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Influence of Galectin-9/Tim-3 Interaction on Herpes Simplex Virus-1 Latency

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After HSV-1 infection, CD8+ T cells accumulate in the trigeminal ganglion (TG) and participate in the maintenance of latency. However, the mechanisms underlying intermittent virus reactivation are poorly understood. In this study, we demonstrate the role of an inhibitory interaction between T cell Ig and mucin domain-containing molecule 3 (Tim-3)–expressing CD8+ T cells and galectin 9 (Gal-9) that could influence HSV-1 latency and reactivation. Accordingly, we show that most Kb-gB tetramer-specific CD8+ T cells in the TG of HSV-1–infected mice express Tim-3, a molecule that delivers negative signals to CD8+ T cells upon engagement of its ligand Gal-9. Gal-9 was also upregulated in the TG when replicating virus was present as well during latency. This could set the stage for Gal-9/Tim-3 interaction, and this inhibitory interaction was responsible for reduced CD8+ T cell effector function in wild-type mice. Additionally, TG cell cultures exposed to recombinant Gal-9 in the latent phase caused engagement of its ligand Gal-9. Gal-9 was also upregulated in the TG when replicating virus was present as well during latency. Moreover, additional recombinant Gal-9 to ex vivo TG cultures induced enhanced viral reactivation compared with untreated controls. Our results demonstrate that the host homeostatic mechanism mediated by Gal-9/Tim-3 interaction on CD8+ T cells can influence the outcome of HSV-1 latent infection, and manipulating Gal-9 signals might represent therapeutic means to inhibit HSV-1 reactivation from latency. The Journal of Immunology, 2011, 187: 5745–5755.

Herpesviruses are highly successful pathogens in part because they have found ways to resist removal from the host once they have caused infection. They persist by adopting an alternative gene expression program termed latency (1–3). Latency is defined as lifelong retention of functional viral genome without production of infectious virions (4). During HSV-1 latency, transcription of viral genome is limited to latency-associated transcript, whereas the lytic genes are maintained in a transcriptionally repressed state (5). Studies in mice with HSV-1 showed that latently infected ganglia have a persistent inflammatory response that is assumed responsible for maintaining latency and precluding virus from completing a normal replication cycle (2, 6–8). Similar inflammatory responses have occasionally been observed in human ganglia (9), although the consequence of such immune responses is almost impossible to evaluate in human systems. In the mouse model, the phenotype expressed by the ganglionic inflammatory cells is considered to indicate that the response is being driven by the frequent or continuous presence of viral Ags produced by HSV-1–infected neurons (10). Indeed, substantial evidence shows that the CD8+ T cells in the inflamed trigeminal ganglion (TG) express the activation phenotype and that most of them were Kb-gB tetramer specific (7, 10). Moreover, when TG isolated from HSV-1–infected mice were cultured ex vivo, the infected neurons rapidly reactivated the virus when CD8+ T cells were depleted from cultures (10, 11). Additionally, CD8+ T cells with defective function, such as the inability to mediate cytotoxicity, were less able to maintain latency both in vivo and ex vivo (8). Hence, CD8+ T cells are considered to be critically involved in maintaining HSV-1 latency, and it could be that a similar process operates to maintain latency in humans, the relevant host. A number of recent investigations have indicated that the extent and functional efficiency of the inflammatory response is influenced by numerous host-derived counterinflammatory events (12). One such system is mediated by a family of glycoproteins termed the galectins (13). Some of these are upregulated during inflammation and can deliver signals that modify the function of cells that express specific galectin receptors (14, 15). One system of particular interest is mediated by an ubiquitously expressed lectin, galectin 9 (Gal-9), binding to its specific receptor T cell Ig and mucin domain-containing molecule 3 (Tim-3) that is expressed on activated T cells (16). This interaction between Gal-9 and Tim-3 expressed on effector CD4+ and CD8+ T cells can result in their apoptosis or a change in their functional properties (14, 15). In some inflammatory diseases, Gal-9/Tim-3 interaction was shown to control the extent of tissue damage that occurs during autoimmune as well as in a viral-induced immunoinflammatory disease (15, 16). However, the Gal-9/Tim-3 interaction can also reduce the efficiency of protective immune response and may also be responsible in part for the functional exhaustion of T cells observed in some chronic infections (17, 18).

