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Herpes Virus Entry Mediator (HVEM) and Ocular Herpes: More Than Meets the Eye

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ABSTRACT

Herpes Virus Entry Mediator (HVEM) and Ocular Herpes:
More Than Meets the Eye

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Herpes stromal keratitis (HSK) is a potentially blinding inflammatory condition initiated by corneal infection with herpes simplex virus (HSV). As its name suggests, the host receptor herpes virus entry mediator (HVEM) facilitates HSV entry through interactions with a viral envelope glycoprotein. HVEM also bridges several signaling networks, binding ligands from both TNF- and Ig-superfamilies with diverse, and often opposing, outcomes. While it has long been established as an entry receptor for HSV-1, HVEM has only recently emerged as a host factor important for pathogenesis of ocular herpetic disease. HVEM KO mice are protected from severe systemic disease, including neurologic morbidity, viral spread, and epithelial lesion development. To investigate the contribution entry makes to this process, I infected mice with a mutant HVEM entry-null virus; to my surprise, I found that HVEM-mediated pathogenesis does not require the gD-HVEM interaction, indicating its effects are separable from viral entry. In addition, adoptive transfer experiments show that susceptibility to disease coincides with the presence of HVEM on radiation-resistant cell type(s). In contrast to previous reports, the majority of HVEM in the cornea is found on monocyte-lineage cells rather than on corneal epithelial cells, suggesting HVEM on resident macrophages may account for pathogenesis in our model. Characterization of corneal-specific disease revealed HVEM increases inflammatory cytokine expression and stromal immune cell infiltrates, and exacerbates corneal nerve damage. Together, these findings demonstrate HVEM has a central and multifaceted role in the immunopathogenesis of HSK, impacting not only viral replication and spread, but also chronic
inflammation. Systemic treatment with immune modifying nanoparticles (IMPs) successfully disrupted immune cell trafficking to the cornea, reducing corneal sensitivity loss and disease after HSV-1 infection. HVEM is broadly expressed, intersects two important immunologic signaling networks, and impacts autoimmunity, infection, and inflammation. I hope that by unpacking the complex range of effects mediated during by this receptor, I can offer insights applicable to a wide set of disease states.
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Dedicated to my family,

Rick, Carolyn, Sam, George, Anna, Gelesia,

Henry, Sylvie, Simon, and Bennet
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CHAPTER 1

Introduction to the Dual Roles of HVEM: Entry and Immunomodulation

Introduction

Herpes simplex virus (HSV) types 1 and 2 infect the majority of the world’s population by adulthood (1), making them among the most ubiquitous pathogens in the human species. Although these closely related viruses cause genital herpes at near equal rates, HSV-1 is responsible for the vast majority of ocular herpetic infections (2-4). Asymptomatic people as well as those with active herpes lesions, including corneal ulcers and orolabial cold sores, shed HSV-1 in tears and saliva (5-7). After reaching a new host through close personal contact, the virus invades the nervous system from the typical infection site, the oral epithelium, along sensory branches of the trigeminal nerve (8). HSV-1 establishes lifelong latency in the trigeminal ganglia (TG), and is consistently found in a majority of human TGs obtained at autopsy (9, 10). Reactivations of HSV-1 in the TG can lead to anterograde movement of the virus along the ophthalmic branch of the trigeminal nerve, resulting in recurrent infection of virtually all the superficial tissues of the eye, including the cornea, conjunctiva, and eyelid (5, 11, 12). While reactivation accounts for the majority of human disease, murine studies of HSK typically model primary infection as mice do not efficiently or reliably reactivate from latency (5).

Ocular herpes infections can lead to epithelial ulceration of the cornea, uveitis, and retinitis, but most commonly (roughly 20% of causes) cause herpes stromal keratitis or HSK (13). HSK is characterized by chronic inflammation of the corneal stroma, leading to corneal thickening, opacification, scarring, and potentially blindness (14, 15). HSK produces 40,000 new cases of severe vision loss or blindness each year, and is a leading cause of infectious blindness globally (16). The development of HSK requires two components of seemingly equal importance: actively replicating herpesvirus as well as a competent immune response. Immune-
mediated damage has been established as the main cause of HSK. Athymic mice do not develop the syndrome after infection, although they are highly vulnerable to neurologic HSV-1 disease (17). Replication-competent virus, while absolutely required to initiate disease (18-20), does not persist in the cornea by the time a second, pathologic wave of immune infiltrates, including CD4<sup>+</sup> T cells and polymorphonuclear cells (PMN), invade the stroma (21, 22). Multiple lines of evidence indicate that host infiltrates are responsible for the inflammation and subsequent damage incurred during HSK (19, 23-26). How these immune responders remain activated in the absence of virus remains a mystery, although most evidence suggests nonspecific, bystander activation is responsible, leading to the pathogen-provoked inflammation observed in HSK (14, 22, 27).

Herpes virus entry mediator (HVEM, also called tumor necrosis factor superfamily-member receptor 14 [Tnfrsf14]) is a host factor with an established role as a viral entry receptor and emerging one as an ocular immunomodulator in herpes keratitis. HVEM has a multitude of roles in mucosal response to a variety of pathogens (28-32), and has been found to influence HSV pathogenesis in the eye during most stages of infection, including entry/acute viral replication, early/innate responses, chronic inflammation, and even viral latency. The combination of HVEM and HSV produce devastating inflammation of the cornea, and illustrate the enmeshed virus-host interactions characteristic of HSK.

**HVEM is a viral entry receptor**

*Entry mechanics of HSV.* Herpesviruses are enveloped viruses with double-stranded, linear DNA genomes enclosed in an icosahedral capsid (33). Glycoproteins studding the viral envelope are essential for entry into host cells, a complex process requiring at least four separate glycoproteins, including gB, gH/gL, and gD (34). Fusion between viral and host membranes requires binding of gD to a host surface receptor (34). Several surface proteins
have been identified as gD receptor targets including nectin-2, HVEM, and nectin-1, although the latter two are considered the most biologically relevant entry receptors for HSV (35-38). Nectin-1 is involved in cell-cell adhesion and is a member of the immunoglobulin super-family (39). The complex immunomodulatory functions of HVEM, a member of the tumor necrosis factor (TNF) superfamily, are discussed in the next section.

The crystal structures of gD bound to both nectin-1 and HVEM have been solved (40, 41). Binding of HVEM or nectin-1 occurs on distinct regions of gD dimers (42), although either receptor binding gD triggers similar conformational changes in the structure of the viral glycoprotein, exposing the C-terminal pro-fusion domain (43). This allows gD to interact with gB trimers bound to a gB-specific receptor and the heterodimer gH-gL, whose cellular receptor remains unidentified (34, 44). The sum of these interactions is the insertion of the gB fusion loops into the cellular plasma membrane (or, in some circumstances, endosomal membrane) and refolding of the gB trimer into a post-fusion form (36). Subsequent mixing of viral and host membranes and the formation of a fusion pore through which the viral capsid and tegument proteins can be deposited completes the entry process (45).

While HVEM and nectin-1 exhibit similar kinetics and binding affinities to gD in vitro (44, 46), early in vivo studies showed a greater importance for nectin-1 in pathogenesis of HSV, especially in terms of invasion of and spread throughout the nervous system in intravaginal and intracranial models of HSV-2 infection (47, 48). Remarkably, nectin-1 KO mice inoculated with HSV-2 directly into the hippocampus do not develop encephalitis, even though HVEM is expressed in the brain; intracranially inoculated nectin-1 KOs also lack demonstrable virus by immunofluorescence (47). Although largely dispensable for HSV-2 infection of the brain (47), vagina (48), or eye (49), HVEM has been shown to promote HSV-1 pathogenesis specifically in the setting of ocular infection (50, 51). The reliance of HSV-1 on HVEM is tissue-specific in addition to serotype-specific, as HSV-1 infection of the vagina proceeds normally in the absence
of HVEM (49). In order to understand why this receptor is required for pathogenesis of ocular herpetic infections, both the entry and immunomodulatory functions of HVEM must be explored.

**HVEM-mediated entry in the eye.** HVEM expression has been evaluated in a number of ocular tissues. Studies of cultured human cell lines by real-time PCR (RT-PCR) and immunofluorescence (IF) or flow cytometry indicate retinal pigment epithelial (RPE) cells (52), corneal fibroblasts (53), trabecular meshwork cells (54), and conjunctival and corneal epithelial cells (55, 56) express HVEM mRNA and membrane-bound protein, respectively. Use of HVEM blocking antibodies (54, 56) or HVEM siRNA knockdown (55) reduces viral entry of some, but not all, of these cell types in vitro, suggesting HVEM is in use as an entry receptor. However, widespread expression of nectin-1 and sufficiency of nectin-1 in vivo for infection of the murine cornea and TG preclude the notion that HVEM is the primary receptor in the eye (50, 57-59).

This is not to say that HVEM is not important during ocular HSV-1 infection. Despite the presence of a suitable alternate entry receptor in the cornea, HSV-1 infection of Tnfrsf14−/− (HVEM knockout, KO) mice results in lower viral loads in eye swabs, with a subsequent decrease in titer from the TG, brain, and periocular skin (POS), and reduced rates of reactivation from the TG compared to wild type (WT) controls (49, 50). Along with this replication defect, HVEM KO mice are protected from systemic clinical symptoms of HSV-1, including periocular lesion development and neurologic morbidity (49, 50). While it is clear that HVEM contributes to immunopathology during HSK, the precise mechanisms underlying this process remain largely unknown. Evidence gleaned from other disease models/organs, in which HVEM signaling has been more thoroughly explored, provides clues about how HVEM may promote chronic inflammation in the eye.
HVEM alters host immune responses to HSV

HVEM signaling in the host. HVEM, also designated CD270, is a bi-directional receptor that can bind ligands of both the TNF superfamily and the immunoglobulin (Ig) family (Fig. 1) (32). Outside of the eye, HVEM expression is broad: although its expression fluctuates throughout maturation, HVEM is found on most types of leukocytes, including T and B cells, dendritic cells (DCs), NK cells, and myeloid cells (60). HVEM is also expressed by nervous tissue, and is present at high levels in gut and lung epithelia (30, 61).

Identified HVEM ligands include LTα (lymphotoxin α), LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to HVEM on T cells), BTLA (B and T lymphocyte attenuator), and CD160 (62-64). Immature DCs, monocytes, and activated T cells express membrane-bound or soluble forms of LTα and LIGHT (60). These trimeric TNF family ligands typically enhance activation or differentiation of a variety of immune cell types upon binding cysteine-rich domain (CRD) 2 and 3 of HVEM (65-68). BTLA is an Ig superfamily member found on T and B cells, DCs, and myeloid cells (63). CD160 is a dimeric, GPI-anchored protein with an Ig-like fold prototypically expressed by NK cells, but is also found on subsets of CD4+ and CD8+ T cells (64). Both BTLA and CD160 communicate a co-repressive signal upon binding HVEM CRD1 in most described cases (69-73). However, the outcome of HVEM signaling differs depending on whether the ligand is in soluble or membrane-bound form, whether the interaction occurs in cis or trans, and the specific identity of the cells involved (32, 64, 74, 75). Viral gD also binds CRD1 and most directly competes with BTLA for HVEM binding, although there is evidence LIGHT and LTα interactions with HVEM are also affected by the presence of gD (76). Because the TNF- and Ig-ligand domains occur on different faces of the receptor, combinations of HVEM and its ligands may result in ternary complexes (68, 69, 77-79).
As a receptor, HVEM engagement by LIGHT, CD160, BTLA, or viral gD recruits members of the TNFR-associated factor (TRAF) family to the cytoplasmic tail of HVEM, ultimately activating nuclear factor-κB (NF-κB) signaling (80, 81). NF-κB is a transcription factor usually sequestered in the cytoplasm by proteins such as IκBa/β; upon their degradation, NF-κB translocates to the nucleus to initiate transcription of DNA, cytokine production, and cell survival (82).

HVEM activates pro-survival signaling through NF-κB even when its binding partner is a co-repressive ligand, such as BTLA or CD160, meaning that cell-cell interactions through these molecules can impart two opposing messages. For example, T cells receiving co-suppressive signals through BTLA cease proliferating, while Btla−/− T cells stimulated by soluble BTLA-Fc induce NF-κB signaling via HVEM and exhibit increased survival (81). NF-κB induced by the binding of gD to HVEM has anti-apoptotic effects in vitro, and loss of NF-κB activation (and nuclear translocation), which is sensitive to blocking with anti-gD antibodies, reduces viral yield 80-90% and increases apoptosis (83-87). Therefore, HSV may benefit from HVEM activation through pro-survival signals that prevent apoptosis of infected cells. This process may be especially important in corneal epithelial cells, where there is some indication that activation of apoptotic pathways precedes their blockage by NF-κB, a series of events that may in fact be required for efficient replication in this cell type (88). Understanding the intricacies of the HVEM signaling network is challenging given the number of possible ligands involved, lack of predictability in whether the outcome of an interaction will be pro- or anti-inflammatory, and possibility of competing, opposing signals arising from the same receptor-ligand interaction. Nevertheless, I have found increasing evidence that HVEM modulates aspects of the innate immune response to HSV in the cornea to ultimately worsen inflammation, although much of the underlying mechanisms remain to be elucidated.
Figure 1. HVEM signaling is bi-directional. HVEM is a TNF-receptor superfamily member that can interact with Ig-like ligands (CD160 and BTLA), TNF ligands (LIGHT and LTα), and an HSV glycoprotein, gD. HVEM is expressed on a broad range of cell types, including T cells, B cells, DCs, NK cells, macrophages, PMN, neurons, and epithelial cells. BTLA and LIGHT are also found on most leukocytes, including B and T cells, granulocytes, NK cells, etc. CD160 is more restricted in its expression, and is found on subsets of CD4+ and CD8+ T cells, NK cells, and intraepithelial lymphocytes (IELs). BTLA and CD160 bind cysteine-rich domain (CRD) 1 of HVEM, while LIGHT and soluble LTα bind CRD2 and 3. The HSV glycoprotein gD also binds HVEM on CRD1, and competes directly with BTLA (and, presumably, CD160) for HVEM binding. Outcomes can vary from co-repressive signals delivered through CD160 and BTLA in trans to co-stimulatory signals through LIGHT. BTLA and LIGHT can also associate with HVEM in cis, forming heterotrimeric complexes, the conformation most commonly seen on resting T
cells. *Cis* binding between BTLA and HVEM holds HVEM in an inactive state, without NK-κB activation. In contrast, binding of HVEM by BTLA in *trans* or by any of its other ligands leads to activation of NF-κB signaling. The cytoplasmic portion of HVEM has been shown to recruit TRAF family members. Subsequent activation of IKKβ/α, phosphorylation and degradation of the NF-κB chaperone IκB, and activation and nuclear translocation of NF-κB results in increased transcription of inflammatory factors and pro-survival signals within the HVEM-expressing cell.
Overview of HSK pathogenesis. In the primary murine model of HSK, actively replicating HSV-1 in the cornea can be detected by plaque assay for up to 5-6 days post infection (dpi), although viral DNA, detected by PCR, persists for several weeks (13, 89). Immediately after infection (<24 hours), infected and uninfected epithelial cells secrete chemotactic, angiogenic, and lymphangiogenic factors facilitating stromal infiltration by innate immune cells (19, 90-93). These early responders, including neutrophils (PMN), macrophages, natural killer (NK) cells, DCs, and γδ T cells are critical for initiating adaptive responses and, in the case of PMN and macrophages particularly, restricting viral replication and spread to the CNS (94-100). After infection is controlled, populations of PMN subside (13); however, a larger, secondary influx of PMN and CD4^+ T cells arrive 7-14 dpi, causing chronic inflammation and damage of the cornea (21, 22, 101). In the analogous condition in humans, stromal inflammation, neovascularization, and scarring may subsequently lead to vision loss, requiring corneal transplantation (102). Sensation to mechanical stimuli and heat is also lost in human HSK patients and reflects damage to corneal nerves (103). In mice, corneal nerve retraction, most likely caused by the inflammatory milieu rather than by viral lysis of neurons, occurs prior to second-wave infiltration of the stroma, and may contribute to later inflammation as loss of blinking causes desiccation (101, 104). Consistent with its broad leukocytic expression and host of immunomodulatory functions, HVEM has been shown to influence a variety of innate and adaptive aspects of HSK immunopathogenesis as well as other autoimmune, infectious, and inflammatory conditions (31).

HVEM and innate immune responses. The innate immune system is responsible for nonspecific, immediate countermeasures to limit the spread of and damage from a pathogen. Key features of this response include the secretion of cytokines and chemokines, removal of infected cells, and antigen presentation. My studies have found that HVEM, or HVEM ligands,
are involved in several aspects of the innate response to HSV-1 in the cornea, consistent with previous work indicating HVEM influences innate immune effectors in a variety of ways.

One of the first indications that herpesviruses may alter early innate immune responses to infection via HVEM came from the vaginal model of genital HSV-2 infection. Mice infected with an HVEM entry-null HSV-2 (generated through a targeted deletion in the HVEM-binding interface of viral gD, Δ7-15) have significantly higher levels of the cytokine IL-6 and chemokines CXCL9, CXCL10, and CCL4 in vaginal washes than do mice infected by HSV-2 that could engage HVEM (105). These findings imply HSV-2 targets HVEM signaling to suppress cytokine induction. In vitro, siRNA-mediated knockdown of HVEM from cultured telomere-immortalized human corneal epithelial cells increases production of IFN-γ, MIP-1α (CCL3), and MIP-1β (CCL4) after HSV-1 challenge (106). It is important to keep in mind that a major limitation of this single-cell type, in vitro system is that HVEM stimulation occurred solely with HSV gD, while in vivo, binding by natural ligand(s) could be of equal or great importance.

The mechanism by which HVEM influences cytokine secretion has not been defined. Elegant studies of innate responses to bacteria in lung and gut mucosal epithelia have identified a role for host HVEM signaling in the induction of IL-6 and other cytokines (29). In this model, CD160 on innate-like intraepithelial lymphocytes (IELs) activates HVEM, which is highly expressed by the intestinal epithelium, resulting in NF-κB-mediated Stat3 activation and increased expression of genes and peptides related to epithelial immunity (29, 30). During HSK, the corneal epithelium secretes IL-6 and other cytokines in response to HSV-1 infection (91, 107), and it has been reported that corneal epithelial HVEM expression increases after infection (108). Though these data suggest the corneal epithelium could contribute to pathogenesis via HVEM, studies reported here do not support the latter finding, and instead indicate HVEM is mostly restricted to leukocytes in the eye.
Corneal and conjunctival macrophages are required for viral restriction in the first 48 hours after infection (96, 99, 100, 109). However, control of viral replication comes at a price: macrophages contribute to CD4\(^+\) T cell activation during ocular HSV-1 infection (100), likely through antigen presentation, and macrophage-associated cytokines like MIP-1\(\alpha\) and MIP-1\(\beta\) further corneal infiltration and damage (110). Similarly, depletion of PMN allows HSV to replicate to higher levels in the cornea and increases mortality due to CNS invasion (94), but also reduces HSK severity and incidence even when CD4\(^+\) T cells are still present (21, 95). Macrophages and PMN also promote vascularization of the cornea, a required step in HSK development, through secretion of VEGF and matrix degradation enzymes (92, 111). All of these effects could be influenced by HVEM signaling on macrophages and neutrophils, potentially through HVEM activation directly or through interactions with its ligands.

LIGHT binding of HVEM on macrophages and neutrophils provides an activating signal, increasing phagocytic activity and production of inflammatory/anti-bacterial factors, including nitric oxide (NO), reactive oxygen species (ROS), IL-8, and TNF-\(\alpha\) (112) via changes in intracellular calcium sequestration (113). Agonistic binding of HVEM on neutrophils also increases respiratory burst and degranulation, providing further explanation for the increased bactericidal activity of PMN via HVEM (114). In vivo, secretion of type I interferons from splenic cells, especially macrophages, partially requires HVEM; in HVEM-deficient mice, loss of type I interferons reduces lymphocyte bystander activation and immunopathology after Listeria infection (115).

In contrast, HVEM-BTLA interactions reduce activation of innate cell populations, including macrophages, inflammatory monocytes, and PMN (31, 70, 71). During acute experimental sepsis, HVEM-BTLA interactions on innate populations worsen organ injury, bacterial burden, and mortality (116). Although both HVEM and BTLA are expressed on recruited myeloid cells in this model, BTLA-directed co-repressive signals rather than HVEM-
directed pro-survival/NF-κB signals more easily explain the finding of reduced myeloid activation and survival. Our laboratory is currently investigating the corneal expression of both LIGHT and BTLA on resident and infiltrating cells during HSV-1 infection in order to understand what role these ligands play in HVEM-mediated disease.

