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# Interactions between Herpesvirus Entry Mediator (TNFRSF14) and Latency-Associated Transcript during Herpes Simplex Virus 1 Latency

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**Herpesvirus entry mediator (HVEM) is one of several cell surface proteins herpes simplex virus (HSV) uses for attachment/entry. HVEM regulates cellular immune responses and can also increase cell survival. Interestingly, latency-associated transcript (LAT), the only viral gene consistently expressed during neuronal latency, enhances latency and reactivation by promoting cell survival and by helping the virus evade the host immune response. However, the mechanisms of these LAT activities are not well understood. We show here for the first time that one mechanism by which LAT enhances latency and reactivation appears to be by upregulating HVEM expression. HSV-1 latency/reactivation was significantly reduced in *Hvem*<sup>-/-</sup> mice, indicating that HVEM plays a significant role in HSV-1 latency/reactivation. Furthermore, LAT upregulated HVEM expression during latency *in vivo* and also when expressed *in vitro* in the absence of other viral factors. This study suggests a mechanism whereby LAT upregulates HVEM expression potentially through binding of two LAT small noncoding RNAs to the HVEM promoter and that the increased HVEM then leads to downregulation of immune responses in the latent microenvironment and increased survival of latently infected cells. Thus, one of the mechanisms by which LAT enhances latency/reactivation appears to be through increasing expression of HVEM.**

The herpes simplex virus 1 (HSV-1) infects its human host through multiple routes, stimulating strong immune responses that resolve the acute infection but prove unable to prevent the virus from establishing latency in peripheral sensory neurons or preventing reactivation from latency (1–4). The latent phase of HSV infection is characterized by the presence of viral genome without detectable infectious virus production except during intermittent episodes of reactivation from latency (2, 5–7). During HSV-1 neuronal latency in mice, rabbits, and humans, the only viral gene that is consistently expressed at high levels is the latency-associated transcript (LAT) (3, 5). The primary LAT RNA is ~8.3 kb in length. A very stable 2-kb intron is readily detected during latency (1, 4, 6, 8). LAT is important for wild-type (WT) levels of spontaneous and induced reactivation from latency (9, 10). The LAT region plays a role in blocking apoptosis in rabbits (11) and mice (12). Antiapoptosis activity appears to be the critical LAT function involved in enhancing the latency-reactivation cycle because LAT-deficient [LAT(-)] virus can be restored to full wild-type reactivation levels by substitution of different antiapoptosis genes (i.e., baculovirus inhibitor of apoptosis protein gene [cpiAP] or cellular FLICE-like inhibitory protein [FLIP]) (13–15).

Experimental HSV-1 infection in mice and rabbits shows that HSV-1 establishes a latent phase in sensory neurons (2, 5–7). Although spontaneous reactivation occurs in rabbits at levels similar to those seen in humans, spontaneous reactivation in mice occurs at extremely low rates (16). During latency, in addition to LAT, some lytic cycle transcripts and viral proteins appear to be expressed at very low levels in ganglia of latently infected mice (17, 18), suggesting that very low levels of reactivation and/or abortive reactivation can occur in mice.

HSV-1 utilizes several routes of entry to initiate the infection of cells including herpesvirus entry mediator (HVEM; TNFRSF14), nectin-1, nectin-2, 3-O-sulfated heparan sulfate (3-OS-HS), paired immunoglobulin-like type 2 receptor  $\alpha$  (PILR $\alpha$ ) (19–21), nonmuscle myosin heavy chain IIA (NMHC-IIA) (22), and myelin-associated glycoprotein (MAG) (23). This apparent redundancy of HSV-1 receptors may contribute to the ability of HSV-1 to infect many cell types (19, 21, 24–28). The virion envelope glycoprotein D (gD) of HSV-1 is the primary viral protein that engages the HVEM molecule (25, 26, 29).

HVEM is a member of the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) that regulates cellular immune responses, serving as a molecular switch between proinflammatory and inhibitory signaling that aids in establishing homeostasis (30, 31). HVEM is activated by binding the TNF-related ligands, LIGHT (TNFSF14) and lymphotoxin- $\alpha$ , which connect HVEM to the larger TNF and lymphotoxin cytokine network (30). HVEM also engages the immunoglobulin superfamily members CD160 and B and T lymphocyte attenuator (BTLA) (32, 33). HVEM as a ligand for BTLA activates tyrosine phosphatase SHP1 that suppresses antigen receptor signaling in T and B cells (32, 34). BTLA and HVEM are coexpressed in hematopoietic cells, forming a complex in *cis* that restricts HVEM activation by its ligands in the

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microenvironment (34). HVEM is broadly expressed in the hematopoietic compartment but is also expressed in epithelial cells in many organs. For example, HVEM expressed in intestinal mucosa cells limits the inflammatory action of T cells and innate effector cells through activation of BTLA (35). HVEM activates NF- $\kappa$ B survival programs that appear necessary for survival of long-term memory T cells that arise from persistent inflammatory processes (36). These observations define the HVEM pathway as a communication network formed between cells in the immune system and tissues in the surrounding microenvironment to achieve homeostasis.

The HSV-1 virion envelope gD forms a complex with HVEM which mimics the BTLA-HVEM interaction (37), allowing the virus to directly access NF- $\kappa$ B-dependent cell survival pathways through HVEM, providing a strong selective pressure. However, given the diversity in entry routes, the evolution of the gD-HVEM interaction in the context of the acute phase of infection seems less critical as a selective pressure, leading us to consider a role for HVEM in viral latency and reactivation.

We report here that HSV-1 latency and reactivation from latency are significantly impaired in mice deficient in the HVEM gene. The experiments demonstrate that two small noncoding RNAs (scnRNAs) in the LAT gene (38) induce HVEM expression in trigeminal ganglia of latently infected mice. In addition, the effect of LAT on latency is dramatically lost in mice deficient in HVEM. Replacement of LAT with a viral ortholog of the cellular inhibitor of apoptosis (cIAP) restores viral latency but not HVEM expression. Moreover, the signature of immune T cells and cytokines recruited into the trigeminal ganglia is selectively altered in *Hvem*<sup>-/-</sup> mice. These results indicate that LAT regulates viral latency and reactivation at least in part by increasing HVEM expression, which in turn increases survival of cells harboring latent virus and limits effector T cell activation. These results identify a LAT-HVEM relationship as a novel mechanism that manipulates homeostatic pathways involved in HSV-1 latency.

## MATERIALS AND METHODS

**Virus and mice.** Plaque-purified HSV-1 strains, the wild-type McKrae expressing LAT [LAT(+)], dLAT2903 [LAT(-)], and other LAT(-) viruses, were grown in rabbit skin (RS) cell monolayers in minimal essential medium (MEM) containing 5% fetal calf serum (FCS), as described previously (9, 39). Four different LAT(-) viruses, all derived from HSV-1 McKrae, were used: (i) dLAT2903 has both copies of the LAT promoter (one in each viral long repeat) and the first 1,667 nucleotides (nt) of the LAT transcript deleted (9); (ii) dLAT-gK<sup>3</sup> has LAT nt 76 to 1499 in both copies of LAT replaced by the open reading frame (ORF) encoding HSV-1 glycoprotein K (resulting in the virus containing three copies of gK [gK<sup>3</sup>]) (40); (iii) dLAT-CD80 contains the complete murine CD80 ORF in place of LAT nt 76 to 1499 in both copies of LAT; and (iv) dLAT-cpIAP contains the complete baculovirus inhibitor of apoptosis protein gene (cpIAP) ORF in place of LAT (15). C57BL/6 and C57BL/6-*Hvem*<sup>-/-</sup> mice (33) were used in this study. C57BL/6 mice were purchased from Jackson Laboratories, while the knockout mice were bred in-house. Animal research protocols were approved by the Institutional Animal Care and Use Committees.