In this study, we sought to determine whether the Gal-9/Tim-3 signaling system could influence the maintenance of HSV-1 la-
tency. To evaluate this hypothesis, we compared the inflammatory responses and T cell composition in the TG of wild-type (WT) and Gal-9 knockout (KO) mice. We also measured the stability of latency by both ex vivo approaches. We show that the TG inflammatory responses in the two mouse strains were similar, with most K8-gB tetramer-specific CD8+ T cells in both strains being Tim-3+ and expressing activation markers. However, the K8-gB tetramer-specific CD8+ T cell population in the Gal-9 KO TG showed heightened levels of some functional parameters compared with WT TG cells. Of notable interest, latency was more stable ex vivo in Gal-9 KO compared with WT mice. Moreover, the addition of exogenous recombinant (r) Gal-9 to WT TG cultures induced apoptosis of most of the Tim-3+ cells and enhanced viral reactivation than control cultures. Our results are compatible with the notion that the Gal-9/Tim-3 regulatory system may help regulate latency and that manipulating signals to Tim-3 could be a useful approach to stabilize latency.

Materials and Methods

Mice and virus
Female 6- to 8-wk-old C57BL/6 mice were purchased from Harlan Sprague Dawley Laboratory (Indianapolis, IN). Animals were housed in the animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care at the University of Tennessee, and all experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Gal-9 KO mice on C57BL/6 background were provided by GalPharma (Kagawa, Japan). HSV-1 strain RE was grown on Vero cells expressing HSV-1 ocular infection and clinical scoring

Corneal infections of C57BL/6 mice were conducted under deep anesthesia induced by i.p. injection of tribromoethanol (Avertin). Mice were scarified on cornea with a 27-gauge needle, and a 3 μl vol was applied to the eye. The eyes were examined on different days postinfection (p.i.) with a slit-lamp biomicroscope (Kowa, Tokyo, Japan). The viral reactivation was titrated and stored in aliquots at −80°C until further use.

HV-1 ocular infection and clinical scoring

Female 6- to 8-wk-old C57BL/6 mice were purchased from Harlan Sprague Dawley Laboratory (Indianapolis, IN). Animals were housed in the animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care at the University of Tennessee, and all experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Gal-9 KO mice on C57BL/6 background were provided by GalPharma (Kagawa, Japan). HSV-1 strain RE was grown on Vero cells expressing HSV-1 ocular infection and clinical scoring

Ex vivo apoptosis assay

TG single-cell suspensions isolated from draining cervical lymph nodes, spleen, and TG samples of mice ocularly infected with HSV-1 were collected at different time points, and aliquots of single-cell suspensions were stained for different cell surface markers that included CD45, CD3, CD8, K8-gB tetramer, CD44, CD69, and Tim-3 on CD8+ T cells. For macrophage analysis, TG suspensions were stained for CD45, CD11b, F4/80, and Gal-9. Additionally, the TG suspensions were also stained for F0x3 expressing CD4+ T cells. The cells were fixed in paraformaldehyde (MultiScience) as described (11).

Preparation of TG for immunofluorescence staining

TG of naive and infected mice were removed on day 14 p.i., embedded in Tissue-Tek OCT (Sakura Finetek) for cryosectioning, and stored at −80°C. Transverse sections were cut 15 μm thick and air dried for 15 min. Representative sections of TG were incubated in Dako serum-free protein block for 2 h at room temperature (RT). Anti-NeuN, clone A60 (Millipore) and anti–Gal-9 Ab (0.5 μg/ml; GalPharma) were incubated in protein block at RT for 1 h. After three rinses for 5 min each in PBS, slides were incubated for 1 h at RT with secondary Ab labeled with Alexa Fluor 488 (green) and Alexa Fluor 594 (red) (Invitrogen, Carlsbad, CA). Sections were washed three times with PBS, air dried, and mounted with ProLong Gold antifade mounting medium (Invitrogen), covered with a coverslip, and visualized under a fluorescence microscope.

Flow cytometry

Single-cell suspensions from draining cervical lymph nodes, spleen, and TG samples of mice ocularly infected with HSV-1 were collected at different time points, and aliquots of single-cell suspensions were stained for different cell surface markers that included CD45, CD3, CD8, K8-gB tetramer, CD44, CD69, and Tim-3 on CD8+ T cells. For macrophage analysis, TG suspensions were stained for CD45, CD11b, F4/80, and Gal-9. Additionally, the TG suspensions were also stained for F0x3 expressing CD4+ T cells. The cells were fixed in paraformaldehyde (MultiScience) as described (11).

