Galectin-1 Suppresses Autoimmune Retinal Disease by Promoting Concomitant Th2- and T Regulatory-Mediated Anti-Inflammatory Responses[1]

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Intraocular inflammatory diseases are a common cause of severe visual impairment and blindness. In this study, we investigated the immunoregulatory role of galectin-1 (Gal-1), an endogenous lectin found at sites of T cell activation and immune privilege, in experimental autoimmune uveitis (EAU), a Th1-mediated model of retinal disease. Treatment with rGal-1 either early or late during the course of interphotoreceptor retinoid-binding protein-induced EAU was sufficient to suppress ocular pathology, inhibit leukocyte infiltration, and counteract pathogenic Th1 cells. Administration of rGal-1 at the early or late phases of EAU ameliorated disease by skewing the uveitogenic response toward nonpathogenic Th2 or T regulatory-mediated anti-inflammatory responses. Consistently, adoptive transfer of CD4$^+$ regulatory T cells obtained from rGal-1-treated mice prevented the development of active EAU in syngeneic recipients. In addition, increased levels of apoptosis were detected in lymph nodes from mice treated with rGal-1 during the efferent phase of the disease. Our results underscore the ability of Gal-1 to counteract Th1-mediated responses through different, but potentially overlapping anti-inflammatory mechanisms and suggest a possible therapeutic use of this protein for the treatment of human uveitic diseases of autoimmune etiology. ***The Journal of Immunology,* 2006, 176: 6323–6332.**

Intraocular inflammatory diseases are a major cause of ocular disease accounting for ~10% of legal blindness in the United States (1). There are several different diseases with such characteristics as pars planitis, sympathetic ophthalmia and idiopathic posterior uveitis (2). Experimental autoimmune uveitis (EAU),[5] an organ-specific T cell-mediated disease characterized by inflammation and subsequent destruction of the neural retina, closely resembles human uveitic diseases of autoimmune etiology and can be induced by immunization with one of several retinal Ags in adjuvants in rodents and nonhuman primates (3, 4).

Previous studies demonstrated that a dominant Th1 response underlies the pathogenesis and genetic susceptibility to EAU (5). Susceptible mouse and rat strains are dominant Th1 responders to uveitogenic retinal Ags and uveitogenic effector T cells display a Th1-like cytokine profile (5–7). In addition, it has been shown that manipulating the immune system to up-regulate Th2- and T regulatory cytokine responses can prevent inflammatory responses and protect against the development of EAU (5). These strategies include induction of oral tolerance (8), anterior chamber-associated immune deviation (9), adoptive transfer of Ag-specific Th2-polarized cells (10), administration of IL-10 (11), and treatment with cholera toxin (12) or the calcitonin gene-related peptide (13).

Galectins, a growing family of carbohydrate-binding proteins, have recently attracted the attention of immunologists as novel regulators of immune cell homeostasis (14–20). Galectin-1 (Gal-1), a member of this protein family, is expressed at sites of T cell activation and immune privilege (21–23), and has the potential to regulate the inflammatory response (24–33). How Gal-1 exerts its anti-inflammatory effect is poorly understood, primarily because of its pleiotropic nature where it has been shown to affect T cell activation and proliferation (24, 26), T cell adhesion to the extracellular matrix (34) and NO production (35). Foremost, however, has been the ability of Gal-1 to promote apoptosis of activated T cells through binding to specific carbohydrate ligands on cell surface glycoconjugates (25, 36, 37). In this context, Ishida et al. (22) have reported the ability of retinal pigment epithelium to suppress T cell activation in vitro through secretion of Gal-1, suggesting its possible role in the establishment of immune privilege (22, 38, 39). Furthermore, we have recently provided evidence in vivo showing

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[5] Abbreviations used in this paper: EAU, experimental autoimmune uveitis; Gal-1, galectin-1; IRBP, interphotoreceptor retinoid-binding protein; PTX, pertussis toxin; DTH, delayed-type hypersensitivity; i.d., intradermally.

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that Gal-1 plays a pivotal role in conferring immune privilege to tumor cells (27).

In the present study, we demonstrate that systemic administration of rGal-1 either early or late during the course of EAU ameliorates clinical ocular pathology and counteracts pathogenic Th1 cells in vivo. Treatment with rGal-1 skews the balance of the uveitogenic response toward nonpathogenic Th2 and T-regulatory cytokine profiles. Thus, Gal-1 triggers different anti-inflammatory mechanisms to achieve immunosuppression and thwart pathogenic Th1 responses in vivo.

Materials and Methods

Animals

Six- to 8-wk-old B10.RIII mice were obtained from the animal facilities at the University of São Paulo (São Paulo, Brazil). All animals were housed under specific pathogen-free conditions and treated according to Association for Research in Vision and Ophthalmology guidelines for animal care and use. The Ethics Committee on the Use and Care for Laboratory Animals at the Institute of Biomedical Sciences from the University of São Paulo approved all the procedures used in this study.

Ag and reagents

Interphotoreceptor retinoid-binding protein (IRBP) was isolated from bovine retinas as described previously using Con A-Sepharose affinity chromatography and fast performance liquid chromatography (40). Pertussis toxin (PTX) and CFA were purchased from Sigma-Aldrich. Recombinant Gal-1 (rGal-1) and the C2S/rGal-1 (a variant of rGal-1 in which Cys^2 was substituted to make it more stable under nonreducing conditions) were produced and purified as previously described (41). In brief, *Escherichia coli* BL21 (DE3) were transformed with expression plasmids constructed using pET expression systems (Novagen) and production of rGal-1 was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. Soluble fractions were obtained for subsequent purification by affinity chromatography on an asialofetuin-agarose column and stored at −20°C in 4 mM 2-ME to keep its sugar-binding activity under reducing conditions. LPS content of the purified samples was tested using a Gel Clot *Limulus* Test (Cape Cod).

Induction of EAU

Mice were immunized s.c. at the base of the tail with 50 μg of IRBP in 0.2 ml of CFA (v/v). At the same time, mice were injected i.p. with 0.40 μg of PTX in 0.1 ml of saline solution as an additional adjuvant.

Experimental design

Animals were injected i.p. with 50 μg of rGal-1 (a dose of 1.6 mg/kg), 50 μg C2S/rGal-1 or saline solution (vehicle) in 0.1 ml during the afferent (days 2, 4, 6) or efferent (days 14, 16, 18) phases following immunization (day 0). In another set of experiments, rGal-1 was injected in both the afferent and efferent phases of EAU. To determine whether the anti-inflammatory activity of rGal-1 relies on its carbohydrate-binding activity, rGal-1 was injected in different groups of mice following preincubation with 30 mM lactose.

Histopathological score

Eyes were collected and prepared for histopathological evaluation at the end of each experiment (day 21 after immunization). The eyes were immersed for 1 h in phosphate-buffered glutaraldehyde 4%, transferred into phosphate-buffered formaldehyde 10% for 24 h, and replaced with ethanol 70% until processing. Fixed and dehydrated tissue was embedded in paraffin wax, and 4–6-μm sections were cut through the papillary-optic nerve plane. Sections were stained using H&E. Presence or absence of disease was evaluated in a double-blinded fashion by examining six sections cut at different levels for each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, according to a semiquantitative system described previously (3), considering the lesion type, size, and number. In brief, the minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroids, or retina (EAU grade 0.5). Progressively higher grades were assigned for the presence of discrete lesions in the tissue such as vasculitis, granuloma formation, retinal folding and/or detachment, and photoreceptor damage.

