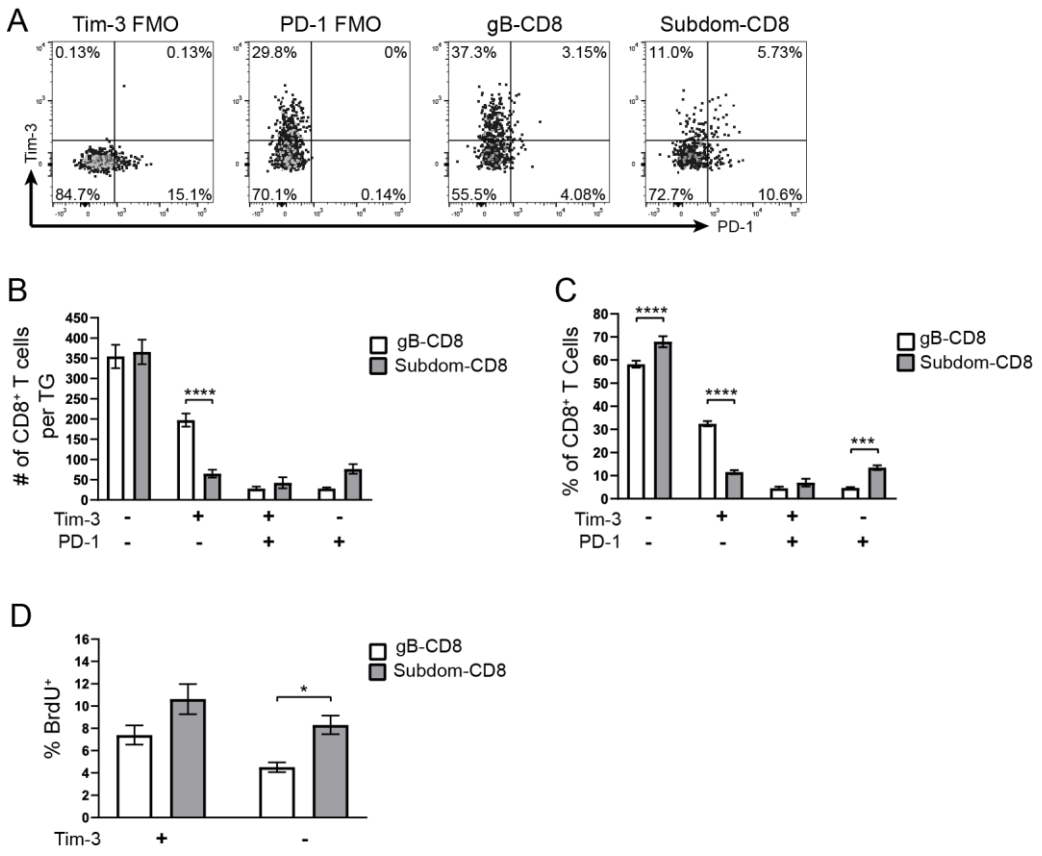



**Figure 9. Tim-3 is primarily expressed on PD-1$^-$-CD8$^+$ T cells in the latently infected TG.**
TGs from latently infected mice (33-35 dpi) were removed, processed into single cell suspensions, and stained for viability, CD45, CD8, gB tetramer, Tim-3, and PD-1 and analyzed by flow cytometry. To assess *in vivo* T cell proliferation, mice received i.p. injections of 1 mg of BrdU 48 and 24 h prior to sacrifice and BrdU uptake was measured by flow. Representative dot plots from one mouse showing the expression of the indicated checkpoint molecules on the gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells are shown along with the FMO gating controls (A). In the graphs, the bars represent the mean number of cells, % of CD8$^+$ T cells, or % BrdU+ ± SEM with the gB-CD8$^+$ T cell population represented by white bars and the Subdom-CD8$^+$ T cell population represented by grey bars. Differences in (B-D) were assessed by two-way ANOVAs with Tukey's posttests, *P ≤ 0.05, *** P ≤ 0.001, **** P ≤ 0.0001. (A) Expression of Tim-3 and PD-1. (B) Total number of CD8$^+$ T cells/TG in each of Tim-3/PD-1 quadrants from (A), n=11, experiment was repeated an additional eight times with similar results. (C) Percentage of CD8$^+$ T cells in each of Tim-3/PD-1 quadrants from (A), n=11, experiment was repeated an additional eight times with similar results. (D) gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell populations were each gated into Tim-3$^+$ and Tim-3$^-$ populations and the percentage of cells that were BrdU$^+$ in each subgroup are shown, n=25, from two pooled independent experiments.

### 3.5.3 Tim-3$^+$ gB-CD8$^+$ T cells are multifunctional after *ex vivo* stimulation

This study and previous work (281) showed that Tim-3 was primarily expressed on PD-1 negative cells (in both gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells). Therefore, we were next

interested in determining if Tim-3 was instead preferentially expressed on functional cells. After stimulation with cognate antigen, the most functionally competent cells can produce and release multiple factors, including IFN-γ, TNF-α and lytic granules containing GzmB, while functionally exhausted $CD8^+$ T cells gradually lose this multifunctionality (299). To determine if the Tim-$3^+$ cells in our model were functional, we stained latently infected TGs with anti-Tim-3 antibody and subsequently stimulated the TGs with gB peptide pulsed B6WT3 cells for six hours along with fluorescently labeled anti-CD107a and brefeldin A. We then used flow cytometry to identify cells that produced IFN-γ and/or TNF-α and confirmed multifunctionality by assessing lytic granule release ($CD107a^+$) in responding cells (Figure 10A). When Tim-3 expression was quantified on the responding-$CD8^+$ T cells (cells that produced IFN-γ and/or TNF-α after gB peptide stimulation) we found that ~35% expressed Tim-3 (Figure 10B). When we calculated the multifunctionality of Tim-$3^+$ cells, we found that ~70% of the Tim-$3^+$ cells were multifunctional (IFN-$\gamma^+$ TNF-$\alpha^+$ $CD107a^+$) (Figure 10C), which suggested that Tim-3 expression was not associated with impaired cells but instead appeared to be associated with functionally competent cells.

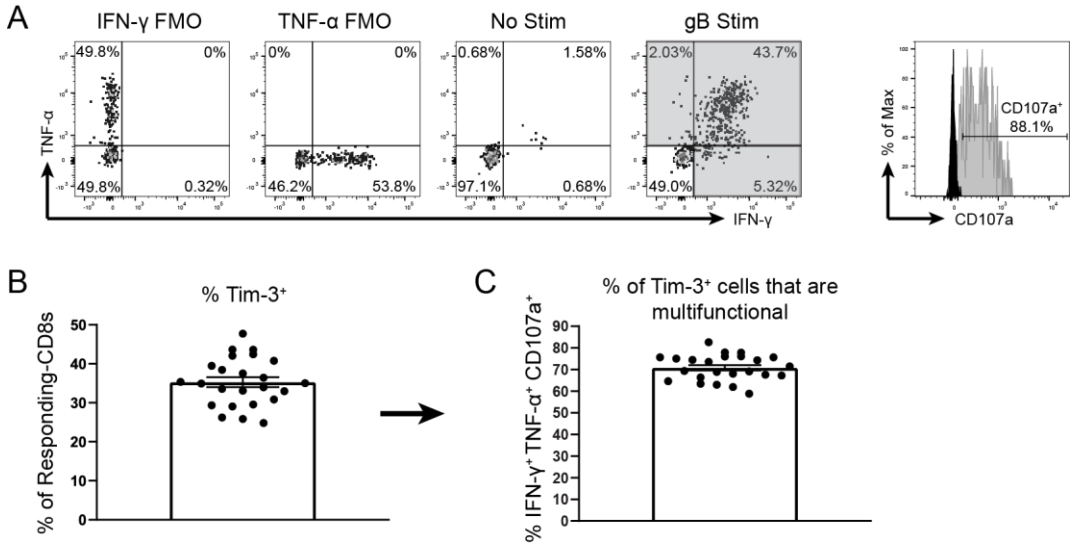



**Figure 10. Tim-3$^+$ gB-CD8$^+$ T cells are multifunctional after *ex vivo* stimulation.**
TGs from latently infected mice (33-35 dpi) were removed, processed into single cell suspensions, stained with anti-Tim-3 antibody, and stimulated for 6 h with gB peptide pulsed B6WT3 cells in the presence of anti-CD107a and brefeldin A. Cells were then collected and stained for viability, CD45, CD8, IFN-γ, and TNF-α. In graphs, bar represents mean ± SEM. n=24, from two pooled independent experiments. (A) Representative dot plots of IFN-γ and TNF-α response from an individual TG that received no peptide stimulation (plot 3rd from left) and an individual TG that received gB peptide stimulation (plot 4th from left). Shaded quadrants make up the "Responding-CD8$^+$ T cells". Gating for CD107a is shown at far right with FMO represented in black and a representative sample in grey. (B) Percentage of Responding-CD8$^+$ T cells that are positive for Tim-3. (C) Percentage of Tim-3$^+$ Responding-CD8$^+$ T cells that are multifunctional (IFN-γ$^+$ TNF-α$^+$, CD107a$^+$).

### 3.5.4 Tim-3$^+$ cells are functional *in vivo*

Although our data from Figure 10 established that Tim-3 expressing cells were highly functional after peptide stimulation, we were also interested in further investigating their ability to produce GzmB, which marks activated CD8$^+$ T cells and contributes to maintenance of viral latency in host neurons by cleaving the essential HSV-1 protein ICP4 (162). When CD8$^+$ T cells from latently infected TGs were assessed for GzmB expression directly *ex vivo*, significantly higher percentages of gB-CD8$^+$ T cells expressed GzmB than Subdom-CD8$^+$ T cells (Figure 11A), consistent with previously published data (169). When GzmB expression was measured in the Tim-3$^+$ gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells, we found that the majority of cells in both

groups were positive for GzmB; however, a higher frequency of Tim-3$^+$ gB-CD8$^+$ T cells expressed GzmB$^+$ than Tim-3$^+$ Subdom-CD8$^+$ T cells (Figure 11B). The percentage of Tim-3$^+$ gB-CD8$^+$ T cells that were GzmB$^+$ was similar to the percentage of cells that were multifunctional after stimulation (Figure 10C), which supported the hypothesis that Tim-3$^+$ cells are functional.

As we had unexpectedly found that the Tim-3$^+$ Subdom-CD8$^+$ T cells were significantly less functional in terms of GzmB production than the Tim-3$^+$ gB-CD8$^+$ T cells, we assessed whether PD-1 could be modulating this difference, despite the low levels of co-expression that we observed (Figure 9). When PD-1$^+$ cells were assessed for GzmB expression, we found that 60% of PD-1$^+$ gB-CD8$^+$ T cells expressed GzmB while only 20% of PD-1$^+$ Subdom-CD8$^+$ T cells expressed GzmB (Figure 11C). This corroborated our previous study, which showed a lower overall level of PD-1 expression on gB-CD8$^+$ T cells than on Subdom-CD8$^+$ T cells (282). Therefore, the disparity in functionality between PD-1$^+$ gB-CD8$^+$ T cells and PD-1$^+$ Subdom-CD8$^+$ T cells may be due to the differences in expression levels of PD-1.

With Tim-3$^+$ or PD-1$^+$ Subdom-CD8$^+$ T cells exhibiting a reduced ability to produce GzmB compared to Tim-3$^+$ or PD-1$^+$ gB-CD8$^+$ T cells, we then proceeded to look more specifically at: 1) Tim-3$^-$PD-1$^-$, 2) Tim-3$^+$PD-1$^-$, 3) Tim-3$^+$PD-1$^+$, and 4) Tim-3$^-$PD-1$^+$ cells with respect to their ability to produce GzmB. In the gB-CD8$^+$ T cells, the subgroup with the highest percentage of cells that produced GzmB$^+$ was that which expressed Tim-3 alone (Figure 11D). Cells co-expressing Tim-3 and PD-1 produced slightly less GzmB, although the difference was not significant. The Tim-3$^-$PD-1$^+$ and Tim-3$^-$PD-1$^-$ subgroups had significantly lower percentages of GzmB$^+$ cells than the groups expressing Tim-3. Like the gB-CD8$^+$ T cells, Subdom-CD8$^+$ T cells expressing Tim-3 only had the highest percentage of cells that produced GzmB; however, unlike for gB-CD8$^+$ T cells, we observed a significant decrease in the GzmB expression frequency in both

the Tim-3$^{+}$PD-1$^{+}$ and Tim-3$^{-}$PD-1$^{+}$ subgroups compared to the Tim-3$^{+}$PD-1$^{-}$ group (Figure 11E). We conclude from these data that Tim-3 is associated with HSV-1-specific T cell functionality, while PD-1 is associated with impairment at latency, with cells expressing both checkpoint molecules exhibiting an intermediate functional phenotype.

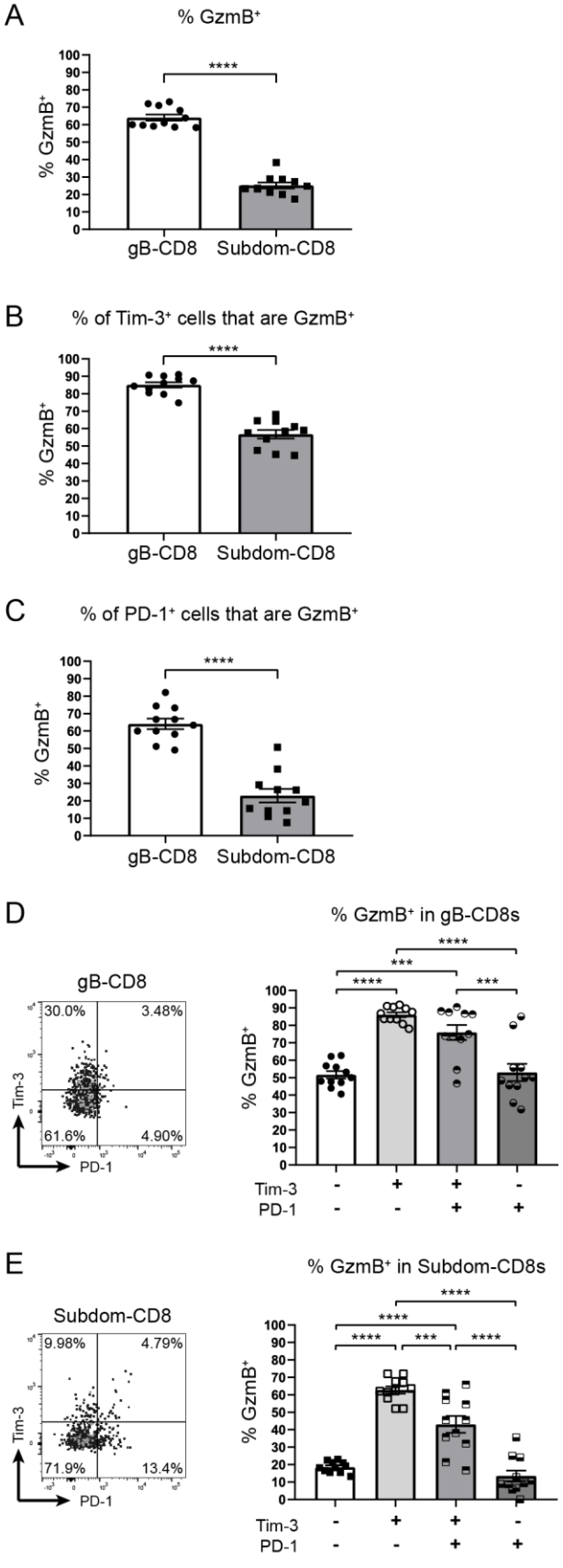

A
% GzmB+
% GzmB+
gB-CD8
Subdom-CD8
****
B
% of Tim-3+ cells that are GzmB+
C
% of PD-1+ cells that are GzmB+
D
gB-CD8
30.0%
3.48%
61.6%
4.90%
Tim-3
PD-1
% GzmB+ in gB-CD8s
***
Tim-3
PD-1
E
Subdom-CD8
9.98%
4.79%
71.9%
13.4%
% GzmB+ in Subdom-CD8s

**Figure 11. Tim-3$^+$ cells are functional *in vivo*.**
TGs from latently infected mice (33-35 dpi) were removed, processed into single cell suspensions, and stained for viability, CD45, CD8, gB tetramer, Tim-3, PD-1, and GzmB and assessed by flow. In the graphs, the bars represent the mean frequency of cells ± SEM with the gB-CD8$^+$ T cell population represented by circles/white bars and the Subdom-CD8$^+$ T cell population represented by squares/grey bars in graphs that contain both groups. n=11, experiment was repeated an additional five times with similar results. Differences were assessed by unpaired t tests (A-C) or one-way ANOVAs with Tukey's posttests (D-E). *** $P \leq 0.001$, **** $P \leq 0.0001$. (A) Percentages of cells in gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups that are GzmB$^+$. (B) Percentages of Tim-3$^+$ cells in gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups that are GzmB$^+$. (C) Percentages of PD-1$^+$ cells in gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups that are GzmB$^+$. (D) Percentages of gB-CD8$^+$ T cells in Tim-3/PD-1 quadrants that are GzmB$^+$. (E) Percentages of Subdom-CD8$^+$ T cells in Tim-3/PD-1 quadrants that are GzmB$^+$.

### 3.5.5 Viral gene expression controls Tim-3 expression in the latently infected TG

Even though lytic viral proteins have not been reliably detected in the latently infected TG, our laboratory previously showed that only HSV-1-specific CD8$^+$ T cells are retained within the TG during latency and that retention requires antigen expression, leading to the conclusion that there are consistent low-levels of lytic viral gene expression during latent HSV-1 infection (145, 168). Since we observed that Tim-3 expression was associated with functionality, we were next interested in determining if changes in antigen availability during latency could alter Tim-3 expression on virus-specific CD8$^+$ T cells. To investigate this, we manipulated the virus-specific CD8$^+$ T cell repertoire and viral gene expression by using genetically modified strains of HSV-1 expressing different levels of the $gB_{498\text{-}505}$ epitope and assessed Tim-3 expression on TG-resident CD8$^+$ T cells during latent infection.

