Title

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Journal

Journal of Virology, 92(24)

ISSN

0022-538X

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Publication Date

2018-12-15

DOI

10.1128/jvi.01451-18

Peer reviewed

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

PATHOGENESIS AND IMMUNITY

Herpes Simplex Virus 1 Latency and the Kinetics of Reactivation Are Regulated by a Complex Network of Interactions between the Herpesvirus Entry Mediator, Its Ligands (gD, BTLA, LIGHT, and CD160), and the Latency-Associated Transcript

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ABSTRACT Recently, we reported that the herpesvirus entry mediator (HVEM; also called TNFRSF14 or CD270) is upregulated by the latency-associated transcript (LAT) of herpes simplex virus 1 (HSV-1) and that the absence of HVEM affects latency reactivation but not primary infection in ocularly infected mice. gD has been shown to bind to HVEM. LIGHT (TNFSF14), CD160, and BTLA (B- and T-lymphocyte attenuator) also interact with HVEM and can interfere with HSV gD binding. It was not known if LIGHT, CD160, or BTLA affected the level of latency reactivation in the trigeminal ganglia (TG) of latently infected mice. To address this issue, we ocularly infected LIGHT$^{-/-}$, CD160$^{-/-}$, and BTLA$^{-/-}$ mice with LAT(+) and LAT(−) viruses, using similarly infected wild-type (WT) and HVEM$^{-/-}$ mice as controls. The amount of latency, as determined by the levels of gB DNA in the TG of the LIGHT$^{-/-}$, CD160$^{-/-}$, and BTLA$^{-/-}$ mice infected with either LAT(+) or LAT(−) viruses, was lower than that in WT mice infected with LAT(+) virus and was similar in WT mice infected with LAT(−) virus. The levels of LAT RNA in HVEM$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and BTLA$^{-/-}$ mice infected with LAT(+) virus were similar and were lower than the levels of LAT RNA in WT mice. However, LIGHT$^{-/-}$, CD160$^{-/-}$, and BTLA$^{-/-}$ mice, independent of the presence of LAT, had levels of reactivation similar to those of WT mice infected with LAT(+) virus. Faster reactivation correlated with the upregulation of HVEM transcript. The LIGHT$^{-/-}$, CD160$^{-/-}$, and BTLA$^{-/-}$ mice had higher levels of HVEM expression, and this, along with the absence of BTLA, LIGHT, or CD160, may contribute to faster reactivation, while the absence of each molecule, independent of LAT, may have contributed to lower latency. This study suggests that, in the absence of competition with gD for binding to HVEM, LAT RNA is important for WT levels of latency but not for WT levels of reactivation.

IMPORTANCE The effects of BTLA, LIGHT, and CD160 on latency reactivation are not known. We show here that in BTLA, LIGHT, or CD160 null mice, latency is reduced; however, HVEM expression is upregulated compared to that of WT mice, and this upregulation is associated with higher reactivation that is independent of LAT but dependent on gD expression. Thus, one of the mechanisms by which BTLA, LIGHT,

Received 22 August 2018 **Accepted** 25 September 2018

Accepted manuscript posted online 3 October 2018

Citation Wang S, Ljubimov AV, Jin L, Pfeffer K, Kronenberg M, Ghiasi H. 2018. Herpes simplex virus 1 latency and the kinetics of reactivation are regulated by a complex network of interactions between the herpesvirus entry mediator, its ligands (gD, BTLA, LIGHT, and CD160), and the latency-associated transcript. J Virol 92:e01451-18. https://doi.org/10.1128/JVI.01451-18.

Editor Jae U. Jung, University of Southern California

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and CD160 null mice enhance reactivation appears to be the increased expression of HVEM in the presence of gD. Thus, our results suggest that blockade of HVEM-LIGHT-BTLA-CD160 contributes to reduced HSV-1 latency and reactivation.

KEYWORDS ocular, latency, reactivation, virus replication, corneal scarring, eye diseases

Herpes simplex virus 1 (HSV-1) has the ability to infect most cell types, possibly due to the presence of at least seven known receptors, including herpesvirus entry mediator (HVEM) as well as nectin-1, nectin-2, 3-O-sulfated heparan sulfate (3-OS-HS), paired immunoglobulin-like type 2 receptor (PILRα), nonmuscle myosin heavy chain IIA (NMHC-IIA), and myelin-associated glycoprotein (MAG) (1–11). The role of HSV-1 receptors has been studied in detail *in vitro* (5, 6, 12), and we also have examined the roles of these seven HSV-1 receptors in latency reactivation (13). HVEM, which is expressed in many tissues (14), was originally identified as the receptor for the virion envelope glycoprotein D (gD) of HSV-1 (6). Physiologically, HVEM has been shown to bind cellular ligands derived from two distinct lineages: the Ig superfamily member B and T lymphocyte attenuator (BTLA) (15, 16) and the tumor necrosis factor (TNF)-related cytokines LIGHT (TNFSF14), lymphotoxin-α (LT-α), CD160 (17, 18), and synaptic adhesion-like molecule 5 (SALM5) (19). The binding of LIGHT or LT-α to HVEM provides a costimulatory signal, whereas the binding of BTLA or CD160 to HVEM sends a coinhibitory signal (20) and SALM5 limits inflammation in the central nervous system (CNS) via its interaction with HVEM (19). BTLA negatively regulates T cell activation (18, 21–25). LIGHT is a potent costimulatory molecule that is expressed predominantly on the surface of activated dendritic cells and T cells, and its upregulation enhances T cell-mediated immunity and promotes autoimmune disease and inflammation (17, 26–29). CD160 is essential for natural killer-mediated gamma interferon (IFN-γ) production (30) and, through HVEM, stimulates innate immunity in the intestine (31).

The HVEM interactions with these immune factors regulate proinflammatory and inhibitory signaling (23) and activate NF-κB survival programs (32). The HVEM pathway normally acts as a communication node that promotes homeostasis during immune responses. Thus, the interaction of gD with HVEM may not only promote HSV-1 internalization but also affect the course of HSV-1 infection. During HSV-1 infection, the interaction of HSV-1 gD with HVEM mimics the BTLA-HVEM interaction (33), whereas LIGHT and CD160 engage different sites on the HVEM molecule (18, 20, 21, 34). It has been demonstrated that gD competes with the cellular ligands for binding to HVEM and that this downregulates HVEM expression on the surface of infected cells (35), whereas latency-associated transcript (LAT) of HSV-1 enhances HVEM expression (13).

The delicate balance of T cell activation and establishment of latency reactivation via the BTLA-LIGHT-CD160-HVEM pathway has not been examined in the context of latent herpesvirus infection and LAT. Although HVEM has been shown to play a role in HSV-1 latency reactivation (13), very little is known regarding the potential roles of LIGHT, BTLA, and CD160 in HSV-1 infectivity. To test whether LIGHT, BTLA, or CD160 influences primary or latent HSV-1 infection, we compared the effects of ocular infection of LIGHT$^{-/-}$, BTLA$^{-/-}$, CD160$^{-/-}$, HVEM$^{-/-}$, and wild-type (WT) mice with LAT(+) and LAT(−) viruses. We have found that, compared to WT mice infected with LAT(+) virus, there were lower levels of latency in the LIGHT, BTLA, and CD160 null mice, and these lower levels occurred independently of infection with LAT(+) and LAT(−) viruses. In marked contrast to the absence of HVEM or LAT, however, the absence of LIGHT, BTLA, and CD160 reduced the time of reactivation, independent of LAT, to that of WT mice infected with LAT(+) virus. Upregulation of HVEM in the TG of LIGHT$^{-/-}$, BTLA$^{-/-}$, or CD160$^{-/-}$ mice was independent of the presence of LAT. Thus, this study defines a novel link between HVEM, its ligands (i.e., gD, BTLA, LIGHT, and CD160), and LAT during latent infection but not primary infection. The schematic diagram of our overall model of latency reactivation with regard to the LAT-gD-HVEM-BTLA-LIGHT-CD160 nexus is summarized in Fig. 10.

RESULTS

Virus replication in the eyes of infected mice. To assess the effects of HVEM ligands BTLA, LIGHT, and CD160 on primary virus replication in the eye, we used a set of knockout mice with global gene deficiencies that included BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice as well as WT and HVEM$^{-/-}$ mice as controls. The mice were ocularly infected with 2×10^5 PFU/eye of LAT(+) or LAT(−) virus as described in Materials and Methods. To determine if the HVEM cellular ligands affect HSV-1 replication during primary ocular infection, we collected tear films from 20 eyes/group daily on day 1 to day 5 postinfection (p.i.), and the amount of virus in each eye was determined using a standard plaque assay. We found previously that replication of HSV-1 in the eyes of ocularly infected HVEM$^{-/-}$ mice was not affected by the presence or absence of LAT (13). To determine if LAT has any effect on HSV-1 replication during primary ocular infection in the absence of HVEM ligands, we compared the viral titers associated with primary infection with LAT(+) or LAT(−) viruses. The viral titers after LAT(+) or LAT(−) infection were not significantly different in either BTLA$^{-/-}$ mice (Fig. 1A, $P > 0.05$) or CD160$^{-/-}$ mice (Fig. 1C, $P > 0.05$). In contrast, in LIGHT$^{-/-}$-infected mice, the virus titers on days 2 and 3 p.i. were significantly lower in the LAT(−)-infected mice than in the LAT(+)-infected mice (Fig. 1B, $P < 0.05$). As we reported previously (13), we did not detect any differences in virus titers between LAT(+)- and LAT(−)-infected HVEM$^{-/-}$ mice (Fig. 1D, $P > 0.05$) or LAT(+)- and LAT(−)-infected WT mice (Fig. 1E, $P > 0.05$). Collectively, these results confirm that similar to HVEM, BTLA and CD160 are not required for primary infection and the mechanism does not require LAT expression. In contrast, the data suggest that LIGHT contribute to viral replication during primary infection and that the underlying mechanism is LAT dependent.