Delayed-type hypersensitivity (DTH)

Two days before the termination of each experiment, mice received 10 μg of IRBP in 10 μl intradermically (i.d) in the pinna of the left ear. The other ear was injected with the same volume of PBS (vehicle). Ear swelling was measured at the termination of the experiment (48 h later) with a dial thickness gauge. DTH results were expressed as Ag-specific swelling, calculated as the difference between the thickness of the IRBP-injected ear and the thickness of vehicle-injected ear.

Cell proliferation assay

Inguinal and iliac lymph nodes were collected and pooled within each group at the end of each experiment (21 days after immunization). Triplicate cultures of 5×10^5 cells/well were stimulated with 30 μg/ml IRBP in 96-well plates in DMEM containing 10% FCS (Invitrogen Life Technologies) and supplemented with 10^{-5} M 2-ME, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 μg/ml gentamicin, 1 mM sodium pyruvate, and 20 mg/ml α-methyl-mannopyranoside to quench any residual ConA remaining from the chromatography preparation of IRBP. The cell cultures were incubated for a total of 96 h and pulsed with [^{3}H]thymidine (0.5 μCi/well) for the last 18 h of incubation. The radioactivity incorporated was quantified in a liquid scintillation counter. Results were expressed as cpm ± SD

ELISA for IRBP-specific Abs

The levels of anti-IRBP IgG and the specific IgG1 and IgG2a/c isotypes were determined by ELISA as previously described (3). Briefly, 96-well microtiter plates (Costar) were coated with IRBP at 1 μg/ml for 4 h at room temperature, blocked with 1% PBS-BSA (Sigma-Aldrich) for 1 h, washed twice with PBS, and incubated for 2 h with serum samples. Plates were then washed and developed with HRP-conjugated rat anti-mouse IgG, IgG1 and IgG2a/c (Southern Biotechnology Associates) for 1 h at room temperature. Plates were washed and incubated with substrate (0.1 M citric acid, 0.2 M Na_2HPO_4 (pH 5.0), 20 μl of H_2O_2, and 20 mg of *o*-phenylendiamine). The concentration of anti-IRBP Abs was estimated using standard curves constructed by coating wells with anti-Ig Ab against the appropriate isotype and adding polyclonal Ig standards of the corresponding isotype.

Determination of cytokine production

Draining lymph node cells harvested 21 days after immunization were cultured in 24-well plates (10^6 cells/well) and stimulated with 30 μg/ml IRBP. Supernatants were collected for cytokine analysis after 48 h and stored at −80°C until assayed. The levels of IFN-γ, IL-5, IL-10, and IL-12 were assessed by ELISA using Ab pairs from BD Biosciences or ELISA kits from Pierce Biotechnology. TGF-β_1 was measured by ELISA using the A75-2 capture Ab and the A75-3 biotinylated detection Ab (BD Biosciences). All kits and Ab pairs were used according to the manufacturer's instructions.

Adoptive transfer experiments

B10.RIII mice were immunized with 50 μg of IRBP and PTX as described above and injected with 50 μg of rGal-1 or control vehicle on days 14, 16, and 18 after immunization. On day 21, eyes were removed to check the effects of rGal-1 treatment by histopathology. Lymph nodes cells were then removed and sorted by CD4 expression using anti-CD4 Ab (BD Biosciences). Previous to adoptive transfer experiments, these cells were analyzed for CD25 expression using an anti-CD25 Ab (BD Biosciences) and Foxp3 expression using an anti-Foxp3 Ab (eBioscience). B10.RIII mice that had been immunized with IRBP 9 days before, received i.v. injections of 5×10^4 $CD4^+$ cells obtained from rGal-1-treated, vehicle-treated, or naive mice. Eyes and lymph node cells from these animals were obtained 12 days after adoptive transfer (21 days after immunization). Eyes were evaluated for histopathological signs of EAU and lymph node cells were cultured in vitro to evaluate IRBP-specific proliferation as described above.

Immunoblot analysis

Following different treatments, inguinal and iliac lymph nodes were removed at different time periods (days 4, 6, and 8 for mice injected at the afferent phase of EAU and days 16, 18, and 20 for mice injected at the efferent phase of the disease), and lymph node cells were processed for immunoblot analysis as described (28). Equal amounts of protein (30 μg) were resolved by SDS-PAGE, blotted to polyvinylidene difluoride membranes (Amersham Biosciences), and probed with anti-GATA3 (Santa Cruz Biotechnology), anti-Foxp3 (eBioscience) or an anti-β-actin polyclonal Abs (Santa Cruz Biotechnology). Bound Abs were detected with a

