THE ROLE OF PROGRAMMED DEATH LIGAND-1 (PD-L1/B7-H1) IN REGULATING
THE IMMUNE RESPONSES AGAINST HERPES SIMPLEX VIRUS-1 (HSV-1)

by

Sohyun Jeon

Bachelor of Science in Life Science, Ewha Womans University, 2007

Submitted to the Graduate Faculty of
School of Medicine in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2013
Copyright permission was granted for:


Copyright 2013. The American Association of Immunologists, Inc.


Copyright 2011. The American Association of Immunologists, Inc.

Copyright permission letters are on file with Sohyun Jeon

Copyright © by Sohyun Jeon

2013
Herpes simplex virus type 1 (HSV)-specific CD8+ T cells provide immunosurveillance of trigeminal ganglion (TG) neurons that harbor latent HSV-1 and prevent viral reactivation. Recurrent reactivations of HSV-1 from latency in the TG can result in severe corneal immunopathology and progressive scarring, leading to a blinding disease called Herpetic Stromal Keratitis. Therefore it is of significant clinical relevance to develop immunotherapies that are designed to boost the immune responses that prevent or reduce the frequency of HSV-1 reactivations. Programmed Death-1 (PD-1) is an inhibitory receptor that is expressed on many types of activated immune cells and regulates their cytokine production and numbers, with high levels of PD-1 inducing apoptosis and low expression inhibiting proliferation. Our goal was to investigate the role of a PD-1 ligand, PD-L1/B7-H1, during HSV-1 infection, and test whether blocking PD-L1 will enhance the innate immune response that clears the initial viral infection, and the adaptive immune response that prevents reactivation from latency. We used a mouse model of HSV-1 infection in which virus infection of the corneal epithelium leads to formation of a lesion; and simultaneous transmission of the virus to the TG results in establishment of HSV-1 latency in a portion of TG neurons. We found that PD-L1 was expressed on HSV-1-infected corneal epithelial cells during acute HSV-1 infection and was preferentially up-regulated by IFN-γ on HSV-1-infected neurons during latency. Using B7-H1/PD-L1−/− mice during acute HSV-1 infection, we found that 1) PD-L1 expressed on corneal epithelium and bone
marrow-derived CD45<sup>+</sup> cells in the cornea inhibits efficient recruitment of Gr-1<sup>+</sup> cells (monocytes/neutrophils) by suppressing chemokine production, 2) PD-L1 expressed on corneal epithelium inhibits γδ T cell infiltration into the infected cornea, and 3) PD-L1 promotes proper DC maturation during T cell priming in the draining lymph nodes. During latent HSV-1 infection, PD-L1 expressed on TG neurons inhibits the survival but not proliferation and cytokine production of TG-resident subdominant CD8<sup>+</sup> T cells. B7-H1<sup>+</sup> mice had an enlarged population of PD-1<sup>high</sup> subdominant CD8<sup>+</sup> T cells that did not provide enhanced protection from HSV-1 reactivation, suggesting that PD-L1 blockade may not be an attractive option as immunotherapy against HSK.
# TABLE OF CONTENTS

ACKNOWLEDGMENT ........................................................................................................... X

1.0 INTRODUCTION ............................................................................................................ 1

1.1 HERPES SIMPLEX VIRUS-1 (HSV-1) ........................................................................... 1

1.1.1 Epidemiology and Disease ...................................................................................... 1

1.1.2 Virus Life Cycle ........................................................................................................ 3

1.2 IMMUNE RESPONSES AGAINST HSV-1 ................................................................. 5

1.2.1 Innate immunity against HSV-1 during acute infection .......................................... 5

1.2.2 Adaptive immunity against HSV-1 during latent infection ...................................... 9

1.3 IMMUNE REGULATION BY INHIBITORY RECEPTORS ...................................... 14

1.3.1 T cell Exhaustion ..................................................................................................... 14

1.3.2 Programmed Death-1 (PD-1) Signaling ................................................................. 16

1.3.3 The role of PD-L1 in regulating adaptive immune responses ............................. 18

1.3.4 The role of PD-L1 in regulating innate immune responses .................................. 20

1.3.5 PD-1-PD-L1 in the eye ............................................................................................ 22

2.0 STATEMENT OF THE PROBLEM ........................................................................... 24

3.0 SPECIFIC AIMS ......................................................................................................... 25

4.0 MATERIALS AND METHODS .................................................................................. 27
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>MICE</td>
<td>27</td>
</tr>
<tr>
<td>4.2</td>
<td>VIRUS AND CORNEAL INFECTIONS</td>
<td>28</td>
</tr>
<tr>
<td>4.3</td>
<td>REAGENTS FOR FLOW CYTOMETRY</td>
<td>28</td>
</tr>
<tr>
<td>4.4</td>
<td>TISSUE PREPARATION</td>
<td>28</td>
</tr>
<tr>
<td>4.5</td>
<td>GENERATION OF BONE MARROW CHIMERIC MICE</td>
<td>28</td>
</tr>
<tr>
<td>4.6</td>
<td>SORTING NEURON SUBPOPULATIONS</td>
<td>30</td>
</tr>
<tr>
<td>4.7</td>
<td>QUANTIFICATION OF VIRAL GENOME COPIES</td>
<td>30</td>
</tr>
<tr>
<td>4.8</td>
<td>MEASUREMENT OF CD8 T CELL PROLIFERATION</td>
<td>31</td>
</tr>
<tr>
<td>4.9</td>
<td>MEASUREMENT OF CD8 T CELL APOPTOSIS</td>
<td>31</td>
</tr>
<tr>
<td>4.10</td>
<td>IN VIVO TREATMENTS</td>
<td>32</td>
</tr>
<tr>
<td>4.11</td>
<td>EX VIVO STIMULATIONS</td>
<td>32</td>
</tr>
<tr>
<td>4.12</td>
<td>EX VIVO TRIGEMINAL GANGLIA NEURON CULTURES</td>
<td>33</td>
</tr>
<tr>
<td>4.13</td>
<td>DETECTION OF INFECTIOUS VIRUS FROM CORNEA</td>
<td>33</td>
</tr>
<tr>
<td>4.14</td>
<td>WHOLE MOUNT FLUORESCENT MICROSCOPY</td>
<td>34</td>
</tr>
<tr>
<td>4.15</td>
<td>MEASUREMENT OF MESSENGER RNA</td>
<td>34</td>
</tr>
<tr>
<td>4.16</td>
<td>GATING STRATEGIES AND STATISTICAL ANALYSIS</td>
<td>35</td>
</tr>
<tr>
<td>4.17</td>
<td>IN VITRO DC-T CELL INTERACTION ASSAY</td>
<td>36</td>
</tr>
<tr>
<td>4.18</td>
<td>SCORING HSK</td>
<td>37</td>
</tr>
<tr>
<td>4.19</td>
<td>MEASUREMENT OF NEURON APOPTOSIS</td>
<td>37</td>
</tr>
<tr>
<td>5.0</td>
<td>THE ROLE OF PD-L1 DURING LATENT HSV-1 INFECTION</td>
<td>38</td>
</tr>
<tr>
<td>5.1</td>
<td>PD-1 and PD-L1 expression in the trigeminal ganglia</td>
<td>39</td>
</tr>
<tr>
<td>5.2</td>
<td>Preferential up-regulation of PD-L1 on HSV-1-infected neurons</td>
<td>41</td>
</tr>
<tr>
<td>5.3</td>
<td>Differences between gB-Tet+ and gB-Tet- CD8 T cells</td>
<td>43</td>
</tr>
</tbody>
</table>
5.4 Frequency of gB-Tet- CD8 T cells in the absence of PD-L1 ........................................ 45
5.5 Increased survival of gB-Tet- CD8 T cells in B7-H1 KO mice ............................... 48
5.6 Functionality of CD8 T cells in B7-H1 KO mice.................................................. 50
5.7 HSV-1 reactivation in B7-H1 KO mice.................................................................... 52
5.8 Discussion ............................................................................................................. 54

6.0 THE ROLE OF PD-L1 DURING ACUTE HSV-1 INFECTION.................................. 59
6.1 PD-L1 expression in the cornea.............................................................................. 60
6.2 Enhanced viral clearance in the cornea of B7-H1 KO mice ................................. 62
6.3 Increased Gr-1+ cells in infected cornea of B7-H1 KO mice.............................. 63
6.4 Biphasic functions of PD-L1 in the cornea............................................................ 66
6.5 The role of PD-L1 in regulating γδ T cells in the HSV-1-infected cornea .... 67
6.6 Discussion ............................................................................................................. 72

7.0 IMPORTANT ASPECTS TO CONSIDER IN USING PD-L1 BLOCKADE...... 79
7.1 Characterization of DC phenotype in B7-H1 KO mice........................................ 80
7.2 Effects of PD-L1 blockade on effector T cell responses ..................................... 83
7.3 Effects of PD-L1 blockade on neuron apoptosis.................................................. 84
7.4 Effects of PD-L1 blockade on Herpetic Stromal Disease .................................... 85
7.5 Effects of PD-L1 and IL-10 combinatorial blockade.......................................... 86
7.6 Discussion ............................................................................................................. 89

8.0 SUMMARY AND CONCLUSIONS ........................................................................ 96

9.0 FUTURE DIRECTIONS............................................................................................. 101
9.1 Other inhibitory molecules on HSV-1-specific CD8 T cells.............................. 101
9.2 The role of PD-L1 in regulating CD4 T cell response ........................................ 102
9.3 The mechanism of PD-L1 in regulating immune cell in the cornea........ 103

APPENDIX A ........................................................................................................... 104

BIBLIOGRAPHY ........................................................................................................ 104
LIST OF TABLES

TABLE 1. DELINEATING THE BIPHASIC FUNCTIONS OF PD-L1 IN THE CORNEA WITH BONE MARROW CHIMERIC MICE ................................................................. 67
LIST OF FIGURES

Figure 1. The murine model of ocular HSV-1 infection...................................................3

Figure 2. The 1:1 ratio of gB-specific CD8 T cells and subdominant gB-nonspecific CD8 T cells in the TG following HSV-1 infection .................................................................11

Figure 3. The immunodominance hierarchy of HSV-1-specific CD8 T cells within the TG of C57BL/6 mice at 8 DPI..................................................................................12

Figure 4. T cell exhaustion..............................................................................................15

Figure 5. “Reinvigorating” the CTL response by PD-L1 blockade.................................20

Figure 6. PD-1 is preferentially expressed on subdominant epitope-specific CD8 T cells in latently infected trigeminal ganglia..............................................................40

Figure 7. PD-L1/B7-H1 expression on neurons in the trigeminal ganglia post HSV-1 infection.........................................................................................................................41

Figure 8. IFN-γ regulates PD-L1 expression on trigeminal ganglion neurons...............43

Figure 9. Elevated HSV-1 genome copy number in PD-L1+ neurons..........................44

Figure 10. Differences between immunodominant gB(498-505)-specific CD8+ T cells and subdominant epitope-specific CD8+ T cells.........................................................45

Figure 11. The number of gB-Tet- CD8+ T cells in B7-H1−/− TG is preferentially increased.....47

Figure 12 Significantly increased number of PD-1hi subdominant CD8+ T cells in B7-H1−/− TG..................................................................................................................48

Figure 13 PD-1/B7-H1 regulates survival of subdominant CD8+ T cells.......................49

Figure 14 B7-H1 on neurons is partially responsible for controlling the survival of PD-1+ gB-Tet− CD8+ T cells.....................................................................................................50

Figure 15 Expanded subdominant CD8+ T cells in B7-H1−/− mice are not functional......52
Figure 16. Decreased genome copy number in B7-H1<sup>−/−</sup> TG..........................................................54
Figure 17. B7-H1 KO mice do not show better protection against HSV-1..............................54
Figure 18. CD45<sup>+</sup> cell infiltration to HSV-1-infected cornea.................................................61
Figure 19. PD-L1 expression on naïve and HSV-1-infected cornea.............................................62
Figure 20. Enhanced viral clearance from the cornea of B7-H1<sup>−/−</sup> mice.................................63
Figure 21. Increased recruitment of Gr-1<sup>+</sup> CD45<sup>+</sup> cells into B7-H1<sup>−/−</sup> cornea at 1 DPI........65
Figure 22. Increased chemokine and cytokine levels in B7-H1<sup>−/−</sup> cornea at 1 DPI....................66
Figure 23. Gr-1<sup>int</sup> inflammatory monocytes but not Gr-1<sup>hi</sup> neutrophils are responsible for enhanced viral clearance in the B7-H1<sup>−/−</sup> cornea..........................................................67
Figure 24. PD-L1 on corneal epithelium is absolutely necessary for suppressing innate immune response at 4 DPI.................................................................68
Figure 25. PD-L1 suppresses the γδT cell response at 3 dpi.........................................................70
Figure 26. PD-L1 on T cells is responsible for CD80 expression on DCs......................................80
Figure 27. Systemic anti-PD-L1 treatments during clonal expansion results in increased number of CD8 T cells.................................................................82
Figure 28. Systemic anti-PD-L1 treatments during clonal expansion does not affect neuron survival...........................................................................................................84
Figure 29. Subconjunctival anti-PD-L1 treatments do not affect HSK incidence and severity...85
Figure 30. Systemic anti-IL-10R treatments in B7-H1<sup>−/−</sup> mice rescues proliferation and cytotoxicity of subdominant CD8 T cells but not IFN-γ production.................................87
ACKNOWLEDGMENT

First and foremost, I would like to thank God without whom I could not have done anything.

I would like to thank my PhD mentor, Dr. Bob Hendricks, for his guidance, his unwavering optimism and passion for research. I am grateful for his patience and wisdom, both of which are qualities that I have learned to be the most important in being a great scientist.

I thank my thesis advisors, Drs. Pawel Kalinski, Kip Kinchington, Kyle McKenna, and Angus Thomson for their precious pieces of advice and helpful suggestions and I thank my current and former lab mates, Tony St. Leger, K-Ann Buela, Alex Rowe, and Moira Geary who celebrated and commiserated with me through both successful and failed experiments.

I thank my loving parents, Songyoung Shim and Jin Seok Jeon who have inspired me, encouraged me to become a scientist and have always supported me throughout my life. I want to thank my grandma, Oksun Kim, who taught me love and strength. She is probably very proud of me. I also thank my brother, Tae Jun Jeon, for his sense of humor and love that helped me get through hard times in graduate school.

Lastly I would like to thank my wonderful boyfriend, Greg Frank, for always being there for me no matter what. I also cannot express with words how grateful I am for the precious friendships that I have built with my lovely friends and classmates. I thank them for making me feel at home and helping me find happiness in a country far away from my own.
1.0 INTRODUCTION

1.1 HERPES SIMPLEX VIRUS-1 (HSV-1)

1.1.1 Epidemiology and Disease

Herpes Simplex Virus-1 (HSV-1) is a prevalent viral pathogen that infects at least 58% of the US population and up to 98% in other areas of the world (1-3). HSV-1 causes pathology at a variety of anatomical sites including the eye where it can infect the cornea, eyelids, conjunctiva, uveal tract, and retina leading to various ocular diseases. HSV-1 corneal infections are the leading infectious cause of blindness in the USA (4). During an initial primary infection of the orofacial region including the cornea, HSV-1 replicates briefly and gains access to the nerve endings present in the primary infection site and travels to the trigeminal ganglia (TG) where it establishes a latent (quiescent) infection within neurons leading to a life-long relationship with the host.

In most individuals, latent HSV-1 in TG neurons is not a significant problem, but in others certain stimuli such as stress, compromise in immunity, or genetic factors (3, 5, 6), lead to viral reactivation from the latent state with axonal transport of infectious virions back to the initial infection site or an alternative site that is innervated by infected neurons. For instance,
viral reactivation in the ophthalmic branch of latently infected TG can lead to viral shedding at the cornea surface even following a non-ocular primary infection (7).

Clinical symptoms of primary ocular HSV-1 infection tend to present themselves mainly in young adults (8, 9). During primary infection, viral replication in the corneal epithelium can damage epithelial cells and result in epithelial lesions (10). Although primary disease is indeed a serious concern, recurrent disease presents more severe problems as previous bouts of keratitis resulting from frequent viral reactivation significantly increases the risk of future recurrences. Recurrent HSV-1 corneal disease can present as: Epithelial Keratitis (56.3%) and Herpetic Stromal Keratitis (HSK) (29.5%) (11).

Epithelial keratitis is a manifestation of the viral cytopathic effect in epithelial cells. Patients with epithelial keratitis often suffer from pain, photophobia, tearing, redness, and blurred vision (12). Epithelial keratitis can, however, progress to HSK that involves inflammation within the corneal stroma that leads to stromal opacity, edema, and neovascularization with varying degrees of residual scarring (13-15).

HSK can be subdivided into two categories: necrotizing disease that involves epithelial defects and active viral replication in the epithelium and non-necrotizing disease that is also known as immune HSK that mainly involves immunopathology in the stroma without active viral replication (10, 16). Repeated HSV-1 reactivation that causes frequent HSK recurrences can lead to irreversible scarring to the cornea threatening its clarity and resulting in blindness.

In this thesis, a C57BL/6 mouse model of HSV-1 corneal infection was used to study the immune responses against HSV-1 (Fig 1). Following scarification, 1x10^5 pfu HSV-1 KOS was topically applied to each cornea, resulting in virus replication in epithelial cells for up to 4~5 days. An innate immune response clears virus from the cornea by 6 days post infection (dpi) but
some virions gain access to the nerves innervating the cornea and travel to the TG starting around 3~4 day post infection (dpi). Migratory Dendritic Cells (DC) that infiltrate the cornea can pick up HSV-1 antigen and travel through the lymphatic vessels to present the antigen to HSV-1-specific CD4+ and CD8+ T cells in the draining lymph nodes (DLN). Following activation, HSV-1-specific T cells that recognize specific HSV-1 epitopes will undergo robust proliferation in the lymph nodes and travel to the TG to surround HSV-1-infected neurons and prevent HSV-1 reactivation for the life of the infected animal. In C57BL/6 mice HSV-specific CD8+ T cells achieve peak levels in the TG around 8 dpi, and then gradually undergo contraction over the next 3 weeks to finally maintain a stable memory population. The infected animal is considered latently infected after 30 dpi.

**FIGURE 1** The murine model (C56BL/J strain) of ocular Herpes Simplex Virus-1 (HSV-1) infection
The role of HSV-1-specific CD8⁺ T cells in maintaining HSV-1 latency in the TG has been well characterized in C57BL/6 mice by our group (5, 17-23) and therefore a C57BL/6 mouse model of HSV-1 infection by KOS Hendricks strain (KOS-H) is a useful tool for studying various immune mechanisms in the TG. While there are no obvious manifestations of HSK in C57BL/6 mice following infection with KOS-H, primary infection with another strain called “RE Lausch” (RE-L) does lead to HSK development and mimics clinical features of HSK seen in humans (24).

In our model, the immune responses against HSV-1 occur in three main organs: the cornea where the initial viral infection is controlled by the innate immune cells, the draining lymph nodes where T cell priming takes place, and the TG where HSV-1 latency is established and maintained by CD8⁺ T cells. This thesis examines these three aspects of HSV-1 infection by studying (1) the role of PD-L1 in controlling the innate immune response in the cornea (Chapter 6), (2) the role of PD-L1 in regulating DC maturation and CD8 T cell priming in the draining lymph nodes (Chapter 7) and (3) the role of PD-L1 in regulating CD8 T cell responses in the TG during latency (Chapter 5).

1.1.2 Virus Life Cycle

HSV-1 is a double-stranded DNA virus whose genome is 152 kb in size and encodes 84 proteins. HSV-1 consists of 4 different structures; 1) viral genome, 2) capsid, 3) tegument, and 4) envelop. The viral gene expression of HSV-1 can be categorized into 4 different classes, which are immediate early (α), early (β), leaky-late (γ1), and true-late (γ2).

During lytic infection, HSV-1 expresses viral genes in a highly organized and sequential manner. Within 1~2 hours after infection, Immediate Early (IE) genes that encode ICP0, ICP4,
ICP22, ICP27, ICP47, and US1.5 are expressed without requiring any *de novo* viral protein synthesis. Most of these IE proteins, mainly ICP0, ICP4, and ICP24, are critically involved in viral transcription and transactivation of Early genes.

Between 4~8 hours following infection, Early (E) genes also known as β-genes are expressed. These genes initiate viral DNA replication, a process required to activate the Late (L), or γ genes. The γ-genes can be subdivided into the “leaky-late” γ1 genes and the “true-late” γ2 genes depending on when the genes are expressed and whether DNA replication is required for their expression. A good example of a “leaky-late” gene is a glycoprotein B (gB), which is expressed in low amounts before DNA replication and then increases exponentially in expression after DNA replication. The γ2 genes can only be expressed following viral DNA synthesis. Most of these γ-genes encode structural proteins that are essential for final assembly of the virion. As the virions exit the host cell, the lytic life cycle of HSV-1 is completed and the host cell such as an infected neuron inevitably undergoes lysis.

When HSV-1 enters latency, the viral genome is retained in the host neuron’s nucleus as a non-integrated episome that is associated with nucleosomes. In latently infected neurons, most viral gene transcription is silenced except for the Latency Associated Transcripts (LATs), which are produced abundantly during latency as the name implies. These transcripts do not encode protein and are stable nuclear RNAs without poly-A tails or 5’ caps that may be microRNA by-products of bigger unstable viral transcripts (25). Functionally, they can promote latency by inhibiting HSV-1 lytic gene expression (26), and blocking virus-induced neuronal apoptosis (27). The LATs lie anti-sense to a lytic gene, ICP0, to silence its expression (28) and can also influence facultative heterochromatin assembly (29) as maintenance of HSV-1 latency seems to
involve epigenetic regulation (30, 31). Some studies have proposed that LATs also have a role in influencing the life styles (lytic or latent) of HSV-1 in different neuronal populations (32).