Viral latency in BTLA-, LIGHT-, and CD160-deficient mice. To investigate the potential effects of BTLA, LIGHT, or CD160 on latency, we repeated the above-described experiment but harvested the TG on day 28 p.i. and assessed latency based on expression of gB as determined by quantitative PCR (qPCR) analysis using primers from the gB region of the HSV-1 genome. Consistent with previous reports and the higher levels of latency associated with infection of WT mice with LAT(+) virus (13, 36, 37), there was significantly more HSV-1 gB DNA in the TG from WT mice infected with LAT(+) than LAT(−) virus (Fig. 2, WT, $P < 0.0001$). Also, consistent with our previous report, there were significantly fewer latent genomes in the TG of the HVEM$^{-/-}$ mice infected with LAT(+) virus than in the TG of WT mice infected with LAT(−) virus (Fig. 2, HVEM$^{-/-}$, $P < 0.0001$). Indeed, the amount of latency in LAT(+)-infected HVEM$^{-/-}$ mice was similar to that observed in LAT(−)-infected WT mice and the levels of latency detected in HVEM$^{-/-}$ mice infected with LAT(−) virus were even lower than those found in WT mice infected with LAT(−) virus (Fig. 2, $P < 0.0001$). Similarly, the amounts of latency in the TG of LAT(+)-infected BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice were significantly lower than that in LAT(+)-infected WT mice (Fig. 2, $P < 0.0001$) and similar to those found in LAT(−)-infected WT mice (Fig. 2, $P > 0.3$). There were, however, no significant differences between the levels of latency associated with infection with LAT(+) virus or LAT(−) virus in the BTLA$^{-/-}$ (Fig. 2, BTLA$^{-/-}$, $P = 0.6$), LIGHT$^{-/-}$ (Fig. 2, LIGHT$^{-/-}$, $P = 0.3$), and CD160$^{-/-}$ (Fig. 2, CD160$^{-/-}$, $P = 0.7$) mice. Thus, BTLA, LIGHT, and CD160 appear to play a role in decreasing the amount of latency in the TG of mice independently of the LAT status of the virus possibly due to reduce competition with gD for binding to HVEM.

As LAT is the only gene product consistently detected in abundance during latency in HSV-1-infected mice, rabbits, and humans (38–42), we also analyzed the TG obtained at day 28 after ocular infection with LAT(+) virus for expression of LAT RNA. Total RNA was analyzed using quantitative reverse transcription-PCR (qRT-PCR) with LAT primers as described in Materials and Methods. LAT(+) latently infected HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice all had lower levels of LAT RNA than LAT(+) latently infected WT mice (Fig. 3, $P < 0.0001$). This was consistent with the above-described data showing lower levels of latency, as

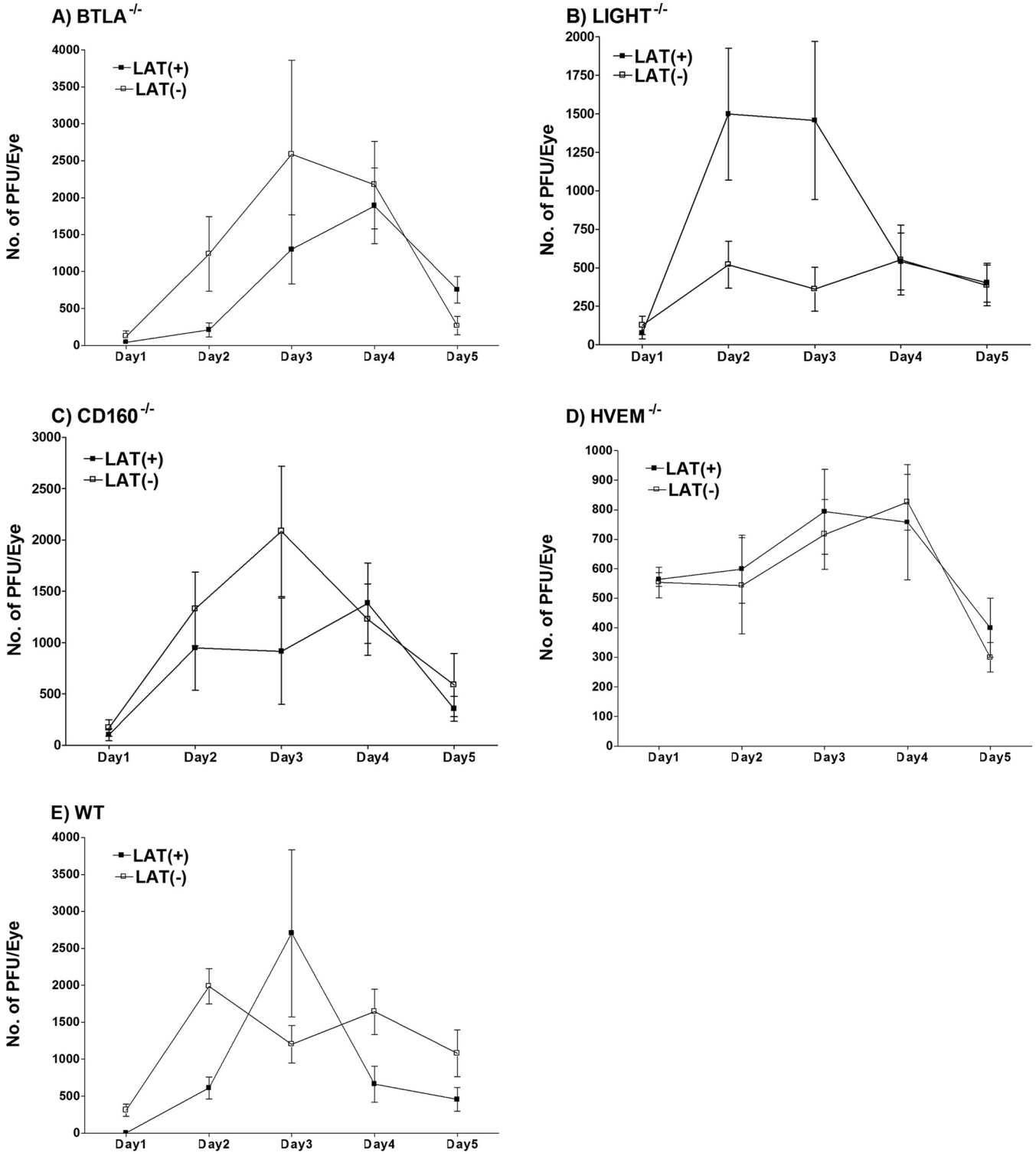

FIG 1 Virus titers in eyes following ocular infection of mice. BTLA$^{-/-}$ (A), LIGHT$^{-/-}$ (B), CD160$^{-/-}$ (C), HVEM$^{-/-}$ (D), and WT (E) mice were infected ocularly with LAT(+) or LAT(−) virus, and the amount of infectious HSV-1 in tear films was determined daily by standard plaque assays as described in Materials and Methods. For each time point, the virus titer (*y* axis) represents the average of the titers from 20 eyes ± standard errors of the means (SEM).

judged by the number of viral genomes (Fig. 2), and the reports that LAT levels during latency are related to the amount of latency (37, 43, 44). Moreover, similar to the results of analysis of gB DNA (Fig. 2, above), there were no significant differences in the levels of LAT RNA among the HVEM$^{-/-}$-, BTLA$^{-/-}$-, LIGHT$^{-/-}$-,

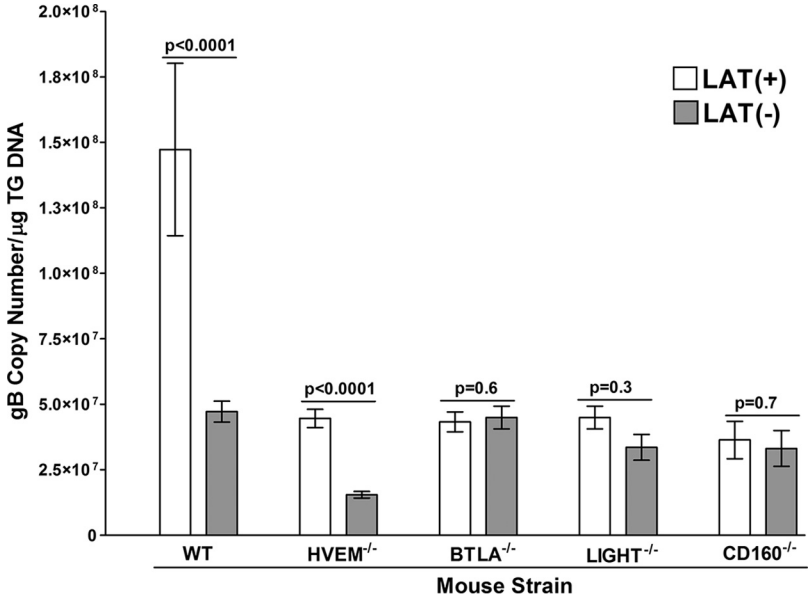

FIG 2 Effects of BTLA, LIGHT, CD160, and HVEM on the levels of gB in TG of latently infected mice. WT, BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and HVEM$^{-/-}$ mice were ocularly infected with LAT(+) or LAT(−) virus as described for Fig. 1. On day 28 pi, TG were harvested from the surviving latently infected mice. qPCR was performed on each individual mouse TG. In each experiment, an estimated relative copy number of gB was calculated with standard curves generated using pAC-gB1 (89). Briefly, the DNA template was serially diluted 10-fold so that 5 μl contained from 10^3 to 10^{11} copies of gB 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 was determined. GAPDH expression was used to normalize the relative expression of gB DNA in the TG. Each point represents the means ± SEM from 20 TG.

