Ocular herpes simplex virus 1 (HSV-1) infection leads to a potentially blinding immunoinflammatory syndrome, herpes stromal keratitis (HSK). Herpesvirus entry mediator (HVEM), a widely expressed tumor necrosis factor (TNF) receptor superfamily member with diverse roles in immune signaling, facilitates viral entry through interactions with viral glycoprotein D (gD) and is important for HSV-1 pathogenesis. We subjected mice to corneal infection with an HSV-1 mutant in which HVEM-mediated entry was specifically abolished and found that the HVEM-entry mutant produced clinical disease comparable to that produced by the control virus. HVEM-mediated induction of corneal cytokines, which correlated with an HVEM-dependent increase in levels of 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 a wild-type (WT) or HVEM knockout (KO) strain, 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.

**ABSTRACT**

Ocular herpes simplex virus 1 (HSV-1) infection leads to a potentially blinding immunoinflammatory syndrome, herpes stromal keratitis (HSK). Herpesvirus entry mediator (HVEM), a widely expressed tumor necrosis factor (TNF) receptor superfamily member with diverse roles in immune signaling, facilitates viral entry through interactions with viral glycoprotein D (gD) and is important for HSV-1 pathogenesis. We subjected mice to corneal infection with an HSV-1 mutant in which HVEM-mediated entry was specifically abolished and found that the HVEM-entry mutant produced clinical disease comparable to that produced by the control virus. HVEM-mediated induction of corneal cytokines, which correlated with an HVEM-dependent increase in levels of 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 a wild-type (WT) or HVEM knockout (KO) strain, 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 1 (HSV-1) infection can disrupt this immune privilege, provoking a host response that ultimately brings about the majority of the damage seen with the immunoinflammatory 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, is a critical component contributing 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.
frquently lead to vision loss, necessitating corneal transplantation (15, 16).

HSV has a complex entry mechanism requiring the expression of multiple envelope glycoproteins (17). Glycoprotein D (gD) interacts with several cellular receptors to facilitate entry. The most biologically relevant in animal models are herpesvirus entry mediator (HVEM) and nectin-1 (18–20). In vivo studies using HVEM (Tnfrsf14−/−) and/or nectin-1 (Pvrll−/−) receptor knockout (KO) mice have revealed 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 (18, 21). 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 (22, 23). This unique dependence of HSV-1 on 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 (24, 25).

HVEM, a member of the tumor necrosis factor (TNF) receptor superfamily, is a bidirectional receptor with multiple immunomodulatory functions depending on cell type and ligand (26–28). HVEM interaction with B and T lymphocyte attenuator (BTLA) or CD160 is typically coinhibitory, 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 LTA (lymphotoxin α) is proinflammatory (29–33). 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 in trans (29–33). HVEM influences immune responses to a variety of pathogens, including viral, bacterial, and helminthic agents, in the vagina, intestine, lung, and other tissues (34–36). In some instances, the pathogen utilizes HVEM to dampen innate responses, while in others HVEM functions to control infection and limit disease progression (26, 34–37). HVEM has also been implicated in a number of autoimmune and inflammatory disorders, including bacterial colitis, atopic dermatitis, and acute graft-versus-host disease (26, 27, 35, 38).

