The Absence of Lymphotoxin- α , a Herpesvirus Entry Mediator (HVEM) Ligand, Affects Herpes Simplex Virus 1 Infection *In Vivo* Differently than the Absence of Other HVEM Cellular Ligands

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ABSTRACT Previously, we reported that the absence of herpesvirus entry mediator (HVEM) decreases latency but not primary infection in ocularly infected mice. Recently, we reported that similar to the absence of HVEM, the absence of HVEM ligands (i.e., LIGHT, CD160, and B and T lymphocyte attenuator [BTLA]) also decreased latency but not primary infection. Similar to LIGHT, CD160, and BTLA, another member of tumor necrosis factor (TNF) superfamily, lymphotoxin- α (LT α), also interacts with HVEM. To determine whether LT α decreases latency in infected mice, we ocularly infected LT $\alpha^{-/-}$ mice with latency-associated transcript-positive [LAT(+)] and LAT(-) viruses using similarly infected wild-type (WT) mice as controls. In contrast to WT C57BL/6 mice, LT $\alpha^{-/-}$ mice were highly susceptible to ocular herpes simplex virus 1 (HSV-1) infection, independent of the presence or absence of LAT. Survival was partially restored by adoptive transfer of CD4⁺, CD8⁺, or total T cells. Infected LT $\alpha^{-/-}$ mice had significantly higher corneal scarring than WT mice, and adoptive T cell transfer did not alter the severity of eye disease. In contrast to results in WT mice, the amount of latency was not affected by the absence of LAT. The amount of LAT RNA in LT $\alpha^{-/-}$ mice infected with LAT(+) virus was similar to that in WT mice, and adoptive T cell transfer did not alter LAT RNA levels in LT $\alpha^{-/-}$ infected mice. Increased latency in the absence of LT α correlated with upregulation of HVEM, LIGHT, CD160, and BTLA transcripts as well as with an increase in markers of T cell exhaustion. The results of our study suggest that LT α has antipathogenic and anti-inflammatory functions and may act to protect the host from infection.

IMPORTANCE Recently, we evaluated the effects of HVEM and its ligands (LIGHT, CD160, and BTLA) on HSV-1 infectivity. However, the effect of LT α , another member of the TNF superfamily, on HSV-1 latency and eye disease is not known. Here, we demonstrate increased latency and corneal scarring in LT $\alpha^{-/-}$ infected mice, independent of the presence of LAT. In addition, infected mice were highly susceptible to HSV-1 infection, and survival was partially but not significantly restored by adoptive T cell transfer. These results suggest that the absence of LT α affects HSV-1 infectivity differently than the absence of HVEM, LIGHT, CD160, and BTLA.

KEYWORDS ocular, virus replication, corneal scarring, latency, survival

Herpes simplex virus-1 (HSV-1) has at least seven known cellular receptors that facilitate its ability to infect most cell types (1–11). Among these receptors, the herpesvirus entry mediator (HVEM) was the first to be identified and is the receptor for the virion envelope glycoprotein D (gD) of HSV-1 (6). Previously, we reported that the latency-associated transcript (LAT) upregulates HVEM *in vivo* and *in vitro* but not the other six HSV-1 cellular receptors (12). We have shown that HSV-1 latency/reactivation

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but not primary infection was significantly reduced in HVEM^{-/-} mice. In addition to gD, B and T lymphocyte attenuator (BTLA), LIGHT (TNFSF14), CD160, lymphotoxin- α (LT α), and synaptic adhesion-like molecule 5 (SALM5) also bind to HVEM (13–18). We recently reported that similar to HVEM^{-/-} mice (12), mice lacking LIGHT, BTLA, or CD160 showed less latency reactivation than wild-type (WT) mice infected with LAT-negative [LAT(-)] virus (19). Our published results show that the inability of BTLA, LIGHT, and CD160 to compete with gD for binding to HVEM reduced latency reactivation but not primary infection.

Similar to LIGHT and CD160, LT α is derived from the tumor necrosis factor (TNF) lineage (15, 16). These molecules bind to distinct sites on HVEM (18), providing costimulatory signals (20). LT α , which exists as a soluble homotrimer and in membrane-bound heterotrimeric complexes with LT β (21), plays an important role in secondary lymphoid organ formation during development (22). LT α is produced by a variety of lymphocytes, especially activated CD4 and CD8 T cells (23). Mice deficient in LT α lack Peyer patches, all lymph nodes, follicular dendritic cells (FDC), and germinal centers (24–26).

HSV-1 gD competes with the cellular ligands for binding to HVEM, consistent with our recent finding that T cell activation and latency reactivation are reduced via the LIGHT-BTLA-CD160-HVEM pathway and that latency reactivation is reduced in the presence of LAT (19). Although LT α is structurally related to LIGHT and although both bind to HVEM (18, 20), whether LT α and LIGHT have similar effects on HSV-1 latency is not known. To determine whether LT α and LIGHT have similar functions, we compared ocular infection of LT α ^{-/-} mice with LAT(+) and LAT(-) viruses. We found the following: (i) LT α ^{-/-} mice are highly susceptible to HSV-1 infection, and adoptive transfer of CD4⁺, CD8⁺, or total T cells did not completely restore protection; (ii) absence of LT α enhanced corneal scarring (CS), and T cell transfer did not change the level of corneal scarring; (iii) LT α ^{-/-} mice had higher levels of latency, which was independent of LAT expression; (iv) expression levels of BTLA, CD160, HVEM, and LIGHT were upregulated in trigeminal ganglia (TG) of infected LT α ^{-/-} mice, and these higher levels were further increased in the presence of LAT; and (v) increased latency in LT α ^{-/-} mice was associated with significantly higher expression of exhaustion markers. Thus, although our previous studies showed a link between HVEM, its ligands, and LAT (12, 19), here we show that the LT α response to HSV-1 infection differs from that of its HVEM binding counterparts.