**Ocular infection.** Mice were infected via the ocular route with  $2 \times 10^5$  PFU of virus suspended in 2  $\mu$ l of tissue culture medium (supplemented with 5% serum). Viruses were administered as an eye drop without prior corneal scarification.

**Titration of virus in tears of infected mice.** Tear films were collected from both eyes of 10 mice per group on days 1 to 4 postinfection (p.i.) using a Dacron-tipped swab (41). Each swab was placed in 0.5 ml of tissue

culture medium and squeezed, and the amount of virus was determined by a standard plaque assay on RS cells.

**In vitro explant reactivation assay.** Mice were sacrificed at 30 days p.i., and individual trigeminal ganglia (TG) were removed and cultured in tissue culture medium as we described previously (42). Aliquots of medium were removed from each culture daily for up to 10 days and plated on indicator cells (RS cells) to assay for the appearance of reactivated virus. As the medium from the explanted TG cultures was plated daily, the time at which reactivated virus first appeared in the explanted TG cultures could be determined.

**C1300 and Neuro2A studies.** C1300 cells stably expressing the LAT region from LAT nt -361 to +3225 were described previously (43). LAT-expressing C1300 cells and controls were grown in MEM supplemented with 10% fetal bovine serum (FBS) in the presence of 1  $\mu$ g/ml puromycin. Control C1300 cells were grown without antibiotic. Neuro2A cells expressing the LAT region from LAT nt -361 to +1499 were described previously (44) and grown as described above but with 1 mg/ml G418 antibiotic. These two LAT(+) stable cell lines were made using different cells, at different times, in two different labs, by two different people, and using different LAT(+) plasmids. Thus, the similar results seen here with both LAT(+) cell lines are extremely unlikely to be due to cloning artifacts or contamination.

**sncRNA1 and sncRNA2 transfection.** Construction of sncRNA1 and sncRNA2 in the plasmid vector pSilence was described previously (45). Neuro2A cells were transfected with plasmid DNA and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Expression of HVEM mRNA was determined by quantitative real-time PCR (qRT-PCR) analysis of total cellular RNA. sncRNA1 and sncRNA2 expression levels were normalized to expression with cells transfected with empty pSilence vector. The experiment was repeated three times.

**Immunostaining of TG.** The trigeminal ganglia (TG) of naive and infected mice were removed at necropsy at 30 days postinfection (p.i.), embedded in optimal cutting temperature compound (OCT) (Tissue-Tek; Sakura, Torrance, CA) for cryosectioning, and stored at -80°C. Transverse sections were cut 15  $\mu$ m thick and air dried for 15 min. Representative sections (spaced 50  $\mu$ m apart) throughout the TG were fixed for 2 h in 4% paraformaldehyde at 4°C, followed by a 30-min incubation in Dako Serum-Free Protein Block. Rat anti-mouse HVEM clone 10F3 antibody (46) was incubated in protein block at 4°C overnight. After three rinses for 5 min each in 1 $\times$  phosphate-buffered saline (PBS), slides were incubated for 1 h at 25°C with secondary antibody labeled with Alexa Fluor-488 (green) (Invitrogen, Carlsbad, CA). Slides were washed three times with PBS, air dried, and mounted with Prolong Gold with 4',6'-diamidino-2-phenylindole (DAPI) mounting medium (Invitrogen). The fluorophores were imaged in separate channels with a Zeiss ApoTome-equipped Axio Imager Z1 (Carl Zeiss Microimaging). Images were then analyzed using ImageJ software, release 1.40g.

**Immunostaining of cell cultures.** Neuro2A cells expressing LAT or control cells were grown to confluence in two-chamber culture slides (BD Falcon, San Jose, CA). Culture slides were fixed for 10 min in ice-cold methanol, followed by 1 min in ice-cold acetone and finally blocked for 30 min in Dako Serum-Free Protein Block. Rat anti-mouse HVEM clone 10F3 antibody was incubated in protein block at 4°C overnight. After three rinses for 5 min each in phosphate-buffered saline (PBS), slides were incubated for 1 h at 25°C with Alexa Fluor-488 (Invitrogen, Carlsbad, CA). Slides were again washed three times with PBS, air dried, and mounted with Prolong Gold with DAPI mounting medium (Invitrogen). The fluorophores were imaged in separate channels with a Zeiss ApoTome-equipped Axio Imager Z1 (Carl Zeiss Microimaging). Images were then analyzed using ImageJ software, release 1.40g. Each experiment was repeated three times.

**Flow cytometry.** Neuro2A cells expressing LAT or control cells were grown to confluence, and the cells were harvested, washed, resuspended in fluorescence-activated cell sorting (FACS) buffer, and incubated for 15

min at 4°C with purified 2.4G2 antibody (Fc block; BD Biosciences, San Diego, CA), followed by subsequent incubation with phycoerythrin (PE)-HVEM antibody (eBioscience, San Diego, CA) at 4°C for 1 h and then by fixation with BD Cytotfix/Cytoperm solution for 20 min at 4°C. The cells were washed again and analyzed using FACScan instrumentation (Becton, Dickinson). The experiment was performed in duplicate.

**DNA extraction and PCR analysis for HSV-1 gB DNA.** DNA was isolated from homogenized individual TG using a commercially available DNeasy Blood and Tissue Kit (Qiagen, Stanford, CA) according to the manufacturer's instructions. PCR analyses was done using gB specific primers (forward, 5'-AACGCGACGCACATCAAG-3'; reverse, 5'-CTGGTACGCGATCAGAAAGC-3'; and probe, 5'-FAM-CAGCCGAGTAC-TACC-3', where FAM is 6-carboxyfluorescein). The amplicon length for this primer set is 72 bp. Relative copy numbers for the gB DNA were calculated using standard curves generated from the plasmid pAc-gB1. In all experiments glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of transcripts.

**RNA extraction, cDNA synthesis, and TaqMan RT-PCR.** TG from individual mice were collected on day 3, 5, or 30 p.i., immersed in RNAlater RNA stabilization reagent, and stored at -80°C until processing. LAT-expressing C1300 cells and Neuro2A cells as well as their controls were grown to confluence in six-well plates. QIAzol RNA reagent (Qiagen) and 1-bromo-2 chloropropane (BCP) were used to extract RNA from each well or individual TG. Total RNA extraction was carried out as we have described previously (40, 47). Following RNA extraction, 1,000 ng of total RNA was reverse transcribed using random hexamer primers and murine leukemia virus (MuLV) reverse transcriptase from a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's recommendations.

