Immunomodulatory Effects of Galectin-1 on an IgE-Mediated Allergic Conjunctivitis Model

Claudia Bosnic Mello,1 Lisandra Ramos,2 Alexandre Dantas Gimenes,2 Teresa Raquel de Moraes Andrade,2 Sonia Maria Oliani,1 and Cristiane Damas Gil1,2

1UNESP - Universidade Estadual Paulista, Laboratório de Imunomorfologia, Departamento de Biologia, São José do Rio Preto, São Paulo, Brazil
2UNIFESP - Universidade Federal de São Paulo, Laboratório de Histologia, Departamento de Morfologia e Genética, São Paulo, Brazil

Correspondence: Cristiane Damas Gil, Departamento de Morfologia e Genética, UNIFESP - Universidade Federal de São Paulo, Rua Botucatu 740, Ed. Lemos Torres, 3º andar, 04023-900 São Paulo, São Paulo, Brazil; cristiane.gil@unifesp.br.

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PURPOSE. Galectin (Gal)-1, a lectin found at sites of immune privilege with critical role in the inflammation, has been poorly investigated in the ocular inflammatory diseases. Here, we evaluated the therapeutic potential of Gal-1 in ocular allergy using a model of ovalbumin (OVA)-induced AC.

METHODS. OVA-immunized BALB/c male mice were challenged with eye drops containing OVA on days 14 through 16 with a subset of animals pretreated intraperitoneally with recombinant Gal-1 (rGal-1) or dexamethasone (Dex).

RESULTS. Recombinant Gal-1 and Dex administration on days 14 through 16 was effective in reducing the clinical signs of allergic conjunctivitis (AC), plasma anti-OVA IgE levels, Th2 (IL-4 and IL-13), and eotaxin/RANTES levels in the lymph nodes. Four hours after the last OVA challenge, rGal-1 markedly increased Gal-1 endogenous levels in the conjunctiva, and provoked eosinophilia, which persisted at 24 hours. Recombinant Gal-1 had no effect on eosinophil activation, as evidenced by the similar pattern of peroxidase eosinophil expression between cells of rGal-1–treated and untreated AC groups. Conjunctival migrated eosinophils and neutrophils exhibited high levels of Gal-1 and β2-integrin, with points of colocalization, in the rGal-1–treated groups. These different effects observed for rGal-1 were correlated with elevated levels of activated ERK and p38 at 4 hours, and diminished levels of activated JNK and p38 at 24 hours in the eyes.

CONCLUSIONS. Gal-1 has an important role in ocular allergic inflammation and represents a potential target for the development of new therapeutic strategies in eye diseases.

Keywords: eosinophil, β2-integrin, ocular allergy, Th2 cytokines, ultrastructural immunogold labelling, mitogen-activated protein kinases

Allergic conjunctivitis (AC) is an inflammation of the conjunctiva triggered by IgE-mediated (type I hypersensitivity reaction) and/or non-IgE-mediated (type IV, delayed hypersensitivity) immune response to allergens. In addition to conjunctiva, AC affects the ocular surface, including the lids, cornea, and tear film, with symptoms and clinical signs that vary from eye redness, chemosis, and itching to irreversible injury in the cornea, affecting visual function. The pharmacological treatment of AC includes antihistamines, mast cell membrane stabilizers, nonsteroidal anti-inflammatory drugs, and corticosteroids, which are used in the more severe forms and have a greater risk of adverse effects such as increased IOP and cataractogenesis. Thus, the discovery of new pharmacological agents that have high efficacy in controlling the inflammatory response with fewer side effects is critical.

In this scenario, we highlight galectin-1 (Gal-1), a 14.5-kDa, β-galactoside-binding, mammalian lectin with anti-inflammatory properties exhibited in models of chronic inflammation and autoimmunity. In experimental autoimmune diseases, Gal-1 alters cytokine secretion by reducing levels of IFN-γ, TNF-α, IL-2, and IL-12, and increasing IL-5 and IL-10, thereby regulating lymphocyte roles. Additionally, recombinant Gal-1 (rGal-1) administration inhibits human neutrophil migration through endothelial cells upon in vitro inflammatory stimulus with IL-8 or TNF-α, respectively. Neutrophil extravasation into the peritoneal cavity can also be negatively regulated by rGal-1 administration after 4 hours of carrageenan-induced peritonitis in rodents. This Gal-1 antimigratory effect has been associated with the expression of adhesion molecules (L-selectin and β2-integrin) on the leukocyte membrane, but not on the endothelium (E-selectin, ICAM-1, VCAM-1).

