RESEARCH ARTICLE

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

Rebecca G. Edwards, Sarah J. Kopp, Andrew H. Karaba,* Douglas R. Wilcox, Richard Longnecker

Department of Microbiology and Immunology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA

* Present address: Andrew H. Karaba, Department of Medicine, The Johns Hopkins University, Baltimore, Maryland, USA.

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.**

Received 16 September 2015 **Accepted** 24 September 2015 **Published** 20 October 2015

Citation Edwards RG, Kopp SJ, Karaba AH, Wilcox DR, Longnecker R. 2015. Herpesvirus entry mediator on radiation-resistant cell lineages promotes ocular herpes simplex virus 1 pathogenesis in an entry-independent manner. mBio 6(5):e01532-15. doi:10.1128/mBio.01532-15.

Editor Peter Palese, Icahn School of Medicine at Mount Sinai

Address correspondence to Richard Longnecker, r-longnecker@northwestern.edu.

This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Herpes simplex virus 1 (HSV-1), a ubiquitous human pathogen, can infect the ocular tissues, resulting in the chronic inflammatory syndrome herpes stromal keratitis (HSK) (1, 2). HSK is characterized by ocular opacity, neovascularization, and edema and produces an estimated 40,000 new cases of severe vision impairment or blindness worldwide each year (3). 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 (1, 2, 4, 5).

In the murine cornea, actively replicating HSV-1 can be detected for 5 to 6 days after infection (4). Secreted factors from infected and uninfected epithelial cells recruit a variety of leukocytes, including neutrophils (polymorphonuclear leukocytes [PMN]), macrophages, NK cells, dendritic cells, and γδ T cells, into the adjacent stromal tissue beginning around 18 h postinfection (hpi) (6–10). The predominately neutrophilic infiltrate mediates viral clearance, and by 5 days postinfection (dpi), PMNs in the cornea decline to preinfection levels (11, 12). A secondary, pathogenic wave of PMNs and $CD4^+$ T cells infiltrates the cornea beginning around 7 dpi and peaking at 14 to 21 dpi (4). Vascular endothelial growth factor receptor (VEGF)-mediated ingrowth 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 (13, 14). Stromal scarring and neovascularization may subse-

quently 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 (*Pvrl1*$^{-/-}$) 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 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 (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 LTα (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-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.

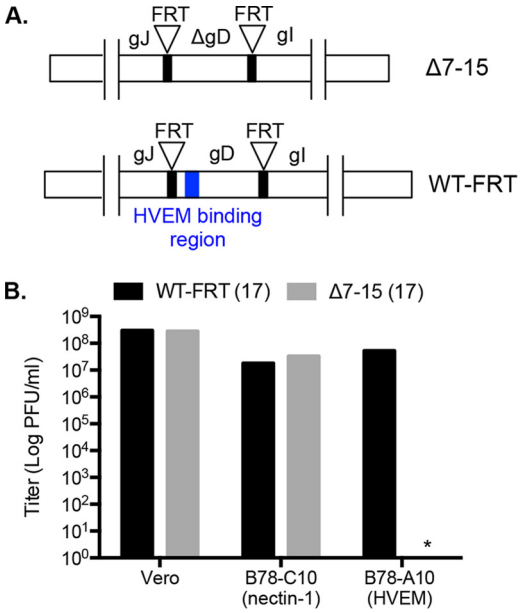

FIG 1 The gDΔ7-15 mutation specifically abolishes cellular entry via HVEM by HSV-1. (A) Schematic diagram of the HVEM-binding HSV-1/gDΔ7-15 mutant (Δ7-15) and of the control virus containing the native gD gene flanked by FRT sites, HSV-1/gDWT-FRT (WT-FRT). WT-FRT was used to generate the Δ7-15 strain, the HVEM entry mutant, via FLP-mediated recombination. The Δ7-15 mutant 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 replicated in B78 cells stably expressing the nectin-1 entry receptor to an average titer of 3.3×10^7 PFU/ml. No detectable replication was observed in HVEM receptor cells (*, below detection limit).

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–

49). 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

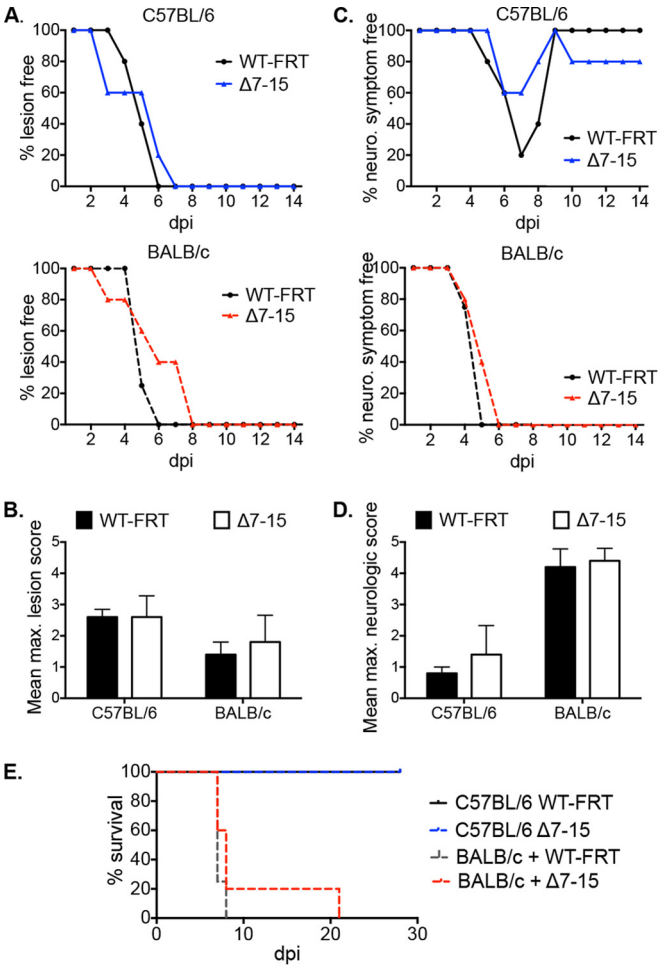

FIG 2 HVEM entry capacity is dispensable for clinical symptoms after corneal inoculation with HSV-1 in multiple strains of mice. HSV-resistant C57BL/6 or HSV-susceptible BALB/c mice were inoculated with 2.0×10^6 PFU/5 μl of the HSV-1/17 viruses per eye and scored daily (using a 0-to-5 scale, with 5 representing 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 postinfection (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 C and D with Holm-Sidak's multiple-comparison test; log-rank test for panel E]).