DCs, another professional antigen presenting cell (APC) type that express HVEM, are susceptible to HSV infection (117, 118). Immature Langerhans-like epithelial DCs reside in the limbus, corneal periphery, and even central cornea in naïve mice, although central corneal DCs express no MHC class II (119). Resident (or very early-infiltrating) DCs are required for viral control in the acute phase, as they direct newly extravasated NK cells and inflammatory monocytes toward the central cornea (98). Without local DCs, increased viral proliferation, inflammatory infiltrates, scarring, and keratitis occur, as well as severe corneal nerve loss (120). Interestingly, corneal nerve loss is associated with decreased nerve infection: lacking DCs, the CNS is spared at the expense of increased corneal damage (120). HSV gD binding activates NF-κB signaling in DCs, directly inducing DC maturation, as well as maturing uninfected neighboring DCs by upregulating type I IFN secretion and expression of co-stimulatory molecules (121, 122). LIGHT binding has similar effects, and has also been shown to increase the ability of DCs to prime of cytotoxic T cell responses (123). It seems possible that maturation of corneal DCs through HVEM could lead to increased activation of CD4⁺ T cells, worsening pathogenesis of HSK, although this has not yet been investigated directly.

NK cells express the HVEM ligands LIGHT, CD160, and BTLA. As mentioned previously, LIGHT on licensed NK cells participates in DC maturation through HVEM signaling (64, 124). In this way, cross-talk between LIGHT-expressing NK cells and resident or early-arriving HVEM-expressing DCs could lead to NK migration to the central cornea and cytokine release (98). CD160 is found mostly on NK cells but also some CD4⁺ and CD8⁺ subsets (see adaptive immune responses section) (75, 125). Unlike in T cells, where it is co-repressive (64),
CD160-HVEM binding increases NK activation in the presence of type I IFN, IL-2, or virally-infected cells, leading to inflammatory cytokine production and infected cell lysis (75, 126). The specific role of NK cells during HSK development remains somewhat undefined, although they are among the earliest infiltrates to appear in the infected corneal stroma, arriving with PMN within 1 day after infection (127). In vivo NK depletion decreases the severity and incidence of HSK in susceptible BALB/c mice (97), corneal scarring and mortality in C57BL/6 mice (128), and corneal neutrophil migration in a reconstituted SCID mouse model (129). NK cells could contribute to HVEM-mediated pathogenesis in a variety of ways, such as by activating other HVEM-expressing leukocytes via LIGHT, or by increasing their own production of inflammatory cytokines via CD160. The impact of HVEM signaling in NK cells during HSK development, like that of HVEM in most innate immune cell types, merits further investigation.

HVEM and adaptive immune responses. Adaptive immune responses develop over the course of days, rather than hours, and are mediated by lymphocytes, including CD4\(^+\) and CD8\(^+\) T cells (130). Although CD8\(^+\) T cells are classically associated with viral infections, helper Th1 CD4\(^+\) T cells are considered the major immunopathologic cell type in HSK (13, 14, 24). Investigators have consistently found that without functional CD4\(^+\) T cells, HSK does not develop (17, 131, 132). Corneal CD4\(^+\) T cells remain activated in the absence of replicating virus, likely through bystander activation (133), in which CD4\(^+\) T cells become non-specifically activated due to the surrounding inflammatory milieu. Consistent with this hypothesis, CD4\(^+\) T cells do not have to be viral-antigen-specific to cause HSK, although virus-specific CD4\(^+\) T cells may initiate the process (22, 133-136). While a variety of other mechanisms have been offered, including auto-antigen unmasking (137) and viral molecular mimicry (138), these hypotheses have fallen out of favor after it was shown that the peptides proposed to produce auto-reactive
CD4+ T cells do not induce HSK in mice, and are not recognized by T cells isolated from patients with HSK (136, 139).

The majority of HVEM on naïve T cells occurs in cis-complexes with BTLA, with or without LIGHT as a part of the complex (140). BTLA provides a co-repressive signal on CD4+ T cells in cis or in trans (64, 70). In the cis complex, BTLA holds HVEM in an inactive state, preventing NF-κB activation (140). BTLA has been reported to increase in the HSV-1-infected cornea, although the identity of BTLA+ corneal cells and the effect endogenous BTLA expression has on pathogenesis were not thoroughly investigated (141). Intravenous treatment with a recombinant BTLA-expressing plasmid prior to and during ocular HSV-1 infection reduces HSK symptoms, the overall number of corneal CD4+ T cells, and the proportion expressing IFN-γ, although no experiments were performed to determine which cell types absorb the plasmid and how these effects are mediated (141).

Like BTLA, CD160 negatively regulates CD4+ T cells in trans, while LIGHT is a potent stimulator of CD4+ T cells when HVEM on APCs is delivered in trans, though CD160 and BTLA binding of CRD1 domain can attenuate this (64). The expression of LIGHT and CD160 in the cornea has not been addressed, and is currently under active investigation by members of the Longnecker laboratory.

A specialized set of anti-inflammatory CD4+ T cells, FoxP3+ regulatory T cells (Tregs), reportedly expand during HSV-1 infection through gD-HVEM interactions, leading to slightly diminished corneal pathology in HVEM KO corneas late after infection (142). While this instance of decreased HSK symptoms in HVEM KO corneas conflicts with a multitude of studies from our laboratory (49-51), differences in viral strain and inoculation dose could produce variability in findings. Due to the complexity of HVEM signaling, and its widespread expression on nearly every leukocytic population implicated in the development of HSK, it would be surprising if only one HVEM-associated cell type or function influenced ocular herpes immunopathogenesis.
While I am confident HVEM-mediated inflammation is the more potent effect, it is plausible that HVEM could have contradictory roles, and that HVEM on Tregs may provide some relief during HSK. Further investigation of this discrepancy is required.

While less important for HSK development, CD8+ T cells control viral spread into the nervous system (143, 144), and suppress viral reactivation from the TG (8). The latency state, characterized by suppression of all viral products, except the long noncoding RNA latency-associated transcript (LAT), occurs in the TG in humans and mice (145). LAT is not required for latency (146, 147), although LAT(-) viruses establish latency and reactivate less efficiently (148). Most human cases of HSK result from reactivation of a latent infection rather than from primary infection (16). Unfortunately, significant gaps in our understanding of latency and reactivation persist because the vast majority of studies are performed in murine models of primary infection (149). However, it has been established that CD8+ memory T cells infiltrate latently infected TGs in humans and mice and reside in close association with neurons (150, 151). Rather than causing apoptosis of neurons (152), granzyme B released from CD8+ T cells enters neighboring latently infected neurons and cleaves viral ICP4 (infected cell polypeptide 4). ICP4 is needed for viral replication (153), and its degradation by granzyme B inhibits reactivation from latency (154).

Interestingly, HVEM has been shown to influence CD8+ memory T cell recall responses (155), and directly impact viral latency in the TG (156). In the vaginal model of HSV-2 infection, mice primed with an HSV-2 that cannot utilize HVEM for entry (Δ7-15 mutation) and rechallenged with WT virus 32 days later had fewer virus-specific CD8+ recruited to the vaginal mucosa during the recall response (155). In addition, several vaccine studies found that constructs lacking gD-HVEM interactions produced stronger memory immune responses (157, 158). Together, these data suggest gD could interfere with natural inhibitory cis BTLA-HVEM signaling on CD8+ T cells. More recently, investigators demonstrated BTLA delivered in trans to
HVEM on CD8⁺ T cells promotes their survival and the development of memory immune responses (159), providing an alternative explanation why lack of HVEM activation (through gD, in the case of HSV-2) may lead to fewer surviving memory CD8⁺ T cells. This development illustrates how challenging it is to predict the relative in vivo importance of cis- vs trans-interactions of the HVEM signaling network, and the need for further study of HVEM and its ligands in CD8⁺ T cell responses.

Finally, the close association of CD8⁺ cells, which typically express a variety of HVEM ligands (73), and neurons of the TG, which express HVEM (108), raises the question of a direct effect of HVEM signaling on HSV latency. HVEM KO mice have lower rates of latency and reactivation than WT mice, although the HVEM KO strain also has lower titers in the eye initially, likely leading to decreased seeding of the TG (50, 156). Recently, investigators reported that LAT upregulates HVEM expression in vivo and in vitro, potentially through binding of the HVEM promoter by small noncoding RNAs derived from LAT (156). Increased HVEM expression could alter immune responses to reactivation; alternatively, HVEM-mediated NF-κB activation could enhance survival of neurons undergoing reactivation. Because human disease is mostly caused by reactivation, our laboratory is actively pursuing murine models of recurrent disease (160) to study what contribution HVEM may make to this process.

**HVEM signaling impacts a variety of human diseases, offering targets for therapy**

HVEM is implicated in a wide range of autoimmune, inflammatory, and infectious processes that impact an astonishing diversity of human syndromes (31). Because of this, the HVEM signaling network is a rich area of research for the discovery of new therapies. HVEM/LIGHT/BTLA signaling and therapeutic opportunities within that network have been most extensively studied in models of graft-versus-host disease (68, 77, 161-164). Graft-versus-host disease (GVHD) is an immunologic syndrome affecting transplant recipients in which engrafted
donor T cells attack host tissues, causing rampant damage (165). Ideally, therapies specifically target pathologic functions of donor T cells without causing global immune suppression (165). Investigators have had success in decreasing symptoms of GVHD in mice by targeting both BTLA-HVEM and LIGHT-HVEM interactions with blocking antibodies (162-164). Blockade of LIGHT-HVEM signaling has also been shown to increase the survival of solid allografts, including pancreatic islets and cardiac transplants (166, 167). Recently, lymphoma B cells from patients with mutations in the HVEM gene (TNFRSF14) were found to have increased alloantigen-presenting capacity than controls, corresponding to higher levels of GVHD in patients undergoing allogeneic hematopoietic stem cell transplantation (168).

In murine experimental autoimmune uveitis (EAU), a model of human autoimmune conditions with ocular manifestations like Behçet disease and sarcoidosis, HVEM was shown to worsen disease severity by inducing pathogenic Th1- and Th17-type T cell responses (169). Similar to HVEM KOs, LIGHT and BTLA KOs are protected from severe disease during EAU, suggesting these ligands in combination with HVEM promote pathogenesis (169). Once the HVEM ligand or ligands responsible for ocular herpetic pathogenesis is/are identified, the appropriate blocking antibody can be tested in prevention or treatment of HSK. If LIGHT and/or BTLA are the ligands involved in HSK as well, targeting HVEM signaling with antibodies or small molecule inhibitors could produce novel therapies applicable to a variety of ocular inflammatory conditions.

**HVEM is a pro-inflammatory factor that drives HSK development**

In a mouse model of HSK, HVEM has been shown to worsen systemic disease, increasing viral spread, neurologic disease and periocular lesion development, and reactivation from TG explants (49, 50). I have found that HVEM also increases disease of the cornea, where it increases viral loads, immune cell infiltrates, expression of inflammatory cytokines, and
corneal sensitivity loss to mechanical stimulation (51). Loss of HVEM-entry capacity through mutations in viral gD produces no change in these outcomes, indicating HVEM interacts with one or several of its natural ligands to mediate immunopathology (51). HVEM is required on a radiation-resistant cell type (51), and because its expression in the cornea is limited to leukocytes, I hypothesize that corneal macrophages are the main mediators of disease through HVEM signaling. Consistent with this, ablation of HVEM from hematopoietic lineages reduces corneal infiltrates and viral loads in the cornea. These findings lead me to propose that, independently of viral entry, HVEM on corneal macrophages orchestrates an immune response to HSV resulting in chronic inflammation, corneal nerve damage, and eventually HSK development. Disruption of this process through treatment with immune-modifying nanoparticles (IMPs) alleviates systemic disease and improves a clinical measure of corneal health, the corneal blink threshold, suggesting this therapy may be useful in the treatment of HSK in patients.
CHAPTER 2

Materials and Methods

Ethics statement. These experiments were performed in strict adherence with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Committee on the Ethics of Animal Experiments of the Northwestern University approved the protocols (Protocol Numbers: 2012-1738 and IS00001532). Procedures were performed under anesthesia with ketamine/xylazine or isoflurane anesthesia. Minimization of suffering was prioritized.

Cells and viruses. African green monkey kidney cells (Vero) were used for all plaque assays and virus propagation unless otherwise indicated. HSV-1 strain 17 was obtained from Dr. David Leib (Dartmouth Medical School, Hanover, NH) and strain F was obtained from Dr. Bernard Roizman (University of Chicago, Chicago, IL). Cre-recombinase-expressing HSV-1(KOS) viruses were obtained from the Tscharke group (Viruses and Immunity, Australian National University). KOS0152 contains HCMV IE-Cre expression cassette in the UL43 gene (170). KOS/pCMV/eGC contains an expression cassette in an intergenic region between UL3 and UL4 but produces an EGFP-Cre fusion protein (170).

Vero cells were cultured in Dulbecco's modification of Eagle medium with 10% fetal bovine serum and 0.1% penicillin-streptomycin (DME) at 37°C. VD60 cells used in the generation of HSV-1/FRT and HSV-1/Δ7-15 are a derivative of Vero cells which express HSV-1 gD upon introduction of the HSV genome (171). They were cultured in DME at 37°C. B78H1 cells are a murine melanoma cell line that was cultured in DME at 37°C. B78-A10s and B78-C10s are derivatives of the B78H1 line that stably express HVEM and nectin-1 respectively (39, 42). They were cultured in DME at 37°C and received G418 (at a final concentration of 400
µg/mL) every other passage.

Viruses were propagated in Vero cells cultured in Dulbecco’s modification of Eagle medium with 1% fetal bovine serum and 0.1% penicillin-streptomycin (DMEV). When the entire monolayer showed cytopathic effect (CPE) the cell culture media and cells were collected and frozen at -80°C. The harvest was then thawed at room temperature and spun at 2380 g for 5 min. The supernatant was collected and spun again at 7711 g for 60 min. The supernatant was discarded and the pellet was resuspended in 6 mL of DMEV, sonicated 2 times for 30 sec., and spun at 214 g for 5 min. The supernatant was aliquoted, titered, and used for infection of mice.

Plasmids. Plasmids were maintained in the XL1-Blue (Stratagene) strain of Escherichia coli grown in Luria broth. Plasmid pOG44 (Life Technologies) contains the FLP recombinase gene. Plasmid pMY168 (38) contains a portion of the HSV-1/KOS genome encoding gJ, gD, and gI in the multiple cloning site of pUC19 except that the coding sequence of gD has been replaced with the gene encoding EGFP flanked by 48 base pair FRT sites. Plasmid pMY171 (38) contains the coding sequence of HSV-1 gD flanked by FRT sites in the multiple cloning site of pUC19. Plasmid pAK02 was constructed by inserting a PCR fragment containing the gD coding sequence with the Δ7-15 mutation flanked by partial FRT sites that was obtained by amplifying this region of the genome of HSV-1/KOStk- Δ7-15 generated by members of Patricia Spear’s laboratory at Northwestern University into pMY171 after excision of the gD coding sequence and partial FRT sites within pMY171 with restriction enzyme Xba1 (New England Biolabs). All laboratory-generated plasmids were confirmed by sequencing using the appropriate primers listed below after isolation of plasmid DNA using the QIAprep Spin Miniprep Kit (Qiagen).
**Primers.** All primers were obtained from Integrated DNA Technologies and designed using Clone Manager software. The primers used for plasmid sequencing are listed in the following table.

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple cloning site of pUC19-based plasmids</td>
<td>pUC19 MCS</td>
<td>CTG CAA GGC GAT TAA GTT GG</td>
<td>TTA TGC TTC CGG CTC GTA TG</td>
</tr>
<tr>
<td>HSV-1 gJ, gD, gl including intergenic regions</td>
<td>pMY171F</td>
<td>CAG GCT GCG CAA CTG TTG GGA AG</td>
<td>CGA AGA CGG AAG CAG TTT CG</td>
</tr>
<tr>
<td>HSV-1 gJ, gD, gl including intergenic regions</td>
<td>pMY171R</td>
<td>CTT TAT GCT TCC GGC TCG TAT G</td>
<td></td>
</tr>
<tr>
<td>Sequencing pMY171</td>
<td>pMY171F</td>
<td>CAG GCT GCG CAA CTG TTG GGA AG</td>
<td>CGA AGA CGG AAG CAG TTT CG</td>
</tr>
<tr>
<td>Sequencing pMY171</td>
<td>pMY171R</td>
<td>CTT TAT GCT TCC GGC TCG TAT G</td>
<td></td>
</tr>
<tr>
<td>gJ region</td>
<td>gJF/R</td>
<td>CAC TTT ATG CTT CCG GCT CGT ATG</td>
<td>ATG ACC GAA CAA CTC CCT AAC C</td>
</tr>
<tr>
<td>Beginning of gD region</td>
<td></td>
<td>TGT GAC ACT ATC GTC CAT ACC</td>
<td>AAG CTG TAT ACG GCG ACG GTG</td>
</tr>
<tr>
<td>End of gD region</td>
<td></td>
<td>TGC CGC AGC GTG CTC CTA AAC</td>
<td>CGC TAC CGA CTT ATC GAC TG</td>
</tr>
<tr>
<td>gI region</td>
<td></td>
<td>TTG GGA TGG GAC CTT AAC TC</td>
<td>CCA GAA GAG GCA AAG TCA ACA C</td>
</tr>
</tbody>
</table>

**Construction of HSV-1/FRT and HSV-1/Δ7-15 viruses.** The generation of these viruses followed a procedure previously described (38). Capsid DNA was isolated from HSV-1(F) and HSV-1(17) as previously described (172). Confluent cultures of Vero cells in 850 cm² roller bottles were infected with the appropriate strain of HSV-1 at a multiplicity of infection (MOI) of 10 and incubated for 24 hours. The culture media was aspirated and replaced with 10 mL of sterile phosphate buffered saline (PBS) and the culture was scraped down with a sterile cell scraper. The harvest was centrifuged at 750g for 10 minutes, the supernatant was removed, fresh sterile PBS was added, and the culture was centrifuged again for 10 minutes at 750g. The
The supernatant was removed again and the pellet was suspended in LCM buffer (13% 1M KCl, 3% 1M Tris at pH 7.4, 0.5% 1M MgCl$_2$, 0.1% 0.5M EDTA, 0.5% NP40, and 0.43% β-mercaptoethanol in deionized H2O; percentages are by volume), extracted twice with Freon, and the top layer was collected each time. The extract was loaded on top of an LCM based glycerol gradient (5% glycerol in LCM buffer by volume on top of 45% glycerol in LCM buffer by volume) and centrifuged at 25,000 RPM for 1 hour at 4°C using an SW41 ultracentrifuge rotor. The supernatant was removed and the pellet was rehydrated in 0.5 mL of TNE (50 mM Tris at pH 7.4, 100 mM NaCl, 10 mM EDTA) for 10 minutes at room temperature. The rehydrated pellet was then added to a solution of 8.5 mL TNE, 0.5 mL 10% SDS, and 10 µg of proteinase K, extracted twice with phenol/chloroform and the top layer was collected each time. 100% ethanol at -20°C and 3 M sodium acetate were added to the extract and the entire solution was kept at -80°C overnight. The following day the solution was centrifuged for 30 minutes at 14,000g at 4°C. The supernatant was discarded, the pellet was washed with 4°C 100% ethanol, dried at room temperature for 10 minutes, and resuspended in 0.5 mL of TE buffer (10 mM Tris, 1 mM EDTA). 150 µL of the isolated capsid DNA preparation was transfected into subconfluent Vero cells along with 1 µg of plasmid pMY168 (38) which contains the portion of the HSV-1/KOS genome flanking the coding sequence for gD with the gene for EGFP inserted into the coding sequence of gD flanked immediately by 48 base pair FRT sites using 7 µL of lipofectamine 2000 (Life Technologies) in Opti-MEM® media. After 18 hours the transfection media was aspirated and replaced with DMEV. Green fluorescent viral plaques were observed between 3 and 6 days later and harvested. Isolates underwent at least 4 rounds of plaque purification on VD60 cells to ensure pure cultures of green fluorescent HSV-1 viruses containing the gene for EGFP flanked by 48 base pair FRT sites in place of the gD coding sequence. Capsid DNA from these viruses was isolated as described above and transfected into VD60 cells as described above along with 1 µg of pOG44 (Life Technologies), which contains the FLP recombinase gene. After 3 to 6
days nonfluorescent plaques were observed, harvested, and plaque purified on VD60 cells to obtain HSV-1 strains with a single 48 base pair FRT site in place of the gD coding sequence. DNA from these viruses was isolated as described above and transfected into Vero cells as described above along with pOG44 and either pMY171 (38) containing the wild-type HSV-1 gD coding sequence, pAK02 containing the HSV-1 gD coding sequence with the Δ7-15 deletion. Selection of recombinant viruses was performed on Vero cells, at least 3 rounds of plaque purification were performed on each isolate, and final viral stocks were titered on Vero cells, B78-A10s, and B78-C10s to confirm entry phenotype. Final recombinant viral isolates as well as intermediates were sequenced to confirm the proper deletion and/or insertion by isolating viral DNA using the High Pure Viral Nucleic Acid Kit 40 (Roche) and the appropriate primers listed above. To generate stocks for animal infections the viruses were grown as described above.