The differences in the mRNA expression levels of nectin-1, nectin-2, HVEM, PILR $\alpha$ , 3-O-sulfated heparin sulfate, NMHC-IIA, BTLA, and LIGHT were evaluated using commercially available TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA) with optimized primers as described below. In all experiments GAPDH was used for normalization of transcripts.

Primer probe sets consisted of two unlabeled PCR primers and the FAM dye-labeled TaqMan minor groove binder (MGB) probe formulated into a single mixture. All cellular amplicons included an intron-exon junction to eliminate signal from genomic DNA contamination. The assays used in this study were as follows: (i) HVEM, Mm00619239\_m1 (amplicon size, 65 bp); (ii) nectin-1, ABI Mm00445392\_m1 (amplicon size, 71 bp); (iii) nectin-2, ABI Mm00436144\_m1 (amplicon size, 65 bp); (iv) PILR $\alpha$ , ABI Mm00463324\_m1 (amplicon size, 77 bp); (v) heparin sulfate-3-O-sulfotransferase, ABI Mm00479621\_m1 (amplicon size, 65 bp); (vi) NMHC-IIA (Myh9), ABI Mm01197036\_m1 (amplicon size, 61 bp); (vii) LIGHT, ABI Mm00444567\_m1 (amplicon size, 68 bp); (viii) BTLA, ABI Mm00616981\_m1 (amplicon size, 71 bp); and (ix) GAPDH, ABI assay Mm999999.15\_G1 (amplicon length, 107 bp). Additionally, a custom-made primer and probe set was used for LAT as follows: forward primer, 5'-GGGTGGGCTCGTGTACAG-3'; reverse primer, 5'-GGACGGGTAAGTAAACAGAGTCTCTA-3'; and probe, 5'-FAM-ACACCAGC-CGGTCTTT-3' (amplicon length, 81 bp).

Quantitative real-time PCR (qRT-PCR) was performed using an ABI ViiA 7 Sequence Detection System (Applied Biosystems, Foster City, CA) in 384-well plates as we described previously (40, 47). Real-time PCR was performed in triplicate for each tissue sample. The threshold cycle ( $C_T$ ) values, which represent the PCR cycles at which there is a noticeable increase in the reporter fluorescence above baseline, were determined using SDS, version 2.2 software.

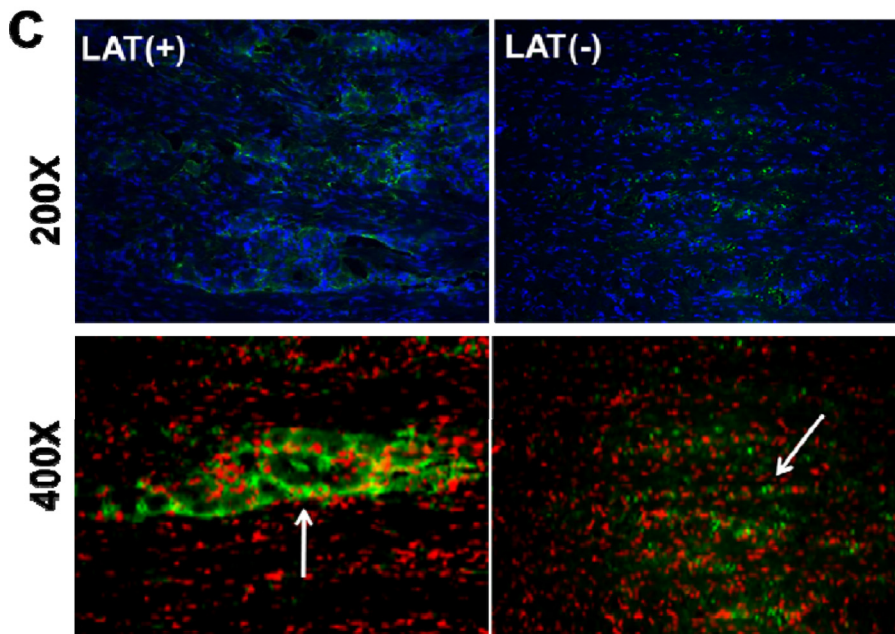
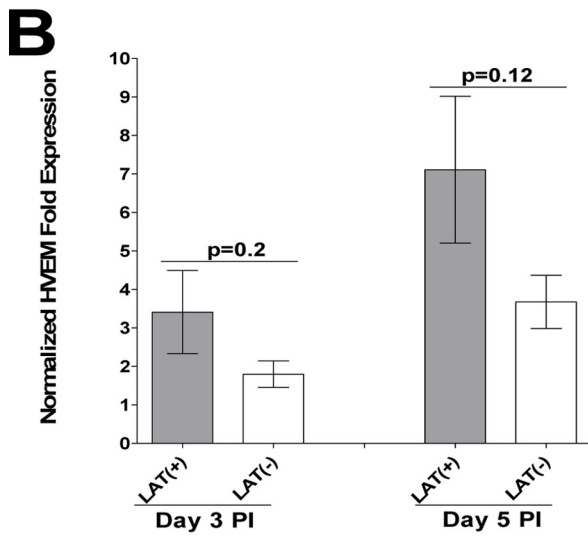
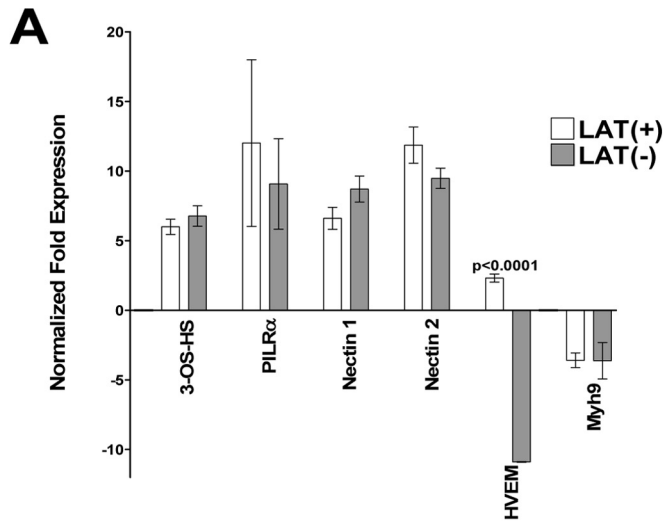
**Statistical analysis.** Student's *t* test and analysis of variance (ANOVA) were performed using the computer program Instat (GraphPad, San Diego, CA). Results were considered statistically significant at a *P* value of <0.05.

