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Galectin-1 Reduces the Severity of Herpes Simplex Virus-Induced Ocular Immunopathological Lesions

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

Stromal keratitis is a chronic immunopathological lesion of the eye caused by HSV-1 infection that can result in blindness. Because the inflammatory lesions are primarily orchestrated by Th1 cells, and to a lesser extent by Th17 cells, inhibiting their activity represents a useful form of therapy. In this study we evaluated the therapeutic potential of galectin-1 (gal-1), an endogenous lectin that in some autoimmune diseases was shown to suppress the functions of Th1 and Th17 cells. Treatment was begun at different times after ocular infection with HSV and the outcome was assessed clinically as well as for effects on various immune parameters. Treatment with recombinant gal-1 significantly diminished stromal keratitis lesion severity and the extent of corneal neovascularization. Treated mice had reduced numbers of IFN-γ- and IL-17-producing CD4+ T cells, as well as neutrophil infiltration in the cornea. Furthermore, disease severity was greater in gal-1 knockout mice compared with their wild-type counterparts. The many effects of gal-1 treatment include reduction in the production of proinflammatory cytokines and chemokines, increased production of IL-10, and inhibitory effects on molecules involved in neovascularization. To our knowledge, our findings are the first to show that gal-1 treatment represents a useful approach to control lesion severity in a virally induced immunopathological disease. The Journal of Immunology, 2012, 188: 4631–4643.

Materials and Methods

Mice and virus

Female C57BL6/J mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Breeder pairs of gal-1 knockout (GKO) mice (Lgals1–/–) were obtained from The Jackson Laboratory (Bar Harbor, ME) and additional mice were bred in the Walters Life Sciences animal facility at the University of Tennessee, Knoxville. The animals were housed in American Association of Laboratory Animal Care-approved facilities at the University of Tennessee, Knoxville. All investigations followed guidelines of the Institutional Animal Care and Use Committee. The HSV-1 RE Tumpey strain was used in all procedures. Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA), titrated, and stored in aliquots at –80°C until used.

Corneal HSV infection

Corneal infections of all mouse groups (6–10 wk old) were conducted under deep anesthesia with tribromoethanol. The mice were scarified

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Abbreviations used in this article: gal-1, galectin-1; GKO, gal-1 knockout; MMP, matrix metalloproteinase; PI, propidium iodide; PMN, polymorphonuclear neutrophil; SK, stromal keratitis; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

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lightly on their corneas with a 27-gauge needle and a 3-μl drop containing 1 × 10⁶ PFU HSV-1 RE Tumpey was applied to the eye. In some experiments, animals were infected with a lower dose of virus (5 × 10⁵ PFU HSV-1 RE Tumpey) for comparing disease in GKO mice and control mice.

Detection of infectious virus on HSV-infected corneas
Mouse corneas were swabbed with sterile swabs (Fisher HealthCare) at 3, 6, and 8 d after infection. The swabs were then placed in 0.5 ml RPMI 1640 and frozen at −80˚C until used for assays. Samples were added to confluent Vero cells, incubated for 75 min at 37˚C, and overlaid with 1% methylcellulose. The cultures were incubated for 72 h, fixed with buffered formalin, stained with crystal violet, and the viral cytopathic effect was examined.

Gal-1 administration
The stable form of recombinant human gal-1 was provided by Dr. Toshiro Niki (GalPharma, Kagawa, Japan), and the clinical severity of keratitis of individually scored mice was recorded by a blinded observer. The scoring system was as follows: 0, normal cornea; 1, mild corneal haze; 2, moderate corneal opacity or scarring; 3, severe corneal opacity by iris visible; 4, opaque cornea and corneal ulcer; 5, corneal rupture and necrotizing stromal keratitis. The severity of angiogenesis was recorded as described previously (12). In reference to the angiogenic scoring system, the method relied on quantifying the degree of neovessel formation based on three primary parameters: 1) the circumferential extent of neovessels (because the angiogenic response is not uniformly circumferential in all cases); 2) the centripetal growth of the longest vessels in each quadrant; and 3) the length of the longest vessel in each quadrant, which was identified and graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of ∼0.4 mm (radius of the cornea is ∼1.5 mm). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovessel index (range, 0–16) for each eye at a given time point.