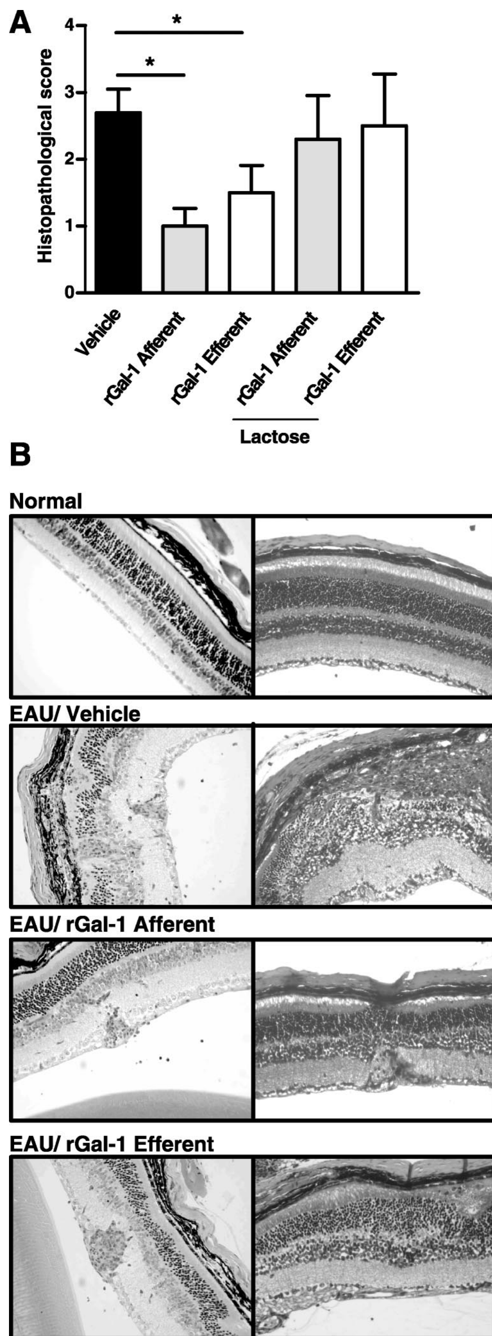

FIGURE 1. Administration of rGal-1 early or late during the course of EAU prevents ocular pathology. B10.RIII mice were immunized with 50 μg of IRBP on day 0 and injected (i.p.) with rGal-1 (50 μg), rGal-1 preadsorbed with 30 mM lactose or saline solution (vehicle control) during the afferent (days 2, 4, and 6) or efferent phases (days 14, 16, and 18) of the disease. Eyes were collected for histopathology on day 21 after IRBP administration. *A*, EAU scores were assigned by histopathological examination of the eyes on a scale from 0 to 4 according to the extent of inflammation and tissue damage as described in *Materials and Methods*. Data are a compilation of three independent experiments with $n = 5$ mice/group; *, $p < 0.05$ vs vehicle-treated control group. *B*, Histopathological features representative of the EAU scores found under different treatments: a normal retinal architecture corresponding to nonimmunized naive mice is shown (*first panel*); mice immunized with IRBP and treated with vehicle control (*second panel*) show ocular lesions characterized by cells infiltrating the vitreous, retinal folds, disorganization of the photoreceptor layer and choroids, granulomas, and vasculitis (disease grade 3); mice treated with rGal-1 on the afferent phase of EAU (*third panel*) exhibit few cells infiltrating the vitreous and vasculitis (disease grade 1) and mice treated with rGal-1 on the efferent phase of the disease (*fourth panel*) show vasculitis, few cells infiltrating the vitreous, retinal fold and slight disorganization of the photoreceptor layer (disease grade 1.5). Representative photographs (H&E staining) of average histopathological scores for each experimental group are shown. Magnification: ×200.

peroxidase-labeled anti-rabbit IgG (Bio-Rad) and developed using enhanced chemiluminescence (Amersham Biosciences). Films were analyzed with Scion Image Analysis software.

Apoptosis assays

Following different treatments, inguinal and iliac lymph nodes were removed at different time periods (days 4, 6 and 8 for mice injected at the afferent phase of EAU and days 16, 18, and 20 for mice treated at the efferent phase of the disease). Lymph node cells were subsequently analyzed for their susceptibility to Ag (IRBP)-induced apoptosis by measuring the nuclear DNA content by flow cytometry as described (28). In brief, cells corresponding to animals treated with rGal-1 at the afferent or efferent phases of EAU were cultured in 24-well plates (Corning) in complete medium at a density of 2×10^6 cells/well in the absence or in the presence of IRBP (30 μg/ml). Cells were recovered after 24 h, washed with ice-cold PBS, and processed for apoptotic cell detection. Cell pellets were gently resuspended in 1 ml hypotonic fluorochrome solution: 50 μg/ml propidium iodide (Sigma-Aldrich) diluted in 0.1% sodium citrate plus 0.1% Triton X-100 and kept at 4°C for 3 h in the dark. The propidium iodide fluorescence emission of individual nuclei was filtered through a 585/42 nm filter and measured by a FACScan cytometer (BD Biosciences). Positive controls of apoptosis included lymph node cells cultured in vitro with rGal-1 (50 μg/ml).

Apoptosis detection in lymph nodes and retinal sections of rGal-1- and vehicle-treated mice was performed as previously described (27) using a peroxidase-conjugated in situ TUNEL assay (Intergen), according to the manufacturer's instructions. Quantitative studies in stained sections were performed in a blinded fashion on five or more fields in each sample.

Statistical analysis

Results are representative of at least three independent experiments. Statistical analysis was performed using the Student's *t* test. Results were considered statistically significant when $p < 0.05$. Histopathological scores were analyzed using Sendecor and Cochran's test for linear trend in proportion.

Results

Administration of rGal-1 early or late during the course of EAU prevents ocular pathology and decreases leukocyte infiltration

The immunoregulatory activity of rGal-1 was examined during the course of EAU. Disease was induced in B10.RIII mice as described in *Materials and Methods*. Groups of five animals were treated i.p. with either rGal-1 or saline solution during the afferent (days 2, 4, and 6) or efferent (days 14, 16, and 18) phases of the disease. Mice from the control group (EAU/vehicle treated) became clinically ill and showed moderate to severe signs of EAU by histopathologic examination on day 21 (mean EAU score: 2.7). Grading of the eyes showed that treatment with rGal-1 during the afferent phase significantly ameliorated the severity of the disease (mean histopathological score of 1.00, $p = 0.03$ when compared with the control group) (Fig. 1*A*). In addition, rGal-1 also suppressed ocular pathology, although at a lesser extent, when administered at the efferent phase of the disease (mean histopathological score 1.4; $p = 0.04$ when compared with the control group). Interestingly, treatment during both the afferent and efferent phases of EAU resulted in a similar clinical outcome to that found when rGal-1 was injected only during the afferent phase of the disease (data not shown). Although the control group (EAU/vehicle-treated) showed ocular lesions characterized by cells infiltrating the vitreous, retinal folds, disorganization of the photoreceptor layer and choroids, granulomas and vasculitis (Fig. 1*B*, *second panel*), mice treated with rGal-1 during the afferent phase of EAU

showed substantial reduction of the leukocyte infiltrate in the vitreous and reduced signs of vasculitis (Fig. 1*B*, *third panel*). In contrast, mice treated with rGal-1 during the efferent phase showed few cells infiltrating the vitreous and slight disorganization of the photoreceptor layer (Fig. 1*B*, *fourth panel*). Interestingly, we also found a marked reduction in EAU incidence in mice treated with rGal-1 at the afferent (4:8) or efferent (5:9) phases of the disease, compared with mice injected with control vehicle (10:10). In addition, similar anti-inflammatory effects were observed when animals were treated with C2S, a Gal-1 variant with increased stability (41) (data not shown). However, there was a dramatic reduction of the anti-inflammatory activity of rGal-1 when this protein was preadsorbed with 30 mM lactose before the in vivo administration during the afferent or efferent phases of the disease (Fig. 1*A*), suggesting that the carbohydrate-binding activity actively participates in the immunoregulatory effects of this protein. Therefore, treatment with rGal-1 ameliorates autoimmune retinal inflammation whether administered early or late during the course of T cell mediated retinal disease.

Administration of rGal-1 suppresses DTH responses and Ag-specific T cell proliferation

Because treatments with rGal-1 during the afferent or efferent phases of the disease resulted in almost completely blockade of the development of EAU, we next investigated the effects of this immunoregulatory protein in T cell mediated immunity by analyzing the in vivo DTH response to IRBP (Fig. 2*A*) and the. Ag-specific proliferative response (Fig. 2*B*). Ear challenge for DTH was tested 19 days after immunization in mice treated with rGal-1 or vehicle control at the afferent or efferent phases of the disease as described in *Materials and Methods*. Swelling of the ear was significantly less prominent in mice treated with rGal-1 during the afferent phase of the disease ($p < 0.0001$) when compared with the vehicle-treated control group (Fig. 2*A*). In contrast, differences in DTH responses were less pronounced when rGal-1 was administered to mice at the efferent phase of the disease ($p = 0.013$). In addition, lymph node cells from mice treated with rGal-1 at the afferent or efferent phases of the disease showed a substantial decrease in the proliferative response to IRBP on day 21 after immunization as compared with lymph node cells from control mice ($p < 0.0001$ and $p = 0.0092$ for mice treated during the afferent or efferent phases, respectively). Thus, rGal-1 treatment significantly inhibits Ag-specific T cell responses during the course of the retinal autoimmune disease. Interestingly, early treatment during the afferent phase of EAU was significantly more effective than treatment during the efferent phase of the disease in promoting inhibition of Ag-specific T cell immunity.

