The Latency-Associated Transcript Inhibits Apoptosis via Downregulation of Components of the Type I Interferon Pathway during Latent Herpes Simplex Virus 1 Ocular Infection

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ABSTRACT The herpes simplex virus (HSV-1) latency-associated transcript (LAT) has been shown to inhibit apoptosis via inhibiting activation of proapoptotic caspases. However, the mechanism of LAT control of apoptosis is unclear, because LAT is not known to encode a functional protein, and the LAT transcript is found largely in the nucleus. We hypothesized that LAT inhibits apoptosis by regulating expression of genes that control apoptosis. Consequently, we sought to establish the molecular mechanism of antiapoptosis functions of LAT at a transcriptional level during latent HSV-1 ocular infection in mice. Our results suggest the following. (i) LAT likely inhibits apoptosis via upregulation of several components of the type I interferon (IFN) pathway. (ii) LAT does not inhibit apoptosis via the caspase cascade at a transcriptional level or via downregulating Toll-like receptors (TLRs). (iii) The mechanism of LAT antiapoptotic effect is distinct from that of the baculovirus inhibitor of apoptosis (cpIAP) because replacement of LAT with the cpIAP gene resulted in a different gene expression pattern than in either LAT$^+$ or LAT$^-$ viruses. (iv) Replacement of LAT with the cpIAP gene does not cause upregulation of CD8 or markers of T cell exhaustion despite their having similar levels of latency, further supporting that LAT and cpIAP function via distinct mechanisms.

IMPORTANCE The HSV-1 latency reactivation cycle is the cause of significant human pathology. The HSV-1 latency-associated transcript (LAT) functions by regulating latency and reactivation, in part by inhibiting apoptosis. However, the mechanism of this process is unknown. Here we show that LAT likely controls apoptosis via downregulation of several components in the JAK-STAT pathway. Furthermore, we provide evidence that immune exhaustion is not caused by the antiapoptotic activity of the LAT.

KEYWORDS HSV-1, IFN, LAT, latency, ocular, TLR, reactivation

Herpes simplex virus 1 (HSV-1) infections are the cause of several diseases with mild to severe pathology, including oral and genital ulcers, virus-induced corneal scarring (CS) and blindness, and encephalitis (1–8). Initial infection occurs via the oral or genital mucosa or the eye, where the virus replicates. After initial replication, innate immune response begins, which helps in virus clearance from the eye. The virus then enters sensory neurons via microtubule-dependent retrograde transport and establishes lifelong latency in the trigeminal ganglia (TG), from where it can periodically reactivate, leading to immunopathology. CS is mainly caused by the immune response to reactivation of the latent virus and not the initial infection (9). It is well known that the latency-associated transcript (LAT), the only transcript that is detectable at high levels during HSV-1 latency, promotes latency reactivation (10–12).

LAT may promote reactivation, at least in part, via inhibiting apoptosis and hence promoting cell survival both *in vitro* and *in vivo* (13–19). Antiapoptotic activity of LAT

is sufficient for reactivation, because replacement of LAT with any of three antiapoptotic genes can rescue reactivation to wild-type (WT) levels (20, 21). However, while restoring the antiapoptotic function of LAT by replacing LAT with antiapoptotic genes restores reactivation, these viruses still have reduced virulence, suggesting that LAT has other functions besides inhibiting apoptosis (22). The antiapoptotic function of LAT may be related to immune exhaustion seen during latency. Previously we found that latent HSV-1 infection leads to immune exhaustion in a LAT-dependent manner (9, 23–25). We showed that PD-1 and TIM-3, markers of immune exhaustion, were elevated in TG of latently infected mice, and this correlated with severity of CS (9). Later, we and others demonstrated that this was dependent on LAT, because mice latently infected with HSV-1 lacking LAT had significantly less immune exhaustion (23, 24).

The mechanism of LAT control of apoptosis *in vivo* is unclear. LAT has been shown to inhibit both the extrinsic and intrinsic pathways of apoptosis. For example, stable expression of LAT reduced activation of the intrinsic (i.e., mitochondrial) pathway of apoptosis by inhibiting AKT dephosphorylation (26). Furthermore, LAT reduced activation of apoptotic caspases 3, 8, and 9. *In vitro*, caspase 8 and caspase 9 cleavage was inhibited in Neuro 2A cells infected with WT virus, but not a LAT null mutant virus (14), suggesting that LAT functions via inhibiting activation of proapoptotic caspases. These data suggest that LAT controls activity of proapoptotic proteins. However, it is not known if LAT functions at the level of transcription, because it has not been shown that LAT encodes a functional protein (reviewed in references 22 and 27), and during latency, LAT is largely found in the nucleus (28). Therefore, it is possible that LAT inhibits apoptosis via a transcriptional mechanism, in addition to inhibiting cleavage of proapoptotic caspases.

In this study, we set out to establish the molecular mechanism of HSV-1 LAT inhibition of apoptosis and if antiapoptotic activity is necessary for immune exhaustion. We focused on components of the caspase cascade, Toll-like receptors (TLRs) and the type I interferon (IFN) pathway, because HSV-1 has been shown to activate these pathways (27). TLRs, which are an important part of the innate immune system, recognize a variety of pathogen-associated molecular patterns, and TLR2, TLR3, TLR7, and TLR9 were shown to be activated during an acute infection with HSV-1 (29–32). Another branch of the innate immune system, the type I IFN pathway (consisting mainly of IFN-α and -β), is activated by several HSV-1 glycoproteins (33). However, the HSV-1 LAT transcript was shown to delay expression of IFN-α and -β during an acute infection (34). Activation of the type I IFN pathway is transduced via Janus kinases (JAKs) and may result in transcription and translation of interferon-stimulated genes (ISGs), production of inflammatory cytokines, and growth arrest or apoptosis (35, 36), although the specific response may be dependent on the cell type (37, 38). ISGs exert antiviral activity via various mechanisms, including inhibiting viral entry, replication, egress, or budding (39). HSV-1, on the other hand, has developed mechanisms to inhibit several ISGs (40–43).