RESULTS

Virus replication in the eyes of LT α ^{-/-} infected mice. LT α binds to HVEM and may compete with HSV-1 gD for binding to HVEM as we reported recently for other HVEM ligands (i.e., LIGHT, BTLA, and CD160) (19). Therefore, we used LT α ^{-/-} mice to assess the effect of LT α binding to HVEM on primary virus replication in the eye of ocularly infected mice in the presence and absence of LAT. WT mice were used as controls. LT α ^{-/-} and WT mice were ocularly infected with 2×10^5 PFU/eye of LAT(+) or LAT(-) virus as described in Materials and Methods. To determine if LT α affects HSV-1 replication during primary ocular infection, we collected tear films from 20 eyes/group daily on days 1 to 5 postinfection (p.i.). The amount of virus in each eye was determined using a standard plaque assay. We found previously that except for LIGHT^{-/-} infected mice, replication of HSV-1 in the eyes of ocularly infected HVEM^{-/-}, BTLA^{-/-}, and CD160^{-/-} mice was not affected by the presence or absence of LAT (12, 19). To determine if LAT affects ocular virus replication in the absence of LT α , we compared viral titers following primary infection with LAT(+) or LAT(-) virus. The viral titers after LAT(+) or LAT(-) virus infection did not differ significantly between WT and LT α ^{-/-} mice (Fig. 1) ($P > 0.05$) on days 1, 3, and 5. However, on days 2 and 4 p.i., WT mice infected with LAT(-) virus had higher virus titers than the other three groups (Fig. 1) ($P < 0.05$), but by day 5 p.i., virus titers in all groups declined and were similar (Fig. 1) ($P > 0.05$). Higher virus replication in the eye of LAT(-) virus-infected mice on days 2 and 4 could be due to the effect of LAT on expression of type I interferon (IFN) genes

FIG 1 Virus titers in eyes following ocular infection of mice. WT and LT $\alpha^{-/-}$ 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 assay as described in Materials and Methods. At each time point, the virus titer represents the average of the titers from 20 eyes $\pm$ standard error of the mean. Viral titers did not differ significantly between WT and LT $\alpha^{-/-}$ mice after LAT(+) or LAT(−) virus infection on days 1, 3, and 5, while on days 2 and 4 p.i., WT mice infected with LAT(−) virus had significantly higher virus titers than the other three groups.

in the eyes of infected mice. Recently, we reported that LAT downregulates components of the type I IFN pathway in ocularly infected mice (27). As we reported previously (12, 19), we did not detect any difference in virus titers between LT $\alpha^{-/-}$ mice infected with LAT(+) and LAT(−) virus (Fig. 1) ($P > 0.05$). These results confirm that, similar to HVEM, BTLA, and CD160 and in contrast to the HEVM binding partner LIGHT, LT α is not affected by the absence of LAT.

Survival of LT $\alpha^{-/-}$ infected mice. C57BL/6 mice are refractory to ocular infection with 2×10^5 PFU/eye of HSV-1 strain McKrae, LAT(+) virus, in the absence of corneal scarification. Mice on a C57BL/6 genetic background with knockout of HVEM (HVEM $^{-/-}$) and its ligands (i.e., BTLA, LIGHT, and CD160) also are refractory to HSV-1 infection (12, 19). Thus, since LT α is an HVEM family member, survival of LT $\alpha^{-/-}$ mice was monitored following ocular infection in both eyes with 2×10^5 PFU/eye of LAT(+) or LAT(−) virus as described above, and survival data from four separate experiments were combined. Nine of 31 (29% survival) LT $\alpha^{-/-}$ mice infected with LAT(+) virus survived ocular infection, and 13 of 32 (41% survival) survived ocular infection with LAT(−) virus; these differences were not statistically significant (Fig. 2) ($P > 0.05$; Fisher exact test). In contrast, and as expected, 53 of 55 (96% survival) WT mice infected with LAT(+) virus and 53 of 56 (95% survival) WT mice infected with LAT(−) virus survived ocular infection (Fig. 2). Survival differences between LT $\alpha^{-/-}$ mice infected with LAT(+) or LAT(−) virus and WT mice infected with either virus were highly significant (Fig. 2) ($P < 0.0001$; Fisher exact test). These results suggest that LT $\alpha^{-/-}$ mice are susceptible to HSV-1 infection and that the absence of LAT slightly, but not statistically significantly, enhances neurovirulence in infected mice.

These results further suggest that in contrast to WT mice and our previous studies with HVEM $^{-/-}$, BTLA $^{-/-}$, LIGHT $^{-/-}$, and CD160 $^{-/-}$ mice (12, 19), LT $\alpha^{-/-}$ mice are highly susceptible to HSV-1 infection. Thus, we next asked whether transfer of either CD4 $^{+}$ or CD8 $^{+}$ T cells alone could restore protection to ocularly infected recipient LT $\alpha^{-/-}$ mice, as described in Materials and Methods. Recipient LT $\alpha^{-/-}$ mice were ocularly infected as described above with 2×10^5 PFU/eye of LAT(+) virus, and survival was monitored for 28 days. Fourteen of 22 (64%) of LT $\alpha^{-/-}$ mice that received CD4 $^{+}$