and CD160$^{-/-}$-infected mice (Fig. 3, $P > 0.05$). These results suggest that, similar to the absence of HVEM or LAT, the absence of BTLA, LIGHT, or CD160 influences the amount of latency that is established and/or maintained. Thus, these results suggest that for WT levels of latency, the presence of HVEM, BTLA, LIGHT, or CD160

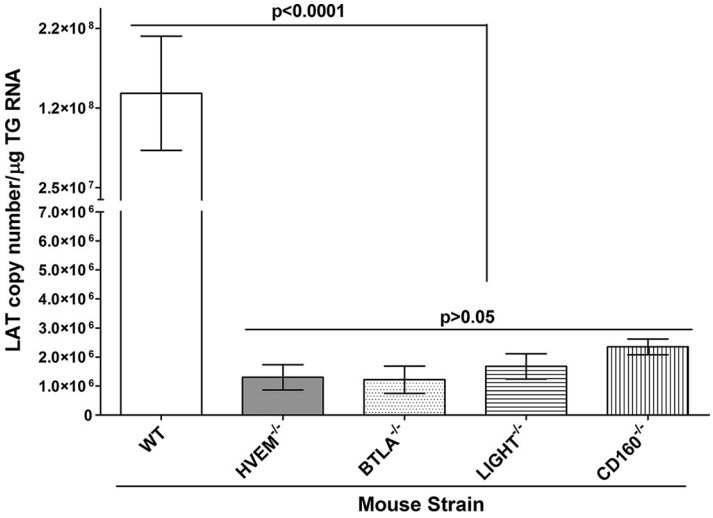

FIG 3 Effects of BTLA, LIGHT, CD160 and HVEM on the levels of LAT in TG of latently infected mice. BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, HVEM$^{-/-}$, and WT mice were ocularly infected with LAT(+) virus as described for Fig. 1. On day 28 p.i., TG were harvested from the surviving latently infected mice. qRT-PCR was performed on each individual mouse TG. In each experiment, an estimated relative copy number of LAT was calculated using standard curves generated from pGem-LAT5317. Briefly, the DNA template was serially diluted as described for Fig. 2, and the copy number for each reaction was determined. GAPDH expression was used to normalize the relative expression of LAT RNA in the TG. Each point represents the means ± SEM from 20 TG.

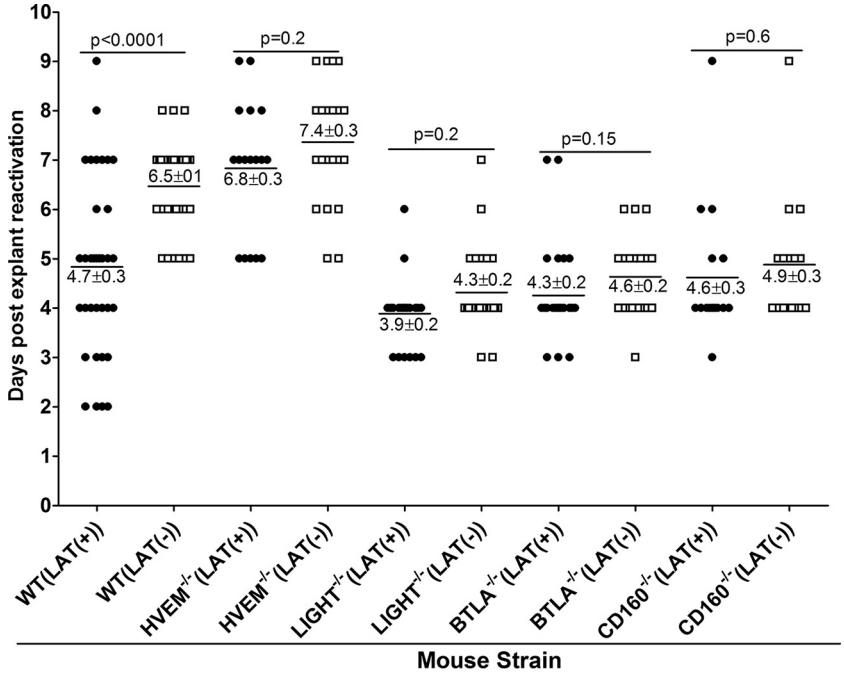

FIG 4 Effect of LAT and TNF superfamily genes on the kinetics of induced reactivation in explanted TG from latently infected mice. WT, BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and HVEM$^{-/-}$ mice were infected ocularly with LAT(+) or LAT(−) virus as described for Fig. 1. Twenty-eight days p.i., individual TG were harvested from infected mice. Each individual TG was incubated in 1.5 ml of tissue culture media at 37°C. Aliquots of media were removed from each culture daily for up to 10 days and plated on indicator cells (RS cells) to assess the appearance of reactivated virus. The results are plotted as the number of TG that reactivated daily. Numbers indicate the average time at which the TG from each group first showed CPE ± SEM. Reactivation is based on 37 TG for WT mice infected with LAT(+) virus, 33 TG for wt mice infected with LAT(−) virus, 18 TG for HVEM$^{-/-}$ mice infected with LAT(+) virus, 22 TG for HVEM$^{-/-}$ mice infected with LAT(−) virus, 27 TG for LIGHT$^{-/-}$ mice infected with LAT(+) virus, 25 TG for LIGHT$^{-/-}$ mice infected with LAT(−) virus, 27 TG for BTLA$^{-/-}$ mice infected with LAT(+) virus, 23 TG for BTLA$^{-/-}$ mice infected with LAT(−) virus, 16 TG for CD160$^{-/-}$ mice infected with LAT(+) virus, and 17 TG for CD160$^{-/-}$ mice infected with LAT(−) virus.

is required. However, an effect of the absence of BTLA, LIGHT, or CD160 on latency similar to that of HVEM is immune mediated, whereas the changes in the level of reactivation are receptor mediated.

Explant reactivation in TG of latently infected mice. It has been shown that the amount of latency as determined by qRT-PCR or qPCR of viral RNA or DNA, respectively, in total TG extracts is approximately 3-fold higher in WT mice infected with LAT(+) versus LAT(−) virus and that this affects the level of reactivation (37, 44–47). We therefore tested whether the lower latency in the TG of HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice affected the timing of explant reactivation from latency. TG were obtained from mice that were ocularly infected with LAT(+) or LAT(−) virus as described above and virus reactivation analyzed by explanting individual TG from infected mice on day 28 p.i. as described in Materials and Methods. As we reported previously, upon infection with LAT(+) virus, the times of reactivation were significantly slower in HVEM$^{-/-}$ mice than in WT mice [Fig. 4; WT infected with LAT(+)- versus HVEM$^{-/-}$-infected mice, $P < 0.001$] and were similar to the times found in WT mice infected with LAT(−) virus [Fig. 4; WT infected with LAT(−)- versus HVEM$^{-/-}$-infected mice, $P > 0.05$]. Surprisingly, the time to reactivation was significantly shorter for both LAT(+)-infected and LAT(−)-infected LIGHT$^{-/-}$ mice [Fig. 4; LIGHT$^{-/-}$, 3.9 ± 0.2 days for LAT(+) versus 4.3 ± 0.2 days for (LAT(−), $P = 0.2$], BTLA$^{-/-}$-infected mice [Fig. 4, BTLA$^{-/-}$, 4.3 ± 0.2 days for LAT(+) versus 4.6 ± 0.2 days for LAT(−), $P = 0.15$], and CD160$^{-/-}$-infected mice [Fig. 4, CD160$^{-/-}$, 4.6 ± 0.3 days for LAT(+) versus 4.9 ± 0.3 days for LAT(−), $P = 0.2$]. Although in BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$

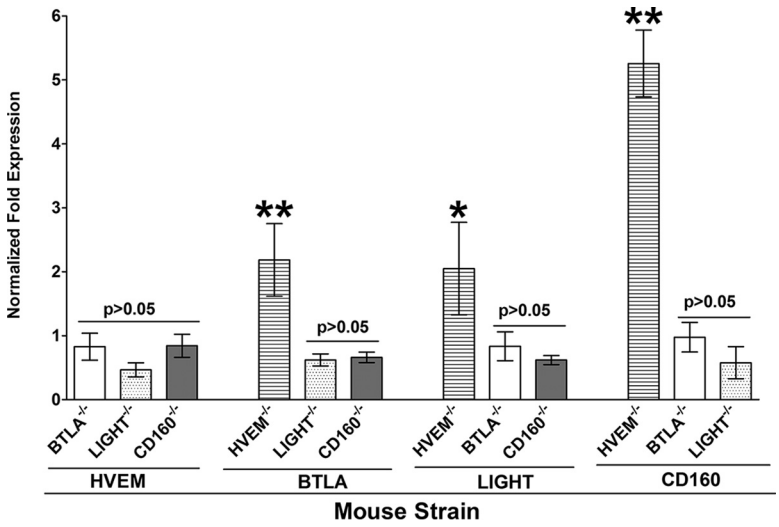

FIG 5 Expression of HVEM, LIGHT, BTLA, and CD160 in TG of uninfected knockout mice. TG from WT, BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and HVEM$^{-/-}$ mice were harvested, RNA from each TG was isolated, and qRT-PCR was performed on each individual mouse TG. The levels of expression of HVEM, LIGHT, BTLA, and CD160 transcripts in TG of each knockout mouse strain were normalized to the level of each transcript in the TG of WT mice and are shown as fold increase or decrease compared to the levels in the WT mice. GAPDH expression was used to normalize the relative expression of each transcript in the TG of infected mice. Each point represents the means ± SEM from 10 TG. Asterisks indicate significant difference: *, $P < 0.05$; **, $P < 0.01$.

mice the duration of reactivation in both LAT(+) and LAT(−) mice were similar to that of WT mice infected with LAT(+) virus (Fig. 2, $P > 0.5$), all were significantly lower than those in WT mice infected with LAT(−) virus (Fig. 2, $P < 0.001$). Finally, BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice infected with LAT(+) or LAT(−) virus had lower duration of reactivation than HVEM$^{-/-}$ mice infected with LAT(+) virus (Fig. 2, $P < 0.001$).

