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

Pradeep B. J. Reddy,* Sharvan Sehrawat,† Amol Suryawanshi,* Naveen K. Rajasagi,* Sachin Mulik,* Mitsuomi Hirashima,‡ and Barry T. Rouse*‡

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 apoptosis of most CD8+ T cells. Furthermore, Gal-9 knockout TG cultures showed delayed and reduced viral reactivation as compared with wild-type cultures, demonstrating the greater efficiency of CD8+ T cells to inhibit virus reactivation in the absence of Gal-9. Moreover, the addition of 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.

**Abbreviations used in this article: Gal-9, galectin 9; GrB, granzyme B; KO, knockout; p.i., postinfection; qPCR, quantitative real-time PCR; r, recombinant; RT, room temperature; TBST, TBS containing 0.05% Tween 20; TG, trigeminal ganglion; Tim-3, T cell Ig and mucin domain-containing molecule 3; WT, wild-type.**

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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 showed that the TG inflammatory responses in the two mouse strains were similar, with most K<sup>+</sup>-gB tetramer-specific CD<sup>8</sup> <sup>+</sup> T cells in both strains being Tim-3<sup>+</sup> and expressing activation markers. However, the K<sup>+</sup>-gB tetramer-specific CD<sup>8</sup> <sup>+</sup> 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<sup>+</sup> 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-week-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<sup>−/−</sup> mice on C57BL/6 background were provided by GalPharma (Kagawa, Japan). HSV-1 strain RE was grown on Vero cells obtained from American Type Culture Collection (no. CCL81). The virus was harvested and stored in aliquots at −80°C until further use.

#### 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 drop containing 1 × 10<sup>7</sup> PFU HSV in 3 µl vol was applied to the eye. The eyes were examined on different days postinfection (p.i.) with a slit-lamp biomicroscope (Kowa, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded as described elsewhere (19, 20). Mice with a corneal score of ≥2 were used for all experiments.

#### Abs and reagents

PerCP-conjugated anti-mouse CD8α (53-6-7), allophycocyanin-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-mouse CD44 (IM7), FITC-conjugated anti-mouse CD69 (H1.2F3), allophycocyanin-conjugated anti-mouse CD45 (50-F11), FITC-conjugated anti-mouse CD107a (1D4B), FITC-conjugated anti-mouse TNF-α, allophycocyanin-conjugated anti-mouse IFN-γ, FITC-conjugated anti-annexin V, anti-CD3 (145-2C11), anti-CD28 (37.51), and Golgi transport inhibitor (brefeldin A) were purchased from BD Biosciences. PE-conjugated anti-mouse Tim-3 Ab was obtained from R&D Systems (Minneapolis, MN). PE-conjugated anti-Fox3 (FJK-16s) and granzyme B (GrB)-PE (16G6) were purchased from eBioscience. Allophycocyanin-conjugated and PE-conjugated H-2Kb/-gB<sub>498–505</sub> (SSIEFARL) tetramers were provided by the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA). Recombinant human and mouse Gal-9 and anti–Gal-9 Abs (1A2 and 108A2) were provided by GalPharma. The Ab-stained cells were acquired with a FACSCalibur (BD Biosciences) and the data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

#### Preparation of TG single-cell suspensions

At various days after HSV-1 infection, mice were euthanized by exsanguination, TG were excised and subjected to collagenase type I treatment (Sigma-Aldrich, St. Louis, MO) at a concentration of 3 mg/ml for 90 min at 37°C. After incubation, the TG were dispersed into single cells by trituration. The cells from multiple TG were pooled and equal numbers of cells were plated in 48-well tissue culture plates. The cells were cultured in DMEM with 10% FCS and 10 U/ml recombinant murine IL-2 (R&D Systems) 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 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, K<sup>+</sup>-gB tetramer, CD44, CD69, and Tim-3 on CD8<sup>+</sup> 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 Foxp3 expressing CD4<sup>+</sup> T cells. The cells were fixed in paraformaldehyde (Thermo Scientific) as described (11). TG were cultured in U-bottom 96-well plates and left untreated or stimulated with gB<sub>498–505</sub> (SSIEFARL) peptide (1 µg/ml) and incubated for 5 h at 37°C in 5% CO<sub>2</sub>. Brefeldin A (10 µg/ml) was added for the duration of the culture period to facilitate intracellular cytokine accumulation. After this period, cell surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD Pharmingen) to enumerate the number of IFN-γ<sup>+</sup>, TNF-α<sup>+</sup>, and GrB-producing CD8<sup>+</sup> T cells as previously described (17). The fixed cells were resuspended in FACS buffer (PBS with 3% heat-inactivated serum) and analyzed flow cytometrically. A CFSE proliferation assay was performed with TG single-cell suspensions isolated from WT and Gal-9 KO mice on day 32 p.i. TG single-cell suspensions were labeled with CFSE (Molecular Probes, Eugene, OR) and cultured in 96-well U-bottom plates. The cells were stimulated with or without CD3/CD28 (0.5 µg/well) for 72 h and, after the incubation, cells were stained with anti-CFSE mAb and analyzed by flow cytometry.

#### Ex vivo apoptosis assay

TG single-cell suspensions from 14 and 32 d.p.i. mice were incubated for 5 h with rGal-9 in the absence or presence of α-lactose in 96-well flat-bottom plates in 5% CO<sub>2</sub>. Autofluor in the presence of 5% CO<sub>2</sub>. After the incubation period the cells were transferred to 96-well U-bottom plates and the cells were stained for annexin V using a kit from BD Biosciences. Additionally, cells were also costained for CD8, Tim-3, and K<sup>+</sup>-gB tetramer. Stained cells were analyzed immediately by flow cytometry.