FIG 2 Survival of ocularly infected mice. WT and $LT\alpha^{-/-}$ mice were ocularly infected with LAT(+) or LAT(-) virus as described in the legend of Fig. 1. An additional group of $LT\alpha^{-/-}$ mice received $CD4^{+}$, $CD8^{+}$, or total T cells, as described in Materials and Methods, 2 weeks before infection with LAT(+) virus. Survival of infected mice was determined 28 days p.i. Fifty-three of 55 WT mice infected with LAT(+) virus survived infection, 53 of 56 WT mice infected with LAT(-) virus survived infection, 9 of 31 $LT\alpha^{-/-}$ mice infected with LAT(+) virus survived infection, 13 of 32 $LT\alpha^{-/-}$ mice infected with LAT(-) virus survived infection, 14 of 22 $LT\alpha^{-/-}$ mice that received $CD4^{+}$ T cells and that were infected with LAT(+) virus survived infection, 11 of 22 $LT\alpha^{-/-}$ mice that received $CD8^{+}$ T cells and that were infected with LAT(+) virus survived infection, and 6 of 10 $LT\alpha^{-/-}$ mice that received total T cells and that were infected with LAT(+) virus survived infection. The difference in survival rates between $LT\alpha^{-/-}$ mice infected with the LAT(+) and LAT(-) viruses was not statistically significant. Similarly, the difference in survival rates between WT mice infected with the LAT(+) and LAT(-) viruses was not statistically significant. In contrast, survival rate differences between $LT\alpha^{-/-}$ mice infected with LAT(+) or LAT(-) virus and WT mice infected with either virus were highly significant. Finally, transfer of $CD4^{+}$ T cells, $CD8^{+}$ T cells, or total T cells partially restored protection compared to levels of either infected WT mice or to infected $LT\alpha^{-/-}$ mice without adoptive transfer, but the differences were not statistically significant.

T cells survived ocular infection while 11 of 22 (50%) of $LT\alpha^{-/-}$ mice that received $CD8^{+}$ T cells survived ocular infection, but differences between the two groups were not statistically significant (Fig. 2) (for mice receiving $CD4$ versus those receiving $CD8$, $P > 0.05$). Although transfer of $CD4^{+}$ or $CD8^{+}$ T cells alone partially restored protection compared to that of either infected WT mice (Fig. 2) ($P > 0.05$) or infected $LT\alpha^{-/-}$ mice without adoptive transfer (Fig. 2) ($P > 0.05$), these differences did not reach statistical significance.

Transfer of $CD4^{+}$ or $CD8^{+}$ T cells alone as described above did not completely restore protection to recipient $LT\alpha^{-/-}$ mice. Thus, we next tested the ability of total T cells to restore protection to recipient $LT\alpha^{-/-}$ mice. Total T cells were transferred to recipient $LT\alpha^{-/-}$ mice, and at 2 weeks posttransfer, recipient mice were infected with LAT(+) virus as described above. Six of 10 (60%) $LT\alpha^{-/-}$ recipient mice survived ocular infection (Fig. 2, +T cells). Survival in mice that received total T cells was similar to that of mice that received $CD4^{+}$ T cells, and survival rates for both were higher than the rate for mice that received $CD8^{+}$ T cells. However, survival differences between recipient mice with each other and with no transfer $LT\alpha^{-/-}$ mice were not statistically significant (Fig. 2) ($P > 0.05$). Overall, our results suggest that $LT\alpha^{-/-}$ mice are highly susceptible to HSV-1 infection and that T cell transfer partially restores protection against ocular infection.

Effects of $LT\alpha$ on corneal scarring in ocularly infected mice. During primary infection we found more blepharitis and dermatitis in $LT\alpha^{-/-}$ infected mice than in WT

FIG 3 Corneal scarring (CS) in surviving mice infected with LAT(+) virus. From 10 to 55 WT and LT $\alpha^{-/-}$ mice, as described in the legend of Fig. 2, were ocularly infected with LAT(+) virus. Some LT $\alpha^{-/-}$ mice received CD4 $^{+}$, CD8 $^{+}$, or total T cells before infection with LAT(+) virus. CS in all infected mice that survived ocular infection was assessed on day 28 p.i. on a scale of 0 to 4. CS is based on mean $\pm$ standard error of the mean of 64 eyes for WT mice, 18 eyes for LT $\alpha^{-/-}$ mice, 28 eyes for LT $\alpha^{-/-}$ mice that received CD4 $^{+}$ T cells, 22 eyes for LT $\alpha^{-/-}$ mice that received CD8 $^{+}$ T cells, and 12 eyes for LT $\alpha^{-/-}$ mice that received total T cells.

mice, and adoptive transfer of CD4 $^{+}$, CD8 $^{+}$, or total T cells to LT $\alpha^{-/-}$ mice did not enhance blepharitis and dermatitis compared to levels in their naive but infected counterparts (data not shown). The severity of corneal scarring in surviving mice on day 28 p.i. was scored in a masked fashion using a scale of 0 to 4, as described in Materials and Methods. As shown in Fig. 3, corneal scarring in WT mice infected with LAT(+) virus was significantly less than that in LT $\alpha^{-/-}$ infected mice (Fig. 3, LT $\alpha^{-/-}$) ($P < 0.01$). Similarly, transfer of CD4 $^{+}$, CD8 $^{+}$, or total T cells to LT $\alpha^{-/-}$ mice did not change corneal scarring compared with that in LT $\alpha^{-/-}$ mice that did not receive any T cells (Fig. 3) ($P > 0.05$). Thus, the absence of LT α has a major effect on HSV-1-induced corneal scarring, and transfer of CD4 $^{+}$, CD8 $^{+}$, or total T cells did not change the severity of corneal scarring compared with that in WT or LT α mice that did not receive any T cells.