**Viral plaque assay.** A standard plaque assay on Vero cells (unless otherwise noted) was used to determine viral titer as previously described (50). Serial 1:10 dilutions were made of each sample or viral stock to be analyzed in PBS-GCS (PBS with a concentration of 100 µg/mL of CaCl₂, 100 µg/mL of MgCl₂, 1% glucose, and 1% heat-inactivated fetal bovine serum). The dilutions were added in duplicate to two wells of a 6-well dish of confluent cells and rocked at 37°C for 2 hours. After 2 hours the inocula were aspirated off the cells and replaced with DMEV containing 3% methylcellulose (w/v) and placed in a 5% CO₂ incubator at 37°C for 72 hours. Plates were washed twice with PBS, fixed with methanol at room temperature for 5 minutes, and stained with Giemsa (Sigma-Aldrich) in water (1:10) for 1 hour. The plates were rinsed twice with water and placed upside down to dry. Plaques were counted and averaged between the two duplicate wells to determine the viral titer of the sample or viral stock.
Animal procedures. Animals were cared for and procedures were performed following institutional and National Institutes of Health guidelines. The Animal Care and Use Committee at Northwestern University approved all procedures. Mice were maintained in a specific pathogen-free environment, and were transferred to a containment facility after infection. C57BL/6 and C57BL/6 with the CD45.1 allele from Jackson Labs (WT), \( \text{Tnfrsf14}^{-/-} \) (HVEM KO), and BALB/c 8-to 16-week-old male mice were used in our experiments. Chimeric mice were produced as follows: WT (C57BL/6 expressing CD45.1 allele) or HVEM KO (C57BL/6 background expressing CD45.2 allele) recipient animals were subjected to a lethal dose of radiation (2 doses of 6 Gy separated by a 3 hour interval) to ablate the bone marrow (BM). Recipients were reconstituted with \( \sim 10 \) million cells harvested from the BM of donor animals via retro-orbital injection within 24 hours of irradiation. After 10 weeks, completeness of the transfer was verified by analyzing the proportion of CD45.1 and CD45.2 positive cells in peripheral blood by flow cytometry (cutoff: \( \geq 95\% \) donor genotype).

In addition, conditional HVEM KOs were generated with Tg(Vav1-cre)A2Kio mice or Tg(KRT14-cre)1Amc/J mice, which express Cre-recombinase in all hematopoietic lineages or in epithelial cells, including those of the cornea, respectively (Jackson). Cre-expressing mice were crossed with transgenic HVEM\textsuperscript{fl/fl} mice, which have loxP sites flanking exon 3 of the \textit{Tnfrsf14} gene, to generate homozygous floxed mice with a Cre allele (Kronenberg laboratory, La Jolla Institute of Allergy and Immunology). Genotypes were confirmed via PCR as follows. Tail snips were digested for 8 hours at 55°C in tail lysis buffer (200 mM Tris pH 8.0, 200 mM NaCl, 10 mM EDTA, 0.4% SDS, 0.2 mg/ml proteinase K) and resuspended in TE buffer. PCR was performed with KOD Hot Start Master Mix (EMD Millipore) or AccuStart II PCR Supermix (Quanta Bio) and run with the following protocol: 95°C – 2 min; 30 cycles of 95°C – 20 sec, melt temp – 10 sec, 70°C – 45 sec; hold at 4°C. The following primers were used:
Gene | Forward primer | Reverse primer | Melt temp | Product size
--- | --- | --- | --- | ---
HVEM | GTA GAC CAA ATC AGA CCT GGG AAG C | ACC TCC CAA GTC TGA CCC TCT | 53°C | WT – 347 bp
 |  |  |  | Fl – 456 bp
Vav-Cre | GGT GTT GTA GTT GTC CCC ACT | CAG GTT TTG GTG CAC AGT CA | 64°C | 390 bp
Cre (for K14) | GCG GTC TGG CAG TAA AAA CTA TC | GTG AAA CAG CAT TGC TGT CAC TT | 56.4°C | 100 bp
+ Int. control | CTA GGC CAC AGA ATT GAA AGA TCT | GTA GGT GGA AAT TCT AGC ATC ATC C |  | 324 bp

Corneal inoculation and titer collection. Animals were inoculated with 2 x 10⁶ PFU of HSV-1 in 5 µl DMEM as previously described (49, 50). Mice were anesthetized with an i.p. injection of a 20:3 ketamine:xylazine solution. Corneas were lightly abraded 10 times with a 25-gauge needle in a crosshatch pattern, and 2 x 10⁶ PFU of virus in 5 µl DMEM was dropped onto each cornea. Mice were weighed and given a lesion score from 0 to 5 (0 = no lesions; 1 = small area of broken skin < 0.5cm; 2 = area of broken skin 0.5-1 cm; 3 = broken skin, bleeding, scabbing, or pustules; 4 = broken skin > 1 cm with multiple pustules or scabbing; 5 = severe scabbing or bleeding with pustules) and a neurological score from 0 to 5 (0 = no symptoms; 1 = ruffled fur, hunched posture, normal movement; 2 = hunched posture, slow to move; 3 = hunched, some movement, labored breathing; 4 = hunched, labored breathing, little to no movement; 5 = moribund or dead).

Eye swabs were collected after light isoflurane anesthesia. The eye was gently proptosed and a sterile cotton swab moistened with DMEM was wiped three times around the circumference of the eye and twice across the center of the cornea in an “X” shape as previously described (49, 50). Swabs were then placed into 1 mL of DMER media (DMEM containing 5% (vol/vol) FBS, 1% gentamicin, 1% ciprofloxacin, and 1% amphotericin B) and
stored at -80°C. In order to determine titers, samples were thawed and vigorously vortexed for 30 seconds. Some animals were sacrificed 5 dpi, and the POS, TG, and brains were collected as previously described. In brief, POS biopsies were taken using a sterile punch (Sklar Instruments). TG and brains were dissected, and all samples were placed in 1 mL DMER, homogenized, sonicated, and stored at -80°C until titration. Brain samples were centrifuged prior to titration to remove debris.

For the TG explants and reactivation studies, mice were euthanized as described above on day 30 post-infection (+/- 2 days). TG were bisected and co-cultured with a monolayer of Vero cells. Cultures were examined each day for CPE and after 7 days of culture the TG and cells were homogenized as described above and titered on fresh Vero cells.

**Cytokine/chemokine analysis.** Corneal cytokines were analyzed with a custom MILLIPLEX® MAP Kit Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA) following the manufacturer’s instructions. Corneas were dissected and pooled (n = 3 mice or 6 corneas per sample) in cold PBS + protease inhibitor cocktail, homogenized for 30 seconds with a bead beater, and immediately loaded into the 96-well prepared plate. Analyte-specific antibody-coated magnetic microspheres were mixed with the sample. After exposure to a biotinylated detection antibody and incubation with streptavidin reporter, the amount of each captured factor was quantified by a Luminex® compact analyzer (Luminex, Austin, TX). Two quality controls were run with each assay, and all anylates fell within quality control ranges.

**Immunohistochemistry.** Whole eyes were collected at the indicated time points after infection, rinsed with PBS, and floated in 10% formalin + neutral buffered PBS for 24 hours. Eyes were then transferred to 70% ethanol and stored at 4°C until paraffin embedding. Serial 4-
µm-thick sections were mounted on glass slides. The Northwestern University Mouse Histology and Phenotyping Laboratory provided naïve murine spleen controls.

The following antibodies and concentrations were used for IHC staining: anti-HSV antigen (Dako) polyclonal antibody diluted 1:5000; anti-Ly6G (Gr-1) monoclonal antibody diluted 1:500 (BD 551459); anti-CD3 monoclonal antibody (Abcam ab16669l) diluted 1:2000; anti-HVEM rabbit polyclonal antibody (Patricia Spear, Northwestern University) diluted 1:200; and anti-HVEM mouse monoclonal antibody (clone HMHV-1B18, BioLegend) diluted 1:200.

Antigen retrieval was performed manually using the Vectastain ABC Kit (Vector Labs) for anti-Ly6G, anti-CD3, and anti-HSV staining, or with Biocare decloaker for 5 min at 100°C followed by citrate buffer (pH 6.0) for anti-HVEM staining. Secondary antibodies labeled with HRP were visualized after treatment with chromagen diaminobenzidine (DAB, Vector Labs). Slides were washed, counterstained with Gill’s Hematoxylin, and imaged on the EVOS XL core cell imaging system.

Flow cytometry. Corneal pairs and spleens from individual mice were collected in cold PBS. Corneas were digested in 0.7 mg/ml Liberase (Roche) in RMPI media for 1 hr in a 37°C, 5% CO₂ incubator. Using a 1 ml syringe plunger, corneas were homogenized on top of a 100-µm mesh, washed with cold PBS, strained through a 40-µm mesh, and collected into a small volume. Spleens were prepared similarly to the corneas, but with a red blood cells (RBC) lysis step between straining steps. After obtaining live cell counts, all of each cornea sample and a portion of each spleen sample was incubated with a 1:1000 dilution of Live/Dead Fixable Aqua Dead Cell Stain Kit (Thermo Scientific) in PBS in the dark at RT for 30 min. Samples were washed with PBS and incubated with Fc block (0.5-1.0 µg/sample anti-mouse CD16/CD32 [eBioscience] in PBS + 1% fetal bovine serum + 0.1% sodium azide [FACS buffer]) for 5 min at 4°C in the dark. Conjugated antibodies (2µg/ml final µl per sample) added directly to Fc block
were incubated for 1 hr at 4°C in the dark. The following antibodies (and isotype controls) were used: HVEM-APC (HMHV-1B18), Ly6G Brilliant Violet 421 (1A8), CD8a Brilliant Violet 421 (53-6.7), E-cadherin-PE (DECMA-1), CD31-PB (390), IgG2α isotype control-Brilliant Violet (421), BioLegend; CD45-FITC (30-F11), CD3-APC eFluor 780 (17A2), CD11b-PECy7 (M1/70), CD11c-PE (N418), Ly6C-PerCP-Cy5.5 (HK1.4), CD4-PE (GK1.5), CD3e-PECy7 (145-2C11), Armenian hamster IgG isotype control-APC (eBio299Arm), rat IgG2βκ isotype control-PerCP eFluor 710 (eB149/10HS), rat IgGα isotype control-APC eFluor 780 (eBR2a), ICAM-1-FITC (YN1/1.7.4), rat IgG2βκ isotype control-FITC (eB149/10H5), eBioscience; NK1.1-APC Cy7 (PK136), BD Biosciences. Samples were washed and resuspended in 200 µl FACS buffer. Samples were collected on a FACS Canto II; the entire corneal pair sample was run, while spleen sample collection was stopped at 100,000 live cells, and data analysis was performed with FlowJo 10.1 software.

Corneal sensitivity. A Luneau Cochet-Bonnet aesthesiometer (Western Ophthalmology, #WO-7760) was used to determine the blink threshold of the central cornea. Animals were scruffed, and the length of the monofilament was varied from 6.0 cm to 0.5 cm and touched perpendicularly to the surface of the central cornea until the first inflection point. A positive response was recorded with ≥2 blinks out of 3 attempts. Absence of a blink response at 0.5 cm was scored as a 0. The same examiner performed all measurements.

Immune-modifying nanoparticles (IMPs) treatment. Negatively-charged IMPs derived from poly(lactic-co-glycolic acid, PLGA) were produced by Phosphorex. IMPs were diluted to a previously-established maximally effective concentration of 4.7 mg/ml in filtered PBS (0.94 mg total/mouse/injection); 200 µl IMPs or PBS vehicle control were delivered intravenously for 5
days (starting 3 dpi) via tail-vein injection (173). A control cohort of animals were sacrificed <24 hours after the final dose for analysis of spleens.

**BTLA antibody treatment.** An activating mouse monoclonal anti-CD272 (BTLA) antibody, 8F4 (BioXCell #BE2010), or a mouse IgG1 isotype control antibody, MOPC-21 (BioXCell #BE0083) were diluted in sterile PBS. Each mouse received 200 µg of either antibody via i.p. injection mice at the time of infection (day 0) on the opposite flank as anesthesia delivery. Mice were dosed again 2 dpi.

**Statistics.** Geometric means were compared using the unpaired two tailed t test or one-way ANOVA with Holm-Sidak correction for multiple comparisons. Variance between groups for over time was analyzed with two-way ANOVA with Holm-Sidak correction for multiple comparisons. Kaplan-Meier mortality curves were compared using the log-rank test. TG reactivation rates were compared with the Chi squared test with 1 degree of freedom. Associations between corneal blink threshold and viral load or CD45⁺, PMN, or macrophage cell number were assessed via linear regression. All statistics were calculated using the GraphPad Prism 6.0f software.
CHAPTER 3

HVEM on Radiation-Resistant Cell Lineages Promotes Ocular Herpes Simplex Virus 1 Pathogenesis in an Entry-Independent Manner*


Abstract

Ocular herpes simplex virus type 1 (HSV-1) infection leads to a potentially blinding immuno-inflammatory syndrome, herpes stromal keratitis (HSK). Herpes virus entry mediator (HVEM), a widely expressed TNF-receptor superfamily member with diverse roles in immune signaling, facilitates viral entry through interactions with the viral glycoprotein gD and is important for HSV-1 pathogenesis. We corneally infected mice with an HSV-1 mutant in which HVEM-mediated entry was specifically abolished, and found that the HVEM-entry mutant produced comparable clinical disease to the control virus. HVEM-mediated induction of corneal cytokines, which correlated with an HVEM-dependent increase in corneal immune cell infiltrates, was also gD-independent. Given the complexity of HVEM immune signaling, we used hematopoietic chimeric mice to determine which HVEM-expressing cells mediate HSV-1 pathogenesis in the eye. Regardless of whether the donor was WT or HVEM KO, HVEM KO recipients were protected from ocular HSV-1, suggesting that HVEM on radiation-resistant cell types, likely resident cells of the cornea, confers wild type-like susceptibility to disease. Together, these data indicate that HVEM contributes to ocular pathogenesis independently of entry, and point to an immunomodulatory role for this protein specifically on radiation-resistant cells.
Importance

Immune privilege is maintained in the eye in order to protect specialized ocular tissues, such as the translucent cornea, from vision-reducing damage. Ocular herpes simplex virus type 1 (HSV-1) infection can disrupt this immune privilege, provoking a host response that ultimately brings about the majority of the damage incurred in the immuno-inflammatory syndrome herpes stromal keratitis (HSK). Our previous work has shown that HVEM, a host TNF-receptor superfamily member that also serves as a viral entry receptor, contributes to ocular HSV-1 pathogenesis, although its precise role in this process remains unclear. We hypothesized that HVEM promotes an inflammatory microenvironment in the eye through immunomodulatory actions, enhancing disease after ocular inoculation of HSV-1. Investigating the mechanisms responsible for orchestrating this aberrant immune response shed light on the initiation and maintenance of HSK, one of the leading causes of infectious blindness in the developed world.

Introduction

Herpes simplex virus 1 (HSV-1), a ubiquitous human pathogen, can infect the ocular tissues, resulting in the chronic inflammatory syndrome herpes stromal keratitis or HSK (15, 24). HSK is characterized by ocular opacity, neovascularization, and edema, and worldwide produces an estimated 40,000 new cases of severe vision impairment or blindness each year (16). The damage sustained during HSK is immune-mediated rather than arising from viral lytic effects, but the complex virus-host interactions that drive this syndrome are incompletely understood (13-15, 24).

In the murine cornea, actively replicating HSV-1 can be detected for 5-6 days after infection (13). Secreted factors from infected and uninfected epithelial cells recruit a variety of leukocytes, including neutrophils (PMN), macrophages, NK cells, dendritic cells, and γδ T cells into the adjacent stromal tissue beginning around 18 hours post infection (hpi) (21, 96, 97, 127,
The predominately neutrophilic infiltrate mediates viral clearance, and by 5 dpi, PMNs in the cornea decline to pre-infection levels (94, 95). A secondary, pathogenic wave of PMNs and CD4+ T cells infiltrates the cornea beginning around 7 dpi and peaking at 14-21 dpi (13). VEGF-mediated in-growth of abnormal blood and lymph vessels into the usually avascular cornea facilitates the invasion of these leukocytes and is a key step in the establishment of CD4+ T cell-driven chronic inflammation (26, 175). Stromal scarring and neovascularization may subsequently lead to vision loss, necessitating corneal transplantation (176, 177).

HSV has a complex entry mechanism requiring the expression of multiple envelope glycoproteins (34). Glycoprotein D (gD) interacts with several cellular receptors to facilitate entry. The most biologically relevant in animal models are herpes virus entry mediator (HVEM) and nectin-1 (37, 48, 178). In vivo studies using HVEM (Tnfrsf14−/−) and/or nectin-1 (Pvrl1−/−) receptor knockout (KO) mice reveal that HSV infection via intracranial or intravaginal inoculation requires nectin-1 for pathogenesis and neural spread, while HVEM is largely dispensable for infection by these routes (47, 48). More recently, HVEM has been implicated as a serotype-specific mediator of HSV-1 pathogenesis after corneal inoculation: HVEM KO mice have lower viral loads, fewer infectious corneal foci, and milder clinical symptoms than control mice (49, 50). This unique dependence of HSV-1 on HVEM in the eye is not readily explained by the absence of a suitable alternate entry receptor, as murine ocular tissue widely expresses both nectin-1 and HVEM (59, 108).

HVEM, a member of the tumor necrosis factor (TNF)-receptor superfamily, is a bi-directional receptor with multiple immunomodulatory functions depending on cell type and ligand (28, 179, 180). HVEM interaction with B and T lymphocyte attenuator (BTLA) or CD160 is typically co-inhibitory, suppressing T cell activation and proliferation, while HVEM bound by LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to HVEM on T cells) or LTα (lymphotoxin α) is pro-inflammatory (60, 62-64, 181). However, these
outcomes differ depending on the cell type, whether the HVEM ligand is in soluble or membrane-bound form, and whether the interaction occurs in cis or trans (60, 62-64, 181). HVEM influences immune responses to a variety of pathogens, including viral, bacterial, and helminthic agents in the vagina, intestine, lung, and other tissues (29, 155, 182). In some instances, the pathogen utilizes HVEM to dampen innate responses, while in others HVEM functions to control infection and limit disease progression (28, 29, 105, 155, 182). HVEM has also been implicated in a number of autoimmune and inflammatory disorders, including bacterial colitis, atopic dermatitis, and acute graft-vs-host disease (28, 29, 179, 183).

There is little overlap in the nectin-1 and HVEM binding regions of gD; mutations made in the first 32 amino acids of gD abrogate entry via HVEM without affecting the ability of gD to bind to and use nectin-1 as an entry receptor (36, 40-42, 184, 185). We tested whether HVEM-mediated entry promotes ocular HSV-1 pathogenesis by infecting mice with HSV-1/gDΔ7-15 (hereafter referred to as Δ7-15), a mutant virus with a targeted deletion that renders HVEM entry nonfunctional but preserves entry via nectin-1. Ocular infection by Δ7-15 was not attenuated compared to control virus, indicating that the requirement of HSV-1 for HVEM in the eye is gD-independent and therefore unlikely to be related to entry. Inflammatory cytokines were upregulated after infection in WT corneas compared to HVEM KO corneas, and HVEM-mediated entry was dispensable for this process. The stroma of HVEM KO corneas also had fewer immune infiltrates compared to WT early and late after infection, implying HVEM may promote pathogenesis independently of its function as a viral entry receptor by creating an inflammatory ocular environment during HSV-1 infection.

A wide variety of cell types, including epithelial, stromal, and immune cells (T cells, dendritic cells, PMN, macrophages, and others) express HVEM, and given the complex, contradictory nature of its functions (co-stimulatory and co-inhibitory), the contribution HVEM may make to ocular pathogenesis is not obvious (28, 60, 108, 112, 186). We developed
hematopoietic chimeric mice in which HVEM expression was restricted to or ablated from radiation-sensitive bone marrow-derived immune cells, and found that HVEM on radiation-resistant cell types was sufficient to confer wild type-like susceptibility to HSV-1 after corneal inoculation. We propose that HVEM on radiation-resident cells of the cornea, such as the corneal epithelium, promotes the induction of inflammatory cytokines in the eye, resulting in increased immune infiltrates. This immune response persists well after detectable virus has vanished, and likely accounts for the worsened disease observed in WT versus HVEM KO animals.

Results

**HVEM-mediated entry does not alter the development of clinical symptoms or mortality after corneal infection in mice.** HVEM is uniquely important for ocular pathogenesis of HSV-1 (49, 50), but whether this is attributable to its entry effects, immunomodulatory effects, or both is not known (49). To determine if HVEM-mediated entry was required for normal virulence after corneal inoculation, we produced an HSV-1 mutant with a deletion of the HVEM binding region of gD (Fig. 2A). Crystallographic and functional assays have shown that deletion of amino acids 7-15 of the gD N-terminus selectively abolishes HVEM entry without functionally disturbing entry via nectin-1 (38, 105, 187, 188). As shown in Fig. 2A, the Δ7-15 viruses in HSV-1(F) and HSV-1(17) backgrounds were engineered by FLP-mediated recombination as described previously (38, 105). The HSV-1/FRT viruses (WT-FRT) contain the WT protein coding sequence for gD flanked by FRT sites (Fig. 2A) and served as controls.

All viruses were confirmed by sequencing and phenotypically verified by plaque assay on Vero cells, B78-A10s (B78H1 cells stably expressing HVEM), and B78-C10s (B78H1 cells stably expressing nectin-1). As expected, the titers of the WT-FRT viruses were similar on all three cell types indicating that both strains of WT-FRT are capable of infecting cells expressing
either HVEM or nectin-1 (results shown for HSV-1(17) viruses, Fig. 2B). Titers of the Δ7-15 viruses were similar to the WT-FRT viruses on Veros and B78-C10s, but no plaques were observed on B78-A10s, indicating that while both the Δ7-15 viruses can infect cells via nectin-1, they are unable to infect cells that only express HVEM (Fig. 2B). Additionally, 3 nectin-1 KO mice were challenged via corneal scarification with the Δ7-15 viruses as previously described (49, 50), and eye swabs were collected on 1 and 3 days post infection (dpi). No replicating virus was recovered from any of the samples collected from these mice at either time point, confirming that the Δ7-15 viruses are defective in the use of HVEM as an entry receptor in vivo (see Andrew Karaba’s thesis).