Immunohistochemical staining
At the termination of the experiment (day 15 postinfection), eyes were removed from control and gal-1–treated mice and snap-frozen in OCT compound (Miles, Elkhart, IN). Six-micrometer-thick sections were cut and stained with H&E.

Flow cytometric analysis
Single-cell suspensions were prepared from the cornea and draining cervical lymph nodes obtained from mice sacrificed at 15 d postinfection. Cornea were excised, pooled groupwise, and digested with 60 U/ml Liberase (Roche Diagnostic) for 45 min at 37˚C in a humified atmosphere of 5% CO₂. After incubation, the corneas were disrupted by grinding with a syringe plunger on a cell strainer and a single-cell suspension was made in complete RPMI 1640 medium. The single-cell suspensions were stained for different cell surface molecules for FACS analyses. All steps were performed at 4˚C. Briefly, a total of 1 × 10⁶ cells were first blocked with an unconjugated anti-CD32/CD16 mAb for 30 min in FACS buffer. After washing with FACS buffer, the cells were stained with respective fluorochrome-labeled Abs. For 30 min. Finally, the cells were washed three times and resuspended in 1% paraformaldehyde. To enumerate the number of IFN-γ and IL-17-producing T cells, intracellular cytokine staining was performed. In brief, cells were stimulated with anti-CD3 (3 μg/ml) and anti-CD28 (1 μg/ml) for 5 h in the presence of brefeldin A (5 μg/ml) in U-bottom 96-well plates. After this period, cell surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer’s recommendations. The Abs used were anti-CD4 allophycocyanin, anti–IFN-γ PE, and IL-17 PerCP-Cy5.5. The fixed cells were resuspended in 1% paraformaldehyde. The stained samples were acquired in a FACSCalibur (BD Biosciences) and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Protein quantification of corneal lysates by ELISA
The corneal samples were pooled groupwise (five corneas per sample) and homogenized using a tissue homogenizer (Pellet Pestle mortar; Kontes). For lymph node samples, four draining cervical lymph nodes were collected for each mouse sample and ELISA was performed on the homogenized sample. The concentrations of various cytokines and vascular endothelial growth factor (VEGF) was measured by sandwich ELISA kits from eBioscience (IL-6, IL-10, IFN-γ, IL-17) and R&D Systems (VEGF-A) as per the manufacturers’ instructions.

Quantitative PCR
At 15 d after ocular infection, the corneas were isolated, pooled groupwise (five corneas per sample), corneal cells were lysed, and total mRNA was extracted using TRizol LS reagent (Invitrogen). Total cDNA was made with 1 μg RNA using oligo(dT) primer. Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA) with an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). The expression levels of different molecules were normalized to β-actin using ∆Ct calculation. Relative expression between control and experimental groups were calculated using the 2^−△△Ct formula. The PCR primers used, that is, VEGF-A, matrix metalloproteinase (MMP)-2, MMP-9, CXCL1, and CCL2, were described previously (13, 14).

In vitro differentiation of CD4⁺Foxp3⁺ regulatory T cells
Splenocytes isolated from DO11.10 Rag2−/− mice were used as the pre-cursor population for induction of Foxp3 in CD4⁺ T cells as described before (15). Briefly, 5 × 10⁵ of total splenocytes after RBC lysis and washings were cultured in 1 ml volume with previously optimized doses of plate-bonded anti-CD3/anti-CD28 Ab (1 μg), recombinant human IL-2 (100 U/ml), and TGF-β (0–5 ng/ml) for 4 d at 37˚C in a 5% CO₂ incubator in 48-well plates. In some cultures in addition to IL-2, gal-1 either alone or various concentrations of gal-1 in combination with TGF-β (1 ng) were added. After 4 d, cells were harvested, stained for Foxp3 expression, and characterized phenotypically by flow cytometry.

Apoptosis assay (ex vivo)
Draining lymph node cells from HSV-1–infected C57BL/6 mice (day 15 postinfection) were incubated for 5 h at 37˚C with different concentrations of recombinant gal-1 in the absence or the presence of 50 μM lactate in 96-well flat-bottom plate in a humidified incubator in the presence of 5% CO₂ as described before (16). After the incubation period the cells were transferred to 96-well U-bottom plates and the cells were stained for annexin V and propidium iodide (PI; BD Biosciences) as per the manufacturer’s instructions. Additionally, cells were also costained for CD4. Stained cells were immediately acquired using a FACScalibur (BD Biosciences).