#### Western blotting and ELISA for the detection and quantification of Gal-9 expression in TG

TG homogenate samples of naive and HSV-1–infected mice collected at different time points p.i. (10 µg/lane) were resolved on 12% SDS-PAGE and transferred electrophoretically on to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked overnight with 5% BSA and washed five times with TBS containing 0.05% Tween 20 (TBST) and incubated with anti–Gal-9 Ab (GalPharma) at a concentration of 0.5 µg/ml diluted in TBST for 1 h at RT. The membrane was washed five times with TBST and incubated with anti-rat HRP (R&D Systems) at a dilution of 1:10,000 for 1 h at RT. The membrane was developed with chemiluminescent substrate (Immobilon Western chemiluminescent HRP substrate; Millipore) and the image was taken on CL-Xposure x-ray film (Thermo Scientific).

 Ninety-six–well microplates were coated with capture Ab at a concentration of 3000 ng/ml (100 µl/well, anti-Gal-9; GalPharma). After incubation overnight at 4°C, the wells were washed three times with PBST and blocked with 300 µl 5% BSA for 2 h at RT. The wells were washed three times with PBST and TG homogenate samples (100 µl) were added to the wells and incubated for 2 h at RT. The wells were then washed with wash buffer three times and biotinylated anti-Gal-9 detection Ab (0.5 µg/ml; R&D Systems) diluted in reagent diluent (R&D Systems; PBS, 5% Tween 20, 0.5% goat serum) was added to each well and incubated for 1 h at RT.

**GALACTIN-9/TIM-3 INTERACTION AND HSV-1 LATENCY**

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**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. Represen-
The wells were then washed three times and 100 μl streptavidin-HRP (1:500 dilution; eBiosciences) 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 in 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 determined in a standard virus plaque assay on Vero cells. 

**Virus titrations**

Ex vivo reactivation experiments

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 mouse were placed in separate tubes containing 1 ml DMEM/1% FBS, ground with tissue grinders (Wheaton Science Products, Millville, NJ), and thawed twice to disrupt the cells. After centrifugation at 500 × g for 5 min to remove the cell debris, the supernatant of each sample was serially diluted and 250 μl 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.

**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 (d 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/gB99-505 (Kb–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 Kb–gB tetramer-specific CD8+ T cells (Fig. 1M). In WT mice. Initially, up to 95% of Kb–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 p.i., but many Kb–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 × 10⁵ 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, suggested that the upregulation of Gal-9 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 K6-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

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**FIGURE 2.** Gal-9 KO mice show diminished numbers of regulatory T cells in TG. C57BL/6 WT and Gal-9 KO mice were infected ocularly with 1 × 10^5 HSV-1 (strain RE) and TG isolated from infected WT and Gal-9 KO mice were analyzed for presence of Foxp3+CD4+ regulatory T cells. A and B, Representative FACS plots depicting the frequencies of total CD4+ T cells and Foxp3+ T cells in the TG of HSV-1–infected mice on days 14 and 32 p.i., respectively. C and D, Bar diagrams representing the frequencies and absolute numbers of regulatory T cells in the WT and Gal-9 KO mice on days 14 and 32 p.i., respectively. Statistical levels of significance were analyzed by a Student t test. Error bars are means ± SEM. *p < 0.05.
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 Kb-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 Kb-gB tetramer+Tim-3–CD8+ T cells underwent apoptosis when exposed to rGal-9. Furthermore, the apoptosis of Kb-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.
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 undetectable in any of the unmanipulated TG subcultures over the entire test period. In contrast, all subcultures from those lacking CD8+ T 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 mounting stronger in Gal-9 KO mice compared with WT mice.

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 K b–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+

![Table I. Summary of experiments performed with ex vivo TG cultures](image)
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 groups, 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 and are presented as the log_{10} PFU/culture. Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

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.
CD8+ T cells in the TG express Tim-3, and this situation re-
in the TG express Tim-3 well into the time when stable latency
or regulatory T cells. However, cytokines such as IFN-
take up the protein produced by other cells such as macrophages
killing. We are currently attempting to determine whether only
produce Gal-9, and it could be that neuronal produced Gal-9
source of the Gal-9 is likely to be inflammatory macrophages, but,
Gal-9 concentration is increased in the TG during both the pro-
candidate system that affects the stability of latency. Accordingly,
these studies, the signaling system that involves the binding of
activation and neuronal cell destruction (27, 28). As we show in
this study, a host regulatory system
mediated by Gal-9 binding to the Tim-3 receptor expressed by
activated effector T cells could represent one such system that
affects the function of the CD8+ T cells that maintain latency. Accordingly, we show that most of the virus-specific CD8+ T cells
in the TG express Tim-3 well into the time when stable latency
occurs and that the acutely and latently infected TG produced
increased quantities of Gal-9 compared with uninfected TG tis-
se. Moreover, the functional capacity of Kb-gB tetramer-specific
CD8+ T cells in the TG of infected animals unable to produce Gal-
was higher than in WT animals. By ex vivo approaches we could
provide clear-cut evidence that Gal-9/Tim-3 signaling influences
stability of latency. Accordingly, TG cultures from Gal-9 KO
animals with established latency showed delayed and reduced
viral reactivation compared with WT TG cultures. Additionally,
reactivation could be accelerated in WT TG cultures when rGal-9
was added. Conceivably, a rise in the concentration of Gal-9 could
contribute to the reversal of latency, and manipulating Gal-9 sig-
could represent a therapeutic approach to influence the sta-
bility of latency.

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-
tion 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-γ and IL-1β
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 cellular 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 Kβ-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 Kβ-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 situ-
ations 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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References
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