Our results suggest that LAT functions in the activation of the type I IFN pathway via downregulation of the Janus kinases during HSV-1 latency. We found that JAK-1 and JAK-2, as well as several downstream effectors of the JAK-STAT pathway, were downregulated in a LAT-dependent manner. We also found evidence of immune exhaustion in TG of mice infected with WT McKrae but not LAT-deficient virus. Together, these results suggest that the HSV-1 LAT likely affects apoptosis via downregulation of the JAK-STAT pathway components. Additionally, we show here that LAT does not function via the same mechanism as cpIAP, because replacement of LAT with the cpIAP gene resulted in a different gene expression pattern than in mice infected with either WT (LAT$^+$) or LAT$^-$ virus. We also demonstrate that the antiapoptotic activity of LAT is not sufficient to cause immune exhaustion.

RESULTS

HSV-1 latent infection induces expression of proapoptotic genes in a LAT-dependent manner. We and others have shown that LAT is necessary for efficient
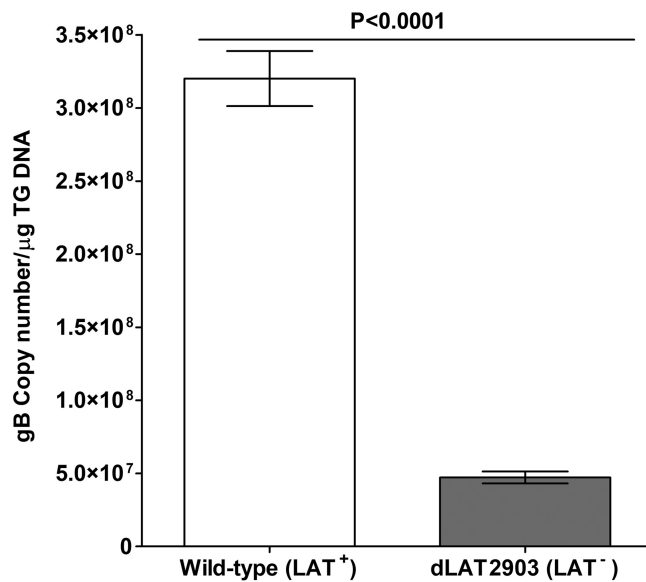
FIG 1 Latency in ocularly infected mice. Mice were infected with 2×10^5 PFU per eye of LAT+ or LAT− virus. Twenty-eight days p.i., TG from infected mice were isolated and quantitative PCR was performed on each individual mouse TG. In each experiment, an estimated relative copy number of the HSV-1 gB for viral DNA was calculated using standard curves generated from pGem-gB1. Briefly, the DNA template was serially diluted 10-fold such 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 viral (gB) DNA in the TG. Each bar is based on 20 TG from 2 separate experiments.

establishment of latency (10–12). To verify that latency is impaired under our experimental conditions, we collected trigeminal ganglia (TG) of C57BL6 mice 28 days after ocular infection with either the HSV-1 WT strain McKrae (LAT+) or the LAT null mutant dLAT2903 (LAT−). The amount of viral DNA in the TG of mice was determined by quantitative PCR (qPCR). Consistent with previous reports, mice infected with LAT+ virus contained approximately 6-fold more viral glycoprotein B (gB) DNA in TG than did mice infected with LAT− virus ($P < 0.0001$ [Fig. 1]). These results suggest that the level of latency is significantly reduced in mice infected with the LAT null mutant, as previously described (10–12).

To determine if LAT controls apoptosis via inhibiting transcription of proapoptotic genes, we measured expression levels of several apoptotic genes. We found that caspases 3, 8, and 9 were mildly elevated in TG of LAT+ mice compared to those of uninfected mice (Fig. 2). This upregulation was dependent on LAT, because mice infected with LAT− virus had significantly lower expression levels of caspase 3, 8, and 9 than did LAT+ virus-infected mice ($P < 0.01$ [Fig. 2]). Proapoptotic markers BID, FasL, and Card10 were also upregulated in TG of mice infected with LAT+ HSV-1 (Fig. 2). However, this upregulation was independent of LAT, because mice infected with LAT− virus had levels of expression of BID, Card10, and FasL similar to those of mice infected with LAT+ HSV-1 ($P > 0.05$ [Fig. 2]). The antiapoptotic BCL2 gene was upregulated in TG of LAT+ virus-infected mice, while it was significantly decreased in LAT− virus-infected mice ($P < 0.01$ [Fig. 2]). The expression level of the Fas-associated death domain (FADD) gene was lower in both LAT+ and LAT− virus-infected mice than in uninfected mice, and the differences between the LAT+ and LAT− virus-infected TG were not statistically significant ($P > 0.05$ [Fig. 2]). These results suggest that LAT is unlikely to regulate apoptosis via downregulation of caspase pathway components.

Upregulation of NF-κB and Toll-like receptors during HSV-1 latent infection occurs independently of LAT. Previous studies have established that NF-κB is upregulated during HSV-1 acute infection (44–46). However, it is not known if this
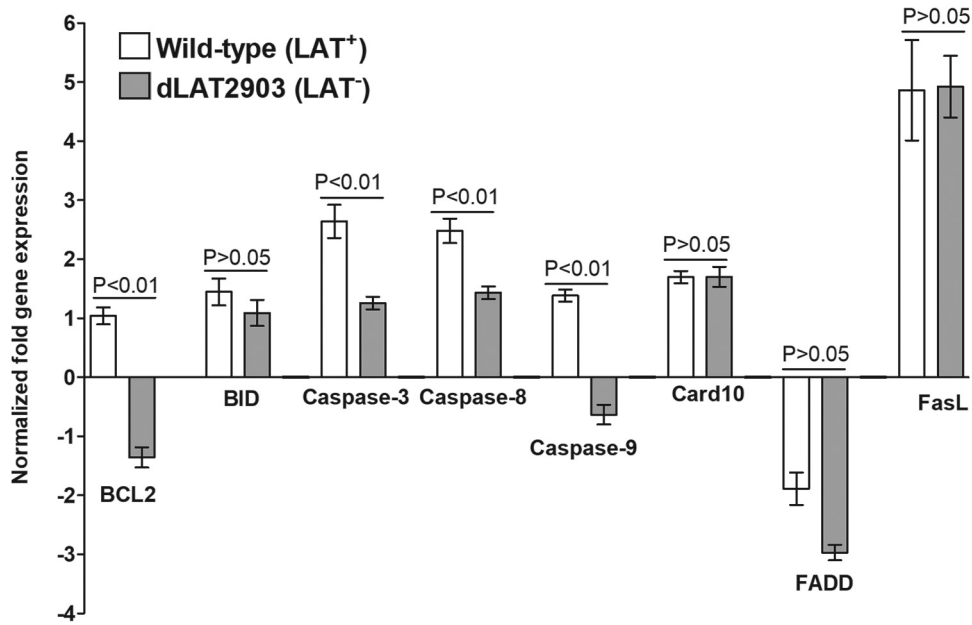
FIG 2 HSV-1 latent infection induces expression of proapoptotic genes. Mice were infected and TG collected as for Fig. 1. On day 28 p.i., mice were euthanized and TG from individual mice collected and combined. qRT-PCR was performed using total RNA. Expression of BCL2, BID, caspase 3, caspase 8, caspase 9, Card, FADD, and FasL in naive mice was used to estimate the relative expression of each transcript in TG, and GAPDH was used to normalize expression of each transcript in infected TG. Each point represents the mean ± SEM from 10 mice.