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-

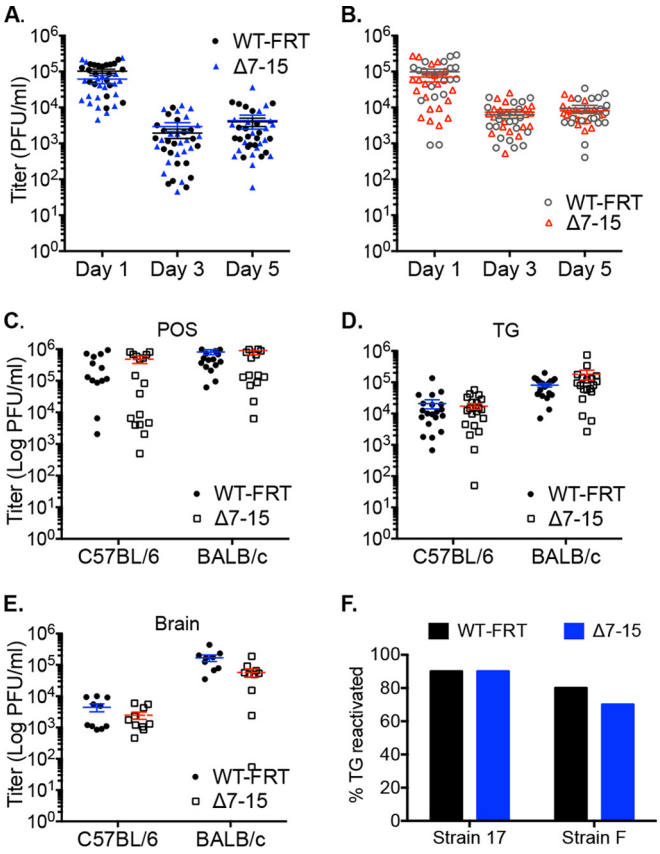

FIG 3 HVEM entry capability is not required for corneal HSV-1 replication or spread to the tissues in multiple strains of mice. HSV-resistant C57BL/6 or HSV-susceptible BALB/c mice were inoculated with 2.0×10^6 PFU/5 μl per eye of the Δ7-15 mutant or the WT-FRT strain (strain 17 background) after corneal 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) 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, $P > 0.05$). (F) At 30 dpi, C57BL/6 mice were sacrificed and TG explants were cocultured on Vero cells for detection of reactivated virus from latency ($n \geq 10$ 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, $P > 0.05$).

fection with strain 17 or F background Δ7-15 mutant 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 mutant or WT-FRT had similar viral loads in the eye swabs at all three time points (data shown for strain 17 background, Fig. 3A 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 (22, 23).

Next, we tested whether spread to relevant organs was reduced by a lack of HVEM entry. We collected periocular skin (POS), trigeminal ganglia (TG), and brains at 5 dpi, homogenized the tissues, and determined titers via plaque assay. Corresponding to titers in the eye swabs, titers in the POS of C57BL/6 or BALB/c mice infected with the Δ7-15 mutant background and those infected with the WT-FRT background did not differ significantly (data shown for strain 17 background; Fig. 3C). Similar results were observed in the TG and brain (strain 17 background; Fig. 3D and E). We also compared titers in the eye swabs, POS, TG, and brain after infection by strain 17 background Δ7-15 mutant and WT-FRT viruses to those seen after infection by the parental HSV-1/17 strain in both mouse strains and found no significant differences in viral replication that would indicate that the recombinants were attenuated (data not shown).

We investigated whether HVEM entry capacity influenced the establishment of latency within the TG. Previous explant reactivation 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 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 compared to WT controls. The previously described experiments indicated 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 a Milliplex multianalyte panel (MAP) mouse cytokine/chemokine magnetic bead panel (Millipore, Billerica, MA) assay system (10, 52–58). Because HVEM KO mice have been reported to have increased T cell reactivity (59), we began by comparing mock-infected WT and HVEM KO corneas to rule out any differences 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. 4A). While most factors were expressed at low levels in the mock-infected samples (≤10 pg/ml), interleukin-1 alpha (IL-1α), CCL2, CXCL2, CXCL9, and, in the HVEM KO, CXCL10 were expressed more highly. The induction of these factors may have been due to scarification alone (56, 58).

We profiled the expression levels of the same panel of cytokines in WT and HVEM KO corneas 5 days after corneal infection with HSV-1(17) virus. 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 severalfold over mock-infected control results, consistent with an infectious, inflammatory process (Fig. 4B). WT corneas expressed IL-6 and the gamma interferon (IFN-γ)-induced T cell chemoattractant CXCL10 (interferon gamma-induced protein 10 [IP-10])