Thus, in contrast to WT and HVEM$^{-/-}$ mice, in BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice the time to explant reactivation did not correlate with the amount of latency that was established, and similar to the latency results, the duration of reactivation was not affected by the absence of LAT. Overall, these results suggest that only the absence of HVEM correlated with the level of latency reactivation.

Effects of the absence of HVEM, BTLA, LIGHT, or CD160 on expression of the other molecules in the TG of uninfected mice. To determine whether knockout of HVEM, BTLA, LIGHT, or CD160 affected the expression of the other molecules, TG from uninfected naive HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and WT mice were isolated, and HVEM, BTLA, LIGHT, and CD160 mRNA expression was determined using qRT-PCR analysis. The results are presented as fold change compared to the baseline mRNA levels in the TG from uninfected WT mice (Fig. 5). There were no significant differences in the levels of HVEM mRNA among BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice (Fig. 5, HVEM, $P > 0.05$). However, the levels of BTLA mRNA were significantly higher in the HVEM$^{-/-}$ mice than in LIGHT$^{-/-}$ or CD160$^{-/-}$ mice (Fig. 5, BTLA, $P < 0.001$), the levels of LIGHT mRNA were significantly higher in HVEM$^{-/-}$ mice than in BTLA$^{-/-}$ or CD160$^{-/-}$ mice (Fig. 5, LIGHT, $P < 0.05$), and the levels of CD160 mRNA were significantly higher in HVEM$^{-/-}$ mice than in BTLA$^{-/-}$ or LIGHT$^{-/-}$ mice (Fig. 5, CD160, $P < 0.001$). Collectively, these results indicate that the absence of BTLA, LIGHT, or CD160 expression did not affect the expression of HVEM or of the other genes, whereas the absence of HVEM increased BTLA, LIGHT, and CD160 expression. That is, HVEM expression influences the expression of BTLA, LIGHT, or CD160, but the absence of expression of BTLA, LIGHT, or CD160 does not influence the expression of HVEM in uninfected mice.

Effect of LAT on BTLA, LIGHT, and CD160 expression in TG of latently infected mice. We have shown previously that HSV-1-associated upregulation of HVEM is LAT

dependent both *in vivo* and *in vitro* (13). Thus, to determine if LAT affects BTLA, LIGHT, or CD160 expression, the TG were harvested at day 28 p.i. from mice ocularly infected with LAT(+) or LAT(−) virus, total RNA extracted, and qRT-PCR carried out to determine HVEM, BTLA, LIGHT, and CD160 mRNA expression. The results are presented as fold change compared to the baseline mRNA levels in TG from uninfected naive mice of each strain (Fig. 6). In WT mice, as we reported previously, the HVEM levels were significantly lower in mice infected with LAT(−) than in those infected with LAT(+) virus (Fig. 6A, WT, $P < 0.001$). The levels of BTLA, LIGHT, and CD160 were higher after HSV-1 infection, and there were no statistically significant differences between the expression levels after LAT(+) and LAT(−) infection (Fig. 6A). Furthermore, there were no significant differences in the levels of BTLA, CD160, or LIGHT between LAT(+)- and LAT(−)-infected HVEM$^{-/-}$ mice (Fig. 6B). Similarly, the presence or absence of LAT did not affect the level of CD160, HVEM, or LIGHT in BTLA$^{-/-}$-infected mice (Fig. 6C, $P > 0.05$), the level of BTLA, CD160, or HVEM in LIGHT$^{-/-}$-infected mice (Fig. 6D), or the level of BTLA, HVEM, or LIGHT in CD160$^{-/-}$-infected mice (Fig. 6E). Thus, in contrast to the HVEM levels in WT mice, LAT does not alter LIGHT, BTLA, or CD160 expression in WT mice or HVEM expression in BTLA$^{-/-}$, LIGHT$^{-/-}$, or CD160$^{-/-}$ mice, LIGHT expression in HVEM$^{-/-}$, BTLA$^{-/-}$, or CD160$^{-/-}$ mice, BTLA expression in HVEM$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice, or CD160 expression in HVEM$^{-/-}$, BTLA$^{-/-}$, or LIGHT$^{-/-}$ mice. Overall, LAT only affected the expression of HVEM in the TG of latently infected WT mice. Thus, the effect of LAT on HVEM upregulation is not required in mice deficient for BTLA, LIGHT, or CD160 due to their reduced competition with gD for binding to HVEM.

CD4, CD8, PD-1, Tim-3, IL-2, IL-21, IFN-γ, and TNF-α mRNA levels during HSV-1 latency in knockout mice. T cell exhaustion is affected by the presence of LAT (37, 48). Thus, we estimated the effects of LAT, HVEM, LIGHT, BTLA, and CD160 on the levels of expression of mRNAs that are considered markers of T cell subtypes (CD4 and CD8) or exhaustion (PD-1, T cell immunoglobulin and mucin domain-containing protein-3 [Tim-3], interleukin-21 [IL-21], IL-2, IFN-γ, and TNF-α). The TG were harvested on day 28 p.i. from the mice, and total RNA was extracted and analyzed using qRT-PCR as described in Materials and Methods. The results are presented as fold change compared to the baseline mRNA levels in TG from uninfected naive mice for each strain of mouse (Fig. 7). Comparison of the mRNA levels in the TG of mice infected with LAT(+) and LAT(−) viruses indicated that during latency there were significantly higher levels of all markers in the LAT(+)-infected WT mice than in the LAT(−)-infected mice (Fig. 7, WT, $P < 0.001$). The levels of CD8 mRNA (Fig. 7, $P < 0.05$), but not those of the other markers (Fig. 7, $P > 0.05$), were significantly higher in the BTLA$^{-/-}$ and CD160$^{-/-}$ mice infected with LAT(+) virus than in those infected with LAT(−) virus. In HVEM$^{-/-}$ mice, there were no significant differences in IL-2, Tim-3, IFN-γ, and TNF-α mRNA levels between the LAT(+)- and LAT(−)-infected mice (Fig. 7, $P > 0.05$). However, the reduction in the levels of CD4, CD8, PD-1, and IL-21 mRNA relative to baseline observed on infection of the mice with LAT(+) virus was significantly lower in the mice infected with LAT(−) virus (Fig. 7, $P < 0.001$). In LIGHT$^{-/-}$ mice, the levels of markers of mRNAs were not significantly different between LAT(+)- and LAT(−)-infected mice, except for those of Tim-3 and IFN-γ, which were lower in the LAT(−)-infected mice (Fig. 7, LIGHT$^{-/-}$, $P < 0.05$). Overall, except for WT mice, our results suggest that alteration of the immune infiltrates in the TG of latently infected HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, or CD160$^{-/-}$ mice did not correlate with the level of latency or duration of reactivation. Thus, the observed differences in the level of latency reactivation between different strains of mice could be a receptor-ligand function rather than an immune function.

Detection of gC and gD transcripts in the TG of latently infected mice. Detection of viral transcripts other than LAT in latently HSV-1-infected mouse and human trigeminal ganglia is always difficult, but Kramer and Coen (49), using RT-PCR, found transcripts of the HSV-1 ICP4 and thymidine kinase (tk) genes in latently infected TG. Feldman et al. (50) confirmed the ICP4 and tk gene expression by *in situ* hybridization and RT-PCR and also detected glycoprotein C (gC) mRNA in the TG of latently infected