In addition to these immunomodulatory roles, the signal transduction events that lead to Gal-1-induced neutrophil and endothelial cell migration, T-cell death, and cell proliferation include the activation of mitogen-activated protein kinases (MAPKs). Mitogen-activated protein kinases, a well-conserved signaling pathways, includes three subtypes: c-jun N-terminal kinases (JNK), extracellular signal-regulated kinases (ERK), and p38 proteins, which are crucial to induce the expression of multiple genes that together regulate the immune responses. This kinase family have been implicated in allergic responses, particularly in asthma, contributing to leukocyte recruitment, proinflammatory cytokine production, and Th2 differentiation. However,
the mechanisms by which Gal-1 modulates cell responses in allergic inflammatory processes remain unclear, especially in ocular models.

Given the increased risk of adverse effects provided by current therapies to treat AC, and the fact that the anti-inflammatory activities of Gal-1 have been poorly explored in ocular inflammatory processes, we evaluated the mechanism of action of this protein in an experimental model of ovalbumin (OVA)-induced AC in mice.

METHODS

Animals

Male BALB/c mice, weighing 20 to 25 g, were randomly distributed into 7 groups (n = 10 animals/group). The animals were housed in a 12-hour light/dark cycle and were allowed food and water ad libitum. All experimental procedures were submitted to and approved by the Ethics Committee in Animal Experimentation of the Federal University of São Paulo (UNIFESP; CEP n° 1984/11) and were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Allergic Conjunctivitis Model and Treatment Protocols

Mice BALB/c were immunized on days 0 and 7 with a subcutaneous injection of 5 µg of ovalbumin (OVA, grade V) and 15 mg/mL of aluminium hydroxide adjuvant diluted in 200 µL of sterile saline according to models reported21,22 with modifications. On days 14, 15, and 16 after an intraperitoneal (i.p.) injection of anaesthesia with ketamine (100 mg/kg) and xylazine (20 mg/kg), mice received the direct instillation of 250 µg of OVA in 10 µL of sterile saline onto the conjunctival sac. Control animals received sterile saline alone.

To determine the therapeutic efficacy of the exogenous administration of Gal-1 in this model, sensitized mice were pretreated on days 14, 15, and 16 with 0.3 µg/animal of recombinant Gal-1 protein (rGal-1; Peprotech EC Ltd., London, UK)23 or dexamethasone (Dex; 1 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA)23 i.p., diluted in 0.1 mL of sterile saline 15 minutes before the instillation of OVA.

Four and 24 hours after the last OVA challenge, mice were anaesthetized to obtain blood through cardiac puncture using a syringe with 10% EDTA, 10% for the analysis of anti-OVA IgE levels and leukocyte quantification. The animals were then euthanized and the eyes and cervical lymph nodes were removed. The eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes at 4°C to obtain organ supernatants. Eyes from mice subjected to different experimental conditions were subjected to histological analysis using Light Microscopy (LM) and electron microscopy (SEM) for each group.

Clinical Score

After 20 minutes of OVA challenge, mice were examined on days 14, 15, and 16 to clinically verify the occurrence and severity of conjunctivitis. Four clinical signs were observed: chemosis, conjunctival hyperemia, lid edema, and tearing. Scoring similar to that described by Magone et al.24 was performed, and each parameter was graded on a scale ranging from 0 to 3+: (0 = absence, 1 = mild, 2 = moderate, and 3 = severe symptoms). Thus, each animal received a total clinical score of ranging from 0 to 12+, and the data were expressed as the mean ± SEM for each group.

Blood Leukocyte Quantification

Aliquots of blood (10 µL) were diluted 1/20 in Turk’s solution (0.1% crystal violet diluted in 3% acetic acid) and differential counting was obtained with a Neubauer chamber using a ×40 objective on a light microscope. For this study, blood cells were distinguished as neutrophils, eosinophils, and monocytes. Data were reported as the mean ± SEM of the number of cells × 10⁷/mL.

Analysis of IgE Anti-Ovalbumin Levels

Whole blood was centrifuged at 600g for 10 minutes to collect the plasma and determine the IgE anti-OVA levels by ELISA under different experimental conditions. The concentration of IgE anti-OVA was measured using a commercially available mouse IgE anti-OVA immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA) with levels estimated according to the manufacturer's instructions. All estimations were made in duplicate and the data expressed the mean ± SEM.