Treatment with rGal-1 modulates anti-IRBP IgG isotype levels

To further investigate the mechanisms underlying the protective effects of rGal-1, we then examined the effects of rGal-1 treatment on anti-IRBP IgG production. As shown in Fig. 3*A*, a marked decrease in Ag-specific IgG levels was observed in mice sera following rGal-1 treatment at the afferent phase of the disease ($p < 0.0001$ compared with control mice). In addition, a slight but statistically significant decrease in anti-IRBP IgG levels was detected when rGal-1 was administered at the efferent phase of EAU ($p = 0.032$ compared with control mice). Furthermore, rGal-1 treatment either early or late during the ongoing retinal disease induced a marked reduction of anti-IRBP IgG2a/c levels (Fig. 3*B*; $p < 0.0001$). In addition, rGal-1 treatment at the efferent phase of the disease resulted in increased levels of Ag-specific IgG1 production (Fig. 3*C*; $p < 0.0001$).

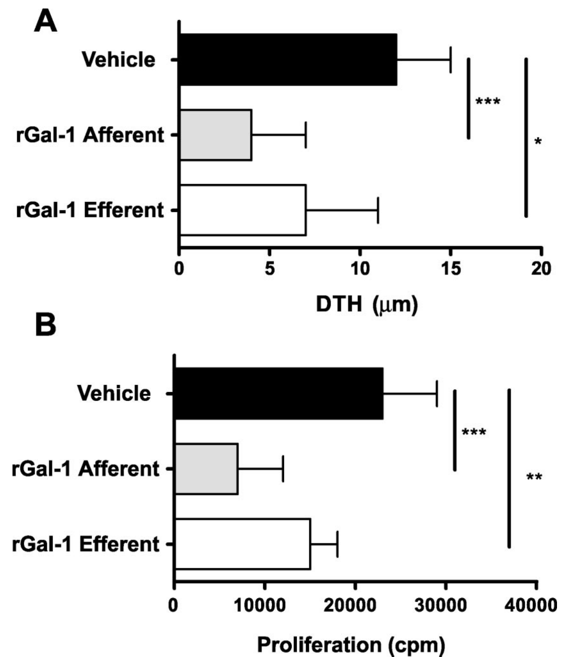

FIGURE 2. Administration of rGal-1 suppresses DTH responses and Ag-specific T cell proliferation. A) IRBP-immunized mice treated with saline solution or rGal-1 at the afferent (days 2, 4, and 6) or efferent phases (days 14, 16, and 18) of EAU were tested for DTH to IRBP. On day 19 after immunization, mice were injected (i.d.) with 10 μg of IRBP into the pinna of the left ear. The right ear was injected with saline solution as a control. Ear swelling was measured after 48 h with a dial thickness gauge. Ag-specific DTH is expressed as the mean difference (micrometer) between the thickness of the IRBP-injected ear and the ear injected with saline solution $\pm$ SD in each experimental group. Data are a compilation of three independent experiments with $n = 5$ mice/group. (***, $p < 0.0001$; *, $p < 0.05$ vs vehicle-treated control group). *B*, Lymphoproliferative response to IRBP was performed 21 days after immunization. Cells from draining lymph nodes (inguinal and iliac) were collected and stimulated in vitro with 30 μg/ml IRBP. Cultures were incubated for 96 h and pulsed with [^{3}H]thymidine for the last 18 h. Results are expressed as mean cpm $\pm$ SD; (**, $p < 0.01$; ***, $p < 0.0001$ vs control vehicle).

Treatment with rGal-1 at early or late phases of EAU shifts the autoimmune response toward nonpathogenic Th2 and T regulatory cytokine profiles

Previous studies have demonstrated that a shift to a Th2 or Th3/Tr1 cytokine profile may account for a decrease in the incidence and severity of EAU (8–13, 42). Because Ag-specific Ab isotypes are good indicators of the cytokines produced during the ongoing autoimmune response (Fig. 3, *B* and *C*), we next investigated the influence of rGal-1 administered early or late during EAU in the regulation of the cytokine balance. Draining lymph node cells from rGal-1-treated or control mice were harvested 21 days after immunization and assayed for cytokine levels in supernatants from IRBP-stimulated cells. Remarkably, treatment with rGal-1 at the afferent phase of EAU resulted in a dramatic decrease in IFN-γ production (Fig. 4*A*) and a marked increase in IL-5 (Fig. 4*B*) and IL-10 (Fig. 4*C*) secretion by lymph node cells. This effect was accompanied by a substantial increase in GATA3 levels at days 4 and 6 following rGal-1 treatment (Fig. 4*F*). Thus, treatment with rGal-1 at early phases of EAU was sufficient to shift the uveitogenic response toward a nonpathogenic Th2 profile. Furthermore, treatment with rGal-1 at the efferent phase of EAU resulted in significant inhibition of IFN-γ (Fig. 4*A*), while no changes were

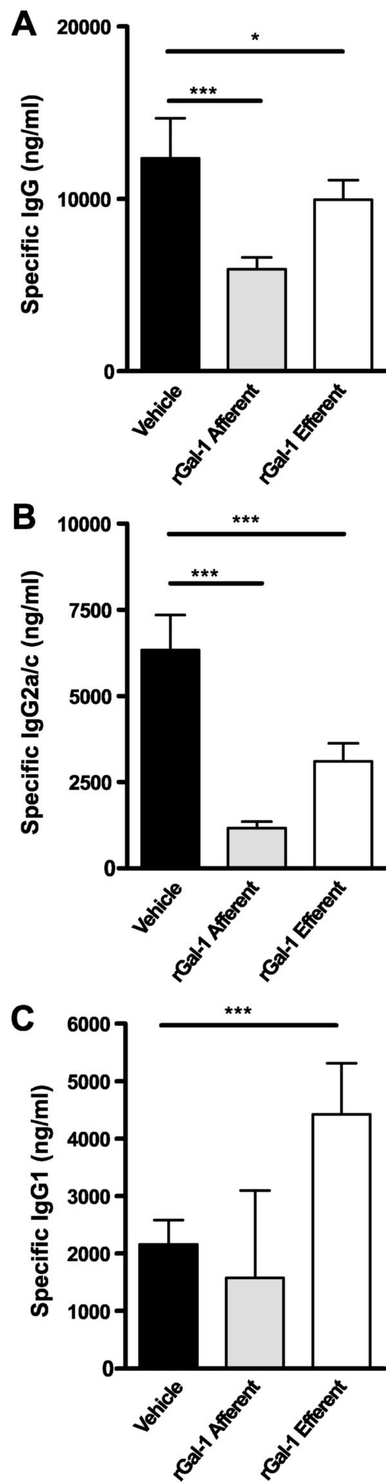

FIGURE 3. Treatment with rGal-1 modulates anti-IRBP IgG isotype levels. Serum levels of anti-IRBP IgG (*A*), IgG2a/c (*B*), and IgG1 (*C*) isotypes in IRBP-immunized mice treated with rGal-1 or vehicle control at the afferent (days 2, 4, and 6) or efferent phases (days 14, 16 and 18) of EAU were determined by ELISA as described in *Materials and Methods*. Results are expressed as the mean concentration (nanograms per milliliter) ± SD in each experimental group. Data are a compilation of three independent experiments with $n = 5$ mice/group. (*, $p < 0.05$; ***, $p < 0.0001$ vs vehicle-treated control group).

detectable in the production of IL-5 (Fig. 4*B*). In addition, administration of rGal-1 during the efferent phase of the ongoing inflammatory disease resulted in a remarkable increase in IL-10 (Fig. 4*C*) and TGF-β_1 (Fig. 4*D*) production, suggesting a pivotal role for rGal-1 in skewing the uveitogenic response toward a T regulatory phenotype. In addition, rGal-1 treatment did not induce any change in IL-12 production either when injected early or late during the course of EAU (Fig. 4*E*). Hence, treatment with rGal-1 suppresses Th1-mediated retinal inflammation by promoting concomitant Th2- and T regulatory cell responses.