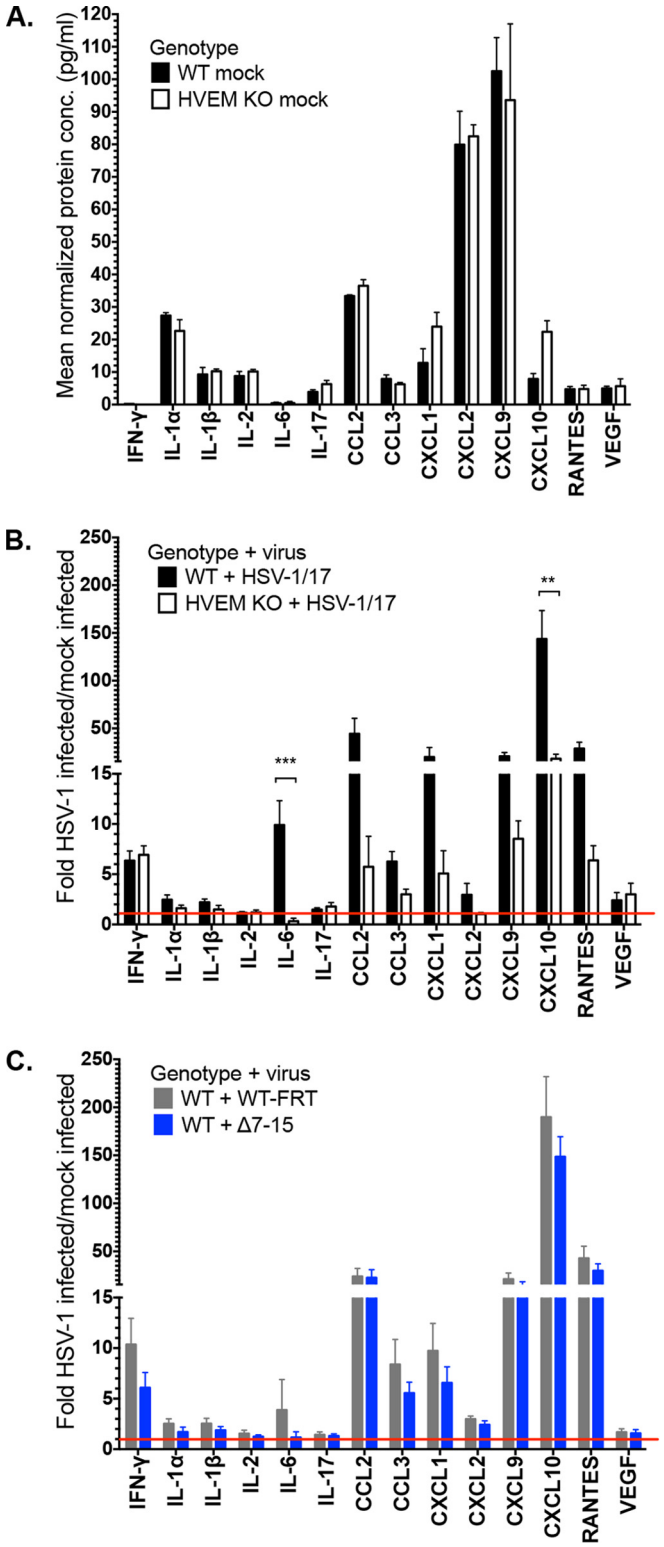

FIG 4 Effects of HVEM depletion or disabled HVEM entry on corneal cytokine expression 5 days after HSV-1/17 infection. (A) Average corneal cytokine protein concentrations (conc.) (in picograms per microliter) 5 days after mock infection in WT and HVEM KO mice. (B) Normalized fold changes in corneal cytokine concentrations of WT or HVEM KO mice infected with 2.0×10^6 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 2.0×10^6 PFU/5 μl per eye of the HVEM-entry Δ7-15

(Continued)

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

Figure Legend Continued

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, with $n \geq 5$ 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).

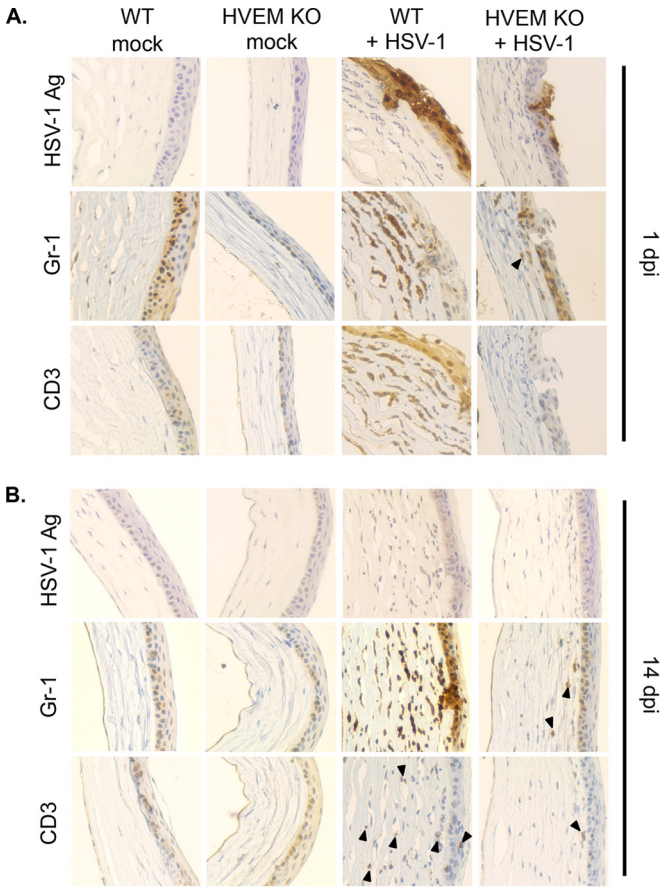

FIG 5 HVEM KO corneas have decreased stromal immune cell infiltrates during acute infection and the chronic phase. (A) Representative immunohistochemical analysis of whole WT or HVEM KO eyes 1 day after mock infection or infection at the corneal surface with 2.0×10^6 PFU/5 μl per eye of HSV-1/17 (original 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$^+$ 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.

stroma remained heavily infiltrated with T cells and especially with granulocytes (Fig. 5B, second row and third rows) whereas HVEM KO stroma contained only rare positive cells (Fig. 5B, black arrowheads). Because the differences between WT and HVEM KO mice 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 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.

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.

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 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.

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

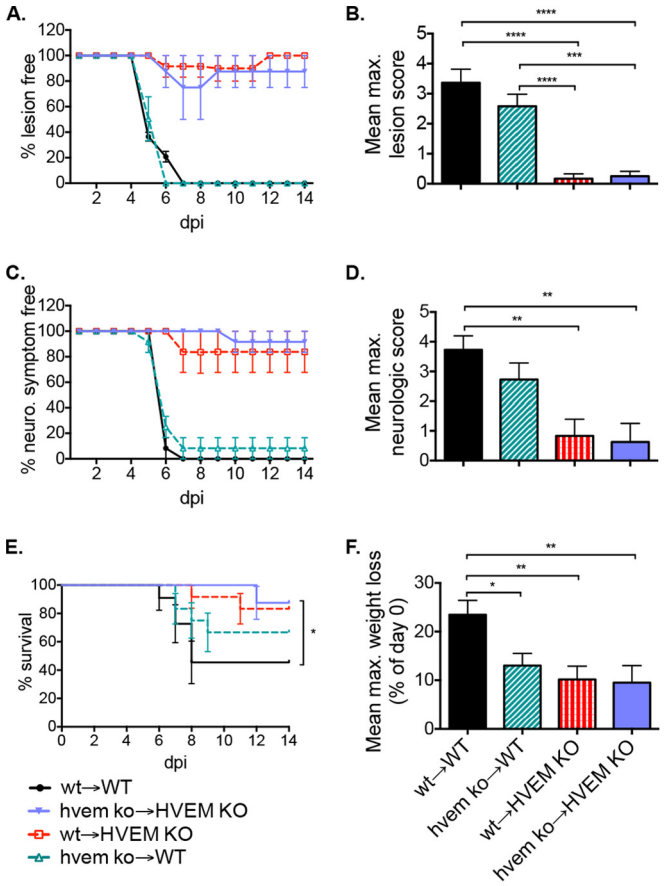

FIG 6 HVEM on radiation-resistant cell types confers wild-type-like susceptibility to clinical HSV-1 symptoms after corneal inoculation. Bone marrow (BM) of WT or HVEM KO mice was ablated with 12 Gy of radiation. Recipients were transplanted with ~10 million WT or HVEM KO cells harvested from donor BM (annotation: donor→RECIPIENT). After 10 weeks of reconstitution and verification 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

disease at a higher rate but also had significantly severer symptoms than HVEM KO recipients (Fig. 6D).