Some of the factors that induce HSV-1 reactivation are exposure to UV light, stress, changes in sex hormones, and immune suppression (6, 33-37), all of which are associated with T cell function (38-42). However, the exact molecular mechanisms of HSV-1 reactivation still need to be understood. One recent theory proposes that during reactivation the virus undergoes the first phase in which viral lytic genes are simultaneously expressed concurrent with degradation of LATs and microRNAs that silence lytic genes, a process called “derepression.” The second phase is characterized by an amplification of these lytic genes by the viral protein VP16, leading to virion formation (43).

1.2 IMMUNE RESPONSES AGAINST HSV-1

1.2.1 Innate Immunity against HSV-1 during acute infection

Dendritic Cells

Despite the popular notion that the cornea is an immune-privileged site devoid of immune cells, a great number of immune cells including CD11c⁺ dendritic cells (DC) can be found within non-infected murine corneas (44-46). They reside mainly in the basal layer of corneal epithelium (44, 47) with heaviest density in the peripheral regions. These cornea-resident DCs may serve as sentinels that sample the environment for any foreign antigens and present them to effector or memory T cells that infiltrate the cornea, similar to the role recently described for DCs in the gut (48).
Following HSV-1 infection, DCs in the cornea detect HSV-1 through pattern recognition receptors (PRR) and contribute to innate immune responses against HSV-1 infection mainly by secreting inflammatory and anti-viral cytokines (49, 50) or providing help to other innate immune cells such as Natural Killer (NK) cells that release cytolytic granules to kill infected cells (51, 52). The main source of type I IFNs during HSV-1 infection seems to be plasmacytoid DCs (pDC), a non-conventional subset of DCs that is recruited to the cornea upon infection (49, 50) whereas IL-12 and IL-15 produced by DCs can help the activation and homeostatic proliferation of NK cells (53-56).

Resident corneal DCs and/or those that infiltrate the cornea during the first 24 h after HSV-1 infection facilitate the recruitment of NK cells and inflammatory monocytes to the central cornea (57). They seem to do this by directly producing or indirectly assisting the expression of a variety of chemokines such as CCL5 (activates NK cells), CCL7 (attracts and activates macrophages), CCL8 (attracts and activates monocytes and NK cells), CXCL9 (attracts NK cells), CXCL10 (attracts macrophages and NK cells), and CXCL11 (attracts NK cells) (57). However, DCs are not responsible for recruiting neutrophils as DC depletion did not lead to changes in neutrophil density in the central cornea or in the levels of CXCL2/MIP-2, the chemokine primarily responsible for neutrophil migration into the HSV-1 infected cornea (58).

Immediately following HSV-1 infection, DCs as one of professional antigen presenting cells (APC) also undergo maturation and up-regulate the co-stimulatory molecules (e.g. CD80, CD86, CD40) and MHC class II that are essential for T cell activation. Although still under investigation, one model proposes that corneal DC carry viral antigens to the draining lymph nodes and directly present them to T cells or serve as a source of viral antigens that are cross-presented by lymph node resident DCs.
Macrophages, inflammatory monocytes, and neutrophils

During HSV-1 acute infection, macrophages in the cornea also are activated through TLR recognition of viral components and immediately become armed with anti-viral cytokines such as type 1 interferons IFN-α and IFN-β (59). Type 1 interferons are crucial for controlling the initial HSV-1 replication as shown by studies using IFN-α/β receptor−/− mice (60) and RAG−/− mice treated with IFN-α/β (61). Macrophages are the main source of Interleukin-12 (IL-12) approximately 8-12 hours after HSV-1 corneal infection (62). IL-12, in synergy with IFN-α/β, can enhance the production of IFN-γ by NK cells (63). IFN-γ released by NK cells subsequently activates macrophages to induce inducible nitric oxide synthase (iNOS), which produces NO to limit HSV replication (64, 65). Macrophages can also facilitate angiogenesis in the cornea by secreting factors such as Vascular Endothelial Growth Factor (VEGF) (66-68) and a tissue-degrading enzyme Matrix Metalloproteinase-9 (MMP-9) (69-71).

Similar to macrophages, inflammatory monocytes (F4/80+ Gr-1int) can also produce antiviral compounds such TNF and NO which are mainly involved in the control of HSV-1 replication in the trigeminal ganglion (72). On the other hand, the role of neutrophils in controlling HSV-1 in the cornea is not clear. Two recent studies demonstrated impaired HSV-1 clearance in mice that were depleted of Gr-1+ cells, which include inflammatory monocytes (Ly6C+) and neutrophils (Ly6G+), but not when they were specifically depleted neutrophils with Ab to Ly6G (73, 74). This data suggests that neutrophils probably do not play a major role in HSV-1 clearance, but since these aforementioned studies were performed using intranasal and flank infections, the role of neutrophils in HSV-1 infected corneas remains unresolved.

Natural Killer (NK) cells
Studies using a murine model of HSV-1 ocular infection indicate that NK cells are indeed activated in the spleen immediately following HSV-1 ocular infection (75). Although uninfected corneas are devoid of NK cells, NK cells migrate into the cornea as soon as 3 dpi in C57BL/6 mice. The chemokine CXCR3 is considered to be important during this process since CXCR3−/− mice show significant reduction in NK cell infiltration (76). As mentioned previously, migration into HSV-1-infected central cornea of NK cells but not their function (cytotoxicity and activation) also seems to be largely dependent on DCs (57).

NK cells can inhibit virus replication through generally lethal release of lytic granule components such as granzyme B, or through nonlethal release of antiviral cytokines, including IFN-γ (77). It is thought that NK cells largely contribute to HSV-1 clearance during primary infection (78-80) and previous studies have established that genetic defects in NK cell responses correlate to susceptibility to herpesvirus infections (81, 82). The C57BL/6 mouse strain is more resistant to lethal HSV-1 infections relative to other susceptible strains, such as Balb/c. This is mainly due to C57BL/6’s unique genetic background containing various allelic variants of genes that confer strong innate protection (83, 84). It is also thought that NK cells may influence the magnitude or the establishment of latent infection (84).

There is conflicting data in the literature about the role of NK cells against HSV-1 infection as some say NK cells play a role (81, 84, 85) while others say they do not (86-88). Habu et al. reported that NK cell activity is crucial for HSV-1 resistance during the early stage of infection, but not after 5 dpi (85). Depletion of NK cells results in significantly increased viral replication (76) and corneal scarring (79). In another study, however, depleting NK cells in SCID mice showed that NK cells may be dispensable for controlling virus replication (88). The contribution that NK cells make seems to be dependent on the type of infection as well since NK
cell depletion has little effect in limiting viral replication in skin infection models but significantly compromises viral clearance from mucosal surfaces (89-92).

NK cells not only contribute to innate viral clearance but also play a major role in shaping the magnitude and quality of adaptive immune responses. In a study using a mouse HSV-1 skin infection model, NK cell depletion resulted in a reduced T cell response against HSV-1, impaired T cell differentiation toward Th1 or Tc1 phenotype and more severe HSV-1 induced lesions (93). These effects were presumably mediated by NK cells licensing APCs to facilitate effective APC-T cell interaction, and by production of cytokines such as IL-15 and IFN-γ that promote CTL responses. Studies by Kastrukoff et al. also show that depletion of both NK cells and CD8 T cells resulted in viral spread in the CNS, but depletion of either NK cells or CD8 T cells did not, suggesting there is redundancy in the immune system that is responsible for restricting virus replication (89).

Furthermore, NK cell-DC interactions are important in maturation of DCs that are responsible for inducing T cell responses. NK cells secrete IFN-γ and TNF-α that are needed for proper DC maturation (94) and recent studies using human NK cells show that NK cells can up-regulate co-stimulatory molecules on DCs and increase production of inflammatory cytokines by DCs (95).

1.2.2 Adaptive Immunity against HSV-1 during latent infection

Adaptive immune responses are initiated in the draining lymph nodes (DLN) within a few days of infection by DC presentation of viral antigens to CD4 and CD8 T cells in the context of MHC. Robust proliferation and expansion of HSV-1-specific T cells takes place and subsequently HSV-1-specific CD8 T cells travel to infected TG at 5~6 dpi. The number of HSV-
1-specific CD8 T cells in the TG peaks at 8 dpi and then undergoes contraction to maintain a small, but stable memory resident CD8 population within the TG long after latency is established. Although there is no virion formation during HSV-1 latency, presentation of viral antigens is suggested by formation of immunologic synapses between CD8+ T cells and neurons (19, 96). These observations have been extended to humans where CD8 T cells with an activated phenotype are retained in the TG surrounding HSV-1-infected neurons (96, 97).

In the C57BL/6 mouse model of corneal HSV-1 infection, 50% of HSV-1-specific CD8 T cells recognize an immunodominant epitope on glycoprotein B (gB498-505) (Fig 2) (98). It has not been established what contributes to this striking immunodominance but it has been suggested that precursor frequency, antigen presentation and/or processing, and the route of infection can play a role. Interestingly, the immunodominance hierarchy in the TG after 8 dpi is constantly maintained at a ratio of 1:1 between gB498-505 CD8 T cells (gB-Tet+CD8) and non-gB498-505 CD8

**FIGURE 2** The 1:1 ratio of gB-specific CD8 T cells and subdominant gB-nonspecific CD8 T cells in the TG following HSV-1 infection The kinetics of the CD8 T cell response was determined by staining CD8 T cells from infected TG at indicated time points with fluorescence conjugated tetramers that detect gB498-505 epitope. Figure courtesy of Brian Sheridan, PhD.
T cells (gB-Tet⁺ CD8). Recently our lab has discovered all of the 18 subdominant epitopes that gB-Tet⁺ CD8 are specific for (Fig 3) (99).

Another interesting point to note is that there are functional and phenotypic differences between gB-Tet⁺ CD8 cells and gB-Tet⁻ CD8 cells. During acute infection, almost all of gB-Tet⁺ CD8 T cells produce IFN-γ, granzyme B (grz B), and have activated phenotype (high expression of CD27, CD69, and low expression of CD62L, CD127) whereas a significantly lower frequency of gB-Tet⁻ CD8 T cells produce IFN-γ, grz B and activated cells (98, 100). These differences are even more striking during latency when only a small portion of gB-Tet⁻ CD8 T cells that is still activated and functional (100).

FIGURE 3 The immunodominance hierarchy of HSV-1-specific CD8 T cells within the TG of C57BL/6 mice at 8 dpi. TG from 8 dpi mice were dispersed into single-cell suspensions. Tissues were then incubated for 6 h with peptide-loaded B6WT3 fibroblasts in the presence of Golgi-Plug, followed by intracellular staining for IFN-gamma. Figure courtesy of Anthony St. Leger, PhD.
Previous studies from our lab have demonstrated that HSV-1-specific CD8 T cells can block HSV-1 reactivation as demonstrated by CD8 T cell depletions of ex vivo cultures of latently infected TG neurons. There are two main mechanisms by which virus-specific CD8 T cells can combat the virus, which are 1) release of anti-viral cytokines such as IFN-γ and 2) release of lytic granule grz B that kills infected targets. When stimulated \textit{ex vivo} with HSV-1 antigens, HSV-1-specific CD8 T cells from latently infected TG can produce and release IFN-γ and grz B suggesting that these indeed are the host CD8 T cells’ strategies to prevent reactivation.

Inside HSV-1-infected neurons, IFN-γ can regulate the expression of over 200 genes that code the host’s cellular proteins (101). It is thought that IFN-γ released from HSV-1-specific CD8 T cells may regulate some of host transcription factors that regulate the production of viral proteins to indirectly influence reactivation or that it can directly block viral reactivation in neurons by inhibiting the expression of viral genes that are essential for virion formation and full reactivation. For instance, IFN-γ can inhibit expression of immediate early genes such as ICP0, and can also block a step that occurs after the expression of some late viral structural genes such as gC and gH genes (18, 22). However, IFN-γ could only block reactivation in 50% of TG neurons, while CD8⁺ T cells can completely block reactivation, suggesting that CD8 T cells utilize a second mechanism to prevent virion formation in latently infected neurons.

The second mechanism by which CD8 T cells can provide protection from HSV-1 reactivation is through grzB. In any viral infection, there has to be a balance between effective viral clearance and protection from severe immunopathology. Since neurons are non-regenerative, it is crucial that they are protected from any lytic damage. Although grzB is better known as a lytic protein that can enter infected cells to cleave caspases and induce cell death, it
is now apparent that grzB released by HSV-1-specific CD8 T cells can block HSV-1 reactivation without killing the infected neurons (23). GrzB can specifically cleave the ICP4 viral protein that is necessary for the expression of early and late HSV-1 proteins and thus successful viral reactivation. Furthermore, LATs produced abundantly during HSV-1 latency can inhibit caspase activation by CD8+ T cells (102, 103) contributing to preservation of neurons at the same time maintaining latency.

Although the role of CD8 T cells in HSV-1 infection has been extensively studied, the role of CD4 T cells is not as clear. CD4 T cells infiltrate the TG and the response peaks at a later time than CD8 T cells (about 10 dpi) maintaining a stable population throughout latency as well. It has been shown that CD4 T cells are not directly involved in the maintenance of HSV-1 latency and that they are not required for maintenance of CD8 T cell memory after 34 dpi (104). gB-Tet+ effector CD8 T cells do undergo contraction more rapidly in the absence of CD4 T cells, but are not functionally compromised. However, CD4 T cell help is critical in regulating the functional avidity of PD-1-mediated exhaustion of HSV-1-specific CD8 T cells in the ganglia. Non-helped gB-Tet+ CD8 T cells showed a progressive functional compromise from 8-35 dpi, which was most pronounced among the high-avidity polyfunctional (IFN/TNF+) cells. When the ligand of PD-1 (PD-L1) was blocked in vivo, it restored function to the non-helped, functionally compromised gB-Tet+ CD8 T cells in the TG (104). Despite the lack of direct involvement of CD4 T cells in maintaining HSV-1 latency, CD4 T cells may be “helping” CD8 T cells establish their proper functionality and possibly play a “back-up” role for protection from virus reactivation in the absence of CD8 T cells.

CD4 T cells seem to be activated during latent HSV-1 infection since at least 16.4% of them express the suppressive cytokine, IL-10. In fact, in a latently infected TG, they are the main
source of IL-10, which preferentially suppresses gB-Tet\(^-\) CD8 T during latency (100). Upon blockade of IL-10R, the functionality of subdominant CD8 T cells was dramatically increased as shown by grzB and IFN-\(\gamma\) staining as well as the numbers and thus increased their ability to block HSV-1 reactivation \(\textit{ex vivo}\). Moreover, there was a significant reduction reactivation frequency when the mice were treated with anti-IL-10R antibody for 3 weeks and then rested without any treatments for another 3 weeks. This suggested that brief immunotherapy involving blockade of IL-10 signaling could effectively inhibit HSV-1 reactivation and thus be an attractive option for controlling recurrent herpetic disease.

1.3 IMMUNE REGULATION BY INHIBITORY RECEPTORS

1.3.1 T cell Exhaustion

![FIGURE 4 T cell exhaustion](image)

<table>
<thead>
<tr>
<th></th>
<th>+++</th>
<th>++</th>
<th>+/-</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-(\gamma)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>CTL</td>
<td>++/-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>IL-2</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proliferation</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
</tbody>
</table>
| Apoptosis      | -   | -  | +/- | ++  | +++
T cell exhaustion is a term describing a dysfunctional state of T cells during high levels of prolonged antigenic stimulation. Exhausted T cells were first identified in a chronic LCMV viral infection (105) but since then they also have been found and characterized in other disease models that such as cancer, bacterial, and parasitic infections (106). During exhaustion, T cells undergo a series of functional loss in a hierarchial manner, which seems to be dependent on the levels of inhibitory molecules expression (Fig 4). Exhausted T cells will first experience loss of proliferative capacity, IL-2 expression, and cytotoxicity. Functions such as TNF production are lost in the intermediate stage whereas a complete or partial IFN-γ loss indicates a more severe and final stage of exhaustion. Following IFN-γ loss, T cells will finally undergo apoptosis that results in their physical deletion.

The exhaustion state is tightly linked to the level T cell antigenic exposure. Higher viral load correlates with more severe exhaustion and epitopes presented in higher levels lead to a more extreme exhaustion and even physical deletion, influencing the immunodominance of virus-specific CD8 T cell repertoire (107). Therefore, the strength of antigenic stimulation determines the state of exhaustion, suggesting the importance of epitope escape in CD8 T cell function. Furthermore, loss of CD4 T cell help and longer duration of infection can also be factors that drive exhaustion.

There are a number of inhibitory molecules that are associated with CD8 T cell exhaustion (108). Many of these have been studied in the context of murine chronic infection models and the phenotype of exhausted CD8 T cells seems to vary between different disease models. There are two main factors that can contribute to exhaustion: cell-extrinsic pathways such as immunoregulatory cytokines and cell-intrinsic molecules such as inhibitory receptors.
Some examples of immunoregulatory cytokines include IL-10, and TGF-β. CTLA-4 and PD-1 are some of the well-known inhibitory receptors. Many of PD-1+ exhausted T cells co-express inhibitory receptors on their surface, such as LAG-3, CD244 (2B4), CD160, and TIM-3 (109, 110). The different patterns and combination of inhibitory molecule co-expression seem to determine the severity of dysfunction, suggesting that many of these regulatory pathways are distinct and not redundant.

A number of studies have utilized these regulatory pathways to “reverse” CD8 T cell exhaustion and restore T cell function (Fig 5). In vivo blockade of these inhibitory molecules results in enhanced pathogen clearance, tumor size reduction and increased survival rate due to increased T cell proliferation and or function (111). In some cases, the process of functional restoration of T cells seems to be far more efficient when targeting different subsets of exhausted T cells by blocking multiple inhibitory pathways (LAG-3, Tim-3, CD160, 2B4, IL-10) at the same time (112-115). However, it also has been shown that blockade of these inhibitory pathways can lead to more severe immunopathology and tissue damage in disease models such as autoimmunity implicating some potential side effects that could arise in treating human patients. Efforts to optimally manipulate these pathways to reverse T cell exhaustion and achieve the maximum therapeutic efficacy without damage to the host are necessary.

1.3.2 Programmed Death-1 (PD-1) Signaling

PD-1 was named due to the discovery of its gene that was up-regulated on a T cell hybridoma undergoing apoptosis (116). PD-1 can be expressed on activated T cells, B cells, NK cells, activated monocytes, and DCs. Low levels of PD-1 can be transiently expressed on effector T cells during priming, but it is better known as a marker for T cell exhaustion although more
recent studies are suggesting that PD-1 expression alone is not necessarily an indicator of complete exhaustion (109, 117-119). PD-1 may exert its inhibitory effects by negatively regulating early activation events that promote cell expansion and survival by CD28 or indirectly through IL-2 (120). It has been demonstrated that PD-1 ligation inhibits the induction of Bcl-XL (121), one of the cell survival factors. PD-1 can also inhibit transcription factors such as GATA-3, T-bet, and Eomes which are crucial for effector T cell function (122). More recently, a study by Zinselmeyer et al. has shown that PD-1 can prevent antiviral CD8 T cells from performing their functions by inducing motility paralysis and prolonging the period of exhaustion (123).

Once PD-1 is engaged by its ligand, it is phosphorylated on its intracellular tyrosines (ITIM and ITSM motifs), which subsequently recruit phosphatases SHP-2 and SHP-1 to the antigen receptor signaling complex. It has been shown that the proximity of PD-1 to the T cell receptor is important for PD-1-mediated inhibition to occur as PD-1 inhibits TCR signaling only in \textit{cis} and not in \textit{trans} (124). Further down the TCR signaling, PD-1 can inhibit PI3K activity and downstream activation of Akt. It also inhibits phosphorylation of many components of the TCR activation signaling complex including CD3ζ, ZAP70, and PKCθ (125).

PD-1 has two ligands, PD-L1 and PD-L2. Unlike PD-1, PD-L1 (CD274, B7-H1) is expressed on a wide variety of cells and tissue including not only hematopoietic cells but also non-hematopoietic cells. PD-L2, on the other hand, is mainly induced on DCs and macrophages. PD-L1 seems to be the major binding partner of PD-1 for PD-1 to exert its inhibitory function but it also has another receptor, B7.1 also known as CD80 the interaction with which results in an inhibitory signaling pathway as well (126). There is evidence that type I IFNs and IFN-γ can induce PD-L1 expression (127, 128). Both constitutive and inducible PD-L1 expression are dependent on IFN regulatory Factor-1 (IRF-1) binding sites (129).
Before discussing the role of PD-1-PD-L1 interaction in regulating T cell responses, it is important to note that PD-1 and PD-L1 have multiple binding partners and that there is bidirectional signaling of PD-1 and PD-L1, which complicates interpretations of studies using different antibodies and knockout mice. Using PD-1$^{-/}$ and B7-H1$^{-/}$ (PD-L1$^{-/}$) mice as opposed to treatment of blocking antibodies such as aPD-L1, aPD-L1, and aPD-1 have shown some contradictory results. Many in vivo studies using blocking antibodies have seen a more dramatic effect with anti-PD-L1 blockade than with anti-PD-1 or anti-PD-L1, which seems to be mainly due to different affinities of the antibodies or to the differential expression of PD-L1 and PD-L2. For instance, one clone of anti-PD-L1 antibody (10F.9G2) blocks both PD-1-PD-L1 interaction and B7.1-PD-L1 interaction while the clone 10F.2H11 specifically blocks only B7.1-PD-L1 interaction (130).