We first used a recently detailed virus (S1L) that contains a single point mutation within the gB epitope that completely abrogates T cell recognition of $gB_{498\text{-}505}$, and therefore prevents the priming and expansion of gB-specific CD8$^+$ T cells, but does not affect viral fitness or the expression profile of other viral genes (168). This lack of gB-specific CD8$^+$ T cells allows RR1-specific CD8$^+$ T cells ($RR1_{982\text{-}989}$ and $RR1_{822\text{-}829}$) to expand and infiltrate the TG in greater numbers than in wild-type (WT) infection (168). We observed that the increase in cell numbers extended

beyond acute infection and into latency (Figure 12A). With this, we were able to assess alterations in Tim-3 expression when there are more $CD8^+$ T cells responding to the same level of viral gene expression, which effectively reduces the chances that any one RR1-specific $CD8^+$ T cell would be antigenically stimulated. Tim-3 expression on RR1-$CD8^+$ T cells was reduced in S1L infection compared to wild-type infection, and there was a concurrent reduction in GzmB expression in the RR1-$CD8^+$ T cells in S1L infected TGs compared to wild-type infected TGs (Figure 12B-C). Despite this, the percentage of Tim-$3^+$ RR1-$CD8^+$ T cells that were $GzmB^+$ remained similar between the two infections (Figure 12D). While it is unknown how the presentation of viral epitopes on MHC molecules may change when the $gB_{498\text{-}505}$ epitope is deleted and how this may influence the total number of cells that are stimulated, overall Tim-3 expression, albeit reduced in the S1L infection, continued to correlate with the expression pattern of GzmB. Therefore, we can conclude from these data that Tim-3 expression is likely linked with recent antigenic stimulation of TG-resident $CD8^+$ T cells.

We next sought to determine if increasing the expression of viral antigen within the TG could result in an upregulation of Tim-3. To study this, we made use of two viruses that express four copies of the gB epitope under the control of the viral promotors ICP0 or gC (296). Compared to the wild-type virus, TGs infected with these viruses maintained a similar frequency of gB-$CD8^+$ T cells during latency; however, significantly higher percentages of gB-$CD8^+$ T cells expressed Tim-3 in the gC- and ICP0-infected TGs (Figure 12E-F). The gB-$CD8^+$ T cells in the ICP0- and gC-infected TGs also had significantly higher percentages of GzmB expression while maintaining similar percentages of Tim-$3^+$ cells that were $GzmB^+$ (Figure 12G-H). Since these viruses express higher levels of the gB antigen but the TGs possess similar numbers of gB-$CD8^+$ T cells to WT infection, these results suggest that increasing antigen availability to the gB-$CD8^+$ T cells increases

the expression of Tim-3 on the gB population as a whole. These data, together with the data from the S1L virus, support the notion that Tim-3 expression correlates with the overall level of T cell stimulation during HSV-1 latency.

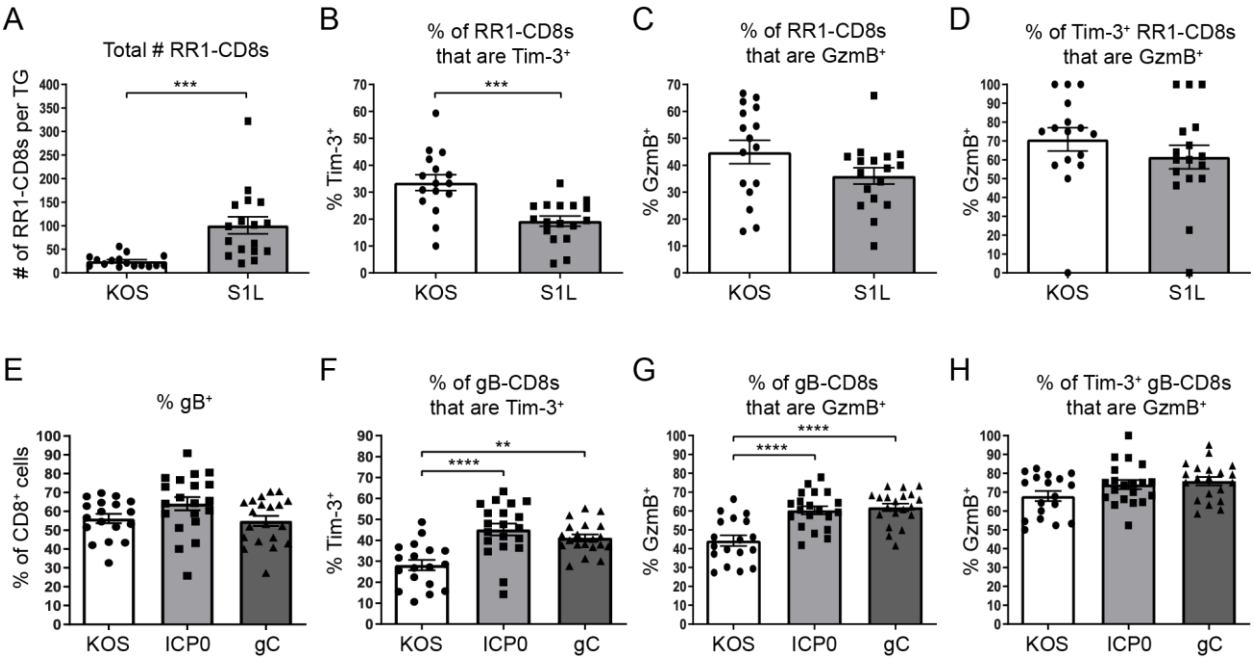



**Figure 12. Viral gene expression controls Tim-3 expression in the latently infected TG.**
TGs from mice latently infected with wild-type KOS or viruses containing mutations (S1L, gC, or ICP0) were removed, processed into single cell suspensions, and stained for viability, CD45, CD8, gB or RR1 tetramer, Tim-3, and GzmB and assessed by flow. Bars represent the mean total number or frequency of cells ± SEM. Differences were assessed by unpaired t tests (A-D) or one-way ANOVAs with Tukey's posttests (E-H). **P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001. (A-D) n=16 (KOS) and 17 (S1L), pooled from two, independent experiments. (E-H) n=18 (KOS), 20 (ICP0), and 20 (gC), pooled from two, independent experiments. (A) Total numbers of cells that are positive for RR1 tetramers in KOS and S1L infected TGs. (B) Percentages of RR1-CD8$^+$ T cells that are positive for Tim-3 in KOS and S1L infected TGs. (C) Percentages of RR1-CD8$^+$ T cells that are positive for GzmB in KOS and S1L infected TGs. (D) Percentages of Tim-3$^+$ RR1-CD8$^+$ T cells that are positive for GzmB in KOS and S1L infected TGs. (E) Percentages of CD8$^+$ T cells that are positive for gB tetramer in KOS, gC, and ICP0 infected TGs. (F) Percentages of gB-CD8$^+$ T cells that are positive for Tim-3 in KOS, gC, and ICP0 infected TGs. (G) Percentages of gB-CD8$^+$ T cells that are positive for GzmB in KOS, gC, and ICP0 infected TGs. (H) Percentages of Tim-3$^+$ gB-CD8$^+$ T cells that are positive for GzmB in KOS, gC, and ICP0 infected TGs.

### 3.5.6 Tim-3$^+$ cells preferentially upregulate genes that are associated with T cell activation

Before regulating functional impairment in models of chronic viral infection or persistent tumors, checkpoint molecules are transiently expressed after T cell activation and may function in

tempering the response to their cognate antigens during acute infection (300). As Tim-3 was preferentially upregulated in functional cells during latent infection, we next assessed whether Tim-3 expression was associated with functionality during acute infection. To do this, we used Nanostring to compare the transcriptional profile of FACS sorted Tim-$3^+$ PD-$1^-$ cells and Tim-$3^-$ PD-$1^-$ cells within the gB-$CD8^+$ T cells and Subdom-$CD8^+$ T cells from TGs at eight days post-infection, when latency has just been established and the immune infiltrate has peaked. As we observed in latency, granzymes were upregulated in Tim-3 expressing cells from both groups, indicating effector functionality (Figure 13A-B). This was further supported by the upregulation of genes such as Notch1, Zap70, and Runx3, all of which are known to contribute to T cell activation (204, 301, 302). When we compared Tim-$3^+$ PD-$1^-$ gB-$CD8^+$ T cells to Tim-$3^+$ PD-$1^-$ Subdom-$CD8^+$ T cells, we found that there was an overall upregulation of genes associated with T cell functionality in the gB-$CD8^+$ T cell population, which was consistent with a higher level of functionality during latency (Figure 13C). This is indicative of very early differences between the gB-$CD8^+$ T cells and Subdom-$CD8^+$ T cells rather than an acquired loss of functionality during latency, as is typical during chronic viral infection.

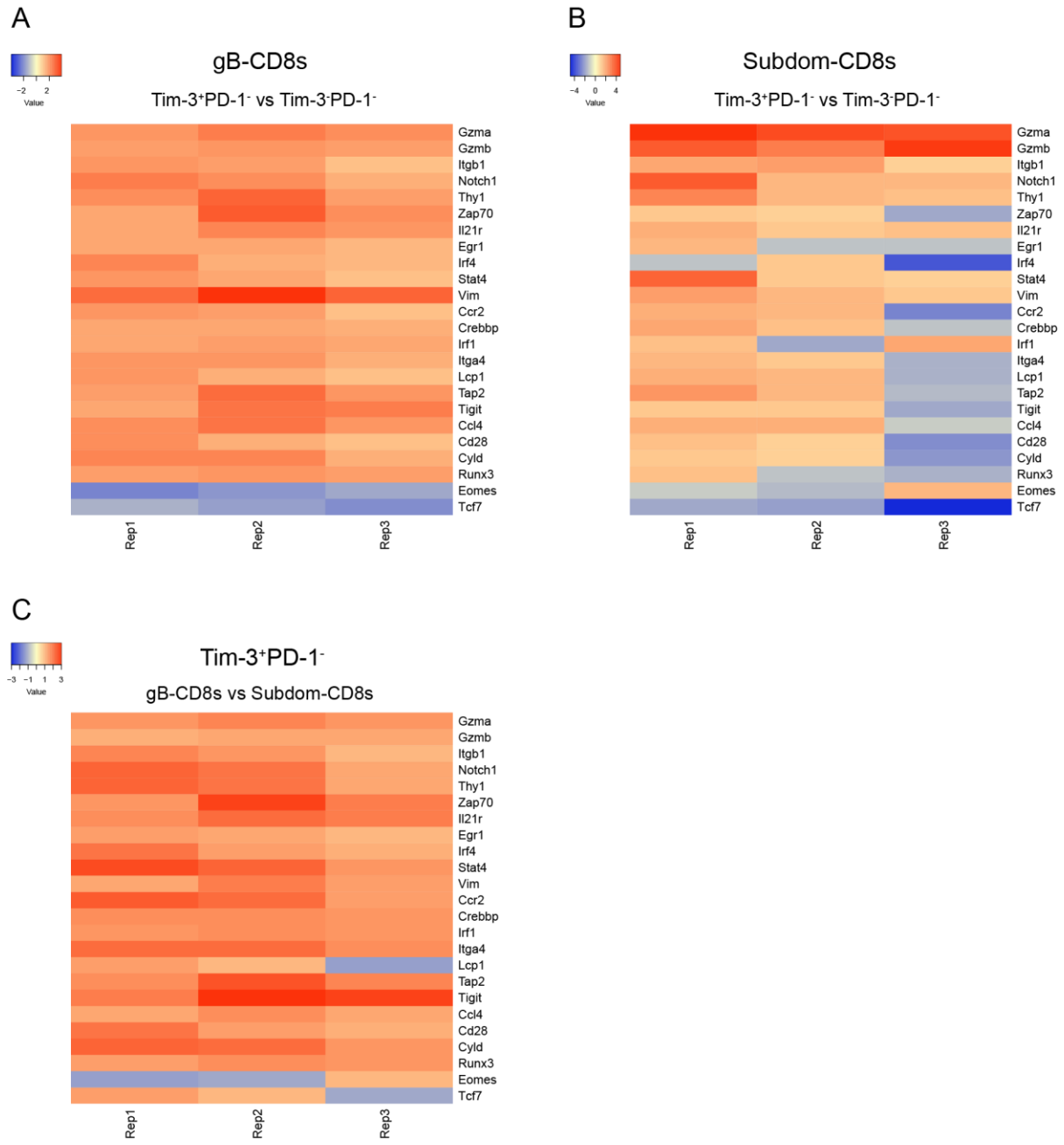



**Figure 13. Tim-3$^+$ cells preferentially upregulate genes that are associated with T cell activation.**
TGs from acutely infected mice (8 dpi) were removed, processed into a single cell suspension, and stained for viable cells, CD45, CD8, gB tetramer, Tim-3 and PD-1. CD8$^+$ T cells were gated on gB-CD8$^+$ T cells or Subdom-CD8$^+$ T cells using gB tetramer and Tim-3$^-$PD-1$^-$, Tim-3$^+$PD-1$^-$, Tim-3$^+$PD-1$^+$, and Tim-3$^-$PD-1$^+$ cells were sorted and their transcriptional profile was assessed using Nanostring. (A) Genes of interest that averaged a fold change of ≥ 1.5 over 3 independent replicates between Tim-3$^+$PD-1$^-$ gB-CD8$^+$ T cells and Tim-3$^-$PD-1$^-$ gB-CD8$^+$ T cells. (B) Fold change of genes of interest between Tim-3$^+$PD-1$^-$ Subdom-CD8$^+$ T cells and Tim-3$^-$PD-1$^-$ Subdom-CD8$^+$ T cells. (C) Fold change of genes of interest between Tim-3$^+$PD-1$^-$ gB-CD8$^+$ T cells and Tim-3$^+$PD-1$^-$ Subdom-CD8$^+$ T cells.

### 3.5.7 Tim-3$^+$ cells are functional at early times post-infection

Since Tim-3 and PD-1 appeared differentially regulated in the TG during latency, and our transcriptional analysis indicated that Tim-3 expression at early times post-infection was associated with the transcription of genes that are generally involved in T cell activation, we next evaluated the relationship between Tim-3, PD-1, and GzmB at early times post-infection. Despite PD-1 being primarily expressed on Subdom-CD8$^+$ T cells during latency, a higher frequency of gB-CD8$^+$ T cells expressed PD-1 than Subdom-CD8$^+$ T cells during the acute response at eight days post-infection (Figure 14A-B). Of note, the expression patterns of PD-1 and GzmB were different between gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells, with most PD-1$^+$ cells in the gB-CD8$^+$ T cell group also expressing GzmB, consistent with transient upregulation after activation. In contrast, there was a distinct population of PD-1$^+$ cells that were GzmB$^-$ in the Subdom-CD8$^+$ T cell group (Figure 14A). Accordingly, a higher percentage of PD-1$^+$ gB-CD8$^+$ T cells expressed GzmB than PD-1$^+$ Subdom-CD8$^+$ T cells (Figure 14C). Furthermore, the levels of PD-1 expression were significantly higher in GzmB$^-$ Subdom-CD8$^+$ T cells than in both GzmB$^+$ Subdom-CD8$^+$ T cells and GzmB$^-$ gB-CD8$^+$ T cells (Figure 14D).

Like PD-1, the overall percentage of cells expressing Tim-3 was elevated at eight days post-infection in both groups, with a significantly higher percentage of cells in the gB-CD8$^+$ T cell group being Tim-3$^+$ than in the Subdom-CD8$^+$ T cell group, similar to what was observed during latency (Figure 14E-F). Tim-3 and GzmB expression appeared to be strongly correlated as over 75% of gB-CD8$^+$ T cells expressed both Tim-3 and GzmB (Figure 14E). Similarly, when we considered only Tim-3$^+$ gB-CD8$^+$ T cells, we found that over 90% of the cells also expressed GzmB (Figure 14G). Even though a majority of Tim-3$^+$ Subdom-CD8$^+$ T cells also co-expressed GzmB, a lower overall frequency of Tim-3$^+$ Subdom-CD8$^+$ T cells expressed GzmB than their

Tim-3$^+$ gB-CD8$^+$ T cell counterparts (Figure 14G). Despite the differences in GzmB expression between gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells, a consistent observation in latent and acute infection was that GzmB was preferentially expressed in Tim-3$^+$ cells, suggesting that Tim-3 did not inhibit functionality during HSV-1 infection (Figure 14H).

During latency, we observed in both the gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells that cells expressing only Tim-3 were the most functional, cells expressing only PD-1 were the least functional, and cells expressing both Tim-3 and PD-1 exhibited an intermediate functionality, suggesting that Tim-3 was associated with functionality and that PD-1 was associated with impairment (Figure 11D-E). At eight days post-infection, the Tim-3$^+$PD-1$^+$ population made up a much more substantial proportion of the total CD8$^+$ T cells in both groups, however, unlike in latency, the relationship between Tim-3, PD-1, and GzmB differed between the gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells (Figure 14I-J). Despite a drop in GzmB expression in Tim-3$^-$PD-1$^+$ cells compared to Tim-3$^+$ cells in the gB-CD8$^+$ T cells, 80% of Tim-3$^-$PD-1$^+$ cells still expressed GzmB (Figure 14K). This is consistent with the idea that Tim-3 is associated with functionality and that PD-1 is transiently upregulated in gB-CD8$^+$ T cells upon T cell activation during acute infection. The Subdom-CD8$^+$ T cell group had a somewhat different response in which both the Tim-3$^+$PD-1$^+$ and Tim-3$^-$PD-1$^+$ groups had significantly fewer GzmB$^+$ cells than the Tim-3$^+$PD-1$^-$ group (Figure 14L). Together, these data support the idea that functional impairment in the Subdom-CD8$^+$ T cell group may not be a result of continuous stimulation during latent HSV-1 infection. Instead, our data suggest that functional impairment may be imprinted on Subdom-CD8$^+$ T cells during acute infection, possibly due to suboptimal priming, resulting in a state of functional impairment that is perpetuated into latency.