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 tended to be higher than those in HVEM KO recipients (Fig. 7D). Titers 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

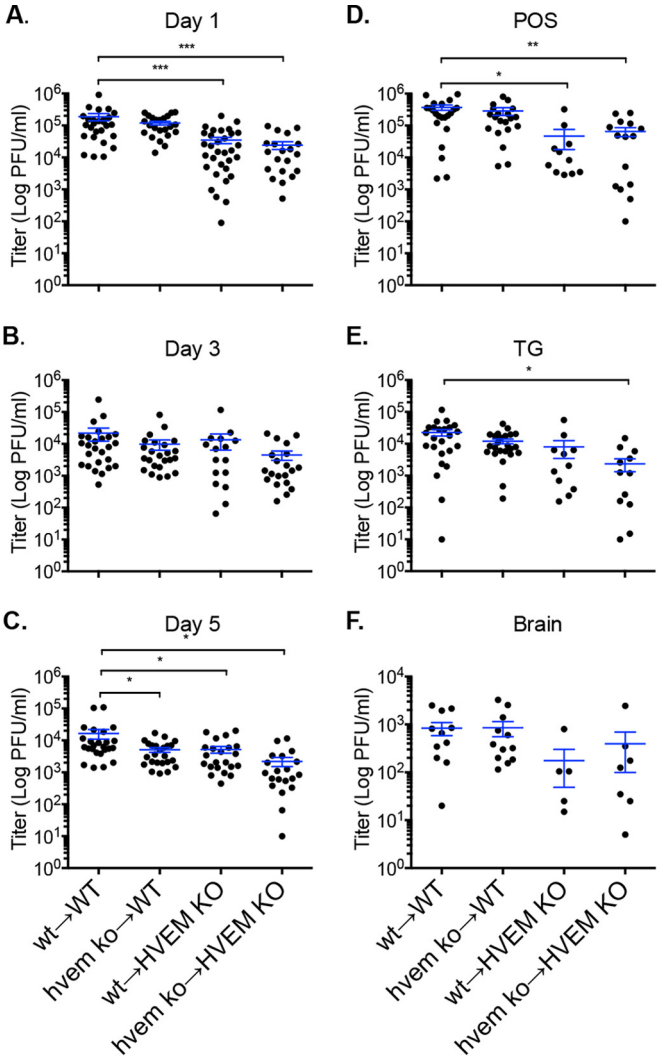

FIG 7 HVEM on radiation-resistant cell types, regardless of radiation-sensitive (donor) genotype, is associated with higher viral loads in the tear film, POS, and TG. Hematopoietic chimeras were inoculated with 2.0×10^6 PFU/5 μl HSV-1/17 per eye after corneal scarification (annotation: donor→RECIPIENT). 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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 $Gr\text{-}1^+$ 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, the presence of HVEM bound by BTLA or CD160 leads to repression of T cell activation and proliferation (corepressor), while interactions with LIGHT or LTα enhance activation and differentiation of many immune cell types (costimulator), although these functions can vary depending on the cellular context and on whether the ligand is expressed in *cis* or in *trans* (60, 70). As a receptor, HVEM engaged by its natural ligands or viral gD activates NF-κB signaling (26, 27, 71). HSV gD has been reported to prevent interactions between HVEM and all its natural ligands (70, 72, 73). Results of experiments performed with the Δ7-15 mutant virus not only

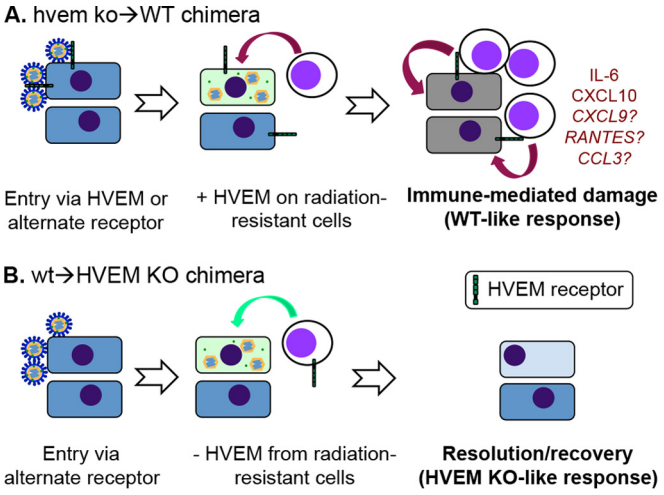

FIG 8 Model of HVEM contribution to HSV-1 pathogenesis in the eye. We have shown that HVEM-expressing, radiation-resistant cell types promote pathogenesis after corneal inoculation of HSV-1 and that HVEM-mediated pathology is entry independent. (A) In hvem ko→WT chimeras, HSV-1 enters through HVEM or 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.

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 we and others 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 the HVEM-expressing cell types that are responsible for HVEM-mediated ocular pathogenesis. Susceptibility to disease after corneal inoculation was shown to segregate 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 corneal epithelial or stromal cells (or another radiation-resistant cell type) increases cytokine production, drawing greater amounts of infiltrates to the cornea and thereby worsening disease after ocular inoculation in the hvem ko→WT chimeras (Fig. 8A). 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 chimeras, because without HVEM, the pathological inflammatory cascade is not initiated (Fig. 8B).

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 costimulatory 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), *Tnfrsf14*$^{-/-}$ 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-orbital 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–neutrally 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 ab16669l) 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.

ACKNOWLEDGMENTS

R.G.E. was supported by a Viral Replication Training grant (T32 AI060523), and R.L. was supported by R01 CA021776. The funders had no role in the design of the studies, the collection or interpretation of the data, or the decision to submit the work for publication.

We thank all the members of the Longnecker laboratory, especially Nanette Susmarski, who graciously provided cell culture assistance. We also acknowledge Osman Cen for his help with generation of hematopoietic chimeras, Valerie Eaton from the laboratory of Steven Miller for her help with flow cytometry, and Lin Li and the Northwestern University Mouse Histology and Phenotyping Laboratory for her assistance with immunohistochemistry.

REFERENCES

1. **Deshpande S, Banerjee K, Biswas PS, Rouse BT.** 2004. Herpetic eye disease: immunopathogenesis and therapeutic measures. Expert Rev Mol Med **6:**1–14. http://dx.doi.org/10.1017/S1462399404007604.
2. **Rowe AM, St. Leger AJ, Jeon S, Dhaliwal DK, Knickelbein JE, Hendricks RL.** 2013. Herpes keratitis. Prog Retin Eye Res **32:**88–101. http://dx.doi.org/10.1016/j.preteyeres.2012.08.002.
3. **Farooq AV, Shukla D.** 2012. Herpes simplex epithelial and stromal keratitis: an epidemiologic update. Surv Ophthalmol **57:**448–462. http://dx.doi.org/10.1016/j.survophthal.2012.01.005.
4. **Biswas PS, Rouse BT.** 2005. Early events in HSV keratitis—setting the stage for a blinding disease. Microbes Infect **7:**799–810. http://dx.doi.org/10.1016/j.micinf.2005.03.003.
5. **Streilein JW, Dana MR, Ksander BR.** 1997. Immunity causing blindness: five different paths to herpes stromal keratitis. Immunol Today **18:**443–449. http://dx.doi.org/10.1016/S0167-5699(97)01114-6.
6. **Thomas J, Gangappa S, Kanangat S, Rouse BT.** 1997. On the essential involvement of neutrophils in the immunopathologic disease herpetic stromal keratitis. J Immunol **158:**1383–1391.
7. **Bauer D, Mrzyk S, van Rooijen N, Steuhl K, Heiligenhaus A.** 2000. Macrophage-depletion influences the course of murine HSV-1 keratitis. Curr Eye Res **20:**45–53. http://dx.doi.org/10.1076/0271-3683(200001)2011-HFT045.
8. **Jager MJ, Bradley D, Atherton S, Streilein JW.** 1992. Presence of Langerhans cells in the central cornea linked to development of ocular herpes in mice. Exp Eye Res **54:**835–841. http://dx.doi.org/10.1016/0014-4835(92)90146-J.
9. **Tamesis RR, Messmer EM, Rice BA, Dutt JE, Foster CS.** 1994. The role of natural killer cells in the development of herpes simplex virus type 1 induced stromal keratitis in mice. Eye **8:**298–306. http://dx.doi.org/10.1038/eye.1994.61.
10. **Inoue T, Inoue Y, Kosaki R, Inoue Y, Nishida K, Shimomura Y, Tano Y, Hayashi K.** 2001. Immunohistological study of infiltrated cells and cytokines in murine herpetic keratitis. Acta Ophthalmol Scand **79:**484–487. http://dx.doi.org/10.1034/j.1600-0420.2001.790511.x.
11. **Tumpey TM, Chen S-H, Oakes JE, Lausch RN.** 1996. Neutrophil-

mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. J Virol **70:**898–904.
12. **Daheshia M, Kanangat S, Rouse BT.** 1998. Production of key molecules by ocular neutrophils early after herpetic infection of the cornea. Exp Eye Res **67:**619–624. http://dx.doi.org/10.1006/exer.1998.0565.
13. **Giménez F, Suryawanshi A, Rouse BT.** 2013. Pathogenesis of herpes stromal keratitis—a focus on corneal neovascularization. Prog Retin Eye Res **33:**1–9. http://dx.doi.org/10.1016/j.preteyeres.2012.07.002.
14. **Suryawanshi A, Veiga-Parga T, Reddy PBJ, Rajasagi NK, Rouse BT.** 2012. IL-17A differentially regulates corneal vascular endothelial growth factor (VEGF)-A and soluble VEGF receptor 1 expression and promotes corneal angiogenesis after herpes simplex virus infection. J Immunol **188:**3434–3446. http://dx.doi.org/10.4049/jimmunol.1102602.
15. **Fine M.** 1959. The surgical treatment of herpetic keratitis. Calif Med **90:**121–125.
16. **Shtein RM, Elner VM.** 2010. Herpes simplex virus keratitis: histopathology and corneal allograft outcomes. Expert Rev Ophthalmol **5:**129–134. http://dx.doi.org/10.1586/eop.10.12.
17. **Connolly SA, Jackson JO, Jardetzky TS, Longnecker R.** 2011. Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat Rev Microbiol **9:**369–381. http://dx.doi.org/10.1038/nrmicro2548.
18. **Taylor JM, Lin E, Susmarski N, Yoon M, Zago A, Ware CF, Pfeffer K, Miyoshi J, Takai Y, Spear PG.** 2007. Alternative entry receptors for herpes simplex virus and their roles in disease. Cell Host Microbe **2:**19–28. http://dx.doi.org/10.1016/j.chom.2007.06.005.
19. **Lopez M, Cocchi F, Menotti L, Avitabile E, Dubreuil P, Campadelli-Fiume G.** 2000. Nectin2α (PRR2α or HveB) and nectin2δ are low-efficiency mediators for entry of herpes simplex virus mutants carrying the Leu25Pro substitution in glycoprotein D. J Virol **74:**1267–1274. http://dx.doi.org/10.1128/JVI.74.3.1267-1274.2000.
20. **Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, Cohen GH, Eisenberg RJ, Rosenberg RD, Spear PG.** 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell **99:**13–22. http://dx.doi.org/10.1016/S0092-8674(00)80058-6.
21. **Kopp SJ, Banisadr G, Glajch K, Maurer UE, Grunewald K, Miller RJ, Osten P, Spear PG.** 2009. Infection of neurons and encephalitis after intracranial inoculation of herpes simplex virus requires the entry receptor nectin-1. Proc Natl Acad Sci U S A **106:**17916–17920. http://dx.doi.org/10.1073/pnas.0908892106.
22. **Karaba AH, Kopp SJ, Longnecker R.** 2011. Herpesvirus entry mediator and nectin-1 mediate herpes simplex virus 1 infection of the murine cornea. J Virol **85:**10041–10047. http://dx.doi.org/10.1128/JVI.05445-11.
23. **Karaba AH, Kopp SJ, Longnecker R.** 2012. Herpesvirus entry mediator is a serotype specific determinant of pathogenesis in ocular herpes. Proc Natl Acad Sci U S A **109:**20649–20654. http://dx.doi.org/10.1073/pnas.1216967109.
24. **Kovacs SK, Tiwari V, Prandovszky E, Dosa S, Bacsa S, Valyi-Nagy K, Shukla D, Valyi-Nagy T.** 2009. Expression of herpes virus entry mediator (HVEM) in the cornea and trigeminal ganglia of normal and HSV-1 infected mice. Curr Eye Res **34:**896–904. http://dx.doi.org/10.3109/02713680903184250.
25. **Valyi-Nagy T, Sheth V, Clement C, Tiwari V, Scanlan P, Kavouras J, Leach L, Guzman-Hartman G, Dermody T, Shukla D.** 2004. Herpes simplex virus entry receptor nectin-1 is widely expressed in the murine eye. Curr Eye Res **29:**303–309. http://dx.doi.org/10.1080/02713680490516756.
26. **Shui J, Kronenberg M.** 2014. HVEM is a TNF receptor with multiple regulatory roles in the mucosal immune system. Immune Netw **14:**67–72. http://dx.doi.org/10.4110/in.2014.14.2.67.
27. **Croft M.** 2014. The TNF family in T cell differentiation and function—unanswered questions and future directions. Semin Immunol **26:**183–190. http://dx.doi.org/10.1016/j.smim.2014.02.005.
28. **Bechill J, Muller WJ.** 2014. Herpesvirus entry mediator (HVEM) attenuates signals mediated by the lymphotoxin beta receptor (LTbetaR) in human cells stimulated by the shared ligand LIGHT. Mol Immunol **62:**96–103. http://dx.doi.org/10.1016/j.molimm.2014.06.013.
29. **Murphy KM, Nelson CA, Šedý JR.** 2006. Balancing co-stimulation and inhibition with BTLA and HVEM. Nat Rev Immunol **6:**671–681. http://dx.doi.org/10.1038/nri1917.