Analysis of Th1/Th2 Cytokines and Chemokines

Cervical lymph nodes were sonicated in a 50 mM Tris-HCl, 150 mM NaCl, and 1% Triton-X, pH 7.4 buffer containing a complete protease inhibitor cocktail tablet (Roche Applied Science, Mannheim, Germany). Subsequently, samples were centrifuged at 10,000g for 20 minutes at 4°C to obtain organ supernatants. For multiplex analysis, 25 µL of the lymph node supernatants were employed using the MILLIPLEX MAP mouse cytokine/chemokine panel (MCYTMAg-70K-PX32; Millipore Corporation, Billerica, MA, USA) and MAGPIX Multiplexing Instrument (Millipore) according to the manufacturer's instructions. Eight analytes were studied: IL-2, IL-4, IL-5, IL-13, IFN-γ, TNF-α, cotaxin, and regulated upon activation normal T cell expressed and presumably secreted (RANTES). The concentration of analytes was determined by MAGPIX Xponent software (Millipore), and the results are reported as the mean ± SEM.

Western Blot Analysis

Eyes from mice subjected to different experimental conditions were sonicated in a 50 mM Tris-HCl, 150 mM NaCl, and 1% Triton-X, pH 7.4 buffer containing complete protease inhibitor cocktail and PhosSTOP tablets (Roche Applied Science). Protein levels were determined by Bradford assay and equalized prior to boiling in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA). To detect Gal-1 and MAPKs, protein extracts (40 µg per lane) were loaded onto a 15% SDS-PAGE together with appropriate molecular weight markers (Bio-Rad Laboratories) and transferred to ECL Hybond nitrocellulose membranes. Reversible protein staining of the membranes with 0.1% Ponceau-S in 5% acetic acid (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to verify even protein transfer. Membranes were incubated 15 minutes in 5% BSA in Tris-buffered saline (TBS) before the addition of rabbit polyclonal antibodies anti-Gal-1 (sc-282848), anti-ERK1/2 (sc-292838), anti-phosphorylated (p)-ERK ½ (sc-16981), anti-JNK (sc-572), anti-p-JNK (sc-6254), anti-p-p38 (sc-3978), anti-phosphorylated (p)-p38 (sc-160469), and anti-p-p38 (sc-17852), and anti-β-actin (sc-130657), all diluted 1:200 in TBS with 0.1% Tween 20 (antibodies were purchased from Santa Cruz Biotechnology). Incubation was followed by 15 minutes of washing with TBS and incubation for 60 minutes at room temperature with peroxidase-conjugated goat anti-rabbit IgG (1:1000; Thermo Fisher Scientific, Inc., Rockford, MI, USA). Membranes were again washed for 15 minutes with TBS, and immunoreactive proteins were detected using an enhanced Supersignal West Pico Chemiluminescent substrate kit (Thermo Fisher Scientific) and film was developed in dark room. Proteins were imaged and quantified using the software ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) to determine the relative expression...
Galectin-1 in Ocular Allergy

Fixation, Processing, and Embedding for Light and Electron Microscopy
For light microscopy analysis, eyes were fixed in 10% buffered formalin (pH 7.4) for 24 hours and, washed in tap water, dehydrated in a decreasing ethanol series, and embedded in paraffin. Sections of 4 μm were obtained in a Leica RM2155 microtome (Leica Microsystems, Nussloch, Germany) and subsequently stained with hematoxylin-eosin for histopathological analysis.

Inflammatory cells were quantified using a high-power objective (×63) on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany). Three semiserial sections of conjunctiva were analyzed per animal and the area was determined using Axiovision software (Carl Zeiss). Values are expressed as the mean ± SEM of the number of cells per 0.1 mm².

For ultrastructural analysis, eyes were fixed in a 4% paraformaldehyde, 0.5% glutaraldehyde, 0.1% sodium cacodylate buffer (pH 7.4) for 24 hours at 4°C. Samples were washed in sodium cacodylate, dehydrated through a graded series of methanol, and embedded in LR Gold (Sigma-Aldrich Corp.). Sections of eyes (70 nm) were cut on an ultramicrotome (Reichert Ultracut; Leica) and placed on nickel grids for immunogold labelling.

Immunohistochemistry
The detection of endogenous Gal-1 in bulbar conjunctiva was conducted in 4-μm sections of paraffin-embedded eyes. After an antigen retrieval step using citrate buffer pH 6.0, the endogenous peroxide activity was blocked and the sections were incubated overnight at 4°C with the primary rabbit polyclonal antibody anti–Gal-1 (1:200; Zymed Laboratories, Cambridge, UK), diluted in 1% BSA. After washing, sections were incubated with a secondary biotinylated antibody (LAB-SA Detection kit, Invitrogen, Paisley, UK). Positive staining was detected using a peroxidase-conjugated streptavidin complex, and color was developed using DAB substrate (Invitrogen). The sections were counterstained with hematoxylin.