$CD4^+$ T cells obtained from rGal-1-treated mice prevent the development of active EAU upon adoptive transfer in syngeneic recipients

The finding that rGal-1 treatment during the efferent phase of EAU promotes a shift toward a T regulatory cytokine profile raised the question whether such regulatory activity could be adoptively transferred into naive recipients in which EAU was induced by active immunization. For this purpose, recipient mice that had been immunized with IRBP 9 days before, received i.v. injections of 5×10^4 $CD4^+$ cells from uveitic mice treated with rGal-1 or vehicle control, or from nonimmunized naive mice. (Fig. 5*A*). Adoptive transfer of $CD4^+$ cells obtained from uveitic mice treated with rGal-1 during the efferent phase of the disease significantly ameliorated the severity of active EAU in syngeneic recipients, as demonstrated by substantial decrease in histopathological score (Fig. 5*B*) and Ag-specific proliferation (Fig. 5*C*). In contrast, $CD4^+$ cells harvested from uveitic mice treated with vehicle control or from naive mice were not effective in transferring protection to uveitic recipient mice (Fig. 5, *B* and *C*). Interestingly, no significant changes were detected in the percentage of $CD4^+$ $CD25^{high}$ cells (<2%) nor in the levels of the transcription factor Foxp3 among different experimental groups (data not shown), suggesting the possibility that rGal-1 treatment would not induce an increase in the number of $CD4^+CD25^{high}$ $Foxp3^+$ regulatory T cells, but would instead regulate the expansion or the suppressive activity of a $CD4^+CD25^-$ regulatory T cell population. Thus, rGal-1 treatment during the efferent phase of the disease favors the activity of $CD4^+$ regulatory T cells which can be adoptively transferred to naive recipient mice and prevent the development of active EAU.

Treatment with rGal-1 during the efferent phase of the disease promotes T cell apoptosis in vivo

It is well known that T cells cycling in response to antigenic stimuli are driven into apoptosis by potent TCR restimulation and that some treatments inhibit EAU by promoting apoptotic death of uveitogenic T cells (43, 44). Because rGal-1 has been shown to trigger apoptosis of activated T cells in vitro (25, 36, 37) and to increase the susceptibility to Ag-induced cell death in vivo (28, 30, 31), we then investigated whether treatment with rGal-1 was able to increase the susceptibility of pathogenic T cells to Ag-induced cell death during the course of the inflammatory disease.

Mice engaged in different protocols were killed 48 h after each treatment with rGal-1 or vehicle control (days 4,6 and 8 for mice treated during the afferent phase of EAU and days 16, 18 and 20 for mice treated during the efferent phase of the disease). Lymph node cells were obtained and pooled as described in *Materials and Methods*, and cultured for 24 h in the presence or absence of IRBP (30 μg/ml) for apoptotic cell detection (Fig. 6*A*). Unexpectedly, we could not observe significant changes in the susceptibility to Ag-induced cell death of lymph node cells from rGal-1-treated vs vehicle-treated mice at any of the time periods studied. Representative results obtained on average days (day 6 on the afferent phase and day 18 on the efferent phase of EAU) are shown in Fig. 6*A*.

Because lymph node cells explanted ex vivo could be indeed survivors and the proapoptotic effects of rGal-1 might take place in