1.3.3 The role of PD-L1 in regulating adaptive immune responses

PD-1 and PD-L1 have been most extensively studied in chronic infection model, notably LCMV infection (111, 131). High PD-1 expression is maintained on virus-specific CD8 T cells during chronic infection and correlates with disease severity as well as the intensity of T cell exhaustion and viral load (132, 133). Upon blockade of PD-L1 in vivo, the ability of exhausted CD8 T cells to proliferate, kill infected targets, and secrete cytokines is restored and maintained while viral burden is substantially decreased (Fig 5). This seems to be the same case for other chronic viral infection models such as HIV, HBV, and HCV where blocking PD-1-PD-L1 interaction reinvigorates not only virus-specific CD8 T cells but also CD4 T cells (134-137). The suppressive role of PD-L1 has also been implicated in bacterial and parasitic infection models as well (138, 139).
PD-L1 also negatively regulates tumor immunity. PD-L1 expression can be found on a variety of tumor tissue and tumor-associated APCs, acting as an immune escape mechanism by tumor cells (140-144). In an immunosuppressive tumor microenvironment, many tumor-infiltrating CD8+ lymphocytes (TIL) express high PD-1 and PD-L1 expression is inversely correlated with the number of TILs, suggesting that PD-L1 can inhibit the migration and infiltration of tumor-specific CD8 T cells. PD-L1 expression also has a link to poor prognosis in many cancer models and PD-L1 blockade in animal models leads to enhanced T cell activation, increased lysis of tumor cells, and decreased cell death of tumor-specific T cells.

While efficient pathogen clearance or tumor control is inhibited by PD-L1, the broad expression of PD-L1 can play a protective role such as host tissue preservation from pathogenic T cell responses (145-147). There are a number of studies indicating that PD-1 is important for induction and maintenance of central and peripheral tolerance. PD-L1 provides a negative signal to PD-1+ thymocytes in the thymus and inhibits positive selection process (148-150). PD-L1 also plays a role in controlling peripheral tolerance by inducing and maintaining regulatory T cells.

**FIGURE 5** “Reinvigorating” the CTL response by PD-L1 blockade.
(Treg), and regulating their function (151-153). In humans, this has implications in autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Multiple Sclerosis (MS), and type I diabetes many of which are linked to polymorphisms in PDCD1 gene (154). In contrast to chronic infection and tumor models, blocking PD-1-PD-L1 interactions in an autoimmune disease setting results in accelerated onset and increased severity of disease as autoreactive T cells are no longer controlled to down-regulate their function by PD-L1. A study using bone marrow chimeras also demonstrates that PD-L1 on non-hematopoietic cells and PD-L1 on hematopoietic cells have different roles in host tissue protection and immune suppression (155).

Up-regulation of PD-L1 by the host cytokines such as IFN-γ suggests that PD-L1 may be an active negative feedback strategy by the host to prevent tissue damage during severe inflammation. Blocking PD-L1 can be an attractive option for treatment of chronic infections as well as cancer therapy but promoting PD-L1 seems to be beneficial in autoimmune diseases and transplantation. In conclusion, PD-1-PD-L1 serves an important function controlling the balance between efficient T cell-mediated pathogen clearance and immunopathology. In HSV-1 corneal infection, the virus infects corneal epithelium as well as neurons in the TG. PD-1 is up-regulated on CD8 T cells in HSV-1 latently infected human TG and PD-L1 is up-regulated on ocular tissue and TG neurons during inflammation. Both corneal and neuronal tissue are non-regenerative and therefore it is crucial that viral clearance is achieved with minimum inflammation-mediated damage to the tissue.
1.3.4 The role of PD-L1 in regulating innate immune responses

While the role of PD-L1 in regulating adaptive immune responses is extensively studied, we are only beginning to understand how PD-L1 affects innate immune responses. However, there are a few studies suggesting that PD-L1 also can regulate innate immune responses. In a study using intranasal influenza infection model, PD-L1 on naïve T cells was shown to regulate DC maturation and function (156). In PD-L1−/− mice, the authors in the study observed reduced CD8 T cell responses against influenza virus and saw that the DCs in the draining lymph nodes were less mature suggesting that immature DCs in the absence of PD-L1 led to suboptimal priming of CD8 T cells. However, it is not clear whether PD-L1 on T cells influenced DC maturation through its ligand CD80 or PD-1 on DCs. Although it is counterintuitive that the absence of PD-L1 could result in diminished CD8 T cell responses, in some cases PD-L1 can also be co-stimulatory as well as inhibitory.

Other studies have shown negative effects that PD-L1 has on APC function. During Listeria monocytogenes infection, PD-1−/− DCs produced higher levels of IL-12 and TNF, providing better protection against bacterial infection compared to Wild type DCs (157). PD-L1 seems to also inhibit macrophage function through PD-1 pathway. In a study using a bacterial sepsis model, severe macrophage dysfunction was associated with PD-1 expression on macrophages and PD-1−/− mice showed an increased survival rate (158). In vitro studies using a mouse macrophage cell line also revealed that PD-1 engagement can down-regulate LPS-mediated IL-12 release (159). A more recent study showed that during visceral Leishmaniasis, PD-1-mediated T cell exhaustion led to impairment of phagocyte function including macrophages’ Reactive Oxygen Intermediate (ROI) production (160).
There is increasing evidence that NK cells are inhibited in terms of function and proliferation through PD-1-PD-L1 interaction. A recent study showed that during chronic HIV infection, not only CD8 T cells but also a small subset of NK cells are PD-1⁺ which was associated with limited NK cell proliferation (161). Other studies using transplant, cancer, and bacterial infection models have demonstrated the role of PD-L1 in inhibiting NK cell function such as IFN-γ production and cytotoxicity by showing a direct link between PD-1 on NK cells and down-regulation of function as well as showing that PD-1 blockade can restore function to NK cells (162-164).

1.3.5 PD-1-PD-L1 interactions in the eye

In the eye, PD-L1 serves functions that are unique to the eye tissue. PD-L1 is expressed in human ocular cell lines and is significantly up-regulated in inflamed ocular tissues (165), suggesting its role in inhibition of immune-mediated ocular inflammation. IFN-γ was able to induce expression of PD-L1 on human ocular cell lines and co-culture with activated T cells decreased cytokine production by T cells but did not induce apoptosis although a previous study suggested that PD-L1-induced apoptosis of T effector cells in the cornea may be a mechanism of immune privilege that promotes corneal allograft transplants (166). Another study using corneal allograft model demonstrated that there are differential functions of recipient vs donor PD-L1 as the host PD-L1 affects T cell priming in the draining lymph nodes whereas the donor PD-L1 locally inhibits T cell infiltration into the donor cornea (167). There have been efforts to utilize this inhibitory effect for corneal allograft survival by overexpressing PD-L1 through Lentiviral vectors (168). In contrast to corneal transplants, there is little data on how PD-L1 influences
development of HSK. One study, however, suggests that PD-L1 inhibits HSK development by suppressing IFN-γ production by CD4 T cells in the draining lymph nodes and inducing their apoptosis (169).

Two studies by Reza Dana’s group showed that PD-L1 can also control corneal angiogenesis and T cell chemotaxis in a suture-induced corneal angiogenesis model and a Dry Eye Disease (DED) model respectively (170, 171). PD-L1−/− corneas had increased expression of chemokines and thus enhanced T cell infiltration, which led to more severe dry eye-associated corneal inflammation and epithelial lesion. PD-L1−/− mice also show higher levels of angiogenesis, but this did not lead to any increased levels of cytokines or neutrophil and macrophage cell recruitment. Interestingly, PD-L1 and CD80 were expressed on both corneal epithelial cells and vascular endothelial cells but PD-1 was not, suggesting that these effects may be partially mediated by PD-L1-CD80 interactions.
2.0 STATEMENT OF THE PROBLEM

Current treatment options for herpetic corneal disease include the use of anti-virals and corneal transplantation. However, the use of anti-virals only acts to suppress viral replication once reactivation occurs and does not prevent HSV-1 reactivation. Also, corneal grafts on beds previously infected with HSV-1 have limited success due to subsequent recurrent reactivation events and the patients have to be on immunosuppressants for life following transplant surgery.

Our lab has previously established the role of TG-resident CD8 T cells in preventing HSV-1 reactivation. Frequent and recurrent HSV-1 reactivations from the ophthalmic branch of the TG eventually lead to corneal scarring and immunopathology in the cornea, resulting in opacity and irreversible damage to non-regenerative corneal tissue. Therefore it is of significant clinical relevance to develop therapies that are designed to decrease the latent viral load and boost the HSV-1-specific CD8 T cell response to prevent HSV-1 reactivation.

The interaction of PD-1 on immune cells and its ligand PD-L1 is known to result in inhibition of immune cell proliferation, function, and survival. Understanding the exact mechanisms of immune regulation mediated by PD-1-PD-L1 interaction following HSV-1 ocular infection will give us insight to developing better therapy options for patients with herpetic eye disease.
3.0 SPECIFIC AIMS

Specific Aim 1: Determine the role of PD-L1 in regulating HSV-1-specific CD8 T cell response within trigeminal ganglia latently infected with HSV-1

Hypothesis: PD-L1 expressed on neurons inhibits the function, proliferation, and survival of PD-1+ HSV-1-specific CD8 T cells in latently infected trigeminal ganglia.

Our preliminary findings indicate that although all of HSV-1-specific CD8 T cells during acute HSV-1 infection produce IFN-γ, some subdominant epitope-specific CD8 T cells show significantly decreased functionality during latent HSV-1 infection. Based on previous studies, engagement of PD-L1 to PD-1 on virus-specific CD8 T cells results in decreased proliferation, cytotoxicity, and functionality of CD8 T cells during chronic viral infection but it is not clear what the role of PD-1 on CD8 T cells during latent viral infection.

Specific Aim 2: Determine the role of PD-L1 in regulating the innate immune cell response within HSV-1-infected cornea during acute HSV-1 infection

Hypothesis: PD-L1 suppresses the chemokine production responsible for innate immune cell recruitment to the cornea and directly inhibits the function and proliferation of PD-1+ innate immune cell populations present within the cornea during acute HSV-1 infection.
HSV-1 clearance from the cornea is mediated mainly by innate immune cells that are recruited to the infected site. The amount of live virions in the cornea during the first few days determines the amount of virus that travel to the TG to establish latency and thus effective control of viral replication in the cornea during primary infection is crucial. Some studies have suggested an inhibitory role of PD-1 and PD-L1 in regulation of innate immune cells but its mechanism is not clear.

Specific Aim 3: Determine the best anti-PD-L1 blocking antibody treatment regimen that will enhance the immune responses against HSV-1 without any side effects

Hypothesis: Blocking PD-1-PD-L1 interaction in vivo during T cell clonal expansion following the initial T cell priming phase results in a robust CD8 T cell response as early as 8 days post-infection.

Many studies using models of chronic infection and cancer have seen improved immune responses, and enhanced clearance of pathogen or tumor cells with PD-L1 blockade. However, a study using influenza infection model has also suggested a costimulatory role of PD-L1, demonstrating that PD-L1 is required for proper maturation of DCs in the draining lymph nodes before and during the initial priming phase. This means that the time, and the route of anti-PD-L1 treatments need to be tightly controlled to prevent any unfavorable outcome in a clinical setting.
4.0 MATERIALS AND METHODS

4.1 MICE

Female 6–8 wk old WT C57BL/6 mice and IFN-γ receptor\(^{-/-}\) (B6.129S7-Ifngr\(^{tm1Agt}\)/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B7-H1\(^{-/-}\) mice were provided by Dr. Lieping Chen (Yale University School of Medicine, New Haven, CT). 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 Institutional Animal Care and Use Committee.

4.2 VIRUS AND CORNEAL INFECTIONS

Wild-type (WT) HSV-1 strain KOS, ICP0-eGFP (generated by Dr. Kinchington) or RE strain Lausch (generously given by Dr. Lausch) was grown in Vero cells, and intact virions were isolated on Optiprep gradients according to the manufacturer’s instructions (Accurate Chemical and Scientific, Westbury, NY). Mice were anesthetized by i.p. injection of 2.5 mg ketamine hydrochloride and 0.25 mg xylazine (Phoenix Scientific, San Marcos, CA) in 0.25 ml HBSS (BioWhittaker, Walkersville, MD). Mice received bilateral topical infection on scarified corneas with a dose of \(1 \times 10^5\) PFU or \(1 \times 10^4\) PFU HSV-1 per cornea.
4.3 REAGENTS FOR FLOW CYTOMETRY

PE-conjugated H-2K^b tetramers conjugated to the gB_{498-505}, RR1_{982-989}, or RR1_{822-829} peptides were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Rat anti-mouse Pacific blue-conjugated anti-CD8α (clone 53-6.7), APC-conjugated anti-IFN-γ (clone XMG1.2), PerCP-conjugated anti-CD45 (clone 30-F11), PE-Cy7-conjugated anti–NK1.1 (clone PK136), and APC-conjugated anti-granzyme B (clone GB11) were purchased from BD Pharmingen. PE-conjugated anti-PD-L1 (clone MIH5), APC-eFluor780-conjugated anti-Thy1.2 (clone 53-2.1), eFluor 450-conjugated anti–CD3 (clone 17A2), allophycocyanin-conjugated anti–CD11c (clone N418), APC-eF780-conjugated anti–Gr-1 (clone RB6-8C5), and eFluor 450-conjugated anti–F4/80 (clone BMB) were purchased from eBioscience. A FITC-conjugated antibody specific for the neuronal nucleus marker (NeuN; clone A60) was purchased from Millipore (Billerica, MA). The PE-Cy7-conjugated anti-PD-1 (clone RMP1-30) was purchased from Biolegend (San Diego, CA). Appropriate isotype control Abs were purchased from BD Pharmingen, eBioscience, or Biolegend. For intracellular staining, Cytofix/Cytoperm and Permeabilization Buffer from BD Biosciences were used. All flow cytometry samples were collected on a FACSAria cytometer and analyzed by FACSDiva software (BD Biosciences). Graphpad Prism software (La Jolla, CA) was used for all statistics.
4.4 TISSUE PREPARATION

Anesthetized mice were injected with 0.3 ml heparin (1000 U/ml) and euthanized by exsanguination. Excised TG were digested in 100 μl per TG of DMEM (Bio Whittaker) containing 10% FCS and 400 U/ml collagenase type I (Sigma-Aldrich) for 1 hour at 37°C, and dispersed into single-cell suspensions by trituration through a p-200 pipette tip.

Corneas were excised, processed under a dissecting microscope to separate the cornea and conjunctival tissue, and then were digested in 100 μl per cornea of DMEM (Bio Whittaker) and 800 U/ml collagenase type I (Sigma-Aldrich) for 1 hour at 37°C, vortexing every 20 minutes, and dispersed into single-cell suspensions by trituration through a p-200 pipette tip.

4.5 GENERATION OF BONE MARROW CHIMERIC MICE

Bone marrow chimeras were created by tail vein transfer of $2 \times 10^6$ bone marrow cells from 6-wk-old WT C57BL/6 mice into 6-wk-old lethally irradiated (2 × 500 rad treatments separated by 4 h rest) WT C57BL/6 recipients (WT→WT), B7-H1−/− recipients (WT→B7-H1−/−) and IFN-γ receptor−/− recipients (WT→IFN-γR−/−) or bone marrow cells from 6-wk-old B7-H1−/− mice into 6-wk-old lethally irradiated WT C57BL/6 recipients (B7-H1−/−→WT). The resulting mice were housed under immunocompromised mouse conditions for 8 wk after the transfer, and neomycin sulfate (2 mg/ml) from Sigma-Aldrich (St. Louis, MO) was put in their drinking water for 2 wk after the transfer. Bone marrow chimeras were fully reconstituted after 8 wk.
4.6 SORTING NEURON SUBPOPULATIONS

TGs were harvested from HSV-1 latently infected C57BL/6 mice at 32 dpi, pooled and dispersed into single cell suspensions as described above. TG cells were stained for surface markers (CD45, Thy1.2, and PD-L1) for 1 hour at 4°C, were fixed and permeabilized using cytofix/perm from BD bioscience and intracellularly stained for NeuN. The neurons (CD45⁺, Thy1.2⁺, NeuN⁺) were sorted into > 95% pure PD-L1⁺ and PD-L1⁻ subpopulations using a FACSaria cytometer. Sorted cells were then used for quantification of HSV-1 gH DNA copies as described below.

4.7 QUANTIFICATION OF VIRAL GENOME COPIES

The number of copies of HSV-1 genome in latently infected TG was determined by real-time PCR quantification of the HSV-1 glycoprotein H (gH) gene in DNA extracts of individual TG or neuronal subpopulations as previously described (23). Briefly, DNA was extracted from dispersed TG cells using DNeasy columns (Qiagen) according to manufacturers’ instructions, and quantified spectrophotometrically. The DNA was mixed with TaqMan Universal PCR Master Mix (Roche, Branburg, NJ) and an HSV-1 gH-specific primer-probe set, custom designed and synthesized by ABI Assays-by-Design service (applied Biosystems, Foster City, CA). Samples, standards, and controls were assayed in 96-well plates with an ABI Prism 7700 sequence detector. ABI Primer Express v1.5a software default settings were used for instrument control and data analysis. The gH sequences were: forward primer (5’–GACCACCAGAAACCCTCTTT-3’), reverse primer (5’-
ACGCTCTCGTCTAGATCAAAGC-3’), and probe (5’-[FAM]TCCGGACCATTTC[NFQ]-3’). The number of copies of gH DNA in each sample was determined from a standard curve generated using known concentrations of gH-containing plasmid standards, and the number of copies of viral gH DNA/TG was calculated based on the total DNA extracted from each TG.

4.8 MEASUREMENT OF CD8 T CELL PROLIFERATION

Latently infected mice received 2 daily intraperitoneal injections of BromodeoxyUridine (BrdU) (1mg/mouse) to measure CD8⁺ T cell proliferation. TG were excised 24 hrs after the second BrdU injection and dispersed cells were stained with anti-CD45, anti-CD8α, and tetramers for 1 h at room temperature. CD8⁺ T cells that divided over the 2-day period were quantified by flow cytometry using a BrdU proliferation assay kit (BD Pharmingen, Cat # 559619) according to manufacturers’ instructions.

4.9 MEASUREMENT OF CD8 T CELL APOPTOSIS

A Caspatag assay kit (Chemicon International, Cat # APT420) was used according to manufacturer’s instructions to quantify cells CD8⁺ T cells undergoing apoptosis. CD8⁺ T cells from TG latently infected with HSV-1 were surface stained with antibodies to CD45, CD8, and fluorescently tagged tetramers containing the immunodominant epitope gB498-505. Following a wash with FACS buffer, cells were incubated with Caspatag FLICA reagent (1μl of the 30X
FLICA reagent/TG) for 1 hour at 37°C and were washed with Caspatag Wash buffer twice at the end of incubation to be collected on flow cytometer.

4.10 IN VIVO TREATMENTS

For in vivo neutralization of IFN-γ, IFN-γ neutralizing antibody (XMG1.2) purchased from BioXcell was injected intraperitoneally (1mg/mouse). For in vivo blockade of PD-L1, anti-PD-L1 antibody (clone 10F.9G2 from BioXCell, West Lebanon, NH) was used. For in vivo NK cell depletions, rabbit anti-ASGM-1 antibody (Wako Pure Chemicals, Osaka, Japan) was used. For local PD-L1 blockade or local NK cell depletions, mice received subconjunctival injections of 40 ng anti-PD-L1 Ab or 10 ul anti-ASGM-1 Ab at -2 dpi, 1 dpi, 4 dpi and at 6 dpi corneas were harvested to confirm blockade/depletion. For systemic blockade, intraperitoneal injections of anti-PD-L1 antibody (200ug/mouse) were given once every 3 days.

4.11 EX VIVO STIMULATIONS OF CD8 T CELLS IN LATENTLY INFECTED GANGLIA

B6WT3 fibroblast targets were infected with HSV-1 (10 pfu/cell for 6 hours) or pulsed with peptides (gB_{498-505}, RR1_{982-989}, or RR1_{822-829}) at a concentration of 0.8ug/ml for 45 min at 37°C/5% CO₂. Cells from each TG were stimulated with 5x10⁵ HSV-1-infected or peptide-pulsed fibroblasts in the presence of RPMI/10% FCS, and Golgi-Plug (BD Biosciences) for 6 h at 37°C/5% CO₂. In some experiments, TG cells were pretreated with PE-conjugated gB_{(498-505)}
tetramers (2µg/ml) for 30 min at 37°C/5% CO₂ prior to stimulation with HSV-1 infected B6WT3 cells. Following stimulation, cells were surface stained for CD8α, permeabilized and fixed with Cytofix/Cytoperm (BD Biosciences), and stained for intracellular IFN-γ and TNF-α.

### 4.12 EX VIVO TRIGEMINAL GANGLION NEURON CULTURES

HSV-1 latently infected TG cells were dispersed and cultured in 48-well tissue culture plates (0.2 TG/well) in culture medium (DMEM containing 10% FCS, 500 U/ml rIL-2 (R & D Systems, Minneapolis, MN), and 50 µm 2-mercaptoethanol (2-ME; Fisher Scientific, Fair Lawn, NJ) with or without anti-CD8α antibody (100µg/ml)) for up to 10 days. Supernatants were serially sampled and replaced with a similar volume of culture medium, and samples were assayed for the presence of infectious virus on Vero cell monolayers as previously described (18).