Densitometric analyses for the Gal-1 immunostaining were performed in the epithelium and lamina propria of bulbar conjunctiva (n = 5 animals/group). The values obtained were between 0 and 255 AU using Axiovision software on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss). The data were expressed as the mean ± SEM.

Postembedding Immunogold Labelling
To detect the colocalization of endogenous Gal-1 with the adhesion molecule β2-integrin (CD11b) in inflammatory cells, ultrathin sections (∼90 nm) from bulbar conjunctivas were incubated sequentially with the following reagents at room temperature: (1) distilled water, (2) 0.1 mol/L phosphate buffer containing 1% egg albumin (PBEA), (3) 0.1 mol/L, PBS containing 5% egg albumin (PBEA) for 30 minutes, (4) the rabbit polyclonal antibody anti-Gal-1 (1:50 in PBEA) and goat polyclonal antibody anti-CD11b (1:100 in PBEA) for 2 hours, with normal rabbit and goat sera as controls, and (5) three washes (5 minutes each) in PBEA containing 0.01% Tween 20.

To detect Gal-1, a goat anti-rabbit IgG antibody (1:50 in PBEA) conjugated to 10-nm colloidal gold (British Biocell, Cardiff, UK) was added. To detect CD11b, a rabbit anti-goat IgG antibody (1:50 in PBEA) conjugated to 15-nm colloidal gold (British Biocell) was added. After 1 hour, the sections were washed extensively in PBEA containing 0.01% Tween 20 and then in distilled water. These sections were stained with uranyl acetate and lead citrate and examined using a Zeiss EM900 electron microscope (Carl Zeiss).

The detection of peroxidase eosinophil (EPX) in the eosinophils of bulbar conjunctiva was performed as described above using the primary goat polyclonal antibody anti–EPX (1:50 in PBEA; Santa Cruz Biotechnologies) and secondary rabbit anti-goat IgG antibody (1:50 in PBEA) conjugated to 15-nm colloidal gold (British Biocell).

Randomly photographed sections of inflammatory cells were used for the immunocytocchemical analysis. The area of the cell compartment was determined with Axiosvision software. The density of immunogold (number of gold particles per micrometer squared) was calculated and expressed for each cell compartment. Values are reported as the mean ± SEM of 10 to 20 electron micrographs analyzed for each cellular type per group.

Statistical Analysis
The data were analyzed using one-way ANOVA followed by Bonferroni t-tests between corresponding time points using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Nonparametric analysis of the scores assigned to the conjunctival symptoms was done using the Mann-Whitney U test. In all cases, a P value less than 0.05 was considered significant.

Clinical Signs of Allergic Conjunctivitis and IgE Anti-OVA Levels Are Reduced by rGal-1
Administration
Initially, we evaluated the clinical response of the animals in the AC model, on days 14 through 16, 20 minutes after OVA instillation in the eyes. Mice from the AC group exhibited moderate to severe signs of chemosis, conjunctival hyperemia, lid edema, and tearing (Figs. 1D–F) with the respect to the controls (Figs. 1A–C). Pharmacological treatments with Dex and rGal-1 produced anti-inflammatory effects decreasing these signs (Figs. 1G–I) compared with the untreated AC group. Score data confirmed our observations with a significant increase in the inflammatory response caused by OVA challenge in AC on days 14 and 16 (Figs. 1M, O). The rGal-1-treated group exhibited significant decrease in the score over the three challenge days compared with the untreated AC group (Figs. 1M–O), whereas Dex reduced scores only on days 14 and 16.

The IgE anti-OVA levels were significantly increased in the plasma of mice after 4 and 24 hours of the last OVA challenge (AC group) compared with controls (Fig. 1P), supporting the efficacy of our experimental model. Pharmacological treatments significantly decreased the levels of this antibody after 4 hours.

rGal-1 Regulates Th2 Cytokines and Chemokine Levels During Ocular Allergy
Given that AC is a classical Th2 ocular surface inflammatory response, we investigated whether our model was consistent with this cytokine profile. We evaluated the cervical lymph nodes, and as expected a marked increase of IL-4 and IL-13 was detected in these organs at 4 (both cytokines) and 24 (only IL-4) hours in the AC group (Fig. 2). In addition, the AC group was characterized with a significant increase of eotaxin and RANTES levels in the lymph nodes at 24 hours. Recombinant Gal-1 and Dex treatments induced similar antiallergic effects.
through an efficient reduction of these Th2 cytokines, as well as chemokines eotaxin and RANTES.