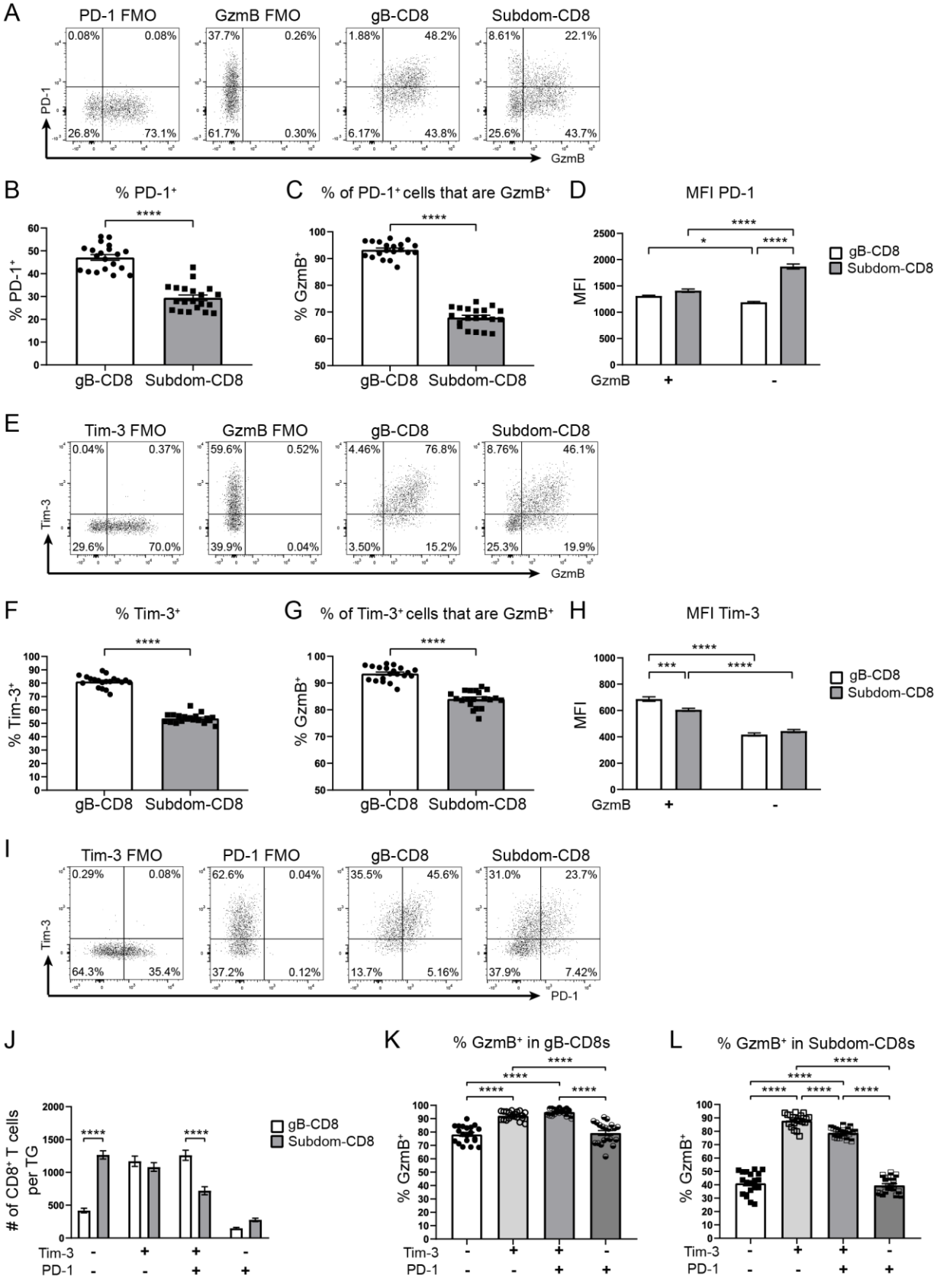

A
PD-1 FMO
GzmB FMO
gB-CD8
Subdom-CD8
PD-1
GzmB
0.08%
0.08%
26.8%
73.1%
37.7%
0.26%
61.7%
0.30%
1.88%
48.2%
6.17%
43.8%
8.61%
22.1%
25.6%
43.7%
B
% PD-1+
% PD-1+
gB-CD8
Subdom-CD8
****
C
% of PD-1+ cells that are GzmB+
% GzmB+
gB-CD8
Subdom-CD8
****
D
MFI PD-1
MFI
GzmB
+
-
gB-CD8
Subdom-CD8
****
*
****
E
Tim-3 FMO
GzmB FMO
gB-CD8
Subdom-CD8
Tim-3
GzmB
0.04%
0.37%
29.6%
70.0%
59.6%
0.52%
39.9%
0.04%
4.46%
76.8%
3.50%
15.2%
8.76%
46.1%
25.3%
19.9%
F
% Tim-3+
% Tim-3+
gB-CD8
Subdom-CD8
****
G
% of Tim-3+ cells that are GzmB+
% GzmB+
gB-CD8
Subdom-CD8
****
H
MFI Tim-3
MFI
GzmB
+
-
gB-CD8
Subdom-CD8
****
***
****
I
Tim-3 FMO
PD-1 FMO
gB-CD8
Subdom-CD8
Tim-3
PD-1
0.29%
0.08%
64.3%
35.4%
62.6%
0.04%
37.2%
0.12%
35.5%
45.6%
13.7%
5.16%
31.0%
23.7%
37.9%
7.42%
J
# of CD8+ T cells per TG
gB-CD8
Subdom-CD8
****
****
Tim-3
PD-1
K
% GzmB+ in gB-CD8s
% GzmB+
Tim-3
PD-1
****
L
% GzmB+ in Subdom-CD8s
% GzmB+
Tim-3
PD-1
****

**Figure 14. Tim-3$^+$ cells are functional at early times post-infection.**
TGs from acutely infected mice (8 dpi) were removed, processed into single cell suspensions, and stained for viability, CD45, CD8, gB tetramer, Tim-3, PD-1, and GzmB and assessed by flow. In graphs, bars represent mean ± SEM with the gB-CD8$^+$ T cell population represented by white bars and the Subdom-CD8$^+$ T cell population represented by grey bars in graphs that contain both groups. n=20, pooled from two independent experiments. Data in panels D, H, and J were analyzed by two-way ANOVAs with Tukey's posttests, data in panels K-L were analyzed by one-way ANOVAs with Tukey's posttests, and data in panels B-C and F-G were analyzed by unpaired t tests. *P ≤ 0.05, *** P ≤ 0.001, **** P ≤ 0.0001. (A) Representative dot plots from one mouse showing the expression of PD-1 and GzmB on gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells are shown along with the FMO gating controls. (B) Percentages of cells in gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups that are PD-1$^+$. (C) Percentages of PD-1$^+$ cells in gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups that are GzmB$^+$. (D) MFI of PD-1 in PD-1$^+$GzmB$^+$ and PD-1$^+$GzmB$^-$ gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups. (E) Representative dot plots from one mouse showing the expression of Tim-3 and GzmB on gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells are shown along with the FMO gating controls. (F) Percentages of cells in gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups that are Tim-3$^+$. (G) Percentages of Tim-3$^+$ cells in gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups that are GzmB$^+$. (H) MFI of Tim-3 in Tim-3$^+$GzmB$^+$ and Tim-3$^+$GzmB$^-$ gB-CD8$^+$ T cell and Subdom-CD8$^+$ T cell groups. (I) Representative dot plots from one mouse showing the expression of Tim-3 and PD-1 on gB-CD8$^+$ T cells and Subdom-CD8$^+$ T cells are shown along with the FMO gating controls. (J) Total numbers of CD8$^+$ T cells/TG in each of the Tim-3/PD-1 quadrants from panel I. (K) Percentages of gB-CD8$^+$ T cells in Tim-3/PD-1 quadrants that are GzmB$^+$. (L) Percentages of Subdom-CD8$^+$ T cells in Tim-3/PD-1 quadrants that are GzmB$^+$.

## 3.6 Discussion

Checkpoint molecules play an important role in balancing the ability of the adaptive immune system to rapidly respond to insult while also preventing damaging immunopathology. Not surprisingly, this control is not limited to the action of a single checkpoint molecule but instead relies on the collective contributions of many such molecules. Checkpoint molecules can be transiently upregulated after T cell activation and periodically thereafter when the cell reencounters its cognate antigen. Expression of these checkpoint molecules can have different effects, ranging from stimulation to inhibition depending on the expression of ligand(s) (300). In most viral infections, this control works efficiently, and the infection is quickly cleared. However, in cases of persistent antigenic exposure, like those found in LCMV or various cancers, the host fails to clear the infection or tumor, resulting in extended stimulation of T cells and aberrant upregulation of checkpoint molecules (217, 263, 292, 298, 303). This process then begins to limit

the T cell response, effectively creating a stalemate between the immune response and the antigenic source.

With both acute and latent lifecycles, HSV-1 infection exhibits characteristics of both a short term, normally cleared viral infection, and a chronic infection. Although actively replicating virus is cleared quickly from the periphery after primary infection, the virus infiltrates and establishes latency in host neurons, where it persists for the life of the host. In its latent state, HSV-1 does not produce infectious virus; however, our laboratory and others have shown that low levels of antigenic expression during latency stimulate ganglion-resident $CD8^+$ T cells throughout the life of the host (92, 304-307). Specifically, we know that $CD8^+$ T cells cluster their TCRs towards latently infected TG neurons and that TG-$CD8^+$ T cells require periodic antigenic stimulation to remain within the TG (92, 145, 304). Based on previous studies, one would expect that these repeated stimulations of virus-specific $CD8^+$ T cells would lead to a loss of function over time (299). Indeed, studies from our laboratory and others have shown that functional impairment of HSV-1 specific $CD8^+$ T cells does occur in the TG and the brain; however, in the TG, this functional impairment is largely limited to the 50% of $CD8^+$ T cells that recognize subdominant epitopes (169, 282, 308). The other 50% of $CD8^+$ T cells that recognize the immunodominant epitope, $gB_{498\text{-}505}$, remain highly functional and actually become more functional throughout latency (158, 169). Similar to chronic viral infection models, PD-1 expression demarks impaired $CD8^+$ T cells in the latently infected TG (Subdom-$CD8^+$ T cells), and functional $CD8^+$ T cells (gB-$CD8^+$ T cells) express little to no PD-1 (282). This disparate phenotype between Subdom-$CD8^+$ T cells and gB-$CD8^+$ T cells led us to investigate how functional impairment may develop in HSV-1 infection. Thus, we sought to identify the expression patterns of other checkpoint molecules to better understand how they may dictate the level of functionality in HSV-1-specific $CD8^+$ T cells.

Using a system in which we can delineate between the largely functional (gB-CD8$^+$ T cell) and functionally impaired (Subdom-CD8$^+$ T cell) populations, we found relatively few differences in the expression of classical checkpoint molecules, with low overall expression levels of most of these markers in both groups. One exception to this was Tim-3, which we observed to be expressed in both populations of CD8$^+$ T cells. Conventionally, Tim-3 has been considered to be an inhibitory checkpoint molecule with a number of studies showing that Tim-3 expression correlates with functionally exhausted cells in chronic viral infections, cancer models, and HSV-1 infections (263, 265, 266, 281, 295, 298). While the evidence for functional compromise in Tim-3$^+$ cells in chronic viral infections and cancer is compelling, the HSV-1 studies assigning functional compromise to Tim-3$^+$ HSV-1-specific CD8$^+$ T cells lacked substantial functional data to support the inhibitory nature of the checkpoint molecule (281, 295). In light of recent evidence that Tim-3 can be stimulatory in some infection models and our previous observation that half of the CD8$^+$ T cells in the latently infected TG remain highly functional, we were interested in identifying whether Tim-3 expression truly implied functional compromise during HSV-1 latency (240, 241, 260, 261). Our study revealed that cells expressing Tim-3 or cells negative for both Tim-3 and PD-1 remained highly functional during latency, while cells expressing only PD-1 appeared functionally compromised. Together, these data suggest that PD-1 expression denotes functional compromise in the latently infected TG, while Tim-3 expression may be an effect of recent antigenic stimulation.

When it became apparent that Tim-3 was not mitigating functionality, we asked if viral factors were governing Tim-3 expression in the latently infected TG. Using genetically modified viruses, we were able to moderate the levels of antigenic stimulation during latency. We first showed that the percentage of cells expressing Tim-3 is reduced when CD8$^+$ T cell numbers are

increased relative to antigen expression. Next, by overexpressing antigen, we were able to show that Tim-3 expression increased on TG-resident $CD8^+$ T cells. Collectively, these data led to the conclusion that Tim-3 expression in the TG is governed, in part, by viral gene expression. In this model, Tim-3 does not appear to regulate T cell functionality; however, our data suggest that Tim-3 expression during latency may be a marker of recent antigenic stimulation. Therefore, our data support the notion that leaky viral gene expression occurs throughout latency and that expression of immune activation markers like Tim-3 on $CD8^+$ T cells may be useful for determining which viral genes are expressed in the TG during latency, as the virus exits latency, and during viral reactivation. It will be intriguing in future studies to use microscopy to assess the expression levels of Tim-3 and PD-1 on either HSV-1-specific $CD8^+$ T cells that have formed immune synapses with neurons, indicating active stimulation, or cells that are not associated with neurons and are patrolling the TG.

While our initial investigations into Tim-3 were focused on latent time points, we became interested in how Tim-3 may contribute to the T cell response during acute infection. Transcriptional analysis, as well as functional studies directly *ex vivo*, showed that, like in latent infection, Tim-3 expression at early times after activation is associated with functionality. This was not unexpected, as many checkpoint molecules are transiently upregulated after T cell activation and may serve to hone the functionality of these responding cells (300). During acute infection, Tim-$3^+$ HSV-1 specific $CD8^+$ T cells were highly functional, with about half of these cells concurrently expressing PD-1. In the Subdom-$CD8^+$ T cell group, there was an unexpected, but distinct, population of PD-$1^+$ cells that did not co-express Tim-3 and had diminished functionality compared to Tim-$3^+$PD-$1^+$ or Tim-$3^+$PD-$1^-$ cells. The progression to T cell exhaustion/impairment is usually described as a stepwise reduction in functionality with

concurrent increased expression of inhibitory checkpoint molecules. However, our results suggest that the T cell impairment observed selectively in Subdom-$CD8^+$ T cells at late times post-infection may be initiated during very early infection and persists into latency. This implies that T cell impairment can result not only from repeated stimulations but also from early defects in development, potentially in priming, that are somehow able to persist despite mechanisms to cull cells with suboptimal performance during activation and expansion. Previous results from our laboratory have shown that regulatory cytokines like IL-10 may play a role in the disparate functionality between populations of $CD8^+$ T cells and it will be of interest to determine how cytokine signaling, in addition to TCR signaling, may affect the expression of checkpoint molecules and subsequent losses of functionality (169).

Checkpoint molecules are generally ascribed inhibitory or stimulatory roles, and for molecules such as PD-1 that have clearly defined negative effects on T cell signaling, their classification as inhibitory is fitting. However, in the case of Tim-3, where downstream signaling and even its ligands have yet to be fully elucidated, classification remains complicated and claims about inhibition/activation should be supported by T cell functionality data. Although there has been clear evidence showing that Tim-3 can indeed function in an inhibitory manner in many viral infections, functional assessment of Tim-$3^+$ $CD8^+$ T cells from the latently infected TG, until now, has never been performed. Our data support an alternative scenario in which Tim-3 is not a dominant inhibitor or an enhancer of $CD8^+$ T cell function but instead marks cells that are responding to antigenic stimulation and are highly functional.

# 4.0 Innate Determinants of Herpes Stromal Keratitis Development in Herpes Simplex Virus 1 Infected Corneas

Kate L. Carroll, Hongmin Yun, Robert L. Hendricks, and Anthony J. St. Leger

## 4.1 Summary

Corneal HSV-1 infection can result in the development of herpes stromal keratitis (HSK), a potentially blinding, immunopathological disease. HSK is characterized by the progressive development of opacity, neovascularization, and, in some cases, hypoesthesia in the cornea after viral reactivation events and can contribute to loss of visual acuity. However, infection with HSV-1 does not lead to the development of HSK in all infected individuals. To investigate why this disparity exists, we used two stains of HSV-1 that either cause a severe HSK (RE) or is nonpathogenic (KOS) in the B6 mouse. By using a bilateral infection model, we determined that the observed difference in pathology could not be explained by differences in the adaptive immune response nor was it a result of excessive viral replication, as KOS-infected corneas had significantly higher titers than RE-infected corneas. Further investigation found that there were significant differences in the magnitude of the innate immune infiltrate between the viruses, with larger numbers of monocytes and neutrophils infiltrating RE-infected corneas. When functionality of the innate immune infiltrate was assessed, a higher percentage of cells in RE-infected corneas expressed the enzyme inducible nitric oxide synthase (iNOS), which contributes to nitric oxide production. Inhibition of iNOS or depletion of monocytes and macrophages delayed the loss of

corneal blink reflex in RE-infected corneas; however, it did not ultimately prevent the development of HSK. These data suggest that HSK development is triggered by an excessive innate immune response early in infection and that the development of treatments that modulate this early response would be useful in preventing HSK development.

## 4.2 Importance

HSK is the leading infectious cause of blindness worldwide. Although not all who contract a corneal HSV-1 infection will develop HSK, those that do face a debilitating loss of vision over time due to accumulation of scar tissue in the cornea. While treatments for HSK do exist, they are often not completely effective in preventing the development of severe disease. Once enough scar tissue has developed as to impair vision, the only clinical recourse that remains is a corneal transplant, which comes with an elevated risk of rejection and is not widely accessible. By studying the differences in the immune response when HSK does or does not develop, we can gain a better understanding of how it is triggered. This will allow us to identify points of intervention that may lead to the development of more effective ways to prevent HSK in those that are susceptible.

## 4.3 Introduction

Visual acuity plays a crucial role in our ability to navigate in and interact with our environment. Although impaired vision or a lack of vision altogether certainly does not prevent one from leading a happy and successful life, it can institute limitations on living alone, restrict

career choices, and make daily tasks more challenging. Our ability to see is the result of complex interactions between our eyes and brain. The eye itself serves as a detector, collecting and focusing light that can then be transformed into electrical signals. These signals are then relayed to and interpreted by the brain to provide us with an image of our environment.

The first structure of the eye that light encounters is the cornea. The cornea is a highly structured, multilayered tissue that provides around two-thirds of the eye's focusing power. This ability to focus and direct light is critically dependent on the shape and transparency of the cornea (309). Although distortions in the shape of the cornea can be corrected by use of special lenses, the development of corneal opacity is much more difficult to manage (310). Once opacity in the cornea has progressed to the point of severely impairing vision, treatment options are mostly limited to corneal transplants, which can face a high risk of graft failure in conditions that have an immune component (311-313). As such, infections that interfere with the clarity of the cornea can have a serious impact on vision.

HSV-1 is well-known for causing oral and genital lesions; however, HSV-1 can also infect other mucosal surfaces such as the cornea. Initial HSV-1 infection of the cornea occurs in corneal epithelial cells and does not usually result in pathology, though some patients do develop a mild conjunctivitis and dendrites that may impair vision (314). Concurrent with viral replication in the cornea, HSV-1 infects the sensory nerves of the TG via axon tips found within the corneal epithelium. Active viral replication in both the cornea and the TG is generally controlled within a few days by a predominantly innate immune response; however, the virus can persist within TG neurons in a latent state indefinitely (133, 135, 285, 286). When HSV-1 latency is interrupted by a stress event, the lytic viral lifecycle is restarted, and infectious viral particles can be transported

back to the cornea. Once in the cornea, the infectious virus can undergo multiple rounds of viral replication, which subsequently induces another response from the host's immune system.

Although an immune response is required for the effective clearance of HSV-1 from the cornea, excessive inflammation can result in tissue damage with eventual scarring. In corneal HSV-1 infection, this immunopathology is called HSK and results in the development of opacity, ingrowth of blood vessels into the central cornea, and in some cases, the development of hypoesthesia (288). HSK progression is generally gradual in most individuals, with each subsequent reactivation event contributing to the severity of the HSK (Figure 15). HSK severity can also be greatly exacerbated by a loss of corneal sensation that results in a reduction or complete loss of blinking of the infected eye (155, 315, 316). This loss prevents tear film redistribution over the surface of the cornea, causing desiccation of corneal tissues and resulting in inflammation referred to as exposure keratitis (317).