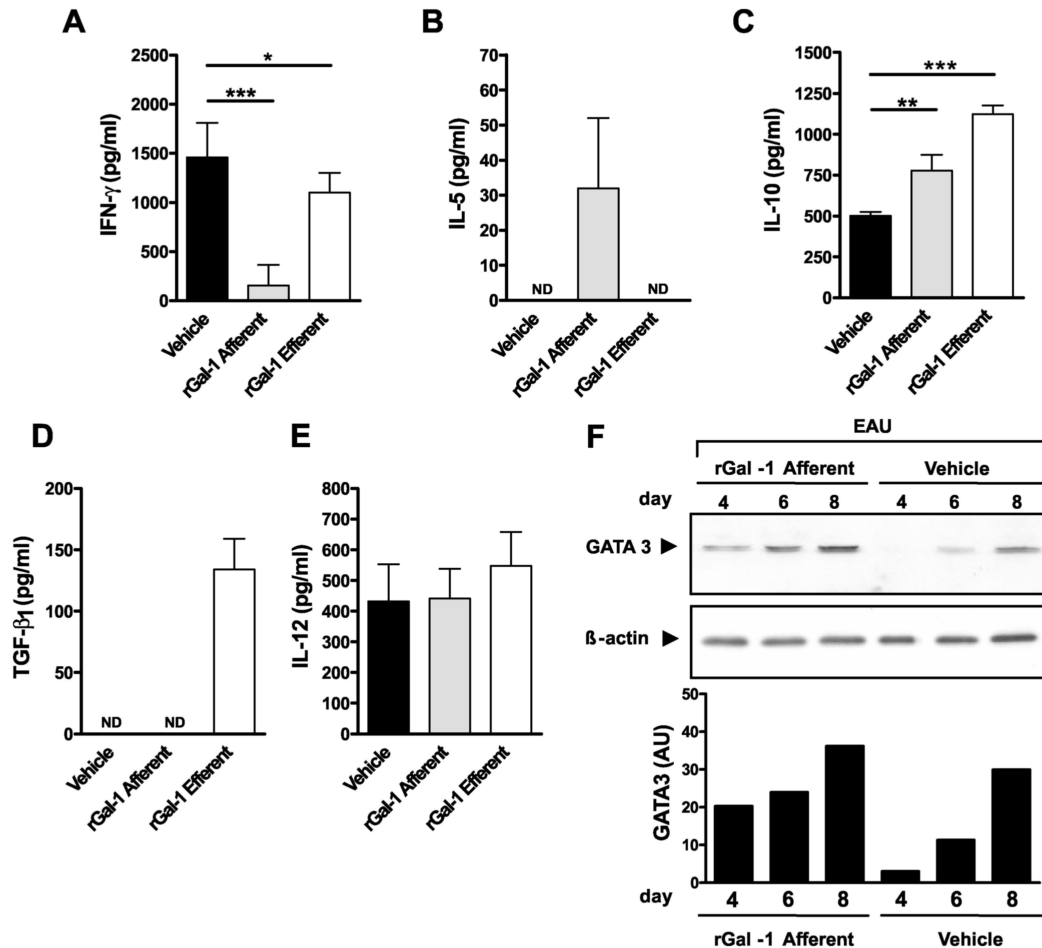

FIGURE 4. Treatment with rGal-1 at early or late phases of EAU shifts the autoimmune response toward nonpathogenic Th2 and T-regulatory cytokine profiles. *A–E*, Draining lymph nodes cells from rGal-1-treated or vehicle-treated mice were harvested at day 21 and stimulated in vitro (10^6 cells/ml) with 30 μg/ml IRBP. After 48 h, IFN-γ (*A*), IL-5 (*B*), IL-10 (*C*), TGF-β_1 (*D*), and IL-12 (*E*) levels were determined in culture supernatants using commercially available ELISA kits or Ab pairs. N.D, not detectable. Results are expressed as mean concentration (picograms per milliliter) $\pm$ SD in each experimental group. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ vs vehicle-treated control group). Data are a compilation of three independent experiments with $n =$ 5 mice/group. *F*, GATA3 expression, during the afferent phase of EAU was analyzed in cell extracts corresponding to draining lymph nodes harvested 48 h after each rGal-1 or vehicle injection (days 4, 6, and 8). GATA3 levels were determined by immunoblot analysis using an anti-GATA3 polyclonal Ab. Equal loading of proteins (30 μg) was evaluated using an anti-β-actin polyclonal Ab. Immunoblot analysis shows GATA3 expression in pooled lymph nodes obtained from three representative mice treated with rGal-1 or vehicle control. Immunoreactive bands were semiquantified by densitometry and expressed as arbitrary units (AU). Data shown by densitometric analysis illustrates one representative of three independent experiments.

vivo, we directly examined the levels of apoptosis in paraffin-embedded lymph nodes sections from rGal-1- and vehicle-treated mice using an in situ TUNEL assay. As shown in Fig. 6, *B* and *C*, a significantly increased number of TUNEL-positive cells was detectable in lymph nodes from mice treated with rGal-1 during the efferent phase of EAU (Fig. 6*B*, *lower right panel*), compared with mice exposed to rGal-1 during the afferent phase of the disease (Fig. 6*B*, *lower left panel*) or treated with vehicle control (Fig. 6*B*, *upper left* and *right panels*). Therefore, treatment with rGal-1 promotes immune cell apoptosis in vivo when administered late during the course of the ongoing inflammatory disease, in agreement with the ability of this protein to kill fully activated T cells. However, TUNEL labeling of retinal samples revealed no changes in the number of tissue-infiltrating apoptotic cells among different experimental groups (data not shown). This finding could be explained by the scarce number of infiltrating cells in the retina which did not allow us the detection of statistically significant differences among different experimental groups. Thus, Gal-1 may trigger different anti-inflammatory mechanisms which might act in concert during different stages of disease evolution to achieve immunosuppression in vivo.

Discussion

In the present study, we have shown that Gal-1, an immunoregulatory β-galactoside-binding protein, can inhibit retinal disease in an experimental model of posterior uveitis. Treatment with rGal-1 resulted in decreased disease scores with maintenance of retinal structures, substantial reduction in leukocyte infiltration and inhibition of T cell-mediated immunity. Furthermore, our findings suggest that rGal-1 can elicit beneficial effects by counterregulating the uveitogenic Th1 response and promoting a shift toward Th2 or T-regulatory cell phenotypes.

Ocular inflammation leads to vision loss through the destruction of delicate tissues along the visual axis (2). The pathogenesis of EAU, and likely also of human autoimmune uveitis, involves cell-mediated destruction of retinal tissues dependent on retinal Ag-specific T cells (5). Because in most cases a Th1-type response has been implicated in the pathogenesis and genetic susceptibility to EAU (6, 7), the established consensus has been that immunoregulatory manipulations designed to enhance the Th2 response at the expense of the Th1 response might be beneficial in the treatment of uveitis (8–12). However, it has been reported that blockade of a