No significant alterations were detected in the IL-2, IL-5, INF-γ, and TNF-α levels of lymph nodes between the untreated AC and the Dex- and rGal-1–treated AC groups (data not shown).

**Effects of rGal-1 on the Allergic Inflammatory Response of the Conjunctiva**

We first evaluated the leukocyte recruitment from blood due to the allergic response (Table). Blood neutrophilia was exhibited by the AC-untreated and Dex-treated groups at 4 and 24 hours,
respectively. The rGal-1 treatment induced a significant increase in monocytes in the initial phase of allergy (4 hours), as well as Dex-treated group, and a reduction in the eosinophil numbers at the two time points compared with the untreated AC group.

Histopathological and quantitative analysis revealed a normal appearance of control conjunctivas (Fig. 3A). In contrast, sensitized and OVA-challenged mice exhibited an intense inflammatory response characterized at 4 hours by neutrophilia (Fig. 3F) and at 24 hours by neutrophilia and eosinophilia (Figs. 3B, E, F). The Dex-treated group also exhibited reduced eosinophil influx at 24 hours (Figs. 3C, E), whereas rGal-1 treatment was associated with eosinophilia into the conjunctiva at 4 and 24 hours (Figs. 3D, E). No effect was detected in the neutrophil counts by either pharmacological treatment which showed similar neutrophilia to the AC group at both time points (Fig. 3F).

The presence of eosinophils in the mouse eyes under different experimental conditions was confirmed by immunoblot analysis that exhibited elevated levels of endogenous peroxidase eosinophil (EPX) at 4 hours in the AC groups (treated or not with Dex or rGal-1) as well as in the untreated AC group at 24 hours compared with the control (Fig. 4A). In this latter time point, eosinophila detected in the rGal-1-treated group was not associated with elevated levels of EPX, but showed similar levels to Dex-treated group (Fig. 4A). Similarly, ultrastructural immunogold labelling revealed a downregulation of EPX expression in the specific cytoplasmic granules of eosinophils induced by rGal-1 at 24 hours and compared with the untreated AC group (Figs. 4B, C).

**Gal-1 Expression is Modulated in the Conjunctiva During the Allergic Inflammatory Response**

To further characterize the Gal-1 expression on mouse ocular tissues during the allergic inflammatory response, we evaluated its levels by immunoblot (Fig. 5A). At 4 hours, the Dex- and rGal-1–treated groups exhibited elevated levels of endogenous

### Table. Quantification of Leukocytes in the Blood

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eosinophils (Cells x 10^5/mL)</th>
<th>Neutrophils (Cells x 10^5/mL)</th>
<th>Monocytes (Cells x 10^5/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.40 ± 0.18</td>
<td>7.5 ± 1.67</td>
<td>2.08 ± 0.51</td>
</tr>
<tr>
<td>AC</td>
<td>0.83 ± 0.16</td>
<td>19.03 ± 2.84†</td>
<td>3.67 ± 0.45</td>
</tr>
<tr>
<td>AC + Dex</td>
<td>0.50 ± 0.17</td>
<td>13.00 ± 1.68</td>
<td>6.47 ± 2.02*</td>
</tr>
<tr>
<td>AC + rGal-1</td>
<td>0.08 ± 0.08§</td>
<td>12.04 ± 2.24</td>
<td>14.95 ± 3.28†</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>0.52 ± 0.10</td>
<td>7.14 ± 0.90</td>
<td>3.45 ± 0.49</td>
</tr>
<tr>
<td>AC + Dex</td>
<td>0.62 ± 0.16</td>
<td>13.33 ± 1.80§§</td>
<td>3.08 ± 0.68</td>
</tr>
<tr>
<td>AC + rGal-1</td>
<td>0.04 ± 0.04§§</td>
<td>8.33 ± 0.67</td>
<td>4.73 ± 0.83</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM of the number of leukocytes x 10^5/mL in mice under different experimental conditions (n = 10 animals/group).

* P < 0.05 versus control.
† P < 0.01 versus control.
‡ P < 0.001 versus control.
§ P < 0.05 versus AC at the same experimental time.
|| P < 0.01 versus AC at the same experimental time.
Galectin-1, whereas the untreated AC group showed similar results at 24 hours. Interestingly, at this later time point, both pharmacological treatments diminished endogenous levels of Gal-1.

