

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 Olini,¹ and Cristiane Damas Gil^{1,2}

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.

Submitted: June 24, 2014

Accepted: December 19, 2014

Citation: Mello CB, Ramos L, Gimenes AD, Andrade TRM, Olini SM, Gil CD. Immunomodulatory effects of galectin-1 on an IgE-mediated allergic conjunctivitis model. *Invest Ophthalmol Vis Sci*. 2015;56:693-704. DOI:10.1167/iovs.14-15100

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 β 2-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, β 2-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.¹ 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.^{1,2} 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.^{1,3} 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-10,⁴⁻⁶ thereby regulating lymphocyte roles. Additionally, recombinant Gal-1 (rGal-1)

administration inhibits human neutrophil⁷ and lymphocyte⁸ 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,⁹ zymosan,¹⁰ or IL-1 β -induced peritonitis⁷ in rodents. This Gal-1 antimigratory effect has been associated with the expression of adhesion molecules (L-selectin and β 2-integrin) on the leukocyte membrane,^{8,10,11} but not on the endothelium (E-selectin, ICAM-1, VCAM-1).⁸

In addition to these immunomodulatory roles, the signal transduction events that lead to Gal-1-induced neutrophil and endothelial cell migration,^{12,13} T-cell death,¹⁴ and cell proliferation,^{15,16} 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.¹⁷ This kinase family have been implicated in allergic responses, particularly in asthma, contributing to leukocyte recruitment, proinflammatory cytokine production, and Th2 differentiation.¹⁸ 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^{19,20} 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 μ g of ovalbumin (OVA, grade V) and 15 mg/mL of aluminium hydroxide adjuvant diluted in 200 μ L of sterile saline according to models reported^{21,22} 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 μ g of OVA in 10 μ L 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 μ g/animal of recombinant Gal-1 protein (rGal-1; Peprotech EC Ltd., London, UK)^{7,10} 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 collected.

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 $\pm$ SEM for each group.

Blood Leukocyte Quantification

Aliquots of blood (10 μ L) 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 $\times 40$

objective on a light microscope. For this study, blood cells were distinguished as neutrophils, eosinophils, and monocytes. Data were reported as the mean $\pm$ SEM of the number of cells $\times 10^5$ /mL.

Analysis of IgE Antiovalbumin 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 $\pm$ 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 μ L of the lymph node supernatants were employed using the MILLIPLEX MAP mouse cytokine/chemokine panel (MCTMAG-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 , eotaxin, 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 $\pm$ 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 μ g 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-28248), anti-ERK1/2 (sc-292838), anti-phosphorylated (p)-ERK $\frac{1}{2}$ (sc-16981), anti-JNK (sc-572), anti-p-JNK (sc-6254), anti-p38 (sc-535), 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^{1.440} (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) to determine the relative expression

(arbitrary units, AU) of Gal-1/ β -actin or phosphorylated/total MAPKs.

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 μ m 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 ($\times 63$) 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 $\pm$ 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- μ m sections of paraffin-embedded eyes. After an antigen retrieval step using citrate buffer pH 6.0, the endogenous peroxidase 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 $\pm$ SEM.

Postembedding Immunogold Labelling

To detect the colocalization of endogenous Gal-1 with the adhesion molecule $\beta 2$ -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 Biotechnology) 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 immunocytochemical analysis. The area of the cell compartment was determined with Axiovision 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 $\pm$ 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-L) 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