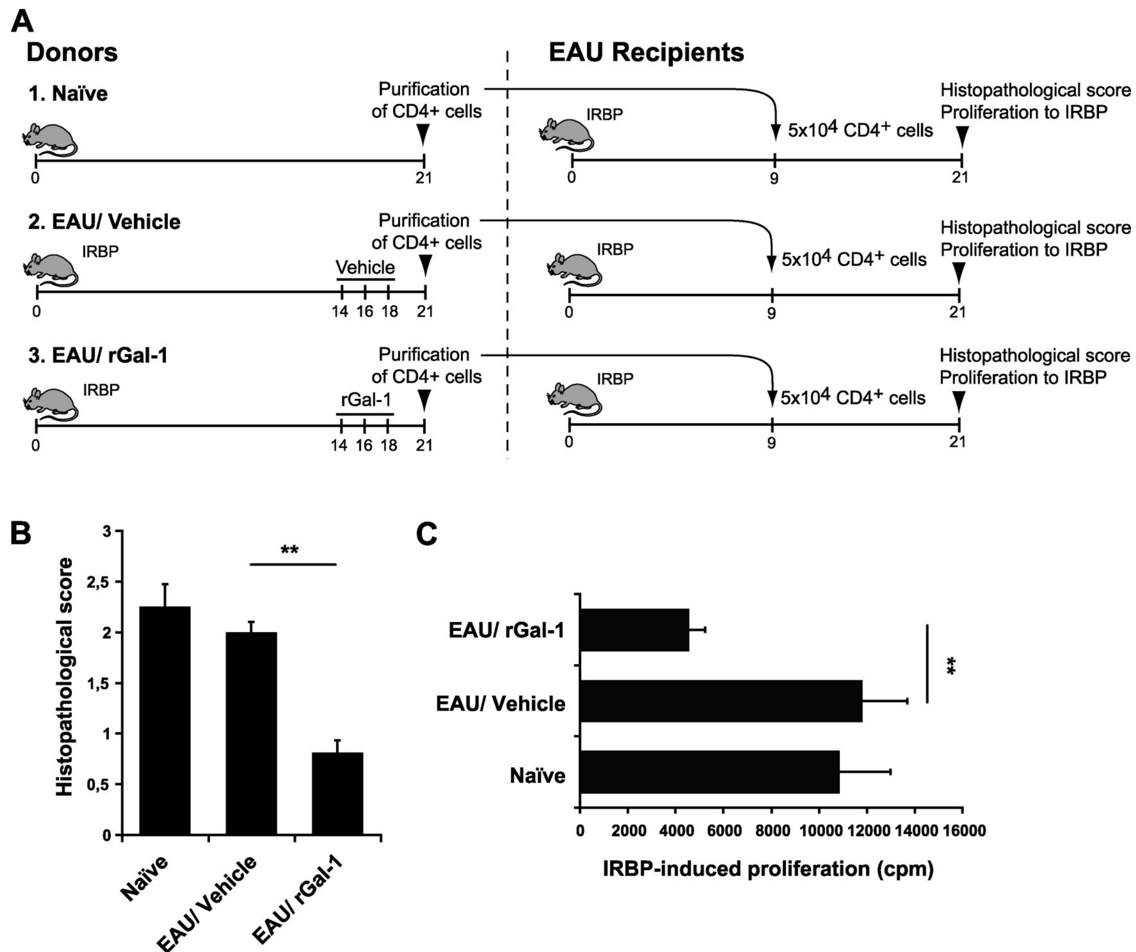

FIGURE 5. $CD4^+$ regulatory T cells obtained from rGal-1-treated mice prevent the development of active EAU upon adoptive transfer to syngeneic recipients. *A–C*, B10.RIII mice were immunized with 50 μg of IRBP and PTX as described in *Materials and Methods* and treated with 50 μg of rGal-1 or vehicle control on days 14, 16, and 18 after immunization. On day 21, eyes were collected to confirm the effects of rGal-1 treatment. Lymph nodes were removed and cells were sorted by CD4 expression. Syngeneic recipient mice that had been immunized with IRBP 9 days before, received i.v. injections of 5×10^4 $CD4^+$ lymph node cells from naive, vehicle-treated or rGal-1-treated mice (*A*). Eyes and lymph node cells from these animals were obtained 12 days after adoptive transfer (21 days after immunization) and evaluated for histopathological signs of EAU (*B*). Data are a compilation of three independent experiments with $n = 5$ recipient mice/group (**, $p < 0.01$ vs vehicle-treated control group). Lymph node cells from recipient mice that received $CD4^+$ cells from naive, rGal-1-treated or vehicle-treated mice were cultured in vitro to evaluate IRBP-specific proliferation (*C*). Cultures were incubated for 96 h and pulsed with [^{3}H]thymidine for the last 18 h. Results are expressed as mean cpm ± SD of three independent experiments (**, $p <$ 0.01 vs vehicle-treated control group).

Th1 response or switching to a Th2 response may not always be advantageous (5, 45–48). The current research is aimed at examining the impact of "non-Th2" immunoregulation in restraining Th1-driven pathology (5, 49). In this regard, "Th3" (TGF-β)- and "Tr1" (IL-10)-mediated regulatory responses have the potential to limit both Th1-and Th2-driven inflammation (5). We demonstrate here that rGal-1 treatment induces the production of substantial amounts of TGF-β_1 and IL-10, which in turn may be responsible for counterregulating the uveitogenic Th1 response. Remarkably, regulatory T cells obtained from rGal-1-treated mice prevent the development of active EAU upon adoptive transfer in syngeneic recipients. Because rGal-1 treatment did not change the number of $CD4^+CD25^{high}$ $Foxp3^+$ cells, it is highly suggestive that this protein favored the expansion of a subpopulation of IL-10- and/or TGF-β_1-secreting $CD4^+CD25^-$ T regulatory cells.

In addition to the critical role of TGF-β (particularly TGF-β_2) in the establishment of immune privilege in the eye (38, 50, 51), this regulatory cytokine may also affect systemic responses involved in EAU (42). It has been demonstrated that pregnancy ameliorates induction and expression of EAU through selective inhibition of uveitogenic Th1 responses with only marginal enhancement of Th2 responses but elevated systemic levels of TGF-β (42). In addition, TGF-β seems to play a key role in down-regulating activation and uveitogenicity of primary Ag-specific effector T cells (52). Our findings suggest that the immunosuppressive effects of rGal-1 observed in EAU, are at least in part, associated with enhanced levels of TGF-β_1 secretion.

In addition to the marked increase in TGF-β_1 secretion, in the present study we demonstrate that IL-10 is markedly increased following rGal-1 treatment during both the afferent or efferent phases of EAU. Moreover, recent findings indicate that exposure of activated T lymphocytes to rGal-1 up-regulates IL-10 mRNA in vitro (53). Interestingly, IL-10 constitutes one of the most critical cytokines mediating a natural recovery from EAU (11). Administration of exogenous IL-10 prevents EAU, whereas administration of an anti-IL-10 Ab exacerbates disease (11). Furthermore, IL-10 is capable of preventing the function of fully differentiated uveitogenic Th1 cells in culture (11), and the expression of IL-10 mRNA in uveitic mouse eyes has been shown to rise during the resolution phase of EAU (54). However, an intriguing observation of our