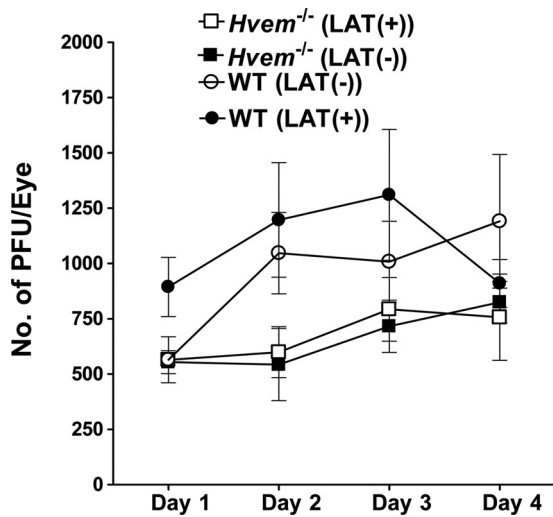
## RESULTS

**HSV-1 receptors and latency.** To investigate the role of HVEM during HSV-1 infection, we utilized a mouse model of viral latency following acute ocular infection with HSV-1 strain McKrae. This strain does not require corneal scarification for efficient ocular infection. We examined mRNA levels of HSV-1 receptors in wild-type (WT) C57BL/6 mice infected with wild-type HSV-1 strain McKrae [LAT(+)] or the McKrae-derived LAT(-) virus dLAT2903 (9). Quantitative RT-PCR analysis of mRNA levels in trigeminal ganglia (TG) at 30 days postinfection (p.i.), when latency is well established, revealed that HVEM mRNA depended on the presence of LAT (Fig. 1A) ( $P < 0.0001$ ). In LAT(+) virus-infected mice HVEM mRNA was increased over uninfected mice, while in LAT(-) virus-infected mice HVEM mRNA was decreased. There were no significant differences in the mRNA levels of nectin-1, nectin-2, 3-O-sulfated heparan sulfate (3-OS-HS), PILR $\alpha$ , or NMHC-IIA in LAT(+) versus LAT(-) virus-infected mice, with nectin-1, nectin-2, 3-OS-HS, and PILR $\alpha$  levels increasing relative to those in uninfected mice with both viruses while NMHC-IIA decreased. In contrast to latent infection, LAT had no statistically significant effect on HVEM mRNA levels during the acute phase of infection (days 3 and 5 p.i.) although there was a trend for increased HVEM mRNA with LAT(+) virus compared to LAT(-) virus (Fig. 1B) ( $P > 0.05$ ).

Immunohistochemical staining of HVEM in TG from mice latently infected with LAT(+) and LAT(-) viruses revealed distinctive patterns of HVEM expression between LAT(+) (Fig. 1C, left panels) and LAT(-) viruses (Fig. 1C, right panels). In LAT(+) TG, HVEM staining localized broadly to large cells with dim nuclei consistent with neurons (Fig. 1C, 200 $\times$ ). In contrast, HVEM staining in LAT(-) TG appeared more punctate and localized to smaller cells (Fig. 1C, 200 $\times$ ). In the bottom panels of Fig. 1C (400 $\times$ ) the HVEM signal appears localized to neurons in LAT(+) TG (bottom left panel), while this signal is considerably reduced and/or absent in LAT(-) TG (bottom right panel). These data suggest that LAT, or a LAT-induced cellular function, regulates the level and pattern of HVEM expression in TG of HSV-1 latently infected mice.

**Viral latency and reactivation in HVEM-deficient mice.** The impact of HVEM on the ability of LAT to increase the amount of latency was investigated in HVEM-deficient (*Hvem*<sup>-/-</sup>) mice. Replication levels of LAT(+) and LAT(-) HSV-1 strains in eyes during the first 4 days of infection were similar to each other and not significantly different between WT and *Hvem*<sup>-/-</sup> mice (Fig. 2). However, there was a trend toward decreased virus replication in *Hvem*<sup>-/-</sup> mice, suggesting that there may be some effect of HVEM on acute HSV-1 infection. This would be consistent with a recent study in which in a corneal scarification model of ocular HSV-1 infection, HVEM affected acute infection (48). The relative amount of latency on day 30 p.i. was determined by quantitative PCR (qPCR) using primers from the gB region of the HSV-1 genome. Consistent with previous reports (12, 49), there was significantly more HSV-1 DNA in TG from WT mice latently infected with LAT(+) virus than in those infected with LAT(-) virus (Fig. 3A, WT) ( $P < 0.0001$ ), which is characteristic of more latency with LAT(+) than LAT(-) virus in WT mice. Strikingly, *Hvem*<sup>-/-</sup> mice infected with LAT(+) virus had significantly fewer latent genomes than WT mice infected with LAT(+) virus (Fig. 3A) ( $P < 0.0001$ ). In fact, the amount of latency in LAT(+) virus-



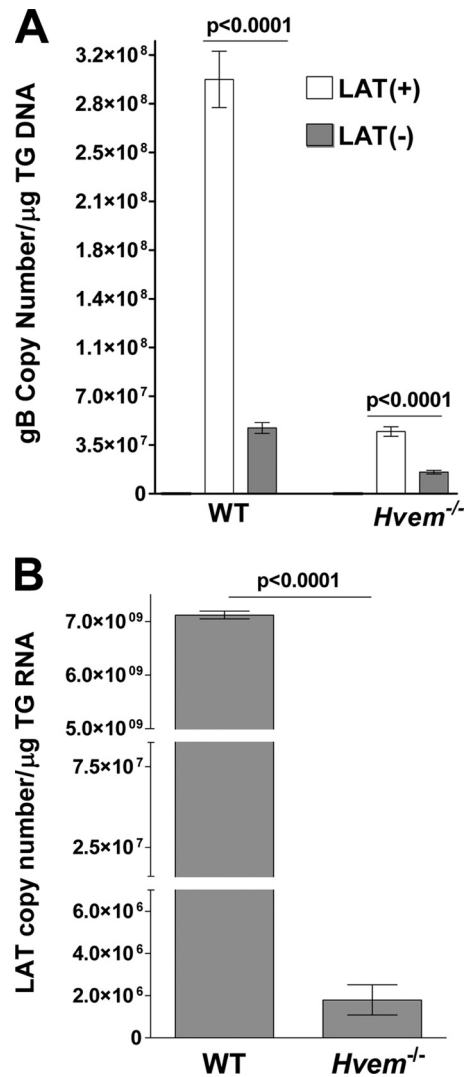


**FIG 2** Virus titers in WT and HVEM<sup>-/-</sup> eyes during primary ocular infection. WT C57BL/6 and C57BL/6 HVEM<sup>-/-</sup> mice were infected ocularly with LAT(+) or LAT(-) virus, and the amount of infectious HSV-1 in tear films was determined daily by standard plaque assays as described in Materials and Methods. For each time point, the virus titer (*y* axis) represents the average of the titers from 20 eyes  $\pm$  standard error of the mean.

infected *Hvem*<sup>-/-</sup> mice was similar to that in LAT(-) virus-infected WT mice. Even less latency was detected in *Hvem*<sup>-/-</sup> mice infected with LAT(-) virus than in WT mice infected with LAT(-) virus (Fig. 3A) ( $P < 0.0001$ ). Thus, HVEM appeared to play a role in increasing the amount of latency in TG of mice infected with both LAT(+) and LAT(-) viruses. As expected, since LAT(+) virus produced less latency, as judged by the number of viral genomes in *Hvem*<sup>-/-</sup> mice compared to that of WT mice, and since LAT levels during latency are related to the amount of latency, LAT(+) latently infected *Hvem*<sup>-/-</sup> mice also had less LAT than WT mice (Fig. 3B) ( $P < 0.0001$ ). These results suggest that HVEM and LAT both influence the amount of latency that is established and/or maintained.