### 4.13 DETECTION OF INFECTIOUS VIRUS FROM CORNEA

Mouse corneas were swabbed with sterile Weck-Cel surgical spears (Beaver Visitec, Waltham, MA) at various time points following HSV-1 infection; spears were then placed in 0.5 ml HBSS and frozen at −80°C until assayed. Samples were added to confluent Vero cells, incubated for 1 h at 37°C, and overlaid with 0.5% methylcellulose. The cultures were incubated for 72 h, fixed with formalin, stained with crystal violet, and a viral cytopathic effect was detected with the aid of a dissecting microscope.
4.14 WHOLE MOUNT FLUORESCENT MICROSCOPY

Whole corneas were excised, washed in PBS 4% FBS, fixed in 1% paraformaldehyde for 2 h, washed once more in PBS 4% FBS, incubated in Fc Block (BD, San Jose, CA) for 1 h, and then incubated with staining antibodies in PBS overnight at 4°C. The next day, corneas were extensively washed in PBS and then were flattened by making radial incisions and mounted. For intracellular chemokine staining, corneas were fixed in Cytofix/Cytoperm for 2 h, washed in Permeabilization buffer from BD Biosciences prior to staining. Images were acquired on an Olympus Fluoview 1000× confocal microscope with a 0.85 NA 20× oil objective. Images were acquired by sequential scanning to avoid fluorescence crossover, and Z stacks were acquired at Nyquist sampling frequency through the tissue. All image reconstructions were made using Olympus Fluoview.

4.15 MEASUREMENT OF MESSENGER RNA

Total RNA was extracted using Qiagen’s RNA extraction kit according manufacturer’s protocol. Samples of total RNA (100 ng) were submitted to the University of Pittsburgh Genomics and Proteomics Core laboratory (GPCL) for analysis using the Nanostring system (Nanostring Technologies, 530 Fairview Avenue N, Seattle, WA 98109). The Nanostring system permits the multiplex measurement of nucleic acid sequences in a sample of this size (see Geiss et al 2008). The system is intermediate in throughput, sensitivity and cost between real-time quantitative PCR (RT-QPCR) and microarrays such as the Affymetrix gene chip. Samples are hybridized overnight to a Codeset, a custom mixture of synthetic capture probes and reporter
probes. The two probe types are complementary to different specific sequences in each mRNA target. After hybridization, [capture probe-target-reporter probe] complexes are bound to a solid substrate via the capture probe moiety, while excess probes and targets are washed off. Digital scanning then counts the number of different reporter probes which have been captured (a target-dependent process). Counting of multiple targets in the same sample (multiplexing) can occur because the reporter probes have six distinct fluorescent-labeled sites, each of which can support one of four fluorescent colors. Theoretically this yields $4^6 = 4,096$ unique “molecular barcodes”, each of has been assigned to a distinct target sequence. Fluorescent sequences (barcodes) which are very similar cannot be usefully distinguished, but concurrent measurement of up to 800 different targets is possible in the present system.

4.16 GATING STRATEGIES AND STATISTICAL ANALYSIS

For PE-conjugated gB$_{498-505}$, RR1$_{982-989}$, or RR1$_{822-829}$ tetramer staining, negative controls (without PE-conjugated tetramer stain) were used to gate tetramer-positive cell populations. For all surface and intracellular staining, appropriate isotype control Abs were used to gate positive populations by FACSDiva software (BD biosciences). For experiments with two groups, unpaired student’s t test was used to determine statistical significance and ANOVA was used for experiments with more than two groups by Graphpad Prism software.
4.17 IN VITRO DC-T CELL INTERACTION ASSAY

Bone marrow was isolated from the femurs and tibiae of either wild type or B7-H1− mice. RBCs were lysed with ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2). Cells were washed and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all purchased from Invitrogen, Carlsbad, CA) supplemented with 1,000 U/ml granulocyte-macrophage colony-stimulating factor (Schering Plough, Kenilworth, NJ), as previously described (172). On day 7, CD11c+ DCs were isolated using EasySep CD11c negative selection kit (Miltenyi Biotech), according to the manufacturer’s protocol. The DCs were then matured for 24 h in the presence of 250 ng/ml lipopolysaccharide (Sigma-Aldrich), 1,000 U/ml interleukin-4 (IL-4) (Schering Plough), and 1,000 U/ml IFN-γ (Peprotech). All maturation conditions included 1,000 U/ml granulocyte-macrophage colony-stimulating factor to sustain cell viability. gB(498-505) peptides were loaded onto the mature DCs for one hour at 37°C and washed with cold PBS. gB(498-505)-specific CD8+ T cells were obtained from the spleens of gBT transgenic mice (bred in house) whose T cells are specific for the HSV-1 immunodominant epitope gB(498-505). gB-T CD8 T cells were isolated using Easysep CD8 T cell selection kit and then were stained with CFSE according to manufacturer’s protocol (Invitrogen). CFSE-labeled gB-specific CD8 T cells were then incubated with the gB-peptide loaded-DCs in the presence of anti-PD-L1 blocking antibody or without at 37°C. Following incubation for 2 days, cells were harvested and stained for flow cytometry.
### 4.18 SCORING HSK

HSK was monitored by slit lamp examination at indicated time points after HSV-1 RE-Lausch corneal infection in C57BL/6 mice. For each cornea, at least two masked observers concurred on all HSK scores. Corneas were divided regionally into 4 quadrants and each quadrant was scored based on opacity. HSK score was determined on the basis of opacity as 1+, mild corneal haze; 2+, moderate opacity; 3+, severe opacity completely obscuring the iris; or 4+, corneal perforation. Total of 4 scores in 4 quadrants were added for a HSK score range of 0 (no disease) to 16 (most severe) per cornea.

### 4.19 MEASUREMENT OF NEURON APOPTOSIS

A Caspatag assay kit (Chemicon International, Cat # APT420) was used according to manufacturer’s instructions to quantify CD45⁻ Thy1.2⁺ neurons that are undergoing apoptosis. TG from either PBS-treated wild type mice or anti-PD-L1-treated mice (treated systemically with 100µg/mouse at 5, and 7 DPI) were harvested at 8 DPI, collagenased to be dispersed into a single cell suspension, and surface stained with antibodies to CD45, and Thy1.2. Following a wash with FACS buffer, cells were incubated with Caspatag FLICA reagent (10µl of the 30X FLICA reagent/TG) for 1 hour at 37°C and were washed with Caspatag Wash buffer twice at the end of incubation to be collected on flow cytometer.
5.0  THE ROLE OF PD-L1 IN HSV-1 LATENT INFECTION

Following HSV-1 corneal infection in C57BL/6 mice, the virus establishes latency within neurons in the TG. CD8 T cell response peaks at 8 dpi and then it undergoes contraction to maintain a stable TG-resident population that actively prevents HSV-1 reactivation. All CD8 T cells in the TG are HSV-1-specific and half recognize the immunodominant gB498-505 epitope, while the gB498-505-tetramer negative population recognizes 18 different subdominant epitopes on 11 different HSV-1 proteins. It is unclear what establishes this striking immunodominance hierarchy. A previous study suggests that the inhibitory receptor PD-1) may play a role in maintaining the immunodominance of virus-specific CD8 T cell repertoire (107). Also, our previous observation indicated that gB-TetCD8 T cells in latently infected TG express less IFN-γ and grzB compared to gB-Tet+ CD8 T cells. PD-1 and its ligand PD-L1 have extensively been characterized as an indicator of CD8 T cell exhaustion in chronic infection models but their role during latent viral infections remains unclear. We sought to determine whether PD-1-PD-L1 interactions in the latently infected TG plays a role in maintaining the ratio between gB-Tet+ and gB-Tet- CD8 T cells in the TG and whether PD-L1 blockade could be an efficient strategy to restore gB-Tet- CD8 T cell function for better protection against HSV-1 reactivation.
5.1 PD-1 AND PD-L1 EXPRESSION IN THE TG

To investigate the role of PD-1 and PD-L1 in TG latently infected with HSV-1, we first determined their expression patterns at different time points throughout infection. Based on our previous observation that gB-Tet+ CD8 T cells show partially exhausted phenotype during latency, we hypothesized that PD-1 levels during latency would be higher on gB-Tet+ CD8 T cells. Indeed, gB-Tet+ CD8 T cells not only exhibited a higher frequency of PD-1+ cells but also showed a higher level of PD-1 expression/cell relative to their immunodominant gB-Tet+ CD8 counterparts in latently infected TG (Fig 6).

FIGURE 6. PD-1 is preferentially expressed on subdominant epitope-specific CD8 T cells in latently infected trigeminal ganglia. Trigeminal ganglia (TG) were excised from mice latently infected with HSV-1 (30 dpi) and then dispersed into single-cell suspensions. Cells were stained with H-2Kb tetramers containing the immunodominant gB498-505 epitope (gB), or subdominant epitopes on HSV-1 RR1 (RR1982-989, RR1822-829) and infected cell protein 8 (ICP8171-179, ICP876-883) with fluorescently tagged Abs to CD45, CD8, and PD-1 and analyzed on FACSaria using FACSDiva software. (A) Representative dot plots and a bar graph showing the mean percentage ± SEM of PD-1+ cells in HSV-1 epitope-specific CD8 T cell populations (n = 5 mice). (B) Mean PD-1 MFI for each HSV-1 epitope-specific CD8 T cells (n = 5 mice). All data are from at least two independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Since these CD8+ T cells interact closely with neurons in infected TG (19, 96) we also asked if TG neurons constitutively express the PD-1 ligand, PD-L1/B7H1 or are induced to express the ligand following HSV-1 infection. The frequency of PD-L1/B7H1 positive neurons was very low (<5%) in non-infected TG, but increased significantly following infection reaching expression on nearly 70% of neurons by 7 dpi (Fig 7C). The frequency of PD-L1/B7H1 positive

FIGURE 7. PD-L1/B7-H1 expression on neurons in the trigeminal ganglia post HSV-1 infection. HSV-1 infected TG were excised at various times after infection and dispersed neurons were stained with antibodies against CD45, Thy1.2, PD-L1, and intracellular NeuN. A and B, Representative dot plots showing gating strategies for quantification of PD-L1+ neurons at 8 dpi. C, Bars represent the mean percentage ± SEM of PD-L1+ cells within the CD45− Thy1.2+ NeuN+ neuron population (n= 5 mice/time). D, Representative graph shows the average ± SEM number of CD8+ T cells or PD-L1+ neurons in the TG at various times following infection (n = 3-7 mice/time). Three independent experiments produced similar results.
neurons decreased from acute (8 dpi) to latent (>30 dpi) infection, but remained significantly higher than that observed in non-infected TG (Figure 7C and D).

5.2 PREFERENTIAL UP-REGULATION OF PD-L1 ON HSV-1-INFECTED NEURONS

It was unclear if PD-L1/B7H1 expression was directly or indirectly regulated by HSV-1 infection. The latter possibility was suggested by the fact that more neurons expressed PD-L1/B7H1 at 7 dpi than would be expected to be HSV-1 infected (Fig 7C), and by the close association between CD8+ T cell infiltration and neuronal PD-L1/B7H1 expression in the TG (Fig 7D). Since TG-resident CD8+ T cells produce IFN-γ when stimulated by HSV antigens (98) and IFN-γ can up-regulate PD-L1/B7H1 expression on oligodendroglia (173), we hypothesized that PD-L1/B7H1 expression on neurons is indirectly regulated by IFN-γ in infected TG. Indeed we found that PD-L1/B7H1 expression on TG neurons at 8 dpi was significantly, but not completely reduced when mice were treated with IFN-γ neutralizing antibody at 4 and 6 dpi (Fig 8A).

To investigate the role of IFN-γ in regulating neuronal PD-L1/B7H1 expression in a cleaner system, bone marrow chimeras were created by transferring wild type (WT) bone marrow into irradiated WT or IFN-γ receptor knockout (IFN-γR−/−) mice. The WT recipients of WT bone marrow showed the expected up-regulation of PD-L1/B7H1 expression on TG neurons at 8 dpi and 32 dpi (Fig 8B). In contrast, the IFN-γR−/− recipients of WT bone marrow that were incapable of expressing IFN-γR on neurons showed no up-regulation of neuronal PD-L1/B7H1
expression, demonstrating that neuronal PD-L1/B7H1 expression in HSV-1 infected TG is regulated by IFN-γ.

Based on the assumption that latently infected TG neurons would be more likely to be exposed to IFN-γ we proposed that PD-L1/B7H1 positive neurons would contain a higher load of latent virus than their non-infected counterparts. In fact, when latently infected TG neurons were FACS sorted into PD-L1 positive and negative subpopulations, the PD-L1 positive neurons were found to contain more copies of latent HSV-1 genomes per 100 cells than the PD-L1 negative

FIGURE 8 IFN-γ regulates PD-L1 expression on trigeminal ganglion neurons. A, Mice received intraperitoneal injections (1 mg/ mouse) of either anti-IFN-γ or control antibody at 4 and 6 dpi, TG were excised at 7 dpi and dispersed cells stained for PD-L1/B7H1, CD45, Thy1.2, and NeuN. Bar graph shows the mean ± SEM percentage of PD-L1/B7H1 positive PD-L1⁺ CD45⁻ Thy1.2⁺ NeuN⁺ neurons. B, TG were obtained from non-infected (naïve) mice or from bone marrow chimeric mice that expressed IFN-γR on all cells (WT to WT) or only on bone marrow-derived cells (WT to IFN-γR⁻/⁻), and dispersed TG were stained as in A and analyzed by flow cytometry. The bar graph shows the mean ± SEM percentage of PD-L1/B7H1 positive neurons. Data are representative of two independent experiments (n = 5 mice (10 TG)/group/experiment). *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001
population (Fig 9). However, two points merit mention. First, PD-L1+ neurons contained only 30 copies of viral genome per 100 cells. Assuming 1 copy of viral genome/infected neuron (quite possibly an underestimate) our data suggest a maximum of 30% of PD-L1+ neurons harbor latent virus. Second, since PD-L1+ neurons represent approximately 30% of total TG neurons during latency, the distribution of total latent virus is approximately equivalent in PD-L1+ and PD-L1− neurons.

5.3 DIFFERENCES BETWEEN GB-TET+ AND GB-TET- CD8 T CELLS

HSV-specific CD8+ T cells consistently maintain a 1:1 ratio of cells specific for an immunodominant gB498-505 epitope (here referred to as gB-Tet+ CD8) to those recognizing subdominant epitopes (here collectively referred to as gB-Tet− CD8) (Fig 4 and Fig 10A) (98).
despite the fact that the latter population exhibits a significantly higher rate of cell division than gB-Tet* CD8 in latently infected TG (Fig 10B). The rate of proliferation varied among TG

FIGURE 10. Differences between immunodominant gB_{498-505} specific CD8+ T cells and subdominant epitope-specific CD8+ T cells. Mice latently infected with HSV-1 (30 dpi) received 2 intraperitoneal BrdU treatments (1mg/mouse) over a 2 day period and then trigeminal ganglia were excised and dispersed into single cell suspensions. Cells were stained with H-2Kb tetramers containing the immunodominant gB_{498-505} epitope (gB, A-D), or subdominant epitopes on HSV-1 ribonucleotide reductase 1 (RR1_{982-989}, RR1_{822-829}) and infected cell protein 8 (ICP8_{171-179}, ICP8_{876-883}) (B), with fluorescently tagged antibodies to CD45, CD8, PD-1, CD127 (D), and BrdU (B) or Granzyme B (GrzB) (D), or with a fluorescently labeled pan caspase inhibitor (caspatag) to identify apoptotic cells (C) and analyzed on a FACSARia using FACSDiva software. A, Bars represent the mean percentage of gB tetramer positive (gB) and negative (subdominant) CD8+ T cells/TG ± SEM (n = 10 mice). B, Bars represent the mean percentage of BrdU+ cells in HSV-1 epitope -specific CD8+ T cell populations ± SEM (n= 5 mice/group). C, Comparison of apoptosis within gB-Tet+ CD8 and subdominant CD8+ cells (n = 5 mice). D, Representative dot plots showing gB tetramer and GrzB staining (far left & left), GrzB and PD-1 staining (right) and CD127 and PD-1 staining (far right) (n= 5 mice).
resident CD8+ T cells specific for individual subdominant epitopes, but all those analyzed except the RR1982-specific cells showed a significantly higher rate of proliferation than the gB-Tet+ CD8s. The subdominant CD8s also exhibited a significantly higher rate of apoptosis than that seen in the gB-Tet+ CD8 population (Fig 10C). Thus, the increased rate of cell division appears to be balanced by a corresponding increase in the rate of apoptosis among gB-Tet' CD8+ T cells, maintaining the 1:1 ratio of gB-Tet+ to gB-Tet' CD8 T cells. Also, a significantly lower percentage of gB-Tet' CD8 showed granzyme B expression, which had an inverse correlation with PD-1 expression (Fig 10D) suggesting selective functional impairment in the PD-1+ cells. Furthermore, these PD-1+ gB-Tet' CD8 T cells seemed to be an effector cell population experiencing exhaustion as suggested by their lack of CD127 expression. To conclude, the increased proliferation and decreased survival and function of TG-resident gB-Tet' CD8+ T cells suggested a state of partial exhaustion among these cells, and strongly correlated with increased PD-1 expression (Fig 6).

5.4 FREQUENCY OF GB-TET- CD8 T CELLS IN THE ABSENCE OF PD-L1/B7-H1

Since gB-Tet' CD8+ T cells in latently infected TG express more PD-1 and undergo apoptosis at a higher rate, we asked if PD-1 interaction with PD-L1/B7H1 regulates the frequency of gB-Tet' CD8+ T cells within latently infected TG. As demonstrated above, latently infected TG of wild type mice contain equivalent numbers of immunodominant gB-Tet+ CD8 and subdominant gB-Tet' CD8+ T cells (Fig 10A). However, latently infected TG of mice that are genetically deficient in B7H1 (B7H1−/− mice) exhibit a significantly increased number of gB-
Tet CD8 and a slight, but significant reduction in gB-Tet⁺ CD8 (Fig 11A). This results in a significant increase in the frequency of gB-Tet⁻ cells (Fig 11B). The immunodominant gB-Tet⁺ CD8 population consistently comprised approximately 50% of the TG resident CD8⁺ T cell population in infected TG of wild type mice from acute infection through latency (Fig 11C). In contrast, the immunodominant gB-Tet⁺ CD8 population represented a slightly reduced proportion of the TG-resident CD8⁺ T cells in acutely infected TG (8 dpi), and progressively declined during latency (Fig 11C).

**FIGURE 11.** The number of gB-Tet⁺ CD8⁺ T cells in B7-H1⁻/⁻ TG is preferentially increased. TG from wild type or B7- H1⁻/⁻ C57BL/6 mice were obtained at 30 dpi (A, B) or at indicated times after infection (C), and dispersed cells were stained for CD45, CD8, gB(498-505)-tetramer (gB-Tet), and PD-1, and analyzed by flow cytometry. A, bars represent the mean ± SEM number of gB-Tet⁺ and subdominant CD8⁺ T cells in TG of WT and B7-H1⁻/⁻ mice (n = 10 mice). B, Bars represent the mean ± SEM percentage of subdominant CD8⁺ T cells (n = 10 mice). C, Line graph showing the mean ± SEM percentage of gB-Tet⁺ CD8⁺ T cells at indicated time points in TG of WT and B7-H1⁻/⁻ mice (n = 5 mice).
Interestingly, during latency 80% of the TG-resident gB-Tet+ CD8 expressed PD-1 in B7H1−/− mice compared to less than 20% in wild type mice (Fig 12A), and the level of PD-1 expression (MFI) was also significantly higher (Fig 12B). In contrast the frequency and level of expression of PD-1+ on immunodominant gB-Tet+ CD8 was only slightly increased in B7H1−/− mice.

FIGURE 12 Significantly increased number of PD-1hi subdominant CD8+ T cells in B7-H1−/− TG. TG from wild type or B7- H1−/− C57BL/6 mice were obtained at 30 dpi and dispersed cells were stained for CD45, CD8, gB(498-505)-tetramer (gB-Tet), and PD-1, and analyzed by flow cytometry. D, Representative dot plots showing PD-1 staining on gB-Tet+ and subdominant CD8+ T cells in TG of Wild type and B7H1−/− mice. Bars indicate the mean ± SEM percentage of PD-1+ gB-Tet+ and subdominant CD8 T cells from WT and B7-H1−/− mice (n= 10 mice). E, Bar graph showing PD-1 MFI for the gB-Tet+ and subdominant CD8+ T cells in wild type and B7-H1−/− TG (n = 10 mice). All data are pooled from three independent experiments. *p ≤0.05, **p ≤ 0.01, and ***p ≤ 0.001
5.5 INCREASED SURVIVAL OF GB-TET- CD8 T CELLS IN B7-H1 KO MICE

The increased number and frequency of subdominant gB-Tet' CD8+ T cells in the latently infected TG of B7H1−/− mice could be due to a selective increase in proliferation and/or survival of gB-Tet' CD8 relative to gB-Tet+ CD8+ T cells. We found that the rate of cell division (BrdU incorporation) among gB-Tet' CD8 was slightly reduced in B7-H1−/− mice (Fig 13A), but their

FIGURE 13 PD-1/B7-H1 regulates survival of subdominant CD8+ T cells. At 30 dpi wild type and B7-H1−/− mice received 2 daily intraperitoneal injections of BrdU (1mg/mouse). At 32 dpi TG were excised and dispersed cells stained for CD45, CD8, gB(498-505)-specific tetramer (gB-Tet), and PD-1 and for BrdU (A) or caspatag (B, C). A, Bars represent the mean percentage ± SEM of BrdU+ cells in gB-Tet+ and subdominant CD8 T cells from wild type and B7-H1−/− mice (n= 5 mice). B & C, Bars represent the mean percentage ± SEM of caspatag+ cells within the indicated cell populations (n= 5 mice). Data are representative of two independent experiments. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001
reduced rate of proliferation was over compensated by a 51% reduction in their rate of apoptosis based on caspatag staining (Fig 13B and C). In contrast, neither the rate of proliferation nor the rate of apoptosis of gB-Tet⁺ CD8 was significantly affected in B7-H1⁻ mice.