Statistical analysis
Statistical significance was determined by a Student t test unless noted otherwise. GraphPad (Prism 5) software was used for statistical analysis. A p value of ≥0.05 was regarded as a significant difference between groups: *p < 0.05, **p < 0.005, ***p < 0.0005. Error bars represent mean ± SEM.

Results
Administration of gal-1 reduces the severity of SK lesions
To evaluate whether the administration of gal-1 could influence the severity of SK lesions, two types of experiments were performed in mice ocularly infected with HSV. Animals were either treated i.p. with gal-1 starting at day 3 (priming phase/preclinical) until day 12 postinfection or were given the reagent injected i.p. starting at day 6 (effector phase/clinical phase) until day 12 postinfection. Mice were sacrificed at day 15 postinfection to evaluate different parameters. The results demonstrated that following systemic administration of gal-1 starting early (day 3 postinfection), significant inhibition of SK lesions (p < 0.005) was observed in the treated mice when compared with untreated controls (Fig. 1A, 1B). Lesions were more severe in control mice with a higher
FIGURE 1. Effect of gal-1 treatment started during the preclinical phase (day 3 postinfection) on SK severity and cellular infiltration. C57BL/6 mice infected with $1 \times 10^4$ PFU HSV-1 RE were given gal-1 i.p. once daily starting from day 3 until day 12 postinfection. The disease severity and immune parameters were evaluated at day 15 postinfection. (A) Disease progression is shown. (B and C) SK lesion severity and angiogenesis at day 15 postinfection are shown. (D) At the indicated time points, eyes of HSV-infected mice were swabbed with a sterile swab and assayed for infectious virus by standard plaque assay. (E) Three corneas were pooled per sample groupwise and digested by Liberase and stained with Abs. Representative histograms show percentage of leukocytes (CD45+), neutrophils (11b+ Ly6G+), and CD4+ T cells in the inflamed cornea of control and gal-1–treated animals at day 15 postinfection. (F–H) Average number of (F) CD45+ cells, (G) 11b+Gr1hi cells, and (H) CD4+ T cells per cornea at indicated time point are shown. (I) Representative plots show percentage of CD4+ cells producing IFN-γ or IL-17 following stimulation with CD3/CD28 in the cornea of infected animals. Plots shown were gated on CD4+ T cells. (J and K) Average number of CD4 cells producing (J) IFN-γ and (K) IL-17 in the cornea are shown. The level of significance was determined by a Student t test (unpaired). Error bars represent means ± SEM ($n = 4–5$ corneas; $n = 6–7$ mice/group). Experiments were repeated at least three times. *$p \leq 0.05$, **$p \leq 0.005$. 
FIGURE 2. Effect of gal-1 treatment started during the clinical phase (day 6 postinfection) on SK severity and cellular infiltration. C57BL/6 mice infected with $1 \times 10^4$ PFU HSV-1 RE were given gal-1 i.p. once daily starting from day 6 until day 12 postinfection. The disease severity and immune parameters were analyzed at day 15. (A) Disease progression is shown. (B and C) SK lesion severity and angiogenesis at day 15 postinfection are shown. (D and E) Mice were terminated at day 15 postinfection, and eyes were processed for cryosection. H&E staining was performed on 6-μm sections. The figure shows image of the sections taken at ×20 original magnification. (F) Three corneas were pooled per sample groupwise and digested by Liberase and stained with Abs. Representative histograms show percentage of leukocytes (CD45+), neutrophils (11b+Ly6G+), and CD4+ T cells in the inflamed cornea of control and gal-1–treated animals at day 15. (G-I) Average number of (G) CD45+ cells, (H) 11b+Gr1hi, and (I) CD4+ T cells per cornea at indicated time point are shown. Experiments were repeated at least three times. The level of significance was determined by a Student t test (unpaired). Error bars represent mean ± SEM ($n = 5$ corneas; $n = 8$–10 mice/group). **$p \leq 0.005$, ***$p \leq 0.0005$. 