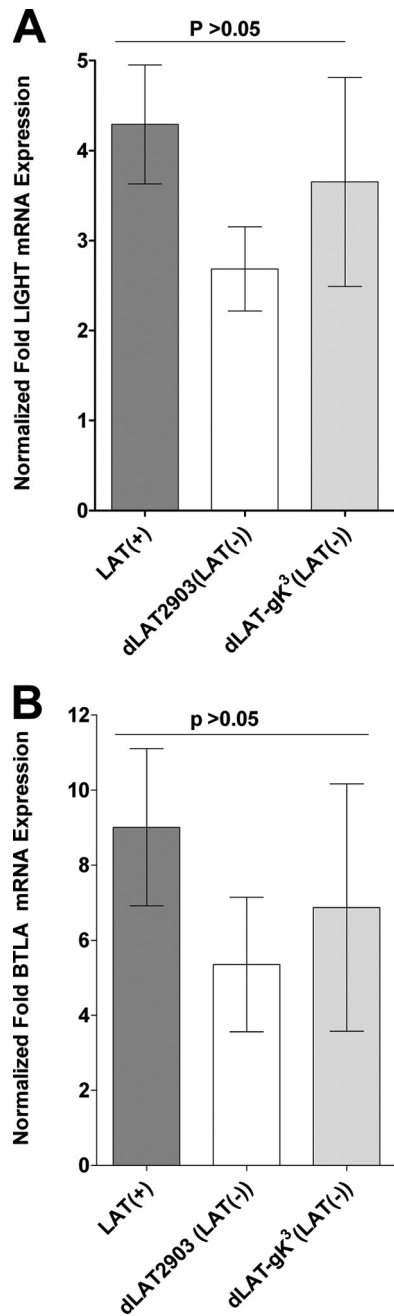
In contrast to the differences in the level of HVEM expression between LAT(+) and LAT(-) viruses (Fig. 1A), mRNA levels of LIGHT and BTLA were not significantly altered in WT mice latently infected with LAT(+) virus versus LAT(-) dLAT2903 or versus LAT(-) dLAT-gK<sup>3</sup> virus (Fig. 4A and B). We have previously shown that HVEM expression is independent of BTLA or LIGHT (34).

Although spontaneous reactivation from latency is too low to study in mice, induced reactivation is routinely analyzed by explanting individual TG into tissue culture medium and monitor-



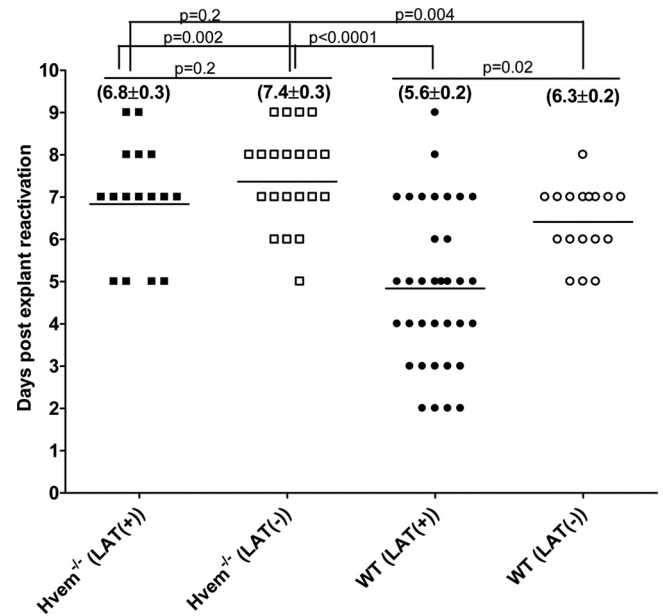
**FIG 3** Effect of LAT and HVEM on HSV-1 latency and reactivation in TG of latently infected mice. WT and HVEM<sup>-/-</sup> mice were ocularly infected with HSV-1 strain McKrae [LAT(+)] or dLAT2903 [LAT(-)] as described in the legend of Fig. 1. On day 30 p.i., TG were harvested from the latently infected surviving mice. Quantitative PCR and RT-PCR were performed on each individual mouse TG. In each experiment, an estimated relative copy number of gB or LAT was calculated using a standard curve generated from pGem-gB1 or pGEM-5317, respectively. Briefly, DNA template was serially diluted 10-fold such that 5  $\mu$ l contained from 10<sup>3</sup> to 10<sup>11</sup> copies of gB or LAT and then subjected to TaqMan PCR with the same set of primers. By comparing the normalized threshold cycle of each sample to the threshold cycle of the standard, the copy number for each reaction product was determined. GAPDH expression was used to normalize the relative expression of gB DNA in the TG. Each bar represents the mean  $\pm$  standard error of the mean from 56 TG for WT mice and from 20 TG for HVEM<sup>-/-</sup> mice.

**FIG 1** Effect of LAT on HVEM expression in TG of infected mice. (A) Effect of LAT on expression of HSV-1 receptors in latently infected mice. C57BL/6 mice were ocularly infected with HSV-1 strain McKrae [LAT(+)] or dLAT2903 [LAT(-)]; the TG from surviving mice were isolated individually on day 30 postinfection, and quantitative RT-PCR was performed using total RNA. Nectin-1, nectin-2, HVEM, PILR $\alpha$ , NMHC-IIA, and 3-O-sulfated heparin sulfate (3-OS-HS) expression in naive mice was used to estimate the relative expression of each transcript in TG. GAPDH expression was used to normalize the relative expression of each transcript in TG of latently infected mice. Each bar represents the mean  $\pm$  standard error of the mean from 20 TG. (B) Expression of HVEM in TG of WT infected mice during primary infection. C57BL/6 mice were infected ocularly with McKrae [LAT(+)] or dLAT2903 [LAT(-)], and expression of HVEM in TG was determined on days 3 and 5 p.i. as described above. GAPDH expression was used to normalize the relative expression of each transcript in TG of latently infected mice. Each point represents the mean  $\pm$  standard error of the mean from 10 TG. (C) Upregulation of HVEM in TG of mice infected with LAT(+) virus. C57BL/6 mice were infected as described above. At 30 days p.i., TG from mice latently infected as indicated were isolated and stained with HVEM antibody as described in Materials and Methods. Nuclei are stained with DAPI (blue), and HVEM is stained in green. With LAT(+) virus infection, staining appears mostly at the surface of large cells (arrow), likely neurons. With LAT(-) virus infection, staining is mostly of small nonneuronal-like cells (arrow). Magnifications are indicated at the right of the panels.



**FIG 4** Effect of LAT on LIGHT and BTLA expression in TG of latently infected WT mice. WT C57BL/6 mice were ocularly infected with HSV-1 strain McKrae [LAT(+)], dLAT2903 [LAT(-)], or dLAT-gK<sup>3</sup> [LAT(-)]. TG were isolated individually on day 30 postinfection, and quantitative RT-PCR was performed using total RNA. LIGHT and BTLA expression in naive WT mice was used to estimate the relative expression of each transcript in TG. GAPDH expression was used to normalize the relative expression of each transcript in TG of latently infected mice. Each point represents the mean  $\pm$  standard error of the mean from 8 TG.