In latently infected TG, PD-L1/B7H1 is expressed on CD45⁺ bone marrow-derived cells as well as on neurons (data not shown), and the relative contribution of expression on these two cell types to the survival of the gB-Tet⁻ CD8⁺ T cells is unclear. Therefore, mice capable of

FIGURE 14 B7-H1 on neurons is partially responsible for controlling the survival of PD-1⁺ gB-Tet⁻ CD8⁺ T cells. TG were obtained at 30 dpi from bone marrow chimeric mice that were capable of expressing B7-H1 on all cells (WT to WT) or only on bone marrow derived cells (WT to B7-H1⁻). Dispersed cells were stained for PD-1 and with gB_{498-505}-tetramers (gB-Tet) and analyzed by flow cytometry. A, Bars represent mean ± SEM absolute numbers (left) or percentage (right) gB-Tet⁺ and gB-Tet⁻ CD8⁺ T cells. B, Bars represent the mean ± SEM percentage of PD-1⁺ gB-Tet⁺ and gB-Tet⁻ CD8⁺ T cells. C, Bars represent the mean ± SEM of Caspatag⁺ gB-Tet⁺ and gB-Tet⁻ CD8⁺ T cells (left) and the percentage of caspatag⁺ PD-1⁺ gB-Tet⁺ and gB-Tet⁻ CD8⁺ T cells (right). All data are representative of two independent experiments. Bars represent mean ± SEM (n = 5 mice). *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.
expressing B7-H1 on bone marrow-derived cells, but not on neurons were created by transferring WT bone marrow into irradiated B7-H1−/− recipients. These mice showed a selective increase in TG-resident gB-Tet CD8+ T cells relative to controls (Fig 1A), although the increase was much less dramatic than that seen in B7-H1−/− mice (Fig 11). The selective lack of B7-H1 on neurons also resulted in an increased frequency of PD-1+ gB-Tet CD8+ T cells in latently infected TG (Fig 1B), though again the difference was not as dramatic as that seen in the B7-H1−/− mice (Fig 12). The increased size of the gB-Tet− CD8+ T cell population in ganglia of chimeras lacking B7-H1 only on neurons was associated with a decreased rate of apoptosis (Fig 1C). Thus, B7-H1 expression on non-hematopoetic cells is at least partially responsible for the selective accumulation of gB-Tet− CD8 in latently infected TG of B7-H1−/− mice.

5.6 FUNCTIONALITY OF CD8 T CELLS IN B7-H1 KO MICE

Dispersed TG cells from latently infected wild type or B7-H1−/− mice were stained directly ex vivo with tetramers containing the immunodominant gB498-505 epitope, stimulated with HSV-1 infected B6WT3 cells for 6 hrs, and then stained for intracellular IFN-γ. Flow cytometry analysis of CD8+ T cells from TG of wild type mice showed that IFN-γ was produced by 80% of the immunodominant gB-Tet+ CD8 but only approximately 30% of the subdominant gB-Tet− CD8 following stimulation (Fig 15A). This is consistent with our previous observation that in wild type mice gB-Tet+ CD8 retain functionality, whereas the subdominant cells become partially exhausted (98, 100). In latently infected TG from B7-H1−/− mice, the frequency of gB-Tet+ CD8 is reduced to approximately 10% of the total CD8+ T cells due primarily to a dramatic
increase in gB-Tet’ cells (Fig 11). The overall frequency of IFN-γ producing CD8+ T cells in TG of B7-H1− mice was reduced to approximately 12%, with approximately 80% of gB-Tet+ CD8 responding, but less than 3% of gB-Tet’ CD8 responding.

**FIGURE 15 Expanded subdominant CD8+ T cells in B7-H1− mice are not functional.** Dispersed TG cells from wild type and B7-H1− mice (30 dpi) were (A) pre-stained with gB(498-505) tetramers (gB-Tet) before stimulation and then stained for CD45, CD8, and intracellular IFN-γ following 6 hrs of stimulation. Bars represent the mean ± SEM percentage of IFN-γ+ CD8+ T cells/TG (n = 10 mice); or (B) stimulated for 6 hours with B6WT3 cells pulsed with gB(498-505) peptide (B left) or pooled RR1(982-989) and RR1(822-829) peptides (B right) followed by staining for CD45, CD8, and intracellular IFN-γ; or (C) stained directly for CD45, CD8, gB-Tet and intracellular GrzB. Bars represent the mean ± SEM percentage of GrzB+ cells in gB-Tet+ or subdominant CD8 populations (n = 5 mice). Symbols represent the mean ± SEM frequency of reactivation at each time point (n = 15 mice). Data for A, B are pooled from at least two independent experiments and data for C is representative of two independent experiments. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.
The absolute number of IFN-γ producing subdominant RR1(982-989) and RR1(822-829)-specific CD8+ T cells was equivalent in the TG of wild type and B7-H1−/− mice (Fig 1B right), suggesting that the cells that survive as a result of disrupting the PD-1/B7H1 interaction are largely non-functional. This is further supported by the observation that the percentage of grzB+ gB-Tet− CD8 is significantly decreased in B7-H1−/− mice (Fig 1C) as well as that of IFN-γ+ subdominant gB-Tet− CD8 (Fig 1A).

5.7 HSV-1 REACTIVATION IN B7-H1 KO MICE

In WT C57BL/6 mice, HSV-1 reactivation from latency in ex vivo TG cultures correlates directly with viral genome copy number, and inversely with the number of immunodominant CD8+ T cells in the TG at the time of excision cultures (23, 174). The B7-H1−/− TG showed a slight, but significant reduction in HSV-1 genome copy number during acute and latent infection (Fig 16). The B7-H1−/− TG also showed a slight, but significant reduction in reactivation frequency compared to WT TG in cultures in which CD8+ T cell function was compromised (Fig 17 Left). The B7-H1−/− TG contained significantly more CD8+ T cells at the time of excision (Fig 11), but the increase represented non-functional subdominant gB-Tet− CD8+ T cells. Therefore, we predicted that the increased number would not result in increased protection from reactivation in TG cultures in which CD8+ T cell function was intact. Indeed, CD8+ T cells reduced the reactivation by an identical amount (31% reduction compared to cultures with anti-CD8 mAb) in WT and B7-H1−/− TG (Fig 17 Right).
FIGURE 16. Decreased genome copy number in B7-H1<sup>−/−</sup> TG. Dispersed TG cells from wild type and B7-H1<sup>−/−</sup> mice (8 dpi and 34 dpi) were subject to DNA extraction and HSV-1 genome copy number was determined by quantitative PCR. Bars represent the mean ± SEM genome copy number/TG (n = 5 mice). Data are pooled from at least two independent experiments. *p ≤ 0.05.

FIGURE 17. B7-H1 KO mice do not show better protection against HSV-1. TG of wild type and B7-H1<sup>−/−</sup> mice (30 dpi) were dispersed and cultured with (left) or without (right) anti-CD8 mAb. Culture supernatants were serially sampled and assayed for infectious virus on monolayers of Vero cells to detect HSV-1 reactivation from latency. Data are pooled from at least two independent experiments. *p ≤ 0.05.
5.8 DISCUSSION

The capacity of immunodominant gB\textsubscript{498-505}-specific CD8\textsuperscript{+} T cells to prevent HSV-1 reactivation from latency is well established (18, 19). These cells have been the primary focus of such studies because of their high frequency and known HSV-specificity. However, it now appears that essentially all of the CD8\textsuperscript{+} T cells in the infected TG are HSV-specific, while only half recognize the gB\textsubscript{498-505} epitope (98, 99). Our recent study demonstrated that the subdominant CD8\textsuperscript{+} T cells in latently infected TG exhibit partial functional exhaustion associated with IL-10-mediated inhibition of their rate of cell division, functionality, and ability to inhibit HSV-1 reactivation from latency (100).

Protracted expression of the inhibitory PD-1 receptor has been associated with CD8\textsuperscript{+} T cell functional exhaustion in several tumor models and models of chronic viral infection (119, 133, 134, 136, 175-178). Approximately 10-20\% of both immunodominant and subdominant HSV-specific effector CD8\textsuperscript{+} T cells that infiltrate the TG during acute infection (8 dpi) express high levels of PD-1 (data not shown). During latency, the frequency and level of expression of PD-1 is maintained on subdominant TG-resident CD8\textsuperscript{+} T cells, but declines among the immunodominant CD8\textsuperscript{+} T cells. The reason for the differential loss of PD-1 on immunodominant memory CD8\textsuperscript{+} T cells is not clear, but two possible explanations arise from our previous studies. We demonstrated that depletion of CD4\textsuperscript{+} T cells during the priming of the HSV-specific CD8\textsuperscript{+} T cell response resulted in a higher frequency of PD1\textsuperscript{+} immunodiminant gB-Tet\textsuperscript{+} CD8 in acutely infected TG (8 dpi) that persisted in the memory population up to 56 dpi (104). The increased PD-1 expression on the gB-Tet\textsuperscript{+} CD8 was associated with reduced functionality. Since subdominant HSV-specific CD8\textsuperscript{+} T cells appear to be generated 1 day earlier than the immunodominant gB-Tet\textsuperscript{+} CD8 (98), and induction of the CD8\textsuperscript{+} T cell response
typically precedes that of CD4⁺ T cells (unpublished observations), it is possible that the subdominant CD8⁺ T cells are generated with less CD4⁺ T cell help. This might result in higher PD-1 expression and lower functionality as seen in non-helped immunodominant gB-Tet⁺ CD8.

An alternative explanation for the elevated PD-1 expression on TG resident subdominant memory CD8⁺ T cells might be that the epitopes recognized by these cells are expressed at a higher level in latently infected neurons. This would be consistent with the observation that these cells exhibit a higher level of proliferation than the immunodominant cells. Overexposure to subdominant epitopes could lead to exhaustion characterized by up-regulation of PD-1 and ultimate physical deletion.

CD8⁺ T cells in latently infected TG interact closely with neurons; the only cells that retain latent virus (19, 96). Since PD-1 inhibition of TCR signaling reportedly requires expression of the TCR ligand and PD-1 ligand on the same cell (124), we examined PD-L1/B7-H1 expression on neurons in infected TG. The frequency of PD-L1/B7-H1 positive neurons increased dramatically during acute infection; and remained higher than non-infected TG during latency. Since the number of PD-L1/B7-H1⁺ neurons exceeded the likely number of infected neurons in acutely infected TG, and their number declined in parallel with the declining numbers of TG-resident CD8⁺ T cells, we hypothesized that neuronal expression of PD-L1/B7-H1 might be regulated by a soluble mediator produced by CD8⁺ T cells. IFN-γ is persistently present in HSV-1 acutely and latently infected TG (179-183), and was shown to regulate PD-L1 expression on neurons in the brains of coronavirus infected mice (173). Indeed, we found IFN-γ has a requisite role in regulating PD-L1 expression on neurons in infected TG.

The ratio of viral genomes to neurons was 3-fold higher among PD-L1⁺ neurons compared to PD-L1⁻ neurons, consistent with the notion that neurons with a higher load of latent
virus are more likely to stimulate an IFN-γ response (presumably by CD8^+ T cells). However, even the PD-L1^+ neurons contained only 30 genome copies per 100 cells, suggesting that a maximum of 30% of PD-L1^+ neurons harbor latent virus. Presumably, the ≥ 70% of PD-L1^+ neurons that did not harbor latent virus were exposed to IFN-γ that diffused away from an encounter between a latently infected neuron and a CD8^+ T cell or other IFN-γ producing cell as previously described (184).

Although PD-L1 negative neurons contain 3-fold fewer viral genomes than PD-L1 positive neurons, there are approximately 3-fold more PD-L1 negative neurons. Therefore, approximately half of latent HSV-1 genomes are harbored in neurons that have not been recently exposed to IFN-γ. This finding is consistent with the notion that a significant proportion of latent viral genomes are maintained in a latent state without immune intervention.

The process of exhaustion appears to advance through stages marked by progressively reduced capacity for lytic granule-mediated target cell lysis, reduced production of cytokines, and ultimately physical deletion of the exhausted cells (119). Cells at advanced stages of exhaustion are marked by increased levels of PD-1 expression (176). Our recent study demonstrated that immunodominant gB-Tet^+ CD8 remain fully functional as assessed by cytokine production and lytic granule release when stimulated with gB_498-505 peptide pulsed targets directly ex vivo, whereas a significant proportion of subdominant CD8^+ T cells lose functionality (100). We further demonstrated that in vivo blockade of the IL-10 receptor had little effect on the immunodominant gB-Tet^+ CD8, but significantly increased the proliferation and size of the subdominant CD8^+ T cell pool as well as the number of subdominant CD8^+ T cells capable of producing cytokines and expressing GrzB in latently infected TG. Here we show a higher frequency and higher level of expression of PD-1 in subdominant relative to
immunodominant CD8⁺ T cells in latently infected TG. Interfering with the PD-1/PD-L1 interaction resembles blockade of the IL-10 receptor in selectively increasing the size of the subdominant CD8⁺ T cell pool in latently infected TG. However, PD-1/PD-L1 blockade differs from IL-10 receptor blockade in that it increases the size of the subdominant CD8⁺ T cell pool by increasing survival rather than increasing proliferation. Moreover, the cells that survive as a result of PD-1/PD-L1 blockade express high levels of PD-1 and are not functional, resulting in a dramatic decrease in the frequency of functional subdominant CD8⁺ T cells in latently infected TG.

Several observations suggest that subdominant CD8⁺ T cells in HSV-1 latently infected TG are selectively undergoing functional exhaustion and physical deletion. First, the subdominant CD8⁺ T cells express less GrzB than the immunodominant cells, and GrzB expression is inversely related to PD-1 expression. Second, the ability to produce IFN-γ is maintained by virtually all immunodominant, but only a small portion of subdominant CD8⁺ T cells in latently infected TG. Third, although the TG-resident subdominant CD8⁺ T cells proliferate at a higher rate than the immunodominant cells, the two populations maintain a strict 1:1 ratio through an increased rate of apoptosis in the subdominant population. Finally, disrupting the PD-1/PD-L1 interaction results in a dramatic accumulation of TG-resident subdominant CD8⁺ T cells accompanied by a decrease in their rate of apoptosis. The surviving cells show a high frequency and level of expression of PD-1, a phenotype that is suggestive of cells in the late stages of exhaustion that would normally be deleted. Moreover, disrupting the PD-1/PD-L1 interaction does not result in an increased frequency or absolute number of functional CD8⁺ T cells as assessed by IFN-γ production or GrzB expression directly ex vivo. Similar results have been shown in HCV-infected patients who received PD-1/PD-L1 blockade.
Based on these findings, we propose that the subdominant TG-resident CD8$^+$ T cells are constantly progressing through stages of exhaustion ranging from functional compromise to physical deletion, with IL-10R regulating the former and PD-1 regulating the latter.

TG-resident CD8$^+$ T cells can inhibit HSV-1 reactivation from latency in ex vivo TG cultures through release of IFN-$\gamma$ and lytic granules containing GrzB (18, 23). Since immunodominant gB-Tet$^+$ CD8 selectively retain these functions during latency, it is likely that gB-Tet$^+$ CD8 play a primary role in preventing HSV-1 reactivation. Disruption of the PD-1/PD-L1 interaction in latently infected TG of B7-H1$^{-/-}$ mice had little effect on the immunodominant gB-Tet$^+$ CD8 T cell, and only resulted in an increase in non-functional subdominant CD8 T cells. Thus, we observed an identical level of CD8$^+$ T cell protection from reactivation in ex vivo cultures of TG from wild type and B7-H1$^{-/-}$ mice. While a portion of CD8$^+$ T cells in human TG express PD-1 (186), our findings suggest that blocking PD-1 function alone would probably not have therapeutic efficacy. However, combined blockade of PD-1, IL-10R, and perhaps other inhibitory receptors seems to have exciting potential for preventing HSV-1 reactivation from latency and recurrent herpetic disease.
6.0 THE ROLE OF PD-L1 IN CONTROLLING INNATE IMMUNE RESPONSES DURING HSV-1 ACUTE INFECTION

The uninfected mouse cornea has antigen presenting cells (APC) such as macrophages and DCs. In our mouse model of HSV-1 ocular infection, more immune cells are recruited to the corneal stroma and epithelium where viral replication takes place following infection. HSV-1 replicates in the corneal epithelium briefly for about 5 days and is cleared mainly by innate immune cells such as inflammatory monocytes and NK cells that are recruited to the cornea. Because the amount of replicating virus found in the cornea during this initial stage of infection dictates the amount of virus that establishes latency in the TG, enhancing the innate immune response that controls the virus in the cornea potentially can reduce HSV-1 reactivation frequency. Although the role of PD-1-PD-L1 interactions in regulating adaptive immune responses has been extensively characterized, it still is not completely understood what role PD-L1 plays in regulating innate immune response in acute viral infection. Based on the observation that the number of viral genome copies was lower in B7-H1−/− mice at 8 DPI in the TG (Fig 16), we speculate that the innate immune response in the cornea is enhanced in the absence of PD-L1. PD-L1 is expressed on a variety of innate immune cells as well as parenchymal tissue including the cornea (127, 145, 187-189) and therefore we hypothesize that PD-L1 suppresses the innate immune response in HSV-1-infected cornea.
6.1 PD-L1 EXPRESSION IN THE CORNEA

The normal, uninfected cornea has corneal-resident CD45+ immune cells in its epithelium and stroma (47) but as early as 18 hour after infection there is an influx of CD45+ cells migrating to the central cornea. In order to detect and quantify a very small number of CD45+ cells in mouse cornea by flow cytometry, we pooled 6 infected corneas into one sample from 9 uninfected or bilaterally infected mice. Upon HSV-1 infection of the epithelium, more CD45+ cells including DCs, macrophages, monocytes, and neutrophils immediately are recruited to the cornea and the number of CD45+ cells in the HSV-1-infected cornea peaks at 4 dpi (Fig 18A left). DCs and PMNs started their migration earlier at day 2 whereas NK cells and macrophages migrated into the cornea later around day 4 (Fig 18A right).

**FIGURE 18** CD45+ cell infiltration into HSV-1-infected cornea. Mice were infected with 1x10⁵ pfu/cornea and corneas were harvested at indicated times following infection to be dispersed into single cell suspension by incubation with collagenase. 6 corneas were pooled per sample (n = 3) and stained for CD45, NK1.1, CD11c, Gr-1, and F4/80. (A) Total numbers of CD45+ cells in naïve cornea and within the cornea at different times following infection. Bars represent the mean ± SEM absolute numbers of cells (left and right). (B) Bars represent the mean ± SEM percentage PD-L1+ cells within indicated cell populations.
PD-L1 is known to be up-regulated on both hematopoietic parenchymal tissue. Approximately 60–80% of all CD45+ cells in the cornea at day 2 expressed PD-L1 and even higher percentage expressed PD-L1 at day 4 (Fig 18B). It was interesting to note, however, that in the spleen, the percentage of PD-L1+ cells was significantly lower and the MFI on these same innate immune cell populations was much lower as well. To determine if the corneal epithelium expresses PD-L1, we took corneas non-infected mice or at various times after HSV-1 infection, stained for PD-L1, and did whole mount cornea imaging. PD-L1 is constitutively expressed on corneal epithelial cells (190) and our confocal imaging revealed that PD-L1 expression was low and restricted to the epithelium in non-infected corneas. However, PD-L1 was up-regulated as early as 1 dpi, peaked at 4 dpi, and declined by 7 dpi, but was maintained at a higher level of expression than that of the uninfected cornea (Fig 19).

**FIGURE 19 PD-L1 expression on non-infected and HSV-1-infected cornea.** Corneas from non-infected and HSV-1 corneally infected mice (n = 3) were harvested at indicated times after infection, stained for PD-L1 by fluorescently tagged anti-mouse PD-L1 antibody, and mounted on microscopy slides. Confocal images were acquired and analyzed using MetaMorph software. Two independent experiments showed similar results.
6.2 ENHANCED VIRAL CLEARANCE IN THE CORNEA OF B7-H1 KO MICE

To investigate what role PD-L1 has on viral control in the HSV-1 infected cornea, we first compared the amount of live virus found in the tear film of wild type mice and B7-H1−/− mice that lack PD-L1 expression. There was a significant decrease in the amount of replicable HSV-1 as early as 1 dpi in corneas of B7-H1−/− mice compared to those of wild type mice (Fig 20A). This significant decrease was observed from 1 dpi to 4 dpi at which time many of B7-H1−/− had already cleared the virus while live virus was still found in corneas of all wild type mice.