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percentage of infected eyes exhibiting a lesion score of ≥3 when compared with gal-1–treated mice. A similar pattern was observed with the extent of corneal neovascularization, wherein gal-1–treated mice showed significantly lower angiogenesis scores (Fig. 1C, p < 0.002). Almost similar virus titers were observed in the gal-1–treated (starting at day 3 postinfection) and control animals and the virus was completely cleared from the eyes by day 8 postinfection (Fig. 1D). The experiments were terminated on day 15 postinfection and corneas were pooled and digested with Liberase to measure various immune parameters by flow cytometry. As shown in Fig. 1E–H, the numbers of total leukocytes (CD45+ cells), neutrophils (CD11b+Ly6G+), and CD4+ T cells were significantly lower in the corneal samples obtained from gal-1–treated mice when compared with control mice. Additionally, the numbers of CD4+ T cells producing IFN-γ and IL-17 were significantly reduced in gal-1–treated mice compared with controls (Fig. 1I–K).

We next examined whether gal-1 treatment of mice has any effect on the disease progression when treatment is begun in the clinical phase, that is, 6 d postinfection, a stage when replicating virus is mostly cleared on the HSV-infected corneas. As shown in Fig. 2A–C, a similar reduction in lesion severity was observed when gal-1 treatment was begun at day 6 postinfection. The results demonstrate that SK lesions and the extent of corneal

FIGURE 3. Gal-1 administration during clinical phase (6 d postinfection) reduces Th1 and Th17 cell responses in the cornea and draining lymph nodes. C57BL/6 mice infected with 1 × 10^4 PFU HSV-1 RE were given gal-1 i.p. once daily starting from day 6 until day 12 postinfection. (A) Three corneas were pooled per sample groupwise and digested by Liberase, and single-cell suspensions were stained with Abs (n = 6 corneas; n = 8–10 mice/group). Representative plots show percentage of CD4+ T cells producing IFN-γ or IL-17 following stimulation with CD3/CD28 in the cornea of infected animals. Plots shown were gated on CD4+ T cells. (B and C) Average numbers of CD4 cells producing (B) IFN-γ and (C) IL-17 in the cornea are shown. (D) Representative FACS plots show percentage of CD4+ T cells producing IFN-γ or IL-17 following stimulation with CD3/CD28 in the draining lymph nodes of infected animals. Plots shown were gated on CD4+ T cells. (E and F) Average number of CD4+ T cells producing (E) IFN-γ and (F) IL-17 in the draining lymph nodes are shown (n = 3–4 mice/group). Experiments were repeated at least three times and the level of significance was determined by a Student t test (unpaired). Error bars represent mean ± SEM. *p ≤ 0.05. **p ≤ 0.005.
FIGURE 4. Effect of local administration of gal-1 on SK severity and cellular infiltration. C57BL/6 mice infected with $1 \times 10^4$ PFU HSV-1 RE were given gal-1 subconjunctively on day 6, 8, 10, and 12 postinfection. The disease severity and immune parameters were analyzed at day 15 postinfection. (A) Disease progression is shown. (B and C) SK lesion severity and angiogenesis at day 15 are shown. (D) Three corneas were pooled per sample groupwise and digested by Liberase, and cell suspensions were stained with Abs. Representative histograms show percentage of leukocytes (CD45+), neutrophils (Ly6G+Gr1hi), and CD4+ T cells in the inflamed cornea of control and gal-1–treated animals at day 15. (E–G) Average number of (E) CD45+ cells, (F) Ly6G+Gr1hi, and (G) CD4+ T cells per cornea at indicated time point are shown. (H) Representative plots show percentage of CD4+ T cells producing IFN-γ or IL-17 following stimulation with CD3/CD28 in the corneas of infected animals. Plots shown were gated on CD4+ T cells. (I and J) Average number of CD4+ T cells producing (I) IFN-γ and (J) IL-17 in the cornea are shown. Experiments were repeated at least three times and the level of significance was determined by a Student t test (unpaired). Error bars represent mean ± SEM ($n$ = 5 corneas; $n$ = 5–6 mice/group). *$p \leq 0.05$, **$p \leq 0.005$. 

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neovascularization were suppressed in animals treated with gal-1 even when treatment was started at day 6 postinfection (Fig. 2A–C). Immunohistochemical analyses showed that cornea from untreated mice were inflamed and contained a massive infiltrate of inflammatory cells compared with gal-1–treated mice (Fig. 2D, 2E). The numbers of infiltrating leukocytes and polymorphonuclear neutrophils (PMNs) into the infected cornea were reduced in the mice treated with gal-1 compared with untreated control mice (Fig. 2F–H). Additionally, the numbers of CD4^+ T cells were reduced in gal-1–treated animals (Fig. 2I). Also, the numbers of CD4^+ T cells producing IFN-γ or IL-17 cells were significantly reduced in the corneas obtained from treated mice compared with controls (Fig. 3A–C). Furthermore, our results also demonstrated that gal-1 treatment significantly reduced the numbers of Th1 and Th17 cells in the draining lymph nodes of treated mice compared with control mice (Fig. 3D–F). These studies show that administration of gal-1 early or late diminishes HSV-1–induced corneal immunopathology and leukocyte infiltration.