transcription factor is also upregulated during latency and, if so, whether this is dependent on LAT. We measured expression of NF-κB in latently infected mouse TG and found that NF-κB is upregulated similarly in TG of LAT$^+$ and LAT$^-$ virus-infected mice ($P > 0.05$ [Fig. 3]). Thus, similar to the case with acute infection (44–46), NF-κB
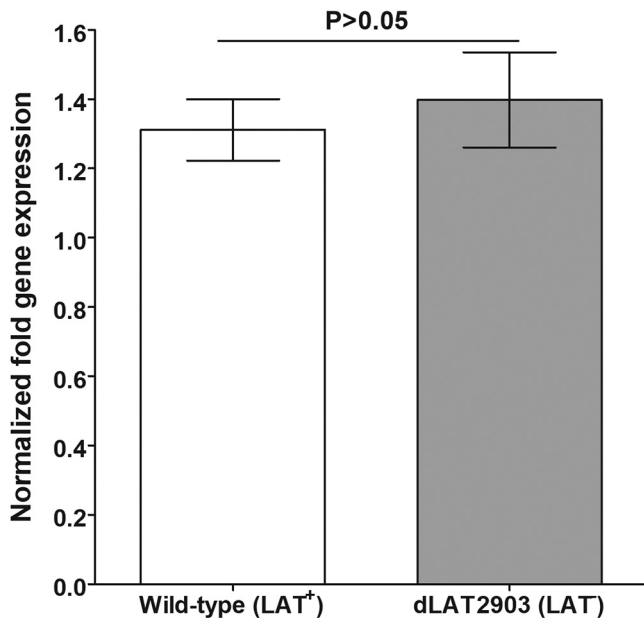

FIG 3 NF-κB expression is upregulated during latent HSV-1 infection. Mice were infected and TG collected as for Fig. 1. On day 28 p.i., mice were euthanized and TG from individual mice collected and combined. qRT-PCR was performed using total RNA. NF-κB transcript levels in naive mice were used to estimate the relative expression of NF-κB in TG, and GAPDH was used to normalize expression of each transcript. Each point represents the mean ± SEM from 10 mice.
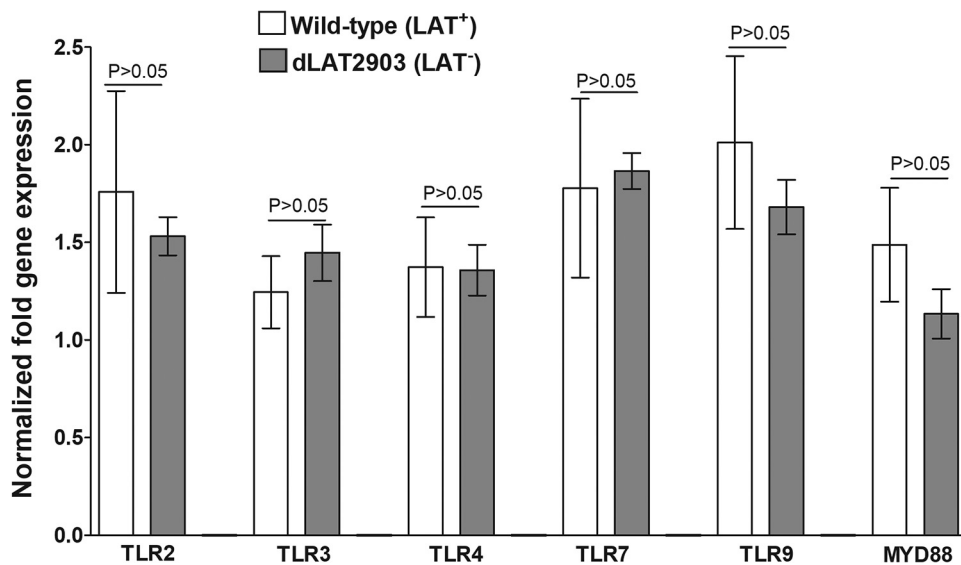
FIG 4 Effect of LAT on TLR expression during latent infection with HSV-1. Mice were infected and TG collected as for Fig. 1. On day 28 p.i., mice were euthanized and TG from individual mice collected and combined. qRT-PCR was performed using total RNA. Expression of TLR2, TLR3, TLR4, TLR7, TLR9, and MyD88 in naive mice was used to estimate the relative expression of each transcript in TG, and GAPDH was used to normalize expression of each transcript. Each point represents the mean ± SEM from 10 mice.

expression during latency is upregulated, and this upregulation is independent of LAT.

To test if HSV-1 escapes immune detection and apoptosis via downregulation of TLRs during latency, we measured expression levels of several TLRs. We found that TLR2, -3, -4, -7, and -9, as well as the TLR adaptor protein MyD88, were upregulated in TG of both LAT$^+$ and LAT$^-$ virus-infected mice (Fig. 4). However, levels of TLR2, -3, -4, -7, and -9 and MyD88 were similar between mice infected with LAT$^+$ and LAT$^-$ viruses ($P > 0.05$ [Fig. 4]). These results demonstrate that the TLR pathway is activated during HSV-1 latency. However, LAT does not mediate its antiapoptotic response via downregulating TLRs during latency.