FIGURE 20 Enhanced viral clearance from the cornea of B7-H1−/− mice. Wild type and B7-H1−/− mice were infected with 1x10⁵ pfu/cornea and tear film was collected at indicated times. Infectious virions were quantified by standard plaque assay using Vero cells (A and C). (A) Bars represent the mean ± SEM plaque forming units (pfu) per cornea (n = 5). Three experiments gave similar results. (B) TG from HSV-1-infected wild type and B7-H1−/− mice were excised at indicated times and dispersed into single cells for DNA extraction and quantification of HSV-1 genome copy numbers by quantitative PCR. Bars represent the mean ± SEM genome copy number/TG (n = 5 mice). Data are pooled from at least two independent experiments. (C) Wild type mice were subconjunctivally treated with either PBS or anti-PD-L1 blocking mAb (40 ng/eye) at -1 dpi and 2 dpi. Bars represent the mean ± SEM plaque forming units (Plaque Forming Units) per cornea (n = 5). Two experiments gave similar results. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.
For the first 2~3 days following infection, some virions inevitably gain access to nerve termini in the cornea to travel to the trigeminal ganglia (TG) despite the efforts of the innate immunity against them. Therefore, the amount of virus that replicates and establishes latency in the TG is determined by the amount of virus replicating in the cornea and the innate immune response that controls it. As expected, the viral genome copy number observed in the TG at 8 dpi was also significantly lower in B7-H1−/− mice (Fig 20B) and this effect carried over to latency (191).

Local in vivo PD-L1 blockade in the cornea by subconjunctival treatments of anti-PD-L1 antibody significantly increased viral clearance to a level similar to that seen in B7-H1−/− mice, suggesting that the local PD-L1 expression in the cornea and not the periphery is crucial for inhibiting viral clearance (Fig 20C). This result also confirmed that the effects we observed in B7-H1−/− mice were due to the absence of PD-L1 signaling and not artifacts of knockout mice.

6.3 INCREASED GR-1+ CELLS IN INFECTED CORNEA OF B7-H1 KO MICE

Based on the observation that viral clearance is significantly enhanced in the absence of PD-L1, we hypothesized that PD-L1 inhibits the recruitment of CD45+ cells that mediate viral clearance at 1 dpi. Indeed, infection of wild type or B7-H1−/− mice with a recombinant HSV-1 that expresses eGFP from the ICP0 promoter (ICP0-eGFP) revealed a significant increase CD45+ cells and Gr-1+ cells and more efficient elimination of virus at 1 dpi in corneas of B7-H1−/− mice (Fig 21) suggesting that Gr-1+ cells are able to infiltrate the cornea more efficiently in the absence of PD-L1. Also, B7-H1−/− corneas showed a significant decrease in the size of
fluorescent viral lesions compared to those of wild type mice. Interestingly, the lesions in the B7-H1⁻/⁻ cornea were restricted to the central cornea whereas those in corneas of wild type mice had viral lesions in the paracentral and peripheral part of the cornea as well.

**FIGURE 21 Increased recruitment of Gr-1⁺ CD45⁺ cells into B7-H1⁻/⁻ cornea at 1 DPI.** Wild type and B7-H1⁻/⁻ mice were infected with 1x10⁵ pfu of HSV-1 ICPeGFP/cornea and their corneas were surface stained with fluorescently tagged antibodies to CD45 and Gr-1. Images were obtained with Olympus confocal microscope 10x objective lens and processed by Olympus Fluoview. Images show viral lesion (green) with cells positive for CD45 (blue), Gr-1 (red) in wild type (left) and B7-H1⁻/⁻ (right) corneas at 1 dpi.

Previous studies suggest that PD-L1 may play a role in chemotaxis of immune cells into the cornea (171). We hypothesized that PD-L1 may be inhibiting the production of chemokines that are responsible for recruiting Gr-1⁺ cells to the cornea following HSV-1 infection. As we expected, the mRNA levels of several chemokines and inflammatory markers were increased in B7-H1⁻/⁻ corneas compared to wild type at 1 DPI (**Fig 22**). Chemokines that attract Gr-1⁺ cells such as CXCL1 and CCL3, as well as the receptors that mediate Gr-1⁺ cell migration such as CXCR2 and PECAM1 are up-regulated in the absence of PD-L1. CD3 and CXCR3 were also
significantly increased, suggesting that T cell infiltration may be occurring earlier in these B7-H1<sup>-/-</sup> corneas compared to wild type corneas.

Gr-1<sup>+</sup> cell population has two different cell subsets: inflammatory monocytes (Gr-1<sup>int</sup> Ly6C<sup>+</sup>) and neutrophils (Gr-1<sup>hi</sup> Ly6G<sup>+</sup>). In order to determine which population is responsible for enhanced HSV-1 clearance from the B7-H1<sup>-/-</sup> cornea, we either depleted the entire Gr-1<sup>+</sup> population including monocytes and neutrophils using αGr-1 antibody (clone RB6-8C5), or selectively depleted Gr-1<sup>hi</sup> Ly6G<sup>+</sup> neutrophils using αLy6G antibody (clone IA8). As expected, B7-H1<sup>-/-</sup> mice treated with αGr-1 had an increased amount of replicating virus in their cornea at 2 dpi compared to PBS-treated B7-H1<sup>-/-</sup> mice (Fig 23). However, we found no significant difference in the amount of replicating virus in the corneas of PBS-treated B7-H1<sup>-/-</sup> mice and B7-H1<sup>-/-</sup> mice treated with αLy6G suggesting that neutrophils do not participate in HSV-1 clearance.
from the cornea. We also conclude that Gr-1\textsuperscript{int} inflammatory monocytes are responsible for the enhanced viral clearance seen in B7-H1\textsuperscript{−/−} mice.

![Graph showing viral clearance in B7-H1\textsuperscript{−/−} mice](image)

**FIGURE 23** Gr-1\textsuperscript{int} inflammatory monocytes but not Gr-1\textsuperscript{hi} neutrophils are responsible for enhanced viral clearance in the B7-H1\textsuperscript{−/−} cornea. Wild type and B7-H1\textsuperscript{−/−} mice were subconjunctivally treated with either PBS, αGr-1 (clone RB6-8C5), or αLy6G (clone IA8) on the day of infection and their tear film was collected at 2 dpi to quantify the amount of viral replication by standard plaque assay. Bars represent the mean ± SEM plaque forming units (PFU) per cornea (n = 5). *p ≤ 0.05.

### 6.4 BIPHASIC FUNCTIONS OF PD-L1 IN THE CORNEA

Since PD-L1 can be expressed on hematopoietic CD45\textsuperscript{+} cells such as DCs, macrophages, NK cells, and PMNs in the cornea but also on parenchymal tissue such as corneal epithelial cells (Fig 19), we wanted to determine on which compartment PD-L1 expression inhibits viral clearance. First, we created bone marrow chimeric mice by transferring either wild type or B7-H1\textsuperscript{−/−} bone marrow cells into irradiated wild type mice (Table 1). There was no significant difference in the amount of live virus found in the tear film of these mice at any time point following infection suggesting that PD-L1 expression on parenchymal cells such as corneal epithelial cells alone is sufficient to inhibit viral clearance (Figure 24).
Next, we transferred wild type bone marrow cells into either irradiated wild type or irradiated B7-H1− mice to determine whether PD-L1 on hematopoietic cells is also sufficient to inhibit viral clearance. In WT→B7−H1− mice, we did not see enhanced viral clearance at 2 dpi but saw a significant drop in viral titers at 4 dpi suggesting that PD-L1 on hematopoietic cells can also inhibit viral clearance at 2 dpi while PD-L1 expression on corneal epithelial cells is absolutely necessary to inhibit viral clearance starting at 4 dpi (Fig 24) when PD-L1 expression on corneal epithelium is at its peak (Fig 18).

These results suggest that PD-L1 on both corneal epithelial cells and CD45+ cells can down-regulate chemokine production thus inhibiting cell recruitment and viral clearance during

<table>
<thead>
<tr>
<th>Bone marrow Donor</th>
<th>Recipients</th>
<th>Hematopoietic</th>
<th>Non-hematopoietic</th>
<th>Viral clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>B7-H1−</td>
<td>PD-L1</td>
<td>No PD-L1</td>
<td>Enhanced starting at 4 DPI</td>
</tr>
<tr>
<td>B7-H1−</td>
<td>Wild type</td>
<td>No PD-L1</td>
<td>PD-L1</td>
<td>No difference</td>
</tr>
</tbody>
</table>

Table 1 Delineating the biphasic functions of PD-L1 in the cornea with bone marrow chimeric mice.
the first 3-4 dpi. However, after 3-4 dpi, it seems that PD-L1 expression on corneal epithelial cells is essential for inhibiting viral control.

6.5 THE ROLE OF PD-L1 IN REGULATING γδ T CELLS IN THE HSV-1-INFECTED CORNEA

Since we saw a significant decrease in replicating virus at 4 dpi from corneas of mice that lack PD-L1 expression only on parenchymal cells, we hypothesized that PD-L1 on corneal epithelial cells may be responsible for down-regulating NK cell function. NK cells start to migrate into the cornea at 3 dpi so we harvested corneas from either wild type or B7-H1−/− mice at 3 dpi and stained for CD45, T cell marker CD3, NK cell marker NK1.1 and activation markers CD69 and granzyme B. In B7-H1−/− mice, we found a dramatic increase in the number of CD45+ cells, which was almost 3 times the size of the CD45+ cell population found in wild type mice (data not shown). However, the number of total NK cells and activated NK cells (as shown by their expression of CD69, grzB, or NKp46) in B7-H1−/− corneas was not significantly different from that of wild type mice (data not shown), suggesting that PD-L1 does not regulate the proliferation and function of NK cell in HSV-1-infected cornea. Our previous studies also revealed that NK cell depletion reduced viral clearance starting at 4 dpi, but the virus was still cleared in their absence. Thus NK cells appear to be an important, but apparently not the only mechanism of viral clearance.
In B7-H1−/− mice, there was a significant increase in the number of CD3+ γδT+ cells most of which were CD69+ (Fig 25) suggesting that PD-L1 expressed on corneal epithelial cells can directly suppress γδ T cell activation and may control their numbers through PD-1 expressed on T cells. An increased number of T cells in the absence of PD-L1 also supports our finding in Fig 24 since γδT cells must interact with infected corneal epithelial cells in order to be activated and function. We speculate that PD-1+ γδT cells are suppressed after encountering PD-L1 on corneal epithelial cells when they are being presented viral antigen by infected epithelial cells. Therefore, PD-L1 on corneal epithelial cells seems to be responsible for regulating the number of γδT cells and their activation status at 3 DPI and after 3 DPI.

FIGURE 25 PD-L1 suppresses the γδT cell response at 3 dpi. Corneas of wild type and B7-H1−/− mice were harvested at 3 dpi, collagenased to be dispersed into a single cell suspension, and stained with fluorescently tagged antibodies for CD45, CD3, GL3, and CD69. Bars represent the mean ± SEM absolute numbers of cells per 4 pooled corneas (n = 3). Two experiments gave similar results. *p ≤ 0.05
6.6 DISCUSSION

Innate immune cells are recruited to the cornea shortly after HSV-1 infection where they participate in viral clearance, and DCs also infiltrate the draining lymph nodes to induce adaptive immunity. The number of CD45$^+$ cells in the corneal stroma and epithelium is dramatically increased as early as 1 dpi. and peaks at 4 dpi, which strongly correlates with high PD-L1 expression in the corneal epithelium. This suggests that inflammatory cytokines secreted by the cornea-infiltrating CD45$^+$ cells, such as IFN-$\gamma$, can up-regulate the ligand of inhibitory receptor PD-1 (PD-L1/B7-H1) to down-regulate the innate immune response against HSV-1 in the cornea. Here, we find that PD-L1 expressed on CD45$^+$ and CD45$^-$ cells in the cornea can inhibit the recruitment of neutrophils and inflammatory monocytes by down-regulating chemokine levels during the first 2~3 days following HSV-1 infection and that after 4 dpi, PD-L1 on corneal epithelial cells is responsible for suppressing $\gamma$Ø T cell recruitment and activation.

In a wild type cornea, the first responders following HSV-1 infection are Gr-$1^{hi}$ neutrophils, and Gr-$1^{int}$ inflammatory monocytes while DCs, NK cells and macrophages seem to be recruited later around 3~4 days following infection. We have previously reported that CD11c$^+$ DCs are necessary for efficient NK cell recruitment to the central cornea in Balb/c mice (57). This delayed NK cell response starting at 4 dpi in the cornea seems consistent with previous studies that suggest DC-NK interactions to be crucial for NK cell function and migration to the infected site (53-56, 192). Although we observe a small population of macrophages within the naïve cornea (47), we do not see a significant increase in their number until 4 dpi. We speculate that the monocytes that come into the cornea following HSV-1 infection requires IFN-$\gamma$ produced
by NK cells to differentiate into macrophages within the infected site as demonstrated in a study using Toxoplasma infection (193).

PD-L1 is significantly up-regulated in inflamed ocular tissues, suggesting its role in inhibition of ocular inflammation (165). It is interesting to observe that a high percentage (60~90%) of hematopoietic cells such as DCs, Gr-1+ cells and NK cells in the cornea up-regulated PD-L1 on their surface while less than 30% of those cells expressed PD-L1 in the spleen (Fig 18B). PD-L1 expression can be induced by inflammatory cytokines such as type I and type II IFNs (165). The high PD-L1 expression we see specifically in the cornea may be due to the fact that it is the infected tissue with active viral replication occurring and that there are a number of immune cells producing type I and II IFNs. Although it has been shown that the main source of type I IFNs during HSV-1 infection is plasmacytoid DCs, we could not detect any pDCs within HSV-1-infected cornea (57). However, IFN-γ can be produced by macrophages, NK cells, and γδ T cells that are recruited to the cornea at 3~4 dpi. This time point is also the peak of PD-L1 expression on corneal epithelium (Fig 19), suggesting that IFN-γ may up-regulate PD-L1 on corneal epithelial cells. Additional experiments using IFN-γ neutralization should be performed to determine whether this is the case.

In B7-H1+ mice, we observed a significantly lower viral replication in the cornea as early as 1 dpi (Fig 20A). These mice continuously exhibited enhanced viral clearance from the cornea until 4 dpi when most mice did not contain live virus in their corneas whereas all of wild type mice still had active viral replication. This effect even dictated the number of viral copies found in the TG at 8 dpi and carried over to latent phase (Fig 20B). Our subconjunctival anti-PD-L1 treatments (at -1 dpi, 2 dpi, 3 dpi) that blocked PD-L1 locally in the cornea also resulted in
enhanced viral clearance as early as 1 dpi, suggesting that the local expression of PD-L1 in the cornea is responsible for inhibiting the efficient clearance of HSV-1.

A previous study using anti-PD-L1 mAb treatments during ocular HSV-1 infection reported significantly increased viral replication in the cornea (194). However, there are a number of differences between this study and our study, which include the virus strain used (KOS versus McKrae), the route of antibody treatment (subconjunctival versus intraperitoneal), and the treatment period (-1, 2, dpi vs 2, 4, 6 dpi). These differences contribute to our strikingly contrary findings, including that they observed increased viral replication in the cornea of anti-PD-L1 treated mice at 7 dpi, the time point at which we do not find any live virus in the tear film, whereas we observed enhanced viral clearance from 1 dpi to 4 dpi in our B7-H1+/− mice and anti-PD-L1 treated mice. However, consistent with their findings, we also have found evidence that systemic anti-PD-L1 treatment can negatively regulate the function of DCs to prime an effective T cell response in the draining lymph nodes, which will be discussed in detail in Chapter 7.

Innate immune cells including inflammatory monocytes, and NK cells mediate the clearance of HSV-1 from the cornea. We found that the enhanced viral clearance seen in B7-H1−/− mice at 1 dpi correlated with the increased infiltration of CD45+ Gr-1+ cells at 1 dpi as shown by confocal imaging (Fig 21). Unfortunately, it was difficult to stain for Gr-1 and obtain enough Gr-1+ cells at 1 dpi to more accurately compare their numbers between wild type and B7-H1−/− corneas by flow cytometry, which we believe is due to the difficulty of disrupting the corneal epithelium with collagenase, which is where many of Gr-1+ cells are recruited.

We show that the enhanced viral clearance in the B7-H1−/− cornea is due to the increase in Gr-1+ inflammatory monocytes and not Gr-1+ neutrophils by using αGr-1 to deplete both
monocytes and neutrophils and and αLy6G antibodies specifically depletd neutrophils (195). Selective depletion of neutrophils did not result in significant changes in corneal viral load compared to the control group suggesting that increased neutrophil chemotaxis is not responsible for the enhanced viral clearance in B7-H1+ corneas. Instead it appears the PD-L1 inhibits early viral clearance by reducing infiltration of Gr-1 int inflammatory monocytes into the cornea.

The increased infiltration of CD45+ Gr-1+ cells in B7-H1−/− corneas appears to result from elevated levels of chemokines as shown by mRNA levels at 1 dpi. This suggests that the chemokine expression within the cornea and Gr-1+ cell migration to the infected site are rapid responses that occur within hours of infection and they seem to take place much faster in the absence of B7-H1. We also show that in B7-H1−/− mice, Gr-1+ cells are much more efficiently recruited to the central cornea where there is a significantly decreased viral lesion. Additional experiments will be performed to address which cell subsets are the primary source of chemokines that are responsible for attracting Gr-1+ cells to the infected site.

Our experiments using bone marrow chimeric mice show that early viral clearance at 2 dpi is not influenced by a lack of PD-L1 from either bone marrow derived or parenchymal cells, but is significantly enhanced when PD-L1 is lacking on both types of cells. We conclude that PD-L1 on either hematopoietic or non-hematopoietic cells can inhibit the expression of chemokines that bring in Gr-1+ cells to control viral replication in the cornea during the first two days after infection. On the other hand, PD-L1 on corneal epithelial cells is necessary to down-regulate the recruitment or the function of immune responses that are responsible for controlling viral replication after 4 dpi.

We have observed recruitment of NK cells into the cornea and their participation in viral clearance at 4 dpi. Previous studies have noted an inverse correlation between PD-L1 expression
on NK cells and their expression of GrzB and IFN-γ. One study reported that PD-1-PD-L1 blockade can lead to increased number of NK cells as well as more active and functional NK cells (161) (162-164). Considering these findings, we hypothesized that NK cells may be responsible for the enhanced viral clearance seen in B7-H1−/− mice at 4 dpi and that NK cell function may be inhibited by PD-L1. However, we found that the number of NK cells and their activation and function (CD69 and GrzB staining) were not statistically different in B7-H1−/− mice from wild type mice at 3 and 4 dpi (data not shown). This was not surprising, however, due to the low frequency of PD-1+ NK cells in the cornea (data not shown).

On the other hand, the total number of T cells (NK1.1−CD3+ cells) was significantly increased in the corneas of B7-H1−/− mice at 3 dpi. As opposed to neutrophils and inflammatory monocytes, T cells must interact with the infected corneal epithelial cells in order to function. The mechanism of PD-L1 regulation of T cell infiltration into infected corneas is not clear, but we speculate that PD-L1 on corneal epithelial cells may directly inhibit T cell function, proliferation or survival by binding to PD-1 on T cells. It has been suggested in other models such as corneal allograft transplants, that PD-L1-induced apoptosis of T effector cells in the cornea is an important mechanism of immune privilege that promotes corneal allograft survival (166). Another possibility is that PD-L1 may indirectly regulate the number of T cells infiltrating the cornea at 3 dpi by inhibiting the expression of chemokines that are responsible for T cell recruitment. CXCR3 and CXCL1/KC have been found to play a role in directly and indirectly recruiting T cells to HSV-1 infected cornea respectively and we indeed see significantly increased levels of both of these chemokines at 1 dpi (76, 196-199). Other groups have also observed increased chemokine expression and enhanced T cell infiltration in PD-L1−/− corneas (170, 171), which are consistent with our data.
What is unique about our data, however, is that the increased T cell population is \( \gamma \delta \) T cells. Although the role of PD-1 in regulation of \( \alpha \beta \) T cells has been extensively studied, the role of PD-1 in regulation of \( \gamma \delta \) T cells is largely unexplored. In tumor models, PD-1 is expressed on human \( \gamma \delta \) T cells and PD-L1 can inhibit their cytotoxicity and IFN-\( \gamma \) production, which can be reversed by anti-PD-L1 mAb treatment or \( \gamma \delta \) TCR-mediated signaling (200, 201). Our data show that there are more \( \gamma \delta \) T cells in the HSV-1-infected cornea in the absence of PD-L1 while we see very few recruited to the cornea at 3 dpi in a wild type setting, suggesting that PD-L1 may inhibit the production of the chemoattractant responsible for recruiting \( \gamma \delta \) T cells. Also, most \( \gamma \delta \) T cells we found in B7-H1\(^{-/}\) corneas were CD69\(^+\) suggesting that they had been recently stimulated and activated. We speculate their physiological role may be to provide protection against HSV-1 infection by serving as an early source of IFN-\( \gamma \) or IL-17. Previous studies have shown \( \gamma \delta \) T cell depletion during intravaginal HSV-2 infection significantly impaired Th1 response and decreased their survival rate (202). In our HSV-1 ocular infection model, depletion of \( \gamma \delta \) T cell resulted in increased HSV-1-induced epithelial lesion and increased mortality from viral encephalitis (203). More experiments examining which cytokines these cells produce in B7-H1\(^{-/}\) mice and how local \( \gamma \delta \) T cell depletion affects viral replication in the cornea will be useful.