FIGURE 5. Effect of gal-1 treatment on inflammatory and angiogenic factors in the corneas and draining lymph nodes of HSV-1–infected animals. C57BL/6 mice infected with 1 × 10^4 PFU HSV-1 RE were given gal-1 i.p. once daily starting from day 6 until day 12 postinfection. Mice were sacrificed at day 15 postinfection and corneal and lymph node extracts were collected for measuring inflammatory factors using either sandwich ELISA or quantitative PCR. (A) IFN-γ, (B) IL-17, (C) IL-6, (D) IL-10, and (E) VEGF-A protein levels in three different pooled corneal samples each consisting of five cornea in control and gal-1–treated animals were quantified using ELISA (n = 3 samples/group). (F–J) Quantitative PCR was used to measure the expression of proangiogenic factors (F) VEGF-A, (G) MMP-2, (H) MMP-9, and chemokines (I) CXCL1 and (J) CCL2 in pooled corneal samples each consisting of five cornea in control and gal-1–treated animals (n = 3 samples/group). (K–M) ELISA was used to measure (K) IFN-γ, (L) IL-17, and (M) IL-10 protein levels in draining lymph node samples obtained from control and gal-1–treated mice at day 15 postinfection (n = 3 mice). The level of significance was determined by a Student t test (unpaired). Error bars represent mean ± SEM. ^p ≤ 0.05, ^^p ≤ 0.005, ^^^p ≤ 0.0005.
FIGURE 6. GKO mice exhibit increased severity of disease. C57BL/6 or GKO mice infected with $5 \times 10^3$ PFU HSV-1 and disease severity and immune parameters were analyzed at day 15 postinfection. (A) Disease progression is shown. (B and C) SK lesion severity and angiogenesis at day 15 postinfection are shown. (D) Representative histograms show percentage of leukocytes (CD45+), neutrophils (11b+Gr1hi), and CD4+ T cells in the inflamed cornea of control and gal-1–treated animals at day 15 postinfection. (E–G) Average number of (E) CD45+ cells, (F) 11b+Gr1hi cells, and (G) CD4+ T cells per cornea at indicated time point are shown. (H) Representative plots show percentage of CD4+ T cells producing IFN-γ or IL-17 following stimulation with CD3/CD28 in the corneas of infected animals. Plots shown were gated on CD4+ T cells. (I and J) Average number of CD4+ T cells producing (I) IFN-γ and (J) IL-17 in the cornea are shown. Experiments were repeated twice and the level of significance was determined by a Student t test (unpaired). Error bars represent mean ± SEM (n = 4 corneas; n = 7–8 mice/group). *p ≤ 0.05, **p ≤ 0.005.
One of the drawbacks of systemic therapy to control a local inflammatory disease is that the overall immune response is compromised. A desirable approach would be to treat the disease locally. Therefore, we tested the therapeutic potential of local administration of gal-1. In animals given gal-1 locally starting on day 6 postinfection (clinical phase), SK lesions were reduced by almost 2-fold on average compared with controls, with these differences being significant (Fig. 4A, 4B, \( p < 0.009 \)).

The extent of neovascularization was also significantly reduced in the mice treated subconjunctively with gal-1 (Fig. 4C, \( p < 0.02 \)). The numbers of total leukocytes (CD45+ cells), PMNs, and CD4+ T cells recovered from corneas were significantly reduced in the treated groups compared with untreated controls (Fig. 4D–G). Additionally, the frequencies and numbers of CD4+ T cells producing either IFN-\( \gamma \) or IL-17 were reduced 5- to 6-fold as a consequence of local gal-1 treatment (Fig. 4H–J). From these studies it is evident that one of the possible mechanisms by which gal-1 acts is by suppressing the recruitment of PMNs and CD4+ T cells into the HSV-infected cornea. Notably, these studies show that gal-1 negatively regulates Th1 and Th17 cells, which are considered to be the chief mediators of SK immunopathology. Taken together, our results demonstrated that exogenous administration