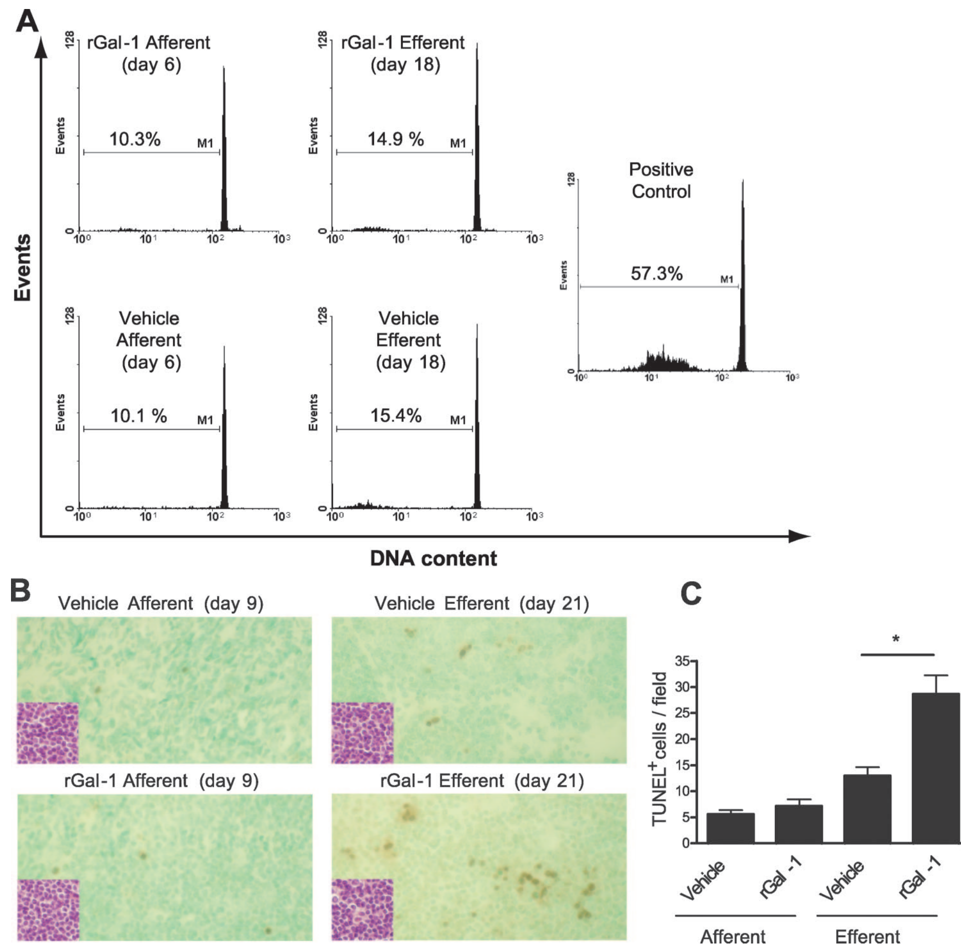

FIGURE 6. Impact of rGal-1 treatment on lymph node cell apoptosis during the course of EAU. *A*, Draining lymph nodes from rGal-1-treated or vehicle-treated mice were removed at different time periods (days 4, 6, and 8 for mice injected at the afferent phase of EAU and days 16, 18, and 20 for mice injected at the efferent phase of the disease). Lymph nodes cells were incubated during 24 h in the presence of 30 μg/ml IRBP to evaluate Ag-induced cell death. The number of apoptotic cells was determined by evaluating the percentage of hypodiploid nuclei by propidium iodide staining and flow cytometry. Representative results at days 6 and 18 are shown. A similar tendency was observed at the rest of the treatment period during both the afferent or efferent phases of EAU. Lymph node cells of immunized mice incubated with IRBP (30 μg/ml) and rGal-1 (50 μg/ml) in vitro were used as an internal positive control of apoptosis. *B* and *C*, Examination of apoptotic cells in situ following rGal-1 treatment. Lymph nodes from rGal-1-treated or vehicle-treated mice were removed at different time periods (day 9 or 21 for mice injected at the afferent or efferent phase of EAU, respectively), paraffin-embedded, and processed for apoptosis detection using a peroxidase-conjugated in situ TUNEL assay. Representative photographs of lymph nodes from mice treated with control vehicle or rGal-1 during the afferent phase of EAU (*left panels*), and lymph nodes from mice treated with control vehicle or rGal-1 during the efferent phase of the disease (*right panels*) are shown. *Insets*, H&E staining of contiguous sections. Magnification: ×400 (*B*). Quantitative studies were performed in a blinded fashion on five or more fields in each sample (*C*). The number of TUNEL-positive cells per field (mean ± SD) was determined (*, $p < 0.05$ vs vehicle-treated mice).

study is the consistent increase in IL-10 production (mainly when rGal-1 was injected at the efferent phase of the disease) in the absence of IL-12 down-regulation. Although the expression of these cytokines was initially considered to be mutually exclusive, this cross-regulation was not found to be consistent throughout all the studies (55, 56). In this regard, Gerosa et al. (55) showed that IL-12 can induce human T cells to secrete both IFN-γ and IL-10. In addition, Bliss and colleagues reported that the Th1-promoting effect of IL-12 in vivo was not accompanied by a long lasting suppression of Th2 development (56). Alternatively, other factors might influence the lack of cross-regulation between these cytokines in vivo including the time frame required for this cross-talk and/or the different source of these cytokines.

During the past few years compelling evidence has been accumulated regarding the immunoregulatory activity of Gal-1 (14, 16). This endogenous lectin has the potential to influence T cell homeostasis by promoting growth arrest and apoptosis of activated T cells (24, 25, 27, 36, 37), inducing partial T cell activation (26), blocking proinflammatory cytokine secretion (32–34) and/or favoring turnover of activated leukocytes (57). In addition, therapeutic administration of rGal-1 suppresses chronic inflammation in experimental models of collagen-induced arthritis (28), autoimmune encephalomyelitis (29), Con A-induced hepatitis (30) and hapten-induced colitis (31). In addition this protein showed immunosuppressive activity in a murine model of GvHD (32). In some of these models, rGal-1 treatment was accompanied with significantly increased levels of apoptosis of Ag-specific T cells. Remarkably, in the present study we found significant levels of apoptosis in vivo only in draining lymph nodes from mice treated with rGal-1 at the efferent phase of EAU. This finding is worthwhile to be discussed in terms of the ability of Gal-1 to preferentially kill fully activated effector T cells.

Of particular interest, we found that treatment with rGal-1 early during the course of EAU (afferent phase) skews the balance of the immune response toward a Th2-mediated cytokine profile, while treatment during the efferent phase of the disease concomitantly

favors a bias toward a regulatory T cell phenotype. This finding highlights the pleiotropic nature of Gal-1 and suggests that the influence of particular downstream therapeutic effector mechanisms is likely to depend of the timing of the treatment (i.e., early or late during the course of an ongoing inflammatory disease). In this regard, a recent report indicates that TGF-β_1 uses distinct mechanisms to restrain pathogenic Th1 responses (58). The authors showed that this immunosuppressive cytokine inhibits IFN-γ expression in CD4$^+$ T cells at priming or recall stages of an ongoing immune response by modulating different mechanisms involving the transcription factors STAT4 or T-bet (58). In addition, a recent study reported that CCR1/CCR5 receptor antagonists may have differential therapeutic effects depending on the time of administration during the afferent or efferent stages of EAU (59). Furthermore, other studies reported the efficacy of specific antagonists at the afferent or efferent phases of ocular inflammation. For example, Sartani et al. (60) reported the efficacy of anti-TNF-α therapy only at the afferent phase of EAU, whereas Martin et al. (61) demonstrated the effects of a peptide inhibitor of α_4 integrin when administered at the efferent phase of the ocular inflammatory disease. Our findings suggest that whatever multiple effects Gal-1 may exert on the immune system (i.e., induction of apoptosis, inhibition of T cell activation or regulation of proinflammatory cytokine secretion), the in vivo data showed in this study provides compelling evidence that it will also impinge on critical stages of T cell differentiation, namely, modulation of Th1/Th2 cytokine balance and the generation of TGF-β- and IL-10-producing regulatory T cells.

Because of its ability to inhibit T cell effector functions, it has been speculated that endogenous Gal-1 expression in tissues such as testis (23) and the eye (21, 22, 62) could function as a novel mechanism of immune privilege. In the mammalian retina, Gal-1 is preferentially expressed by the retinal pigment epithelium, the outer limiting membrane, and the outer plexiform layer (21). Interestingly, Ishida and colleagues (22) demonstrated that cells from the retinal pigment epithelium, suppress T cell activation, at lest in part, through Gal-1-mediated mechanisms. The authors reported that only retinal pigment epithelium from Gal-1 knock out mice showed reduced capacity to inhibit T cell activation (22), suggesting its potential role in the establishment of ocular immune privilege. Accordingly, we hypothesize that Gal-1 might act as an immunosuppressive cytokine within the ocular microenvironment where it may endow T cells with the capacity to suppress the inflammatory response. Further studies are warranted to evaluate the functional cross-talk between Gal-1 and other critical mediators of immune privilege including IL-10 and TGF-β_2 (63). In this regard, we have recently shown that Gal-1 contributes to immune privilege of tumor cells in vivo by tilting the balance toward an immunosuppressive environment at the tumor site (27).

In addition to the immunoregulatory effects of Gal-1, we cannot rule out the possibility that this protein might have direct effects in the modulation of retinal architecture. In this regard, Uehara et al. (21) reported retinal detachment and vacuolation of the outer plexiform layer when an anti-Gal-1 Ab was injected intravitreously into rat eyes, suggesting that endogenous Gal-1 may also be involved in the adhesion of the photoreceptor and outer plexiform layers by interacting with specific glycoconjugates (21).

In summary, our data show that treatment with rGal-1, either early or late during the development of EAU, can protect from T cell mediated retinal inflammation through different anti-inflammatory mechanisms which may act in concert to achieve immunosuppression in vivo. In addition, our data strongly suggest that administration of exogenous rGal-1 may have therapeutic potential in inflammatory and autoimmune ocular diseases.

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Disclosures

The authors have no financial conflict of interest.

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