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The wells were then washed three times and 100 µl streptavidin-HRP (1:500 dilution; eBioscience) was added and incubated for 1 h at RT. The plate was washed and developed with TMB substrate (R&D Systems) followed by addition of stop solution. Absorbance of each sample was determined at 450 nm. A standard curve ranging from 5 µg to 156.25 ng of rGal-9 (GalPharma) was generated to calculate the Gal-9 concentration in the unknown samples.

Ex vivo reactivation experiments

TG were pooled and single-cell suspensions were plated into 48-well tissue culture plates (1.5 TG/well). The cells were cultured with DMEM (Sigma-Aldrich) containing 10% FCS (Atlanta Biologicals, Atlanta, GA) and 10 U/ml recombinant murine IL-2 (R&D Systems) as described by Liu et al. (11). CD8+ T cells were depleted from the TG suspension by immunomagnetic separation using anti-CD8-coated Dynabeads (Dynal, Oslo, Norway). The efficiency of CD8+ T cell depletion was routinely >96% as determined by flow cytometry. In another experiment, the suspensions of TG isolated from 14 and 32 d.p.i. mice were directly incubated with rGal-9. These cultures were incubated in a 5% CO2 humidified incubator at 37˚C for 8 d. Cell culture supernatant was collected at 24-h intervals and the viral titers were determined. Gal-9 concentration was calculated using the 2^-ΔΔCt method and relative quantification between control and infected mice at different time points was performed using the 2^-ΔΔCt formula. The primers used were as follows: GAPDH, 5'-GTTGTCCGAAACACTCAGAT-3' (forward) and 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse); Gal-9, 5'-GGTGCAGCGGAGGCGCTGG-3' (forward) and 5'-ATAATGATGCACACCCGAGAAG-3' (reverse).

RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis

Individual TG from naive and infected mice were collected at different time points and total mRNA was isolated from TG using TRizol LS reagent (Invitrogen). cDNA was prepared using 1 µg RNA using oligo(dT) primers (Promega, Madison, WI). Quantitative real-time PCR (qPCR) analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with an iQ5 real-time PCR detection system (Bio-Rad). The expression level of Gal-9 was normalized to GAPDH with the ΔCt method and relative quantification between control and infected mice at different time points was performed using the 2^-ΔΔCt formula. The primers used were as follows: GAPDH, 5'-AGGTCCGTAGTGAAGGATTG-3' (forward) and 5'-TGTAAGACCATGTAGTTGAGGTCA-3' (reverse); Gal-9, 5'-GGTGCAGCGGAGGCGCTGG-3' (forward) and 5'-ATAATGATGCACACCCGAGAAG-3' (reverse).

Virus titrations

For determination of viral replication in cornea and TG, mice were sacrificed on d 2, 4, 6, and 8 after corneal infection. Eyeballs and TG of each sample were serially ground with tissue grinders (Wheaton Science Products, Millville, NJ), and homogenized in PBS (pH 7.4). The homogenate was serially diluted and 20 µl of each sample was added to confluent monolayer of Vero cells in 12-well plates (Corning) and incubated for 90 min. After incubation, the monolayers were covered with 2% methylcellulose in DMEM/2.5% FBS and incubated for 3 d at 37˚C and 5% CO2. For the reactivation experiments the culture supernatant was collected after TG culture initiation at 24-h intervals and the number of released infectious virions was determined in a standard virus plaque assay on Vero cells.

Statistical analysis

Most of the analyses to determine the levels of significance were performed using a Student t test with GraphPad software. A p value <0.05 was considered significant. Results are expressed as means ± SEM or otherwise stated.