**FIGURE 7.** HSV-1–infected GKO mice have higher numbers of Th1 and Th17 cells and a lower proportion of regulatory T cells (Tregs) in the draining lymph nodes. C57BL/6 or GKO mice infected with \( 5 \times 10^3 \) PFU HSV-1 and disease severity and immune parameters were analyzed at day 15 postinfection. (A) Representative plots show percentage of CD4+ T cells producing IFN-\( \gamma \) or IL-17 following stimulation with CD3/CD28 in the infected animals. Plots shown were gated on CD4+ T cells. (B and C) Average number of CD4+ T cells producing (B) IFN-\( \gamma \) and (C) IL-17 in the draining lymph nodes are shown. (D–F) Histograms show frequencies of (D) Tregs (CD4+Foxp3+), (E) CD103+ Foxp3 cells, and (F) CTLA+ Foxp3 cells. Plots shown were gated on CD4+ T cells (\( n = 3–4 \) mice/group). Experiments were repeated twice. (G) Mice were sacrificed at day 15 postinfection and corneal and lymph node extracts were collected for measuring inflammatory factors using sandwich ELISA. (H) IL-6, (I) IL-10, and (J) VEGF-A protein levels in three different pooled corneal samples each consisting of five corneas per group in control and GKO animals were quantified using ELISA. (K) IL-10 protein levels in draining lymph node samples (four lymph nodes per sample) obtained from control and GKO mice at day 15 postinfection. The level of significance was determined by a Student \( t \) test (unpaired). Error bars represent mean \( \pm \) SEM. *\( p \leq 0.05 \), **\( p \leq 0.005 \).
of gal-1 either systemically or locally diminished the severity of SK lesion and the extent of corneal neovascularization.

**Effect of gal-1 treatment on cytokines, chemokines, and angiogenic factors involved in SK**

Pooled corneal extracts were collected on day 15 postinfection from control and gal-1–treated (started 6 d postinfection) animals and sample pools were analyzed for IFN-γ, IL-17, IL-6, IL-10, and VEGF-A protein levels using ELISA. The data show that gal-1 treatment reduced the production of IFN-γ by 2-fold in the treated mice compared with control mice (Fig. 5A). The levels of IL-17 were reduced by an average of 43% in the gal-1–treated group when compared with the control group (Fig. 5B). Importantly, the levels of IL-6, a key proinflammatory cytokine involved in SK pathology (17), were reduced by 4-fold in gal-1–treated mice compared with the control group (Fig. 5C). Consistent with previous findings (18), gal-1 treatment induced production of IL-10 that was on average 40% higher when compared with control animals (Fig. 5D). Also, the levels of VEGF-A were reduced ~50% in the corneas from gal-1–treated animals compared with untreated control mice (Fig. 5E). Besides VEGF-A, we also examined the expression of MMP-2 and MMP-9, molecules that are also associated with the neovascularization process in the eye (19). The quantitative PCR data demonstrated that levels of VEGF-A were reduced 2-fold, MMP-2 levels were reduced by 4-fold, and MMP-9 levels were reduced by 6-fold in corneal tissues obtained from gal-1–treated group when compared with the control group (Fig. 5F–H). Also, the expression of two important chemokines involved in neutrophil and monocyte migration, CXCL1 and CCL-2, were found to be reduced in the corneal samples from gal-1–treated mice compared with the control group (Fig. 5G).