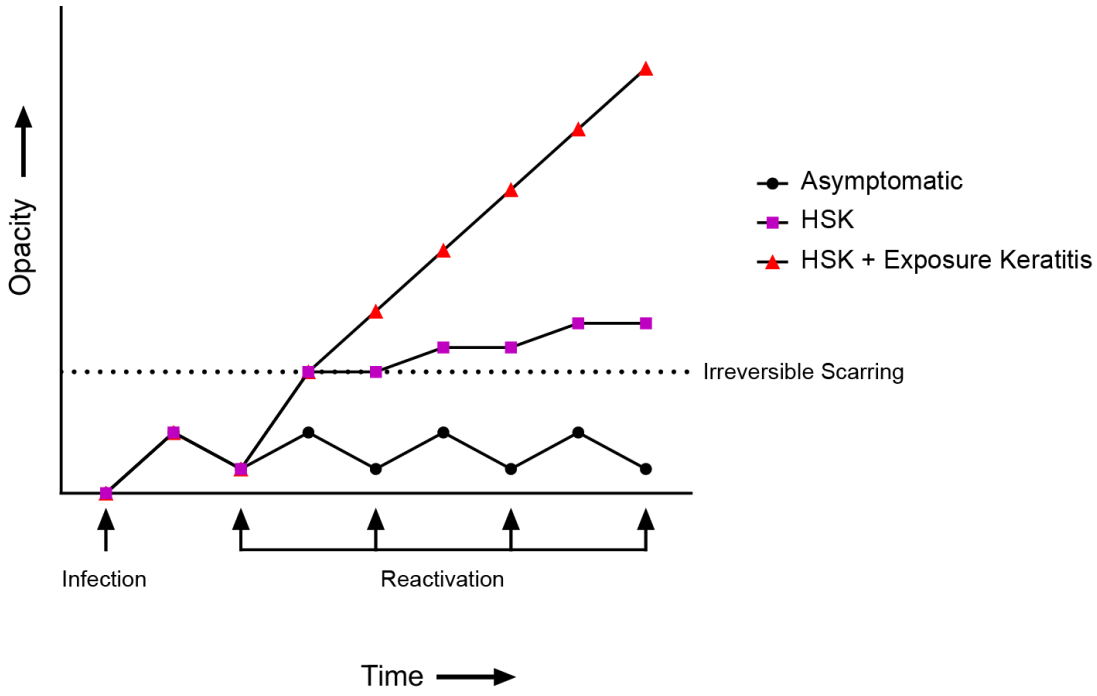



**Figure 15. Model of HSK disease progression.**
Initial HSV-1 infection generally does not cause pathology however, when the virus reactivates, susceptible individuals can develop inflammation that results in irreversible scarring (dotted line) and HSK (purple squares) while non-susceptible individuals remain asymptomatic (black circles). When corneal blink reflex is lost in individuals with HSK and exposure keratitis develops, HSK severity is exacerbated (red triangles).

HSK development in the cornea is often attributed to $CD4^+$ T cells and studies using CD4 knockout mice or depletion of $CD4^+$ T cells from the cornea have shown that a milder HSK develops in these mice after HSV-1 infection (146, 149, 151, 155). Despite this evidence, it is unclear how exactly $CD4^+$ T cells mediate HSK, especially since it is unknown if these cells are virus specific. Recent work from our laboratory has established that corneal $CD4^+$ T cells have a role in maintaining HSK through a mechanism that appears to prevent sensory nerves from reinnervating corneas that have lost sensation, further perpetuating exposure keratitis (155). Although $CD4^+$ T cells exacerbate HSK, $CD4^+$ T cell depletion in the cornea did not completely prevent the development of opacity or the initial loss of corneal sensory nerves (155). These data suggest that initial HSK development and the loss of corneal nerves is mediated by soluble factors and/or cell types other than $CD4^+$ T cells and likely occurs shortly after viral replication is initiated.

As one of the most prevalent human pathogens, HSV-1 infects a large proportion of the adult population. However, only a fraction of these individuals goes on to develop HSK, with about 50,000 new and recurring cases within the United States every year. Although this discrepancy could be explained by failure of the virus to establish latency specifically in the TG of some individuals, studies that have monitored viral shedding in the tear film have found evidence of virus in both symptomatic and asymptomatic individuals (99, 318). This raises the question of why only some people with active viral replication in the cornea go on to develop HSK. We have investigated this question by using two genetically similar strains of HSV-1 in the B6 mouse model (Supplemental Table 1). The strain KOS causes little-to-no pathology in these mice, while the strain RE causes severe HSK (319). We have assessed differences in viral titer and immune responses between corneas infected with the two viruses and determined that HSK susceptibility results from early events in the innate immune response in the local environment of the cornea. Our data demonstrate that the RE strain induces a more robust innate immune infiltrate composed of neutrophils and monocytes and that these cells have increased expression of the enzyme iNOS, which appears to play a role in regulating the loss of corneal sensation observed in severe HSK. These results suggest that early intervention in the immune response during HSV-1 reactivation events is necessary to limit the damaging immunopathology that occurs during HSK development.

## 4.4 Materials and Methods

### 4.4.1 Mice

Female wild-type C57BL/6 mice were obtained from Jackson Laboratories at six weeks of age. Mice were bilaterally infected at seven to eight weeks of age with $1x10^5$ pfu of HSV-1 RE in one eye and $1x10^5$ pfu of HSV-1 KOS in the contralateral eye after being anesthetized with 2.5 mg of ketamine and 0.25 mg of xylazine i.p. Viral inoculant was added to corneas scarified with a 30 G needle in a crosshatch pattern and 6.25 µg of antisedan was administered i.p. after infection. All animal work was performed in accordance with guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee and under an approved protocol.

### 4.4.2 Viruses

HSV-1 strains RE and KOS were prepared as previously described (Section 3.4.2).

### 4.4.3 Reagents

Vero cells (ATCC) were maintained in DMEM containing 10% FBS, 1% Penicillin and Streptomycin, and 1% Gluta-MAX. RPMI (Lonza Cat# 12167Q), DMEM (Lonza Cat# 12614Q), Penicillin-Streptomycin (Gibco Cat# 15140122), GlutaMAX (Gibco Cat# 35050061), HEPES (Gibco Cat# 15630080), Sodium Pyruvate (Gibco Cat# 11360070), Non-essential Amino Acids (Gibco Cat# 11140050), HBSS (Gibco Cat# 24020117), Formalin (Cat# SF100-4), Gentian Violet (Ricca Chemical Cat# 323016), and flat-bottomed, tissue culture treated 48 well plates (Corning

Cat# 353078) were purchased from FisherScientific. Ketamine, xylazine, and antisedan were purchased from Henry Schein. Collagenase Type I (Cat# C0130), Methylcellulose (Cat#M0387), and Aminoguanidine (Cat#A7009) were purchased from MilliporeSigma. Cellulose Eye Spears (Cat#0008685) were purchased from Beaver-Visitec International. Fc Block (Clone 2.4G2), anti-Ly6C-FITC (Clone AL-21), anti-Gr-1-PE (Clone RB6-8C5), and anti-CD45-PerCP (Clone 30-F11) were purchased from BD Biosciences. Anti-F4/80-PE-Cy7 (Clone BM8) was purchased from Biolegend. Aqua Live Dead (Cat# L34957), anti-CD11b-eF450 (Clone M1/70), and anti-iNOS-APC (Clone CXNFT) were purchased from Invitrogen. The BD Biosciences Cytofix/Cytoperm kit (Cat# 554714) was used for all intracellular staining. The Applied Biosystems High-Capacity cDNA Reverse Transcription kit (Cat# 4368814), TaqMan™ Universal PCR Master Mix (Cat# 4364338), PCX (Cat# Mm00500992_m1), IL-6 (Cat# Mm00446190_m1), and CXCL1 (Cat# Mm04207460_m1) primers were purchased from Thermo Fisher. The RNeasy Mini Kit (74106) was purchased from Qiagen. Clodronate-liposomes and PBS-liposomes (Cat# CP-015-015) were purchased from Liposoma BV.

### 4.4.4 Clinical Scoring

Mice were scored for HSK development at the timepoints indicated in the figure legends using a previously described scoring method (155). Briefly, corneal opacity was scored on a scale of 0 to 4 in increments of 0.5, with a score of 0 being a completely clear cornea and a score of 4 being a completely opaque and perforated cornea. Corneas were scored for opacity in four quadrants with the scores represented in the graph being an average of the scores in the four quadrants. Corneal reflex was scored by dividing the cornea into five sections and assessing the mice for their ability to blink when each of the sections was lightly touched with a pair of forceps,

with no blink being negative and a blink being positive. The graphs for corneal reflex show reflex on a scale of 0 to 5, with scores representing the total number of sections that had a positive corneal reflex.

### 4.4.5 Corneal Swabs and Plaque Assays

Corneal swabs of HSV-1-infected mice were taken by hydrating eye spears in 500 µl of HBSS and brushing them over the exposed cornea five times in each of the cardinal directions, for a total of 20 times. Swabs were then frozen at -80º C in 1.5 ml tubes until plaque assay. For plaque assays, $2x10^4$ Vero cells/well were plated in triplicate for each swab dilution on flat bottomed, 48 well plates one day prior to use and incubated at 37ºC with 5% $CO_2$ overnight. Swabs were thawed and diluted in HBSS and 100 µl of each dilution was added to Vero cell monolayers in triplicate and incubated at 37ºC with 5% $CO_2$ for 1 h. After incubation, each well was overlaid with 400 µl of plaque assay media (DMEM containing 2% FBS, 1% penicillin and streptomycin, 1% Gluta-MAX, 1% sodium pyruvate, 1% HEPES, 1% nonessential amino acids, and 0.5% methylcellulose) and incubated for 2 days at 37ºC with 5% $CO_2$. After incubation, overlay was removed, and plaque assay was developed by adding 100 µl of formalin to each well for 20 min at room temperature followed by the addition of 100 µl of gentian violet for an additional 20 min at room temperature. Plates were then washed, counted, and viral titers were determined.

### 4.4.6 *In Vivo* Treatments

For monocyte/macrophage depletions, RE-infected mice received subconjunctival injections of 100 µl of clodronate-liposomes or PBS-liposomes at one, three, and five dpi. For

iNOS inhibition, mice received a total of 400 mg/kg aminoguanidine i.p. daily (two 100 mg/kg doses and one 200 mg/kg dose), starting one day prior to infection. Mice also received topical aminoguanidine (0.05 mg/eye) 3x/day starting one day prior to infection and on days 1-10 post-infection with HSV-1 RE.

### 4.4.7 RT-qPCR

Corneas were dissected from eyes and added to 750 µl of RLT buffer containing 2-mercaptoethanol and a 3 mm stainless steel bead in a 2 ml tube. Samples were disrupted and homogenized in a mixer mill (Retsch MM300) using five, 2 min shakes at 20 Hz, with rests between shakes to prevent sample overheating. Lysates were then centrifuged in a microcentrifuge at full speed for 3 mins before transfer of supernatant to new tubes. Lysates were frozen at -80ºC until RNA extraction. RNA extraction was performed using a Qiagen RNeasy Mini kit according to manufacturer protocol. RNA concentration was assessed with a Nanodrop and cDNA was synthesized using an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit according to the manufacturers protocol, with the RNA from each sample diluted so that each reaction received the same amount of input RNA. After cDNA synthesis, duplicate qPCR reactions for each sample were assembled using 10 µl of master mix (10 µl of TaqMan Universal PCR Master Mix and 1 µl of TaqMan primer probes for IL-6, CXCL1, or PCX per sample) and 8 µl of diluted cDNA per reaction. Primer probes used span intron-exon boundaries and do not amplify genomic DNA as validated with -RT controls. qPCR samples were run on an Applied Biosystems StepOnePlus instrument using the cycle parameters set forth in the TaqMan Universal PCR Master Mix protocol.

### 4.4.8 Flow Staining

Excised corneas were digested in 100 µl of collagenase diluted in flow media (RPMI with 5% FBS, 1% Penicillin and Streptomycin, and 1% Gluta-MAX) to a concentration of 420 U/ml for 45 min at 37ºC. Corneas were then triturated into single cell suspensions and filtered through 35 µm filter topped tubes before being moved to 96 well plates for flow staining. Samples were blocked with Fc block in PBS for 10-15 min at 4ºC before surface markers and viability dye diluted in PBS was added to samples and incubated for an additional 30 min at 4ºC. After surface staining, samples were washed with FACS buffer (PBS with 1% FBS and 0.1% sodium azide) and fixed with Cytofix/Cytoperm for 20 min at 4ºC. For intracellular staining, samples were washed with perm wash and intracellular antibodies were diluted in perm wash and incubated with cells for 30 min at 4ºC. After intracellular staining, samples were washed in perm wash and resuspended in FACS buffer.

### 4.4.9 Data Analysis

Flow samples were run on a BD FACSAria flow cytometer. Flow analysis was performed in FlowJo (BD) and gates were set on fluorescence minus one controls. RT-qPCR data was analyzed with Step One Software (Applied Biosystems) and the $2^{-\Delta\Delta CT}$ method was used to determine fold changes over mock infection with PCX serving as the housekeeping control. Graphs were designed and statistical analysis was performed in Prism (GraphPad). Sample size and statistical tests used are indicated in figure legends.

## 4.5 Results

### 4.5.1 HSK development in the B6 mouse is HSV-1 strain specific and contingent on the local corneal environment

Although humans are the primary host of HSV-1, mice can also be readily infected and undergo a seemingly similar course of infection, with a short acute infection followed by viral latency in neurons. HSK development in mice is also clinically similar to what is observed in human patients, with the exception of how quickly severe HSK develops in mice (288). In humans, the severity of HSK generally increases gradually over the course of multiple reactivation events. In mice, HSK develops shortly after the acute infection is cleared. Despite this, HSK in both mice and humans is defined by edema, an ingrowth of blood vessels, and the development of opacity. We have previously shown that while HSK can develop in B6 mice after corneal HSV-1 infections, it is viral strain specific (319). For instance, infection with the HSV-1 strain KOS generally does not result in the development of HSK, although some KOS-infected corneas can experience a mild and transient opacity. In contrast, infection with the RE strain of HSV-1 causes the development of severe HSK. Taking advantage of the striking differences in pathology caused by the two viruses, we used KOS and RE infection in the B6 mouse model to identify differences in the immune response after HSV-1 infection.

HSK is usually described in the literature as a $CD4^+$ T cell-mediated disease, with CD4 knockout mice developing a mild and transient HSK, although recent work has suggested that these cells have more of a maintenance role than an initiating one (146, 155). Differences in an adaptive immune response could potentially explain the divergent pathology observed between KOS and RE infections. Although previous experiments using KOS and RE had compared the

viruses in mice infected with only one strain of virus (319), we addressed this question by bilaterally infecting (RE in one eye and KOS in the other) mice with RE and KOS. This design allowed us to directly compare the disease caused by both viruses in the same animal while also ascertaining if the adaptive immune response was causing the differences in pathology, as the combined adaptive immune response primed by both viruses would be available to both corneas. Surprisingly, the HSK phenotypes of both KOS and RE were completely maintained in bilaterally infected animals. KOS-infected corneas remained optically clear for weeks after infection while RE-infected corneas developed severe HSK (Figure 16A-C). These results suggested that the disparity in HSK development is likely due to differences in the local corneal environment rather than differences in the circulating adaptive responses.

A component of HSK that is sometimes overlooked is the loss of corneal sensation that can occur. This symptom has long been observed in the clinic in HSK patients and contributes to the severity of HSK as it leads to a reduction in the frequency of blinking (155, 320). Blinking (corneal blink reflex) is an important function of the eyelids that contributes to tear film redistribution and prevention of corneal desiccation. Corneal desiccation itself can cause inflammation, which can worsen pathology associated with the underlying inflammation from viral reactivation (155, 317). When we assessed corneal blink reflex in bilaterally infected mice over time, we found that similar to previously published work (319), KOS-infected corneas underwent a partial loss of corneal blink reflex while RE-infected corneas experienced a rapid and complete loss of corneal sensation by five dpi (Figure 16C). Work from our laboratory and others has shown that this loss of corneal sensation during HSV-1 infection correlates with a retraction of sensory nerves from the cornea (155, 315, 316). When unilaterally RE- or KOS-infected corneas were assessed for the presence of nerves using confocal imaging of whole mount corneas, we found that KOS-infected corneas

had partial retraction of nerve sensory endings from the corneal epithelial layers and nerve plexus from the corneal stroma, while RE-infected corneas had a complete loss of both nerve sensory endings and plexus (Supplemental Figure 1). Full loss of corneal blink reflex in RE and partial loss of corneal blink reflex in KOS is observed in both unilateral and bilateral infections with KOS and RE (compare Figure 16C with Figure 1F from (319)). Thus, it is likely that the observed loss of corneal blink reflex in bilaterally infected mice (Figure 16C) coincides with full (RE) or partial (KOS) corneal nerve retraction.

The dramatic loss of corneal blink reflex in RE-infected corneas at such an early time post-infection further indicates that the difference between the two viruses is not likely mediated by the adaptive immune response, as it takes nearly a week for those cell types to infiltrate the infected corneal tissues. The maintenance of HSK phenotypes in bilaterally infected mice also supports the use of this model in dissecting the local immune differences in the cornea. Collectively, these data suggest that the differences in pathology induced by KOS and RE infections are probably not due to differences in the adaptive immune response but rather are mediated by a local, innate immune response.