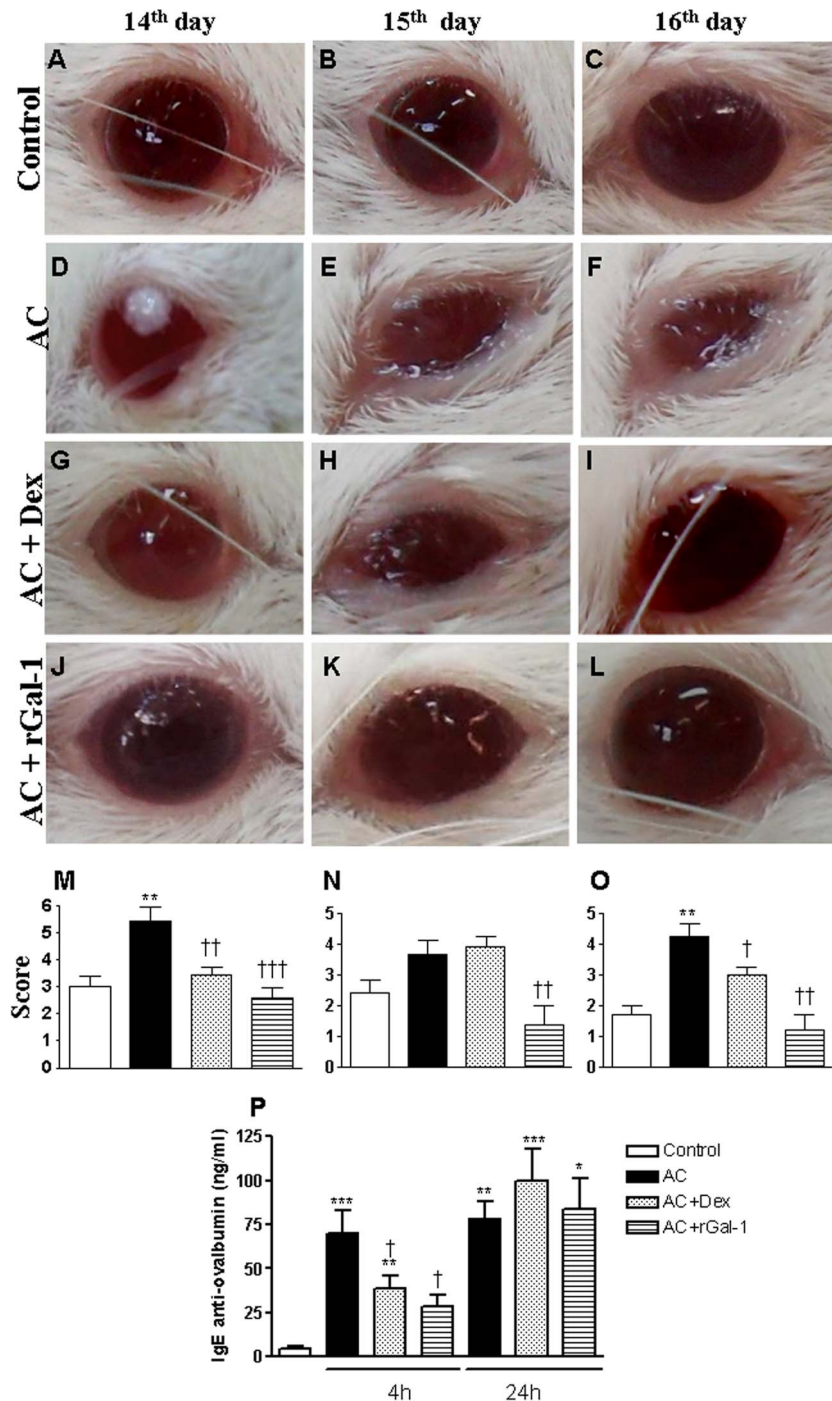


FIGURE 1. Clinical score of allergic conjunctivitis. Mice were challenged with OVA (AC) or sterile saline (control; [A-C]), and the clinical appearance was evaluated after 20 minutes on days 14 through 16. The AC group exhibited chemosis, lid edema, conjunctival hyperemia, and tearing (D-F), which were reduced after Dex (G-I), and rGal-1 (J-L) treatments. (M-O) Data represents mean $\pm$ SEM of clinical scores ($n = 10$ animals/group). ** $P < 0.01$ versus control; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus AC. Antiovalbumin IgE levels (P). Data represent mean $\pm$ SEM of IgE (ng/mL) in the mice plasma ($n = 10$ /group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control; † $P < 0.05$ versus AC at the corresponding experimental time.

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, IFN- 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,