LAT controls apoptosis via downregulating the type I IFN pathway. The type I interferon (IFN) signaling pathway is activated in response to viral infections. Activation of this pathway results in initiation of the inflammatory response and transcription of antiviral genes such as IFN-stimulated genes (ISGs). Depending on cell type and timing of IFN exposure, activation of the type I IFN pathway may result in stimulation or inhibition of apoptosis (reviewed in reference 47). HSV-1 has several methods to inhibit type I IFN signaling. For example, the HSV-1 immediate early (IE) protein 27 (ICP27) was shown to inhibit IFN signaling via blocking activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) in Vero cells (48). To test if LAT can inhibit apoptosis during latency via the JAK-STAT pathway, we measured expression levels of JAK-1 and JAK-2, the tyrosine kinases directly upstream of STAT. Indeed, we found that both JAK-1 and JAK-2 expression levels were downregulated in mouse TG latently infected with LAT$^+$ virus but not in LAT$^-$ HSV-1-infected mice and that these differences were highly significant ($P < 0.0001$ [Fig. 5]).

As we observed LAT-mediated downregulation of JAK-1 and JAK-2, we predicted that downstream effector gene expression is also affected. To test this hypothesis, we measured the expression levels of several known IFN-stimulated genes that were shown to be regulated by HSV-1 during an acute infection (40–43). We found that several interferon-stimulated genes (ISGs), such as ISG56 (or IFIT1) and the genes for interferon regulatory factor 1 (IRF-1), myxoma resistance protein 1 (Mx-1), and class II major histocompatibility complex transactivator (CIITA), were downregulated in mice infected with LAT$^+$ virus but upregulated in mice infected with LAT$^-$ virus (Fig. 6).
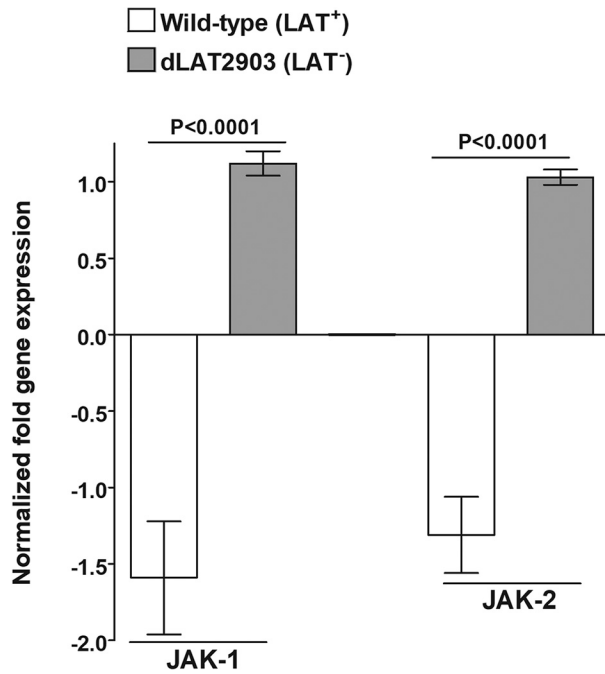
FIG 5 LAT regulates expression of JAK-1 and JAK-2. Mice were infected and TG collected as for Fig. 1. On day 28 p.i., mice were euthanized and TG from individual mice collected and combined. qRT-PCR was performed using total RNA. Expression of JAK-1 and JAK-2 in naive mice was used to estimate the relative expression of the same transcript in TG of infected mice, and GAPDH was used to normalize expression of each transcript. Each point represents the mean ± SEM from 10 mice.

These differences between LAT$^+$ and LAT$^-$ virus-infected TG were statistically significant ($P < 0.0001$ [Fig. 6]). In contrast, the level of oligoadenylate synthetase 3 (OAS-3) was elevated in LAT$^+$ virus-infected mice, while it was lower in TG of LAT$^-$ virus-infected mice ($P < 0.0001$ [Fig. 6]). The expression levels of ISG15, ISG54, and comple-
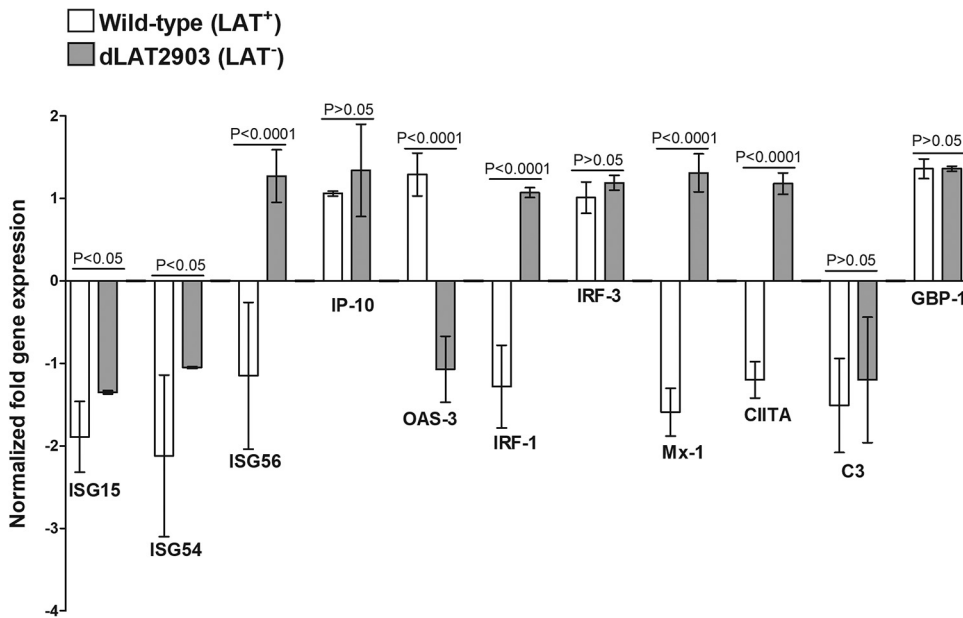
FIG 6 LAT downregulates several type I IFN pathway components. Mice were infected and TG collected as for Fig. 1. On day 28 p.i., mice were euthanized and TG from individual mice collected and combined. qRT-PCR was performed using total RNA. Expression of ISG15, ISG54, ISG56, IP-10, OAS-3, IRF-1, IRF-3, Mx-1, CIITA, C3, and GBP-1 in naive mice was used to estimate the relative expression of each transcript in TG, and GAPDH was used to normalize expression of each transcript. Each point represents the mean ± SEM from 10 mice.