Viral latency in LT $\alpha^{-/-}$ mice infected with LAT(+) and LAT(−) viruses. Previously we reported that the absence of BTLA, CD160, HVEM, and LIGHT is associated with a significant reduction in the amount of latency in the TG of infected mice, independent of the viral LAT status (12, 19). Because LT α is an HVEM ligand, we investigated the potential effect of LT α on latency. TG from surviving WT and LT $\alpha^{-/-}$ mice infected with LAT(+) and LAT(−) viruses as described in the legend of Fig. 2 were harvested on day 28 p.i., and levels of latency were determined based on expression of HSV-1 gB by quantitative PCR (qPCR), as described in Materials and Methods. Similar to published studies (12, 28), we found significantly more HSV-1 gB DNA in the TG from WT mice infected with LAT(+) than with LAT(−) virus (Fig. 4; WT, $P < 0.0001$). However, in contrast to our results with BTLA, CD160, HVEM, and LIGHT (12, 19), we found similar levels of latent genomes in the TG of LT $\alpha^{-/-}$ mice infected with LAT(+) or LAT(−) virus (Fig. 4; LT $\alpha^{-/-}$, $P > 0.05$). Indeed, the amount of latency in LT $\alpha^{-/-}$ mice infected with LAT(+) or LAT(−) virus was similar to that in LAT(+) virus-infected WT mice (Fig. 4) (WT versus LT $\alpha^{-/-}$, $P > 0.05$) but significantly higher than that in WT mice infected with LAT(−) virus (Fig. 4) ($P < 0.01$). Thus, LT α appears to play a role in controlling the amount of latency in the TG of infected mice, independent of the presence or absence of LAT.

FIG 4 Effects of LT α on the level of gB in TG of latently infected mice. WT and LT $\alpha^{-/-}$ mice were ocularly infected with LAT(+) or LAT(−) virus as described in the legend of Fig. 1. On day 28 p.i., TG were harvested from the surviving latently infected mice. qPCR was performed on each individual mouse TG. In each experiment, the estimated relative copy number of gB was calculated from standard curves generated using pAC-gB1 (79). Briefly, the DNA template first was serially diluted 10-fold so that 5 μ l contained from 10³ to 10¹¹ copies of gB and then subjected to TaqMan PCR with the same set of primers. The copy number for each reaction product was determined by comparing the normalized threshold cycle of each sample to the threshold cycle of the standard. 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 of 20 TG from WT mice infected with LAT(+) or LAT(−) virus, 18 TG from LT $\alpha^{-/-}$ mice infected with LAT(+) virus, and 20 TG from LT $\alpha^{-/-}$ mice infected with LAT(−) virus.

LAT expression in TG of LT $\alpha^{-/-}$ mice infected with LAT(+) virus with and without T cell transfer. The above results suggest that gB expression levels are similar in TG of LT $\alpha^{-/-}$ mice infected with LAT(+) or LAT(−) virus and in WT mice infected with LAT(+) virus; however, in WT mice gB expression was dependent on LAT (Fig. 5). Because LAT RNA is abundant during latency (29–31), we analyzed the TG obtained 28 days after ocular infection with LAT(+) virus for LAT RNA expression. Some LT $\alpha^{-/-}$ infected mice also received adoptive transfer of CD4⁺, CD8⁺, or both T cell types, as described in Materials and Methods. Total RNA from the TG of surviving mice was isolated and analyzed by quantitative reverse transcription-PCR (qRT-PCR) using LAT primers, as described in Materials and Methods. LAT RNA levels were similar in LT $\alpha^{-/-}$ mice latently infected with LAT(+) virus with no T cell transfer to those in WT mice similarly infected with LAT(+) virus (Fig. 5, WT versus LT $\alpha^{-/-}$ no transfer; $P > 0.05$, Fisher's exact test). LAT RNA levels were also similar in WT mice and LT $\alpha^{-/-}$ mice latently infected with LAT(+) virus with or without transfer of CD4⁺, CD8⁺, or both types of T cells (Fig. 5) ($P > 0.05$). This is consistent with the above data showing similar levels of latency, as judged by the number of viral genomes (Fig. 4), and with reports that LAT levels during latency are related to the amount of viral genome (28, 32, 33). However, our results with LT $\alpha^{-/-}$ infected mice are in contrast to levels of latency in HVEM^{−/−}, BTLA^{−/−}, LIGHT^{−/−}, and CD160^{−/−} mice that all had lower levels of LAT RNA than WT mice latently infected with LAT(+) virus (12, 19). Thus, in contrast to our finding of decreased latency in the absence of HVEM, BTLA, LIGHT, CD160, or LAT (12, 19), the absence of LT α increases latency, influencing the amount of latency that is established and/or maintained.

Effect of LT α absence on expression of the other members of the TNF superfamily in TG of uninfected mice. To determine whether the absence of LT α affects expression of BTLA, CD160, HVEM, and LIGHT molecules, TG from uninfected naive LT $\alpha^{-/-}$ and WT mice were isolated. BTLA, CD160, HVEM, and LIGHT mRNA expression

FIG 5 Effects of LT α on levels of LAT in TG of latently infected mice with and without T cell transfer. LT $\alpha^{-/-}$ and WT mice were ocularly infected with LAT(+) virus as described in the legend of Fig. 1. Some LT $\alpha^{-/-}$ mice received CD4⁺, CD8⁺, or total T cells, as described in Materials and Methods. On day 28 p.i., TG were harvested from surviving latently infected mice. qRT-PCR was performed on each mouse TG. In each experiment, the estimated relative copy number of LAT was calculated using standard curves generated from pGem-LAT5317. Briefly, the DNA template was serially diluted as described in the legend of Fig. 4, and the copy number for each reaction product was determined. GAPDH expression was used to normalize relative expression of LAT RNA in the TG. Each point represents the mean $\pm$ standard error of the mean of 20 TG from WT mice, 20 TG from LT $\alpha^{-/-}$ mice that received CD4⁺ T cells, 20 TG from LT $\alpha^{-/-}$ mice that received CD8⁺ T cells, and 12 TG from LT $\alpha^{-/-}$ mice that received total T cells.