In conclusion, our findings indicate that PD-L1 plays an inhibitory role in which PD-L1 on either CD45\(^+\) or CD45\(^-\) cells in the cornea can limit Gr-1\(^+\) inflammatory monocyte recruitment and/or function by suppressing chemokine production early during acute HSV-1 infection. After 3 dpi, high levels of PD-L1 on the surface of corneal epithelium can bind to \( \gamma \delta \) T cells possibly through PD-1 to negatively regulate their proliferation and/or survival. These results suggest that the broad expression of PD-L1 in the corneal tissue allows it to serve two distinct roles depending on when it is expressed and which cells express it.
7.0 IMPORTANT ASPECTS TO CONSIDER IN USING PD-L1 BLOCKADE

The previous observation that PD-L1 inhibits Gr-1$^+$ cell recruitment and $\gamma\delta$ T cell function suggests that PD-L1 has many different functions in a variety of tissue, not only suppressing adaptive immune responses within the latently infected TG but also regulating the innate immune response in the cornea during acute HSV-1 infection. Based on previous studies, PD-L1 can be co-stimulatory as well as inhibitory in some cases and PD-L1 on naïve T cells is required for proper conditioning of DCs during influenza infection. This naturally leads to the question of whether PD-L1 plays any role in antigen presentation and T cell priming in the draining lymph nodes during the initial 3-4 days following HSV-1 infection. Before using PD-L1 blockade in a clinical setting for therapy/prophylactic vaccination, it is important to determine the appropriate timing and the route of treatment that do not lead to any off-target effects. In this section, due to a number of complex layers in PD-L1’s multiple roles, we utilize in vivo anti-PD-L1 Ab treatments for specific periods of time rather than using B7-H1$^{+/}$ mice to better delineate PD-L1’s divergent functions during different phases of infection. We also investigate the effect of anti-PD-L1 Ab treatments on the severity and onset of HSK, explore the possibility that PD-L1 blockade may lead to tissue damage during acute infection, and assess the effects of PD-L1 blockade in combination with blockade of another inhibitory molecule, IL-10, during latent HSV-1 infection.
7.1 CHARACTERIZATION OF DC PHENOTYPE IN B7-H1 KO MICE

Despite the increased accumulation of PD-1\(^+\) CD8\(^+\) T cells in the TG of B7-H1\(^/-\) mice during latent HSV-1 infections, the numbers of TG CD8\(^+\) T cells at 8 dpi were comparable between wild type and B7-H1\(^/-\) mice and the number of gB-Tet\(^+\) CD8\(^+\) T cells was slightly decreased (Fig 26A). PD-L1 is strongly expressed on virtually all neurons in acutely infected TG, and PD-1 is expressed on approximately 60% of gB-Tet\(^+\) and gB-Tet\(^-\) CD8\(^+\) T cells in the TG as early as 6 dpi (data not shown). Collectively these observations suggest that PD-1 engagement by PD-L1 does not provide the net inhibitory signal to effector CD8\(^+\) T cells that it does to memory CD8\(^+\) T cells. We speculate that the inhibitory PD-1/PD-L1 signaling in CD8\(^+\) effector T cells may be overwhelmed by strong positive signaling they recently received during priming. This suggested that either PD-1 was not yet expressed on TG CD8\(^+\) T cells for PD-L1 to bind to and send inhibitory signals or there was a different role played by PD-L1 in regulating CD8\(^+\) T cell response during priming phase of the infection. The former possibility was ruled out as we found PD-1 to be expressed on almost 60% of gB-Tet\(^+\) and gB-Tet\(^-\) CD8\(^+\) T cells in the TG as early as 6 dpi (data not shown) as a result of early T cell activation.

A previous study suggested that PD-L1 may provide co-stimulatory as well as inhibitory signals, and that its expression on naïve T cells is required for proper maturation of DCs during viral infection (156). We hypothesized that PD-L1 is required for up-regulation of costimulatory molecules on DCs that present gB\(_{498-505}\) immunodominant epitope to CD8\(^+\) T cells in the draining lymph nodes. Indeed, frequency CD11c\(^+\) DCs that expressed the important co-stimulatory molecules CD40 and CD80 as well as the level of expression of these molecules per cell (MFI) was significantly reduced between in the draining lymph nodes of B7-H1\(^/-\) mice between 3 and 8 dpi (Fig 26B).
FIGURE 26 PD-L1 on T cells is responsible for CD80 expression on DCs. Wild type and B7-H1−/− mice were infected with HSV-1 KOS-H 1x10⁵ pfu/cornea with or without gB peptides (1ug/eye) on their corneas. TG were harvested at 8 DPI, dispersed into single cells by collagenase, and stained for CD45, CD8, and PE-conjugated gB498-505 tetramers for flow cytometry (A and C). Draining lymph nodes of infected mice were harvested at indicated time points to be dispersed into single cells and stained for CD45, CD80, and CD11c for flow cytometry (B). For (D), in vitro DC-T cell cross-presentation assay was performed as described in Materials and Methods. (A) Bars represent the mean ± SEM absolute numbers in the TG at 8 DPI (n = 5) (B) Dots represent the mean ± SEM percentage of CD80⁺CD11c⁺ DCs/DLN (n = 5). (C) Bars represent the mean ± SEM percentage of gB-Tet⁺ CD8 T cells in the TG at 8 DPI (n = 5). (D) Bars represent the mean ± SEM percentage of CFSE- gB-specific CD8 T cells that recently underwent proliferation (n = 5). *p ≤ 0.05 **p ≤ 0.01, and ***p ≤ 0.001.
The immature phenotype of DCs in B7-H1−/− mice could result in defective antigen processing/presentation and insufficient costimulation during T cell priming, leading to a poor generation of CD8 T cell response. In order to determine whether defective antigen processing and presentation were responsible for the decrease in gB-specific CD8 T cell response in the TG of B7-H1−/− mice at 8 DPI, we put immunodominant gB498-505 peptides (1ug/eye) on the eyes along with HSV-1 upon infection to bypass the need for antigen processing. This way, the peptides will be directly loaded on DCs to be presented to gB-specific CD8 T cells.

Interestingly, the percentage of gB-Tet+ CD8 T cells in the TG at 8 DPI was significantly increased when peptides were added to the eye in wild type and B7-H1−/− mice compared to their respective controls with no peptides (Fig 26C). However, even with the addition of gB peptides, B7-H1−/− mice failed to show an increase in percentage of gB-Tet+ CD8 T cells that was seen in wild type mice (almost 70%) that received gB peptides. This strongly suggested that PD-L1 is controlling the gB-Tet+ CD8 T cell response through a DC function other than antigen processing/presentation.

Therefore we next determined whether PD-L1 on CD8 T cells promoted gB-specific CD8 T cell proliferation via co-stimulation with CD80 on DCs. CD80 is an important co-stimulatory molecule expressed on professional antigen presenting cells such as DCs and interacts with CD28 on T cells. Using an in vitro DC-T cell CFSE assay, we compared the proliferation of gB-specific CD8 T cells that were incubated with either control IgG or anti-PD-L1 Ab and with gB-peptide loaded wild type DCs derived from wild type bone marrow. When PD-L1 was blocked on these gB-specific CD8 T cells, CD80 expression on DC was significantly decreased which led to a significant decrease in proliferation of gB-specific CD8 T cells, suggesting that during T cell priming, PD-L1 (presumably on T cells) is crucial for up-regulating CD80 expression on DCs,
which in turn provides proper costimulation for proliferation of gB-specific CD8 T cells (Fig 26D).

### 7.2  EFFECTS OF PD-L1 BLOCKADE ON EFFECUTOR T CELL RESPONSES

Since PD-L1 is important for proper maturation of DCs during T cell priming, anti-PD-L1 treatment would not be an attractive adjuvant for prophylactic vaccination or an option for patients with primary infection. Therefore we wanted to block PD-L1 in vivo shortly after the initial priming phase and determine the role PD-L1 plays in generating effector CD8 T cell

![Graph A](image1)

**FIGURE 27** Systemic anti-PD-L1 treatments during clonal expansion results in increased number of CD8 T cells. Wild type mice were treated intraperitoneally with either control IgG (100µg/mouse) or anti-PD-L1 Ab (100µg/mouse) at 5 dpi and 7 dpi and their TG were harvested at 8 dpi, and dispersed into a single cell suspension to be stained for CD45, CD8, and gB_{498-505} Tetrarimers (A and B). Mice were treated intraperitoneally with either control IgG (100µg/mouse) or anti-PD-L1 Ab (100µg/mouse) at 4 dpi and 6 dpi and BrdU was injected intravenously 4 hours prior to harvesting their draining lymph nodes at 7 DPI. Lymph node cells were stained for BrdU, CD45, CD8, and CD4 (C). (A) Bars represent the mean ± SEM absolute numbers in the TG at 8 DPI (n = 5). (B) Bars represent the mean ± SEM percentage of gB-Tet+ CD8 T cells in the TG at 8 DPI. (C) Bars represent the mean ± SEM percentage of BrdU+ cells in CD8+ and CD4+ populations at 7 DPI (n = 5).
response in the presence of mature, properly conditioned DCs. When we treated mice with anti-PD-L1 mAb at 5 and 7 DPI and harvested TGs at 8 DPI, we observed a dramatic increase in total CD8 T cell numbers as well as increase in both gB-Tet⁺ and gB-Tet⁻ CD8 T cell populations (Fig 27A). This is a result very different from what we previously observed in B7-H1⁻/⁻ mice as we saw a significant decrease in the number and the frequency of gB-Tet⁺ CD8 T cells in B7-H1⁻/⁻ mice (Fig 12 and Fig 26A) whereas there was no change in gB frequency with PD-L1 blockade (Fig 27B). When we blocked PD-L1 starting at 4 dpi and then looked for BrdU incorporation at 7 dpi, there was a significant increase in the percentage of BrdU⁺ CD8 and CD4 T cells in the draining lymph nodes, suggesting that PD-L1 inhibits the proliferation and generation of effector CD4 and CD8 T cells as early as 4 dpi (Fig 27C). We conclude that reagents that block PD-L1 could be effective adjuvants, but the timing of their administration will be crucial.

7.3 EFFECTS OF PD-L1 BLOCKADE ON NEURON APOPTOSIS

During acute infection of HSV-1, almost all CD8 T cells in the TG express granzyme B and IFN-γ (98, 99) both of which are known to be able to induce apoptosis and cause damage to neurons. Studies have shown that PD-L1 on infected parenchyma can provide host tissue protection by inhibiting cytotoxicity and functionality of T cells (145, 155). A concern is that the increased size of the CD8⁺ T cell population observed in TG when mice were treated with anti-PD-L1 starting at 5 dpi might lead to immunopathology in the TG.

To determine whether PD-L1 blockade led to apoptosis of neurons in the TG, we treated mice with anti-PD-L1 at 5 and 7 dpi and harvested TG at 10 dpi to look for active caspases in neurons by Caspatag assay. Compared to control mice, anti-PD-L1 Ab-treated mice did not show
a significant change in the total number of CD45 \(^{-}\) Thy1.2\(^{+}\) neurons (data not shown) and the percentage of apoptotic (caspatag\(^{+}\)) neurons was also similar between control and treated group (Fig 28). We conclude that the adjuvant effect of PD-L1 blockade late in priming does not lead to increased pathology in the TG.

![CD45\(^{-}\) Thy1.2\(^{+}\) Neurons](image)

**FIGURE 28** Systemic anti-PD-L1 treatments during clonal expansion does not affect neuron survival. Wild type mice were treated intraperitoneally with either control IgG (100ug/mouse) or anti-PD-L1 Ab (100ug/mouse) at 5 dpi and 7 dpi and their TG were harvested at 8 dpi, and dispersed into a single cell suspension to be stained for CD45, Thy1.2, and active caspases according to the Caspatag assay protocol described in Materials and Methods. Bars represent the mean ± SEM percentage of Caspatag\(^{+}\) cells in CD45\(^{-}\) Thy1.2\(^{+}\) neuronal populations at 8 DPI (n = 10). This is pooled data from two independent experiments. Unpaired student t test was performed to determine statistical significance.

### 7.4 EFFECTS OF PD-L1 BLOCKADE ON HERPETIC STROMAL DISEASE

We next wanted to test whether local treatment of anti-PD-L1 Ab in the cornea during the first 5 days of primary HSV-1 infection would influence the onset or severity of HSK. Subconjunctival treatment limits the effect of anti-PD-L1 mAb to the cornea, which should not
influence DC maturation (which seems to involve interaction with PD-L1\(^+\) T cells in the lymph nodes) and should not influence T cell priming. However, local PD-L1 blockade could augment the effector phase of the CD4\(^+\) T cell response that orchestrates HSK in the cornea. However, subconjunctival treatment with anti-PD-L1 did not significantly impact the incidence, time of onset, or severity of HSK in mice that received corneal infections with the RE Lausch strain of HSV-1 that induces HSK in C57BL/6 (Fig 29A). It is possible that the enhanced clearance of HSV-1 from the corneas of these mice that should correlate with reduced HSK was offset by an enhancement of the effector phase of the CD4\(^+\) T cell response in the cornea, resulting in no net change in HSK.

**FIGURE 29 Subconjunctival anti-PD-L1 treatments do not affect HSK incidence and severity.** Mice were subconjunctivally treated with either PBS or anti-PD-L1 (1, 2, 5 dpi) and infected with either 1x10\(^4\) or 1x10\(^5\) HSV-1 RE-Lausch pfu/cornea were examined for Herpes Stromal Keratitis (HSK). Score 0 is no disease and score 16 is the most severe disease. Scoring HSK is described in detail in Materials and Methods.
7.5 EFFECTS OF PD-L1 AND IL-10 COMBINATORIAL BLOCKADE

Our previous study using IL-10R antibody to block IL-10, an inhibitory cytokine that can inhibit the function and proliferation of CD8 T cells, showed that IL-10R treatment increased the proliferation of functional subdominant CD8 T cells in the TG and improved protection from HSV-1 reactivation from latency in ex vivo TG cultures. In contrast, we showed in Chapter 5 that B7-H1\(^{-/-}\) mice do not show better protection against HSV-1 reactivation due to the increased survival and accumulation of dysfunctional PD-1\(^{high}\) cells (Fig 15). Therefore we hypothesized that blocking IL-10R in B7-H1\(^{-/-}\) mice during latent HSV-1 infection may result in restoring functionality of the PD-1\(^{high}\) dysfunctional subdominant CD8 T cells in the TG.

We treated wild type or B7-H1\(^{-/-}\) mice with either PBS or IL-10R Ab for 3 weeks starting at after establishment of viral latency (35 dpi) to compare CD8 T cell numbers and functionality. Both B7-H1\(^{-/-}\) mice and wild type IL-10R Ab treated mice had significantly higher percentage and number of subdominant gB-Tet\(^{-}\) CD8 T cells in the TG that contributed to a dramatic increase in overall CD8 T cell numbers. More importantly, treatment of B7-H1\(^{-/-}\) mice with anti-IL-10R Ab resulted in a nearly two-fold increase in the size of the TG-resident CD8\(^{+}\) T cell population when compared with untreated B7-H1\(^{-/-}\) mice or wild type mice treated with anti-IL-10R alone (Fig 30A). The anti-IL-10R treatment of B7-H1\(^{-/-}\) mice also resulted in a significant increase of GrzB\(^{+}\) subdominant CD8\(^{+}\) T cells in the latently infected TG (Fig 30B) whereas no synergistic effect was seen with IFN-\(\gamma\) production by subdominant CD8 T cells (Fig 30C). We conclude that simultaneous blockade of the inhibitory PD-1 and IL-10R receptors can synergistically augment the size and functionality of both the immunodominant and subdominant CD8\(^{+}\) T cell populations in latently infected TG.
FIGURE 30 Systemic anti-IL-10R treatments in B7-H1−/− mice rescue proliferation and cytotoxicity of subdominant CD8 T cells but not IFN-γ production. Wild type and B7-H1−/− mice were treated with either PBS or anti-IL-10R Ab for 3 weeks during HSV-1 latent infection (from 35 DPI to 49 DPI) and were sacrificed at 49 DPI. TG were harvested and (A and B) stained with gB (498-505) tetramers (gB-Tet), CD45, CD8, and intracellular grzB. Bars represent the mean ± SEM absolute numbers of CD8+ T cells or grzB+ cells/TG (n = 5 mice); or (C) stimulated for 6 hours with B6WT3 cells pulsed with gB (498-505) peptide or RR1 (982-989) peptides followed by staining for CD45, CD8, and intracellular IFN-γ. Bars represent the mean ± SEM percentage of IFN-γ+ cells in gB-Tet+ or RR1 (982-989)+ CD8+ T cell populations (n = 5 mice).
7.6 DISCUSSION

PD-L1 is constitutively expressed at low levels on certain tissue and cells such as the cornea and naïve T cells and can be up-regulated further by cytokines such as IFN-γ on a wide variety of cells including DCs and neurons during HSV-1 infection. The fact that PD-L1 is widely expressed and its level of expression is differentially regulated following infection enables PD-L1 expression on different cells to play multiple divergent roles in regulating innate and adaptive immunity. Previous studies have shown that PD-L1 expressed on naïve T cells is required for mounting proper virus-specific CD8 T cell response by maturing DCs in an influenza infection setting (156).

However, PD-L1 is better known as an inhibitory molecule that binds PD-1 on T cells to exert its effects on “exhausted” T cells during chronic infection and blocking PD-1/PD-L1 interaction by anti-PD-L1 blocking mAb has been frequently used to “reinvigorate” CTL responses in cancer and chronic infection models (134-137). This makes PD-L1 blockade a promising option for cancer and chronic viral infections where the antigenic stimulation can be overwhelmingly high for CD8 T cells. Therefore it is crucial to investigate whether blocking PD-L1 may result in unfavorable outcomes for the patients since PD-L1 has also been known to play a stimulatory role during T cell priming. Here we found that systemic anti-PD-L1 treatments: a) when administered before and during the early priming phase (0~4 dpi) can lead to a diminished CD8 T cell response; when administered after the early priming phase (5 dpi) augments expansion of CD8+ effector T cells, but does not result in neuron apoptosis, when administered locally in the cornea during the first five dpi does not change the incidence or the severity of HSK; and that simultaneous blockade of PD-1/PD-L1 and IL-10 signaling during latency synergistically augments the size and functionality of CD8+ T cells in latently infected TG.
In the draining lymph nodes of B7-H1−/− mice, there was a significant decrease in CD80+ DCs and lower CD80 expression per cell but no difference in CD86+ DCs. Why does PD-L1 regulate only CD80 and not CD86? The easy explanation could be that PD-L1 binds to CD80 and not CD86, which may lead to down-regulation of CD80 on DCs as a negative feedback mechanism when CD80 does not meet its ligand, PD-L1. However, establishing such a mechanism will require additional experiments using either CD80−/− mice or PD-1−/− mice.

The biology of differences between CD80 (B7.1) and CD86 (B7.2) is more complicated and is very much dependent on context (204). They are similar in that they both are expressed on the same cell types such as DCs, and both bind to the same receptors CD28 and CTLA-4. Studies have shown, however, that they may play opposing roles as CD80 is a more potent ligand for CTLA-4, an inhibitory receptor, and CD86 binds preferentially to CD28, a costimulatory receptor on T cells. Interestingly, in vivo studies have shown that blocking CD80 leads to enhancement of immune responses (205) whereas CD86 blockade attenuated immune responses (206). In contrast, our results show that the decrease in CD80+ DCs resulted in fewer gB-specific CD8 T cells in the TG (Fig 26A) suggesting that CD80 on DCs is important for gB-specific CD8 T cell generation during priming in the lymph nodes.

When we incubated PD-L1+ gB-specific CD8 T cells with gB peptide-loaded bone marrow derived DCs from either wild type or B7-H1−/− mice, we observed a significant decrease in proliferation of gB-specific CD8 T cells that were incubated with B7-H1−/− DCs and treated with PD-L1 Ab, suggesting that PD-L1 on T cells contributes to gB-specific CD8 T cell proliferation (Fig 26D). We believe this was due to a decreased CD80 expression of DCs as there was a significant reduction in CD80 expression when we examined the DCs that were incubated with PD-L1 Ab-treated gB-T cells (data not shown). Therefore, PD-L1 on T cells
increases CD80 expression on DC, which in turn leads to an increase in gB-specific CD8 T cell proliferation, presumably by providing a costimulatory signal through CD28.

The observation that the number of gB-specific CD8 T cells, but not subdominant CD8 T cells, is decreased at 8 dpi again raises the question of what the differences are between the immunodominant gB-specific and subdominant CD8 T cells. We speculate that gB-specific CD8 T cells may need mature DCs that express both CD80 and CD86 for priming whereas the need for CD80 in generating subdominant CD8 T cells may be absent or lower. There have been studies suggesting that cross-presentation by gamma-inducible lysosomal thiol reductase (GILT) in DCs is absolutely required for processing and presenting gB immunodominant epitope while the subdominant epitope on RR1 is not cross-presented (207). However, our data in Fig 26C indicate that PD-L1 influences only DC maturation and not antigen processing/presentation by DCs, making the decreased cross-presentation capacity an unlikely reason for the decreased gB-specific CD8 T cell response we see in B7-H1−/− mice.

In previous sections we have utilized B7-H1−/− mice to determine the role PD-L1 plays during HSV-1 infection but interpreting data obtained from using B7-H1−/− mice can be difficult as PD-L1 has different roles depending on time and location of its expression. Since PD-L1 plays a crucial role in providing costimulation to CD8 T cell responses through DC maturation in a very early phase following infection, we blocked PD-L1 shortly following priming phase to examine the role PD-L1 plays in generating CD8 T cell responses without affecting DC maturation in the draining lymph nodes or the early innate immune response in the cornea.