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 (39–44). We tested whether HVEM-mediated entry promotes ocular HSV-1 pathogenesis by infecting mice with HSV-1/gDΔ7-15 (here referred to as the Δ7-15 mutant), a mutant virus with a targeted deletion that renders HVEM entry nonfunctional but preserves entry via nectin-1. Ocular infection by the Δ7-15 mutant was not attenuated compared to infection by a 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 wild-type (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 results early and late after infection, implying that 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 (costimulatory and coinhibitory), the contribution HVEM may make to ocular pathogenesis is not obvious (24, 26, 29, 45, 46). We developed hematopoietic chimeric mice in which HVEM expression was restricted to or ablated from radiation-sensitive bone marrow (BM)-derived immune cells and found that HVEM on radiation-resistant cell types was sufficient to confer WT-like susceptibility to HSV-1 after corneal inoculation. We propose that HVEM on radiation-resistant 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 (22, 23), but whether this is attributable to its entry effects, immunomodulatory effects, or both is not known (23). 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. 1A). Crystallographic and functional assays have shown that deletion of amino acids 7 to 15 of the gD N terminus selectively abolishes HVEM entry without functionally disturbing entry via nectin-1 (37, 47–
As shown in Fig. 1A, the Δ7-15 viruses in HSV-1(F) and HSV-1(17) backgrounds were engineered by FLP-mediated recombination as described previously (37, 49). The HSV-1/FRT viruses (WT-FRT) contain the WT protein coding sequence for gD flanked by FRT sites (Fig. 1A) and served as controls.

All viruses were confirmed by sequencing and phenotypically verified by plaque assay on Vero cells, B78-A10 cells (B78H1 cells stably expressing HVEM), and B78-C10 cells (B78H1 cells stably expressing nectin-1). As expected, the titers of the WT-FRT viruses were similar on all 3 cell types, indicating that both strains of WT-FRT are capable of infecting cells expressing either HVEM or nectin-1 (results are shown for HSV-1(17) viruses in Fig. 1B). Titers of the Δ7-15 viruses were similar to the titers of the WT-FRT viruses on Vero cells and B78-C10 cells, but no plaques were observed on B78-A10 cells, indicating that while both the Δ7-15 virus strains can infect cells via nectin-1, they are unable to infect cells that express only HVEM (Fig. 1B). Additionally, 3 nectin-1 KO mice were challenged via corneal scarification with the Δ7-15 viruses as previously described (22, 23), and eye swabs were collected on 1 and 3 days postinfection (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 (data not shown).

We monitored 10- to 12-week-old male C57BL/6 wild-type (WT) mice infected with the WT-FRT strain or Δ7-15 mutant from both strain 17 backgrounds (Fig. 2) and strain F (data not shown) after corneal scarification as previously described (22, 23). We also inoculated 10- to 12-week-old male BALB/c mice, which are exceptionally sensitive to ocular HSV-1, with the Δ7-15 mutant or WT-FRT (strain 17 background) to ensure that the resistance of the C57BL/6 strain to ocular HSV-1 infection did not mask subtle differences between the viruses (Fig. 2). 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 are shown for HSV-1(17) in Fig. 2A and B]. Both Δ7-15 and WT-FRT viruses produced lesions, and by 5 to 7 dpi all mice were symptomatic.

Neurologic symptoms began around 5 dpi, and by 7 dpi, 40% to 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. 2C). The severities 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. 2D). Mice infected with either Δ7-15 strain lost a percentage of day 0 body weight similar to that seen with mice infected with the WT-FRT strain (data not shown). All C57BL/6 mice survived to 28 dpi, when the experiment ended (Fig. 2E). This high percentage of survival is consistent with previously reported data (22). The majority of BALB/c mice succumbed to infection by day 30 regardless of the HVEM entry capacity of the virus (Fig. 2E).

Unlike the extremely mild disease observed in HVEM KO mice (22, 23), the Δ7-15 virus produced disease comparable to that seen with the WT-FRT virus (Fig. 2). To ensure that our findings were not due to attenuation of our FLP recombinase-generated mutants, we also compared the results of infection of C57BL/6 and BALB/c mice by the strain 17-derived Δ7-15 mutant and the WT-FRT virus to those seen with 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 that attenuation did not occur during the recombination process (data not shown). 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 tissue viral 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 in-
Next, we tested whether spread to relevant organs was reduced 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 × 10^6 PFU/5 μl per eye of the Δ7-15 mutant or the 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 to E) Titters determined at 5 dpi using samples from the periorcular 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). (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 experimental study results from our laboratory showed that TG from WT mice reactivated at 4 times the rate of those from HVEM KO mice (22). After 30 days, rates of reactivation after corneal inoculation of C57BL/6 mice with WT-FRT or Δ7-15 virus from either strain did not differ significantly, as measured by the ex vivo reactivation assay, suggesting that HVEM-mediated entry was not required to successfully seed the TG (Fig. 3F). 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 or 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 (36). Therefore, disruption of HVEM entry by mutation of gD does not phenocopy the results observed in infection of HVEM KO mice with a wild-type virus.