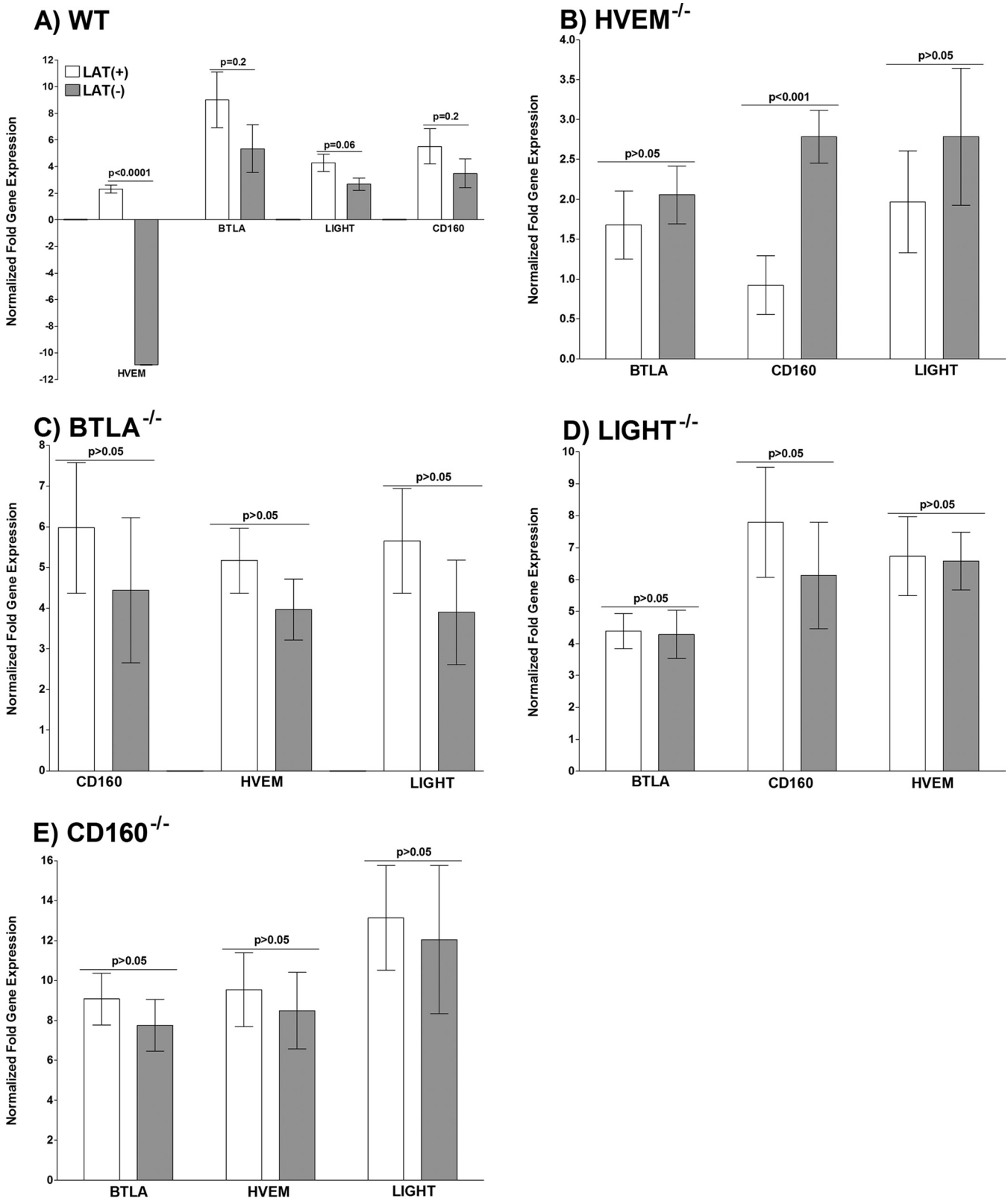

FIG 6 Effect of LAT on HVEM, BTLA, LIGHT, and CD160 expression in TG of latently infected knockout mice. WT (A), HVEM$^{-/-}$ (B), BTLA$^{-/-}$ (C), LIGHT$^{-/-}$ (D), and CD160$^{-/-}$ (E) mice were ocularly infected with LAT(+) or LAT(−) virus. TG were isolated individually on day 28 p.i., and qRT-PCR was performed using total RNA. BTLA, LIGHT, HVEM, and CD160 expression in naive mice for each strain 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 TGs of latently infected mice. Each point represents the means ± SEM from 10 TG. Asterisks indicate significant difference: *, $P < 0.05$; **, $P < 0.01$.

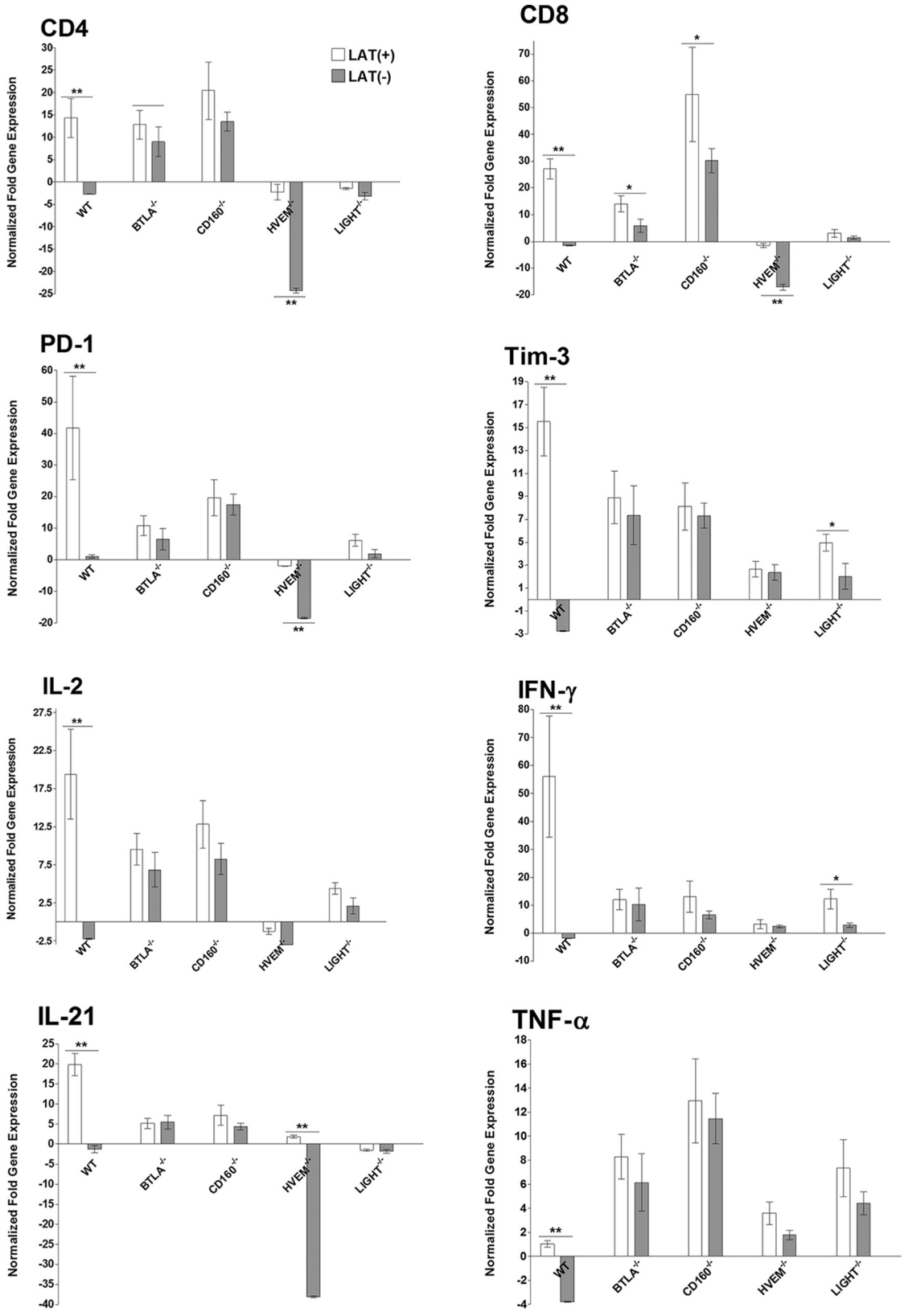

FIG 7 Effect of LAT, BTLA, LIGHT, CD160, and HVEM on T cell exhaustion in the TG of latently infected knockout mice. TG from latently infected WT, BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and HVEM$^{-/-}$ mice, as described for Fig. 1, were individually isolated on day 28 p.i., and qRT-PCR was performed using total RNA as described in Materials and Methods. CD4, CD8, PD-1, Tim-3, IL-2, IFN-γ, IL-21, and TNF-α expression in naive mice from each line was used as a baseline to estimate the relative expression of each transcript in TG of latently infected mice. GAPDH expression was used to normalize the relative expression of each

(Continued on next page)

mice. Furthermore, viral proteins were detected in the TG of latently infected mice (37, 50–52). Feldman et al. (50) used the term "spontaneous molecular reactivation" to describe this detection of subclinical reactivation in the absence of detectable infectious virus in the TG of latently infected mice. Both ICP0 and ICP4 transcripts were detected in human TG (53), whereas no viral proteins have been detected in HSV-1 latently infected human ganglia. However, analyses of latently infected TG have shown that neuron-interacting CD8 T cells are directed to at least 11 viral proteins in mice (54) and 14 viral proteins in humans, and that ICP47 and VP16 proteins in human TG are recognized by CD4 T cells (55). Since both gC protein and gC mRNA were detected in TG of latently infected mice (37, 50), the presence of gC transcript on day 28 p.i. was determined by qRT-PCR using primers from the gC region of the HSV-1 genome. As expected, we detected the gC transcript in TG of all infected mice (Fig. 8A). There were no significant differences in the levels of gC transcripts in LAT(+)- versus LAT(−)-infected WT (Fig. 8A, $P = 0.4$), HVEM$^{-/-}$ (Fig. 8A, $P = 0.2$), or BTLA$^{-/-}$ (Fig. 8A, $P = 0.4$) mice. CD160$^{-/-}$ mice infected with LAT(+) virus had higher gC expression than CD160$^{-/-}$ mice infected with LAT(−) virus (Fig. 8A, $P = 0.05$), while LIGHT$^{-/-}$ mice infected with LAT(+) virus had significantly lower gC expression than CD160$^{-/-}$ mice infected with LAT(−) virus (Fig. 8A, $P = 0.04$). Overall, LAT(+)-infected HVEM$^{-/-}$ mice had lower gC copy numbers than LAT(+)-infected WT, BTLA$^{-/-}$, CD160$^{-/-}$, or LIGHT$^{-/-}$ mice.