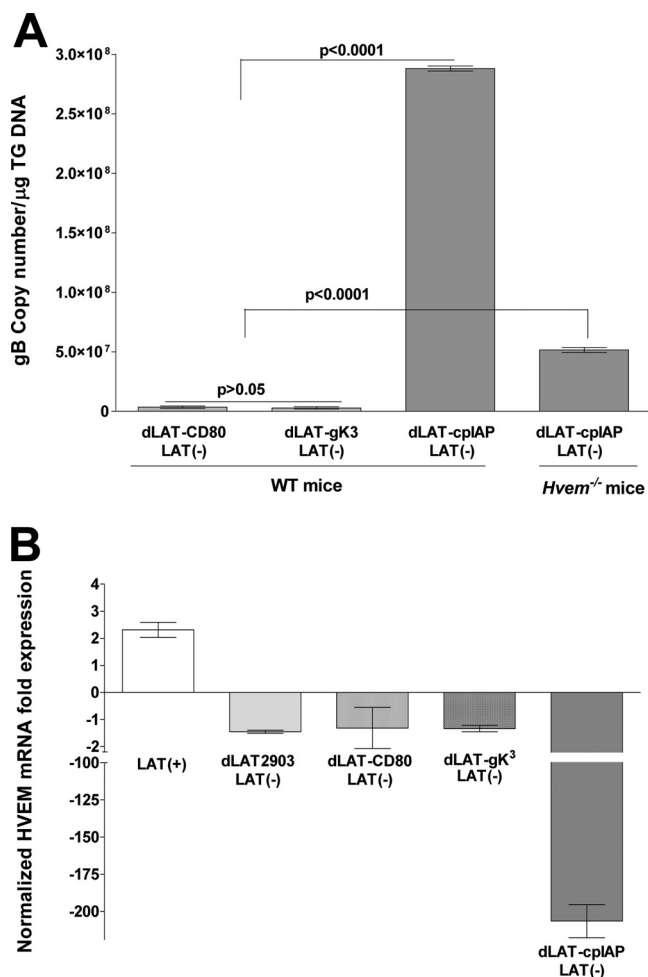
ing the time required for production of infectious virus (9, 49–52). Consistent with previous studies, the time to reactivation in WT mice was significantly shorter with LAT(+) virus than with LAT(-) virus ( $5.6 \pm 0.2$  days versus  $6.3 \pm 0.2$  days;  $P = 0.02$ ) (Fig. 5). The time to reactivation was significantly delayed in



**FIG 5** Effect of HVEM on kinetics of induced reactivation in explanted TG from latently infected mice. At 30 days postinfection individual TG were harvested from HVEM<sup>-/-</sup> or WT mice. Each individual TG was incubated in tissue culture medium, and a 10- $\mu$ l aliquot was removed from each culture daily and used to infect RS cell monolayers for 10 days, as described in Materials and Methods. The RS cells were monitored daily for the appearance of cytopathic effect for up to 5 days to determine the time of first appearance of reactivated virus from each TG. The results are plotted as the number of TG that reactivated daily. Numbers indicate the average time that the TG from each group first showed cytopathic effect  $\pm$  standard error of the mean. For each group, 20 TG from 10 mice were used.

Hvem<sup>-/-</sup> mice [ $6.8 \pm 0.3$  days with LAT(+) virus,  $P = 0.002$ ;  $7.4 \pm 0.3$  days with LAT(-) virus,  $P = 0.004$ ]. Although in Hvem<sup>-/-</sup> mice LAT(+) virus appeared to reactivate faster than LAT(-) virus, this difference did not reach statistical significance ( $P = 0.2$ ). The alterations in latency and reactivation in Hvem<sup>-/-</sup> mice were largely independent of significant immunopathogenesis, as monitored by corneal scarring at day 30 p.i. or by mouse survival (data not shown).

**Mechanisms involved in LAT-HVEM regulation.** To define the mechanism of LAT-HVEM regulation, we utilized recombinant HSV-1 in which LAT is replaced with genes involved in cell survival or immune modulation. Mice were infected with HSV-1 containing either the antiapoptosis gene from *Cydia pomonella* granulosis virus (dLAT-cpiAP) (15), the CD80 T cell activating coreceptor (dLAT-CD80) (unpublished data), or, as a control, the HSV-1 envelope glycoprotein gK (dLAT-gK<sup>3</sup>) (40). The amount of latency as judged by qPCR of viral DNA in mice latently infected with dLAT-cpiAP was similar to that of wild-type HSV-1 (compare Fig. 3A and 6A). This was expected since we previously showed that this virus has a WT [LAT(+)] reactivation phenotype (15). In contrast, dLAT-gK<sup>3</sup> and dLAT-CD80 did not support wild-type levels of latent virus (Fig. 6A) ( $P < 0.0001$ ) and, like LAT(-) virus, dLAT-gK<sup>3</sup> and dLAT-CD80 did not upregulate HVEM mRNA (Fig. 6B). In Hvem<sup>-/-</sup> mice dLAT-cpiAP had reduced latency, similar to LAT(+) virus in Hvem<sup>-/-</sup> mice (compare Fig. 3A and 6A). However, in contrast to LAT(+) virus, dLAT-cpiAP did not upregulate HVEM mRNA levels in latently



**FIG 6** Effect of recombinant viruses expressing foreign genes in place of LAT on latency and HVEM expression. (A) gB DNA. WT C57BL/6 and C57BL/6-HVEM<sup>-/-</sup> mice were ocularly infected with dLAT-cpIAP. As controls, some of the WT mice were similarly infected with dLAT-CD80 or dLAT-gK<sup>3</sup>. On day 30 postinfection, TG were harvested from the latently infected surviving mice, and quantitative PCR was performed on each individual mouse TG. In each experiment, an estimated relative copy number of gB was calculated using standard curves. GAPDH expression was used to normalize the relative expression of gB DNA in the TG. Each point represents the mean  $\pm$  standard error of the mean from 10 TG. (B) HVEM mRNA. C57BL/6 mice were ocularly infected with the HSV-1 McKrae [LAT(+)] strain or the LAT(-) dLAT2903, dLAT-CD80, dLAT-gK<sup>3</sup>, or dLAT-cpIAP strain; the TG of surviving mice were isolated individually on day 30 postinfection, and quantitative RT-PCR was performed using total RNA. HVEM expression in naive mouse TG was used to estimate the relative expression of HVEM transcript in TG of infected mice. GAPDH expression was used to normalize the relative expression of each transcript in TG of latently infected mice. Each point represents the mean  $\pm$  standard error of the mean from 10 TG.

infected WT mice. In fact, dLAT-cpIAP appeared to drastically reduce HVEM mRNA (Fig. 6B). These results suggest that LAT had a direct effect on HVEM mRNA levels, rather than the effects on HVEM mRNA being the result of an increased latent viral load in TG with LAT(+) compared to LAT(-) viruses.

The increased HVEM mRNA levels in LAT(+) virus-infected mice, but not those of other receptor mRNAs, prompted us to investigate whether LAT could regulate HVEM expression in the absence of other viral genes. HVEM mRNA levels were analyzed

by qRT-PCR in two neuroblastoma lines, C1300 and Neuro2A, that stably express LAT (43, 44). In both LAT(+) cell lines (Fig. 7A and B) HVEM mRNA expression was significantly upregulated compared to cell lines containing the empty vector suggesting a direct effect of LAT on HVEM gene expression. To estimate relative HVEM protein levels, the Neuro2A cells were stained with mouse HVEM antibody. There appeared to be more HVEM-positive cells in the LAT(+) than in the LAT(-) cell line (Fig. 7C). In addition, more high-intensity HVEM-positive cells were also detected in the LAT(+) than in the LAT(-) cell line using flow cytometry (Fig. 7D). Thus, LAT appeared to upregulate expression of HVEM in neuronal-derived C1300 and Neuro2A cells in the absence of other viral genes.