We monitored 10-12 week old male C57BL/6 wild type (WT) mice infected with WT-FRT or Δ7-15 from both strain 17 backgrounds (Fig. 3) and strain F (see Andrew Karaba’s thesis) after corneal scarification as previously described (49, 50). We also inoculated 10-12 week old male BALB/c mice, which are exceptionally sensitive to ocular HSV-1, with Δ7-15 or WT-FRT (strain 17 background) to ensure the resistance of the C57BL/6 strain to ocular HSV-1 infection did not mask subtle differences between the viruses (Fig. 3). Mice were monitored daily for changes in weight and signs of HSV-1 disease as described in Materials and Methods. The development and severity of lesions did not differ significantly between HVEM entry competent- and HVEM entry null-viruses in either mouse strain (data shown for HSV-1(17), Fig. 3A and B). Both Δ7-15 and WT-FRT viruses produced lesions, and by 5-7 dpi all mice were symptomatic.
The gDΔ7-15 mutation specifically abolishes cellular entry via HVEM by HSV-1. (A) Schematic diagram of the HVEM-binding mutant HSV-1/gDΔ7-15 (Δ7-15), and the control virus containing the native gD gene flanked by FRT sites, HSV-1/gDWT-FRT (WT-FRT). WT-FRT was used to generate Δ7-15, the HVEM entry mutant, via FLP-mediated recombination. Δ7-15 has an 8 amino acid deletion in glycoprotein gD that specifically abolishes entry interactions with the HVEM entry receptor. (B) Characterization of the recombinant strain 17 viruses by plaque assay. The Δ7-15 virus replicates in B78 cells stably expressing the nectin-1 entry receptor to average titer of 3.3x10⁷ PFU/ml. No detectable replication was observed in HVEM receptor cells (* = below detection limit).
Fig 3. HVEM entry-capacity is dispensable for clinical symptoms after corneal inoculation with HSV-1 in multiple strains of mice. HSV-resistant C57BL/6 or HSV-susceptible BALB/c mice were inoculated with $2.0 \times 10^6$ PFU/5 µl of the HSV-1(17) viruses per eye and scored daily (using a 0-to-5 scale, with 5 representing the greatest severity) for the development of epithelial lesions and neurologic symptoms ($n = 4$ to 6 per group; data representing the results of one representative experiment are shown). (A) Percentages of mice from each group with no lesions on the indicated day post infection (dpi).

(B) Average maximum epithelial lesion scores (means ± standard errors of the means [SEM]). max., maximum. (C) Percentages of mice from each group with no neurologic (neuro.) symptoms on the indicated dpi. (D) Average maximum neurologic scores (means ± SEM). (E) Survival for each group. For each mouse strain, no significant differences were detected between the viruses ($P > 0.05$ [two-way ANOVA for panels A and C or two-tailed $t$ test for panels B and D with Holm-Sidak’s multiple-comparison test; log-rank test for panel E]).
Neurologic symptoms began around 5 dpi, and by 7 dpi 40-80% of the C57BL/6 and 100% of the BALB/c mice displayed at least some neurologic morbidity, including ruffled fur, hunched posture, postural instability, and absence of movement (Fig. 3C). The severity of neurologic symptoms also did not differ depending on the capacity of the virus to use HVEM as an entry receptor within each mouse strain (Fig. 3D). Mice infected with either strain of Δ7-15 lost a similar percentage of day 0 body weight as mice infected with WT-FRT (Fig. A1). All C57BL/6 mice survived to 28 dpi, when the experiment ended (Fig. 3E). This high percent survival is consistent with previously reported data (50). The majority of BALB/c mice succumbed to infection by day 30 regardless of HVEM-entry capacity of the virus (Fig. 3E).

Unlike the extremely mild disease observed in HVEM KO mice (49, 50), the Δ7-15 virus produced disease comparable to that of the WT-FRT (Fig. 3A-E). To ensure that our findings were not due to attenuation of our FLP-recombinase generated mutants, we also compared infection in C57BL/6 and BALB/c mice by strain 17-derived Δ7-15 and WT-FRT to the parental HSV-1/17 strain from which both viruses were made. There were no significant differences in any measure of clinical disease between the parental strain and either of the modified viruses, indicating attenuation during the recombination process did not occur (Fig. A2). Collectively, these data suggest that the entry interaction between gD and HVEM is not required to produce clinical symptoms during ocular HSV-1 infection.
HVEM-mediated entry does not influence viral tissue loads after corneal challenge in mice. To test whether HVEM-mediated entry was required to establish HSV-1 infection in the eye and/or facilitate spread to other organs, we assessed viral titers after infection with strain 17 or F background Δ7-15 or WT-FRT viruses in relevant tissues. Eye swabs collected 1, 3, and 5 dpi were analyzed via plaque assay on Vero cells to determine viral loads in the tear film. C57BL/6 or BALB/c mice infected with Δ7-15 or WT-FRT had similar viral loads in the eye swabs at all three time points (data shown for strain 17 background, Fig. 4A and B), indicating that the gD-HVEM entry interaction is dispensable for the primary establishment of infection in the murine cornea. This is in contrast to previous results with receptor KO animals, where HSV-1 viral titers and infectious corneal foci were limited in the absence of HVEM (49, 50).

Next, we tested whether spread to relevant organs was reduced by a lack of HVEM entry. We collected the periocular skin (POS), trigeminal ganglia (TG), and brains at 5 dpi, homogenized the tissues, and determined titer via plaque assay. Corresponding to titers in the eye swabs, titers in the POS of C57BL/6 or BALB/c mice infected with either background of Δ7-15 or WT-FRT did not differ significantly (data shown for strain 17 background, Fig. 4C). Similar results were observed in the TG and brain (strain 17 background, Fig. 4D and 4E). We also compared titers in the eye swabs, POS, TG, and brain after infection by strain 17 background Δ7-15 and WT-FRT to that of the parental HSV-1(17) strain in both mouse strains, and found no significant differences in viral replication that would indicate the recombinants were attenuated (Fig. A3).
Fig 4. HVEM entry-capability is not required for corneal HSV-1 replication or spread to the tissues in multiple strains of mice. HSV-resistant C57BL/6 or HSV-susceptible BALB/c mice were inoculated with $2.0 \times 10^6$ PFU/5 µl per eye of the Δ7-15 mutant or WT-FRT strain (strain 17 background) after corneal scarification. (A) C57BL/6 eye swabs collected 1, 3, and 5 dpi. (B) BALB/c eye swabs collected at the same time points (means ± SEM). (C-E) Titers determined at 5 dpi using samples from the periocular skin (POS) (C), trigeminal ganglia (TG) (D), and brains (E) (means ± SEM). Data representing the results of two independent experiments are shown (total $n$ per group = 10). For each mouse strain, no significant differences in titers were detected between the viruses in eye swabs or tissues (two-tailed $t$ test with Holm-Sidak’s multiple-comparison test, $P > 0.05$). (F) At 30 dpi, C57BL/6 mice were sacrificed and TG explants were cocultured on Vero cells for detection of reactivated virus from latency ($n = 10$ TG for each group). No significant differences were observed in levels of TG reactivation between the mice
infected with the Δ7-15 mutant and those infected with the WT-FRT strain from either viral background (chi-square test with 1° of freedom, P > 0.05).
We investigated whether HVEM entry-capacity influenced the establishment of latency within the TG. Previous explant reactivation studies from our laboratory showed that TG from WT mice reactivated at 4 times the rate of those from HVEM KO (50). After 30 days, rates of reactivation after corneal inoculation of C57BL/6 mice with WT-FRT or Δ7-15 from either strain did not differ significantly, as measured by the ex vivo reactivation assay, suggesting HVEM-mediated entry was not required to successfully seed the TG (Fig. 4F). Together, these results indicate that disruption of the gD-HVEM entry interaction does not significantly impact the establishment of viral infection at the cornea or POS, nor hinder spread within the nervous system. This is consistent with studies from the vaginal model of HSV-2 infection, which found that viral replication in the vaginal mucosa was minimally affected by the Δ7-15 deletion in HSV-2 (155). Therefore, disruption of HVEM entry by mutation of viral gD does not phenocopy the results observed in infection of HVEM KO mice with a wild type virus.

**HVEM KO corneas have decreased cytokine induction after HSV-1 infection when compared to WT controls.** The previous experiments indicate that the requirement of HSV-1 for HVEM in the murine eye is entry-independent. We hypothesized that the immunomodulatory functions of HVEM may contribute to pathogenesis after corneal inoculation. We examined the expression of a number of cytokines known to impact the development of HSK in pooled corneal samples from WT or HVEM KO mice using the MILLIPLEX® MAP Kit Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA) assay system (25, 110, 127, 189-193). Because HVEM KO mice have been reported to have increased T cell reactivity (194), we began by comparing mock-infected WT and HVEM KO corneas to rule out any differences present between the genotypes unrelated to HSV-1 infection. Lack of HVEM did not significantly influence the baseline expression of any of the cytokines examined in mock-infected corneas 5 days after scarification (Fig. 5A). While most factors were expressed at low
levels in the mock-infected samples (≤10pg/ml), IL-1α, CCL2, CXCL2, CXCL9, and, in the HVEM KO, CXCL10 were expressed more highly. The induction of these factors may be due to scarification alone (110, 192).

We profiled the expression of the same panel of cytokines in WT and HVEM KO corneas 5 days after corneal infection with HSV-1(17). To control for the scarification process, we compared the results from infected samples as fold changes over mock-infected samples. The majority of factors were elevated several fold over mock-infected controls, consistent with an infectious, inflammatory process (Fig. 5B). WT corneas expressed IL-6 and the IFN-γ-induced T cell chemoattractant CXCL10 (interferon gamma-induced protein 10; IP-10) at over 9- and 125-fold the level in HVEM KO corneas, respectively. Several other chemokines, including CXCL9 (monokine induced by gamma interferon; MIG), CCL3 (macrophage inflammatory protein 1α; MIP-1α), and RANTES (regulated on activation, normal T cell expressed and secreted; CCL5) were also elevated in WT corneas compared to HVEM KO corneas, but this upregulation did not achieve statistical significance after correction for multiple comparisons.

A previous study in the vaginal HSV-2 model showed that disruption of the gD-HVEM entry interaction through deletion of amino acids 7-15 in HSV-2/333gD led to decreased induction of IL-6, CXCL9, and CXCL10 in vaginal washes early after infection (105). To test whether the changes we observed in WT and HVEM KO corneas required HVEM-mediated entry, we analyzed the cytokine profile of WT mice infected with Δ7-15(17) or WT-FRT(17) control (Fig. 5C). Consistent with the disease and viral load data, changes in the induction of the examined factors caused by the two viruses were not significantly different, suggesting that gD-HVEM entry is not required for HVEM-mediated induction of certain inflammatory cytokines in the cornea during HSV-1 infection. Together, these findings demonstrate that HVEM expression by the host produces changes in the magnitude of the cytokine response to infection independently of the gD-HVEM entry interaction.
Fig 5. Effects of HVEM depletion or disabled HVEM entry on corneal cytokine expression 5 days after HSV-1/17 infection. (A) Average corneal cytokine protein concentrations (conc.) (in picograms per microliter) 5 days after mock infection in WT and HVEM KO mice. (B) Normalized fold changes in corneal cytokine concentrations of WT or HVEM KO mice infected with 2.0x10^6 PFU/5 µl per eye of HSV-1(17) compared to those of mock-infected controls 5 dpi. (C) Normalized fold changes in corneal cytokine concentrations of WT mice infected with 2.0x10^6 PFU/5 µl eye of the HVEM-entry Δ7-15 mutant virus or the control WT-FRT virus compared to that of mock-infected controls 5 dpi. The values are mean results ± SEM from two independent experiments, n = 5 pooled samples (6 corneas per sample) per group.
Values above the red line represent increased expression compared to that seen with mock-infected controls. Statistically significant differences are indicated as follows: **, $P < 0.01$; ***, $P < 0.001$ (two-tailed $t$ test with Holm-Sidak’s adjustment for multiple comparisons).
Immune infiltrates are reduced in HVEM KO corneas at the onset of infection and after virus has been cleared. We hypothesized that HVEM KO corneas would exhibit other changes indicative of a diminished immune response to HSV-1. To assess whether the HVEM-mediated increase in cytokine expression was reflected by changes in inflammatory infiltrates, we performed immunohistochemical analysis of corneas taken from several time points after HSV-1 infection. Whole eyes from mock-infected or HSV-1(17)-infected HVEM KO or WT animals were collected after corneal inoculation during early and acute infection (1 or 5 dpi) or the height of HSK (14 dpi). Previously, we found that, on average, WT corneas have more than twice the number of infectious foci than HVEM KO corneas, and foci in WT corneas also tended to be larger (50). Consistent with these findings, Fig. 6A qualitatively demonstrates that HVEM KO corneas had fewer, smaller infectious foci in the corneal epithelium than WT corneas immediately after infection (1 dpi). By 5 dpi, HSV antigen was only rarely detectable in the corneas of both genotypes (Fig. A4), and by 14 dpi no virus was found in the cornea (Fig. 6B, top row).

Serial sections adjacent to infectious foci were stained with anti-Ly-6G (Gr-1) to visualize monocytes and granulocytes, including peripheral neutrophils, or anti-CD3 to identify T cells. The stroma of HVEM KO corneas qualitatively displayed markedly less Gr-1⁺ or CD3⁺ staining 1 dpi compared to that of WT (Fig. 6A, second and third rows). Interestingly, at 14 dpi, after HSV-1 antigen was no longer detectable in the eye, WT stroma remained heavily infiltrated with T cells and especially with granulocytes (Fig. 6B, second row and third rows), while HVEM KO stroma contained only rare positive cells (Fig. 6B, black arrowheads). Because the differences between WT and HVEM KO persisted well beyond the end of initial infection, these data suggest that HVEM not only influences the establishment of infection, but may also play a role in the maintenance of an inflammatory microenvironment in the cornea in the absence of viral replication. We hypothesize that this apparent difference in immune infiltrates in HVEM KO corneas is due to the absence of HVEM, which is known to play a role in the regulation of immune responses. Further studies are needed to confirm this hypothesis and to understand the mechanisms by which HVEM regulates immune responses to HSV-1 infection.
corneas when compared to WT corneas may be responsible, in part, for the exacerbated pathogenesis observed in WT animals compared to HVEM animals, and will be a future focus of our studies.
Figure 6. HVEM KO corneas have decreased stromal immune cell infiltrates during acute
infection and the chronic phase. (A) Representative immunohistochemical analysis of whole WT or HVEM KO eyes 1 day after mock infection or infection at the corneal surface with $2.0 \times 10^6$ PFU/5 µl per eye of HSV-1(17) (original magnification, 400x). Paraffin-embedded eyes were serially sectioned and stained for HSV-1 or markers of immune cell infiltration. Gr-1 stains granulocytes, including PMN, macrophages, and monocytes, while CD3 is specific for T cells. A representative image of an HSV-infected region in the WT corneal epithelium is adjacent to a stromal region floridly positive for Gr-1 and CD3 (third column), while in an HVEM KO section, fewer Gr-1+ and CD3+ cells were found in the stroma despite the presence of HSV antigen (Ag) (fourth column). The mock-infected sections contained no specific HSV staining and only occasional Gr-1+ cells. (B) Representative images of WT or HVEM KO eyes 14 days after mock infection or infection with HSV-1 after corneal scarification. HSV antigen was absent from the cornea at this time, as expected. The WT corneal stroma remained infiltrated with numerous Gr-1+ and CD3+ cells, while the HVEM KO stroma contained rare positive cells (representative positive cells are indicated with black arrowheads). Control mock-infected sections were negative for all markers, except for occasional Gr-1+ cells.
HVEM on radiation-resistant cell types contributes to clinical disease after corneal HSV-1 inoculation. To further investigate how HVEM promotes the inflammatory corneal environment after HSV-1 infection, we sought to characterize which subsets of HVEM-expressing cells mediate ocular pathogenesis. HVEM is expressed broadly in both hematopoietic and non-hematopoietic organs, including the murine eye, sensory neural tissue, and on a wide variety of leukocytes, including T cells, B cells, NK cells, PMN, DCs, and myeloid cells (108, 112, 186). Furthermore, both the cell type expressing HVEM and the ligand with which it interacts influences whether the HVEM signal is co-stimulatory or co-inhibitory (32, 60, 73, 75, 112, 195). HVEM on corneal resident cells could interact with natural HVEM ligands on infiltrating cells to promote inflammation and disease; alternatively, HVEM expressed on infiltrating immune cells could provide signals that aggravate disease.

To distinguish between these possibilities, we produced four groups of hematopoietic chimeric mice by transplanting WT or HVEM KO bone marrow (BM) cells into lethally irradiated WT (C57BL/6, CD45.1 allele) or HVEM KO (on C57BL/6 background, CD45.2 allele) mice (annotation: donor⇒RECIPIENT). In this process, cells that are sensitive to radiation, including most BM-derived immune cells, are ablated from recipient animals and replaced via transplantation of BM tissue from donor animals. The majority of cell types, including resident cells of the cornea such as the epithelium and stroma, are resistant to radiation, and are not replaced by donor tissue. After a recovery period of 10 weeks, reconstitution efficiency was evaluated by flow cytometry of peripheral blood lymphocytes for the CD45 alleles. Chimeras with ≥95% reconstitution were then infected with HSV-1(17) via corneal scarification and monitored for 14 days.

Mice with HVEM on radiation-resistant cell types (WT recipients) began exhibiting lesions 5 dpi, and by 7 dpi all mice from these groups had lesions (Fig. 7A). In contrast, mice lacking HVEM on radiation-resistant cell types (HVEM KO recipients) were relatively protected,
as only one animal from each genotype developed a lesion (Fig. 7A). These differences were significant: wt→WT mice had higher incidence of lesions than hvem ko→HVEM KO and wt→HVEM KO for days 5-14; hvem ko→WT also had a significantly higher incidence of lesions than hvem ko→HVEM KO for that same time period. The two mixed chimeras also differed significantly from each other in lesion incidence: hvem ko→WT mice had a higher incidence of lesions than wt→HVEM KO mice for 6-14 dpi. Lesions in the WT recipient mice were also more severe than the rare lesions that occurred in the HVEM KO recipient animals (Fig. 7B).

We also scored the animals for neurologic symptoms. Like lesion incidence, neurologic symptom incidence segregated with HVEM expression on radiation-resistant cell types. WT recipients began developing symptoms 6 dpi, and by the following day all or nearly all mice from these groups had at least mild symptoms (Fig. 7C). In contrast, signs of neurologic disease were rare or absent in the HVEM KO recipient groups: HVEM KO recipients had a significantly lower incidence of neurologic disease than WT recipient groups for all time points after and including day 6. Neurologic symptom severity was tracked by day, and mice whose symptoms were severe enough to require euthanasia were assigned a score of 5. WT recipients not only developed neurologic disease more frequently, but also had significantly severer symptoms than HVEM KO recipients (Fig. 7D).
Fig 7. HVEM on radiation-resistant cell types confers WT-like susceptibility to clinical HSV-1 symptoms after corneal inoculation.

Bone marrow (BM) of WT or HVEM KO mice was ablated with 12 Gy of radiation. Recipients were transplanted with ~10 million WT or HVEM KO cells harvested from donor BM (notation: donor→RECIPIENT). After 10 weeks of reconstitution and verification of transfer completeness via flow cytometry, chimeric animals were infected via corneal scarification with 2.0×10^6 PFU/5 µl per eye of HSV-1(17) (two independent experiments, total n = 8 to 12 per group). Clinical symptoms were monitored for 14 days. (A and C) Incidence of epithelial lesion development (A) or neurologic (neuro.) symptoms (C) over time. Both HVEM KO-recipient groups had a significantly lower lesion incidence than WT-recipient groups from 5 to 14 dpi (two-way ANOVA with Holm-Sidak’s multiple-comparison test, P < 0.001) and significantly lower neurologic morbidity from 6 dpi to the end of the experiment at 14 dpi (two-way ANOVA with Holm-Sidak’s multiple-comparison test, P < 0.0001). (B and D) Mean maximum (max.) epithelial lesion score (B) or neurologic score (0 to 5, with 5 representing the greatest severity) (D) reached on any day (two-
tailed t test with Holm-Sidak's multiple-comparison test). (E) Survival for each group (log-rank test). (F) Maximum weight loss, expressed as a percentage of starting weight (two-tailed t test with Holm-Sidak's multiple-comparison test). Values are means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
In addition to developing less frequent, milder lesions and neurologic symptoms, HVEM KO recipient mice were also relatively protected against HSV-1 induced mortality (Fig. 7E). In this case, the mixed chimeras (wt→HVEM KO and hvem ko→WT) had intermediate phenotypes and differences either between them or when compared to control groups did not reach statistical significance. However, the total 14-day wt→WT mortality rate (50%) was significantly higher than that of hvem ko→HVEM KO (15%). This was also consistent with data indicating wt→WT animals lost significantly more weight than all other groups, more than double that of either HVEM KO recipient group (Fig. 7F). The wt→WT controls also exhibited more weight loss than hvem ko→WT group (13.01 ± 2.51%). Because hvem ko→WT mice had lower weight loss and mortality compared to wt→WT mice, absence of HVEM on BM-derived radiation-sensitive cells may also limit these HSV-1-induced outcomes specifically when HVEM is expressed on all other cell types. Collectively, the results from these experiments indicate that HVEM expression on radiation-resistant cell lineages confers susceptibility to wild type-like clinical disease after inoculation with HSV-1 at the corneal surface. In contrast, HVEM on radiation-sensitive, BM-derived lineages has limited impact on clinical measures, except perhaps in subtle contributions to mortality and weight loss, as hvem ko→WT mice were slightly protected in these measures compared to wt→WT controls.