FIG 7 Replacement of LAT with the cpIAP gene rescues latency in LAT cells. Mice were infected with LAT+ or dLATcpIAP (LAT−) virus as for Fig. 1. Twenty-eight days p.i., TG from infected mice were isolated and 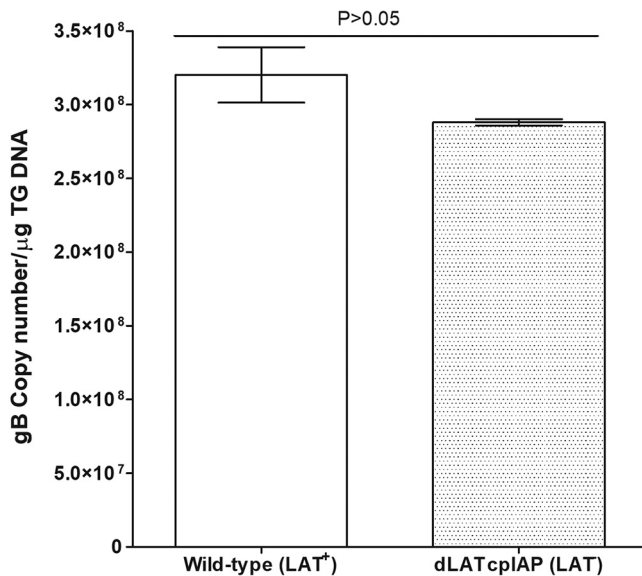quantitative PCR was performed on each individual mouse TG. In each experiment, an estimated relative copy number of the HSV-1 gB for viral DNA was calculated using standard curves generated from pGem-gB1 as for Fig. 1. By comparing the normalized threshold cycle of each sample to the threshold cycle of the standard, the copy number for each reaction was determined. GAPDH expression was used to normalize the relative expression of viral (gB) DNA in the TG. Each bar is based on 20 TG.

ment component 3 (C3) were lower in infected than in uninfected TG, but similar in both LAT+ and LAT− infected TG ($P < 0.05$ for ISG15 and ISG54 and $P > 0.05$ for C3 [Fig. 6]). Finally, interferon-inducible cytokine 10 (IP-10), IRF-3, and guanylate binding protein 1 (GBP-1) expression levels increased in TG of infected mice independent of the presence or absence of LAT, and the difference between LAT+ and LAT− virus-infected mice was not statistically significant ($P > 0.05$ [Fig. 6]). Overall, these results indicate that LAT may control apoptosis via downregulating JAKs and several, but not all, IFN-regulated genes.

LAT and cpIAP function via distinct mechanisms. LAT antiapoptotic activity enhances HSV-1 reactivation because replacement of LAT with either the baculovirus inhibitor of apoptosis (cpIAP), cellular FLIP, or bovine herpesvirus 1 (BHV-1) latency-related (LR) gene rescued the reactivation defect seen in LAT− virus-infected mouse TG to WT levels (15, 20, 49, 50). To verify that antiapoptotic activity is sufficient to rescue latency in the absence of LAT, we ocularly infected mice with either WT HSV-1 strain McKrae or a mutant virus in which LAT was replaced with the baculovirus cpIAP gene (dLATcpIAP). At 28 days after ocular infection, we collected mouse TG and measured gB levels as an indicator of viral load using qPCR. There was no significant difference in gB copy numbers of mice infected with WT and dLATcpIAP viruses, suggesting similar levels of latency ($P > 0.05$ [Fig. 7]). This lack of difference could reflect an increased number of neurons surviving due to the antiapoptotic activity of cpIAP.

To examine if cpIAP and LAT control apoptosis using a similar mechanism, we measured expression levels of caspase 8, BCL2, BID, and FADD in TG of mice latently infected with WT HSV-1 and dLATcpIAP virus. Mice infected with virus containing cpIAP had significantly lower expression levels of caspase 8, BCL2, BID, and FADD genes than mice infected with WT virus ($P < 0.0001$ [Fig. 8]). These results suggest that while both LAT and cpIAP can regulate expression of apoptotic genes, they function via distinct mechanisms.

Previously we have shown that HSV-1 latent infection is correlated with CD8+ T cell exhaustion in a LAT-dependent manner (9, 23). We hypothesized that the
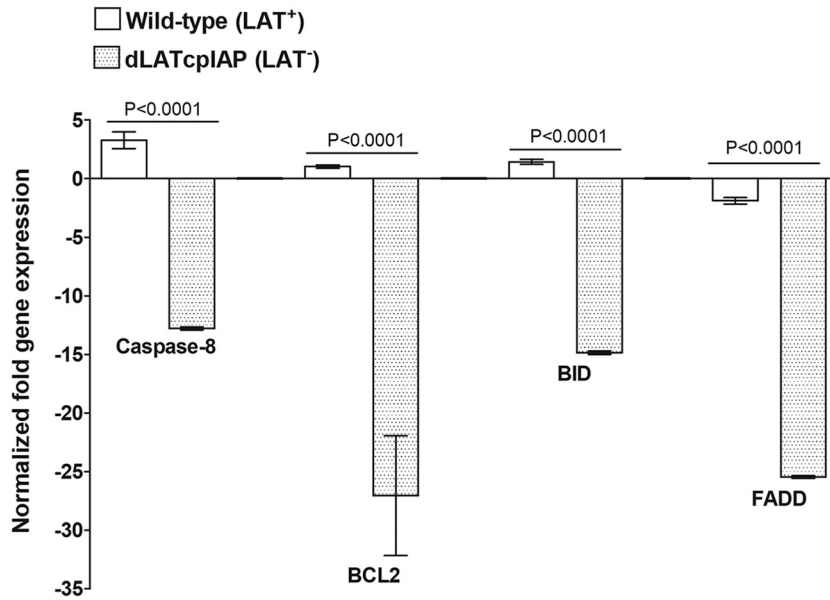
FIG 8 LAT and cpIAP function via distinct mechanisms. Mice were infected with LAT⁺ or dLATcpIAP (LAT⁻) virus as for Fig. 1. On day 28 p.i., mice were euthanized and TG from individual mice collected and combined. qRT-PCR was performed using total RNA. Expression of caspase 8, BCL2, BID, and FADD in naive mice was used to estimate the relative expression of each transcript in TG, and GAPDH was used to normalize expression of each transcript. Each point represents the mean ± SEM from 10 mice.

antiapoptotic activity of LAT is the mechanism that leads to CD8⁺ T cell exhaustion. If this is the case, we predicted that replacement of LAT with a different antiapoptotic gene will rescue the loss of CD8⁺ T cell exhaustion associated with LAT deficiency. To test this hypothesis, we infected mice ocularly with WT McKrae, LAT⁻, or dLATcpIAP (LAT⁺) virus. We found that expression levels of CD8, PD-1, and TIM-3 mRNAs were significantly lower in TG of mice infected with either the LAT⁻ virus or a recombinant virus in which LAT was replaced with the cpIAP gene (dLATcpIAP) than in LAT⁺ virus-infected mice ($P < 0.0001$ [Fig. 9]). Because replacement of LAT with cpIAP does not restore expression levels of CD8, PD-1, and TIM-3 to those of LAT⁺ virus, we conclude that LAT and cpIAP function via distinct mechanisms and that exhaustion seen in infection with WT HSV-1 is not due to the antiapoptotic function of LAT.