In contrast to gC, the presence of gD transcripts or protein in latently infected TG has not been reported in either mice or humans. Due to its binding to HVEM and possible competition with BTLA, LIGHT, and CD160, we examined the presence of gD transcripts in the TG of WT and knockout mice infected with LAT(+) and LAT(−) viruses as described above. The presence of gD transcript on day 28 p.i. was determined by qRT-PCR using primers from the gD region of the HSV-1 genome. We detected the gD transcript in all infected mice (Fig. 8B). There were no significant differences in the levels of gD transcripts among WT, HVEM$^{-/-}$, BTLA$^{-/-}$, and CD160$^{-/-}$ mice infected with LAT(+) virus (Fig. 8B, $P > 0.05$). The levels of gD transcripts, however, were significantly higher in the TG of the infected LIGHT$^{-/-}$ mice than those in the infected HVEM$^{-/-}$, BTLA$^{-/-}$, and CD160$^{-/-}$ groups and in WT mice infected with LAT(−) virus (Fig. 8B, $P < 0.05$) but were not significantly different from those of WT mice infected with LAT(+) virus (Fig. 8B, $P > 0.05$). There were no significant differences in the levels of gD in the TG from mice infected with LAT(+) compared to those infected with LAT(−) virus in any of the groups (Fig. 8B, WT, $P = 0.4$). After 40 cycles of RT-PCR we did not detect any gC or gD expression in uninfected HVEM$^{-/-}$, BTLA$^{-/-}$, CD160$^{-/-}$, LIGHT$^{-/-}$, or WT mice, and these negative data from all groups are shown as the uninfected control (Fig. 8A and B, uninfected). Thus, the levels of gC and gD transcripts in TG of all infected mice were significantly higher than those in the TG of uninfected mice (Fig. 8A and B, $P < 0.05$). These results demonstrate that gC and gD transcripts are present in the TG of the latently infected mice. In addition, the levels of gD transcripts were higher in TG of infected mice than the levels of gC transcripts in the same mice. This is consistent with a previous report that gD level was significantly higher than gC level (56).

Effects of HVEM, BTLA, LIGHT, and CD160 on survival and corneal scarring (CS) in ocularly infected mice. The survival of mice was recorded at day 28 p.i. There were no significant differences in the survival of WT, HVEM$^{-/-}$, and BTLA$^{-/-}$ mice irrespective of infection with LAT(+) or LAT(−) virus (Table 1, $P > 0.7$ by Fisher exact test). Although LIGHT$^{-/-}$ and CD160$^{-/-}$ mice infected with LAT(+) virus appeared to be more susceptible to infection than their counterparts infected with LAT(−) virus or WT,

FIG 7 Legend (Continued)
transcript. Each point represents the means ± SEM from 10 TG. CD4, CD8, PD-1, Tim-3, IFN-γ, IL-2, IL-21, and TNF-α transcripts are indicated in the figure. A single asterisk indicates significant difference ($P < 0.05$) between LAT(+) (white bar) and LAT(−) (gray bar) viruses; double asterisks indicate significant difference ($P < 0.01$) between LAT(+) (white bar) and LAT(−) (gray bar) viruses. ns, not significant.

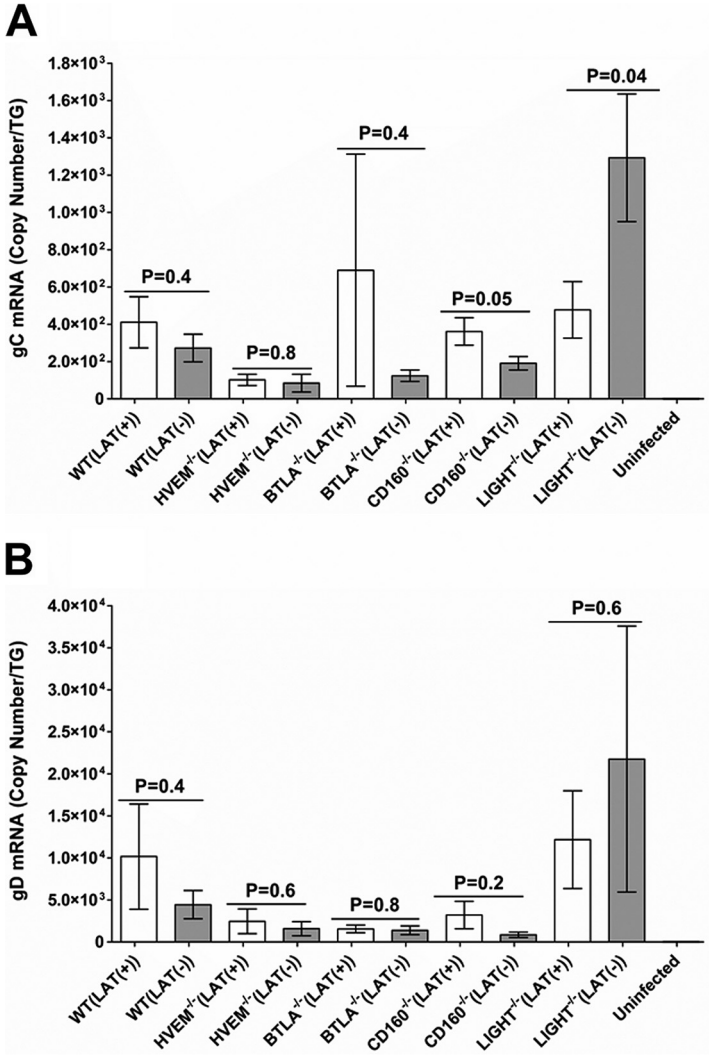

FIG 8 Detection of gC and gD mRNAs in TG of latently infected mice. RNA isolated from mice described in the legend to Fig. 7 were used to detect gC and gD mRNAs in TG of latently infected mice, and mRNA from TG of uninfected mice for each group was used as a negative control. qRT-PCR was performed on each individual mouse TG. In each experiment, an estimated relative copy number of gC and gD was calculated using standard curves generated from pAC-gC1 (92) and pAC-gD1 (93), respectively. Briefly, DNA template was serially diluted as described for Fig. 3, and the copy number for each reaction was determined. GAPDH expression was used to normalize the relative expression of gC and gD RNA in the TG. Each point represents the means ± SEM from 10 TG, and the data are presented as copy number per TG. (A) gC transcript; (B) gD transcript.

HVEM$^{-/-}$, and BTLA$^{-/-}$ mice, these differences were not statistically significant (Table 1, $P > 0.05$ by Fisher exact test). Higher mortality in LIGHT$^{-/-}$ and CD160$^{-/-}$ mice infected with LAT(+) virus than in mice infected with LAT(−) virus could be due to higher virus replication in the eye of LAT(+)-infected mice (Fig. 1).

TABLE 1 Survival of ocularly infected WT, HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice[a]

Virus	No. of mice surviving/total no. (%)				
	WT	HVEM$^{-/-}$	BTLA$^{-/-}$	LIGHT$^{-/-}$	CD160$^{-/-}$
McKrae [LAT(+)]	45/48 (94)	29/31 (93)	27/29 (93)	18/24 (75)	24/29 (83)
dLAT2903 [LAT(−)]	36/40 (90)	21/23 (91)	25/26 (96)	20/21 (95)	26/26 (100)
P value	0.7	1.0	1.0	0.1	0.05

[a]WT, HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice used throughout the studies were ocularly infected with 2×10^5 PFU/eye of McKrae [LAT(+)] or dLAT2903 [LAT(−)] virus, and survival was determined 28 days p.i.

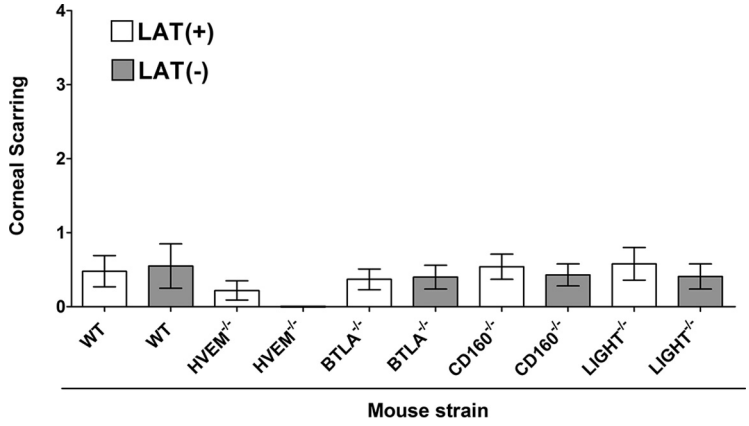

FIG 9 Corneal scarring (CS) in surviving ocularly infected mice. WT, HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice were ocularly infected with 2×10^5 PFU/eye of virus and survival determined 28 days p.i. (Table 1). The CS in all infected mice that survived ocular infection was assessed on day 28 p.i. based on a scale of 0 (no scarring) to 4 (severe ulcer). CS is based on means ± SEM from 90 eyes for WT mice infected with LAT(+) virus, 72 eyes for WT mice infected with LAT(−) virus, 58 eyes for HVEM$^{-/-}$ mice infected with LAT(+) virus, 42 eyes for HVEM$^{-/-}$ mice infected with LAT(−) virus, 54 eyes for LIGHT$^{-/-}$ mice infected with LAT(+) virus, 50 eyes for LIGHT$^{-/-}$ mice infected with LAT(−) virus, 36 eyes for BTLA$^{-/-}$ mice infected with LAT(+) virus, 40 eyes for BTLA$^{-/-}$ mice infected with LAT(−) virus, 48 eyes for CD160$^{-/-}$ mice infected with LAT(+) virus, and 52 eyes for CD160$^{-/-}$ mice infected with LAT(−) virus.

During primary infection, we did not observe any detectable differences in the level of blepharitis or dermatitis between HVEM$^{-/-}$, BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and WT mice infected with LAT(+) or LAT(−) virus (not shown). The severity of corneal scarring in surviving mice on day 28 postinfection was scored in a masked fashion using a 0 to 4 scale as described in Materials and Methods. As shown in Fig. 9, no corneal scarring was found in HVEM$^{-/-}$ mice infected with LAT(−) virus. Corneal scarring was found in HVEM$^{-/-}$ mice infected with LAT(+) virus as well as BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and WT control mice infected with either LAT(+) or LAT(−) virus, and there were no significant differences in the extent of corneal scarring among these groups of mice (Fig. 9, $P > 0.5$). Thus, neither the lack of BTLA, LIGHT, or CD160 nor the presence or the absence of LAT appeared to have a major effect on HSV-1-induced mouse survival or corneal scarring.