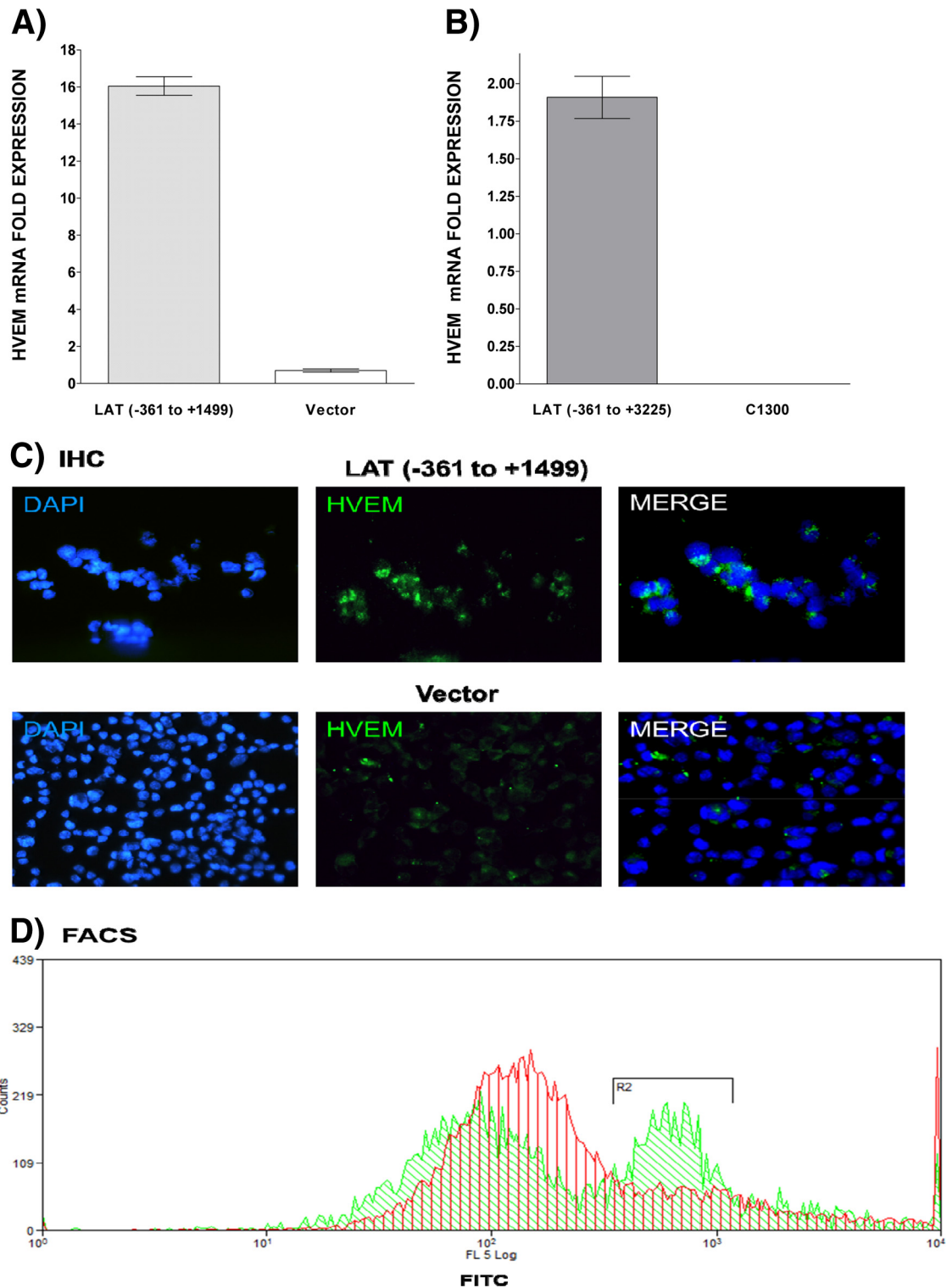
Previously, we showed that two small noncoding RNAs (sncRNAs) (38) that do not appear to be miRNAs and that are located within the region of LAT involved in the spontaneous reactivation phenotype and the blocking of apoptosis (the first 1.5 kb of LAT) affect both viral infection and apoptosis (45). Neuro2A cells were transfected with sncRNA1 or sncRNA2 as we described previously (45) and harvested at 8, 12, 24, and 48 h posttransfection. HVEM expression in empty vector-transfected control cells was used to normalize the relative expression of HVEM. Both sncRNA1 and sncRNA2 transiently increased HVEM mRNA expression at 8 and 12 h posttransfection, with sncRNA2 having a greater effect at 8 h than sncRNA1 (Fig. 8).

## DISCUSSION

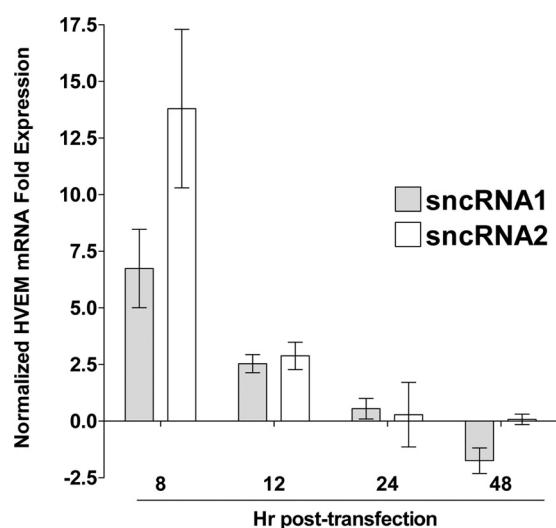
During HSV-1 latency, LAT is the only viral gene product consistently detected in abundance in infected mice, rabbits, and humans (1, 3, 5, 6, 10, 53). LAT is important for high, WT levels of spontaneous (9) and induced (10) HSV-1 reactivation from latency. The results presented here indicate that the HSV-1 LAT gene targets HVEM in its capacity to help establish and maintain viral latency. Our results using an HSV-1 mouse ocular infection model indicate that LAT manipulates HVEM expression, which in turn increases virus latency and enhances the latency-reactivation cycle in the trigeminal ganglia. Moreover, HVEM appears essential to maintaining a normal immune signature in the TG, suggesting its importance for host immunity during latency. These results indicate that LAT-HVEM forms a critical pathogen-host axis contributing to viral latency.