levels were determined using qRT-PCR analysis. The results are presented as fold change compared to baseline mRNA levels in the TG from uninfected WT mice (Fig. 6). There were no significant differences in BTLA, CD160, HVEM, and LIGHT mRNA expression levels in LT $\alpha^{-/-}$ mice (Fig. 6). Although BTLA mRNA expression was highest while HVEM was lowest in LT $\alpha^{-/-}$ mice, these differences were not significant compared with levels in WT mice (Fig. 6) ($P > 0.05$). Overall, these results indicate that the absence of

FIG 6 Expression of HVEM, LIGHT, BTLA and CD160 in TG of uninfected LT $\alpha^{-/-}$ mice. TG from WT and LT $\alpha^{-/-}$ mice were harvested, RNA from each TG was isolated, and qRT-PCR was performed on each individual mouse TG. Levels of HVEM, LIGHT, BTLA, and CD160 transcript expression in TG of LT $\alpha^{-/-}$ mice were normalized to the level of each transcript in the WT mouse TG and are shown as fold increase or decrease compared to the levels in the WT mice. GAPDH expression was used to normalize relative expression of each transcript in the TG of infected mice. Each point represents the mean $\pm$ standard error of the mean of 10 TG.

FIG 7 Effect of LAT and LT α on HVEM, BTLA, LIGHT, and CD160 expression in TG of latently infected mice. WT and LT $\alpha^{-/-}$ 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. The BTLA, LIGHT, HVEM, and CD160 expression levels in naive mice for each strain were used to estimate the relative expression level 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 normalized fold gene expression calculated from the mean $\pm$ standard error of the mean of 10 TG. Differences between groups were analyzed using ANOVA.

LT α expression did not affect mRNA expression of BTLA, CD160, HVEM, and LIGHT in naive mice.

Effects of LAT and LT α on BTLA, LIGHT, CD160, and HVEM expression in TG of latently infected WT and LT $\alpha^{-/-}$ mice. We have shown that upregulation of HVEM but not BTLA, CD160, or LIGHT is LAT dependent (12, 19). Thus, to determine if the absence of LT α and LAT affects BTLA, CD160, HVEM, or LIGHT expression, the TG from LAT(+) or LAT(-) virus-infected LT $\alpha^{-/-}$ and WT mice were harvested at day 28 p.i. as described in the legend of Fig. 1. Total RNA was extracted, and qRT-PCR was used to quantitate BTLA, CD160, HVEM, and LIGHT mRNA expression levels. The results are presented as fold change compared to baseline mRNA levels in TG from uninfected naive mice of each strain (Fig. 7). In WT mice, levels of BTLA, CD160, and LIGHT were the same in LAT(+) and LAT(-) virus-infected WT mice (Fig. 7, WT; $P > 0.05$), while HVEM levels were affected by the presence of LAT (Fig. 7, WT HVEM; $P < 0.01$) as we reported previously (12, 19). In contrast, levels of BTLA, CD160, HVEM, and LIGHT were significantly higher in LT $\alpha^{-/-}$ mice infected with either LAT(+) or LAT(-) virus (Fig. 7; $P < 0.01$). In addition, levels of BTLA, CD160, HVEM, and LIGHT were significantly higher in the TG of mice infected with LAT(+) virus than with LAT(-) virus (Fig. 7; $P < 0.01$). Overall, the absence of LT α significantly affected expression of BTLA, CD160, HVEM, and LIGHT in infected mice, and this upregulation was further affected by the presence of LAT. Thus, LT α is required for control of BTLA, CD160, HVEM, and LIGHT expression in infected mice.

CD4, CD8, PD-1, Tim-3, IL-2, IL-21, IFN- γ , and TNF- α mRNA levels during HSV-1 latency in LT $\alpha^{-/-}$ mice. Previously, we showed that the degree of latency affects T cell exhaustion (12, 19, 28). The above results suggest similar levels of latency in TG of LT $\alpha^{-/-}$ and WT mice although the absence of LAT decreased latency in WT mice while upregulating latency in LT $\alpha^{-/-}$ mice in a LAT-independent manner. Thus, we determined the effect of LT α on expression of mRNAs that are considered markers of T cell subtypes (CD4 and CD8) or exhaustion (PD-1, Tim-3, interleukin-21 [IL-21], IL-2, IFN- γ , and TNF- α). TG were harvested on day 28 p.i. from infected WT and LT $\alpha^{-/-}$ 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 baseline mRNA levels in TG from uninfected naive mice for each mouse strain (Fig. 8). By comparing mRNA levels in the TG of infected LT $\alpha^{-/-}$ mice with those of infected WT mice, we found significantly higher levels of all markers in LT $\alpha^{-/-}$ mice during latency than in WT

FIG 8 Effect of LAT and LT α on T cell exhaustion markers in the TG of latently infected mice. TG from latently infected WT and LT $\alpha^{-/-}$ mice as described in the legend of 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 levels in naive mice from each line were used as baselines to estimate the relative expression levels of each transcript in TG of latently infected mice. GAPDH expression was used to normalize the relative expression of each transcript. Each point represents the normalized fold gene expression calculated from the mean $\pm$ standard error of the mean from 10 TG. For all statistical tests, *P* values of ≤ 0.01 were considered statistically significant and are marked by a single asterisk (*).

infected mice (Fig. 8) ($P < 0.001$). Thus, despite similar levels of latency in WT and LT $\alpha^{-/-}$ mice, the exhaustion marker levels were significantly higher in LT $\alpha^{-/-}$ mice than in WT mice. Therefore, differences between infected LT $\alpha^{-/-}$ and WT mice could be an immune function rather than a receptor-ligand function, and LT α is required to reduce the immune infiltrates in the TG to better control of disease.