DISCUSSION

Following infection with HSV, the virus establishes latency in the sensory neurons of an infected host (40–43, 57). In both rabbit and human, latent virus spontaneously reactivates, travels back to the original site of infection, and causes recurrent disease in which free virus can be detected in the tears (58–62). In mice, however, spontaneous reactivation of HSV-1 does not occur, although subclinical reactivation is suggested by reports of very low levels of lytic transcripts and proteins in the latently infected ganglia (37, 49, 50). HSV-1 LAT is consistently detected in abundance during latency and in the neurons of infected humans, mice, and rabbits (38–42). It plays a major role in the establishment of latency reactivation, and we and others have shown an approximately 3-fold increase of latency in the TG of WT mice infected with LAT(+) virus compared with mice infected with LAT(−) virus (37, 38, 44, 46). Previously, we have shown that LAT enhances HVEM expression *in vivo* and *in vitro* (13). HVEM expression *in vitro* was increased in a C1300 cell line expressing LAT −361 to +3225 and in a Neuro2A cell line expressing LAT −361 to +1499. We mapped the HVEM upregulation region of LAT to HSV-1 genomic nucleotides (nt) 119756 to 119717 and 120145 to 120179 using small noncoding RNA1 (sncRNA1) and sncRNA2, respectively. Notably, both HSV-1 latency and explant reactivation were significantly lower in HVEM$^{-/-}$ mice infected with LAT(+) virus than in infected WT mice. Collectively, these results indicated that LAT has a significant effect on HVEM expression during latent periods *in vivo* and that LAT is sufficient to upregulate HVEM in the absence of other viral genes *in vitro*. Thus, one of

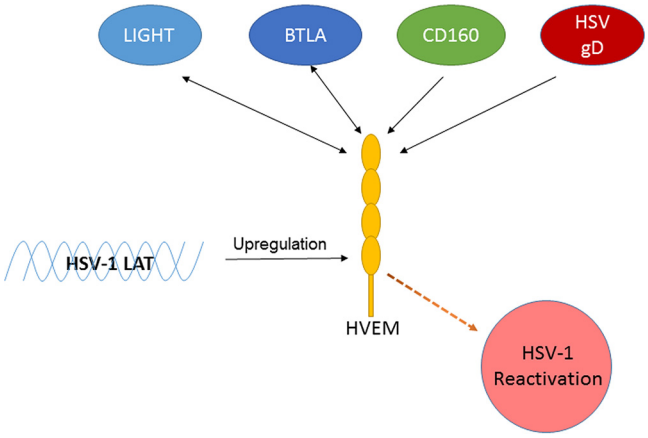

FIG 10 HVEM-gD-LAT model. Schematic diagram depicts our proposed model of HSV-1 latency reactivation in WT mice involving upregulation of HVEM by LAT and the binding of HVEM to its ligands (i.e., gD, CD160, BTLA, and LIGHT). (Upper) HVEM ligands. Arrows indicate their binding to HVEM. (Lower) Upregulation of HVEM by HSV-1 LAT (left) and enhancement of HSV-1 reactivation (right). Overall, in our model of latency reactivation we have shown that in the absence of HVEM and independent of the presence of LAT, the level of latency declined and the duration of reactivation increased, whereas in the absence of BTLA, LIGHT, or CD160 and independent of the presence of LAT, latency was reduced but the time of reactivation was enhanced.

the mechanisms by which LAT enhances latency/reactivation is by increasing the expression of HVEM in infected TG. In the present study, we investigated the roles of HVEM ligands (i.e., gD, BTLA, LIGHT, and CD160) using BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice in HSV-1 latency reactivation.

The schematic diagram of our overall model of latency reactivation with regard to LAT-gD-HVEM-BTLA-LIGHT-CD160 nexus is summarized in Fig. 10.

In this study, we did not observe any differences in the level of virus replication in the eye of BTLA$^{-/-}$, CD160$^{-/-}$, HVEM$^{-/-}$, or WT mice infected with LAT(+) compared to their counterparts infected with LAT(−) virus on days 1 to 5 p.i. The patterns of virus replication and peak of virus titers in BTLA$^{-/-}$ mice were similar to that of WT mice, and CD160$^{-/-}$ and LIGHT$^{-/-}$ mice showed patterns similar to those of HVEM$^{-/-}$ mice, having the lowest levels of virus replication. The results with HVEM$^{-/-}$ mice are similar to those of previous studies reporting lower virus replication after ocular infection with HSV-1 strain 17 (10). Since, in addition to HVEM, HSV-1 uses at least six other receptors, our results suggest that these receptors compensate for reduced binding of gD to HVEM due to higher virus load during primary infection but not during latency. The absence of BTLA, LIGHT, CD160, or HVEM had no effect on the level of CS or survival in ocularly infected mice. In contrast to this study, it was reported previously that HVEM contributes to more severe eye disease (63–65). This discrepancy between our results and those of the previous study may be due to the use of different strains of virus and corneal scarification. Previously, we have shown that C57BL/6 mice are refractory to HSV-1-induced CS (66). Thus, since all of the mice used in this study are on a C57BL/6 background, the absence of a significant level of CS in HVEM$^{-/-}$, BTLA$^{-/-}$, CD160$^{-/-}$, and LIGHT$^{-/-}$ mice, as we have reported for BALB/c mice (67), was not unexpected.

In this study, we did not detect any differences in the mRNA levels of BTLA, LIGHT, CD160, or HVEM in the TG of BTLA$^{-/-}$, LIGHT$^{-/-}$, CD160$^{-/-}$, and HVEM$^{-/-}$ mice latently infected with LAT(+) compared to LAT(−) virus. In contrast and as we reported previously (13), in the absence of LAT [i.e., LAT(−) virus], the levels of HVEM mRNA in the TG of WT mice were significantly downregulated compared to those for LAT(+) virus, whereas the presence or absence of LAT had no effect on the levels of BTLA, LIGHT, or CD160 in the TG of latently infected mice. Thus, only HVEM is affected by the presence of LAT. Previously, we demonstrated that two different neuron-derived cell lines that stably express LAT upregulate HVEM expression (13). We also mapped LAT sequences that upregulated HVEM expression to two specific regions of LAT. These two

regions of LAT are involved in the inhibition of productive infection and inhibition of apoptosis (68). The antiapoptotic activity of LAT is crucial with respect to how LAT enhances the reactivation phenotype, since the WT reactivation phenotype can be restored to a LAT(−) mutant by replacing the LAT gene with various antiapoptotic genes (69, 70). This study confirms and extends our previous data that the upregulation of HVEM function is unique to LAT. HVEM is the receptor for HSV gD (2), and LIGHT, BTLA, and CD160 also bind to HVEM (6, 17, 20, 71). Previously it was shown that LIGHT and BTLA influence the use of HVEM by gD (35). In this study, we have shown that BTLA, LIGHT, and CD160 are required for upregulation of latency, but in contrast to that for HVEM, this upregulation of latency is independent of LAT. In BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice infected with LAT(+) virus, the level of latency was reduced compared to that of LAT(−) virus. Thus, our results suggest that an interaction of LAT-HVEM but not LAT with BTLA, LIGHT, or CD160 leads to higher latency. In contrast to HVEM, which is affected by the presence of LAT, BTLA, LIGHT, and CD160 become upregulated in infected TG independent of the presence or absence of LAT. The reduced latency in BTLA$^{-/-}$, LIGHT$^{-/-}$, and CD160$^{-/-}$ mice, independent of LAT, is probably due to negative effects of these molecules on immune responses in TG of infected mice. For example, BTLA negatively regulates T cell activation (18, 21–25), whereas LIGHT promotes autoimmune disease and inflammation (17, 26–29). Furthermore, the absence of BTLA, LIGHT, HVEM, or CD160 had no negative effect on latent infection, suggesting that their function is not essential or is redundant.

In addition to latency, our results indicate that BTLA, CD160, HVEM, and LIGHT signaling play a significant role in HSV-1 reactivation. Time of reactivation of latent virus in TG explant cultures was significantly reduced in LIGHT$^{-/-}$, BTLA$^{-/-}$, and CD160$^{-/-}$ mice independent of infection with LAT(+) or LAT(−) virus compared to HVEM$^{-/-}$-infected mice. Our results demonstrate that while the absence of HVEM increases reactivation time, the absence of LIGHT, BTLA, or CD160, in contrast, decreases reactivation time. The levels of BTLA, LIGHT, and CD160 in naive HVEM$^{-/-}$ mice were significantly higher than those of naive WT mice, suggesting that HVEM expression has an inhibitory effect on LIGHT, BTLA, and CD160 expression. In contrast to WT mice, the increased levels of HVEM in infected LIGHT$^{-/-}$, BTLA$^{-/-}$, and CD160$^{-/-}$ mice were independent of the presence or absence of LAT. Furthermore, an increase of HVEM expression in LIGHT$^{-/-}$, BTLA$^{-/-}$, and CD160$^{-/-}$ mice to a level that is even higher than that of HVEM expression in WT mice following infection with LAT(+) virus may be responsible for higher reactivation in infected LIGHT$^{-/-}$, BTLA$^{-/-}$, or CD160$^{-/-}$ mice. Finally, the above-described factors may contribute to reduced competition with gD by LIGHT, BTLA, or CD160 for binding to HVEM, thereby contributing to faster reactivation, similar to that of WT mice infected with LAT(+) virus.