Results

Inflammatory responses in the TG of HSV-1–infected WT and Gal-9 KO mice

To study the inflammatory responses in TG, WT and Gal-9 KO mice were ocularily infected with HSV-1. TG and draining lymph nodes of those mice that developed stromal keratitis lesions were evaluated for phenotypic characterization of various cell types by flow cytometry. The initial inflammatory response in the TG was similar in cellular composition in both strains of mice at the early time point (day 4 p.i.). As depicted in Fig. 1A, both WT and Gal-9 KO mice showed similar infiltration of total leukocytes. Among these cells, macrophages constituted 70–80% (Fig. 1A). However, we did not observe T lymphocytes (both CD4 and CD8) on d 4 p.i. (Fig. 1B). Subsequent analysis on day 8 p.i. showed marked infiltration of both CD4+ (40%) and CD8+ T (60%) lymphocytes in the TG (Fig. 1C, 1D). CD8+ T cell numbers per TG were marginally higher in WT than in Gal-9 KO animals (Fig. 1E). Although total CD8+ T cell numbers were higher at day 8 p.i. in both WT and Gal-9 KO mice, subsequent analysis on days 10, 14, and 32 p.i. revealed a gradual reduction in total cell numbers (Fig. 1E). We also noted that 65% of the total CD8+ T cell population in WT TG expressed the Tim-3 marker on day 8 p.i., with these frequencies staying approximately the same throughout the 32-d observation period (Fig. 1F). In both Gal-9 KO and WT mice, ~40–50% of the CD8+ T cells were found to be HSV-1–specific H-2Kb-gB 9-505 (K-b-gb tetramer) as measured by tetramer staining at different time points p.i. and most of these tetramer+ cells expressed Tim-3 marker (85%) on days 14 and 32 p.i. (Fig. 1G, 1H). Similar to the results observed by others (10, 21), a large proportion of TG resident cells were also positive for the activation markers CD69 (Fig. 1I, 1J) and CD44 (Fig. 1K, 1L) in both strains, indicating that only activated effector cells were retained in the TG of infected mice. Draining lymph node populations were also examined for the kinetics of Tim-3 expression on K-b-gb tetramer-specific CD8+ T cells (Fig. 1M) in WT mice. Initially, up to 95% of K-b-gb tetramer+CD8+ T cells were Tim-3+ on day 7, but gradually the percentage of Tim-3+ cells decreased and were almost nonexistent by day 32 p.i. The CD4+ T cell population in the TG was also in part Tim-3+ (36%) as shown in Fig. 1N, with minimal differences between Gal-9 KO and WT mice on day 14 p.i. Taken together, our observations show that a high proportion of TG resident CD8+ T cells express Tim-3, which remains so for at least 32 d p.i., but many K-b-gb tetramer-specific CD8+ T cells in the draining lymph node expressing Tim-3+ marker during the acute response become Tim-3- by day 32 p.i.

The TG suspensions were also stained for the presence of regulatory T cells on days 14 and 32 p.i. The Foxp3 regulatory T cell population was ~2-fold higher in WT mice compared with Gal-9 KO in the early stages of infection (day 14, Fig. 2A, 2C). However, these differences in regulatory T cell numbers among the two strains were not significant by day 32 p.i., as shown in Fig. 2B, 2D.

Gal-9 is upregulated in the TG after HSV-1 infection

The current observation that most TG resident CD8+ T cells express Tim-3, and our previous finding that exposure of Tim-3+ CD8+ T cells to Gal-9 delivered an inhibitory signal or apoptosis of target cells (17), could indicate that endogenously produced Gal-9 in TG acts likewise and serves to limit the magnitude of TG resident CD8+ T cell effector function. To study this possibility we examined TG for Gal-9 expression after HSV-1 infection. As shown in Fig. 3A, Gal-9 protein was readily detectable in TG samples of infected mice by Western blotting at different days p.i. Additionally, Gal-9 protein concentration was also quantified by ELISA and mRNA levels were analyzed by qPCR. Although Gal-9 expression was very low in uninfected mice (90 ng/mg), subsequent analysis on day 4 p.i. showed a significant increase in Gal-9 expression (1000 ng/mg) (Fig. 3B). The increased Gal-9 levels persisted for at least 2 wk and subsequently declined by day 32 p.i. However, even on day 32 p.i., the concentration of Gal-9 protein was much higher than in the uninfected mice. Similarly, qPCR analysis of Gal-9 mRNA expression showed increased expression on day 4 p.i. (~90-fold), with peak expression on day 6 p.i. (~119-fold) followed by a gradual decline (Fig. 3C). Because macrophages are known to secrete Gal-9 (22) and these cells migrate to TG early after HSV-1 infection, next we analyzed whether TG
FIGURE 1. Tim-3 expression is upregulated on Kb-gB tetramer-specific CD8+ T cells after HSV-1 infection. C57BL/6 WT and Gal-9 KO mice were ocularly infected with $1 \times 10^5$ HSV-1, and TG isolated from four mice at each time point were processed and analyzed by flow cytometry. A. Representative FACS plots depicting the infiltration of macrophages on day 4 p.i. stained for CD45, CD11b, and F4/80 markers in collagenase-digested TG from WT and Gal-9 KO mice. B. Representative FACS plots depicting the absence of T cells on day 4 p.i. stained for CD45 and CD3 markers in WT mice. C. Representative FACS plots showing the infiltration of CD8+ and CD4+ T cells in the TG of WT mice at day 8 p.i. gated on total (Figure legend continues).
infiltrating macrophages could express Gal-9. Flow cytometry analysis showed that most macrophage populations (90%) expressed Gal-9 (Fig. 3D). Hence, we propose that the upregulation of Gal-9 protein appears to correlate with the macrophage infiltration into TG (Fig. 3E), perhaps indicative that macrophages could act as the prime source of Gal-9 during the early stages (days 3–10). However, it is conceivable that neurons themselves could be a source of Gal-9. To confirm this, immunofluorescence staining was performed on serial sections of TG from d 14 p.i. mice for Gal-9 expression. As shown in Fig. 3F, we observed colocalization of Gal-9 with NeuN (a neuronal cell marker), indicating that neuronal cells can also act as a source of Gal-9. Taken together, our data indicate that Gal-9 expression is increased in TG after HSV-1 infection, and both infiltrating macrophages as well as neuronal cells can act as a source of Gal-9.