DISCUSSION

Activated T, B, and NK cells express lymphotoxins (LTs) (34, 35), which consist of LT α and LT β molecules (36, 37). LT $\alpha^{-/-}$ and LT $\beta^{-/-}$ mice have some phenotypic similarities, but specific differences have also been noted between these mouse strains, indicating that LT α and LT β have independent functions (38). A unique function of LT α is its ability to bind to HVEM (15). Recently, we reported that the absence of interaction between HVEM and three of its ligands (i.e., BTLA, CD160, and LIGHT) reduced latency reactivation but not primary infection (19). However, the role, if any, of LT α in HVEM signaling during HSV-1 infection remains unknown. In this study, we investigated the roles of LT α during the course of HSV-1 infection using LT $\alpha^{-/-}$ mice. Previously, we showed that HVEM is upregulated by LAT both *in vitro* and *in vivo* (12). In this study, we also demonstrated that BTLA, CD160, HVEM, and LIGHT are upregulated in TG of latently infected LT $\alpha^{-/-}$ mice; however, upregulation of these molecules was higher in the presence of LAT. Levels of HSV-1 latency were similar in LT $\alpha^{-/-}$ mice infected with LAT(+) and LAT(−) virus, similar to that in WT mice infected with LAT(+) virus, and significantly higher than that in WT mice infected with LAT(−) virus, suggesting that LT α has a protective role during HSV-1 infection. LT α induces apoptosis (21, 39) while LAT has antiapoptotic function (40); thus, the absence of an apoptosis function of LT α could have contributed to the similarity of the levels of latency in WT and LT $\alpha^{-/-}$ mice and independent of the presence or absence of LAT.

LT α and LIGHT are members of the tumor necrosis factor superfamily (TNFSF), and both bind to HVEM (15). While LT α enhances binding between HVEM and BTLA (41, 42), LT α and LIGHT have distinct binding effects on HVEM, with HVEM having a stronger binding affinity to LIGHT than to LT α (15). This lower binding affinity of HVEM for LT α may be the reason for LAT(+) and LAT(−) viruses lacking a phenotype with regard to the level of latency in infected LT $\alpha^{-/-}$ mice. The absence of an LAT phenotype in LT $\alpha^{-/-}$ mice compared with that in LIGHT $^{-/-}$ mice may be due to limited expression

of LT α in activated T, B, NK, and LTi cells compared with a broader range of LIGHT expression. Recently, we showed that the absence of LAT significantly reduced latency in LIGHT $^{-/-}$ mice below that seen in WT mice (19). Overall, and in contrast to the absence of BTLA, CD160, HVEM or LIGHT, the absence of LT α and independence of LAT negatively impacted latency, suggesting that LT α may be essential to control latency but not primary infection.

Similar to BTLA $^{-/-}$, CD160 $^{-/-}$, HVEM $^{-/-}$, and WT mice and in contrast to LIGHT $^{-/-}$ mice (19), virus replication levels in the eye of LT α $^{-/-}$ mice infected with LAT(+) virus were similar to those of their counterparts infected with LAT(-) virus on days 1 to 5 p.i. or to levels in WT control mice. However, the absence of LT α had a significant effect on the level of corneal scarring (CS) in ocularly infected mice. Previously, we showed that HVEM $^{-/-}$, BTLA $^{-/-}$, CD160 $^{-/-}$, and LIGHT $^{-/-}$ mice in C57BL/6 backgrounds are refractory to HSV-1-induced CS (19), and C57BL/6 mice are resistant to CS following infection with virulent LAT(+) or LAT(-) virus (43). Because the LT α $^{-/-}$ mice used in this study are on a C57BL/6 background, the presence of significant CS in these mice was unexpected even though these mice had the same level of primary virus replication as WT mice. However, this is consistent with our previous study showing that primary virus titers in the eye and TG did not correlate with levels of viral DNA in latent TG and eye disease (44). Both CD4 $^{+}$ and CD8 $^{+}$ T cells have been implicated in HSV-1-induced CS (45–48). In this study, levels of CS were similar in LT α $^{-/-}$ mice with and without adoptive transfer of CD4 $^{+}$, CD8 $^{+}$, or total T cells from WT mice. Although it is well established that HSV-1-induced CS is due to immune response to the virus, our transfer studies support the idea that exacerbation of CS in LT α $^{-/-}$ mice could be due to cells such as macrophages or NK cells, as reported previously (43, 49–52).