**HVEM on radiation-resistant cell types increases viral titers in eye swabs and tissues after corneal HSV-1 inoculation.** To corroborate the clinical findings observed in the hematopoietic chimeras, we determined the viral loads in eye swabs on 1, 3, 5 dpi and titers in the POS, TG, and brains collected 5 dpi via plaque assay. The wt→WT controls had approximately 1 log higher titers in the tear film 1 dpi than either HVEM KO recipient group, regardless of donor genotype (Fig. 8A). The other WT recipient group, hvem ko→WT, also trended toward higher titers than the HVEM KO recipients. By 3 dpi, titers had decreased in all
four groups, and differences were no longer significant (Fig. 8B). On day 5, viral loads in the tear film of the wt→WT controls were once again significantly higher than in either HVEM KO recipient group by around 1 log (Fig. 8C). At this later time point, the amount of virus present in wt→WT eye swabs was also higher than that of the hvem ko→WT group, suggesting that HVEM on radiation-sensitive cells may also promote viral replication later in infection. In summary, establishment of primary corneal infection immediately after infection (1 dpi) as well as later in infection (5 dpi) was limited in the absence of HVEM on radiation-resistant cell types.

Similar results were observed during analysis of viral loads in the POS and TG at 5 dpi. POS titers from wt→WT controls were subtly but significantly (~1 log) higher than both HVEM KO recipient groups, and titers in hvem ko→WT POS also tended to be higher than those of HVEM KO recipients (Fig. 8D). Titers from the TG of wt→WT controls were ~1 log higher than hvem ko→HVEM KO controls (Fig. 8E). Although wt→HVEM KO mice had slightly lower titers than either of the WT recipient groups, this difference did not reach statistical significance. The decreased viral loads observed in the POS and TG are supportive of the primary replication defect in the cornea, as spread to these sites is dependent on the initial infection in the cornea. Similarly, viral titers in the brain in HVEM KO recipients trended toward lower titers than WT recipients, although this did not reach statistical significance (Fig. 8F). Lower titers in the brains of HVEM KO recipients are likely due to viral spread, as others have found that HVEM is not required for pathogenesis in the brain (47).
Fig 8. HVEM on radiation-resistant cell types, regardless of radiation-sensitive (donor) genotype, is associated with higher viral loads in the tear film, POS, and TG. Hematopoietic chimeras were inoculated with $2.0 \times 10^6$ PFU/5 µl HSV-1(17) per eye after corneal scarification (annotation: donor→RECIPIENT). Data representing the results of two independent experiments are shown (total $n = 6$ to 12 per group). (A to C) Titers of virus from eye swabs collected 1, 3, and 5 dpi. (D to F) Titers in the POS (D), TG (E), and brain (F). Data were evaluated with one-way ANOVA with Holm-Sidak’s multiple-comparison test. Values are means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 
These results indicate that HVEM expression on radiation-resistant cells subtly but significantly increases viral loads in the eye swabs 1 and 5 dpi, and in the POS and TG 5 dpi, supporting our clinical findings. HVEM on radiation-sensitive, BM-derived lineages also moderately increased replication in the eye at later points in infection, as wt→WT titers were higher than hvem ko→WT titers in swabs collected 5 dpi. This suggests that HVEM on radiation-sensitive cell types may also contribute positively to pathogenesis, although more subtly than HVEM on radiation-resistant cell types.

Discussion

In this study, we sought to investigate the contribution HVEM makes to HSV-1 pathogenesis after ocular inoculation (49, 50). Our data indicate that, during ocular infection with HSV-1, a) the gD-HVEM entry interaction is dispensable for normal disease development; b) HVEM-positive corneas have higher amounts of inflammatory cytokines and granulocytic and T cell infiltrates; and c) lack of HVEM expression on radiation-resistant cell types is sufficient to protect against wild type-like disease. HVEM, a TNF receptor superfamily member, has both pro- and anti-inflammatory actions and is known to modulate responses to a wide variety of pathogens in a number of organs (28, 29, 155, 182, 183). We found that HVEM significantly increased levels of the inflammatory cytokine IL-6 and the chemokine CXCL10 in the cornea during acute infection (Fig. 5); this induction did not require the entry mediating function of HVEM. Several other chemokines also followed this trend, including CCL3, CXCL9, and RANTES. Along with increased expression of a variety of chemotactic factors, the corneas of HSV-1 infected WT mice were also more heavily infiltrated by CD3+ T cells and Gr-1+ granulocytic cells during both acute and chronic phases (Fig. 6). This type of infiltrate was consistent with previously described functions of the upregulated chemokines: CCL3, secreted from a variety of cell types, recruits and activates PMN and monocytes, while CXCL9, CXCL10,
and RANTES are T cell chemoattractants known to promote ocular HSV-1 pathogenesis (110, 127, 193, 196). We did not differentiate between subsets of T cells, and cannot rule out the increased levels of CD3\(^+\) cells in WT corneas were regulatory T cells (Tregs), which have been shown to control ocular infections in some models (197-199). This would be somewhat consistent with a previous report that found that HVEM KO mice have decreased expansion of CD4\(^+\)Foxp3\(^+\) Tregs in the draining lymph nodes after footpad injection (142). However, others have shown that numbers of Tregs in the cornea are lower in mice that did not develop HSK, and that Treg depletion does not influence HSK incidence (200). While further characterization of the precise HVEM-mediated changes in the immune infiltrate of the cornea is required, it is clear that, at least in our model, these changes promote rather than ameliorate ocular pathogenesis. My next set of experiments quantifies infiltrating cells and analyzes the expression of HVEM in corneal tissues in order to more precisely determine the contribution HVEM makes to HSV-1-induced ocular pathogenesis.

Collectively, these data indicate that HVEM mediates the development of an inflamed cornea after HSV-1 infection without requiring the gD-HVEM entry interaction. Nectin-1, expressed widely in the murine eye, is available to mediate entry when HVEM is absent, likely explaining why HVEM is not required as an entry receptor (59). We hypothesized that gD-independent immunomodulatory functions of HVEM promote disease after ocular inoculation of HSV-1. HVEM is bound by a number of natural ligands, including LIGHT, LT\(\alpha\), CD160, and BTLA (62, 73, 179). HVEM is a bidirectional receptor: as a ligand, HVEM bound by BTLA or CD160 leads to repression of T cell activation and proliferation (co-repressor), while interactions with LIGHT or LT\(\alpha\) enhance activation and differentiation of many immune cell types (co-stimulator), although these functions can vary depending on the cellular context and whether the ligand is expressed in cis or trans (32, 201). As a receptor, HVEM engaged by its natural ligands or viral gD activates NF-\(\kappa\)B signaling (28, 81, 179). HSV gD has been reported to
prevent interactions between HVEM and all its natural ligands (76, 79, 201). Experiments with the Δ7-15 mutant virus not only indicate that HVEM-mediated entry is not required for ocular pathogenesis, they also suggest that gD competition with host HVEM ligands is not likely to account for the attenuated disease observed in HVEM KO mice, as the Δ7-15 mutation in gD, which others and we have shown abolishes normal entry-related interactions with HVEM, likely limits the ability of the protein to compete with natural ligands for HVEM binding.

Using hematopoietic chimeras, we broadly isolated which HVEM-expressing cell types are responsible for HVEM-mediated ocular pathogenesis. Susceptibility to disease after corneal inoculation segregated with HVEM expression on radiation-resistant cell types, as WT recipients had indistinguishable neurologic and lesion scores, rates of mortality, and viral loads in the tissue, while HVEM KO recipients, regardless of donor genotype, were protected from infection. We conclude that HVEM expression on radiation-resistant cell types, likely on resident cells of the cornea, is required for normal HSV-1 pathogenesis after corneal inoculation.

In our proposed model, HVEM expression on a radiation-resistant cell type (or types) such as the corneal epithelium or resident immune cells increases cytokine production, drawing greater amounts of infiltrates to the cornea, thereby worsening disease after ocular inoculation in the hvem ko→WT chimeras (Fig. 9A). Alternatively, HVEM on radiation-resistant cells may interact with infiltrating immune cells expressing HVEM ligands to increase their activation and secretion of cytokines. In contrast, the wt→HVEM KO chimeras, which lack HVEM on radiation-resistant, resident cells of the eye, are protected from infection or resolve infection in a manner similar to that observed in the HVEM KO, because without HVEM, the pathologic inflammatory cascade is not initiated to the same degree (Fig. 9B).
Fig 9. Model of HVEM contribution to HSV-1 pathogenesis in the eye. We have shown that HVEM-expressing, radiation-resistant cell types promote pathogenesis after corneal inoculation of HSV-1 and that HVEM-mediated pathology is entry independent. (A) In hvem ko→WT chimeras, HSV-1 enters through HVEM or nectin-1 at the initial site of infection. HVEM is not required on radiation-sensitive cells, such as infiltrating immune cells, in order for pathogenesis to occur in a wild-type-like manner. (B) In wt→HVEM KO chimeras, HSV-1 likely enters cells through its other receptor, nectin-1, as gD-HVEM entry is not required for the establishment or spread of infection. Despite the presence of HVEM on radiation-sensitive infiltrating cells, these animals develop attenuated or only mild disease after corneal inoculation, which is similar to what is observed in full HVEM KOs.
Given the wide expression of multiple HVEM ligands on many immune cell types, it is difficult to predict which ligand interacts with HVEM to promote ocular HSV-1 pathogenesis. HVEM on radiation-resistant cells may interact with one of its natural ligands, such as LIGHT or CD160, expressed on infiltrating immune cells, to promote inflammation. LIGHT provides costimulatory signals to murine and human T cells, enhancing proliferation and activation in the context of TCR ligation (65, 66). If this interaction drove pathogenesis our model, the radiation-resistant HVEM-positive cell type would also have to express the MHC receptor, as would occur on antigen presenting cells (APCs). High numbers of CD11b+ macrophages and CD11c+ DCs reside in the cornea, and recent studies have shown these cells increase in number and, in the case of DCs, in MHC class II expression after infection with HSV-1 (119, 120, 202, 203). These cells, although BM-derived, incompletely turn over after irradiation: in one study, 25% of myeloid-lineage cells persisted in the corneal stroma of chimeric mice even after 8 weeks of reconstitution (204). This small but significant population of HVEM-positive, radiation-resistant APCs could interact with LIGHT or other HVEM binding partners on infiltrating immune cells to increase their proliferation, activation, and/or secretion of cytokines. It is also possible that corneal cells, such as epithelial cells or keratocytes, may be responsible for the changes observed in corneal cytokine expression. HVEM is widely expressed by the normal murine corneal epithelium, and its expression in the corneal epithelium and stroma has been reported to increase after HSV-1 infection (108). Infected cells and neighboring uninfected cells of the cornea are believed to be the earliest instigators of HSK, and there is evidence that all of the cytokines that exhibited HVEM-dependent induction after infection in our model can be produced by cells of the cornea, including the epithelium, stroma, or endothelium (25, 91, 93, 107, 110, 192, 205, 206). The HVEM receptor, after binding any of its ligands, activates NF-κB signaling (80, 81). Given that the cytokines we found to be upregulated in an HVEM-dependent manner are also NF-κB target genes (91, 207-217), it is possible that activation of NF-κB
within corneal resident cells through HVEM could be responsible for the increased expression of inflammatory mediators, and subsequently, immune cell infiltrates in WT eyes. HSV also strongly activates NF-κB upon infection, potentially through gD-HVEM interactions, although other viral proteins have also been shown to be important for this process (84, 218). NF-κB activation is required for efficient viral replication and expression of viral proteins, as well as to prevent apoptosis of infected cells (85, 87, 218). If NF-κB activation is the signaling pathway responsible for the HVEM-mediated pathogenesis in our model, our results with the Δ7-15 virus suggest that natural HVEM ligands may be equally capable of activating NF-κB signaling during HSV infection.

In conclusion, we have shown HVEM on radiation-resistant cells types, such as the corneal epithelium, stroma, or long-lived, resident APCs, plays an important immunomodulatory role in the pathogenesis of ocular HSV-1 infections independently of its entry receptor functions. These findings suggest that the contribution made by HVEM during HSV-1 pathogenesis occurs via the innate response, i.e. on residents of the eye, rather than the adaptive immune response. Understanding how HVEM, a receptor with diverse roles in infection, autoimmunity, and inflammation, orchestrates ocular HSV-1 pathogenesis could not only provide avenues for new therapeutics, but could also yield general insights into a variety of immune-mediated ocular diseases.
CHAPTER 4

Corneal Inflammation and Nerve Damage After HSV-1 Are Promoted by HVEM and Ameliorated by Immune-Modifying Nanoparticle Therapy*


Abstract

**Purpose:** To determine cellular and temporal expression patterns of herpes virus entry mediator (HVEM, Tnfrsf14) in the cornea during the course of herpes simplex virus 1 (HSV-1) infection, the impact of this expression on pathogenesis, and whether alterations in HVEM or downstream HVEM-mediated effects ameliorate corneal disease.

**Methods:** Corneal HVEM levels were assessed in C57BL/6 (WT) or Tnfrsf14−/− (HVEM knockout, KO) mice after infection with HSV-1(17). Leukocytic infiltrates and corneal sensitivity loss were measured in the presence, global absence, or partial absence of HVEM. Effects of immune-modifying nanoparticles (IMPs) on viral loads, corneal sensitivity, and corneal infiltrates were measured.

**Results:** Corneal HVEM+ populations, particularly monocytes/macrophages during acute infection (3 dpi) and neutrophils (PMN) during the chronic inflammatory phase (14 dpi), increase after HSV-1 infection. HVEM increases leukocytes in the cornea and decreases corneal sensitivity. Ablation of HVEM from CD45+ cells, or intravenous IMP therapy delivered 3-7 dpi reduce infiltrates in the chronic phase and improve corneal blink response.
Conclusions: HVEM is expressed on two key populations: corneal monocytes/macrophages and PMN. HVEM promotes the recruitment of myeloid cells to the cornea in the chronic phase. The timing of HVEM-associated corneal sensitivity loss suggests it precedes leukocytic infiltration, and therefore may play an active role in recruitment. We propose that HVEM on resident corneal macrophages increases nerve damage and immune cell invasion, and show that prevention of late-phase infiltration of PMN and CD4\(^+\) T cells by IMP therapy can ameliorate sensory nerve loss, clinical symptoms, and mortality caused by HSV-1.

Introduction

Herpes simplex virus 1 (HSV-1) is a neurotropic human pathogen causing a wide range of disease states from mild orolabial lesions, the most common manifestation, to deadly encephalitis and meningitis (33). Another potentially devastating outcome of HSV-1 infection is herpes stromal keratitis (HSK), a recurrent syndrome in which chronic inflammation initiated by the virus produces corneal scarring, opacification, neovascularization, and potentially loss of vision (13, 14, 24). Although actively replicating HSV-1 is required (19, 20, 192), a pathologic immune response driven primarily by neutrophils and CD4\(^+\) T cells develops that is sustained in the absence of replicating virus (21, 22). Understanding the virus-host interactions that together produce this pathogen-provoked immuno-inflammatory syndrome is critical for the development of new, targeted therapies.

Herpes virus entry mediator (HVEM), a tumor necrosis factor (TNF)-receptor superfamily member, is a cellular receptor that stands at the intersection of viral pathogenesis and host immune responses. Originally identified as one of several gD-receptors required for HSV entry (35, 38, 188), HVEM facilitates viral entry in cultured human corneal and conjunctival epithelial cells (55, 219) as well as in primary human monocytes (220) and dendritic cells (117).
While HVEM is required for ocular pathogenesis \textit{in vivo} (50), the immunomodulatory effects of this receptor are more potent than its entry functions: HVEM increases corneal inflammatory cytokine production and leukocytic infiltration independently of viral entry (51, 105, 155). HVEM KO mice have lower viral loads in eye swabs and subsequent spread to the nervous system, and lessened or absent clinical symptoms, including neurologic disease, periocular lesions, and mortality (49-51).

HVEM expression in the anterior eye has previously been localized to the corneal epithelium and stroma (108). HVEM is also found on a wide range of leukocytes, including neutrophils, monocytes, dendritic cells, and CD4$^+$ and CD8$^+$ T cells, although the expression of HVEM on these cells once in the cornea has not been assessed (60, 112). We recently found that HVEM-dependent disease after HSV-1 infection at the corneal surface is mediated by a radiation-resistant cell type or types (51), leading us to hypothesize that HVEM on corneal epithelial cells, stromal fibroblasts, or resident stromal macrophages, which incompletely turn over after irradiation (204), may promote pathogenesis in our adoptive transfer model. We investigated the expression of HVEM on a variety of cell lineages before, during, and after acute HSV-1 infection and found that corneal monocytes/macrophages are the first population to express HVEM, followed by neutrophils (PMN), double-negative (DN) T cells, and CD4$^+$ T cells. Increases in corneal HVEM expression were associated with loss of corneal sensitivity and leukocytic infiltrates, while loss of HVEM$^+$ cells from the cornea, either by genetic ablation or by administration of immune-modifying particles (IMPs), improved disease. We propose that HVEM on corneal resident monocytes/macrophages is integral for the recruitment of PMN and other inflammatory cells to the cornea as well as nerve damage and loss of corneal sensitivity. Treatment with IMPs prevents the mobilization of immune cells to the cornea without hindering viral clearance, and shows promise as a new therapeutic for treatment of chronic inflammation in HSK.
Results

**HSV-1 infection expands HVEM<sup>+</sup> populations in the cornea.** Based on adoptive transfer experiments, we localized HVEM-mediated pathogenesis to a radiation-resistant cell type or types (51). We re-investigated the expression of HVEM in the cornea by immunohistology and flow cytometry, as a previous report indicated that HVEM is widely expressed in the naïve murine corneal epithelium *in vivo*, and that its expression increases in the epithelium and stroma as early as 1 day after infection (108). The same rabbit polyclonal anti-HVEM antibody, R11874 (Patricia Spear, Northwestern University), nonspecifically stained the corneal epithelium of both WT (C57BL/6) and HVEM KO (*Tnfrsf14<sup>−/−</sup>*) 9-12 week old male mice 1 day post infection (dpi) with 2.0x10<sup>6</sup> PFU/5 µl/eye HSV-1 strain 17 or post mock-infection (Fig. 10A-D). In contrast, a commercially available mouse monoclonal antibody to HVEM, HMHV-1B18 (BioLegend), produced little positive staining in the cornea 1 dpi (Fig. 10E-H). The spleen was floridly positive for HVEM after HMHV-1B18 staining (Fig. 10I and J), consistent with previously described expression on B cells, T cells, myeloid cells, DCs, and other leukocytes (60). These findings suggest HVEM expression in the cornea is limited immediately after scarification or infection.

We used flow cytometry to quantify HVEM expression in pairs of corneas from naïve, mock-infected, or infected adult WT or HVEM KO control mice 3 or 14 dpi (Fig. 10K). HVEM KO samples, an isotype control antibody, and fluorescence minus one (FMO) control were used to define the threshold for HVEM positivity. WT corneas contained a significantly higher proportion (Fig. 10K) and greater absolute number (Fig. 10L) of HVEM<sup>+</sup> cells 3 and 14 dpi compared to naïve corneas or mock-infected corneas, and this increase was specific to the cornea, as no such expansion occurred in the spleen (Fig. 10M). The majority of the expanded HVEM<sup>+</sup> population was attributable to an increase in HVEM<sup>+</sup>/CD45<sup>+</sup> cells (compare Fig. 10K to 10N). These findings indicate that HVEM, rather than being highly expressed by the murine corneal
epithelium, is limited to CD45\(^+\) populations and is induced after infection with HSV-1 in a time-dependent manner.