**Gal-9/Tim-3 interaction impairs TG resident CD8+ T cell effector function**

The observations that a majority of the CD8+ T cells in the TG expressed Tim-3 and that levels of Gal-9 were upregulated after HSV-1 infection set the stage for Gal-9/Tim-3 interaction, which might have functional consequences. To measure this possibility, single-cell suspensions of TG samples obtained from WT and Gal-9 KO mice were compared for CD8+ T cell production of various cytokines by intracellular cytokine staining assay following stimulation with gB 498–505 (SSIEFARL) peptide. As shown in Fig. 4A–D, the percentage of total CD8+ T cells that produced either IFN-γ or TNF-α cytokines in the Gal-9 KO group was ∼2-fold higher compared with WT animals (both on days 14 and 32 p.i.). Additionally, dual cytokine (IFN-γ and TNF-α)-producing cells were ∼3-fold more frequent in Gal-9 KO mice as compared with WT mice (Fig. 4C, 4D). Furthermore, the mean fluorescence intensity levels of both cytokines were higher in Gal-9 KO compared with WT mice (Fig. 4E) at both time points measured (days 14 and 32). CD8+ T cell populations from TG cell suspensions were also compared for their proliferative response to TCR stimulation (with anti-CD3/CD28) using CFSE dilution assay. As shown in Fig. 4F, 83% of CD8+ T cells from the Gal-9 KO group proliferated during the 72-h incubation period as compared with 66% in the WT group. Additionally, the percentage of Kβ-gB tetramer*GrB+ cells in the Gal-9 KO group was higher (46%) as compared with the WT group (32%), as shown in Fig. 4G. We also
observed reduced CD107 expression in CD8+ T cells of WT mice (15%) as compared with 30% in Gal-9 KO mice as measured on day 32 p.i. (Fig. 4G). These results imply that the functionality of CD8+ T cells in the TG of Gal-9 KO animals was higher than that in WT mice.

**Gal-9 induces apoptosis of TG resident Tim-3+CD8+ T cells**

Our previous reports have revealed that Gal-9 binding to TIM-3 receptor on some T cell subsets induced apoptosis of target cells (14, 17). To study the functional significance of increased Gal-9 expression and its interaction with Tim-3–expressing TG resident CD8+ T cells, samples were collected from both WT and Gal-9 KO mice on day 14 and 32 p.i. Ex vivo cultures were established from pooled TG and were exposed to range of rGal-9 concentrations (0.125–1 μM) for 5 h. Subsequently, the samples were analyzed by flow cytometry to record the proportion of total CD8+ T cells, Tim-3+CD8+ T cells and tetramer+Tim-3+CD8+ T cells undergoing apoptosis in the presence or absence of rGal-9. When the frequencies of total CD8+ T cells were analyzed, there was a dose-dependent decrease in the percentages of CD8+ T cells in the presence of rGal-9 (Fig. 5A, upper panel). The apoptosis of CD8+ T cells caused by rGal-9 could be partially blocked by adding a molar excess of α-lactose to the cultures (Fig. 5A, lower panel). At the onset of culture, 24% of the CD8+ T cells were annexin V +Tim-3+ (Fig. 5B, upper panel). Addition of rGal-9 to the cultures resulted in an increase of annexin V +Tim-3+ CD8+ T cells. At 1 μM Gal-9 concentration, almost all cells that were TIM-3+ underwent apoptosis. The apoptosis of Tim-3+CD8+ T cells could be blocked in the presence of α-lactose (Fig. 5B, lower panel). In additional experiments, the effects of rGal-9 addition on K b–gB tetramer+CD8+ T cells, almost all of which were Tim-3+ (as described was above) were measured. As shown in Fig. 5C (upper panel), most K b–gB tetramer+Tim-3+CD8+ T cells underwent apoptosis when exposed to rGal-9. Furthermore, the apoptosis of K b–gB tetramer+CD8+ T cells could be blocked in the presence of a competitive inhibitor, α-lactose (Fig. 5C, lower panel). A similar pattern of results was also obtained with CD8+ T cells isolated from d 14 WT and Gal-9 KO TG.