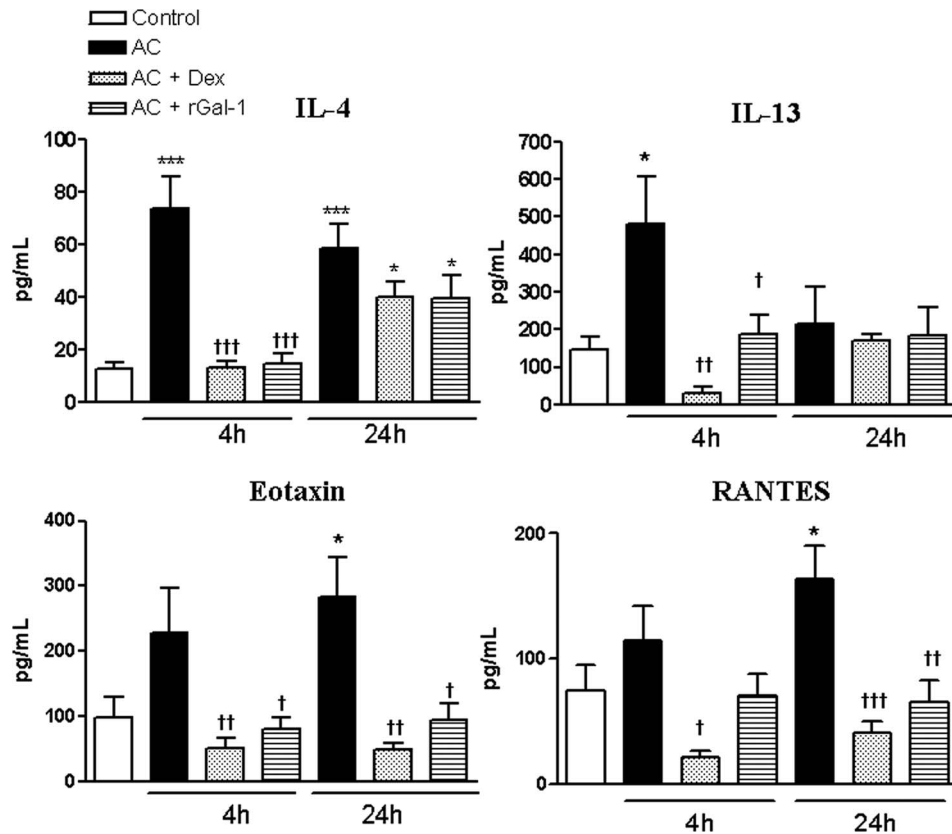


FIGURE 2. Effect of rGal-1 on the IL-4, IL-13, eotaxin, and RANTES production in the cervical lymph nodes. Values are expressed as the mean $\pm$ SEM of the cytokines/chemokines (pg/mL; $n = 5-10$ /group). * $P < 0.05$ and *** $P < 0.001$ versus control; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus AC group at the corresponding experimental time.

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, eosinophilia 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

Groups	Cells $\times 10^5$ /mL		
	Eosinophils	Neutrophils	Monocytes
Control	0.40 $\pm$ 0.18	7.5 $\pm$ 1.67	2.08 $\pm$ 0.51
4 h			
AC	0.83 $\pm$ 0.16	19.03 $\pm$ 2.84†	3.67 $\pm$ 0.45
AC+Dex	0.50 $\pm$ 0.17	13.00 $\pm$ 1.68	6.47 $\pm$ 2.02*
AC+rGal-1	0.08 $\pm$ 0.08§	12.04 $\pm$ 2.24	14.93 $\pm$ 3.28‡
24 h			
AC	0.32 $\pm$ 0.10	7.14 $\pm$ 0.90	3.45 $\pm$ 0.49
AC+Dex	0.62 $\pm$ 0.16	13.33 $\pm$ 1.80*§	3.08 $\pm$ 0.68
AC+rGal-1	0.04 $\pm$ 0.04§	8.33 $\pm$ 0.67	4.73 $\pm$ 0.83

Data represent the mean $\pm$ SEM of the number of leukocytes $\times 10^5$ /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.