The majority of HVEM\(^+\) cells in the acutely infected murine cornea derive from the monocyte/macrophage lineage. To further characterize corneal HVEM expression, we analyzed HVEM\(^+\) corneal isolates from adult WT mice 3 dpi for the pan-leukocyte marker CD45, the endothelial marker CD31, and the epithelial markers E-cadherin or ICAM-1 by flow cytometry. Isotype controls were used to set thresholds for each. Mock-infected corneas contained few HVEM\(^+\) cells, and few of those were CD45\(^+\), while the HVEM\(^+\) cells from infected corneas 3 dpi were mostly CD45\(^+\) leukocytes (Fig. 11A and B). Epithelial cells, defined in this study as CD45\(^-\)/E-cadherin\(^+\)/ICAM\(^+\), and endothelial cells (CD45\(^-\)/CD31\(^+\)) represented small portions of HVEM\(^+\) cells in infected WT corneas (Fig. 11B). The HVEM\(^+\) leukocytes in infected corneal tissue 3 dpi were overwhelmingly CD3\(^-\)/CD11b\(^+\) myeloid cells (Fig. 11C), specifically Ly6G\(^-\)/Ly6C\(^+\)/Ly6C\(^+\) inflammatory monocytes/macrophages (Fig. 11D and E). Few were found to be Ly6G\(^-\)/Ly6C\(^+\)/CD11c\(^-\) neutrophils (PMN) or Ly6G\(^+\)/CD11c\(^+\) myeloid dendritic cells (mDCs).
Fig 10. HVEM expression increases in the murine cornea after HSV-1 infection. (A-J) Representative immunohistological analysis of adult C57BL/6 (WT) or Tnfrsf14−/− (HVEM KO) eyes 1 day after mock- infection (A, B, E, F) or infection (C, D, G, H) at the corneal surface with $2.0 \times 10^6$ PFU/5 µl per eye of HSV-1 strain 17 (original magnification 400x). Formalin-fixed paraffin-embedded eyes were serially sectioned and stained for HVEM with a rabbit polyclonal antibody, R11874 (panels A-D), or with a mouse monoclonal antibody, HMHV-1B18 (Biolegend; panels E-H). (I-J) Naïve murine spleens stained with HMHV-1B18. (K) Percentage and (L) absolute number of HVEM+ cells per cornea pair as determined by flow cytometry ($n = 7-12$ cornea pairs from individual mice, 3 replicates). (M) Percentage HVEM-positive per spleen 3 dpi ($n = 3$). (N) Percentage of CD45+ HVEM+ of total cells per cornea pair 3 dpi ($n = 7-9$, 2 replicates). Values for K-N are means ± SEM. Statistically significant differences are indicated
as follows: **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$ (two-way ANOVA with Holm-Sidak’s adjustment for multiple comparisons).
Fig 11. Acutely-induced HVEM$^+$ cells in the infected murine cornea are mainly CD45$^+$ CD11b$^+$ CD11c$^-$ Ly6C$^+$ Ly6G$^-$ monocytes. 3 dpi, corneas from WT mice infected at the corneal surface with HSV-1(17) were analyzed for expression of HVEM, the pan-leukocyte marker CD45, the endothelial marker CD31, and the epithelial markers E-cadherin and ICAM via flow cytometry. (A) Representative dot plots of live HVEM$^+$ cells from mock and infected corneas for CD45 and CD31. (B) Percentage of total HVEM$^+$ cells from infected WT corneas that are CD45$^+$ leukocytes, CD45$^-$/E-cadherin$^-$/ICAM$^+$ epithelial cells, or CD45$^-$/CD31$^+$ endothelial cells. Corneal isolates were also evaluated for leukocytic lineage markers, including CD3, CD11b, Ly6C, Ly6G, and CD11c. (C) Percentage of HVEM$^+$/CD45$^+$ cells expressing the lymphoid marker CD3 or the myeloid marker CD11b 3 dpi. (D) Percentage of HVEM$^+$/CD3$^-$/CD45$^+$/CD11b$^+$ myeloid
cells categorized as inflammatory monocytes/macrophages lineage (CD11c⁺/Ly6C⁺/Ly6G⁻; I-M), PMN (CD11c⁺/Ly6C⁺/Ly6G⁺), or dendritic cells (CD11c⁺/Ly6G⁻; DCs). (E) Representative dot plot of CD11c vs Ly6C expression of HVEM⁺/CD45⁺/CD11b⁺ myeloid cells from a WT cornea pair 3 dpi. Values for B-E are means ± SEM (n = 8 cornea pairs, 2 replicates).
**HVEM promotes loss of corneal sensitivity and corneal leukocytic infiltration.** To determine whether the increase in HVEM+ cells over the course of HSV-1 infection impacts corneal physiology and function, we determined the corneal touch threshold in WT and HVEM KO mice corneally infected with HSV-1(17) with a Leneau Cochet-Bonnet aesthesiometer as previously described (104). Briefly, the length of the filament was varied from 6 cm – 0.5 cm in increments of 0.5 and touched perpendicularly to the central cornea until the first inflection point. The blink threshold was counted as the length at which the animal blinked ≥2 times out of 3; if no response occurred at 0.5 cm, the eye was scored as 0. After infection, WT mice rapidly and dramatically lost sensitivity in the central cornea compared to HVEM KO mice, in which corneal reflexes were largely maintained (Fig. 12A). Along with experiencing a loss of sensitivity, WT corneas also became more heavily infiltrated by CD45+ leukocytes, as determined by flow cytometry on day 14 (Fig. 12B).

To determine if a correlation between these two factors existed, we performed a linear regression on the average blink threshold length at the end of the experiment, day 14, compared to the number of CD45+ cells in the cornea on that same day (Fig. 12C), and found a significant negative association between these factors ($R^2 = 0.338$, $P = 0.0005$). Characterized by cell type, similar negative correlations also existed between number of PMN (Fig. 12D) and number of monocytes/macrophages (Fig. A5) and the blink threshold length ($R^2 = 0.374$, $P = 0.0002$ and $R^2 = 0.300$, $P = 0.0012$ respectively). We collected eye swabs from infected mice 1, 3, and 5 dpi to determine viral loads in the tear film, and found lower day 1 titers from HVEM KO eye swabs compared to WT (Fig. A6), similar to what we have previously reported (50). Comparison of day 1 (but not day 3 or day 5) titers to the corresponding to the blink threshold (day 14) for each eye individually revealed a significant but small negative correlation (Fig. 12E, $R^2 = 0.145$, $P = 0.0155$).
We next investigated the specific identities of the CD45+ populations in WT and HVEM KO corneas 14 dpi (see Fig. A7 and A8 for gating strategy). HSV-infected-WT corneas tended to contain higher populations of CD4+ and CD8+ T cells on day 14 than HVEM KO corneas, although these differences did not reach statistical significance (Fig. 12F). Levels of mDCs (CD45+/CD3-/CD11b+/Ly6G-/CD11c+), monocytes/macrophages (M; CD45+/CD3-/CD11b+/Ly6G-/CD11c-), and specifically inflammatory monocytes/macrophages (I-M; Ly6C+ monocytes/macrophages) in WT corneas were higher than HVEM KO corneas to the 0.05 significance level, but after correction for multiple comparisons, only the level of PMN remained statistically significantly higher in WT corneas than in HVEM KO corneas (Fig. 12G). At 14 dpi, the majority of HVEM+/CD45+ cells in WT corneas remained CD3-/CD11b+ myeloid cells, although approximately 20% of HVEM+/CD45+ cells were CD3+ (Fig. A9). Most HVEM+/CD11b+ cells were PMN, signaling a switch from the acute phase infection, when corneal monocytes/macrophages represented the greatest HVEM+ population (comparing Fig. 11D to Fig. 12H). Of HVEM+/CD3+ cells, most were CD8+/CD4- (as well as NK1.1-) cells, here called double-negative (DN) T cells, although these could represent γδ T cells. A small population of HVEM+/CD4+ also occurred (Fig. 12I). These data indicate that HVEM promotes an increase in viral replication, as previously reported, as well as increased corneal infiltration, both of which correlated with a loss of central corneal sensitivity.
Fig 12. HVEM-dependent loss of corneal sensitivity is associated with leukocytic infiltration of the cornea. (A) Corneal touch threshold was determined with a Leneau Cochet-Bonnet aesthesiometer every other day beginning prior to infection (day 0) in WT or HVEM KO mice inoculated at the corneal surface with HSV-1(17). Lack of a blink reflex at 0.5 cm was recorded as 0 cm (n = 10 mice per group, 20 individual corneas measured, 2 replicates). (B) Flow cytometry analysis of CD45⁺ infiltrates in WT or HVEM KO corneas 14 dpi (n = 7-8, 2 replicates, two-tailed t test). (C) Relationship between corneal touch threshold on day 14 and the absolute number of corneal CD45⁺ cells, (D) PMN, or (E) day 1 viral loads in the cornea (n = 32, ≥ 3 individual experiments, linear regression for goodness of fit). (F) Absolute number of CD4⁺ T cells (CD45⁺/CD3⁺/CD4⁺/CD8⁻), CD8⁺ T cells (CD45⁺/CD3⁺/CD8⁺/CD4⁻), NKT cells
(CD45⁺/CD3⁻/NK1.1⁺), or double-negative (DN) T cells (CD45⁺/CD3⁺/CD8⁻/CD4⁻/NK1.1⁻) or (G) the absolute number of mDCs (CD45⁺/CD3⁻/CD11b⁺/Ly6G⁻/CD11c⁺), monocytes/macrophages (M; CD45⁺/CD3⁻/CD11b⁺/Ly6G⁻/CD11c⁻), which were then further categorized as inflammatory monocytes/macrophages (I-M; CD45⁺/CD3⁻/CD11b⁺/Ly6G⁻/CD11c⁻/Ly6C⁺) or non-inflammatory monocytes/macrophages (NI-M; CD45⁺/CD3⁻/CD11b⁺/Ly6G⁻/CD11c⁻/Ly6C⁻), and finally PMN (CD45⁺/CD3⁻/CD11b⁺/Ly6C⁻/Ly6G⁻) or in WT vs HVEM KO corneas 14 dpi (n = 11, ≥ 3 individual experiments). (H-I) Percentage of HVEM⁺ corneal cells that of the indicated cell lineage 14 dpi (n = 8, 2 replicates). Values in (A, B, D-F) are means ± SEM, analyzed with two-tailed t test with Holm-Sidak’s correction for multiple comparisons. *, P < 0.05, **, P < 0.01, ***, P < 0.001, ****, P < 0.0001.
Ablation of HVEM from CD45+ lineages reduces myeloid infiltrates late in infection.

We specifically ablated HVEM from CD45+ cells using a Cre/lox system. HVEM^{fl/fl} mice on the C57BL/6 background contain loxP sites flanking exons 3-6 of the HVEM gene. These animals were bred to B6.Tg(Vav1-icre)A2Kio mice (Jackson), which express Cre-recombinase under control of the Vav promoter, resulting in expression in 98-100% of hematopoietic cells with no littermate mosaicism (221). Genotyping was performed by PCR analysis of floxed HVEM and Vav-Cre genes. In addition, we confirmed that homozygous floxed animals expressing Vav-Cre (Vav^{+} HVEM^{fl/fl}) had successful ablation of HVEM from CD45+ cells in comparison to homozygous HVEM^{fl/fl} controls by analysis of peripheral blood (Fig. A10) and infected corneas via flow cytometry for HVEM expression (Fig. 13A). Although CD11b^{+} myeloid cells had lost HVEM expression, this did not impact the presence of CD11b^{+} populations in the cornea 3 dpi (Fig. 13B). Consistent with HVEM KOs, Vav^{+} HVEM^{fl/fl} corneas had significantly lower titers than controls early after infection (Fig. 13C). We also assessed late-phase populations of corneal leukocytes to determine if HVEM-dependent infiltration required HVEM expression on CD45+ cell types. Infiltration of CD4^{+} and CD8^{+} T cells was diminished in Vav^{+} HVEM^{fl/fl} mice compared to controls, although this did not reach statistical significance (Fig. 13D). However, myeloid cell infiltration into the cornea 14 dpi was markedly blunted when HVEM was ablated from CD45+ cells (Fig. 13E). Corneal sensitivity in Vav^{+} HVEM^{fl/fl} mice was also maintained similar to that of HVEM KOs (Fig. A10). These findings suggest that the establishment of CD11b^{+} myeloid cells in the cornea in the acute phase is HVEM-independent, but that the recruitment or maintenance of myeloid lineages in the chronic phase of infection cannot occur effectively without HVEM.
Fig 13. Loss of HVEM from CD45+ lineages reduces myeloid infiltrates during the chronic inflammatory phase. Mice lacking HVEM specifically on CD45+ cells were generated by crossing HVEM<sup>fl/fl</sup> mice with B6.Tg(Vav1-icre)A2Kio mice, which express Cre recombinase throughout the hematopoietic compartment. Adult mice were infected with 2.0 x 10<sup>6</sup> PFU/5 µl per eye of HSV-1(17) after corneal scarification. (A) Confirmation of loss of HVEM expression on CD45+ hematopoietic cells in the cornea 3 dpi in the conditional KOs, which are Cre-expressing homozygous floxed animals (Vav<sup>+</sup> HVEM<sup>fl/fl</sup>), compared to homozygous HVEM<sup>fl/fl</sup> controls. (B) Percentage of CD11b+ myeloid cells of total CD45+ cells 3 dpi in Vav<sup>+</sup> HVEM<sup>fl/fl</sup> or control HVEM<sup>fl/fl</sup> corneas. Values are means ± SEM, n = 2-3, two-tailed t test. (C) Titers in eye swabs collected 1, 3, and 5 dpi in Vav<sup>+</sup> HVEM<sup>fl/fl</sup> or control HVEM<sup>fl/fl</sup> corneas (n = 11-13 mice, 3 replicates). (D) Absolute number of CD4+ T cells, CD8+ T cells, NKT cells, or DN T cells, or (E)
absolute number of inflammatory monocytes/macrophages (I-M), monocytes/macrophages (M), mDCs, non-inflammatory monocytes/macrophages (NI-M), or PMN found in the corneas of conditional KO or control mice 14 dpi (n = 5-10, 3 replicates). All values are means ± SEM, (C-E) evaluated with two-tailed t tests with Holm-Sidak’s correction for multiple comparisons. *, P < 0.05, **, P < 0.01, ***, P < 0.001, ****, P < 0.0001.
Negatively-charged immune-modifying nanoparticle treatment ameliorates disease after ocular HSV-1(17) infection. Negatively-charged, 500-nm-diameter, immune-modifying nanoparticles (IMPs) derived from poly(lactic-co-glycolic acid, PLGA) limit tissue infiltration of inflammatory monocytes and other circulating phagocytic cells by re-routing them to the spleen for degradation (173). IMP therapy is effective in a wide range of inflammatory diseases, including West Nile infection, experimental autoimmune encephalitis, cardiac and kidney reperfusion injury, and others (173). HVEM, which we found localized mostly on corneal inflammatory monocytes/macrophages during acute infection, and on PMN during the chronic phase, promotes infiltration and is associated with sensory loss at the corneal surface. We hypothesized that IMP treatment could prevent the migration of circulating immune cells into the cornea, even in the presence of HVEM, and thus ameliorate corneal disease.

We performed a pilot study in adult male BALB/c mice, which are highly susceptible to neurologic morbidity after corneal HSV-1 infection, to determine if a previously-established dose of 0.94 mg IMPs/mouse via tail vein injection delivered daily for 5 days could improve survival compared to mice receiving the vehicle, phosphate-buffered saline (PBS). In order to target the later, pathologic influx of immune cells without hindering viral clearance, we began treatment 3 dpi, prior to the onset of clinical symptoms like ruffled fur, periorbital swelling, and lesion development, which occur around day 5 (50), but after an innate response had been mounted (13, 95, 98, 222). Treated mice survived at significantly higher rates (40% vs. 0%) compared to mice that received the vehicle (Fig 14A). We next tested the therapeutic potential of IMPs in C57BL/6 mice to prevent loss of corneal sensitivity, reduce corneal infiltration, and improve symptoms after HSV-1 infection. IMP-treated-mice maintained corneal blink thresholds similar to pre-infection levels, while corneal sensitivity in vehicle-treated animals significantly declined by 6 dpi (Fig. 14B). Viral titers in eye swabs collected 1, 3, and 5 dpi did not differ between the treatment groups (Fig. 14C).
Flow cytometry of corneas collected on day 14 revealed that IMP treatment significantly reduced CD45\(^+\) populations (Fig. 14D), particularly in all myeloid populations we investigated (Fig. 14E) and tended to decrease CD4\(^+\) and DN T cells (Fig. 14F). Populations of leukocytes present in the spleens of treated or sham-treated mice were compared <24 hours after administration of the last dose. Spleens from IMP-treated mice contained higher numbers of most leukocytes, especially PMN, compared to PBS-treated mice (Fig. A11), suggesting splenic sequestration may account for the decrease in corneal leukocyte populations in IMP-treated animals.

While the absolute number of HVEM\(^+\) cells in the cornea was lowered by IMP therapy (Fig. 14G), the proportion of HVEM\(^+\) cells in the cornea was similar between treatments (Fig. 14H). IMP-treated mice had a significant decrease in the proportion of corneal HVEM\(^+\) PMN compared to control, while the proportion of HVEM\(^+\) monocytes/macrophages and DCs increased (Fig. 14I). Proportions of lymphoid HVEM\(^+\) populations did not vary significantly between the treatments (Fig. 14J). These data support the notion that HVEM\(^+\) monocyte/macrophages and DCs in the cornea could derive from resident populations rather than from circulating pools, as IMP therapy did not affect, or even increased, their relative representation.
Fig 14. Treatment with negatively-charged immune-modifying nanoparticles (IMPs) ameliorates disease and reduces corneal infiltrates after ocular HSV-1(17) infection. WT C57BL/6 or BALB/c mice were inoculated with $2.0 \times 10^6$ PFU/5 µl per eye of HSV-1(17) after corneal scarification and treated with 200µl of vehicle control (PBS) or negatively-charged IMPs for 5 days starting 3 dpi. (A) Survival of highly-HSV-sensitive BALB/c mice with or without IMP treatment ($n = 5$, Log-rank test). (B) Corneal touch threshold of C57BL/6 mice with or without IMP treatment (determined with a Leneau Cochet-Bonnet aesthesiometer, $n = 10$ individual mice, 20 corneas tested, 2 replicates). (C) Viral loads in the tear film of C57BL/6 mice with or without IMP treatment ($n = 10$). (D-G) Absolute numbers of the indicated cell population present...
in IMP- or sham-treated corneas 14 dpi as determined by flow cytometry. (H) Percentage of total cells that are HVEM$^+$ in treated or sham-treated mice. (I-J) Percentage of HVEM$^+$ cells belonging to each population ($n = 10$ for D-J, 2 replicates, values are means ± SEM, two-tailed $t$ test with Holm-Sidak’s correction for multiple comparisons). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$. 
Discussion

In this study, we show that HSV-1 infection acutely induces HVEM⁺ expression on monocyte/macrophages in the cornea. HVEM expression correlated with loss corneal sensitivity and increased leukocytic infiltration, particularly in the chronic inflammatory phase of herpes stromal keratitis, which was ameliorated by ablation of HVEM from CD45⁺ lineages. Finally, this work demonstrated that IMP-therapy was useful in the treatment of murine HSK, as it prevented circulating leukocytes from invading the cornea and limited corneal sensation loss.

HVEM⁺ populations in the cornea differed in the acute infectious stage from the chronic inflammatory phase, shifting from corneal monocyte-lineage cells to PMN, DN T cells, and, as has been noted by others, CD4⁺ T cells (142). In contrast to a prior report (108), we were unable to detect significant levels of HVEM on corneal epithelial cells. In a previous study using adoptive transfers with WT and HVEM KO mice, we found that that HVEM on radiation-resistant lineage(s) is sufficient to mediate disease after HSV infection (51). The naïve cornea, once thought to be devoid of immune cells, contains numerous CD11b⁺ cells with variable MHC class II expression; the majority lack CD11c and Ly6G and therefore are of the monocyte/macrophage lineage (204, 223). Because a sizable portion (25%) of in vivo corneal resident macrophages do not turn over post-irradiation even after 8 weeks of recovery (204), we hypothesize that this cell type, essentially the only lineage to express HVEM early after infection, is the radiation-resistant population sufficient for HVEM-mediated HSV-1 pathogenesis.

HVEM on corneal macrophages may promote immunopathogenesis by recruiting other inflammatory cells to the eye. HVEM expression increases the levels of macrophage-associated chemotactic factors in the cornea, such as CXCL10 and CCL3, which are known to recruit PMN, monocytes, and T cells during ocular HSV-1 (51, 110, 224). HVEM upregulates these factors independently of viral entry (51), consistent with the observation that, specifically in the cornea, levels of infectious virus do not correlate with chemokine expression (225). In line with this
hypothesis, we found that genetic ablation of HVEM from CD45+ cells through a Cre/lox system prevented infiltration by myeloid cells and tended to limit CD4+ populations.

In human patients with HSK, corneal sensitivity to mechanical stimulation is significantly impaired (103). In our murine model, HVEM promoted loss of corneal sensitivity, indicating damage of corneal nerves (101). Early immune responses rather than direct viral infection are likely to initiate nerve damage, as the global nature of corneal nerve retraction would require the virus to successfully infect every neuron innervating the cornea (101). Additionally, local depletion of early HSV-induced conventional DCs (cDCs) produces more extensive nerve damage, despite decreasing corneal nerve infection, suggesting inflammatory cells or products are responsible (120). Some corneal monocytes/macrophages reside in close proximity to corneal nerve endings (226), raising the intriguing possibility that resident HVEM+ macrophages could aggravate nerve damage during acute infection. Consistent with this theory, WT mice had already begun losing corneal sensitivity compared to HVEM KOs as early as 2-3 dpi, at which time corneas contained significant HVEM+ monocyte/macrophage cells but few other infiltrates, indirectly suggesting nerve damage precedes large-scale leukocyte invasion.