When we blocked PD-L1 by treating mice with anti-PD-L1 mAb at day 5 and 7 following infection and looked at CD8 T cell response in the TG at 8 dpi, we saw a significant increase in both gB-specific and gB-Tet- CD8 T cell populations resulting in a significant
increase in overall CD8 T cells within the TG (Fig 27A and B). Anti-PD-L1 treatments at day 4 and 6 following infection also resulted in enhanced proliferation of CD4 and CD8 T cells in the draining lymph nodes at day 7 (Fig 27C). Previous study using systemic anti-PD-L1 treatment starting at 2 dpi in mice ocularly infected with HSV-1 has observed a decreased number of mature CD80+ DCs (194). This, combined with our data with 4 dpi treatment in Fig 27C, suggests that PD-L1 plays a stimulatory role before day 4 post-infection during the very early phase but after day 4, PD-L1 inhibits T cell proliferation in the draining lymph nodes decreasing the number of CD8 T cells that migrate to the TG.

Despite the abundance of data suggesting that PD-L1 expression on infected parenchymal tissue can protect the host from immunopathology (145, 155), we did not find any evidence of neuron apoptosis following anti-PD-L1 blockade during acute HSV-1 infection as detected by Caspatag assay. This is a good thing as neurons are non-regenerative and neuronal tissue preservation is as crucial as efficient viral clearance by adaptive immune responses. However, there is a possibility that it may have been difficult to detect such a small difference in dying neurons ex vivo because in vivo apoptosis is an efficient process in which the apoptotic bodies are quickly removed by the macrophages within the TG (208). Thus, the apoptotic neurons may have been eliminated in vivo before we could detect them. Moreover, at least 20% of neurons die just from processing TG for flow cytometry following excision and this may have also contributed to masking the difference in neuron apoptosis between control and anti-PD-L1-treated group.

We found that treating mice subconjunctivally with anti-PD-L1 during the first five days following HSV-1 infection does not result in any changes in HSK incidence or severity. Studies have shown a link between the amount of viral replication found in the cornea during HSV-1
acute infection and the incidence and severity of HSK. With subconjunctival anti-PD-L1 treatments, the innate immune response is enhanced and viral replication is significantly decreased as seen in (Fig 20) in Chapter 6, but the effect will be limited only to the corneal region and would not affect DC maturation in the draining lymph nodes. We expected the local treatments may result in delayed or less severe disease but in our experiment virus replication did not correlate with HSK incidence. HSK incidence and severity in B7-H1−/− mice also were not significantly different from those seen in wild type mice (data not shown). It may be a better strategy to systemically block PD-L1 during the generation of effector response after sufficient DC-T cell priming has occurred but this potentially may result in more severe immunopathology in the cornea during HSK (169) as there will be a more robust CD4 T cell response, which is known to be the main contributor in HSK development (209, 210).

As seen in Chapter 5, the absence of PD-L1 did not rescue HSV-1-specific CD8 T cells and rather resulted in an enhanced survival of PD-1high dysfunctional gB-Tet' CD8 T cells that would have undergone deletion in the presence of PD-L1 (Fig 13). On the other hand, IL-10 blockade via IL-10R blocking antibody treatment for 3 weeks during HSV-1 latent infection results in not only increased proliferation but also increased functionality of subdominant CD8 T cells (100). As an attempt to rescue the dysfunctional PD-1high gB-Tet' CD8 T cells in B7-H1−/− mice, we blocked IL-10 by treating mice either with PBS or anti-IL-10R antibody for 3 weeks. With IL-10 blockade in B7-H1−/− mice, the number of CD8 T cells was significantly increased compared to IL-10 blocked wild type mice or PBS treated B7-H1−/− mice, suggesting that PD-L1 and IL-10 dual blockade has synergistic effects in increasing proliferation of CD8 T cells in HSV-1-infected TG. This is consistent with previous data using LCMV infection model that IL-10 and PD-1 pathways work in distinct, independent mechanisms (114). Cytotoxicity as shown
by intracellular granzyme B staining was also significantly increased in IL-10 blocked B7-H1<sup>−/−</sup> mice but the number of IFN-γ<sup>+</sup> CD8 T cells remained the same as IL-10 single treated mice (Fig 30).

Why does IL-10 blockade in B7-H1<sup>−/−</sup> mice result in increased grzB expression and increased number of subdominant CD8 T cells but not IFN-γ<sup>+</sup> cells? Although IL-10 blockade rescues the subsets of partially exhausted CD8 T cells that PD-L1 blockade is not able to rescue in terms of cytotoxicity, proliferation, and IFN-γ production, it may be that IL-10 blockade still cannot rescue the highly dysfunctional PD-1<sup>+</sup> CD8 T cells that survive in B7-H1<sup>−/−</sup> mice because they may already be beyond the point of rescue.

A previous study using a HSV-1 footpad infection model has reported that blocking PD-L1 (only) during priming (-1 and +3 dpi) significantly increases not only the primary CD8 T cell response but also the secondary memory CD8 T cell response upon recall with vaccinia virus encoding gB<sub>498-505</sub> epitope (211). However, our results show that during ocular HSV-1 infection, treating before or during the initial DC-T cell interaction at priming phase may not be the best strategy in enhancing primary or memory CD8 T cell response as it does not result in increased CD8 T cells and rather results in a decreased number of gB-specific CD8 T cells. As we have seen in Chapter 5, absence of PD-L1 throughout the infection leads to an increased CD8 T cell population that consist of decreased gB-specific CD8 T cells and PD-1<sup>high</sup> dysfunctional subdominant CD8 T cells in the TG.

Therefore we conclude that treating systemically after 3~4 dpi during the clonal expansion/generation of CD8 T cell effector response may be the best option but it is not clear whether the continous treatments until latent infection period is necessary or treatments only during clonal expansion is sufficient for enhanced CD8 T cell memory response and better
protection against HSV-1 reactivation. Future experiments will determine how long the increased CD8 T cell population will be retained within the TG with varying periods of treatment.
8.0 SUMMARY AND CONCLUSIONS

Herpetic Stromal Keratitis (HSK) is a blinding disease that results from corneal scarring and immunopathology in the cornea stroma due to frequent reactivations of HSV-1 from latency in the trigeminal ganglia (TG). Almost 98% in the world and at least 58% of the population in the US are infected with HSV-1 and therefore developing therapeutic vaccines that prevent viral reactivations are more realistic and helpful in disease prevention than prophylactic vaccines that will protect individuals from primary infection.

Because HSV-1 reactivation from latency occurs due to compromised immune function, boosting the immune responses against HSV-1 would be an excellent strategy to prevent viral reactivation and thus alleviate the severity of and/or prevent HSK. Our lab has previously established that HSV-1-specific CD8 T cells that interact with latently infected neurons in the TG can prevent reactivation by releasing IFN-γ and non-cytolytic granzyme B. The number of TG-resident immunodominant CD8 T cells within latently infected TG inversely correlates with HSV-1 reactivation from latency ex vivo and latent viral genome copies (23, 174), which is largely determined by not only HSV-1-specific CD8 T cells but also the innate immune response that controls viral replication in the cornea early during the primary infection.

One of the best-characterized inhibitory receptors in immune regulation is PD-1. PD-1 is expressed on a wide range of activated immune cells including the innate immune cells such as neutrophils, inflammatory monocytes, and macrophages in the infected cornea, and the adaptive
immune cells such as CD4 and CD8 T cells in the draining lymph nodes and the TG following infection. We found its ligand PD-L1 to be up-regulated on parenchymal tissue such as corneal epithelial cells, neurons in the TG, and also on CD45+ cells following HSV-1 infection and predicted that in vivo PD-L1 blockade may reduce HSV-1 reactivation frequency by enhancing both innate and adaptive immune responses during HSV-1 infection.

**Conclusion 1)** PD-L1 inhibits Gr-1+ inflammatory monocyte recruitment by down-regulating chemokine levels and suppresses γδ T cell response in the cornea during acute HSV-1 infection.

Our data indicate here that, hours after HSV-1 ocular infection, PD-L1 on CD45+ or CD45− cells can suppress the production of chemokines responsible for recruiting Gr-1+ cells to the infected cornea. HSV-1 infected corneas of B7-H1−/− mice had significantly increased levels of chemokines including CCL3, CXCL1, CXCR2, and CXCR3, which resulted in an increased infiltration of CD45+ Gr-1+ cells into the central cornea at 1 DPI. Furthermore, the amount of viral replication in the cornea was significantly decreased in B7-H1−/− mice at as early as 1 DPI, due to the increased number of Gr-1+ cells. Depletion of neutrophils did not result in any significant difference in viral clearance whereas depletion of both neutrophil and inflammatory monocyte populations resulted in a significant increase in viral titers in the cornea. This suggests that the enhanced viral clearance was due to the increased number of inflammatory monocytes in the cornea.

The enhanced viral clearance from B7-H1−/− cornea is continuously observed until 4 DPI when most B7-H1−/− mice did not contain live virus in their corneas whereas all of wild type mice still did. We found that the expression of PD-L1 on corneal epithelium peaked at 4 DPI when macrophages, NK cells and T cells were starting to come into the cornea. At 3 DPI, we found a
significant increase in the number of activated, CD69\(^+\) \(\gamma\delta\) T cells in B7-H1\(^+\) corneas but the number and the function of NK cells remained equal to wild type.

We conclude that PD-L1 does not regulate NK cell function or proliferation in our model but it can regulate the number of \(\gamma\delta\) T cells that are recruited to the cornea after 3 DPI in two ways: 1) PD-L1 indirectly regulates \(\gamma\delta\) T cell recruitment by suppressing the production of chemokines such as CXCR3 and CXCL1 during the early phase at 1~2 DPI, and 2) PD-L1 on corneal epithelial cells directly regulates T cell survival and activation by interacting with PD-1\(^+\) \(\gamma\delta\) T cells in the cornea after 3 DPI. It is also important to note that it is PD-L1 expressed on CD45\(^-\) corneal epithelial cells that is crucial for regulating the T cell response in the cornea during the late phase (after 4 DPI) since T cells must interact with infected cells to function whereas PD-L1 on both CD45\(^+\) and CD45\(^-\) cells can inhibit Gr-1\(^+\) cell recruitment by suppressing chemokine production during the early phase (before 4 DPI).

**Conclusion 2** PD-L1 expressed on T cells promotes DC maturation and further contributes to proper priming of gB-specific CD8 T cells in the draining lymph nodes during acute HSV-1 infection.

Although PD-L1 seems to play an inhibitory role in regulating innate immune responses in the cornea, we found that PD-L1 expressed on T cells can also be stimulatory by inducing the expression of a co-stimulatory molecule CD80 on DCs before infection and for up to 4 days following infection when T cell priming occurs. We found a significantly decreased number and percentage of CD80\(^+\) DCs in the lymph nodes of B7-H1\(^+\) mice during the first 8 days following HSV-1 infection, which resulted in a slightly decreased number of gB-specific CD8 T cells in the TG throughout HSV-1 infection. We speculate that this DC-T cell interaction between CD80\(^+\) DCs and gB-Tet\(^+\) CD8 T cells in the draining lymph nodes may be crucial for generating a
proper gB-specific CD8 effector T cell response. We continue to see a slightly decreased number of gB-Tet CD8 T cells throughout HSV-1 latent infection.

**Conclusion 3) IFN-γ induces PD-L1 expression on HSV-1-latently infected neurons in the TG and PD-L1 induces apoptosis in PD-1hi gB-TetCD8 T cells in the TG. However, the absence of PD-L1 does not lead to the enhanced functionality of these cells or better protection against HSV-1 reactivation.**

During HSV-1 latent infection, PD-L1 was up-regulated preferentially on neurons that were latently infected. IFN-γ was necessary to up-regulate PD-L1 on these neurons during acute and latent infection, suggesting this may be a negative feedback mechanism of HSV-1-specific CD8 T cells that interact with infected neurons and release IFN-γ to dampen their response. On the other hand, PD-L1’s receptor PD-1 was preferentially expressed on gB-Tet CD8 T cells in the TG and many of these cells had a phenotype indicative of partial exhaustion as shown by IFN-γ and grzB staining whereas majority of gB-Tet CD8 T cells retained full functionality with very little PD-1 expression.

Consistent with the findings from models of chronic infection and cancer, the absence of PD-L1 led to a significant increase in the total number of CD8 T cells during HSV-1 latent infection. There was a preferential increase in the number of PD-1+ gB-Tet CD8 T cells in the latently infected TG of B7-H1− mice due to the increased survival and accumulation of PD-1+ gB-Tet CD8 T cells. Unfortunately, this increased PD-1+ gB-Tet CD8 T cell population had a high expression of PD-1 per cell and was largely dysfunctional. We conclude that the absence of PD-L1 did not “restore” the function or the proliferative capacity of gB-Tet CD8 T cells and instead only rescued the PD-1+ gB-Tet CD8 T cells that were highly dysfunctional from undergoing physical deletion. This finding is contrary to previous observations using chronic
viral infection models because in their systems blockade of PD-L1 or the absence of PD-L1 usually lead to restoration of virus-specific CTL function and proliferation.

Furthermore, there was no significant difference in HSV-1 reactivation frequency between wild type and B7-H1−/− mice. We conclude that B7-H1−/− mice do not show better CD8 T cell-mediated protection against HSV-1 reactivation despite the increase in CD8 T cell numbers and lower latent viral load in their TG. The increase in CD8 T cell number in B7-H1−/− mice was mostly in dysfunctional CD8 T cells and lower latent viral load was an effect that carried over from the enhanced innate immune response that we observed during acute HSV-1 infection.

**Conclusion 4)** Anti-PD-L1 blocking antibody treatments lead to significantly varying results in immune responses against HSV-1 depending on the timing, the duration, and the route of treatment.

Lastly, we wanted to investigate the most effective anti-PD-L1 blocking antibody treatment strategies in enhancing immunity against HSV-1 in order to prevent viral reactivation. Using anti-PD-L1 treatments for *in vivo* PD-L1 blockade rather than using B7-H1−/− mice also allowed us to dissect the many different roles and mechanisms of PD-L1. The summary of our findings using different anti-PD-L1 treatments are: 1) Subconjunctival (local) anti-PD-L1 treatments during the early acute infection (0~4 DPI) enhances the innate immune response that controls viral replication in the cornea but does not lead to any difference in HSK incidence and severity, 2) Intraperitoneal (systemic) anti-PD-L1 treatments and the absence of PD-L1 in B7-H1−/− mice during the early acute infection (0~3 DPI) does not result in an increased CD8 T cell response possibly due to the lower percentage of mature DCs (CD80+ DCs) that can provide co-stimulation for HSV-1-specific CD8 T cells, 3) Systemic anti-PD-L1 treatments that start during T cell clonal expansion (4~7 DPI) result in a significant increase in CD4 and CD8 T cell
proliferation but does not lead to any significant neuron damage in the TG, and 4) Anti-IL-10R treatments in B7-H1−/− mice during latent HSV-1 infection results in enhanced proliferation and increased granzyme B expression of subdominant CD8 T cells, suggesting that combinatorial blockade during latency using anti-PD-L1 blockade and other inhibitory receptor blockade may prove to be an attractive option for enhancing HSV-1-specific CD8 T cell response to prevent reactivation.

In conclusion, systemically blocking PD-1-PD-L1 interaction after the initial DC-T cell interaction and during the generation of T cell effector response (after 4 DPI) not only generates an enhanced CD8 T cell response without affecting DC maturation or neuron apoptosis but also results in enhanced gB-Tet+ CD8 T cell response. Therefore, continuous anti-PD-L1 treatments from 4 DPI to latency will maximize the CD8 T cell response against HSV-1 and may enhance CD8 T cell-mediated protection but more experiments that determine the possible detrimental effects on neurons in the TG, reactivation frequency and further characterization and function studies of CD8 T cell should be done for future studies.
9.0 FUTURE DIRECTIONS

9.1 OTHER INHIBITORY MOLECULES ON HSV-1-SPECIFIC CD8 T CELLS

From our studies using B7-H1−/− mice and in vivo anti-IL-10R blockade (100, 191), we have concluded that PD-L1 regulates the survival of HSV-1 subdominant epitope-specific CD8 T cells whereas IL-10 controls the proliferation of subdominant CD8 T cells in the TG during latent HSV-1 infection. We also have done preliminary studies blocking IL-10 in B7-H1−/− mice and conclude that they act through independent mechanisms. However, there are a number of other inhibitory molecules that can be up-regulated on virus-specific CD8 T cells during infection such as LAG-3, CD244 (2B4), CD160, CTLA-4, and TIM-3 (109, 110) whose functions and mechanisms during latent HSV-1 infection are not completely understood.

A previous study using recombinant galectin-9, the ligand of Tim-3, has suggested that during latency, the majority of gB-Tet+ CD8 T cells express Tim-3, Gal9/Tim-3 interactions induce apoptosis of CD8 T cells in the TG, and adding recombinant Gal-9 to ex vivo latent TG cultures resulted in significantly increased HSV-1 reactivation (212). Because co-expression of inhibitory receptors suggest higher level of exhaustion, blocking multiple inhibitory pathways at the same time to restore functionality to different subsets of exhausted T cells may be more efficient than blocking one pathway alone (112-115). We hypothesize that a carefully controlled blockade of multiple inhibitory pathways can enhance the proliferation, function, and survival of
HSV-1-specific CD8 T cells and further prevent HSV-1 reactivation without causing immunopathology.

9.2 THE ROLE OF PD-L1 IN REGULATING CD4 T CELL RESPONSE

A number of studies have shown that PD-L1 can also inhibit the proliferation and function of PD-1+ CD4+ T cells. Although we have not determined the exact mechanism of PD-L1-mediated control of CD4 T cell responses during HSV-1 infection, our data indicates that systemic anti-PD-L1 treatments at 4 DPI and 6 DPI significantly increased CD4 T cell proliferation in the draining lymph nodes at 7 DPI (Fig 27C), suggesting PD-L1 may be inhibiting at least the proliferation of CD4+ T cells during the generation of effector CD4 T cell response.

However, it is not certain whether this increased CD4 population will migrate to and be retained within the TG during latent HSV-1 infection to help prevent HSV-1 reactivation. We know that the absence of PD-L1 throughout the infection does not result in increased function of CD4 T cells based on the results in which we did not observe any decrease in reactivation frequency in B7-H1−/− mice (Fig 16). Therefore we predict that anti-PD-L1 treatments only during the generation of CD4 T cell effector response may not only increase CD4 T cell proliferation in the latently infected ganglia but also provide help for HSV-1-specific memory CD8 T cells in preventing HSV-1 reactivation.

Another role PD-L1 may play in regulating CD4 T cell responses is by generating and maintaining the regulatory T cell population. In vitro studies using beads bound to recombinant
PD-L1 have suggested this. Although we only see less than 10% of Foxp3⁺ CD4⁺ regulatory T cells within infected TG, blocking PD-L1 in vivo may lead to a significant decrease in this population, contributing to enhanced effector and memory CD4 and CD8 T cell responses within the TG.

9.3 THE MECHANISM OF PD-L1 IN REGULATING RECRUITMENT AND FUNCTION OF IMMUNE CELLS IN THE CORNEA

Although we attempt at dissecting the biphasic functions of PD-L1 in regulating the innate immune response in Chapter 6, a number of questions remain to be answered. What cells are responsible for producing chemokines and cytokines that are suppressed by PD-L1 in the cornea? Future experiments to determine this include confocal imaging of the whole mount cornea staining of chemokines such as CXCL1, CXCR3 and innate immune cell subsets in the cornea. We can also utilize a number of in vivo chemokine blockades in wild type and B7-H1⁻/⁻ corneas to confirm that the increase in chemokine production is indeed responsible for enhanced cell infiltration into the cornea.

Does PD-L1 contribute to down-regulating the function of neutrophils and inflammatory monocytes during the first three days? The production of MPO (myeloid peroxidase) assay can be performed to determine whether there is more production of MPO by neutrophils in the absence of PD-L1. Based on the increased infiltration of Gr-1⁺ cells and the increased viral load with Gr-1⁺ cell depletion but not with neutrophil depletion, we know that the inflammatory monocyte population is responsible for the enhanced viral clearance in B7-H1⁻/⁻ mice. For future
studies, we could neutralize the increased chemokines in B7-H1−/− mice and determine whether it results in decreased recruitment of inflammatory monocytes, and therefore increased viral replication in the cornea.

We have ruled out the possibility that PD-L1 can suppress the function and the number of NK cells in the cornea after 3 DPI. Instead, we have observed a significant increase in the number of γδ T cells infiltrating the cornea at 3 DPI. Are these T cells in the cornea responsible for the late control of virus in the cornea in the absence of PD-L1? We could get at this by depleting γδ T cells locally and determine whether the viral clearance in the B7-H1−/− cornea is decreased.

Does the increased immune cell infiltration during acute infection cause any damage to the corneal tissue during acute infection? Previous studies have demonstrated that viral replication in the cornea is controlled mainly by the innate immune cells such as inflammatory monocytes and NK cells during primary HSV-1 ocular infection. However, in B7-H1−/− mice we see an increased T cell infiltration that occurs earlier in the cornea, suggesting that T cells may be able to contribute HSV-1 clearance from the cornea but are normally inhibited through PD-1-PD-L1 interactions possibly as a corneal tissue protective mechanism. To test this, we can perform TUNEL assay on the wild type and B7-H1−/− corneas and compare tissue damage.
APPENDIX A

LIST OF WORKS


37. Vicetti Miguel RD, Sheridan BS, Harvey SA, Schreiner RS, Hendricks RL, Cherpes TL. 17-beta estradiol promotion of herpes simplex virus type 1 reactivation is estrogen receptor


155. Mueller SN, Vanguri VK, Ha SJ, West EE, Keir ME, Glickman JN, Sharpe AH, Ahmed R. PD-L1 has distinct functions in hematopoietic and nonhematopoietic cells in regulating T cell