To ascertain that the Gal-1 expression was modulated in the bulbar conjunctiva, a key mucous membrane for inflammatory response in this model, we performed immunohistochemistry studies. Data revealed the presence of endogenous Gal-1 in the conjunctiva of control animals localized in the surface epithelial cells and lamina propria (Fig. 5B). After 4 hours of OVA challenge (AC), lower Gal-1 immunoreactivity was observed in the lamina propria, whereas high levels were detected in the epithelium after 24 hours. The pharmacological treatments (AC+Dex and AC+rGal-1) produced intense Gal-1 immunostaining in the epithelium, and only rGal-1 treatment in the lamina propria, in the initial phase of allergic response (4 hours) compared with the untreated AC group, corroborating our ultrastructural immunogold labelling. Our ultrastructural studies focused on the untreated and rGal-1-treated AC groups due to the ease in localizing inflammatory cells in the ultrathin sections of conjunctiva at both time points, 4 and 24 hours.

Gal-1 immunoreactivity was detected in the neutrophils and eosinophils and was localized in the nucleus and cytosol. These inflammatory cells exhibited several points of Gal-1 and β2-integrin colocalization in the plasma membrane and cytosol, especially after rGal-1 treatment (Figs. 6A, B). No immunoreactivity was detected in the section used as a negative control (Fig. 6C). Four hours after OVA challenge and rGal-1 treatment, neutrophils exhibited significantly increased Gal-1 (27 ± 3.7 gold particles per μm²; P < 0.001) and β2-integrin (2 ± 0.2; P < 0.05) endogenous levels compared with cells from the untreated AC group (4.67 ± 1.3 and 1 ± 0.3, respectively). A similar effect was detected in the Gal-1 expression in eosinophils at 24 hours, which revealed significantly increased levels of this lectin after rGal-1 treatment (5.4 ± 0.7; P < 0.001) compared with the untreated AC group (1.5 ± 0.5). Eosinophils also presented elevated levels of β2-integrin (1.2 ± 0.2) after rGal-1 treatment, but were not significantly altered with respect to the AC group (0.87 ± 0.2).

AC Induces MAPK Activation that is Differentially Regulated by rGal-1 Treatment

To understand the downstream molecular signalling pathways involved in the effect of rGal-1 treatment in AC, we performed Western blot analysis to assess the phosphorylation of ERK, JNK, and p38 MAPK using pooled extracts of eyes (n = 5 animals per group) under different experimental conditions. In the early phase of AC (4 hours), rGal-1 and Dex treatments increased p-p38 expression (Fig. 7B) compared with the nontreated AC group. Additionally, OVA-induced AC elevated p-ERK levels at both time points (Fig. 7A) and p-JNK at 24 hours (Fig. 7C). In this late phase, rGal-1 markedly reduced p-p38 and p-JNK (Figs. 7B, C) compared with the nontreated AC group.
DISCUSSION

We evaluated the therapeutic potential and mechanism of action of Gal-1 on ocular inflammation using a classical OVA-induced AC model in mice. Initially, we confirmed the efficacy of our AC model through the detection of clinical signs of conjunctivitis as well as by the increased plasma levels of IgE anti-OVA, Th2 cytokines (IL-4 and IL-13), and chemokines (eotaxin and RANTES) corroborating previous studies. Histopathology of the bulbar conjunctiva characterized the ocular allergic response as neutrophilia at 4 hours and as eosinophilia and neutrophilia at 24 hours. Our histologic findings are consistent with previous reports using AC experimental models induced by different antigens, such as OVA, ragweed pollen, mite, and cat dander extract.

The Dex-treated group exhibited a decrease in the clinical signs of AC as well as in the inflammatory cell recruitment into the conjunctiva, especially in the late phase of the allergic response. These findings are consistent with studies using AC models in mice and guinea pigs induced by OVA and ragweed pollen, respectively. Once the anti-inflammatory...
effects of Dex in the AC model were characterized, we focus our studies on understanding the therapeutic potential of rGal-1. This lectin administration exhibited effective anti-inflammatory action with a significant decrease in the clinical signs of conjunctivitis observed on all challenge days (14, 15, and 16) associated with decreased plasma levels of IgE anti-OVA in the early phase of AC (4 hours). Similar findings were detected in a phospholipase A2-induced edema model in rat paw, in which pharmacological pretreatment with Gal-1 reduced clinical signs and mast cell numbers in the tissue at 4 hours.35 However, in our study no changes in the mast cell numbers in the bulbar conjunctiva were detected under different experimental conditions (data not shown).