![Figure 3](http://www.jimmunol.org/)
FIGURE 4. Gal-9 KO mouse mount stronger CD8+ T cell responses in TG compared with WT mice. K1-gB tetramer-specific CD8+ T cell responses in TG of HSV-1-infected mice were compared among age- and gender-matched HSV-infected WT and Gal-9 KO animals on days 14 and 32 p.i. TG were excised and pooled (n = 4) from WT and Gal-9 KO mice, dispersed into single-cell suspensions, and stimulated for 6 h with or without HSV-1 gB peptide at 1 µg/ml concentrations in the presence of brefeldin A. For optimal surface staining of the CD107 marker, the FITC-conjugated CD107 Ab was added to the culture during stimulation. After incubation, the cells were stained for surface marker CD8 and cytokines IFN-γ, TNF-α, and GrB by intracellular staining and analyzed by flow cytometry. Representative FACS plots depicting IFN-γ and TNF-α production in WT versus Gal-9 KO mice on (A) day 14 and (B) day 32 are shown. C and D, Bar diagrams represent means ± SEM frequencies of TG CD8+ cells producing IFN-γ and TNF-α and the cells producing both IFN-γ and TNF-α. E, Mean fluorescence intensity (MFI) of the cytokines produced is shown. Experiments were repeated three times and the values are means ± SEM. F, Gal-9 KO CD8+ T cells proliferate better than do cells from WT mice. TG cells were stained with CFSE and cultured for 72 h with or without added CD3/CD28. After incubation, the cells were stained with anti-CD8α mAb, and CFSE-staining intensity of CD8+ T cells was analyzed by flow cytometry. A representative histogram shows the extent of CFSE dilution in TG resident CD8+ T cells from WT and Gal-9 KO mice gated on CD45 and CD8+ T cells. G, FACS plots representing the expression of GrB and CD107 by specific TG resident CD8+ T cells in WT and Gal-9 KO mice on day 14 and day 32 p.i. All of the above experiments were repeated three times with similar results and data represent the means ± SEM. *p < 0.05, **p < 0.01.

cultures (data not shown). These data indicate that TG resident CD8+ T cells can undergo apoptosis in the presence of Gal-9.

Influence of Gal-9/Tim-3 interaction on HSV-1 reactivation

With the observations in the previous sections that Gal-9/Tim-3 interaction can either deliver an inhibitory signal or induce apoptosis of CD8+ T cells, we evaluated the consequence of this interaction on HSV-1 latency. To determine whether latency control differs between Gal-9 KO and WT animals, multiple TG cultures were established ex vivo and culture supernatants were tested daily to detect and quantify infectious virus levels over an 8-d period. The cultures were established from infected mice at two time points, days 14 and 32 p.i., a time when infectious virus was detectable in any of the unmanipulated TG subcultures over the entire 8-d test period. In contrast, all subcultures were tested daily for the presence of infectious virus. As is evident, virus reactivation was not detectable in any of the unmanipulated TG subcultures over the entire 8-d test period. One set of unmanipulated (i.e., containing CD8+ T cells) cultures was given 1 µM Gal-9 (the concentration at which most Tim-3+ and K1-gB tetramer+ cells underwent apoptosis, as shown in Fig. 5B, 5C) and the others were left untreated. All subcultures were tested daily for the presence of infectious virus. With the notion that the Gal-9/Tim-3 signaling system does influence the stability of HSV latency.