Similar to their resistance to CS, WT C57BL/6 mice are resistant to the lethal dose of virus that we used in this study. For example, of 111 C57BL/6 mice that we infected with LAT(+) or LAT(-) virus, only 5 mice died, a 96% survival rate, while 41 of the 63 LT α $^{-/-}$ mice infected with LAT(+) and LAT(-) viruses died, a 35% survival rate. We have previously showed that, similar to WT C57BL/6 mice, BTLA $^{-/-}$, LIGHT $^{-/-}$, CD160 $^{-/-}$, and HVEM $^{-/-}$ mice are also refractory to HSV-1 infection. In this study, we found that transfer of CD4 $^{+}$, CD8 $^{+}$, or total T cells partially but not completely restored protection LT α $^{-/-}$ mice. This is consistent with previous reports showing that reconstitution of irradiated LT α $^{-/-}$ mice with wild-type (WT) mouse bone marrow cells restored the ability to form FDC, germinal centers, and adjuvant-independent IgG responses, but these functions were not restored by transfer of spleen T cells (53). Furthermore, B cells alone can restore protection to LT α $^{-/-}$ mice (34), and generation or expression of memory T cells from these mice was not affected (54). Thus, the absence of complete protection after T cell transfer in this study is not unexpected. In previous studies, LT α $^{-/-}$ mice developed CD8 $^{+}$ T cells normally, but these cells were functionally impaired, making the infected animals susceptible to HSV-1 infection (55). CD8 $^{+}$ T cells from LT α $^{-/-}$ mice failed to mature into cytotoxic T cells (CTL) or to express intracellular IFN- γ upon *ex vivo* stimulation with antigen (Ag) (55). We have also shown that transfer of CD4 $^{+}$, CD8 $^{+}$, or total T cells from WT mice to LT α $^{-/-}$ mice did not provide complete protection, suggesting that even T cells from WT mice are not completely functional in the absence of LT α expression in LT α $^{-/-}$ mice. In contrast to LT α $^{-/-}$ mice, RAG mice were completely protected against lethal HSV-1 infection following adoptive T cell transfer (56).

Similar to this study, a previous study has shown that LT α $^{-/-}$ mice have increased susceptibility to infections (57). The level of susceptibility to infection in LT α $^{-/-}$ mice varied with different viral infections. For example, LT α $^{-/-}$ mice were able to successfully clear lymphocytic choriomeningitis virus (LCMV) infection (57). Similarly, respiratory challenge of LT α $^{-/-}$ mice with murine gammaherpesvirus 68 (MHV-68) led to an acute productive infection and latent infection. However, despite reduced immune responses, infected mice were able to clear the infection and control the latent infection but with delayed kinetics compared to those of WT mice (58). In contrast, LT α $^{-/-}$ mice are more susceptible to influenza virus infection than WT mice, with delayed but effective B and T cell responses to infection (59). Similar to WT mice,

LT $\alpha^{-/-}$ mice cleared systemic rotavirus infection within 10 days, while clearance of intestinal infection in LT $\alpha^{-/-}$ mice depended on local generation of mucosal IgA (60). Previously it was reported that humoral immune responses to different antigens is impaired in LT $\alpha^{-/-}$ mice (61). Similarly, LT $\alpha^{-/-}$ mice immunized with attenuated HSV-2 virus showed delayed virus clearance following lethal challenge with a virulent HSV-2 virus, but all challenged mice were protected from disease and death (62).

In this study, we detected significantly higher expression levels of BTLA, CD160, HVEM, and LIGHT mRNAs in the TG of LT $\alpha^{-/-}$ mice latently infected with LAT(+) than with LAT(−) virus, but these transcripts were more highly expressed in LT $\alpha^{-/-}$ mice infected with LAT(−) virus than in WT mice infected with either virus. In contrast, and as we reported previously (19), we did not detect any differences in BTLA, LIGHT, CD160, or HVEM mRNA levels in the TG of BTLA $^{-/-}$, LIGHT $^{-/-}$, CD160 $^{-/-}$, and HVEM $^{-/-}$ mice latently infected with LAT(+) or LAT(−) virus. Levels of BTLA, CD160, HVEM, and LIGHT were higher in TG of LAT(+) virus -infected LT $\alpha^{-/-}$ mice than in LT $\alpha^{-/-}$ mice infected with LAT(−) virus. These higher expression levels in the presence of LAT could be due to higher antiapoptotic function of LAT in the absence of LT α . In addition to the upregulation of BTLA, LIGHT, CD160, or HVEM in the TG of LT $\alpha^{-/-}$ infected mice, markers of T cell exhaustion (CD4, CD8, PD-1, Tim-3, IL-2, IFN- γ , IL-21, and TNF- α) are also upregulated in infected mice (63–69). Previously, we found that increased latency is associated with increased levels of T cell exhaustion (70) and that higher latency in the presence of LAT was associated with higher levels of T cell exhaustion (28, 71). However, during HSV-1 latency not all of the T cells are exhausted (72). In this study, we show a correlation between higher latency in LT $\alpha^{-/-}$ infected mice with higher levels of CD4, CD8, PD-1, Tim-3, IL-2, IFN- γ , IL-21, and TNF- α that was not observed in WT mice. In contrast to our recent study (19), BTLA $^{-/-}$, LIGHT $^{-/-}$, CD160 $^{-/-}$, and HVEM $^{-/-}$ infected mice had lower levels of CD4, CD8, PD-1, Tim-3, IL-2, IFN- γ , IL-21, and TNF- α than WT infected mice. This may be due to lower levels of latency in the infected mice. In general, we consistently find that markers of T cell exhaustion are higher in WT mice than in their knockout counterparts due to higher levels of latency (19, 28). But in this study, infected WT mice had lower expression levels of CD4, CD8, PD-1, Tim-3, IL-2, IFN- γ , IL-21, and TNF- α transcripts than LT $\alpha^{-/-}$ infected mice. The elevated expression of these transcripts in TG of infected mice is independent of the presence of LAT and could be associated with the proinflammatory nature of this gene. LT α was originally identified as a proinflammatory molecule (73). It can induce apoptosis, necroptosis, differentiation, proliferation, and the expression of a variety of inflammatory cytokines and chemokines, depending on its environment (21, 39). Thus, the absence of some or all of these LT α functions may contribute to increased viral pathogenesis in LT $\alpha^{-/-}$ mice.