The rGal-1 and Dex pharmacological treatments also exhibited antiallergic effects through the significant reduction of IL-4 and IL-13 (Th2) in the lymph nodes at 4 hours, which is consistent with the negative regulation of IgE anti-OVA levels and significant improvement in clinical scoring. In the late phase (24 hours), both treatments also decreased eotaxin and RANTES levels in the lymph nodes compared with the AC group. The regulatory effects of Dex on Th2 cytokine (IL-4 and IL-5) expression have been reported in bronchoalveolar lavage...
Galectin-1 in Ocular Allergy

FIGURE 6. Ultrastructural colocalization of Gal-1 and β2-integrin immunogold particles in neutrophil (A) and eosinophil (B) on bulbar conjunctiva of rGal-1–treated mice (24 hours). Colocalized particles (arrows) of Gal-1 and β2-integrin (10- and 15-nm colloidal gold particles, respectively) are highlighted in the squared areas from inflammatory cells and detected on the plasma membranes of neutrophil (Nφ) interacting with endothelial cell (EC) and eosinophil (Eφ). (C) The absence of gold labelling for Gal-1 and β2-integrin in neutrophil incubated with nonimmune rabbit and goat serum. N, nucleus.

and the lungs of animals treated with this drug in a model of OVA-induced asthma.\textsuperscript{36,37} Inhibitory effects of Dex in RANTES and eotaxin levels were also described in in vitro studies using ionomycin-stimulated human mast cells\textsuperscript{38} and IL-4–TNF-α–stimulated human conjunctival epithelial cells.\textsuperscript{39} Indeed, a potent anti-inflammatory role for Gal-1 has been shown with respect to the Th1 cytokines (TNF-α, IFN-γ, IL-2, and IL-12) through experimental models of acute\textsuperscript{10} and systemic\textsuperscript{4–6} inflammation. In ocular inflammation, this protective effect of Gal-1 was demonstrated in experimental models of Th1 responses, such as uveitis\textsuperscript{5} and herpetic stromal keratitis.\textsuperscript{40} In this scenario, our study is the first to demonstrate a negative regulation of Th2 cytokines and an important inhibitory role of systemic humoral response by Gal-1 in an in vivo ocular allergy model, highlighting this lectin as promising target to study delayed hypersensitivity allergic diseases (type IV).

Despite the antiallergic effects observed for rGal-1 treatment, our ocular model revealed an antagonistic role for this lectin associated with leukocyte recruitment. In the initial phase (4 hours), rGal-1 pretreatment caused a significant reduction in blood eosinophil numbers that was most likely due to the increased eosinophil migration to the conjunctiva at this time point. Eosinophils are key cells in the allergic inflammatory response and express Gal-1 protein, whose endogenous levels are positively modulated by rGal-1 treatment, and are associated with high expression of β2-integrin with points of colocalization in the plasma membrane at 24 hours, suggesting a participation of this lectin in the cell attachment to extracellular matrix. In vitro assays corroborate our findings, revealing notably decreased migration and increased adhesion of human eosinophils onto Gal-1–coated support compared with support that is P-selectin–coated.\textsuperscript{41} Additionally, in a concentration and time-dependent manner Gal-1–coated supports markedly activated eosinophils as revealed by an increase in the level of eosinophil cationic protein (ECP) expression. In contrast, no differences in the EPX production, another important marker of eosinophil activation,\textsuperscript{42} were observed in the rGal-1–treated AC group compared with the untreated group as detected by ultrastructural immunogold labelling of EPX.

In addition, in the OVA-induced AC group, rGal-1 treatment did not produce an antimigratory effect on neutrophils into the conjunctiva despite the inhibitory role in neutrophil recruitment described for Gal-1 using in vivo\textsuperscript{9,10} and in vitro\textsuperscript{7,11} models of acute inflammation. As observed for eosinophils, rGal-1 induced high endogenous levels of Gal-1 and β2-integrin (CD11b) in neutrophils, with points of colocalization, which reinforced its role in the participation of leukocyte-extracellular matrix interactions. The modulation of Gal-1 levels was also described in rat neutrophils during carrageenan-induced peritonitis, in which neutrophils exhibited low levels of endogenous Gal-1 in the early phase of inflammation (4 hours) and high levels after 24 hours due to its anti-inflammatory role.\textsuperscript{9} In vitro experiments using human neutrophils activated by platelet-activating factor (PAF) revealed that rGal-1 has no effect on the expression of CD11b at low concentrations (~2.7–27.5 nM) but is capable of inhibiting the capture and rolling of these cells at the endothelium.\textsuperscript{11} However, at high concentrations (~275 nM), Gal-1 increases the adherence of neutrophils stimulated by PAF to the endothelium\textsuperscript{11} and stimulates the chemotaxis of these cells.\textsuperscript{12} In vivo investigations performed in our laboratory have also demonstrated an association of the inhibitory role of rGal-1 in the transmigration of neutrophils into the mesentry, with a significant decrease in the expression of CD11b at 4 hours (early acute inflammation) after zymosan-induced peritonitis.\textsuperscript{10} Altogether, the role of rGal-1 in the context of ocular allergy involves the regulation of eosinophil and neutrophil migration to tissues, which may be counterbalanced by mast cell regulation.