In fact, nerve damage likely contributes to further leukocytic infiltration of the cornea. Neuropeptides released from corneal nerve endings such as substance P and calcitonin gene-related peptide (CGRP) can induce IL-8 transcription in corneal epithelial cells, recruiting PMN (227, 228); substance P has also been reported increase the severity of HSK lesions (229). Another mechanism recently proposed by Yun and colleagues posits that HSV (or, more likely, the initial inflammation associated with viral replication) damages corneal nerves, and subsequent desiccation due to lack of blinking leads to chronic, bilateral leukocytic infiltration (101). We found that large populations of leukocytes in the cornea, especially PMN and monocytes/macrophages, correlated with a maintained lowered blink threshold 14 dpi. When HVEM ablation or treatment with IMPs 3-7 dpi prevented late-phase corneal infiltration, corneal
sensitivity was preserved, suggesting nerve damage is exacerbated or perpetuated by PMN, CD4+, and other chronic-phase infiltrates (101). Coincidentally, these populations were also HVEM+, although expression on these radiation-sensitive populations did not contribute significantly to pathogenesis in our earlier adoptive transfer experiments (51). We find it likely that both early and late inflammatory cells contribute to loss of corneal sensitivity during HSV infection: resident macrophages may secrete cytokines or other factors that damage corneal nerves, decreasing blinking and drying the eye, leading to immune cell infiltration, which in turn damages corneal nerves further.

Regardless of the precise recruitment mechanism of immune cells in the chronic phase, it is well established that their presence in the cornea promotes neo-angiogenesis and neo-lymphangiogenesis (26, 230), opacification (200), and scarring (231, 232). CD4+ T cells also prevent reinnervation of the cornea, sustaining loss of the blink response and desiccation-related inflammation (101). However, loss of immune cells, particularly PMN and macrophages, can be equally devastating, as unchecked viral replication leads to CNS invasion and mortality: depletion of PMN with a monoclonal antibody prior to and during infection increases replication, spread to the skin and brain, and mortality in BALB/c mice (94). Depletion of macrophages with clondronate liposomes injected into the subconjunctiva several days prior to infection severely heightens viral replication, blepharitis, and epithelial keratitis; however, stromal keratitis was mildly improved by macrophage depletion (96, 99, 100). In contrast, delaying macrophage depletion to 2 and 4 dpi does not enhance viral replication (100).

Knowing this, we hypothesized that therapy in the pre-chronic phase (3-7 dpi) with immune-modifying nanoparticles (IMPs), which are efficacious in the treatment of a host of inflammatory disorders (173, 233), could limit HSK symptoms without enhancing viral replication. IMPs are absorbed by circulating engulfing cells; in inflammatory monocytes/macrophages, negatively-charged particles are taken up in an opsonin-independent
manner by the MARCO (macrophage receptor with collagenous structure) receptor (173). This process redirects them to the spleen, where they undergo apoptosis, preventing tissue damage caused by these cells at extrasplenic sites (173). In this study, the corneas of IMP-treated mice contained significantly fewer CD45\(^+\) cells in general; both lymphoid (CD4\(^+\), NKT, and DN) and myeloid cell types (PMN, monocytes, macrophages, and mDCs) were excluded from the cornea, with a concomitant preservation of corneal blink response. Initiation of treatment 3 dpi had no impact on viral loads, and actually improved mortality due to CNS involvement, indicating immune control of viral spread occurs within the first 72 hours after infection; after this, immune populations in the cornea exacerbate rather than ameliorate pathology (14).

Although previous work has focused on the role of IMPs in preventing inflammatory monocyte-driven pathology, in our model IMP therapy was also effective in limiting PMN, CD4\(^+\) and DN T cells from the cornea (173). Interestingly, these were the precise populations that expressed HVEM in the cornea 14 dpi. There is some evidence that IMPs can act directly on these cell types (233), although it is also possible that without inflammatory monocytes/macrophages in the cornea, T cell and neutrophil populations are not mobilized to the cornea. The characteristics and role of CD3\(^+\)/CD4\(^-\)/CD8\(^-\) DN cells during HSK requires a more thorough investigation, although it is clear that IMP therapy limits their presence in the cornea. While acutely-induced populations control viral spread, PMN and macrophages in the chronic phase orchestrate much of HSK-related tissue damage (14, 21, 96). Importantly, CD4\(^+\) T cells are considered the primary pathologic cell type in HSK, and recent findings suggest depletion of this cell type allows for reversal of nerve loss and corneal damage (101). Because IMP treatment significantly limited PMN and CD4\(^+\) T cells in the cornea, this therapy seems highly beneficial to the treatment of HSK inflammation.

In conclusion, we propose that HVEM on resident corneal monocytes/macrophages promotes early nerve damage, leading to increased infiltration of the cornea by a variety of
leukocytes and furthering loss of corneal sensitivity. Interruption of this process, either by HVEM ablation from CD45^+ cells or by treatment with IMPs, prevents the influx of circulating immune cells, maintaining corneal health. Given these promising results, we are hopeful that IMP therapy could be adapted to treat recurrent HSK in human disease.
CHAPTER 5
Discussion and Future Directions

Summary and Contribution

My thesis research focused on understanding why HVEM is required for HSV-1 pathogenesis after corneal inoculation. In contrast to other routes of infection, where HVEM is dispensable (48), ocular infection is attenuated in HVEM receptor KO mice even though an alternate entry receptor, nectin-1, is widely expressed in the murine cornea and was previously shown to be sufficient for corneal infection (49, 50, 58, 59). To learn why HVEM is required, I investigated both the entry and immunomodulatory functions of this receptor in the context of herpetic keratitis.

To assess the contribution entry makes, I utilized a well-characterized mutant HSV-1, HSV-1(17)gDΔ7-15, which is restricted to nectin-1 entry through targeted deletion of the HVEM-specific-binding region of gD (36, 38, 41, 42, 46, 187, 188). My studies confirmed HVEM is not the primary entry receptor in the cornea, as infection of C57BL/6 or BALB/c mouse strains with gDΔ7-15 produces equivalent titers, clinical disease, and inflammatory cytokine levels as the HVEM-entry-competent virus control, WT-FRT (51). Because loss of the gD-HVEM interaction does not recapitulate the attenuated phenotype observed in HVEM KO mice, I concluded that HVEM mediates its effects in the cornea independently of entry.

These findings led me to investigate the impact HVEM, a TNF-receptor superfamily member, may have on the immune response to HSV-1. I found that infected HVEM KO corneas have decreased levels of several inflammatory cytokines, including IL-6 and CXCL10, compared to infected C57BL/6 (WT) controls early after infection. In contrast to the vaginal model, these differences in cytokine production occurred independently of HVEM entry, as levels did not differ between HVEM entry-null or –competent viruses (51). These data suggested the entry-
independent contribution of HVEM to ocular HSV pathogenesis could arise from increasing inflammatory responses the virus. Consistent with this, I found that HVEM KO mice, in addition to having lower levels of viral replication and less severe clinical symptoms, also are protected from cornea-specific immunopathology. Compared to HVEM KOs, WT corneal stroma are more heavily infiltrated with leukocytes (51). A clinically relevant outcome, loss of sensitivity to mechanical pressure, is a surrogate measure of corneal nerve damage (103). I found that HVEM KO mice maintain corneal blink thresholds similar to pre-infection levels, while sensitivity in WT mice drops precipitously after infection.

Because HVEM is expressed so broadly, and has such a diverse range of functions in orchestrating immune responses (68), I sought to characterize which HVEM-expressing cells are responsible for promoting HSV-1 pathogenesis. Adoptive transfer experiments with WT and HVEM KO mice indicate susceptibility to disease occurs when HVEM is present on radiation-resistant cells, such as the corneal epithelium or long-lived immune cell residents of the cornea (51, 204). Although I anticipated finding HVEM highly expressed on the corneal epithelium and stroma (108), my analysis of corneas by IHC and flow cytometry indicated HVEM expression on epithelial or endothelial cells is limited in vivo. Flow cytometry of whole corneas from C57BL/6 mice indicates that the majority of HVEM is located on CD11b+/CD11c−/Ly6C+/Ly6G− monocyte-lineage cells in the acute phase. In the chronic phase (14 dpi), HVEM+ populations of PMN and, to a lesser degree, CD4+ T cells, dominate.

Conditional HVEM KOs, in which a Vav-Cre driver ablated HVEM expression from all hematopoietic lineages, provided direct evidence that HVEM on CD45+ cells increases viral replication in the acute phase and directs inflammatory infiltrates to the cornea in the chronic phase. Conditional KOs had lower viral loads in eye swabs early after infection, although replication in eye swabs or tissues did not differ significantly from controls at later time points. On day 14, the corneas of conditional KOs contained fewer leukocytic infiltrates, especially
myeloid subpopulations. Corneal nerve damage was limited in mice lacking HVEM on leukocytes as well. Together, these findings indicate that HVEM on leukocytes leads to increased corneal infiltration, and that this receptor and/or its downstream effects have physiologically relevant impact on corneal disease; namely, in worsening sensory loss at the corneal surface.

In order to counteract the pathologic outcomes of HVEM signaling, I tested whether treatment with immune-modifying nanoparticles (IMPs) could limit corneal leukocytic infiltration and nerve damage. IMPs reroute inflammatory cells to the spleen for degradation, preventing their infiltration of the cornea. IMPs are taken up by inflammatory monocytes, CD4+ T cells, PMN, and other circulating inflammatory cells and subsequently undergo apoptosis in the spleen (173, 233). IMP treatment was initiated 3 dpi to allow the innate immune response to contain viral replication, as previous studies indicate clodronate liposome-depletion of macrophages or antibody-depletion of PMN prior to or in the first days of infection result in highly increased viral replication and worsened systemic disease (94, 96, 99, 100). IMP treatment 3-7 dpi did not change viral titers. IMP treatment improved survival of BALB/c mice, which are highly susceptible to fatal HSV-1 encephalitis, a process shown to result from PMN- and macrophage-associated immunopathology in the brain (234). It also improved corneal blink responses and decreased a variety of myeloid and lymphoid populations in the corneas of C57BL/6 mice during the chronic inflammatory phase. Although IMP treatment does not target HVEM signaling specifically, it achieves many of the same outcomes observed in the HVEM KO, likely by functioning downstream of HVEM, preventing HVEM-mediated trafficking of leukocytes to the cornea and the subsequent damage they cause. I am hopeful that this breakthrough in the treatment of pathologic inflammation during with IMPs can be applied beyond the murine model to treat HSK in human patients.
Beyond entry: HVEM as a master regulator of corneal immune responses

The initial finding that HVEM worsens disease after HSV-1 inoculation at the corneal surface could be ascribed to a replication defect caused by deficient viral entry in HVEM KO mice (49, 50). In this model, HSV-1 replicates to lower levels in the HVEM KO cornea because it cannot enter cells as efficiently, or cannot infect as many cell types (34). Decreased replication in the cornea leads to decreased seeding of the TG, spread to the brain, neurologic symptoms, and reactivation, while decreased spread to the periorcular skin could reduce lesion scores in HVEM KO mice (49, 50).

However, in light of the data presented here, this hypothesis can no longer explain the phenotype of WT versus HVEM KO mice. Experiments with the HVEM entry-null virus gDΔ7-15 clearly demonstrate that HSV can establish infection, spread to peripheral tissues, cause development of neurologic symptoms and lesions, and induce inflammatory cytokine production with or without HVEM-mediated entry (51). These data directly challenge the model that diminished HSV entry, and subsequent replication and spread, account for the exacerbation in clinical disease observed when HVEM is present.

Several other pieces of evidence uncovered by my research also imply the immunomodulatory functions of HVEM take precedence over entry functions. Corneal blink response and the size of the corneal CD45+ population, both of which were impacted by HVEM expression, were more strongly correlated than corneal blink response and viral titer. In addition, the observed defect in viral replication in conditional KOs lacking HVEM on CD45+ cells was relatively small (only in eye swabs on day 1) compared to the dramatic absence of inflammatory cells in the corneas of these animals. Though titers in the tissues and eye swabs at later time points were indistinguishable from controls, the corneal blink threshold in conditional HVEM KOs was maintained, similar to that of HVEM KOs, while WT corneas rapidly lost all sensation to mechanical pressure. While possible, it seems unlikely that this small
change in titer in a single time point could be responsible for such a dramatic difference in phenotype. I find it more likely that this is another example in which the immunomodulatory effects of HVEM outweigh the entry function, and that HVEM promotes trafficking to the cornea and nerve damage mostly independently of its effect on viral replication, at least as it derives from entry. Whether HVEM promotes inflammation in the cornea regardless of the stimulus, or whether this process requires viral specific-effects, such as activation of corneal macrophages by type I interferons and interleukins secreted from epithelial cells (19, 91, 107, 189, 235), remains to be determined.

If the immunopathology caused by HVEM is completely separable from viral entry, how then can increased viral titers in WT eye swabs and tissues be explained? Previous studies indicate the efficiency of HSV replication is significantly enhanced by activation of NF-κB signaling, as this decreases apoptosis of infected cells (85, 87). HVEM activates NF-κB during infection (83). This can occur through gD binding, although other viral mechanisms to activate this pathway have also been identified (83, 84, 218). In the WT host infected with a gD mutant, the combination of other viral activation mechanisms plus natural HVEM ligands present in the host could potentially compensate for gD-HVEM in activation of NF-κB signaling. In contrast, global loss of HVEM (as in the HVEM KO) would abolish NF-κB activation through any of the HVEM signaling molecules, whether viral- or host-derived. Whether this hypothesis is correct requires further investigation of the effects of HVEM-mediated NF-κB activation on viral replication in vivo.

I have shown through IHC and flow cytometry that HVEM is mostly absent from the corneal epithelium early after infection. Therefore, the cell types most likely to be impacted by an HVEM-dependent increase in survival (via NF-κB activation) that could be responsible for higher viral titers in WT vs HVEM KO corneas are leukocytes or neurons. PMN, DCs, and monocytes/macrophages have all been shown to be susceptible to HSV-1 infection (117, 236,
Flow cytometry could be used to determine whether infected macrophages have higher levels of NF-κB signaling than uninfected macrophages, and whether this is HVEM-dependent (238). To my knowledge, no reports of nectin-1 expression on leukocytes exist. If HVEM does confer a survival benefit to infected leukocytes via NF-κB, I would have anticipated a replication defect after infection with Δ7-15 because leukocytes have not been shown to express another suitable gD receptor. Therefore, entry-independent effects of HVEM on leukocytes are more likely to influence viral replication indirectly, perhaps by improving survival of infected corneal epithelial cells or fibroblasts, although it is not clear how this might occur.

The HVEM status of the TG was not assessed by my experiments, but it has been reported the TG expresses HVEM, and that this expression increases after infection with HSV-1 (108). In the cornea, staining with the same rabbit anti-HVEM polyclonal antibody was nonspecific, indicating the expression of HVEM in the TG should be re-examined. However, if corneal nerves and the TG express HVEM (and nectin-1), this could represent a cell type susceptible to infection with or without HVEM that could receive a survival benefit that increases viral titers in the presence of intact HVEM signaling. Consistent with this hypothesis, the brains and TG of WT mice contain higher levels of virus compared to HVEM KOs, and WT mice have both a higher incidence and increased severity of neurologic symptoms (49, 50).

HSV-2, a virus closely related to HSV-1, does not require HVEM to cause severe disease during ocular infection, even though it has similar capacity to use HVEM for entry in vitro (36, 49). While initially puzzling, especially since the HVEM binding domains of HSV-1 and HSV-2 differ by only one amino acid (41, 42), this finding makes more sense in light of my data showing HVEM pathology is independent of gD binding. Compared to the highly virulent strain HSV-1(McKrae), corneal inoculation with a clinical HSV-2 isolate produces higher TG titers and inflammatory cytokine/chemokine levels in the CNS 7 dpi, correlating with increased corneal lymphangiogenesis, despite the viruses reaching equal titer in the cornea early in infection.
Similar results were obtained when HSV-1 and HSV-2 were compared in a vaginal model of infection (239). It is difficult to know whether a comparison of HSV-1 and HSV-2 illustrates general differences between the serotypes, or merely reflects strain-specific differences. However, if the former is true, these data indicate HSV-2 is inherently more pathogenic than HSV-1 at mucosal sites including the eye, perhaps so much so that the pro-replication and pro-inflammatory effects of HVEM are insignificant during HSV-2 infection.

I have shown through a variety of experiments that HVEM has a prominent, entry-independent role in orchestrating corneal inflammation during HSV infection, the mechanics of which are discussed in the next section. This hypothesis is not surprising based on the literature, as HVEM has been shown to influence immune responses to a variety of pathogens, including other herpesviruses (79), other viruses (159), bacteria (28-30), and helminths (182). Furthermore, because HVEM-mediated pathology is, for the most part, divorced from viral entry and replication, it is also possible that HVEM is a regulator of ocular inflammation in general. In line with this hypothesis, HVEM was recently shown to exacerbate experimental autoimmune uveitis (EAU), a murine model of autoimmune conditions affecting the posterior eye such as Behçet disease and sarcoidosis (169). My findings suggest HVEM signaling may have broad implications for ophthalmic inflammatory conditions in general.

**HVEM on resident corneal macrophages: a double–edged sword?**

Adoptive transfer experiments indicate HVEM on a radiation resistant cell type, like the corneal epithelium, mediates disease (51). However, I was unable to detect substantial quantities of HVEM on the corneal epithelium (or stroma) in vivo either by IHC or flow cytometry, making it unlikely these cell types are the source of the HVEM-mediated cytokine production, clinical disease, or infiltration. Stromal macrophages (and DCs) also incompletely turn over after irradiation (204). Early after infection, I found that the majority of HVEM was expressed on
CD11b⁺/Ly6C⁺ monocyte-lineage cells; therefore, stromal macrophages could be the radiation-resistant HVEM⁺ cell type identified as mediating disease in adoptive transfer experiments (51). Although other HVEM⁺ lineages could also contribute to pathogenesis, albeit in a subtler fashion, monocytes/macrophages are particularly intriguing to me in light of these findings.

Corneal macrophages are the predominant form of immune cell in the cornea immediately after infection, and depletion of these early responding cells via clodronate liposomes increases viral replication (96, 99, 100, 109) while decreasing inflammatory lymphangiogenesis (240). Later depletion of macrophages has little effect on HSK severity, although PMN depletion in the chronic phase reduces the severity of corneal lesions (Suvas S et al., IOVS 2011;52: ARVO E-Abstract 2910). If HVEM on resident corneal macrophages mediates pathology, these findings suggest HVEM-associated alterations in the immune response early after infection are sufficient to initiate a chain of events leading to chronic inflammation. I found that the presence of HVEM is associated with expansion of the corneal leukocyte populations, especially PMN, which was also associated with increased corneal nerve damage. This is consistent with reports that while CD4⁺ T cells are required for disease (241), PMN are the main mediators of corneal lesion development and opacification (21, 242). It seems likely the same inflammatory factors promoting HSK pathology could also cause nerve damage (101). Experiments with conditional HVEM KOs lacking HVEM on CD45⁺ cells indicate that HVEM is not required for the early appearance of CD11b⁺ in the cornea, but is necessary for the recruitment of late-phase infiltrates such as PMN. I propose that HVEM on corneal resident CD11b⁺ populations causes nerve damage and recruits chronic-phase infiltrative cells, like PMN and CD4⁺, worsening HSK (Fig. 15).

Corneal macrophages secrete a number of inflammatory cytokines and angiogenic factors (110), and I showed IL-6 and CXCL10 are upregulated in the acute phase (5 dpi) in an HVEM-dependent manner. IL-6 and CXCL10 are neutrophil and T cell chemoattractants,
respectively, known to promote ocular HSV-1 pathogenesis (110, 127, 193, 196, 243), and increased expression of these chemotactic factors may lead to heavier infiltration by myeloid and lymphoid cells in WT versus HVEM KO corneas (51). IL-6 is also implicated in neovascularization and lymphangiogenesis during HSK through paracrine induction of VEGF (92, 93, 111), suggesting increased vessel development in WT corneas due to higher levels of IL-6 could account for larger populations of infiltrating cells. Monocytes and macrophages produce both IL-6 and CXCL10, which are under transcriptional control of NF-κB and are functionally induced by stimulation of this transcription factor (244, 245). In my model, I propose that HVEM-mediated NF-κB activation in corneal macrophages causes increased production of inflammatory cytokines, facilitating PMN and T cell trafficking to the cornea directly by chemotaxis or indirectly by facilitating new vessel growth. Examining differences in vessel development between WT and HVEM KO corneas will be an important first step in determining the mechanism of HVEM-dependent leukocyte recruitment.

Increased secretion of these cytokines from HVEM⁺ macrophages could also damage nerves, as resident macrophages are closely associated with nerve endings in the corneal stroma (226). Nerve retraction after HSV-1 infection is swift and pervasive, suggesting it is caused by the inflammatory microenvironment of the infected cornea rather than by direct infection of neurons (101, 104). Consistent with this, I observed loss of corneal sensitivity in WT mice early after infection, while HVEM KO mice (and Vav⁺ HVEM<sup>fl/fl</sup> conditional KOs) maintained blink thresholds. Release of neuropeptides like CGRP and substance P from injured neurons has been shown increase cytokine release, PMN infiltration, and HSK severity during ocular herpetic infection (227-229). Loss of the blink response in mice due to nerve retraction has also been shown to contribute to pathology via ocular desiccation and inflammation (101). I found that corneal sensitivity loss in WT mice compared to HVEM KOs preceded the classic second-wave of pathogenic T cells and PMN; therefore, nerve damage, rather than being a byproduct
chronic phase immunopathology, may initiate leukocyte trafficking to the cornea. IMP treatment decreased most types of immune infiltrates in the cornea by increasing their sequestration in the spleen and diminished corneal sensitivity loss, suggesting nerve damage and leukocytic infiltration feed off of each other in a vicious cycle during HSK.