**FIGURE 8.** Gal-1 synergistically induces Tregs in the presence of TGF-β. Splenocytes isolated from DO11.10 TCR transgenic RAG−/− mice were stimulated for 96 h with plate-bound anti-CD3/CD28 (1 μg/ml each), 10 ng/ml IL-2 in the presence of TGF-β, Gal-1, or both as indicated. All histograms shown were gated on CD4+ T cells. (A) Representative plots show Foxp3+CD4+ T cells at various concentrations of TGF-β. (B) Percentage of Foxp3+CD4+ T cells at different concentrations of TGF-β. (C) Representative plots show Foxp3+CD4+ T cells at various concentrations of gal-1 and suboptimal concentration of TGF-β (1 ng). (D) Percentage of Foxp3+CD4+ T cells at different concentrations of gal-1 and suboptimal concentration of TGF-β (1 ng). (E) Gal-1 (5 μM) was added to some cultures in the absence of TGF-β as indicated. CD4+ T cells expressing Foxp3 were measured by intracellular staining. The level of significance was determined with one-way ANOVA using a Tukey multiple comparison test. Error bars represent mean ± SEM. These results are representative of three different experiments. *p ≤ 0.05, **p ≤ 0.005.
1 treated mice (Fig. 5I, 5J). CXCL1 was reduced by 3-fold and CCL2 was reduced by 5-fold in corneas obtained from gal-1–treated animals compared with untreated control mice. Additionally, draining lymph node samples from HSV-infected gal-1–treated and control mice were analyzed for IFN-γ, IL-17, and IL-10 protein levels by ELISA. As shown in Fig. 5K and 5L, the levels of IFN-γ and IL-17 were significantly reduced in gal-1–treated mice compared with control animals. The levels of IL-10 were found to be higher in the draining lymph node samples obtained from gal-1–treated mice (Fig. 5M).

**GKO mice show increased herpes stromal keratitis-related immunopathology**

To further investigate the role of endogenous gal-1 in vivo in a virally induced immunopathological disease, we infected GKO mice and control mice (C57BL/6) ocularly with HSV-1. We observed significant differences in the disease severity, with GKO mice showing greater incidence as compared with control mice (Fig. 6A–C). SK lesions were more severe in GKO mice with a higher percentage of infected eyes exhibiting a lesion score of ≥3 when compared with control mice (Fig. 6A). A similar increase was observed with the extent of corneal neovascularization, where GKO mice showed significantly higher angiogenesis scores (Fig. 6D, p < 0.004). The results show that the numbers of total leukocytes, neutrophils, and CD4+ T cells were significantly higher in the infected corneas obtained from GKO mice compared with the control group (Fig. 6D–G). Additionally, the cornea from GKO mice had a significantly higher number of IFN-γ-producing CD4+ T cells (Fig. 6H, 6I). Although greater numbers of IL-17–producing CD4+ were present in the corneas of GKO mice, the differences were not significant (Fig. 6H, 6I). Furthermore, draining lymph node samples from GKO mice had a higher number of IFN-γ–producing CD4+ T cells than did control mice (Fig. 6A–C). The proportion of Tregs was significantly lower in GKO mice (Fig. 7D, 7E). Notably, the percentage of CD4+ Foxp3+ cells expressing functional markers CD103 or CTLA-4 was lower in GKO mice compared with control mice (Fig. 7F, 7G). Additionally, pooled corneal extracts and draining lymph node samples were collected at day 15 postinfection from control and GKO animals and sample pools were examined for IL-6, IL-10, and VEGF-A protein levels using ELISA. Our data showed that the levels of IL-6 were increased by 2-fold in GKO mice compared with control mice (Fig. 7H). Importantly, the levels of IL-10 were reduced by 2-fold in the corneas obtained from GKO animals compared with control animals (Fig. 7J). Also, we found higher levels of VEGF-A in the corneas from GKO animals compared with control mice (Fig. 7J). A similar reduction in IL-10 levels was observed in draining lymph node samples obtained from GKO mice compared with control mice (Fig. 7K).

**Gal-1 induces Tregs synergistically with TGF-β in vitro**

Because GKO mice show lower frequencies of Tregs, we hypothesized that gal-1 may have a role in the expansion of Tregs. Therefore, to examine the possible effects of gal-1 on the induction of Foxp3+ Tregs in vitro, splenocytes obtained from DO11.10 RAG−/− animals (that lack Tregs) were stimulated in vitro with plate-bound anti-CD3/CD28 in the presence or absence of TGF-β (Fig. 8A, 8B). Addition of gal-1 along with suboptimal concentration of TGF-β caused the induction of Foxp3 CD4+ T cells synergistically. As shown in Fig. 8C and 8D, the percentage of Foxp3-expressing cells increased in the presence of gal-1. However, gal-1 when added alone did not induce Foxp3+ T cells (Fig. 8E). These results show that gal-1 in combination with TGF-β promotes Foxp3+ T cell expansion.