**FIG 3** 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 × 10^6 PFU/5 μl per eye of the Δ7-15 mutant or the 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 to E) Titters determined at 5 dpi using samples from the periorcular 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). (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).
at over 9- and 125-fold the levels in HVEM KO corneas, respectively. Levels of 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 to 15 in HSV-2(333) led to decreased induction of IL-6, CXCL9, and CXCL10 in vaginal washes early after infection (37). To test whether the changes that 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) virus or the WT-FRT(17) control virus (Fig. 4C). Consistent with the disease and viral load data, the changes in the levels of 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.

Levels of 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 (IHC) analysis of corneas taken from several time points after HSV-1 infection. Whole eyes from mock-infected or HSV-1(17) virus-infected HVEM KO or WT animals were collected after corneal inoculation during early and acute infection (1 or 5 dpi) or at the height of HSK (14 dpi). Previously, we found that, on average, WT corneas have more than twice the number of infectious foci that HVEM KO corneas have and that foci in WT corneas also tended to be larger (22). Consistent with these findings, Fig. 5A qualitatively demonstrates that HVEM KO corneas had fewer and smaller infectious foci in the corneal epithelium than WT corneas immediately after infection (1 dpi). By 5 dpi, HSV antigen was detectable only rarely in the corneas of both genotypes (data not shown), and no virus was found in the cornea by 14 dpi (Fig. 5B, 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 with anti-CD3 to identify T cells. The stroma of HVEM KO corneas qualitatively displayed markedly less Gr-1+ or CD3+ staining 1 dpi than the stroma of WT corneas (Fig. 5A, second and third rows). Interestingly, at 14 dpi, after HSV-1 antigen was no longer detectable in the eye, WT...
CD3 staining and only occasional Gr-1 (fourth column). The mock-infected sections contained no specific HSV stainings may be responsible, in part, for the exacerbated pathogenesis of infection but may also play a role in the maintenance of an inflammatory microenvironment in the cornea in the absence of HSV EM KO section, fewer Gr-1 staining 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<sup>®</sup> and CD3<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> and CD3<sup>®</sup> 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<sup>®</sup> cells.

FIG 5 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 × 10<sup>6</sup> PFU/5 μl per eye of HSV-1/17 (original magnification, ×400). 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<sup>®</sup> and CD3<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> and CD3<sup>®</sup> 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<sup>®</sup> cells.

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 epithelial and stromal cells, 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) virus via corneal scarification and monitored for 14 days.

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.

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Mice with HVEM on radiation-resistant cell types (WT recipients) began exhibiting lesions 5 dpi, and all mice from these groups had lesions by 7 dpi (Fig. 6A). 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. 6A). These differences were significant: wt→WT mice had a higher incidence of lesions than hvem ko→HVEM KO and wt→HVEM KO mice for days 5 to 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 to 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. 6B).

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 all or nearly all mice from these groups had at least mild symptoms by the following day (Fig. 6C). 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 observed in WT animals compared to HVEM animals and will be a future focus of our studies.

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 nonhematopoietic organs, including the murine eye and sensory neural tissue, and on a wide variety of leukocytes, including T cells, B cells, NK cells, PMN, dendritic cells (DCs), and myeloid cells (24, 45, 46). Furthermore, both the cell type expressing HVEM and the ligand with which it interacts influence whether the HVEM signal is costimulatory or coinhibitory (29, 45, 60–63). 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.