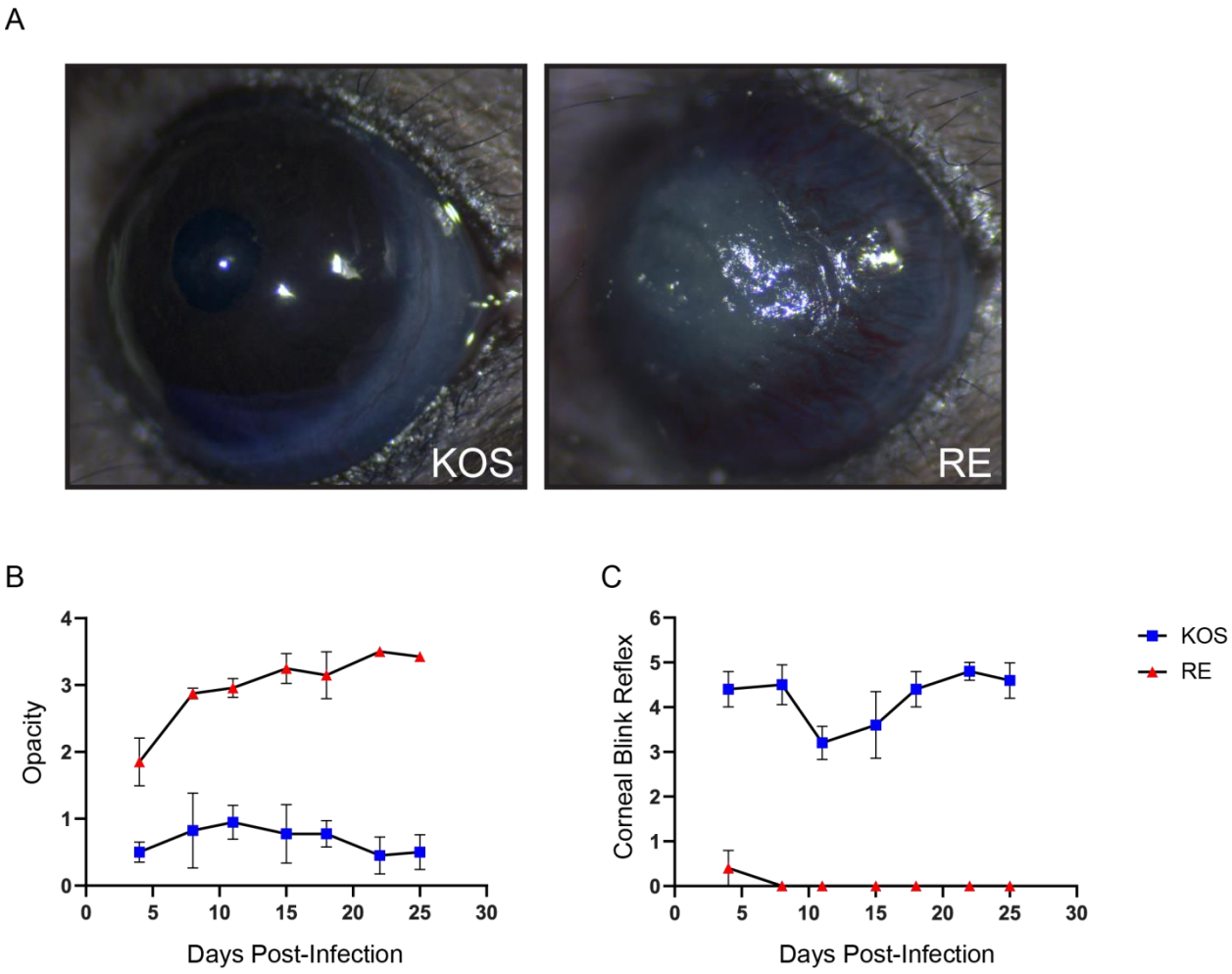



**Figure 16. HSK development in the B6 mouse is HSV-1 strain specific and contingent on the local corneal environment.**
Mice were bilaterally infected with KOS and RE and clinical disease was monitored. In graphs, points represent mean ± SEM with KOS infection represented by blue squares and RE infection represented by red triangles. (A) Images of eyes infected with KOS (left image) or RE (right image) at 21 dpi. (B) Graph of corneal opacity scores in RE- and KOS-infected corneas from 4-25 dpi. (C) Graph of corneal blink reflex scores in RE- and KOS-infected corneas from 4-25 dpi. n=5 corneas/group.

### 4.5.2 HSK severity is independent of corneal viral titers

Another possible cause of the increased susceptibility to HSK in RE infection could be differences in the ability of the two strains to replicate within the cornea. Excessive viral replication could not only potentially induce a more severe immune response but could also directly result in tissue damage. Using our bilateral infection model, we quantified viral titers in KOS- or RE-

infected corneas by taking corneal swabs each day after infection for a week. Unexpectedly, when the amount of virus was quantified at early times post-infection, we found that KOS-infected corneas had significantly higher amounts of infectious virus than RE-infected corneas (Figure 17). This suggests that HSK is not due to tissue damage from viral replication and further implicates the innate immune response in the development of HSK, as the differences in viral titer were evident as early as one day post-infection.

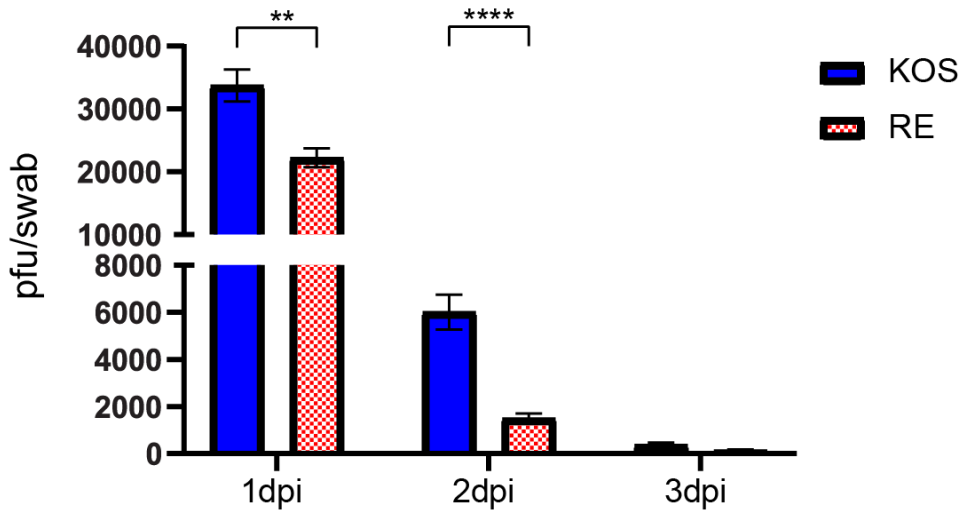



**Figure 17. HSK severity is independent of corneal viral titers.**
Mice were bilaterally infected with HSV-1 strains RE and KOS and corneal swabs were taken starting at 1 dpi. Swabs were diluted and assayed for infectious virus with standard plaque assays and pfu/swab were calculated. Data shown are from two pooled experiments, with a total n of 20. Bars represent mean ± SEM with KOS represented in blue and RE represented in red and white pattern. Differences were assessed by a RM two-way ANOVA with Sidak's posttests to determine significance between viruses at each time point. **P ≤ 0.01, **** P ≤ 0.0001.

### 4.5.3 RE infection induces a more proinflammatory environment at early times post-infection

Together, the maintenance of the HSK phenotype in bilaterally infected mice and the significantly lower viral titers in RE-infected eyes at early times post-infection suggested that an innate immune response could be mediating the differences between the two viruses. Therefore,

we next assessed the chemokine and cytokine environment within the cornea shortly after infection. Previous work using a variety of mouse and HSV-1 strains has shown that numerous inflammatory chemokines and cytokines are upregulated following HSV-1 infection of the cornea. These molecules include: CXCL1, CCL5, CXCL10, IL-6, MIP-1α and MIP-2, which can induce the recruitment of immune cells such as monocytes, neutrophils, and T cells (321-329). Of particular interest are IL-6 and CXCL1 (a homologue of human IL-8), as both IL-6 and IL-8 are upregulated in human corneal cells after HSV-1 infection (330, 331). IL-8 can contribute to neutrophil infiltration by serving as a chemoattractant while IL-6 can cause many downstream effects, including corneal upregulation of VEGF, which promotes vessel ingrowth and an increase in HSK severity (321, 332-334).

When we quantified transcripts of IL-6 and CXCL1 at early times post-infection in bilaterally infected mice relative to mock infection (scratching of the cornea without HSV-1 infection), we found that expression of IL-6 and CXCL1 were both significantly higher in RE-infected corneas than KOS-infected corneas at two and three dpi (Figure 18A-B). This difference was also apparent at one dpi but was significant only for CXCL1. The observed disparity between KOS and RE was most pronounced at two dpi, suggesting that the events leading to the development of HSK are determined very early in infection. This discrepancy in the production of proinflammatory mediators is potentially due to differences in how the two strains of virus trigger innate sensors and indicates that early intervention after HSV-1 infection is likely to be important in preventing HSK development. Of note, the fold change shown in Figure 18 is relative to mock-infected corneas. Although RE-infected corneas had more IL-6 and CXCL1 transcripts than KOS-infected corneas, KOS infection clearly induces the production of proinflammatory mediators, thus a lack of pathology in KOS-infected corneas is not due to a complete lack of immune response.

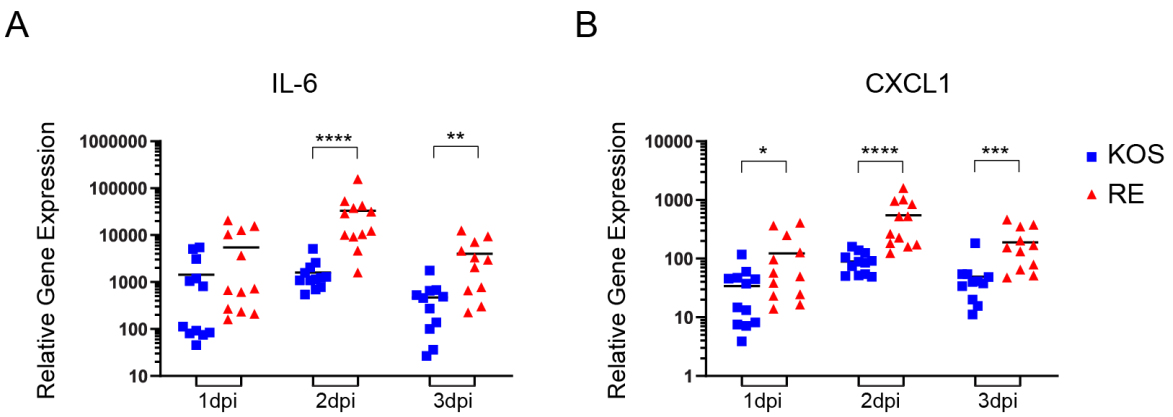
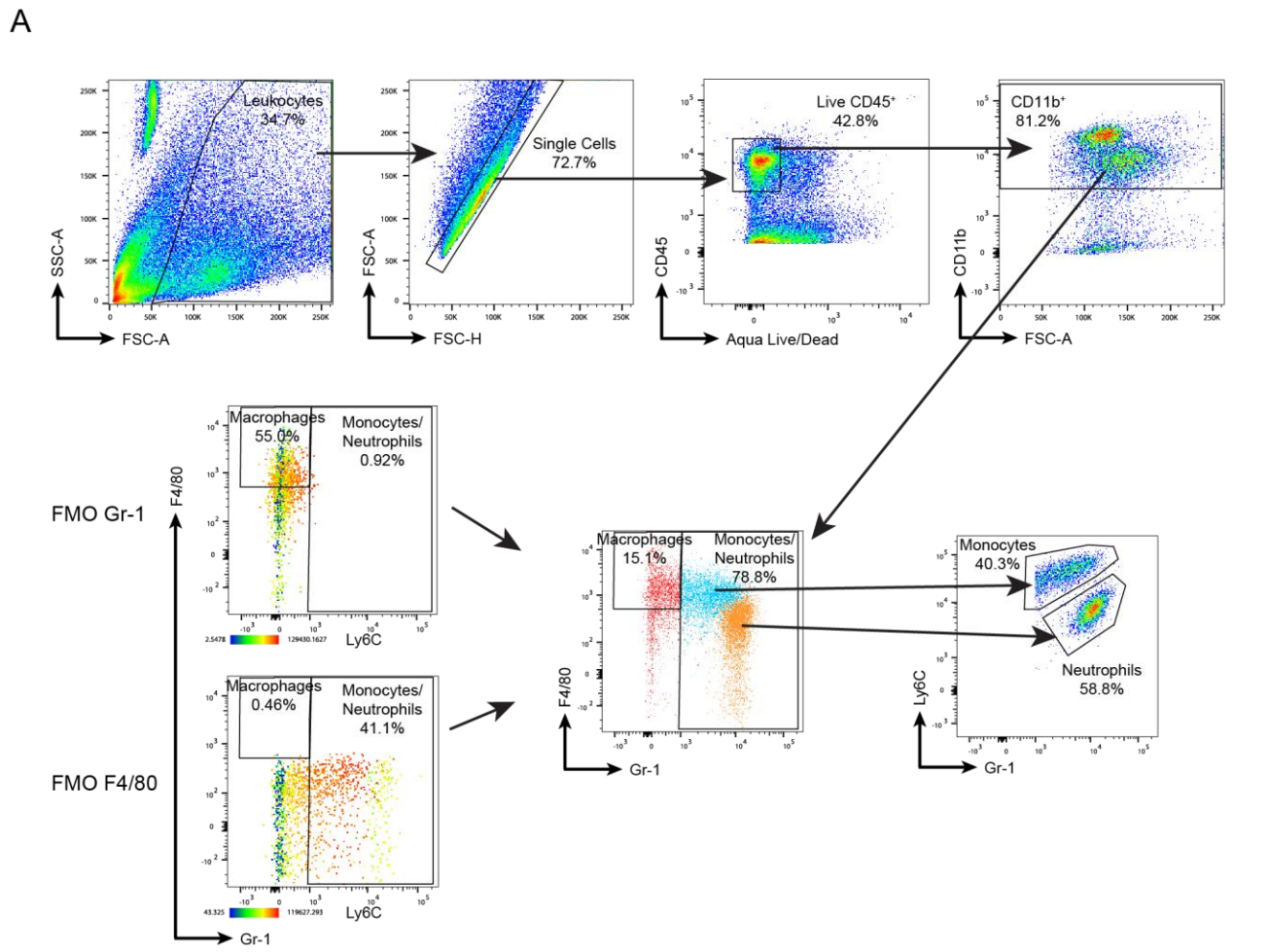



**Figure 18. RE infection induces a more proinflammatory environment at early times post-infection.** Mice were bilaterally infected with HSV-1 strains KOS and RE or mock infected and corneas were excised and RNA was extracted for qRT-PCR at 1, 2, and 3 dpi. CT values were analyzed with the $2^{-\Delta\Delta CT}$ method, normalizing to the reference gene PCX and to mock-infected corneas to determine fold changes. Fold change over mock infection is displayed in graphs with bar at the mean. (A) Fold change in IL-6 expression. (B) Fold change in CXCL1 expression. Data shown are pooled from two independent experiments, with a total n of 12 for each KOS and RE at 1 and 2 dpi, and a total n of 11 for each KOS and RE at 3 dpi. Differences were assessed on ΔΔCT values with unpaired t tests to determine significance. *P ≤ 0.05, **P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

### 4.5.4 RE infection induces a more robust innate immune infiltrate into the cornea

Since the RE strain of HSV-1 induced a more proinflammatory environment in the cornea than did the KOS strain, it seemed likely that the innate immune infiltrate would also be altered between the two viral strains. Much of the early infiltrate into the cornea after HSV-1 infection is comprised of neutrophils and inflammatory monocytes (335). With the differences in cytokines and chemokines between the two strains peaking at two dpi, we decided to compare the immune infiltrate at one, three and six dpi in RE and KOS bilaterally infected corneas and mock-infected corneas. Most of the $CD45^+$ population in the cornea at these early times post-infection was $CD11b^+$ and was separated into three populations: macrophages ($F4/80^+Gr\text{-}1^{lo\text{-}neg}$), monocytes ($F4/80^+Gr\text{-}1^{int}Ly6C^{hi}$), and neutrophils ($F4/80^-Gr\text{-}1^{hi}Ly6C^{int}$) (Figure 19A).

Although mock infection induces an innate immune infiltrate, it is transient and minor when compared to the infiltrate that occurs after HSV-1 infection, regardless of strain. Like the chemokine and cytokine environment (Figure 18), this demonstrates that while KOS infection does not cause pathology, it does induce an immune response, albeit one that does not damage the clarity of the cornea. When the numbers of $CD45^+$ cells and $CD11b^+$ cells per infected cornea were quantified, it was apparent that RE infection induced a significantly larger $CD45^+$ infiltrate into the cornea at all three timepoints and a larger $CD11b^+$ infiltrate at three and six dpi than KOS infection (Figure 19B-C). When the $CD11b^+$ infiltrate was further broken down into macrophages, monocytes, and neutrophils, we found significantly more monocytes at all times points assayed and more neutrophils at three and six dpi in RE-infected corneas compared to KOS-infected corneas (Figure 19E-F). However, the numbers of macrophages remained similar between both strains (Figure 19D). These results indicate that RE induces a more prominent innate immune infiltrate into infected corneas, consistent with our previous findings of an increased proinflammatory environment in RE-infected eyes (Figure 18).