Our results also suggest that in contrast to the absence of HVEM that reduces latency and increases time to reactivation, the absence of BTLA, LIGHT, or CD160 reduces the level of latency but decreases the time to reactivation. This is most likely due to more efficient binding of gD to HVEM that leads to faster reactivation. In this study, we have detected gC and gD transcripts in the TG of all groups, independent of the presence of LAT. However, in contrast to the differences in the level of LAT RNA in TG of WT mice from the knockout mice, the level of gD transcript was higher than that of gC transcript and independent of the presence of LAT. During latent infection due to a low level of virus in the TG, binding of gD to HVEM and to other HSV-1 receptors is required for efficient reactivation; thus, the absence of BTLA, LIGHT, or CD160 reduces their competition with gD for binding to HVEM and enhances reactivation. Consequently, as we have shown here, even this low level of gD expression may facilitate its binding to HVEM and cause subclinical reactivation *in vivo* and faster explant reactivation *ex vivo*. Similar to the negative role of LIGHT in latency reactivation, it was recently shown that LIGHT signaling through HVEM expression is crucial for development of dermatitis (72).

In addition to PD-1, new studies now have demonstrated that elevation of Tim-3 (73, 74), and the absence of IL-21 (75–77), makes cytotoxic T cells (CTL) lose the ability to

produce IL-2, TNF-α, and IFN-γ (78, 79), which mediates T cell exhaustion. Previously, we have shown that increased latency in TG of mice latently infected with HSV-1 is associated with increased levels of mRNA for $CD8^+$ T cells and PD-1 (80). More recently, we found that HSV-1 LAT plays a role in the exhaustion of T cells in TG of latently infected mice (37). In this study, we have shown that higher latency in WT mice infected with LAT(+) virus correlated with higher levels of CD4, CD8, PD-1, Tim-3, IL-2, IFN-γ, IL-21, and TNF-α than those of LAT(−)-infected mice. In contrast, lower latency in $HVEM^{-/-}$ mice correlated with reduced CD4, CD8, PD-1, IL-2, and IL-21 but not Tim-3, IFN-γ, and TNF-α. However, lower latency in $BTLA^{-/-}$, $LIGHT^{-/-}$, and $CD160^{-/-}$ mice did not correlate with any of the transcripts. These results demonstrate that in the presence of LAT and in WT mice, $CD8^+$ T cells are exhausted, and this exhaustion can be restored in the absence of HVEM, LIGHT, BTLA, or CD160, possibly due to lower virus load.

In summary, the results presented here suggest that (i) LAT, HVEM, BTLA, CD160, and LIGHT are all required to achieve wild-type levels of latency; (ii) BTLA, CD160, and LIGHT play an inhibitory role in reducing latency and WT reactivation in a LAT-independent manner; (iii) faster time for explant reactivation in the absence of BTLA, CD160, or LIGHT is due to higher expression of HVEM but is independent of LAT; (iv) the presence of gD is required for reactivation but not for establishment of latency; (v) reduced BTLA, CD160, or LIGHT competition with gD for binding to HVEM contributes to lower latency and faster reactivation; and (vi) in contrast to WT and $HVEM^{-/-}$ mice, the level of latency in $LIGHT^{-/-}$, $BTLA^{-/-}$, or $CD160^{-/-}$ mice did not correlate with time of reactivation. In conclusion, we have documented a novel link between a viral entry pathway and its viral and nonviral ligands, with establishment of latency reactivation through host immune response signaling pathways.

MATERIALS AND METHODS

Ethics statement. All animal procedures were performed in strict accordance with the Association for Research in Vision and Ophthalmology *Statement for the Use of Animals in Ophthalmic and Vision Research* (81) and the NIH *Guide for the Care and Use of Laboratory Animals* (82). Animal research protocol was approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center (protocol number 5030).

Virus and mice. Plaque-purified HSV-1 strains McKrae [wild type; LAT(+)] and dLAT2903 [LAT(−)], derived from McKrae, were grown in rabbit skin (RS) cell monolayers in minimal essential medium (MEM) containing 5% fetal calf serum (FCS), as described previously (44, 83). Throughout the study, McKrae and dLAT2903 viruses are referred to as LAT(+) and LAT(−) viruses, respectively.

All knockout mice were generated from C57BL/6 mice. WT C57BL/6 and $BTLA^{-/-}$ mice were purchased from Jackson Laboratories and bred in-house. The $HVEM^{-/-}$, $LIGHT^{-/-}$, and $CD160^{-/-}$ mice have been described previously (30, 84, 85) and were bred in-house.

Ocular infection. Both male and female mice were infected ocularly with 2×10^5 PFU of LAT(+) or LAT(−) virus in 2 μl of MEM tissue culture medium with 5% FCS, administered as an eye drop without corneal scarification.

Titration of virus in tears of infected mice. WT and knockout mice were infected as described above with LAT(+) or LAT(−) virus. Tear films were collected from both eyes of 8 to 10 mice per group on days 1 to 5 p.i., using a Dacron-tipped swab (67). Each swab was placed in 1 ml of MEM tissue culture medium and squeezed, and the amount of virus was determined using a standard plaque assay on RS cells as we described previously (86).

***In vitro* explant reactivation assay.** Mice were sacrificed at 28 days p.i., and individual TG were removed and cultured in 1.5 ml MEM as we described previously (87). Briefly, a 100-μl aliquot was removed from each culture daily for 10 days and used to infect RS cell monolayers. The RS cells were monitored daily for the signs of cytopathic effect (CPE) for 5 days to determine the time of first appearance of reactivated virus from each TG. As the media from the explanted TG cultures were plated daily, the time at which reactivated virus first appeared in the explanted TG cultures could be determined.

CS. The severity of CS in surviving mice was scored in a masked fashion on a 0 to 4 scale (0, no disease; 1, 25%; 2, 50%; 3, 75%; and 4, 100% corneal staining or involvement) as we described previously (88).

DNA extraction and PCR analysis for HSV-1 gB DNA. DNA was isolated from homogenized individual TG using the commercially available DNeasy blood & tissue kit (Qiagen, Stanford, CA) according to the manufacturer's instructions. PCR analyses were carried out using gB-specific primers (forward, 5′-AACGCGACGCACATCAAG-3′; reverse, 5′-CTGGTACGCGATCAGAAAGC-3′; probe, 5′-6-ca rboxyfluorescein [FAM]-CAGCCGCAGTACTACC-3′). The amplicon length for this primer set is 72 bp. Relative copy numbers for gB were calculated using standard curves generated from the plasmid

pAc-gB1 (89). 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 28 p.i., immersed in RNAlater RNA stabilization reagent (Thermo Fisher Scientific, Waltham, MA), and stored at −80°C until processing. In some experiments, TG from uninfected mice of the same age as infected mice were collected as described above. Total RNA extraction was carried out as we have described previously (90, 91). 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. In mice infected with LAT(+), the level of LAT RNA from latent TG was determined using the following custom-made primers and probe set for LAT: forward primer, 5′-GGGT GGGCTCGTGTTACAG-3′; reverse primer, 5′-GGACGGGTAAGTAACAGAGTCTCTA-3′; and probe, 5′-FAM-ACACCAGCCCGTTCTTT-3′ (amplicon length, 81 bp). The amplicon for the LAT primer set corresponds to LAT nt 119553 to 119634. Relative copy numbers for the LAT were calculated using standard curves generated from the plasmid pGem-LAT5317.

The levels of various transcripts (CD4, CD8, PD-1, Tim-3, IL-2, IL-21, IFN-γ, TNF-β, HVEM, LIGHT, BTLA, and CD160) in TG were evaluated using commercially available TaqMan gene expression assays (Applied Biosystems, Foster City, CA) with optimized primer and probe concentrations. Primer probe sets consisted of two unlabeled PCR primers and the FAM dye-labeled TaqMan MGB probe formulated into a single mixture. Additionally, all cellular amplicons included an intron-exon junction to eliminate signals from genomic DNA contamination. The assays used in this study were the following: (i) HVEM, ABI assay identifier Mm00619239_m1 (amplicon length, 65 bp); (ii) LIGHT, ABI Mm00444567_m1 (amplicon length, 68 bp); (iii) BTLA, ABI Mm00616981_m1 (amplicon length, 71 bp); (iv) CD160, ABI Mm00444461_m1 (amplicon length, 61 bp); (v) CD4, ABI Mm00442754_m1 (amplicon length, 72 bp); (vi) CD8 (α chain), ABI Mn01182108_m1 (amplicon length, 67 bp); (vii) CD8 (β chain), ABI Mm_00438116_m1 (amplicon length, 89 bp); (viii) PD-1 (programmed death 1; also known as CD279), ABI Mm00435532_m1 (amplicon length, 65 bp); (ix) Tim-3, ABI Mm00454540_m1 (amplicon length, 98 bp); (x) IL-21, ABI Mm00517640_m1 (amplicon length, 67 bp); (xi) IL-2, ABI Mm00434256_m1 (amplicon length, 82 bp); (xii) IFN-γ, ABI Mm00801778_m1 (amplicon length, 101 bp); (xiii) TNF-α, ABI Mm00443258_m1 (amplicon length, 81 bp); (xiv) HSV-1 gC, ABI ARCE4XE (amplicon length, 122 bp); and (xv) GAPDH, used for normalization of transcripts, ABI Mm999999.15_G1 (amplicon length, 107 bp). The levels of gD RNA from latent TG were determined using the following custom-made gD primers and probe set: forward primer, 5′-GCGGCTC GTGAAGATAAACG-3′; reverse primer, 5′-CTCGGTGCTCCAGGATAAACTG-3′; and probe, 5′-FAM-CTGGAC GGAGATTACA-3′ (amplicon length, 59 bp).

qRT-PCR was performed using the QuantStudio 5 system (Applied Biosystems, Foster City, CA) in 384-well plates, as we described previously (92, 93), in triplicate for each tissue sample. The threshold cycle values, which represent the PCR cycle at which there is a noticeable increase in the reporter fluorescence above the baseline, were determined using QuantStudio design and analysis software.

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

ACKNOWLEDGMENTS

This study was supported by NIH Public Health Service grants EY13615, EY026944, and EY029160, as well as AI 125955.

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