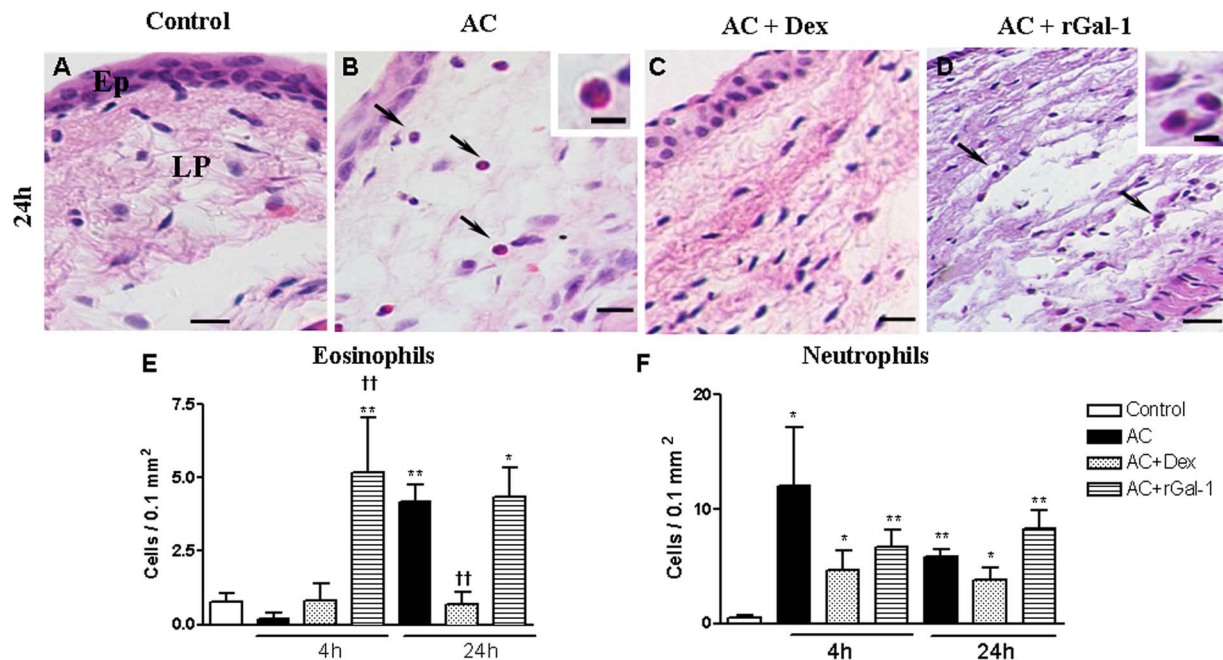


FIGURE 3. Histopathology of the bulbar conjunctiva. (A) Control conjunctiva after 24 hours. Eosinophilia (arrows; inset) characterized the AC (B) and rGal-1-treated groups (D) while the Dex-treated group (C) exhibited histology similar to the control group. Stain: hematoxylin-eosin. Scale bars: 10 μ m (A–C); 20 μ m (D); 5 μ m (insets of [B, D]). Quantitative analyses of eosinophils (E) and neutrophils (F) in the bulbar conjunctiva. Data represent the mean $\pm$ SEM of the cell number analyzed per 0.1 mm² in the bulbar conjunctiva ($n = 5$ /group). * $P < 0.05$ and ** $P < 0.01$ versus control; †† $P < 0.01$ versus AC at the corresponding experimental time. Lamina propria, LP; epithelium, Ep.

Gal-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 immunoblot data. After 24 hours, opposite effects were detected for both treatments Dex increased protein levels in the epithelium and lamina propria, whereas rGal-1 decreased its endogenous expression. No immunostaining was detected in the negative control section. The histologic findings were supported by densitometry analyses of Gal-1 expression in the epithelial cells and lamina propria of the conjunctiva (Fig. 5C).

rGal-1 Regulates β 2-integrin Expression in Neutrophils and Eosinophils

Once detected that rGal-1 treatment increased Gal-1 endogenous expression in the lamina propria of the conjunctiva at 4 hours, favoring neutrophilia and eosinophilia in the tissue, we evaluated Gal-1 and β 2-integrin endogenous expressions in inflammatory cells localized in the conjunctiva using 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 β 2-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 μ m²; $P < 0.001$) and β 2-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 β 2-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.