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HVEM Promotes Ocular HSV-1 Independently of Entry

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. 6E). In this case, the mixed chimeras (wt→HVEM KO and hvem ko→WT) had intermediate phenotypes, and the results of comparisons between those two groups or to control groups did not reach statistical significance. However, the total 14-day wt→WT mouse mortality rate (50%) was significantly higher than that of hvem ko→HVEM KO mice (15%). This was also consistent with data indicating that wt→WT animals lost significantly more weight than all other groups, as wt→WT weight loss was more than double that seen with either HVEM KO recipient group (Fig. 6F). The wt→WT controls also exhibited more weight loss than the hvem ko→WT group (13.01% ± 2.51%). Because the hvem ko→WT mice had lower weight loss and mortality than the 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 at 1, 3, and 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 at 1 dpi than either HVEM KO recipient group, regardless of donor genotype (Fig. 7A). 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. 7B). 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. 7C). At that 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 those from HVEM KO recipient groups, and POS titers in hvem ko→WT mice also trended to be higher than those in HVEM KO recipients (Fig. 7D). Titters from the TG of wt→WT controls were ~1 log higher than titers from the TG of hvem ko→HVEM KO controls (Fig. 7E). Although wt→HVEM KO mice had slightly lower titers than mice in 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 brains of HVEM KO recipients trended toward lower levels than those in the brains of WT recipients, although this did not reach statistical significance (Fig. 7F). Lower titers in the brains of HVEM KO recipients were likely due to viral spread, as others have found that HVEM is not required for pathogenesis in the brain (21).

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 (22, 23). Our data indicate that, during ocular infection with HSV-1, (i) the gD-HVEM entry interaction is dispensable for normal disease development; (ii) HVEM-positive corneas have larger amounts of inflammatory cytokines and granulocytic and T cell infiltrates; and (iii) 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 (26, 34–36, 38). We found that HVEM significantly increased levels of the inflammatory cytokine IL-6 and the chemokine CXCL10 in the cornea during acute infection (Fig. 4); this induction did not require the entry-mediating function of HVEM. Several other chemokines, including CCL3, CXCL9, and RANTES, also followed this trend. 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 the acute and chronic phases (Fig. 5). 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 (10, 57, 58, 64). We did not differentiate between subsets of T cells and cannot rule out the possibility that the increased levels of CD3+ cells in WT corneas represented regulatory T cells (Tregs), which have been shown to control ocular infections in some models (65–67). 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 (68). 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 (69). 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. We plan to pursue this finding in our future studies by quantifying infiltrating cells and analyzing the expression of HVEM and relevant HVEM ligands 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, widely expressed in the murine eye, is available to mediate entry when HVEM is absent, likely explaining why HVEM is not required as an entry receptor (25). 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, LTα, CD160, and BTLA (27, 32, 61). HVEM is a bidirectional receptor: as a ligand, it is expressed in 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 (10, 57, 58, 64). We did not differentiate between subsets of T cells and cannot rule out the possibility that the increased levels of CD3+ cells in WT corneas represented regulatory T cells (Tregs), which have been shown to control ocular infections in some models (65–67). 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 (68). 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 (69). 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. We plan to pursue this finding in our future studies by quantifying infiltrating cells and analyzing the expression of HVEM and relevant HVEM ligands in corneal tissues in order to more precisely determine the contribution HVEM makes to HSV-1-induced ocular pathogenesis.