Furthermore, rGal-1 exhibited a chemotactic effect on blood monocytes 4 hours after the last OVA challenge, as detected in a classic zymosan-induced peritonitis model, the peritoneal mononuclear-phagocytic cell population was increased in rGal-1–treated mice compared with the untreatedAccepted Manuscript
In vitro assays revealed that this chemotactic role of Gal-1 on monocytes was a result of its CRD binding, given that the addition of lactose reduced this effect by 65%, and by activation of the ERK1/2 pathway.43 Monocyte and macrophage physiology can be regulated by Gal-1 in an ERK-dependent pathway with potential implications in the control of innate and adaptive immunity through the regulation of the expression and function of receptors for the Fc portion of IgG (FcγRI) and MHC class II.44

Prompted by the data concerning the modulation of Gal-1 expression in inflammatory cells in the ongoing AC model, we investigated the levels of this lectin in the conjunctiva under different experimental conditions using immunohistochemistry. As expected, Gal-1 immunoreactivity was positive in the surface epithelial cells and lamina propria of the conjunctiva, supporting previous data of this lectin localization in the anterior segment of the human eye.45 Four hours after the last OVA challenge, endogenous Gal-1 levels decreased in the lamina propria of the conjunctiva, followed by an increase in the epithelium in the late phase of the response. Decreased levels of Gal-1 in the lamina propria of the conjunctiva during AC facilitate leukocyte transmigration, whereas the epithelium exists as a potential source of this lectin.

The addition of rGal-1 by pharmacological treatment upregulates its endogenous expression in the epithelium and lamina propria 4 hours after the last OVA challenge and inhibits its increase in the late phase (24 hours), likely as a mechanism of negative feedback. In contrast, the Dex-treated groups exhibited increased significantly Gal-1 in the two compartments of the conjunctiva analyzed, especially after 24 hours. Previous investigations in nasal polyps, including those from our laboratory, also indicate upregulation of Gal-1 mRNA and protein after glucocorticoid treatment (budesonide and bethamethasone), particularly in epithelial and connective tissues.41,46,47 Therefore, in the ocular inflammatory response induced by AC, anti- or promigratory effects on leukocytes are positively associated with high and low levels of Gal-1 in the lamina propria, respectively, which may adversely affect the maintenance of leukocyte-matrix adhesion in the conjunctiva.

Another aspect evaluated by our study was the relationship between rGal-1 treatment and MAPK cascades in the AC model. Immunoblot analysis was performed with antibodies specific for the total and activated/phosphorylated forms of these MAPKs, and our findings revealed that rGal-1 increased p38 activation in the eye mouse extracts in the early phase of AC models (4 hours). In contrast, at 24 hours, rGal-1 was associated with decreased levels of activated JNK and p38 in the eyes compared with the AC untreated group. In vitro and in vivo assays demonstrated the capacity of Gal-1 to induce neutrophil migration through the p38 activation and the sialoglycoprotein CD4313 contrasting with its capacity to inhibit cell trafficking described in models of acute inflammation.9,10 Additionally, Gal-1 enhanced vascular endothelial cell migration through JNK phosphorylation mediated by the neuropilin-1 and VEGF receptors.12 On the other hand, JNK pathway plays a key role in T-cell death regulation14 and chemokine production in the rat pancreatic stellate cells (fibroblasts)15 in response to Gal-1 stimulation. Therefore, the
activation of p38 induced by rGal-1 treatment in the early phase of the AC model should mediate leukocyte-extracellular matrix adhesion. At 24 hours, decreased activation of JNK and p38 induced by rGal-1 ought to contribute to the downregulation of chemokine production (eotaxin and RANTES) and leukocyte activation.

In summary, the results presented herein provide alternative explanatory mechanisms for the immunoregulatory effects of Gal-1 in experimental model of ocular inflammation at the crossroads of allergic responses. This carbohydrate-binding protein can negatively modulate the clinical signs of AC, Th2 (IL-4 and IL-13), eotaxin/RANTES levels, and MAPK-signaling pathway. In contrast, the promigratory role for Gal-1 is recognized in the conjunctiva characterized by eosinophilia due to increased Gal-1, β2-integrin, and activated p38 endogenous expression. Therefore, Gal-1 has an important role in ocular allergic inflammation and represents a potential target for the development of new therapeutic strategies in eye diseases.

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References