Little is known regarding a role of HSV-1 entry receptors in latency and reactivation and the role that LAT may play in this process. In contrast to the other known entry routes for HSV-1 (19–23), HVEM mRNA levels significantly increased in a LAT-dependent fashion in latently infected TG of normal mice. This finding is surprising given the lesser role HVEM plays in viral entry in mucosa, brain, and, as shown here, the ocular infection route. The upregulation of HVEM by LAT(+) virus appeared to be a result of LAT's expression rather than an increase in viral load in the TG during latency or a result of increased unapparent spontaneous reactivation with LAT(+) versus LAT(-) viruses. This conclusion is based on several lines of reasoning. First, the dLAT-cpIAP mutant virus, which establishes latency and reactivates in the same way as LAT(+) virus (15), did not increase HVEM levels. This result suggests that the upregulation of HVEM function is unique and specific to LAT. Second, cell lines stably expressing LAT had increased HVEM levels compared to control cell lines. Third, in transient-transfection experiments, plasmids expressing either of the two LAT sncRNAs (38, 45) significantly upregulated





**FIG 7** Effect of LAT on HVEM expression *in vitro*. (A and B) HVEM mRNA is upregulated in the presence of LAT *in vitro*. C1300 (A) and Neuro2A (B) cells expressing LAT nt -361 to +3225 and -361 to +1499, respectively, were grown to confluence, and quantitative RT-PCR was performed using total RNA. HVEM expression in vector-only control cells was used to estimate the relative expression of HVEM mRNA. GAPDH expression was used to normalize the relative expression. Each bar represents the mean  $\pm$  standard error of the mean from three independent experiments. (C and D) HVEM protein is upregulated in the presence of LAT *in vitro*. Neuro2A cells expressing LAT -361 to +1499 (top) or vector without HSV-1 LAT (bottom) were grown to confluence, stained with HVEM antibody, and subjected to immunohistochemistry (IHC) (C) or FACS (D) analyses as described in Materials and Methods. Nuclei are stained with DAPI (blue). HVEM is shown in green. FACS of Neuro2A cells expressing LAT or containing empty vector. Cells were stained and gated for HVEM, and results are shown as an overlay. Green represents LAT, and red represents an empty vector.



**FIG 8** Effect of LAT sncRNAs on HVEM expression *in vitro*. Neuro2A cells were transfected with sncRNA1 or sncRNA2, and expression of HVEM mRNA was determined as described above. HVEM expression in untransfected control cells was used to normalize the relative expression of HVEM. GAPDH expression was used to normalize relative expression. Each bar represents the mean  $\pm$  standard error of the mean from three independent experiments.

HVEM mRNA levels. Thus, LAT was able to upregulate HVEM expression, independently of other viral factors.

To date, no LAT-encoded protein that regulates the latency-reactivation cycle has been identified, suggesting that LAT regulates the latency-reactivation cycle by exerting its effect as an RNA molecule rather than by directing production of a protein. The HSV-1 LAT locus includes several microRNAs, at least two of which affect expression of a viral protein (54). However, these microRNAs all map outside the first 1.5 kb of the primary 8.3-kb LAT transcript, which is the region of LAT that we previously demonstrated was both sufficient and required for LAT's ability to enhance the reactivation phenotype in mouse or rabbit models of infection (9, 55, 56). Thus, these microRNAs are unlikely to be involved in enhancing latency/reactivation in these animal models. In contrast, we identified two small noncoding RNAs (sncRNAs) that are located within the first 1.5 kb of LAT (38, 45). These LAT sncRNAs do not appear to be microRNAs, based on their sizes and their predicted structures. In this report we show that following transient transfection, both of these sncRNAs can independently upregulate expression of HVEM mRNA. In addition, the RNAhybrid algorithm (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid>) predicts interaction between the mouse HVEM promoter and both of the LAT sncRNAs. The analysis suggests that LAT sncRNA1 can interact with the HVEM promoter at position 493 in the forward direction while sncRNA2 can interact with the HVEM promoter in the reverse direction at position 87. These results suggest a direct impact of LAT RNA on HVEM expression.

Both LAT and HVEM directly contribute to cell survival within their respective contexts. The LAT region plays a role in blocking apoptosis of infected cells in rabbits (11) and mice (12) and in human cells (11). The antiapoptosis activity appears to be a critical function of LAT involved in enhancing the latency-reactivation cycle because the LAT(−) virus can be restored to a full wild-type reactivation phenotype by substitution of different prosurvival/antiapoptosis genes (i.e., baculovirus inhibitor of apoptosis pro-

tein gene [cpIAP] and FLIP [cellular FLICE-like inhibitory protein]) (13, 14). HVEM activation by BTLA or LIGHT contributes to survival of chronically stimulated effector T cells *in vivo* (36, 57). Both LIGHT and BTLA induce HVEM to activate NF- $\kappa$ B (RelA) transcription factors known to enhance survival of activated T cells (34, 58). Furthermore, the LAT sncRNAs can stimulate NF- $\kappa$ B-dependent transcription in the presence of the RNA sensor, RIG-I (59). HVEM, like its related tumor necrosis factor receptor superfamily (TNFRSF) paralogs, utilizes TNF receptor-associated factor 2 (TRAF2) and cellular IAPs as part of the ubiquitin E3 ligases that regulate NF- $\kappa$ B activation pathways (60–62). cpIAP, an ortholog of the cellular IAP E3 ligases (63), and cFLIP, an NF- $\kappa$ B-regulated antiapoptosis gene (64), mimic the activated HVEM signaling pathway. These results lead us to suggest that in addition to upregulating HVEM expression, LAT also promotes active HVEM signaling.

Our results indicate that HVEM signaling plays a significant role in HSV-1 latency. We found that the level of latent viral genomes of LAT(+) virus in *Hvem*<sup>−/−</sup> mice compared to that of WT mice was significantly reduced. Similarly, reactivation of latent virus in TG explant cultures was also significantly reduced in *Hvem*<sup>−/−</sup> mice compared to levels in WT mice, demonstrating that HVEM is a significant factor in increasing HSV-1 latency and reactivation. However, differential replication and spread in the eye and possibly the reactivation efficiencies may influence these results. We found that, in contrast to increasing HVEM expression, LAT did not significantly alter LIGHT or BTLA mRNA levels. This is consistent with the idea that LIGHT and BTLA expression occurs in immune cells in the microenvironment of the latently infected cell and is therefore not affected by LAT expression in latently infected neurons.

We have previously shown that LAT functions as an immune evasion gene (49, 65), as an antiapoptosis gene (11), and as an inhibitor of productive infection (45). All three of these LAT functions would seemingly contribute to enhancing HSV-1 latency and the HSV-1 reactivation phenotype. The results reported here suggest that these important LAT functions contribute to LAT increasing expression of HVEM in latently infected neurons. The results presented here identify HVEM as an important target of LAT that influences latency, reactivation, and survival of ganglion-resident T cells. We found that HVEM is upregulated by two LAT sncRNAs and that in the absence of HVEM (i.e., in *Hvem*<sup>−/−</sup> mice), HSV-1 latency and reactivation significantly decreased. This result suggests that increasing HVEM above a threshold level by LAT leads to more efficient binding of HSV-1 gD to HVEM in the latent microenvironment and therefore enhances HSV-1 latency and reactivation. HSV-1 targets the HVEM pathway by at least two distinct mechanisms—at entry by direct interaction with gD and in latency through LAT-dependent transcriptional regulation—suggesting that HVEM is a critical node of selective pressure in alphaherpesvirus evolution. This concept may apply to other herpesviruses based on the observations that human cytomegalovirus encodes an HVEM-like ortholog (UL144) that specifically engages BTLA (24, 66).

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