30. **Schneider K, Potter KG, Ware CF.** 2004. Lymphotoxin and LIGHT signaling pathways and target genes. Immunol Rev **202:**49–66. http://dx.doi.org/10.1111/j.0105-2896.2004.00206.x.
31. **Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ.** 2008. CD160 inhibits activation of human CD4$^+$ T cells through interaction with herpesvirus entry mediator. Nat Immunol **9:**176–185. http://dx.doi.org/10.1038/ni1554.
32. **Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu G, Ruben S, Murphy M, Eisenberg RJ, Cohen GH, Spear PG, Ware CF.** 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator. Immunity **8:**21–30. http://dx.doi.org/10.1016/S1074-7613(00)80455-0.
33. **Sedy JR, Gavrieli M, Potter KG, Hurchla MA, Lindsley RC, Hildner K, Scheu S, Pfeffer K, Ware CF, Murphy TL, Murphy KM.** 2005. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. Nat Immunol **6:**90–98. http://dx.doi.org/10.1038/ni1144.
34. **Breloer M, Hartmann W, Blankenhaus B, Eschbach M-, Pfeffer K, Jacobs T.** 2015. Cutting edge: the BTLA-HVEM regulatory pathway interferes with protective immunity to intestinal helminth infection. J Immunol **194:**1413–1416. http://dx.doi.org/10.4049/jimmunol.1402510.
35. **Shui J, Larange A, Kim G, Vela JL, Zahner S, Cheroutre H, Kronenberg M.** 2012. HVEM signalling at mucosal barriers provides host defence against pathogenic bacteria. Nature **488:**222–225. http://dx.doi.org/10.1038/nature11242.
36. **Kopp SJ, Storti CS, Muller WJ.** 2012. Herpes simplex virus-2 glycoprotein interaction with HVEM influences virus-specific recall cellular responses at the mucosa. Clin Dev Immunol **2012:**284104. http://dx.doi.org/10.1155/2012/284104.
37. **Yoon M, Kopp SJ, Taylor JM, Storti CS, Spear PG, Muller WJ.** 2011. Functional interaction between herpes simplex virus type 2 gD and HVEM transiently dampens local chemokine production after murine mucosal infection. PLoS One **6:**e16122. http://dx.doi.org/10.1371/journal.pone.0016122.
38. **Krause P, Zahner SP, Kim G, Shaikh RB, Steinberg MW, Kronenberg M.** 2014. The tumor necrosis factor family member TNFSF14 (LIGHT) is required for resolution of intestinal inflammation in mice. Gastroenterology **146:**1752–1762.e4. http://dx.doi.org/10.1053/j.gastro.2014.02.010.
39. **Connolly SA, Landsburg DJ, Carfi A, Wiley DC, Eisenberg RJ, Cohen GH.** 2002. Structure-based analysis of the herpes simplex virus glycoprotein D binding site present on herpesvirus entry mediator HveA (HVEM). J Virol **76:**10894–10904. http://dx.doi.org/10.1128/JVI.76.21.10894-10904.2002.
40. **Connolly SA, Landsburg DJ, Carfi A, Wiley DC, Cohen GH, Eisenberg RJ.** 2003. Structure-based mutagenesis of herpes simplex virus glycoprotein D defines three critical regions at the gD-HveA/HVEM binding interface. J Virol **77:**8127–8140. http://dx.doi.org/10.1128/JVI.77.14.8127-8140.2003.
41. **Connolly SA, Landsburg DJ, Carfi A, Whitbeck JC, Zuo Y, Wiley DC, Cohen GH, Eisenberg RJ.** 2005. Potential nectin-1 binding site on herpes simplex virus glycoprotein d. J Virol **79:**1282–1295. http://dx.doi.org/10.1128/JVI.79.2.1282-1295.2005.
42. **Spear PG, Manoj S, Yoon M, Jogger CR, Zago A, Myscofski D.** 2006. Different receptors binding to distinct interfaces on herpes simplex virus gD can trigger events leading to cell fusion and viral entry. Virology **344:**17–24. http://dx.doi.org/10.1016/j.virol.2005.09.016.
43. **Uchida H, Shah WA, Ozuer A, Frampton AR, Jr., Goins WF, Grandi P, Cohen JB, Glorioso JC.** 2009. Generation of herpesvirus entry mediator (HVEM)-restricted herpes simplex virus type 1 mutant viruses: resistance of HVEM-expressing cells and identification of mutations that rescue nectin-1 recognition. J Virol **83:**2951–2961. http://dx.doi.org/10.1128/JVI.01449-08.
44. **Di Giovine P, Settembre EC, Bhargava AK, Luftig MA, Lou H, Cohen GH, Eisenberg RJ, Krummenacher C, Carfi A.** 2011. Structure of herpes simplex virus glycoprotein D bound to the human receptor nectin-1. PLoS Pathog **7:**e1002277. http://dx.doi.org/10.1371/journal.ppat.1002277.
45. **Heo S-, Ju SA, Lee SC, Park SM, Choe SY, Kwon B, Kwon BS, Kim BS.** 2006. LIGHT enhances the bactericidal activity of human monocytes and neutrophils via HVEM. J Leukoc Biol **79:**330–338. http://dx.doi.org/10.1189/jlb.1104694.
46. **Jung HW, La SJ, Kim JY, Heo SK, Kim JY, Wang S, Kim KK, Lee KM, Cho HR, Lee HW, Kwon B, Kim BS, Kwon BS.** 2003. High levels of soluble herpes virus entry mediator in sera of patients with allergic and