**Gal-1 causes apoptosis of CD4+ T cells isolated from HSV-1–infected mice in vitro**

Prior studies suggest that one of the mechanisms by which gal-1 acts is through the selective apoptosis of activated CD4+ T cells (20). Therefore, we investigated whether CD4+ T cells isolated
from HSV-infected mice are susceptible to killing by gal-1. For this purpose, draining lymph node cells from HSV-infected mice were exposed gal-1 for 5 h. Our results demonstrated that CD4+ T cells as a consequence of HSV infection in vivo could be induced to undergo apoptosis when exposed to gal-1 in vitro (Fig. 9, Supplemental Fig. 1). Addition of lactose, a competitive inhibitor to the culture medium, reduced gal-1–mediated apoptosis of CD4+ T cells (Fig. 9A, lower panel).

**Discussion**

Controlling chronic immunoinflammatory lesions in the eye such as those caused by ocular HSV-1 infection remains a major therapeutic challenge. These SK lesions are thought to be primarily orchestrated by CD4+ T cells of the Th1 subtype with some involvement of Th17 CD4+ T cells (3–5). One therapeutic strategy would be to suppress the function of effector CD4+ T cell subsets and the events they orchestrate, as we demonstrate can be achieved by gal-1 treatment. The beneficial effect of gal-1 was the consequence of multiple mechanisms. These included apoptotic effects on proinflammatory T cells, reduced recruitment of tissue-damaging neutrophils, inhibitory effects on inflammatory cytokine and chemokine production, an increased production of the anti-inflammatory IL-10, as well as an inhibitory effect on the corneal neovascularization needed for inflammatory cells to access the corneal stroma. Our results indicate that gal-1 treatment may represent a useful approach to control HSV-induced ocular lesions, the most common infectious cause of blindness in the Western world (2).

Although others have already shown the potential of gal-1 therapy in models of autoimmunity (9–11), to our knowledge this study is the first to document the useful modulatory effects of gal-1 in an infectious model of inflammatory disease. We show that early therapy was highly effective in diminishing lesions of SK, and that even when the treatment was commenced at the stage when virus had been mostly cleared and early lesions begun was also therapeutically useful. As was initially reported using gal-1 to control autoimmunity, gal-1 could effectively inhibit the participation of the inflammatory CD4+ T cells that orchestrate the lesions of SK. As a consequence, the number of such effector cells and the concentration of their inflammatory products such as IFN-γ and IL-17 and chemokines such as KC were significantly reduced by gal-1 treatment. In contrast, mice unable to produce gal-1, because of gene knockout, experienced more severe SK lesions than did normal HSV-infected animals. The effect of gal-1 on inflammatory T cells was likely a direct one (20–22), which could be demonstrated in vitro with apoptosis studies. Fewer cells producing cytokines and chemokines would also explain why levels of such molecules were diminished in treated animals. Furthermore, treated mice had increased levels of the anti-inflammatory cytokine IL-10, which could have been derived from the Treg population (7, 18, 23). Moreover, others have shown that gal-1 may drive the differentiation of dendritic cells toward a regulatory phenotype, and capable of favoring Treg modulation of Th1 and Th17 cells (18, 24).

As we and others have shown (25), the tissue damage evident in SK appears to be mediated mainly by nonspecific inflammatory cells, particularly neutrophils that are recruited to the corneal stroma. One major outcome of gal-1 therapy was a marked reduction of neutrophil numbers presumably due to a reduction in levels of chemokines such as KC. A critical secondary effect of diminishing neutrophil infiltration was the effect on neovascularization. Thus PMNs may act as a source of VEGF, a major angiogenic factor, as well molecules such as metalloproteinases that facilitate corneal neovascularization (19, 26–28). In addition to inhibiting corneal neovascularization via effects on neutrophil recruitment, gal-1 could be mediating anti-angiogenesis through its inhibitory effects on proangiogenic cytokines such as IL-6 and IL-17A, both of which may act to cause VEGF production (5, 17–29, 31).

In conclusion, gal-1, a normal host molecule, may be acting to modulate the severity of viral immunoinflammatory lesions. In the absence of gal-1, lesions were more severe. It is also evident that the application of additional gal-1 particularly early in the disease process was beneficial and reduced the extent of viral immunoinflammatory lesions. The latter effect occurred because gal-1 could diminish both the effector T cells that orchestrate lesions and inhibit the participation of inflammatory cells mainly responsible for tissue damage. Therefore, gal-1 treatment could represent a useful approach to manage the severity of inflammatory disease caused by an infectious agent.

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**Disclosures**

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