Further evidence for such effects was obtained by comparing the outcome of virus reactivation in cultures of WT TG to companion cultures with added exogenous rGal-9 at the dose found to cause apoptosis of most Tim-3+ TG resident CD8+ T cells (see above section). Three separate experiments were performed at days 14 and 32 p.i. with pools of dispersed TG cells taken from eight mice in each instance. Part of the pool was used to deplete CD8+ T cells using a Dynabead approach. Multiple cultures were then established and incubated for up to 8 d. One set of unmanipulated (i.e., containing CD8+ T cells) cultures was given 1 µM Gal-9 (the concentration at which most Tim-3+ and K1-gB tetramer+ cells underwent apoptosis, as shown in Fig. 5B, 5C) and the others were left untreated. All subcultures were tested daily for the presence of infectious virus. As is evident, virus reactivation was not detectable in any of the unmanipulated TG subcultures over the entire 8-d test period. In contrast, all subcultures from those lacking CD8+ T cells became virus-positive by 48 h, with the virus titers being maximal at ~6 d. These results were consistent with previous findings (11). Notably, virus reactivation was detected in six
of nine subcultures with added rGal-9 at the 48 h time point, and most cultures were positive by 72 h. The maximal viral titers in the Gal-9 supplemented TG cultures were less (∼4-fold) than those with CD8+ T cell-depleted cultures (Fig. 6B). This experiment demonstrates that in the presence of excess Gal-9, CD8+ T cells undergo apoptosis and induce virus reactivation.

A similar result pattern was evident in the experiments performed with 32 d p.i. WT TG cultures comparing untreated with rGal-9 added TG cultures. Reactivation occurred in both sets of cultures by 48 h, but there were differences in viral production levels between the two. Accordingly, cultures with added rGal-9 produced ∼20-fold (Fig. 6C) more virus at day 4 than did the control untreated cultures. From this experiment we demonstrate that addition of excess rGal-9 to latent TG cultures enhanced virus reactivation. Taken together, our results support the contention that the latency inhibiting effects of CD8+ Gal-9 TIM-3 interaction and HSV-1 latency.
T cells are diminished when the Tim-3 + anti-viral T cells bind to Gal-9.

Analysis of HSV-1 replication in cornea and TG of WT and Gal-9 KO mice

To rule out the possibility that reduced virus reactivation in Gal-9 KO TG cultures compared with WT cultures is due to enhanced viral clearance in cornea and TG during acute phase, and hence reduced establishment of latency in TG, we examined the viral replication kinetics in the cornea and TG of WT and Gal-9 KO mice at different times p.i. We found slightly reduced virus replication in Gal-9 KO mice in both cornea (Fig. 7A) and TG (Fig. 7B). However, the differences were not statistically significant, and by day 8, the virus was cleared from cornea and by day 10 from TG of both WT and Gal-9 KO mice, suggesting that there was no difference in the virus clearance in the WT and Gal-9 KO groups.

Discussion

Herpesviruses remain in the host indefinitely p.i., persisting in a nonproductive state called latency. During latency, productive viral proteins are either undetectable or present in very minimal amounts (3, 23). Latency is established in neurons, which abundantly express only a latency associated transcript that encodes some micro-RNAs but seemingly no proteins (24). However, virus may be continually attempting to return to the productive replication cycle, but is constrained from doing so by a sustained viral-specific CD8+ T cell response in the ganglion (10, 11). Several anti-viral mechanisms have been identified that account for latency retention, none of which was lethal to the infected neuron...
CD8+ T cells in the TG expressed Tim-3, and this situation re-
sults. Moreover, the functional capacity of K\(^{\alpha}\)-gB tetramer-specific
occurs and that the acutely and latently infected TG produced
in the TG express Tim-3 well into the time when stable latency
take up the protein produced by other cells such as macrophages
latently infected neurons produce Gal-9, or whether some neurons
killing. We are currently attempting to determine whether only
serves as one of the host regulatory mechanisms to prevent their
expression of Tim-3 leads to functional exhaustion of CD8+ T cells.