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FIG 7 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 × 10⁶ PFU/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.
Given the widespread 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 co-stimulatory signals to murine and human T cells, enhancing proliferation and activation in the context of TCR ligation (74, 75). If this interaction drove pathogenesis our model, the radiation-resistant HVEM-positive cell type would also have to express the major histocompatibility complex (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 that these cells increase in number and, in the case of DCs, in MHC class II expression after infection with HSV-1 (76–79). 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 (80). 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 (24). 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 cells of the epithelium, stroma, or endothelium (54, 56, 58, 81–85). The HVEM receptor, after binding any of its ligands, activates NF-κB signaling (71, 86). Given that the cytokines that we found to be upregulated in an HVEM-dependent manner are also NF-κB target genes (82, 87–97), 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, of 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 (98, 99). NF-κB activation is required for efficient viral replication and expression of viral proteins, as well as to prevent apoptosis of infected cells (99–101). If NF-κB activation is the signaling pathway responsible for the HVEM-mediated pathogenesis in our model, our results obtained 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 that HVEM on radiation-resistant cell types, such as cells of the corneal epithelium or 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 via 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.

MATERIALS AND METHODS

Ethics statement. These experiments were performed in strict adherence to 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 Northwestern University approved the protocol (Protocol no. 2012-1738). Procedures were performed under anesthesia using ketamine/xylazine or under 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 David Leib (Dartmouth Medical School, Hanover, NH), and strain F was obtained from Bernard Roizman (University of Chicago, Chicago, IL). See Text S1 in the supplemental material for details of cell culture and viral propagation.

Viral plaque assay. A standard plaque assay on Vero cells (unless otherwise noted) was used to determine viral titers as previously described (see Text S1 in the supplemental material).

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 mice and C57BL/6 mice with the CD45.1 allele from Jackson Laboratory (WT mice), Trfrsf14+/- mice (HVEM KO mice), 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 irradiation (2 doses of 6 Gy separated by a 3-h interval) to ablate the BM. Recipients were reconstituted with ~10 million cells harvested from the BM of donor animals via retro-vascular injection within 24 h of irradiation. After 10 weeks, the completeness of the transfer was verified by analyzing the proportions of CD45.1-positive and CD45.2-positive cells in peripheral blood by flow cytometry (cutoff, ≥95% donor genotype).

The animals were inoculated with 2 × 10^6 PFU of HSV-1 in 5 μl Dulbecco’s modified Eagle’s medium (DMEM) as previously described and as described in Text S1 in the supplemental material (22, 23).

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 phosphate-buffered saline (PBS)—protease inhibitor cocktail, homogenized for 30 s with a bead beater, and immediately loaded into the prepared 96-well 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 using a Luminex compact analyzer (Luminex, Austin, TX). Two quality controls were run with each assay, and all analytes fell within quality control ranges.

IHC. Whole eyes were collected at the indicated time points after infection, rinsed with PBS, and floated in 10% formalin—neutral buffered PBS for 24 h. 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. Antigen retrieval was performed manually using a Vectastain ABC kit (Vector Labs). The following antibodies and concentrations were used for immunohistochemistry (IHC) staining: anti-HSV antigen (Dako) polyclonal antibody diluted 1:5,000; anti-Ly6G (Gr-1) monoclonal antibody (BD551459) diluted 1:500; and anti-CD3 monoclonal antibody (Abcam ab166991) diluted 1:2,000. Secondary antibodies labeled with horseradish peroxidase (HRP) were visualized after treatment with chromogen diaminobenzidine (DAB; Vector Labs). Slides were washed, counterstained with Gill’s hematoxylin, and imaged on an EVOS XL core cell imaging system.

Statistics. Geometric means of numbers of viral-plaque-forming units per tissue sample, maximum neurologic and lesion scores, maximum weight losses, and concentrations of cytokines were compared using the unpaired Student’s t test or one-way analysis of variance (ANOVA) with Holm-Sidak’s multiple-comparison test. Variance over time between groups with respect to lesion development or neurologic morbidity was analyzed with two-way ANOVA with Holm-Sidak’s multiple-comparison test. Kaplan-Meier mortality curves were compared using the log rank test. TG reactivation rates were compared using the chi-square test with 1° of freedom. All statistics were calculated using GraphPad Prism 6.0f software.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01532-15/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.

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