A
Leukocytes
34.7%
SSC-A
FSC-A
Single Cells
72.7%
FSC-A
FSC-H
Live CD45+
42.8%
CD45
Aqua Live/Dead
CD11b+
81.2%
CD11b
FSC-A
FMO Gr-1
Macrophages
55.0%
Monocytes/
Neutrophils
0.92%
F4/80
Ly6C
FMO F4/80
Macrophages
0.46%
Monocytes/
Neutrophils
41.1%
Ly6C
Gr-1
Macrophages
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F4/80
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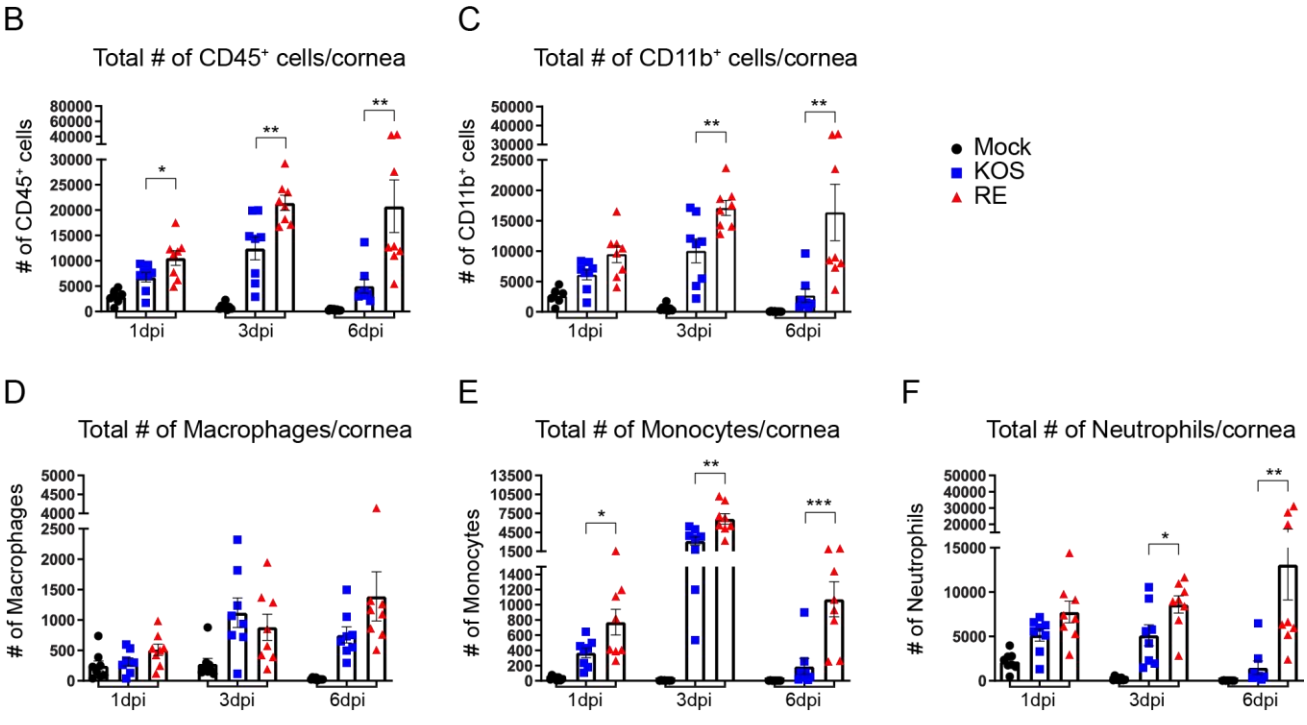
B
Total # of CD45+ cells/cornea
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6dpi
Mock
KOS
RE
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1dpi
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E
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Total # of Neutrophils/cornea
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1dpi
3dpi
6dpi

**Figure 19. RE infection induces a more robust innate immune infiltrate into the cornea.**
Mice were bilaterally infected with KOS and RE or mock infected and corneas were excised at 1, 3, and 6 dpi, processed into single cell suspensions, and stained for viability, CD45, CD11b, Gr-1, F4/80, Ly6C, and iNOS. In graphs, bars represent mean ± SEM with mock infection represented by black circles, KOS infection by blue squares, and RE infection by red triangles. (A) Gating strategy used is as follows: cells were gated by size, doublets were excluded, live $CD45^+$ cells were gated, and $CD11b^+$ cells were gated. $CD11b^+$ cells were gated into macrophage, monocyte, and neutrophil populations by first gating macrophages as $F4/80^+Gr\text{-}1^{lo\text{-}neg}$ cells and gating the monocytes and neutrophils together as $Gr\text{-}1^{int\text{-}high}$ cells. Monocytes and neutrophils were then resolved into separate populations using expression Ly6C with monocytes considered $F4/80^+Gr\text{-}1^{int}Ly6C^{hi}$) and neutrophils considered ($F4/80^-Gr\text{-}1^{hi}Ly6C^{int}$. FMO controls for F4/80 and Gr-1 (with Ly6C expression shown as a heatmap) are shown along with backgating for monocytes (blue) and neutrophils (orange) on the $CD11b^+$ cells plot. (B) Total numbers of $CD45^+$ cells/cornea. (C) Total numbers of $CD11b^+$ cells/cornea. (D) Total numbers of macrophages/cornea. (E) Total numbers of monocytes/cornea. (F) Total numbers of neutrophils/cornea. Data shown are pooled from two independent experiments, with a total n of 8 for each infection type. Differences were assessed with one-way ANOVAs for each time point with Tukey's posttests to determine significance. $*P \leq 0.05$, $**P \leq 0.01$, $*** P \leq 0.001$.

### 4.5.5 RE-infected corneas experience a more functional innate immune infiltrate at early times post-infection

Since RE infection induced a more proinflammatory environment in the cornea, we next wondered if the innate immune infiltrate observed during acute infection in RE-infected corneas also exhibited a higher level of functionality than in KOS-infected corneas. Previous studies of nerve damage in noninfectious models of neuroinflammation have shown that nitric oxide (NO) can impair nerve function and inhibit neuron growth (336, 337). NO production is regulated though the activity of three enzymes, eNOS, nNOS, and iNOS. eNOS and nNOS are expressed by endothelial and neuronal cells respectively, while iNOS is expressed by immune cell types including macrophages, monocytes, and neutrophils after activation. NO production in macrophages is influenced by IFN-γ signaling, which is abundantly produced after HSV-1 infection (338).

When we assayed iNOS expression in the macrophages, monocytes, and neutrophils infiltrating infected corneas, we found that a significantly higher percentage of all of these cell types expressed iNOS in RE-infected corneas than in KOS-infected corneas at three and six dpi

(Figure 20A-C). This difference was particularly interesting in macrophages, where the total number of macrophages did not change between infections (Figure 19D) but the percentage of cells producing iNOS was significantly different starting at three dpi (Figure 20A). These differences in the ability of infiltrating innate immune cells to produce iNOS supports the conclusion that RE infection induces a more proinflammatory environment in the cornea, which influences not only the magnitude of the infiltrating immune cells but also function of these cells in the cornea.

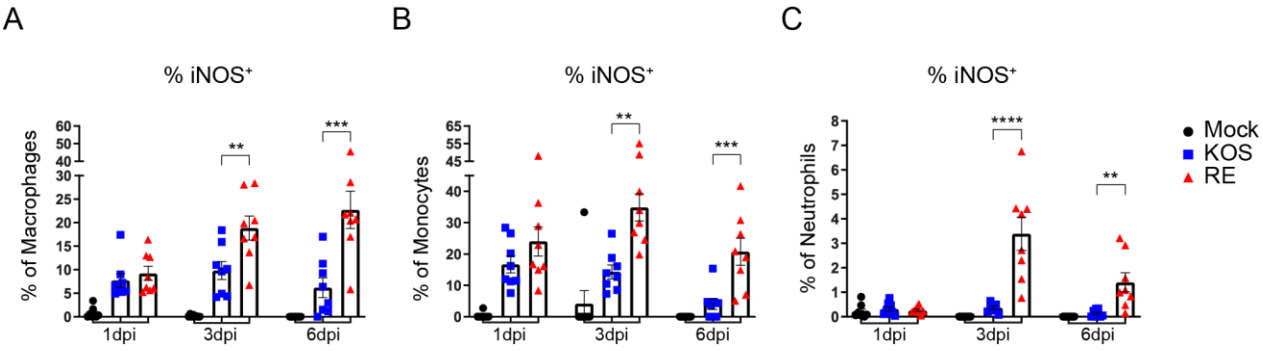



**Figure 20. RE-infected corneas experience a more functional innate immune infiltrate at early times post-infection.**
Mice were bilaterally infected with KOS and RE or mock infected and corneas were excised at 1, 3, and 6 dpi, processed into single cell suspensions, and stained for viability, CD45, CD11b, Gr-1, F4/80, Ly6C, and iNOS. In graphs, bars represent mean ± SEM with mock infection represented by black circles, KOS infection by blue squares, and RE infection by red triangles. Gating strategy used was described in the figure legend for figure 19. (A) % of macrophages positive for iNOS expression. (B) % of monocytes positive for iNOS expression. (C) % of neutrophils positive for iNOS expression. Data shown are pooled from two independent experiments, with a total n of 8 for each infection type. Differences were assessed with one-way ANOVAs for each time point with Tukey's posttests to determine significance. **P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

### 4.5.6 Interference with macrophage/monocyte infiltration or function delays loss of corneal sensitivity

Because we observed that RE infection induces not only a larger innate immune cell infiltrate into the cornea but also an increase in functionality within those cells, our next step was

to ascertain if macrophages and monocytes or production of NO contributes to the loss of corneal nerves and subsequent loss of corneal blink reflex that we observe during RE-induced HSK. Previous work from another laboratory has shown that macrophages can cause a loss of corneal sensitivity after HSV-1 infection. However, it is not clear if the depletion of macrophages in that group's model resulted in sustained retention of corneal sensitivity or merely delayed its loss (339). To determine the role of macrophages and monocytes in our model, we locally depleted these cell types by subconjunctival injection of chlodronate liposomes. Depletion of macrophages and monocytes resulted in a delay in the loss of corneal blink reflex in RE-infected corneas at three dpi; however, corneal blink reflex was still completely lost by seven dpi (Figure 21A). These results agreed with the previous study that macrophages play a role in the loss of corneal sensitivity (339) but suggest that additional cell types may contribute. Similarly, when we blocked the production of NO in infected corneas by administration of the iNOS inhibitor aminoguanidine, we observed a delay in the loss of corneal blink reflex at three and four dpi as compared to corneas from RE-infected mice treated with PBS (Figure 21B). Similar to corneas depleted of macrophages and monocytes, these corneas ultimately lost corneal blink reflex. Collectively, these preliminary data suggest that macrophages and monocytes, as well as NO production by these cells (and others), contribute to the loss of corneal sensitivity in HSV-1-infected corneas, although other cell types and mediators also likely contribute to this phenotype.

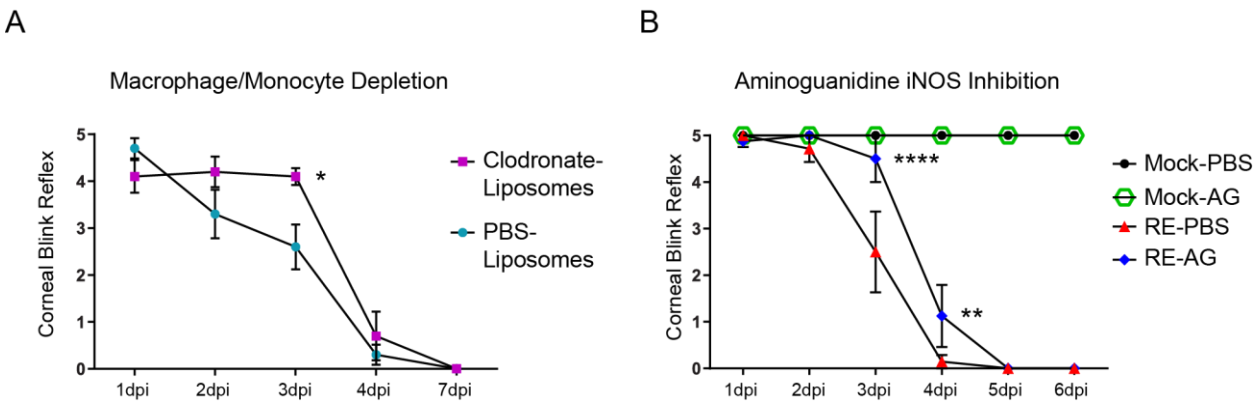



**Figure 21. Interference with macrophage/monocyte infiltration or function delays loss of corneal sensitivity.** RE-infected corneas were depleted of macrophages/monocytes or treated with the iNOS inhibitor aminoguanidine (AG) and scored for corneal blink reflex. (A) Effect of macrophage/monocyte depletion on corneal blink reflex. Mice were bilaterally infected with HSV-1 RE and given subconjunctival injections of 100 µl of clodronate liposomes or PBS-liposomes at 1, 3, and 5 dpi and scored for corneal blink reflex 1-4, and 7 dpi. Macrophage depleted corneas (n=10) are represented by purple boxes while PBS treated controls are represented by teal circles (n=10). There was a significant difference between clodronate-liposome and PBS-liposome treatments at 3 dpi. (B) Effect of aminoguanidine treatment on corneal blink reflex. Mice were bilaterally infected with HSV-1 RE or mock infected and treated with IP injections of 400 mg/kg aminoguanidine (starting 1 day prior to infection thru 6 dpi) and 0.05 mg/eye topical aminoguanidine 3x daily (starting 1 day prior to infection and then one days 1-10 post-infection) and scored for corneal blink reflex 1-6 dpi. Mock-infected corneas from PBS treated mice are represented by black circles (n=6), mock-infected corneas from AG treated mice are represented by green open circles (n=8), RE-infected corneas from PBS treated mice are represented by red triangles (n=8), and RE-infected corneas from AG treated mice are represented by blue diamonds (n=8). There were significant differences between RE-PBS and RE-AG at 3 and 4 dpi. In graphs, points represent mean ± SEM. Graphs in both A and B are each from one preliminary experiment. Differences were assessed by two-way ANOVAs with Sidak (A) and Tukey (B) posttests for significance. *P ≤ 0.05, **P ≤ 0.01, **** P ≤ 0.0001.

## 4.6 Discussion

A transparent cornea is a critical component of clear vision. The body expends a considerable amount of effort to maintain this clarity by tightly restricting the ingrowth of vessels into the cornea and by limiting the infiltration of immune cells. Despite this, infection of the cornea with HSV-1 triggers a rapid immune response that can quickly destroy this finely tuned regulation, resulting in irreversible tissue damage and impaired vision (288). Curiously, HSV-1 infection of the cornea does not always result in HSK and indeed, primary HSV-1 infection of the cornea generally goes unnoticed by the infected individual (314). This phenomenon is also observed

during viral reactivation, with both healthy and diseased corneas testing positive for infectious virus (99, 318). Although individual susceptibility to HSK is likely to be due to several factors, understanding how the immune response differs in corneas that develop HSK or do not develop HSK may give us insight into the key pathways that regulate HSK development.

To assess these differences, we have used a B6 mouse model and two strains of HSV-1 that cause either a severe HSK (RE) or is nonpathogenic (KOS). These two viral strains are genetically very similar, yet their impact on the B6 mouse cornea is vastly different (319). Although an easy explanation for the difference between the two strains would simply be that the KOS strain is less capable of replication in the cornea and therefore does not induce an immune response, we found that KOS-infected corneas have higher titers than RE-infected corneas. KOS infection also clearly induces an immune response as compared to mock-infected corneas. Thus, while mock infection causes a brief and minor increase in immune cell numbers in the scratched cornea, KOS infection induces a larger and more sustained infiltrate that can produce NO. Consequently, the relative absence of pathology in KOS-infected corneas cannot be explained by either a lack of immune response or detectable virus.

The higher viral titers in KOS-infected corneas as compared to RE-infected corneas also suggests that the pathology seen in diseased corneas is not due to direct tissue damage from viral replication. Instead, by using a bilateral model of infection, we were able to determine that a component of the early innate immune response within the cornea serves as the possible triggering event in initiating the differences in HSK development. When we assayed the early innate immune response in bilaterally infected mice, we found significantly higher levels of CXCL1 and IL-6, and larger numbers of inflammatory monocytes and neutrophils in RE-infected corneas than in KOS-infected corneas. The infiltrating innate immune cells also exhibited a higher level of functionality

in RE-infected corneas as measured by the expression of iNOS. Notably, these differences occurred very quickly, with a clear trend towards a more proinflammatory environment in RE-infected corneas evident at one dpi. This early effect was also noticeable in the loss of corneal blink reflex in RE-infected corneas by five dpi, suggesting that corneal nerves likely start retracting shortly after HSV-1 infection. As a loss of corneal blink reflex has been shown to worsen disease (155), treatments that can prevent this early nerve loss will likely be useful in modulating HSK progression.

Current treatments for HSK are largely limited to antivirals, used to control viral replication, administered alongside corticosteroids (11, 103, 104). One study that assessed the efficacy of corticosteroid treatment on recurrent HSK has shown that corticosteroid administration results in a decrease in the duration of inflammation, however, there was ultimately no difference in visual outcomes between the corticosteroid and placebo groups (103). Although corticosteroids are able to globally suppress the immune response and thereby limit the inflammation experienced during HSK, they can also cause severe side effects in the eye, such as the development of cataracts and glaucoma (340). Corticosteroids can also prevent control of viral replication, making the concurrent administration of antivirals a necessary part of HSK treatment. The potential for problematic side effects, combined with the inability of corticosteroids to prevent loss of visual acuity, demonstrates that nonspecifically suppressing inflammation is not enough to truly control HSK development and that more targeted interventions are needed.

Although we have ascertained that the early innate response is a determining factor in whether HSK will develop after HSV-1 infection of the cornea, there is still much to be determined as we look for new ways to control HSK. When we depleted monocytes and macrophages in RE-infected corneas, we observed a delay in the loss of corneal sensation, although the depleted mice

did go on to completely lose sensation and develop opacity. These data suggest that multiple cell types likely contribute to this early phenotype. This adds a layer of complexity in trying to mitigate HSK, as innate immune cell types also play important roles in controlling viral replication (Section 1.1.7.1), and past studies using combined depletions of monocytes, macrophages, and neutrophils in mice have resulted in a lack of viral control and increased mortality (341, 342). Additionally, nonimmune corneal cell types may also play a role in corneal damage via production of soluble proinflammatory molecules, suggesting that neutralization of these molecules or inhibition of signaling cascades may be a more feasible treatment strategy.

When we inhibited production of the proinflammatory mediator NO via the inhibition of iNOS, we observed a delay in the loss of corneal sensation that was slightly more sustained than the delay in monocyte/macrophage depletion. This suggests that NO mediates some amount of nerve damage during HSV-1 infection and that iNOS inhibition could be used in combination with other treatments to prevent corneal nerve damage. However, our efforts to identify additional points of manipulation such as TLR and IL-6 signaling (data not shown) have not resulted in any appreciable improvement in disease. Similarly, studies from other laboratories in which early immune responses were modulated have also had limited success in preventing HSK development (325, 343, 344). Collectively, these experiments suggest that it will take careful dissection of the pathways that are triggered after infection to identify what manipulations can be made to limit the development of HSK. To this end, we are currently performing RNAseq at early times post-infection in bilaterally infected mice, with hopes that a big picture view of changes in the early immune response will provide nuances that will direct our subsequent steps in pinpointing important steps in HSK development.

The development of HSK after a corneal HSV-1 infection can result in serious visual impairment. While treatments exist for HSK, they are few and limited in their ability to prevent the scar tissue formation that leads to blinding opacity. With recent work demonstrating how important maintaining corneal blink reflex is in preventing severe HSK development and our observations as to how quickly this occurs, we propose that focusing on modulating specific, early events in reactivation, rather than a global suppression of inflammation, will be important in the successful development of new treatments for preventing the progression of HSK.

## 5.0 Summary

HSV-1 is the definition of a successful pathogen. The ease with which it is transmitted has guaranteed its widespread infection across the world, and its ability to evade the immune response and persist within the host has all but assured its continued presence within the human population. Although HSV-1 infection does not generally result in mortality, the variety of disease states that HSV-1 can induce constitutes a considerable health burden. Thus, there has been significant interest in the development of vaccines that prevent initial HSV-1 infection as well as therapies that can be used to treat those who are already infected. Many HSV-1 vaccines have been developed in the past several decades, but all have fallen short of providing effective and lasting protection against HSV-1 infection. This has left interventions that prevent viral reactivation and limit the severity of HSV-1 induced pathologies as the only recourse to treat HSV-1 infections. The development of new and improved therapies relies on the continued effort to better understand the host's immune response to HSV-1. To this end, the studies described in this dissertation have used a corneal HSV-1 infection model that induces the immunopathological disease HSK to investigate both the immune responses in the cornea during HSK development and in the TG during viral latency.

We first focused on the immune response within the infected TG by studying the function of virus-specific $CD8^+$ T cells during acute and latent HSV-1 infection. We have previously shown that $CD8^+$ T cells within the TG play a crucial role in controlling viral latency (157, 161), however, this control is compromised by a loss of functionality in $CD8^+$ T cells specific to the subdominant epitopes (145, 169, 282). With how important virus specific-$CD8^+$ T cells are in maintaining the

virus in its latent state, increasing the function of this impaired subset of $CD8^+$ T cells may be a potential way to prevent reactivation.