A confounding consequence of HSV infection is that lifelong
latency always follows primary infection. The latency state may be
periodically reversed, giving rise to recurrent episodes of viral
shedding with or without lesions (29, 30). Such recurrences in
the eye can be devastating to ocular function since multiple episodes
often result in profound vision impairment (31). Several circum-
stances may precipitate viral reactivation, but a common theme
may be that they all influence the balance established between
virus infection of the neuron and its control by one or more
aspects of host immunity. Elegant studies, principally by Hen-
dricks and colleagues (8), have demonstrated that the proteins
involved in the productive replication cycle are periodically
expressed, but the virus-specific CD8\(^{+}\) T cells that are closely
associated with infected neurons inhibit the virus reactivation.
Moreover, the factors responsible for the diminution or decreased
function of the CD8\(^{+}\) T cells are likely to result in virus reac-
tivation and neuronal cell destruction (27, 28). As we show in
these studies, the signaling system that involves the binding of
Gal-9 to the Tim-3 receptor on activated T cells may represent one
candidate system that affects the stability of latency. Accordingly,
Gal-9 concentration is increased in the TG during both the pro-
ductive and latent phases of infection. The principal cellular
source of the Gal-9 is likely to be inflammatory macrophages, but,
as we could show by in vitro studies, neuronal cells might also
produce Gal-9, and it could be that neuronal produced Gal-9
serves as one of the host regulatory mechanisms to prevent their
killing. We are currently attempting to determine whether only
latently infected neurons produce Gal-9, or whether some neurons
take up the protein produced by other cells such as macrophages
or regulatory T cells. However, cytokines such as IFN-\(\gamma\) and IL-1\(\beta\)
can stimulate various cells to produce Gal-9 (32, 33) and may
represent other sources of Gal-9 in the TG at later time points
(after day 10). Kuchroo and colleagues (14) also reported that the
CNS may also produce Gal-9, but the neuronal cell source was not
established. If indeed neurons do produce or take up Gal-9, this
may act in some circumstances to blunt the activity of Tim-3+-
CD8\(^{+}\) T cells that interact with them and favor virus reactivation at
the same time. We are currently investigating this issue.

Our results clearly showed that most of K\(^{\alpha}\)-gB tetramer-specific
CD8\(^{+}\) T cells in the TG expressed Tim-3, and this situation re-
mained in the acute stage as well as during the established phase
of latency. As mentioned above, the infected TG made increased
amounts of Gal-9, and this would set the stage for killing or in-
hibition of CD8\(^{+}\) T cell function and the potential reversal of la-
tency. We could readily demonstrate that the addition of rGal-9 to
Tim-3+ TG CD8\(^{+}\) T cells in vitro resulted in their apoptosis. We
could also show that the K\(^{\alpha}\)-gB tetramer-specific CD8\(^{+}\) T cell
population recovered from the TG of WT animals was less
functional than were TG CD8\(^{+}\) cells from Gal-9 KO animals. The
change in function included their proliferative capacity, levels of
cytokines produced, as well as GrB production, properties that
others had shown accounted in part for the latency maintaining
function of CD8\(^{+}\) T cells (8, 10, 34). Our results are consistent
with the recent reports demonstrating Tim-3 expression on TG
resident CD8\(^{+}\) T cells in HSV-1 infection (35) and that the ex-
pression of Tim-3 leads to functional exhaustion of CD8\(^{+}\) T cells.
Additionally, at least early after HSV infection the TG of Gal-9
KO animals had lesser numbers of regulatory T cells than did WT
TG, which might also favor the retention as well as blunting the
activity of CD8\(^{+}\) T cell effector function before the time when
latency becomes stably established.

Despite some effort, we were not able to demonstrate any re-
producible differences in latency control in vivo between Gal-9 and
WT animals, but we continue with such studies using a range of
models that others claim can cause virus reactivation. Nevertheless,
our ex vivo investigations showed striking support for the notion
that Tim-3/Gal-9 signaling does affect the stability of latency. Thus,
ex vivo cultures of TG from infected Gal-9 KO animals maintained
latency in vitro for longer periods and produced significantly less
virus than did WT cultures. Even more of interest, the addition of
rGal-9 to WT TG cultures caused enhanced viral reactivation than
did control untreated cultures. Such studies led us to hypothesize
that local changes in Gal-9 and perhaps other galectins and host-
derived regulatory proteins (12) could contribute to viral reac-
tivation by blunting the protective function of CD8\(^{+}\) T cells in
close contact with latently infected neurons. The source of the
Gal-9 need not be the TG itself and could arrive via the blood-
stream from other sites. Raised levels of galectins that include
Gal-9 have been reported in some stress situations (36–39), con-
ditions where some heat shock proteins are elevated as well in
some acute infections (40) that include HSV (41, 42). Stress sit-
uations are a well-recognized prequel to HSV reactivation, which
can result in disease, with or without lesions (43, 44). Whether the
human stress scenarios include changes in levels of Gal-9 prior to
HSV reactivation merits investigation. If the change is shown to
occur, then modulating responses to Gal-9, as can be achieved
with some sugars (17), might turn out to be a useful therapeutic
maneuver.

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Disclosures
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