DISCUSSION

Despite some early skepticism, the ability of the HSV-1 LAT to inhibit apoptosis has been well documented in various infection models and is now widely accepted (13–19). Furthermore, antiapoptotic activity of LAT likely plays a role in controlling the latency reactivation cycle. However, the mechanism by which LAT controls apoptosis is unclear. Since LAT is not known to encode a functional protein, we hypothesized that it likely functions at the level of transcription. In this study, we took a systematic approach to test several pathways that have been shown to be activated in response to HSV-1 infections. We demonstrated that various components of type I IFN pathway are downregulated in a LAT-dependent manner.

Viral infections trigger the innate immune response. Viral antigens such as DNA or RNA bind to a pattern recognition receptor (PRR), such as Toll-like receptor 3 (TLR3), the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), or the nucleotide oligomerization domain (NOD)-like receptors (NLRs) (reviewed in reference 51). This results in expression and secretion of IFN-α and -β. In the canonical type I IFN pathway, binding of IFN-α or -β to the interferon α receptor (IFNAR) results in activation of Janus kinase proteins 1 and 2, which then phosphorylate their effector, signal transducer and
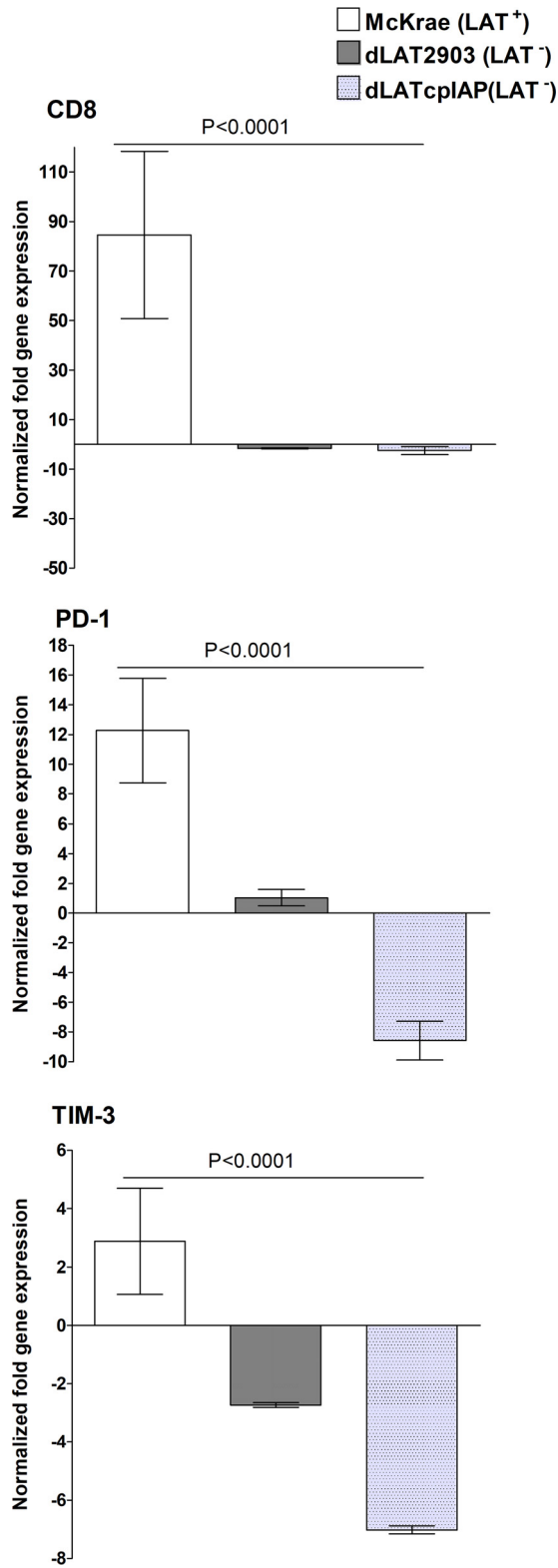
FIG 9 Markers of immune exhaustion are upregulated in LAT[+] virus-infected mice. Mice were infected with LAT[+], LAT[−], or dLATcpIAP (LAT[−]) virus as for Fig. 1. On day 28 p.i., mice were euthanized and TG from individual mice collected and combined. qRT-PCR was performed using total RNA. Expression of CD8, PD-1, and TIM-3 in naive mice was used to estimate the relative expression of each transcript in TG, and GAPDH was used to normalize expression of each transcript. Each point represents the mean ± SEM from 10 mice.

activator of transcription 1 (STAT1), resulting in transcription and translation of interferon-stimulated genes (ISGs) (39). On a cellular level, activation of the type I IFN pathway results in growth arrest and/or apoptosis (35, 36), although this may be cell type specific (37, 38).

HSV-1 inhibits the type I IFN pathway during the lytic cycle (43). A study from the Jones laboratory showed that LAT delays several IFN-α subtypes, including IFN-α1, IFN-α4, IFN-α5, IFN-α6, and IFN-α14, as well as IFN-β, during an acute infection *in vitro*, and IFN-α4 and IFN-β *in vivo* (34). Additionally, the JAK-STAT pathway was shown to have a protective role during acute HSV-1 infection *in vivo* (52). A recent study also reported a correlation between high expression levels of JAK-STAT pathway components and a lack of HSV-1 symptoms in humans (53). We have shown previously that JAK-STAT pathway is involved in blocking HSV-1 replication *in vitro* during an acute infection in macrophages and dendritic cells (54). Finally, *in vitro* studies by the Smiley group showed that several ISGs, including ISG15, ISG56, IP-10 (CXCL10), OAS-3, and Mx-1, were stimulated upon infection with HSV-1 lacking functional ICP0 and VP16 but not with WT HSV-1 (41). These results, and studies by others, suggested that while HSV-1 infection triggers activation of type I IFN pathway, HSV-1 encodes several proteins, including ICP0 and ICP27, that inhibit the type I IFN pathway components during the acute phase of infection (40–43). We hypothesized that LAT could utilize a similar mechanism to inhibit apoptosis during latency in mice. Indeed, we found JAK-1 and JAK-2 to be downregulated in mice latently infected with WT but not LAT-deficient HSV-1. Although we did not directly test if protein levels of JAK-1 and JAK-2 were also affected, we observed a concomitant downregulation of several downstream ISGs (ISG15, ISG54, ISG56, IRF-1, Mx-1, CIITA, and C3) in mice infected with WT McKrae, suggesting that the activity of this pathway was reduced. Further experiments, such as phosphorylation status and nuclear localization of STATs, would help support these findings.