Radiation-resistant HVEM$^+$ corneal macrophages could also influence infiltrating CD4$^+$ T cells. Consistent with a previous report that HVEM is highly expressed by Tregs (142), I found that 14 dpi, a small but real population of the HVEM$^+$ population were CD4$^+$, although I did not assess FoxP3$^+$ expression in these cells. The previous study found a protective role for HVEM on Tregs in draining lymph nodes, a difference from our model that could be explained by differences in viral inoculum/strain or mouse strain. HVEM, which is broadly expressed in the hematopoietic compartment, likely has contradictory roles depending on the cellular context. Ablation of HVEM from Tregs specifically could provide insights on this discrepancy.

In any case, CD4$^+$ T cells are not likely to be the HVEM$^+$ cell responsible for pathology, at least in our adoptive transfer model, because they turn over with radiation and reconstitution. Instead, interactions between HVEM (on other cell types, such as corneal macrophages) and HVEM ligands like LIGHT, BTLA, or CD160 on CD4$^+$ T cells could contribute to the development of HSK. I found that chimeras lacking HVEM from radiation-sensitive cells developed more severe disease than HVEM KO controls. In this experiment, absence of HVEM on CD4$^+$ cells may make BTLA and LIGHT available to interact with HVEM on other cell types, such as long-lived, resident macrophages, activating NF-κB signaling and inflammatory cytokine release during HSK.

However, I find it most likely that HVEM on corneal macrophages influences disease through recruitment of PMN to the cornea, either through nerve damage and subsequent recruitment or by influencing trafficking directly. In IMP-treated mice, the proportion of HVEM$^+$ cells in the cornea did not change, suggesting they were residents or infiltrated the cornea prior
to the start of treatment. Despite the presence of HVEM+ cells in the cornea, IMP treatment reduced sensitivity loss and recruitment of PMN and CD4+ T cells in the chronic phase, along with other leukocytes, and significantly increased PMN in the spleen. This suggests IMPs interrupted the normal development of HSK by sequestering PMN in the spleen, which may have also prevented the infiltration of other cell types, like CD4+ T cells, which were not increased in the spleen but were decreased from the cornea. Previous studies with IMP therapy indicate the majority of their effect is through uptake by the MARCO receptor on inflammatory monocytes, leading to apoptosis in the spleen (173, 233). My research demonstrates that IMPs may be useful for neutrophil-mediated diseases like HSK, as they are capable of rerouting PMN to the spleen away from sites where they cause tissue damage, although more research is required to understand how PMN take up and are affected by nanoparticles.

Conclusions and future directions

Based on previous findings and our most recent data, I propose HVEM on corneal resident macrophages is critical for HSK development, either by interacting with HVEM ligands on other cells, or through increased cytokine production, perhaps via NF-κB activation (Fig. 2). I hypothesize that HVEM on these cells leads to increased corneal nerve damage, immune cell recruitment, and overall severity of disease. Because resident macrophages and nerves are physically associated in the peripheral stroma (226), it is possible that HVEM-mediated secretion of damaging cytokines from macrophages hastens corneal nerve damage, decreasing blinking and desiccating the cornea (104). HVEM on other immune cell types, including CD4+ T cells and PMN, is not likely to be necessary for HSK development, in light of adoptive transfer experiments, but may still be contributory. Resolving pathogenic versus protective functions of HVEM, i.e. on corneal resident macrophages compared to Tregs, is an important next step for the field, as this information is critical for targeted therapy design.
There is some evidence HVEM contributes to latent infections as well (50, 156). Whether this is physiologically significant and how this effect comes about are intriguing lines of investigation for the future. Murine models of HSK are generally based on primary infection, because, unlike humans, mice do not undergo efficient spontaneous reactivations (149). How the immune response during HSK differs between a primary infection and a reactivation from latency represents a significant gap in our knowledge. Investigators recently described an efficient model of latency and reactivation using the immunocompromised Rag-2 KO mouse strain, which lack mature B and T cells (160, 246). Our laboratory is currently crossing Rag-2 KOs to HVEM KOs to answer two questions: 1) what impact does HVEM have on innate immunity in the acute phase, and 2) how does the absence of HVEM impact reactivation from or response to latency?

Another area in need of further study is the expression and role of the host HVEM ligands during HSV-mediated pathogenesis. Studies with the HVEM entry-null Δ7-15 mutant indicate that the gD-HVEM interaction is not required for the inflammation HVEM causes; therefore, attention must be turned to the HVEM ligands BTLA, CD160, LIGHT, and LTα. To my knowledge, only BTLA expression has ever been examined in the cornea (141); expression of these molecules is under investigation by members of the Longnecker laboratory. In addition, KO mice for BTLA, CD160, BTLA, and LIGHT have been generated (126, 247, 248), and are currently being studied in the hopes one (or several) will phenocopy the HVEM KO, implying that ligand is involved in pathology of the cornea. If none of the single ligand KOs exhibits an attenuated phenotype similar to that of the HVEM KO, it may be necessary to produce double or even triple KOs, given that HVEM ligands can interact in heteromeric complexes. It is also possible an as-yet unidentified HVEM ligand could be promoting pathogenesis; if discovered, this could open up a new chapter in HVEM signaling research. Beyond informing a mechanistic understanding of HVEM signaling during ocular herpetic infection, this information will also
facilitate the testing of antibody or small molecule therapies targeted to pathologic HVEM signaling in the cornea.

Both HSK and HVEM signaling are complex processes involving nearly every type of leukocyte; consequently, untangling the role HVEM plays during development of HSK has been and will continue to be a challenge. Advances made in the molecular signaling mechanisms of HVEM and its ligands are extremely useful going forward, but the complications of bidirectional signaling interactions, with complimentary or contradictory messages being delivered at the same time, remain. Unfortunately, this complexity means that findings about HVEM signaling relevant to one disease model cannot necessarily be applied to the next. HVEM signaling warrants further investigation specifically in the context of immunomodulation in the eye, as my research clearly demonstrates a pathogenic and inflammatory role during ocular HSV infection. With luck, these discoveries will be translatable to new therapies for patients with this blinding condition.
Fig 15. Proposed mechanism of HVEM pathogenesis during ocular HSV-1. (A) Early after infection, the majority of corneal HVEM is localized to CD11b⁺ Ly6C⁺ monocytic lineages rather than epithelial or endothelial cells. I propose these HVEM⁺ cells are corneal resident macrophages, as adoptive transfer experiments indicate radiation-resistant cells are sufficient for HVEM-mediated pathogenesis, and macrophages in the cornea incompletely turn over after irradiation. HSV-1 infects the corneal epithelium, causing secretion of type I interferons and other factors that activate corneal resident macrophages, which could induce HVEM expression on these cells early after infection. (B) Because the gD-HVEM interaction is dispensable for pathogenesis, I hypothesize that HVEM on corneal macrophages then interacts with a host ligand, BTLA, CD160, or LIGHT, on an as-yet unidentified cell type. Experiments with WT and HVEM KO mice have shown that the result of this interaction is HVEM-dependent induction of inflammatory cytokines, loss of corneal sensitivity, and immune cell recruitment. It is not yet clear whether this stems from changes in the HVEM⁺ cells directly, such as through NF-κB activation, or through signaling on the HVEM ligand-bearing cell. Whether nerve damage
causes leukocytic recruitment, or whether recruited leukocytes damage corneal nerves, remains a chicken-and-egg problem. Macrophages and corneal nerves are found in close proximity in the peripheral stroma; it is possible increased cytokine release from macrophages damages nerves initially. Neuropeptide release induces the production of inflammatory cytokines from neighboring cells, increases recruitment of PMN, and worsens HSK pathogenesis, although it is not known whether HVEM-mediated inflammation affects this secretion. Nerve damage also desiccates the eye through loss of the blink response, worsening inflammation; both mechanisms could be at work. Alternatively, recruited leukocytes rather than resident corneal macrophages could cause nerve retraction and corneal sensitivity loss by secretion of inflammatory products. I find it likely that both processes influence each other, synergizing to create a cycle of chronic inflammation and corneal nerve damage.
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Figure A1. Weight loss by day after infection with Δ7-15 or WT-FRT. (A) C57BL/6 or (B) BALB/c adult male mice (9-15 weeks) were infected with 2.0x10^6 PFU/5 µl per eye after corneal scarification with HSV-1(17), the HVEM-entry mutant Δ7-15, or the repaired control WT-FRT. Mice were weighed daily, percentage weight loss of day 0 weights is presented as means ± SD (n = 5 per virus).
Figure A2. Clinical symptoms in C57BL/6 mice after inoculation with Δ7-15, WT-FRT, or the parental HSV-1(17). C57BL/6 or BABL/c adult male mice (9-15 weeks) were infected with 2.0x10^6 PFU/5 µl per eye after corneal scarification with HSV-1(17), the HVEM-entry mutant Δ7-15, or the repaired control WT-FRT. Mice were scored daily (using a 0-to-5 scale, with 5 representing the greatest severity) for the development of epithelial lesions and neurologic symptoms (n = 4 to 6 per group; data representing the results of one representative experiment are shown). (A) Percentages of mice from each group with no lesions on the indicated day post infection (dpi). (B) Average maximum epithelial lesion scores (means ± standard errors of the means [SEM]). max., maximum. (C) Percentages of mice from each group with no neurologic (neuro.) symptoms on the indicated dpi. (D) Average maximum neurologic scores (means
SEM). (E) Survival for each group. For each mouse strain, no significant differences were detected between the viruses ($P > 0.05$ [two-way ANOVA for panels A and C or two-tailed $t$ test for panels B and D with Holm-Sidak's multiple-comparison test; log-rank test for panel E]).
Figure A3. Viral loads in C57BL/6 mice after inoculation with Δ7-15, WT-FRT, or the parental HSV-1(17). C57BL/6 or BALB/c adult male mice (9-15 weeks) were infected with 2.0x10^6 PFU/5 µl per eye after corneal scarification with HSV-1(17), the HVEM-entry mutant Δ7-15, or the repaired control WT-FRT. (A) C57BL/6 or (B) BALB/c eye swabs collected 1, 3, and 5 dpi (means ± SEM). (C to E) Titers determined at 5 dpi using samples from the periocular skin (POS) (C), trigeminal ganglia (TG) (D), and brain (E) (means ± SEM). Data representing the results of two independent experiments are shown (total n per group = 10). For each mouse strain, no significant differences in titers were detected between the viruses in eye swabs or tissues (two-tailed t test with Holm-Sidak’s multiple-comparison test, P > 0.05).
Fig A4. Detection of HSV by immunohistochemistry (IHC) in WT and HVEM KO corneas over time. Representative IHC images of whole WT or HVEM KO eyes 1 day after infection at the corneal surface with $2.0 \times 10^6$ PFU/µl per eye of HSV-1(17) (original magnification, 400x). Paraffin-embedded eyes were serially sectioned and stained for HSV-1 1, 5, and 14 dpi. By 5 dpi, HSV was occasionally detected in deeper corneal layers of WT, but not HVEM KO, mice. By 14 dpi, no HSV was detectable in the corneas of either genotype.
Fig A5. Relationship between blink threshold and corneal macrophage population 14 dpi.

Corneal touch threshold was determined with a Leneau Cochet-Bonnet aesthesiometer. The relationship between day 14 corneal touch threshold and the absolute number of corneal macrophages (CD45+/C11b+/CD11c\textsuperscript{low}/Ly6C\textsuperscript{high}) determined by flow cytometry ($n = 32, \geq 3$ individual experiments, linear regression for goodness of fit).
Fig A6. Viral titers in eye swabs from C57BL/6 and HVEM KO mice infected with HSV-1(17). C57BL/6 (WT) or HVEM KO adult male mice were infected with $2.0 \times 10^6$ PFU/5 µl per eye after corneal scarification with HSV-1(17) and eye swabs were collected on days 1, 3, and 5 (means ± SEM, $n = 10$, two tailed $t$ test with Holm-Sidak’s correction for multiple comparisons, $P = 0.004$).
Fig A7. Gating strategy for lymphoid cell populations. Whole corneas were assessed by flow cytometry. Cell populations were gated according to FSC-SSC, then restricted to singlets, live cells, CD45+ cells, CD3+ cells, and then were segregated into CD4 versus CD8 populations or NK1.1+ or NK1.1− populations. Cells negative for CD4, CD8, and NK1.1 were considered DN cells.
Fig A8. Gating strategy for myeloid cell populations. Whole corneas were assessed by flow cytometry. Cell populations were gated according to FSC-SSC, then restricted to singlets, live cells, and CD11b\(^+\) cells. Neutrophils were classified as the Ly6C\(^+\) Ly6\(^+\) population. The rest of cells were then separated into monocytes/macrophages, myeloid DCs, inflammatory monocytes/macrophages, and non-inflammatory monocytes/macrophages populations as shown above.
Fig A9. Percentage of live, CD45⁺ HVEM⁺ cells that are CD11b⁺/CD3⁻ versus CD11b⁻/CD3⁺ in WT corneas 14 dpi. WT adult male mice were infected with 2.0x10⁶ PFU/5 µl per eye after corneal scarification with HSV-1(17), and whole corneas were assessed by flow cytometry 14 dpi. The proportion of live, CD45⁺ HVEM⁺ cells were separated into myeloid- and lymphoid-lineages by CD3 and CD11b staining.
Fig A10. Conditional KO's lacking HVEM from CD45+ lineages have maintained corneal blink thresholds and lower viral loads in the eyes 1 dpi. HVEM on peripheral blood leukocytes in (A) HVEM\textsuperscript{fl/fl} controls or (B) Vav\textsuperscript{+} HVEM\textsuperscript{fl/fl} conditional KO's was evaluated by flow cytometry to verify successful ablation of HVEM from CD45+ lineages. (C) Corneal touch threshold was determined with a Leneau Cochet-Bonnet aesthesiometer every other day beginning prior to infection (day 0) in WT, HVEM KO, or Vav\textsuperscript{+} HVEM\textsuperscript{fl/fl} mice inoculated at the corneal surface with HSV-1\textsuperscript{(17)}. Lack of a blink reflex at 0.5 cm was recorded as 0 cm (n = 8-10 mice per group, 18-20 individual corneas measured, 2 replicates). (D) Viral loads in the POS, TG, and brain of HVEM\textsuperscript{fl/fl} controls or Vav\textsuperscript{+} HVEM\textsuperscript{fl/fl} conditional KO's 5 dpi (means ± SEM, n = 5-10, 2 replicates, two tailed t test with Holm-Sidak's correction for multiple comparisons, P > 0.05). (E) Average maximum neurologic symptom score or (F) lesion score reached on any day (mean ± SEM, n = 12, 2 replicates, two tailed t test, P > 0.05 for both).
Fig A11. Treatment with IMPs significantly increases the number of PMN, tended to increase other leukocytic populations, in the spleens of C57BL/6 mice. WT mice were infected with 2.0x10^6 PFU/5 μl per eye after corneal scarification with HSV-1(17) and treated with either IMPs or vehicle (PBS) intravenously 3-7 dpi. Less than 24 hours after the last dose (day 8), the spleens of a subset of the group were harvested and prepared for flow. (A and B) Absolute number of the indicated cell types in WT treated or mock-treated mice (n = 4, means ± SEMs, two tailed t test with Holm-Sidak’s correction for multiple comparisons). *, P ≤ 0.05.
Fig A12. HSV-1 infection of K14-Cre\(^+\) HVEM\(^{fl/fl}\) mice produces similar corneal HVEM induction as that observed in WT. HVEM\(^{fl/fl}\) mice were crossed with B6.Tg(KRT14-cre)1Amc/J, which express Cre recombinase in epithelial cells of the skin and cornea (personal communication, Dr. Robert Lavker) to generate conditional KOs of HVEM in the corneal epithelium. Adult mice were infected with 2.0 x 10\(^6\) PFU/5 µl per eye of HSV-1(17) after corneal scarification, and HVEM expression on leukocytes (CD45\(^+\)) or epithelial cells (EpCAM\(^+\)) cells was assessed in (A) control or (B) conditional KO mice 3 dpi (representative flow plots shown). Most HVEM is found on CD45\(^+\) cells in both genotypes, and the HVEM\(^+\) CD45\(^-\) populations in control and conditional KOs are similar, suggesting HVEM is not ablated in the K14-Cre\(^+\)
HVEM$^{fl/fl}$ mice. (C) Percentage of HVEM$^+$ cells in mock-infected or infected WT, HVEM KO, K14-Cre$^+$ HVEM$^{fl/fl}$ conditional KO corneas 3dpi. HSV-1 infection of the conditional KO induces HVEM expression similar to WT, indicating that either the ablation of HVEM on the corneal epithelium is unsuccessful, or, more likely, there is little HVEM expression on corneal epithelial cells in vivo, making ablation from these cell types moot. Consistent with this last hypothesis, little HVEM was detected by IHC or flow in corneal epithelial cell types (Fig. 10), and infection of K14-Cre$^+$ HVEM$^{fl/fl}$ mice was similar to HVEM$^{fl/fl}$ controls in terms of clinical symptoms.
Fig A13. Treatment of WT mice with 8F4, a BTLA agonizing anti-body, or the control MOPC-21 mouse IgG1 in WT and HVEM KO mice. HVEM expression worsens disease after HSV-1 infection. This effect could arise via HVEM activation of BTLA signaling, though this is typically associated with repression of T cells, and seems less likely to worsen corneal immunopathology. Nevertheless, to test whether activation of BTLA signaling by an agonizing antibody, 8F4, could restore disease in HVEM KO mice, I infected mice with $2.0 \times 10^6$ PFU/5 µl per eye of HSV-1(17) after corneal scarification. On the day of infection and 2 dpi, mice were given 200 µg/mouse via i.p. injection of the activating α-BTLA antibody or control antibody. (A) Viral titers in eye swabs collected 1, 3, or 5 dpi ($n = 10$, 2 replicates, means ± SEM) or (B) in tissues collected 6 dpi ($n = 5$, 1 replicate, means ± SEM) in HVEM KO mice. (C) Mean maximum lesion score on any day in HVEM KO mice ($n = 4\text{--}5$, 1 replicate, $P > 0.05$, two tailed $t$ test). (D) Mean maximum lesion score on any day in WT mice ($n = 4\text{--}5$, 1 replicate, $P > 0.05$, two tailed $t$ test). Because BTLA agonism seemed to have moderate (but statistically insignificant) protective effects in the HVEM KO, consistent with its established function as a co-repressor, I next tested the efficacy of this
antibody to reduce disease in the more susceptible WT strain. (E) Titers in eye swabs collected 1, 3, or 5 dpi or (F) tissues collected 6 dpi in WT mice. \( n = 4-5, 1 \) replicate, means ± SEM). No significant differences occurred in viral replication in WT mice (two tailed \( t \) test with correction for multiple comparisons, \( P > 0.05 \)). These findings suggest that in the absence of HVEM, BTLA agonism, if that is truly the effect of the 8F4 antibody, may provide some slight protection from disease, perhaps by reducing T cell activation in the cornea. However, this effect, if real, is hard to measure given the overall mild course of pathogenesis in HVEM KO mice. HVEM signaling could still worsen disease through its own downstream effects, such as NF-κB activation, by binding to BTLA. In the WT, solely activating BTLA through antibody treatment could reduce T cell activation without interfering with pathogenic HVEM signaling, causing it to have little effect on disease. If a BTLA-HVEM interaction is important for HVEM-mediated pathogenesis by activation of intracellular HVEM signaling, then disease in a BTLA KO mouse would be attenuated, while treatment with a BTLA agonist, as reported here, would not.
Fig A14. KOS0152, a Cre-expressing HSV-1 strain, has a replication defect in the tissues of WT and HVEM\textsuperscript{fl/fl} mice. In order to test whether HVEM expression is important on infected or uninfected cells, I used a Cre-expressing HSV-1 strain, KOS0152, to knock out HVEM in infected cells specifically. (A-C) Viral loads 3 and 5 dpi in the POS, TGs, and brains of HVEM\textsuperscript{fl/fl} mice infected with KOS0152, a WT control KOS, or KOSeGC, which expresses a GFP-Cre fusion protein (\(n = 9\) mice for day 5, 3-5 mice for day 3, two replicates, means ± SEM, two tailed \(t\) test with correction for multiple comparisons). (D) Viral loads 5 dpi in WT mice (\(n = 9\) mice, 2 replicates, means ± SEM, two tailed \(t\) test with correction for multiple comparisons). Because KOS0152 replicated to lower levels in the WT control, I cannot draw any conclusions from the replication defect observed in HVEM\textsuperscript{fl/fl} mice. I also compared eye swabs, but found no significant differences between KOS and KOS0152 for either WT or HVEM\textsuperscript{fl/fl} mice.