In summary, the results of this study suggest the following: (i) that LT α is required to achieve WT levels of CS and survival; (ii) that LT α affects latency in an LAT-independent manner; and (iii) that LT α inhibits the induction of HVEM and its ligands as well as T cell exhaustion markers. Furthermore, we have shown that the function of the HVEM ligand, LT α , is completely different from the functions of other HVEM ligands (i.e., BTLA, CD160, and LIGHT) and that further studies are warranted to dissect the role of LT α in HSV-1 pathogenesis.

MATERIALS AND METHODS

Virus and mice. Plaque-purified HSV-1 strains McKrae [WT; LAT-positive, or LAT(+)] and dLAT2903 [LAT-negative, or 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 (33, 74). Throughout the study, McKrae and dLAT2903 viruses are referred to as LAT(+) and LAT(−) viruses, respectively.

WT C57BL/6 and LT $\alpha^{-/-}$ mice were purchased from The Jackson Laboratories and bred in-house. Both male and female mice were used in the study. The animal research protocol was approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center (Protocol no. 5030).

Ocular infection. Mice were infected ocularly with 2×10^5 PFU of LAT(+) or LAT(−) virus in 2 μ l of MEM tissue culture medium with 5% FCS, administered in an eye drop without corneal scarification.

Titration of virus in tears of infected mice. Tear films from mice infected as described above were collected from both eyes of 10 mice per group on days 1 to 5 postinfection (p.i.), using a Dacron-tipped

swab (75). 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 (76).

Adoptive transfer of T cell to $LT\alpha^{-/-}$ mice. In an effort to improve survival of $LT\alpha^{-/-}$ mice after ocular infection, $CD4^{+}$, $CD8^{+}$, or total T cells isolated from WT mice were adoptively transferred into $LT\alpha^{-/-}$ mice before infection with HSV-1. Briefly, spleens from WT C57BL/6 mice were pooled, and single-cell suspensions were prepared as described previously (77). $CD4^{+}$ T cells alone, $CD8^{+}$ T cells alone, or total T cells were isolated using magnetic beads as described by the manufacturer (Miltenyi Biotec, San Diego, CA). Recipient mice received a single intraperitoneal injection of either $CD4^{+}$ T cells, $CD8^{+}$ T cells, or total T cells suspended in MEM (300 μ l) equivalent to the amount injected into a single donor mouse. The mice were infected ocularly with McKrae LAT(+) virus 2 weeks after the T cell transfer.

CS. The severity of corneal scarring (CS) in surviving mice was scored in a masked fashion on a scale of 0 to 4 (0, no disease; 1, 25% corneal staining or involvement; 2, 50%; 3, 75%; and 4, 100%) as we described previously (78).

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 were carried out using gB-specific primers (forward, 5'-AACGCGACGCACATCAAG-3'; reverse, 5'-CTGGTACGCGATCAGAAAGC-3'; probe, 5'-FAM-CA GCCGCACTACTACC-3', where FAM is 6-carboxyfluorescein). The amplicon length for this primer set is 72 bp. Relative gB copy numbers were calculated using standard curves generated from the plasmid pAc-gB1 (79). In all experiments, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize 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 also collected as described above. Total RNA extraction and RT-PCR were carried out as we have described previously (80, 81). In mice infected with LAT(+) virus, the level of LAT RNA from latent TG was determined using a custom-made primer and probe set for LAT as follows: forward primer, 5'-GGGTGGGCTCGTGTACAG-3'; reverse primer, 5'-GGACGGGTAAGTAACAGAGTCTCTA-3'; and probe, 5'-FAM-ACACCAGCCCGTCTTT-3' (amplicon length, 81 bp). The LAT amplicon primer set corresponds to LAT nucleotides (nt) 119553 to 119634. Relative LAT copy numbers were calculated using standard curves generated from the pGem-LAT5317 plasmid.

The levels of various transcripts ($CD4$, $CD8$, $PD-1$, $Tim-3$, $IL-2$, $IL-21$, $IFN-\gamma$, $TNF-\beta$, $HVEM$, $LIGHT$, $BTLA$, 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 a FAM dye-labeled TaqMan MGB (minor groove binder) probe formulated into a single mixture. Additionally, all cellular amplicons included an intron-exon junction to eliminate signals from genomic DNA contamination. The following assays were used in this study: (1) for $HVEM$, ABI assay Mm00619239_m1, with amplicon length of 65 bp; (2) for $LIGHT$, ABI Mm00444567_m1, with amplicon length of 68 bp; (3) for $BTLA$, ABI Mm00616981_m1, with amplicon length of 71 bp; (4) for $CD160$, ABI Mm00444461_m1, with amplicon length of 61 bp; (5) for $CD4$, ABI Mm00442754_m1, with amplicon length of 72 bp; (6) for $CD8$ (α chain), ABI Mm01182108_m1, with amplicon length of 67 bp; (7) for $CD8$ (β chain), ABI Mm_00438116_m1, with amplicon length of 89 bp; (8) for $PD-1$ (programmed death 1; also known as $CD279$), ABI Mm00435532_m1, with amplicon length of 65 bp; (9) for $Tim-3$, ABI Mm00454540_m1, with amplicon length of 98 bp; (10) for $IL-21$, ABI Mm00517640_m1, with amplicon length of 67 bp; (11) for $IL-2$, ABI Mm00434256_m1, with amplicon length of 82 bp; (12) for $IFN-\gamma$, ABI Mm00801778_m1, with amplicon length of 101 bp; (13) for $TNF-\alpha$, ABI Mm00443258_m1, with amplicon length of 81 bp; and (14) for $GAPDH$ (used to normalize transcripts), ABI Mm999999.15_G1, with amplicon length of 107 bp.

Statistical analysis. Student's t test, Fisher's 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 .

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