Our results are in agreement with previous reports that showed an immediate early (IE) gene expression-dependent downregulation of these factors during an acute infection (40–43). ISG56, IRF-1, Mx-1, and CIITA were downregulated in a LAT-dependent manner, suggesting that similarly to IE genes during an acute infection, LAT can regulate some, but not all, of the type I IFN pathway during latency. Interestingly, we also found a LAT-dependent upregulation of OAS-3. As OAS-3 functions in limiting virus replication (55, 56), it is tempting to speculate that LAT's mechanism to maintain latency could involve upregulation of OAS-3. Considering that only a small fraction of the cells in TG are latently infected, the fact that we see significant differences in gene expression suggests that gene expression changes within those infected cells must be substantial. This could be tested by examining expression levels of these genes in individual cells, for example, by using RNA fluorescent *in situ* hybridization (FISH).

What property of the LAT inhibits the type I IFN pathway? LAT partially overlaps and lies antisense to the ICP0 gene (57). ICP0, on the other hand, can prevent IFN-mediated repression of HSV-1 during acute infection (52). Because ICP0 is still intact in the LAT null virus dLAT2903 used in this study, the effects seen in this study are not due to lack of ICP0 expression. However, LAT may repress expression of ICP0 during latency (58), although conflicting evidence has also been reported (59). Therefore, it is possible that continued expression of ICP0 in the absence of the LAT could contribute to downregulation of JAK-1 and JAK-2. It would be interesting to test if this is the case by using a LAT-deficient virus in which ICP0 is also disrupted. Unfortunately, ICP0 mutant viruses have lowered virulence, which could be a confounding factor.

An alternative hypothesis could be made that LAT control of type I IFN genes is an indirect effect of lower production of viral particles and, thus, lowered immunogenic response. Previous studies have shown that LAT inhibits expression of lytic genes, possibly via an epigenetic mechanism during latency (60–63). While this is an attractive hypothesis, one would further predict that loss of LAT would phenocopy the ISG

expression pattern seen during lytic cycle. However, based on the data presented here, we cannot completely discount this possibility, and further *in vitro* experiments could help in making such a distinction.

LAT is not known to encode a functional protein to regulate latency reactivation cycle or apoptosis. Therefore, it is possible that LAT exerts its effect as an RNA molecule. The primary LAT transcript of 8.3 kb is spliced to a stable 2-kb LAT and an unstable 6.3-kb LAT. Several microRNAs are encoded within the primary LAT, at least two of which reside in the LAT promoter region (reviewed in reference 8). Additionally, two small noncoding RNAs (sncRNAs) within the 2-kb LAT which are not present in the LAT$^-$ virus have been described (64). These sncRNAs have been implicated in protection from apoptosis (19) and affect upregulation of HVEM (herpesvirus entry mediator) and, thus, increase in reactivation from latency (65). Whether these two sncRNAs mediate this LAT-dependent inhibition of JAK-1 and JAK-2 is under investigation.

Previous work from various groups has demonstrated that LAT controls apoptosis via inhibiting activation of key caspases (14, 17, 18, 26, 66). Interestingly, we found that caspase 3, 8, and 9 transcript levels were reduced in mice infected with LAT null mutant compared to those in mice infected with WT HSV-1. These results are in contrast to several published works reporting a LAT-dependent inhibition of caspase 3, 8, and 9 activation (17, 66, 67). However, as we did not test if these caspases are activated, it is likely that the LAT mediates activation of the caspase pathway only at the level of protein activation, not gene expression. In principle, this could occur via BCL2, because we observed a slight, LAT-dependent upregulation of the antiapoptotic gene *Bcl2* (Fig. 2). BCL2 inhibits apoptosis via regulating the activity of the proapoptotic BCL2 family members, such as BAD, BID, and BIM (68). Additionally, BCL2 inhibits activation of caspase 3 and caspase 9 indirectly via inhibiting cytochrome *c* release from the mitochondria (68).

NF-κB is upregulated early after infection with HSV-1 (44, 45). Upon viral entry, HSV-1 was shown to redirect NF-κB to ICP0 gene to promote viral replication and to block NF-κB recruitment to the promoter of Iκ-Bα *in vitro* (44). Additionally, expression of gD or treatment of cells with soluble gD protected cells from Fas-mediated apoptosis via upregulation of NF-κB (69). Later studies from the Mastino lab established that gD binding to the HVEM mediates upregulation of NF-κB (46). Although the mechanism of NF-κB activation during early infection with HSV-1 has been established, it is not known if NF-κB is also affected during latency and, if so, whether this is LAT dependent. Here we show that while NF-κB transcription is upregulated in latently infected mice, this is not dependent on LAT expression.

TLR2 and TLR3 have been found to mediate apoptotic signaling in response to bacterial or viral infections (70, 71). During acute HSV-1 infection viral glycoproteins are recognized by TLR2 (72). In addition, TLR3 has been shown to play a protective role during HSV-1 acute infection. TLR3 deficiency was identified in two patients with HSV-1 encephalitis (HSE) (73–75), and Lafaille et al. showed that induced pluripotent stem cells (iPSCs) from patients lacking TLR3 were more susceptible to HSV-1 infection (76). TLR3 is necessary for CD8 T cell immunity to HSV-1 (77). Based on these findings, we hypothesized that LAT could inhibit apoptosis via downregulating the TLR pathway during latency. However, we did not observe a downregulation in any of the TLRs tested, suggesting that, at least during latency, LAT does not function via this pathway to inhibit apoptosis.