Study of T cell impairment/exhaustion has revealed that persistently stimulated T cells gradually lose their capacity to function. This loss of function is correlated with an increase in checkpoint molecule expression, and the development and use of checkpoint molecule inhibitors has allowed for the successful reinvigoration of some subsets of exhausted cells. As such, checkpoint molecule blockade therapy may represent a potential solution to the loss of $CD8^+$ T cell function in the TG. A previous study from our laboratory established that the impaired Subdom-$CD8^+$ T cells preferentially express the inhibitory checkpoint molecule PD-1 (282). However, when PD-1 signaling was blocked, these cells did not regain functionality (282). Although this finding was disappointing in terms of the specific use of PD-1/PD-L1 blockade therapy to prevent HSV-1 reactivation, it did not rule out the possibility that an inhibitor to another checkpoint molecule might yet be of use.

With this in mind, we assessed the expression of six additional checkpoint molecules in the latently infected TG. While we determined that CTLA-4 and BTLA were preferentially expressed on the impaired Subdom-$CD8^+$ T cells, their expression even on the Subdom-$CD8^+$ T cell population was quite low, suggesting that blockade of these molecules may not achieve meaningful differences in overall Subdom-$CD8^+$ T cell functionality. Our screen also identified two checkpoint molecules that were preferentially expressed on the functional gB-$CD8^+$ T population to levels that exceeded what we had previously observed for PD-1 expression on the Subdom-$CD8^+$ T population (282).

One of these molecules, Tim-3, is generally described as an inhibitory checkpoint molecule, including in some studies of HSV-1 infection (281, 295), although, several recent reports

have suggested that its role is context dependent (240, 241, 261). Since the functional capacity of gB-CD8$^+$ T cells in the HSV-1-infected TG has been well established, we considered it unlikely that Tim-3 expression on these cells would be indicative of functional impairment and decided to further investigate this phenotype. We observed that Tim-3 expression does not overlap with PD-1 expression on T cells within the latently infected TG and that the cells expressing Tim-3 were some of the most capable of responding to cognate antigen. This indicates that Tim-3 expression is a marker of T cell functionality within the HSV-1 latently infected TG. Furthermore, using HSV-1 strains with mutations that manipulated the ratio of antigen to cognate CD8$^+$ T cells in the TG, we showed that the expression of Tim-3 on T cells was at least partially modulated by viral gene expression. These findings suggest that Tim-3 can be used as a T cell marker of recent antigen stimulation within the TG.

As Tim-3 expression in the latently infected TG was associated with T cell function, we also decided to assess Tim-3 and PD-1 expression during acute infection. Like in latent infection, Tim-3 was expressed on cells that were clearly functional. However, PD-1 expression was unusual, with a distinct PD-1$^+$ population in the Subdom-CD8$^+$ T cell group that was unable to produce GzmB. This suggests that the PD-1-associated impairment of Subdom-CD8$^+$ T cells observed during latency may arise as a result of a priming defect, rather than from sustained antigen stimulation leading to exhaustion. If it is the former, this could explain why the impaired CD8$^+$ T cells were refractory to PD-1 and IL-10R blockade therapy (169, 282).

While the function of Tim-3 in the latently infected TG has not yet been determined, our work shows that it clearly does not mark cells that are incapable of responding to stimulation. This highlights the importance of validating the functional role of checkpoint molecules in each unique system before attempting manipulation. Regardless of what role Tim-3 may play in HSV-1 latency,

our data suggest that T cell impairment during HSV-1 infection is phenotypically distinct from conventional T cell exhaustion described in models of chronic infection such as LCMV. While this does not necessarily preclude checkpoint molecule therapy as a potential treatment for enhancing immune control of HSV-1 latency, it does suggest that much more work is needed to understand how these markers function within HSV-1 infection and if the cells that express them are even able to be rescued.

While management of HSV-1 infection though inhibiting HSV-1 reactivation would obviously be the preferred method to prevent HSV-1 induced pathologies, it is unlikely that these therapies would be completely effective. Thus, there is a need to also develop interventions that help control reactivation events when they do occur. In chapter 4, we investigated how the immune response to HSV-1 infection contributes to the loss of corneal sensitivity and the accumulation of scar tissue that defines HSK.

The development of HSK is well known to be contingent on the immune response after HSV-1 infection. However, it is unclear why only some people who experience viral replication in the cornea develop HSK (99, 318). This suggests that there may be a way to uncouple viral replication in the cornea from the resulting tissue damage that occurs during HSK. We have leveraged a bilateral infection model that utilizes pathogenic and nonpathogenic strains of HSV-1 to identify differences in the immune responses to each strain, with a goal of pinpointing potential therapeutic targets. Using this model, we ruled out the adaptive immune response and excessive viral replication as drivers of HSK development in the pathogenic strain. Instead, we found that HSK development appears to be contingent on an innate immune response that is local to the cornea. Accordingly, we observed notable differences in the production of chemokines and cytokines, the magnitude of the innate immune infiltrate, and the function of the infiltrate between

the pathogenic and nonpathogenic strains as early as one dpi. These differences suggest that the trigger for HSK development occurs shortly after the immune response begins. This is particularly evident in how rapidly sensation is lost from pathogenic corneas. As a loss of corneal blink reflex correlates with more profound corneal scarring and opacity, it is likely that interventions that target early immune responses will be necessary to modulate HSK severity.

In preliminary experiments investigating what components of the early immune response are important in HSK development, we determined that monocytes, macrophages, and NO all contribute to the loss of corneal sensation, as monocyte/macrophage depletion and iNOS inhibition delayed the loss of corneal blink reflex. These results further support the conclusion that the innate immune response initiates loss of corneal sensation and HSK development. Most of the current literature identifies $CD4^+$ T cells as being responsible for HSK development (149, 151), however, recent work from our laboratory showed that a mild HSK develops in mice depleted of $CD4^+$ cells (155). Combining this observation with the data from our bilateral infection model suggests that HSK may be characterized by two distinct phases, with HSK initiation falling under the control of the innate immune response and HSK maintenance being mediated by $CD4^+$ T cells.

Much work remains in understanding the complex immune response that is induced after HSV-1 infection. While this immunity is necessary for effective control of viral latency within the TG, it can be detrimental in the cornea, where excessive inflammation causes irreparable tissue damage. Our studies in the TG suggest that manipulation of $CD8^+$ T cell functionality, while likely not impossible, is more complicated than previously appreciated. Thus, a better understanding of the expression and function of checkpoint molecules in virus-specific-$CD8^+$ T cells will be needed in order to effectively use checkpoint immunotherapy during HSV-1 infection. As for the cornea, we show that the trigger for HSK development occurs early during inflammation, although it

remains to be determined what cell type(s) and pathway(s) are required. This suggests that the development of future HSK treatments should be focused on targeting innate responses, especially those that contribute to nerve damage and retraction.

# 6.0 Future Directions

## 6.1 T cell impairment during HSV-1 infection

### 6.1.1 Checkpoint molecule expression during HSV-1 infection

The loss of $CD8^+$ T cell function during HSV-1 latency compromises the ability of the host to control the virus. Although checkpoint molecule blockade therapies have been successfully used to reverse this loss of function in other models, $CD8^+$ T cells during latent HSV-1 infection have been curiously resistant to these therapies (169, 217, 274, 282, 291). When we attempted to further define checkpoint molecule expression during latency, we were surprised to find that of the six checkpoint molecules we screened, only two were upregulated on impaired $CD8^+$ T cells and even then, they were expressed on only a small proportion of the impaired population. This is decidedly different than what is normally seen during T cell exhaustion when the most exhausted cells express high levels of at least one, but usually multiple checkpoint molecules, suggesting that T cell impairment during HSV-1 infection may not be as straightforward to rectify. However, it is still possible that other checkpoint molecules could be upregulated on impaired cells and thus manipulated to restore function in future studies. It may also be worth attempting combined checkpoint molecule therapies with PD-1, CTLA-4, and BTLA, despite the low levels of CTLA-4 and BTLA expression, as small individual effects may synergize to restore significant function to the Subdom-$CD8^+$ T cell population.

### 6.1.2 Role of Tim-3 in the HSV-1-infected TG

While we did not identify additional inhibitory checkpoint molecules that were strong candidates for individual blockade therapy, we did observe that Tim-3 was preferentially expressed on functional gB-CD8$^+$ T cells. Although expression of Tim-3 is traditionally considered inhibitory, we found that Tim-3 was expressed on the most functional cells in the TG and was clearly not acting in an inhibitory manner. This leads to the obvious question of what is the function of Tim-3 in CD8$^+$ T cells in the TG? Preliminary experiments with Tim-3 knockout mice did not show any significant differences in gB immunodominance, the ability of virus-specific CD8$^+$ T cells to produce GzmB, or the expression of PD-1 on virus-specific CD8$^+$ T cells at latency when compared to infection of WT mice (Figure 22). This suggests that Tim-3 is not required for normal CD8$^+$ T cell function within the TG, although the fact that functional impairment still occurs in the latently infected TG in the knockout mice supports the hypothesis that Tim-3 signaling is not necessarily inhibitory. However, the mice used for these experiments were germ line knockouts and with Tim-3 being expressed on and regulating function in other immune cell types, the interpretation of these data is not straightforward. These experiments were also only evaluated at a single timepoint post-infection and assessed but a few basic parameters. In future studies it would be interesting to follow HSV-1 infection in Tim-3 knockout mice for at least several months after latency is established to determine if there is any decline in functionality of gB-CD8$^+$ T cells and if virus-specific-CD8$^+$ T cells are less capable of preventing viral reactivation.

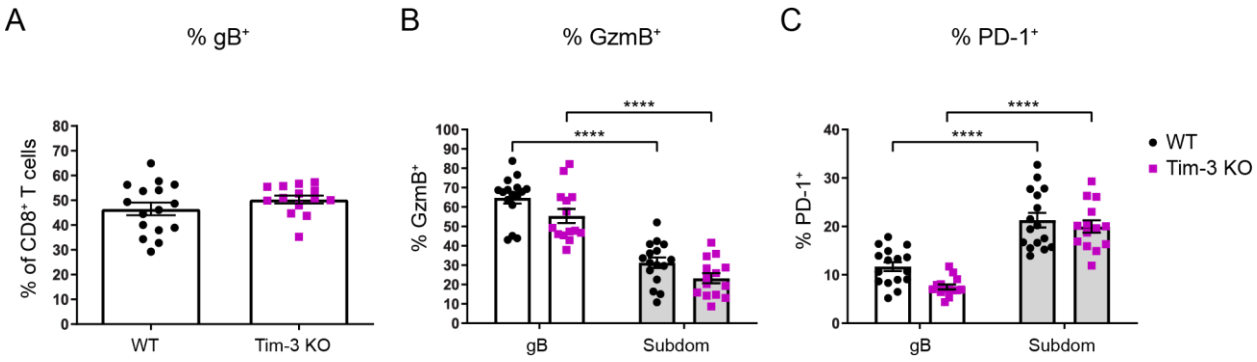



**Figure 22. Tim-3 knockout mice do not have altered CD8$^+$ T cell functionality in the latently infected TG.** TGs from latently infected WT or Tim-3 knockout (KO) mice (35 dpi) were removed, processed into single cell suspensions, and stained for viability, CD45, CD8, gB tetramer, Tim-3, PD-1, and GzmB and assessed by flow. In the graphs, the bars represent the mean frequency of cells ± SEM with WT mice represented by black circles and Tim-3 KO mice represented by purple squares. n=16 for WT mice and n= 14 for Tim-3 KO mice, data shown are from two pooled, independent experiments. Differences were assessed by unpaired t tests (A) or two-way ANOVAs with Tukey's posttests (B-C). **** $P \leq 0.0001$. (A) Percentage of CD8$^+$ T cells that are positive for gB tetramer. (B) Percentage of CD8$^+$ T cells that are positive for GzmB. (C) Percentage of CD8$^+$ T cells that are positive for PD-1.

### 6.1.3 Tim-3 as a marker of viral gene expression

The extent of HSV-1 gene expression during latency has long been a topic of debate and it was initially thought that the virus only expresses the LAT gene products (that are not translated into protein) during latency. However, indirect evidence of gene expression has been shown via the interaction of virus-specific CD8$^+$ T cells with neurons during latent HSV-1 infection (92, 159). Our observations support this model and further link the expression of viral gene products with the expression of Tim-3, as viruses containing mutations that increase expression of gB in the TG also induced increased expression of Tim-3 on gB-CD8$^+$ T cells. This apparent association between TCR engagement and Tim-3 expression suggests that we can use Tim-3 expression as an indirect measure of viral gene expression. Additional support for this correlation could be assessed by determining if Tim-3$^+$ cells form immune synapses with the neurons of the TG and by ascertaining if gB-CD8$^+$ T cells in the blood or lymphoid organs express lower levels of Tim-3, as these cells should have little access to viral gene expression during latency. It would also be

interesting to track Tim-3 expression over the course of infection, including in a reactivation model, to determine if viral gene expression is cyclical or if there is a low level of persistent gene expression that can be upregulated after a reactivation event.

### 6.1.4 2B4 as a marker of functionality in the HSV-1-infected TG

Most of our investigations of $CD8^+$ T cell functionality in the HSV-1-infected TG focused on Tim-3. Nonetheless, we also observed that 2B4 was preferentially upregulated on gB-$CD8^+$ T cells in a manner similar to Tim-3 (Figure 8). Although 2B4 has four identified ITSM domains whose presence normally suggest inhibition of TCR signaling, 2B4 has been shown to be have both stimulatory and inhibitory roles in T cell functionality. This difference in function appears to depend on both the level of expression of 2B4 as well as the expression of the adaptor molecule SAP within the cell cytoplasm (237, 292, 345). Thus, it may be interesting to determine if, like Tim-3, 2B4 serves to mark recently stimulated cells in the HSV-1 infected TG that are highly functional. It would also be of interest to determine if Tim-3 and 2B4 expression overlaps or if they mark separate subsets of functional cells.

### 6.1.5 Initiation of functional impairment in Subdom-$CD8^+$ T cells

Perhaps one of the most intriguing findings of our studies was the observation that Subdom-$CD8^+$ T cells have a distinctly nonfunctional PD-$1^+$ population that is evident at eight dpi. PD-1 is known to be transiently upregulated on T cells during activation and is thus associated with functional cells at that time. This is evident in our own work, with almost half of gB-$CD8^+$ T cells expressing PD-1 at eight dpi (Figure 14). Virtually all the PD-$1^+$ gB-$CD8^+$ T cells at this time

point also express GzmB. In the Subdom-CD8$^+$ T cell group, most of the cells that are PD-1$^+$ co-express GzmB, but it is clear that a small population, marked by PD-1, is unable to produce effector molecules even at this early time point. This lack of function was associated with higher levels of PD-1 expression, mirroring our previous observations of PD-1 expression levels in the latently infected TG, in which the impaired Subdom-CD8$^+$ T cells have a higher PD-1 MFI than the functional gB-CD8$^+$ T cells (282).

These data suggest that the functional impairment observed during HSV-1 latency may not result from persistent stimulation and is not truly exhaustion. Instead, it suggests that this phenotype is acquired during T cell priming. It would be worthwhile to determine if this functionally impaired population at eight dpi persists into latency. If so, it may indicate that reinvigorating these impaired cells is not possible. Although likely not helpful in terms of restoring function, it would be of interest to determine why this nonfunctional PD-1$^+$ population develops at early times post-infection only in the Subdom-CD8$^+$ T cells and how it is able to migrate to and persist within the latently infected TG. Subdom-CD8$^+$ T cell numbers have been shown to peak in the TG one day prior to gB-CD8$^+$ T cells (145). This suggests that these cells are primed before gB-CD8$^+$ T cells in a suboptimal environment that, while able to activate these cells enough for them to traffic into the TG, results in impaired function.

## 6.2 Innate determinants of HSK

### 6.2.1 Development of new HSK treatments

Current treatments for HSK are restricted to administration of antivirals and corticosteroids. Although these serve to control viral replication and curb inflammation in the eye, they are not overly effective at preventing the irreversible impairment of vision that can occur as a result of immunopathology (103, 104), suggesting that more targeted treatments are needed. Our studies have shown that HSK is initiated by an early immune response. Indeed, we observed clear differences in the production of soluble proinflammatory mediators and the size and function of the innate immune infiltrate in pathogenic and nonpathogenic HSV-1 infections as early as one dpi. Although we and others have attempted to mitigate the development of HSK by preventing cytokine signaling and performing immune cell depletions, the differences in pathology compared to untreated mice were only temporary (339, 343, 344). This suggests that we have not yet identified the key regulator(s) that couple viral replication to the initiation of HSK.

To address this, we have decided to take a more global approach to the problem and have performed RNAseq on mock-, KOS-, and RE-infected corneas at two dpi. Although we are currently in the initial stages of our analysis, our RNAseq data confirm our assessment of the upregulation of IL-6, CXCL1, and iNOS in RE-infected corneas compared to KOS-infected corneas. From the data, it is evident that there are several hundred genes that are significantly differently regulated between KOS and RE (Figure 23). Two of the significantly downregulated genes in RE infection as compared to KOS infection, Secreted LY6/PLAUR Domain Containing 1 (Slurp1) and LY6/PLAUR Domain Containing 2 (Lypd2), are members of the lymphocyte antigen-6 (Ly6)/urokinase-type plasminogen activator receptor (uPAR) family (346). Slurp1 has

previously been shown to be expressed within the cornea and appears to have roles in preventing immune cell infiltration (347, 348). Given that Slurp1 is downregulated during RE infection, it may be interesting to supplement Slurp1 via subconjunctival injections during corneal HSV-1 infection to see if it can temper immune infiltrate into the cornea.

In terms of increased gene expression, CXCL14 is highly significantly upregulated in RE infection compared to KOS infection. CXCL14 is secreted by a number of cell types including fibroblasts and has been shown to mediate infiltration of immune cell types such as neutrophils, DCs, and monocytes as well as enhance angiogenesis (349, 350). Blocking CXCL14 signaling may be an additional way to limit immune cell infiltration into the HSV-1-infected cornea. Like CXCL14, protein kinase C and casein kinase substrate in neurons 1 (PACSIN1) was also upregulated in RE-infected corneas. While most of the current literature focuses on PACSIN1's role in neuronal vesicle formation, it has also been identified as an adaptor molecule in plasmacytoid DCs that is necessary for signaling through TLR9/7 (351). In mice that lack PACSIN1, i.v. injection of HSV-1 resulted in a decrease in the production of IFN-α, suggesting that PACSIN1 serves to regulate interferon production during HSV-1 infection (351). Although type I interferons are crucial for the control of viral replication, it is possible that modulating their production may potentially help limit immunopathology in the eye. As our work showed that KOS-infected corneas have increased viral titers in the cornea without accompanying pathology, a slight increase in detectable virus due to manipulation of interferon production may be acceptable. Although, this balance would clearly have to be very finely tuned to prevent out of control viral replication. Further analysis of significant changes in immune regulated genes and utilization of pathway analysis tools using these differences will be useful in identifying regulator(s) of HSK development and provide additional potential targets for new treatment development.