autoimmune diseases. Exp Mol Med **35:**501–508. http://dx.doi.org/10.1038/emm.2003.65.
47. **Manoj S, Jogger CR, Myscofski D, Yoon M, Spear PG.** 2004. Mutations in herpes simplex virus glycoprotein D that prevent cell entry via nectins and alter cell tropism. Proc Natl Acad Sci U S A **101:**12414–12421. http://dx.doi.org/10.1073/pnas.0404211101.
48. **Yoon M, Zago A, Shukla D, Spear PG.** 2003. Mutations in the N termini of herpes simplex virus type 1 and 2 gDs alter functional interactions with the entry/fusion receptors HVEM, nectin-2, and 3-O-sulfated heparan sulfate but not with nectin-1. J Virol **77:**9221–9231. http://dx.doi.org/10.1128/JVI.77.17.9221-9231.2003.
49. **Yoon M, Spear PG.** 2004. Random mutagenesis of the gene encoding a viral ligand for multiple cell entry receptors to obtain viral mutants altered for receptor usage. Proc Natl Acad Sci U S A **101:**17252–17257. http://dx.doi.org/10.1073/pnas.0407892101.
50. Reference deleted.
51. Reference deleted.
52. **He J, Ichimura H, Iida T, Minami M, Kobayashi K, Kita M, Sotozono C, Tagawa Y, Iwakura Y, Imanishi J.** 1999. Kinetics of cytokine production in the cornea and trigeminal ganglion of C57BL/6 mice after corneal HSV-1 infection. J Interferon Cytokine Res **19:**609–615. http://dx.doi.org/10.1089/107999099313749.
53. **Staats HF, Lausch RN.** 1993. Cytokine expression in vivo during murine herpetic stromal keratitis. J Immunol **151:**277–283.
54. **Araki-Sasaki K, Tanaka T, Ebisuno Y, Kanda H, Umemoto E, Hayashi K, Miyazaki M.** 2006. Dynamic expression of chemokines and the infiltration of inflammatory cells in the HSV-infected cornea and its associated tissues. Ocul Immunol Inflamm **14:**257–266. http://dx.doi.org/10.1080/09273940600943581.
55. **Tumpey TM, Cheng H, Yan XT, Oakes JE, Lausch RN.** 1998. Chemokine synthesis in the HSV-1 infected cornea and its suppression by interleukin-10. J Leukoc Biol **63:**486–492.
56. **Su YH, Yan XT, Oakes JE, Lausch RN.** 1996. Protective antibody therapy is associated with reduced chemokine transcripts in herpes simplex virus type 1 corneal infection. J Virol **70:**1277–1281.
57. **Lundberg PS, Cantin EM.** 2003. A potential role for CXCR3 chemokines in the response to ocular HSV infection. Curr Eye Res **26:**137–150. http://dx.doi.org/10.1076/ceyr.26.3.137.14898.
58. **Carr DJ, Tomanek L.** 2006. Herpes simplex virus and the chemokines that mediate the inflammation. Curr Top Microbiol Immunol **303:**47–65.
59. **Wang Y, Subudhi SK, Anders RA, Lo J, Sun Y, Blink S, Wang Y, Wang J, Liu X, Mink K, Degrandi D, Pfeffer K, Fu Y.** 2005. The role of herpesvirus entry mediator as a negative regulator of T cell–mediated responses. J Clin Invest **115:**711–717. http://dx.doi.org/10.1172/JCI200522982.
60. **Ware CF, Šedý JR.** 2011. TNF superfamily networks: bidirectional and interference pathways of the herpesvirus entry mediator (TNFSF14). Curr Opin Immunol **23:**627–631. http://dx.doi.org/10.1016/j.coi.2011.08.008.
61. **Cai G, Freeman GJ.** 2009. The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. Immunol Rev **229:**244–258. http://dx.doi.org/10.1111/j.1600-065X.2009.00783.x.
62. **Sedy JR, Bjordahl RL, Bekiaris V, Macauley MG, Ware BC, Norris PS, Lurain NS, Benedict CA, Ware CF.** 2013. CD160 activation by herpesvirus entry mediator augments inflammatory cytokine production and cytolytic function by NK cells. J Immunol **191:**828–836. http://dx.doi.org/10.4049/jimmunol.1300894.
63. **Harrop JA, McDonnell PC, Brigham-Burke M, Lyn SD, Minton J, Tan KB, Dede K, Spampanato J, Silverman C, Hensley P, DiPrinzio R, Emery JG, Deen K, Eichman C, Chabot-Fletcher M, Truneh A, Young PR.** 1998. Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. J Biol Chem **273:**27548–27556. http://dx.doi.org/10.1074/jbc.273.42.27548.
64. **Menten P, Wuyts A, Van Damme J.** 2002. Macrophage inflammatory protein-1. Cytokine Growth Factor Rev **13:**455–481. http://dx.doi.org/10.1016/S1359-6101(02)00045-X.
65. **Sehrawat S, Suvas S, Sarangi PP, Suryawanshi A, Rouse BT.** 2008. In vitro-generated antigen-specific CD4$^+$ CD25$^+$ Foxp3+ regulatory T cells control the severity of herpes simplex virus-induced ocular immunoinflammatory lesions. J Virol **82:**6838–6851. http://dx.doi.org/10.1128/JVI.00697-08.
66. **Suvas S, Azkur AK, Kim BS, Kumaraguru U, Rouse BT.** 2004. CD4+CD25$^+$ regulatory T cells control the severity of viral immunoinflammatory lesions. J Immunol **172:**4123–4132. http://dx.doi.org/10.4049/jimmunol.172.7.4123.
67. **Veiga-Parga T, Suryawanshi A, Mulik S, Gimenez F, Sharma S, Sparwasser T, Rouse BT.** 2012. On the role of regulatory T cells during viral-induced inflammatory lesions. J Immunol **189:**5924–5933. http://dx.doi.org/10.4049/jimmunol.1202322.
68. **Sharma S, Rajasagi NK, Veiga-Parga T, Rouse BT.** 2014. Herpesvirus entry mediator (HVEM) modulates proliferation and activation of regulatory T cells following HSV-1 infection. Microbes Infect **16:**648–660. http://dx.doi.org/10.1016/j.micinf.2014.06.005.
69. **Divito SJ, Hendricks RL.** 2008. Activated inflammatory infiltrate in HSV-1-infected corneas without herpes stromal keratitis. Invest Ophthalmol Vis Sci **49:**1488–1495. http://dx.doi.org/10.1167/iovs.07-1107.
70. **Steinberg MW, Cheung TC, Ware CF.** 2011. The signaling networks of the herpesvirus entry mediator (TNFRSF14) in immune regulation. Immunol Rev **244:**169–187. http://dx.doi.org/10.1111/j.1600-065X.2011.01064.x.
71. **Cheung TC, Steinberg MW, Oborne LM, Macauley MG, Fukuyama S, Sanjo H, D'Souza C, Norris PS, Pfeffer K, Murphy KM, Kronenberg M, Spear PG, Ware CF.** 2009. Unconventional ligand activation of herpesvirus entry mediator signals cell survival. Proc Natl Acad Sci U S A **106:**6244–6249. http://dx.doi.org/10.1073/pnas.0902115106.
72. **Stiles KM, Whitbeck JC, Lou H, Cohen GH, Eisenberg RJ, Krummenacher C.** 2010. Herpes simplex virus glycoprotein D interferes with binding of herpesvirus entry mediator to its ligands through downregulation and direct competition. J Virol **84:**11646–11660. http://dx.doi.org/10.1128/JVI.01550-10.
73. **Cheung TC, Humphreys IR, Potter KG, Norris PS, Shumway HM, Tran BR, Patterson G, Jean-Jacques R, Yoon M, Spear PG, Murphy KM, Lurain NS, Benedict CA, Ware CF.** 2005. Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway. Proc Natl Acad Sci U S A **102:**13218–13223. http://dx.doi.org/10.1073/pnas.0506172102.
74. **Shi G, Luo H, Wan X, Salcedo TW, Zhang J, Wu J.** 2002. Mouse T cells receive costimulatory signals from LIGHT, a TNF family member. Blood **100:**3279–3286. http://dx.doi.org/10.1182/blood-2002-05-1404.
75. **Wan X, Zhang J, Luo H, Shi G, Kapnik E, Kim S, Kanakaraj P, Wu J.** 2002. A TNF family member LIGHT transduces costimulatory signals into human T cells. J Immunol **169:**6813–6821. http://dx.doi.org/10.4049/jimmunol.169.12.6813.
76. **Hamrah P, Zhang Q, Liu Y, Reza DM.** 2002. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. Invest Ophthalmol Vis Sci **43:**639–646.
77. **Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL.** 2002. Identification of a novel macrophage population in the normal mouse corneal stroma. Invest Ophthalmol Vis Sci **43:**2264–2271.
78. **Liu Y, Hamrah P, Zhang Q, Taylor AW, Reza DM.** 2002. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class-II positive dendritic cells derived from MHC class II-negative grafts. J Exp Med **195:**259–268. http://dx.doi.org/10.1084/jem.20010838.
79. **Hu K, Harris DL, Yamaguchi T, von Andrian UH, Hamrah P.** 2015. A dual role for corneal dendritic cells in herpes simplex keratitis: local suppression of corneal damage and promotion of systemic viral dissemination. PLoS One **10:**e0137123. http://dx.doi.org/10.1371/journal.pone.0137123.
80. **Chinnery HR, Humphries T, Clare A, Dixon AE, Howes K, Moran CB, Scott D, Zakrzewski M, Pearlman E, McMenamin PG.** 2008. Turnover of bone marrow-derived cells in the irradiated mouse cornea. Immunology **125:**541–548. http://dx.doi.org/10.1111/j.1365-2567.2008.02868.x.
81. **Bryant-Hudson KM, Gurung HR, Zheng M, Carr DJJ.** 2014. Tumor necrosis factor alpha and interleukin-6 facilitate corneal lymphangiogenesis in response to herpes simplex virus 1 infection. J Virol **88:**14451–14457. http://dx.doi.org/10.1128/JVI.01841-14.
82. **Li H, Zhang J, Kumar A, Zheng M, Atherton SS, Yu FX.** 2006. Herpes simplex virus 1 infection induces the expression of proinflammatory cytokines, interferons and TLR7 in human corneal epithelial cells. Immunology **117:**167–176. http://dx.doi.org/10.1111/j.1365-2567.2005.02275.x.
83. **Terasaka Y, Miyazaki D, Yakura K, Haruki T, Inoue Y.** 2010. Induction of IL-6 in transcriptional networks in corneal epithelial cells after herpes