LAT mechanism of control of apoptosis is distinct from baculovirus antiapoptotic gene cpIAP. LAT antiapoptotic function is necessary for the HSV-1 latency reactivation cycle, and replacement of LAT with the unrelated, baculovirus antiapoptotic gene cpIAP restores latency and reactivation to WT HSV-1 levels (21). However, it is not known if LAT inhibits apoptosis in a manner similar to that of other viral antiapoptotic genes. The LAT sequence necessary to promote cell survival is contained within a stable 1.5-kb intron, which also functions to promote spontaneous reactivation, whereas the baculovirus cpIAP gene encodes a functional protein (78). Therefore, we hypothesized that the LAT mechanism of action may be

TABLE 1 TaqMan gene expression assays[a]

Gene	Gene expression assay no.	Amplicon size (bp)
CD8α	Mm01182108_m1	67
PD-1	Mm00435532_m1	65
TIM-3	Mm00454540_m1	98
BCL2	Mm00477631_m1	85
BID	Mm00432073_m1	108
Caspase 3	Mm01195085_m1	70
Caspase 8	Mm00802247_m1	96
Caspase 9	Mm00516563_m1	68
FADD	Mm00438861_m1	98
FasL	Mm00438864_m1	84
NF-κB	Mm00476361_m1	70
TLR2	Mm00442346_m1	69
TLR3	Mm01207404_m1	121
TLR4	Mm00445273_m1	87
TLR7	Mm00446590_m1	125
TLR9	Mm00446193_m1	60
Myd88	Mm00440338_m1	90
JAK-1	Mm00600614_m1	72
JAK-2	Mm00434561_m1	74
ISG15	Mm01705338_s1	107
IP-10	Mm99999072_m1	62
CIITA	Mm00482919_m1	93
IRF-1	Mm01288579_m1	95
IRF-3	Mm01203177_m1	55
Card10	Mm00459941_m1	77
ISG56	Mm00515153_m1	80
ISG54	Mm00492606_m1	66
Mx-1	Mm01218004_m1	106
OAS-3	Mm00460944_m1	87
C3	Mm01232779_m1	88
GBP-1	Mm00657086_m1	110
GAPDH	Mm99999915_g1	107

[a]Primers used for RT-PCR assay of each gene transcript in TG of latently infected mice.

different from that of cpIAP. Here we show evidence that replacement of LAT with the baculovirus cpIAP causes a drastic downregulation of caspase 8, BCL2, BID, and FADD transcripts, while WT HSV-1 causes a significantly lesser downregulation of FADD and upregulation of caspase 8, BCL2, and BID (Fig. 2 and 8). These results suggest that LAT and cpIAP inhibit apoptosis using different mechanisms.

Antiapoptotic activity is not sufficient to cause immune exhaustion. We have reported that HSV-1 latency is associated with immune exhaustion and that this is dependent on expression of LAT and subclinical reactivation of virus (23). This finding suggests that instead of true latency, HSV-1 may establish a chronic, low-level infection or cause continuous abortive reactivations. Several of LAT's functions could contribute toward this activity: inhibition of apoptosis, immune evasion, and immune exhaustion. Here we show that apoptosis does not contribute toward establishing a state of immune exhaustion, because replacement of LAT with an unrelated antiapoptotic gene cpIAP did not result in immune exhaustion.

MATERIALS AND METHODS

Virus and mice. Three HSV-1 strains were used in this study: wild-type (WT) HSV-1 McKrae (LAT+), dLAT2903 (LAT−), in which both LAT promoters in each viral long repeat and the first 1,667 nucleotides (nt) of the LAT are deleted (11), and dLATcpIAP (LAT−), which is similar to dLAT2903 except that it contains the complete baculovirus inhibitor of apoptosis protein gene (cpIAP) open reading frame (ORF) in place of the LAT (50). The WT and mutant viruses are depicted in Fig. S1 in the supplemental material. Plaque-purified viruses were grown in rabbit skin (RS) monolayer cells and maintained in 5% fetal bovine serum (FBS) in minimal essential medium (MEM) as described previously (11). WT C57BL/6 female mice (Jackson Laboratories) were infected with 2×10^5 PFU suspended in 2 μl of MEM per eye of each virus without corneal scarification. All animal procedures adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and institutional animal care and use guidelines.

DNA extraction and PCR analysis for HSV-1 genomic DNA. DNA was isolated from homogenized individual TG of infected mice on day 28 postinfection (p.i.) using the commercially available DNeasy blood and tissue kit (catalog number 69506; Qiagen, Stanford, CA) according to the manufacturer's instructions. PCR analyses were done using gB-specific primers (forward, 5′-AACGCGACGCACATCA AG-3′; reverse, 5′-CTGGTACGCGATCAGAAAGC-3′; and probe, 5-FAM-CAGCCGCAGTACTACC-3′, where FAM is 6-carboxyfluorescein). The amplicon length for this primer set is 72 bp. Relative copy numbers for the gB DNA were calculated using standard curves generated from plasmid pAc-gB1 (79).

RNA extraction, cDNA synthesis, and TaqMan RT-PCR. Infected and mock-infected mice were euthanized 28 days p.i., and TG were harvested and suspended in RNAlater (Qiagen) and stored at −80°C until processed. Tissue processing and RNA extraction were done using QIAzol RNA reagent (Qiagen) and 1-bromo-2 chloropropane (BCP) as described previously (80–82). One microgram of 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), using the manufacturer's recommendations. Expression levels of various RNAs were determined using TaqMan gene expression assays (Applied Biosystems). Primer-probe sets consisted of two unlabeled PCR primers and the FAM dye-labeled TaqMan MGB probe in a single mixture. Additionally, all amplicons included an intron-exon junction to eliminate signal from genomic DNA contamination.

The mRNA expression levels of the genes of interest were evaluated using TaqMan gene expression assays (Applied Biosystems) and are listed in Table 1.

Quantitative reverse transcription-PCR (qRT-PCR) was performed using QuantStudio 5 (Applied Biosystems) in 384-well plates, as we described previously (83, 84). Real-time PCR was performed in triplicate for each tissue sample. The threshold cycle (C_T) values, which represent the PCR cycles at which there is a noticeable increase in the reporter fluorescence above baseline, were determined using SDS, version 2.2 software. In all experiments, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of transcripts. Expression in naive mice was used as a baseline control to estimate the relative expression of each transcript in TG of latently infected mice.

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

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.00103-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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