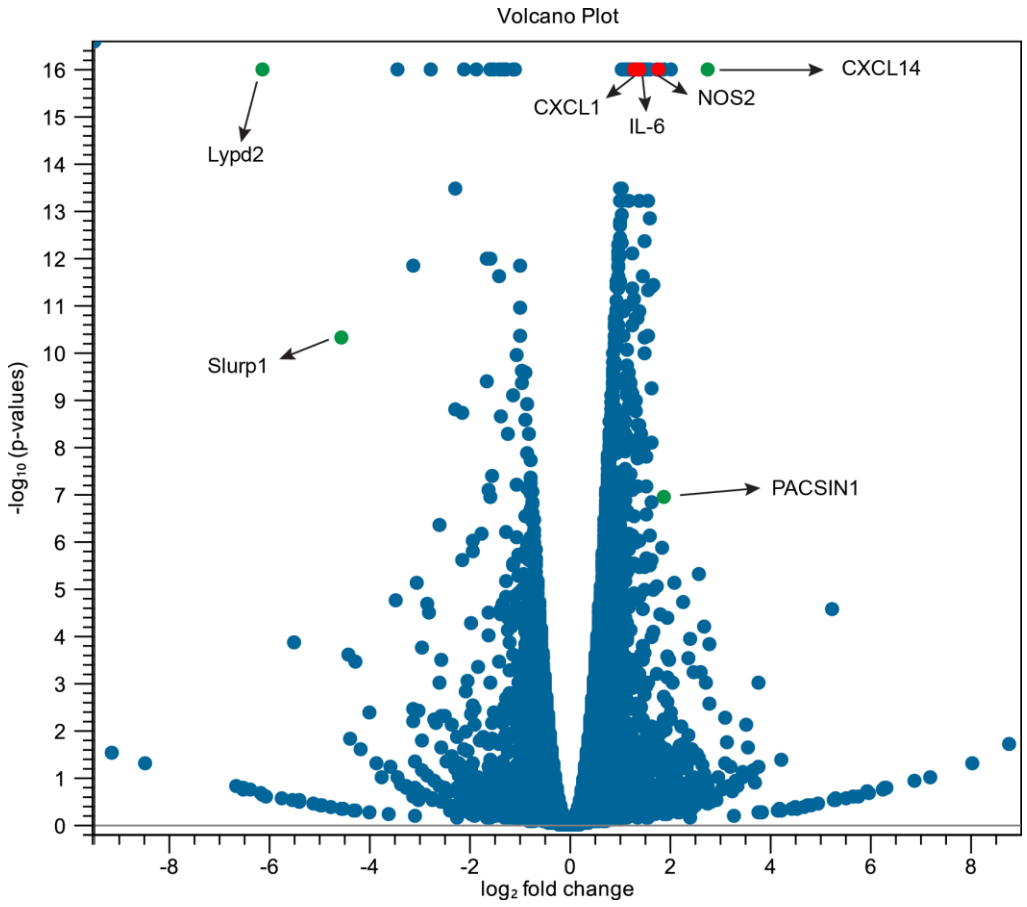



**Figure 23. RNAseq of KOS- and RE-infected corneas.**
Volcano plot of gene expression differences in RE-infected corneas as compared to KOS-infected corneas.

### 6.2.2 Role of innate immune response in sensory nerve retraction

One of the most striking differences between pathogenic and nonpathogenic strains of HSV-1 in the B6 mouse is how quickly and completely corneas infected with the pathogenic strain lose their ability to register corneal sensation and thus their ability to blink. Although humans are less prone to exposure keratitis than mice as they have consensual blink reflex, many patients do progressively lose corneal sensitivity with repeated reactivation events, putting them at risk for the severe inflammation that contributes considerably to scar tissue formation. We and others have

shown that this loss in sensation is due to a loss of corneal sensory nerves which poses the question of why this occurs and how we can prevent or restore this loss of nerves (155, 315, 316).

Although HSK can develop in contexts where corneas have sensation and are able to blink, the disease is generally not nearly as severe as in corneas that have lost sensation (155). This suggests that the loss of reflex increases the severity of HSK but that HSK initiation is not dependent on loss of corneal sensory nerves. This indicates that finding ways to prevent the loss of corneal sensation may be an acceptable option for limiting HSK progression.

We have previously shown that the loss in corneal sensation is maintained by the hyperinnervation of sympathetic nerves that prevent the reinnervation of sensory nerves (352). However, we still do not know what causes the initial sensory nerve retraction from the cornea. In the B6 mouse, this retraction begins shortly after infection, with a complete loss of corneal sensitivity by five dpi, suggesting that there is innate immune involvement. When we depleted macrophages and monocytes or blocked iNOS, we saw a slight delay in the loss of corneal blink reflex in corneas infected with the pathogenic strain. However, sensation was still eventually completely lost and severe HSK developed. This suggests that more than one immune cell type, and additional soluble mediators, are likely to be involved in orchestrating nerve retraction. As interventions that deplete immune cell types are not generally used clinically at this time, it is likely that modulating the soluble proinflammatory mediators will be a more useful solution. Assessment of our RNAseq data with a focus on neuroinflammatory pathways will likely be useful in narrowing down the potential contributors to this phenotype.

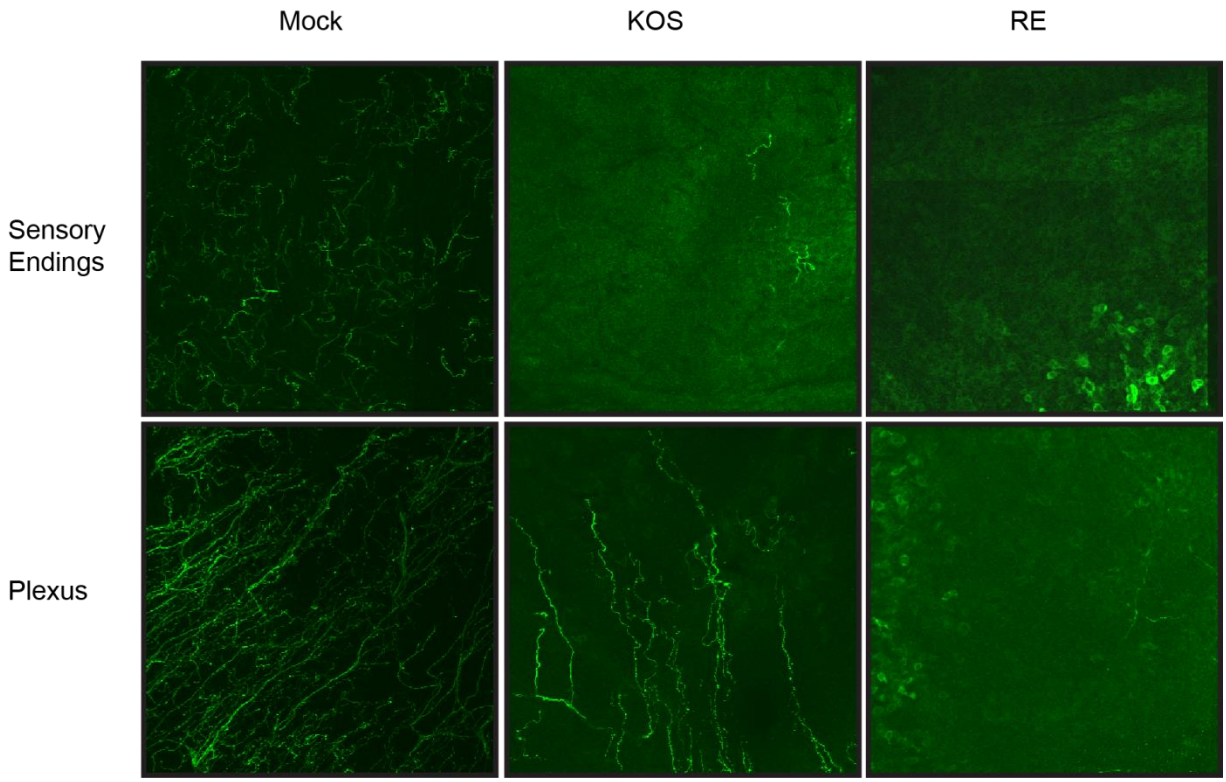
### 6.2.3 Contributions of viral strain to HSK development

We are currently focused on assessing differences in the immune response that leads to the initiation of HSK, as it is relevant to the development of new treatments. However, it is also of interest to determine how two genetically similar viruses bring about such differences. This is an important consideration when thinking about the genetic diversity of the circulating HSV-1 strains in the human population. The strains of HSV-1 that were used in our studies are very closely related, with over 99% of their nucleotide sequence being identical (Supplemental Table 1). With the availability of tools to create recombinant HSV-1 strains, HSV-1 gene swap mutants could be created and assessed for their ability to induce HSK. As the HSV-1 genome is large and encodes a considerable number of proteins, this is not a trivial task. However, given how quickly immune responses diverge after HSV-1 infection, we would hypothesize that viral gene products that trigger early innate immune responses, such as the glycoproteins that interact with TLRs or those that interfere with early detection such as ICP0 (109-111, 353), would be some of the most likely to mediate these differences. Our comparison of KOS and RE gene products (Supplemental Table 1) additionally suggests that the PKR inhibitor ICP34.5 could also be an attractive target, as it is one of the proteins with the most variation between the viruses. The most noticeable difference between KOS and RE ICP34.5 occurs in the proline-alanine-threonine (PAT) repeat region, which has been shown to determine cellular location of ICP34.5 and thus its ability to inhibit PKR signaling (354, 355). Our RNAseq data may also help inform gene swap choices, as differences in viral gene expression at early times post-infection could suggest potential targets.

Identifying what viral genes are necessary for the initiation of HSK development could additionally be advantageous from a screening standpoint. If we are able to localize the observed differences in HSK development to one or two viral gene products, these could potentially be used

as biomarkers to screen the infected population. As HSK development predisposes an individual to experience additional occurrences of the disease, it would be useful to be able to rate patients' risk for HSK and accordingly provide antiviral therapy when appropriate.

# Appendix A – Supplemental Data




**Supplemental Figure 1. Nerves in mock and HSV-1-infected corneas.**
C57BL/6 mice were unilaterally infected with either HSV-1 strains RE or KOS or mock infected. Corneas were excised and fixed at 4 dpi and whole mount staining was performed for β-III Tubulin, which denotes nerves. Images were acquired on an inverted Olympus IX81 FluoView 1000 confocal microscope and images shown are z-stack projections of epithelial (sensory endings) and stromal (plexus) layers of the cornea to show nerve presence.

**Supplemental Table 1. Nucleotide and protein alignments for HSV-1 strains KOS and RE.**
Nucleotide sequences for KOS (Genbank Accession# JQ780693) and RE (Genbank Accession# KF498959) were aligned with NCBI blastn using the megablast algorithm. Protein sequences for KOS and RE were aligned with NCBI global blastp using the Needleman-Wunsch algorithm.

| KOS and RE Nucleotide Sequence Alignment | | | | % identity |
|---|---|---|---|---|
| Whole genome blastn of JQ780693 (KOS) and KF498959 (RE) | | | | 99.2 |
| **KOS and RE Protein Sequence Alignment** | | | | |
| **Gene** | **Gene Product** | **# of mismatches** | **# of gaps** | **% identity** |
| RL1 | neurovirulence protein ICP34.5 | 6 | 18 | 91 |
| RL2 | ubiquitin E3 ligase ICP0 | 6 | 8 | 98 |
| UL1 | envelope glycoprotein L | 1 | 0 | 99 |
| UL2 | uracil-DNA glycosylase | 5 | 0 | 99 |
| UL3 | nuclear protein UL3 | 2 | 0 | 99 |
| UL4 | capsid portal protein | 2 | 0 | 99 |
| UL5 | helicase-primase helicase subunit | 7 | 0 | 99 |
| UL6 | capsid portal protein | 5 | 0 | 99 |
| UL7 | tegument protein | 2 | 0 | 99 |
| UL8 | helicase-primase subunit | 2 | 2 | 99 |
| UL9 | DNA replication origin-binding helicase | 4 | 0 | 99 |
| UL10 | envelope glycoprotein M | 3 | 0 | 99 |
| UL11 | myristylated tegument protein | 1 | 0 | 99 |
| UL12 | deoxyribonuclease | 6 | 0 | 99 |
| UL13 | tegument serine/threonine protein kinase | 8 | 0 | 98 |
| UL14 | tegument protein UL14 | 5 | 0 | 98 |
| UL15 | DNA packaging terminase subunit 1 | 0 | 0 | 100 |
| UL16 | tegument protein UL16 | 0 | 0 | 100 |
| UL17 | DNA packaging tegument protein UL17 | 3 | 0 | 99 |
| UL18 | capsid triplex subunit 2 | 0 | 0 | 100 |
| UL19 | major capsid protein | 1 | 0 | 99 |
| UL20 | envelope protein UL20 | 0 | 0 | 100 |
| UL21 | tegument protein UL21 | 2 | 0 | 99 |
| UL22 | envelope glycoprotein gH | 7 | 0 | 99 |
| UL23 | thymidine kinase | 1 | 0 | 99 |
| UL24 | nuclear protein UL24 | 2 | 0 | 99 |

| | | | | |
|---|---|---|---|---|
| UL25 | DNA packaging tegument protein UL25 | 0 | 0 | 100 |
| UL26 | capsid maturation protease | 5 | 0 | 99 |
| UL26.5 | capsid scaffold protein | 4 | 0 | 99 |
| UL27 | envelope glycoprotein B | 5 | 0 | 99 |
| UL28 | DNA packaging terminase subunit 2 | 1 | 0 | 99 |
| UL29 | single-stranded DNA-binding protein ICP8 | 2 | 0 | 99 |
| UL30 | DNA polymerase catalytic subunit | 5 | 0 | 99 |
| UL31 | nuclear egress lamina protein | 3 | 0 | 99 |
| UL32 | DNA packaging protein UL32 | 1 | 0 | 99 |
| UL33 | DNA packaging protein UL33 | 1 | 0 | 99 |
| UL34 | nuclear egress membrane protein | 0 | 0 | 100 |
| UL35 | small capsid protein | 0 | 0 | 100 |
| UL36 | large tegument protein | 28 | 20 | 98 |
| UL37 | tegument protein UL37 | 1 | 0 | 99 |
| UL38 | capsid triplex subunit 1 | 0 | 0 | 100 |
| UL39 | ribonucleotide reductase subunit 1 | 12 | 0 | 99 |
| UL40 | ribonucleotide reductase subunit 2 | 3 | 0 | 99 |
| UL41 | tegument host shutoff protein VHS | 3 | 0 | 99 |
| UL42 | DNA polymerase processivity subunit | 4 | 0 | 99 |
| UL43 | envelope protein UL43 | 10 | 0 | 98 |
| UL44 | envelope glycoprotein C | 11 | 0 | 98 |
| UL45 | membrane protein UL45 | 1 | 0 | 99 |
| UL46 | tegument protein VP11/12 | 7 | 1 | 99 |
| UL47 | tegument protein VP13/14 | 4 | 0 | 99 |
| UL48 | transactivating tegument protein VP16 | 1 | 0 | 99 |
| UL49 | tegument protein VP22 | 2 | 0 | 99 |
| UL49A | envelope glycoprotein N | 0 | 0 | 100 |
| UL50 | deoxyuridine triphosphatase | 0 | 0 | 100 |
| UL51 | tegument protein UL51 | 1 | 0 | 99 |
| UL52 | helicase-primase primase subunit | 6 | 0 | 99 |
| UL53 | envelope glycoprotein K | 2 | 0 | 99 |
| UL54 | multifunctional expression regulator ICP27 | 3 | 0 | 99 |
| UL55 | nuclear protein UL55 | 0 | 0 | 100 |
| UL56 | membrane protein UL56 | 2 | 0 | 99 |
| RS1 | transcriptional regulator ICP4 | 10 | 11 | 98 |
| US1 | regulatory protein ICP22 | 2 | 0 | 99 |
| US2 | virion protein US2 | 1 | 0 | 99 |
| US3 | serine/threonine protein kinase US3 | 1 | 0 | 99 |
| US4 | envelope glycoprotein G | 4 | 1 | 98 |
| US5 | envelope glycoprotein J | 0 | 0 | 100 |
| US6 | envelope glycoprotein D | 0 | 0 | 100 |

| | | | | |
|---|---|---|---|---|
| US7 | envelope glycoprotein I | 0 | 14 | 96 |
| US8 | envelope glycoprotein E | 4 | 2 | 99 |
| US8A | membrane protein US8A | *33 | 0 | 83 |
| US9 | membrane protein US9 | **5 | 0 | 91 |
| US10 | virion protein US10 | 2 | 3 | 98 |
| US11 | tegument protein US11 | 1 | 3 | 97 |
| US12 | TAP transporter inhibitor ICP47 | 0 | 0 | 100 |

* = KOS US8A has a mutation that eliminates a stop codon found in strains RE, F, and 17.

**= US9 is truncated in both RE and KOS as compared to strains F and 17 via two separate mutations that leaves the RE gene product slightly shorter than the KOS gene product.

## Appendix B – List of Works and Copyright Information

**Carroll KL**, Avery L, Treat BR, Kane LP, Kinchington PR, Hendricks RL, St Leger AJ. 2020. Differential Expression of Immune Checkpoint Molecules on $CD8^+$ T Cells Specific for Immunodominant and Subdominant Herpes Simplex Virus 1 Epitopes. Journal of Virology Volume 94, Issue 2. doi: 10.1128/JVI.01132-19.

Jeon S, Rowe AM, **Carroll KL**, Harvey SAK, Hendricks RL. 2018. PD-L1/B7-H1 Inhibits Viral Clearance by Macrophages in HSV-1–Infected Corneas. The Journal of Immunology doi:10.4049/jimmunol.1700417.

Hendricks RL, Yun H, Rowe AM, and **Carroll KL**. Animal Models of Ophthalmic Diseases. 1st ed. Switzerland: Springer International Publishing; c2016. Chapter 1, Animal Models of Herpes Keratitis; p. 1-10.



**Carroll KL**, Avery L, Treat BR, Kane LP, Kinchington PR, Hendricks RL, St Leger AJ. 2020. Differential Expression of Immune Checkpoint Molecules on $CD8^+$ T Cells Specific for Immunodominant and Subdominant Herpes Simplex Virus 1 Epitopes. Journal of Virology Volume 94, Issue 2. doi: 10.1128/JVI.01132-19.



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