simplex virus type 1 infection. Invest Ophthalmol Vis Sci **51:**2441–2449. http://dx.doi.org/10.1167/iovs.09-4624.

84. **Tran MT, Tellaetxe-Isusi M, Elner V, Strieter RM, Lausch RN, Oakes JE.** 1996. Proinflammatory cytokines induce RANTES and MCP-1 synthesis in human corneal keratocytes but not in corneal epithelial cells. Invest Ophthalmol Vis Sci **37:**987–996.
85. **Susarla R, Liu L, Walker EA, Bujalska IJ, Alsalem J, Williams GP, Sreekantam S, Taylor AE, Tallouzi M, Southworth HS, Murray PI, Wallace GR, Rauz S.** 2014. Cortisol biosynthesis in the human ocular surface innate immune response. PLoS One **9:**e94913. http://dx.doi.org/10.1371/journal.pone.0094913.
86. **Marsters SA, Ayres TM, Skubatch M, Gray CL, Rothe M, Ashkenazi A.** 1997. Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with members of the TNFR-associated factor family and activates the transcription factors NF-kB and AP01. J Biol Chem **272:**14029–14032. http://dx.doi.org/10.1074/jbc.272.22.14029.
87. **Nomi N, Kimura K, Nishida T.** 2010. Release of interleukins 6 and 8 induced by zymosan and mediated by MAP kinase and NF-kappaB signaling pathways in human corneal fibroblasts. Invest Ophthalmol Vis Sci **51:**2955–2959. http://dx.doi.org/10.1167/iovs.09-4823.
88. **Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, Arnold MM, Knipe DM, Finberg RW.** 2004. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc Natl Acad Sci U S A **101:**1315–1320. http://dx.doi.org/10.1073/pnas.0308057100.
89. **Moriuchi H, Moriuchi M, Fauci AS.** 1997. Nuclear factor-kB potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. J Immunol **158:**3483–3491.
90. **Yu H, Jiang W, Du H, Xing Y, Bai G, Zhang Y, Li Y, Jiang H, Zhang Y, Wang J, Wang P, Bai X.** 2014. Involvement of the Akt/NF-kappaB pathways in the HTNV-mediated increase of IL-6, CCL5, ICAM-1 and VCAM-1 in HUVECs. PLoS One **9:**e93810. http://dx.doi.org/10.1371/journal.pone.0093810.
91. **Hiroi M, Ohmori Y.** 2003. Constitutive nuclear factor κB activity is required to elicit interferon-γ-induced expression of chemokine CXC ligand 9 (CXCL9) and CXCL10 in human tumour cell lines. Biochem J **376:**393–402. http://dx.doi.org/10.1042/bj20030842.
92. **Yeruva S, Ramadori G, Raddatz D.** 2008. NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines. Int J Colorectal Dis **23:**305–317. http://dx.doi.org/10.1007/s00384-007-0396-6.
93. **Grove M, Plumb M.** 1993. C/EBP, NF-κB, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1α immediate-early gene. Mol Cell Biol **13:**5276–5289.
94. **Widmer U, Manogue KR, Cerami A, Sherry B.** 1993. Genomic cloning and promoter analysis of macrophage inflammatory protein (MIP)-2, MIP-1α, and MIP-1β, members of the chemokine superfamily of proinflammatory cytokines. J Immunol **150:**4996–5012.
95. **Melchjorsen J, Paludan SR.** 2003. Induction of RANTES/CCL5 by herpes simplex virus is regulation by nuclear factor kappa B and interferon regulatory factor 3. J Gen Virol **84:**2491–2495. http://dx.doi.org/10.1099/vir.0.19159-0.
96. **Paludan SR.** 2001. Requirements for the induction of interleukin-6 by herpes simplex virus-infected leukocytes. J Virol **75:**8008–8015. http://dx.doi.org/10.1128/JVI.75.17.8008-8015.2001.
97. **Takeda S, Miyazaki D, Sasaki S, Yamamoto Y, Terasaka Y, Yakura K, Yamagami S, Ebihara N, Inoue Y.** 2011. Roles played by toll-like receptor-9 in corneal endothelial cells after herpes simplex virus type 1 infection. Invest Ophthalmol Vis Sci **52:**6729–6736. http://dx.doi.org/10.1167/iovs.11-7805.
98. **Teresa Sciortino M, Antonietta Medici M, Marino-Merlo F, Zaccaria D, Giuffre M, Venuti A, Grelli S, Mastino A.** 2007. Signaling pathway used by HSV-1 to induce NF-kappaB activation: possible role of herpes virus entry receptor A. Ann N Y Acad Sci **1096:**89–96. http://dx.doi.org/10.1196/annals.1397.074.
99. **Roberts KL, Baines JD.** 2011. UL31 of herpes simplex virus 1 is necessary for optimal NF-kappaB activation and expression of viral gene products. J Virol **85:**4947–4953. http://dx.doi.org/10.1128/JVI.00068-11.
100. **Patel A, Hanson J, McLean TI, Olgiate J, Hilton M, Miller WE, Bachenheimer SL.** 1998. Herpes simplex type 1 induction of persistent NF-κB nuclear translocation increases the efficiency of viral replication. Virology **247:**212–222. http://dx.doi.org/10.1006/viro.1998.9243.
101. **Goodkin ML, Ting AT, Blaho JA.** 2003. NF-B is required for apoptosis prevention during herpes simplex virus type 1 infection. J Virol **77:**7261–7280. http://dx.doi.org/10.1128/JVI.77.13.7261-7280.2003.