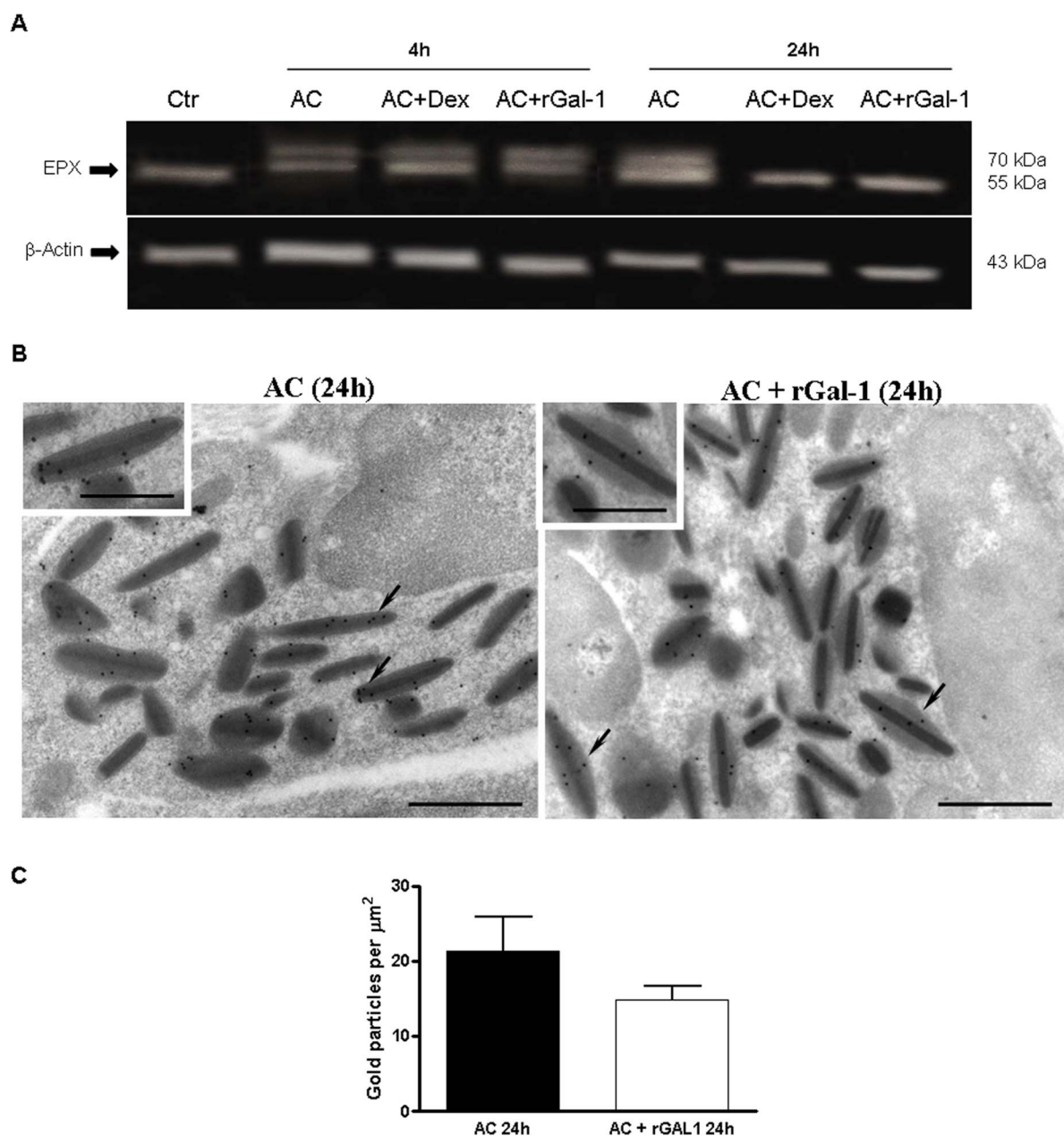


FIGURE 4. Expression of EPX. **(A)** Eosinophil peroxidase endogenous levels were determined in the pooled extracts of mouse eyes ($n = 5$ animals/group) from the control (Ctr) and the allergic conjunctivitis (AC) groups, treated or not with Dex (AC+Dex) or rGal-1 (AC+rGal-1), at 4 and 24 hours. Immunoblot analysis revealed high EPX expression in the AC groups (treated or not with Dex or rGal-1) at 4 hours, while diminished levels were detected in the Dex- and rGal-1-treated groups at 24 hours. β -Actin was used as a protein loading control. The data illustrate one representative of two independent experiments. **(B)** Eosinophil peroxidase expression was detected in the cytoplasmic granules (arrows; detail in insets) of eosinophils from the bulbar conjunctiva of the AC and AC+rGal-1 groups after 24 hours. Scale bars: 1 and 0.5 μm (insets). **(C)** Density of EPX immunogold particles. Data are the mean $\pm$ SEM of gold particles per micrometer squared.

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.^{21,22} 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.^{21,22,25–30} 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,^{21,22} ragweed pollen,^{31–33} mite,²⁸ and cat dander extract.³⁴

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 mice²¹ and guinea pigs³¹ induced by OVA and ragweed pollen, respectively. Once the anti-inflammatory

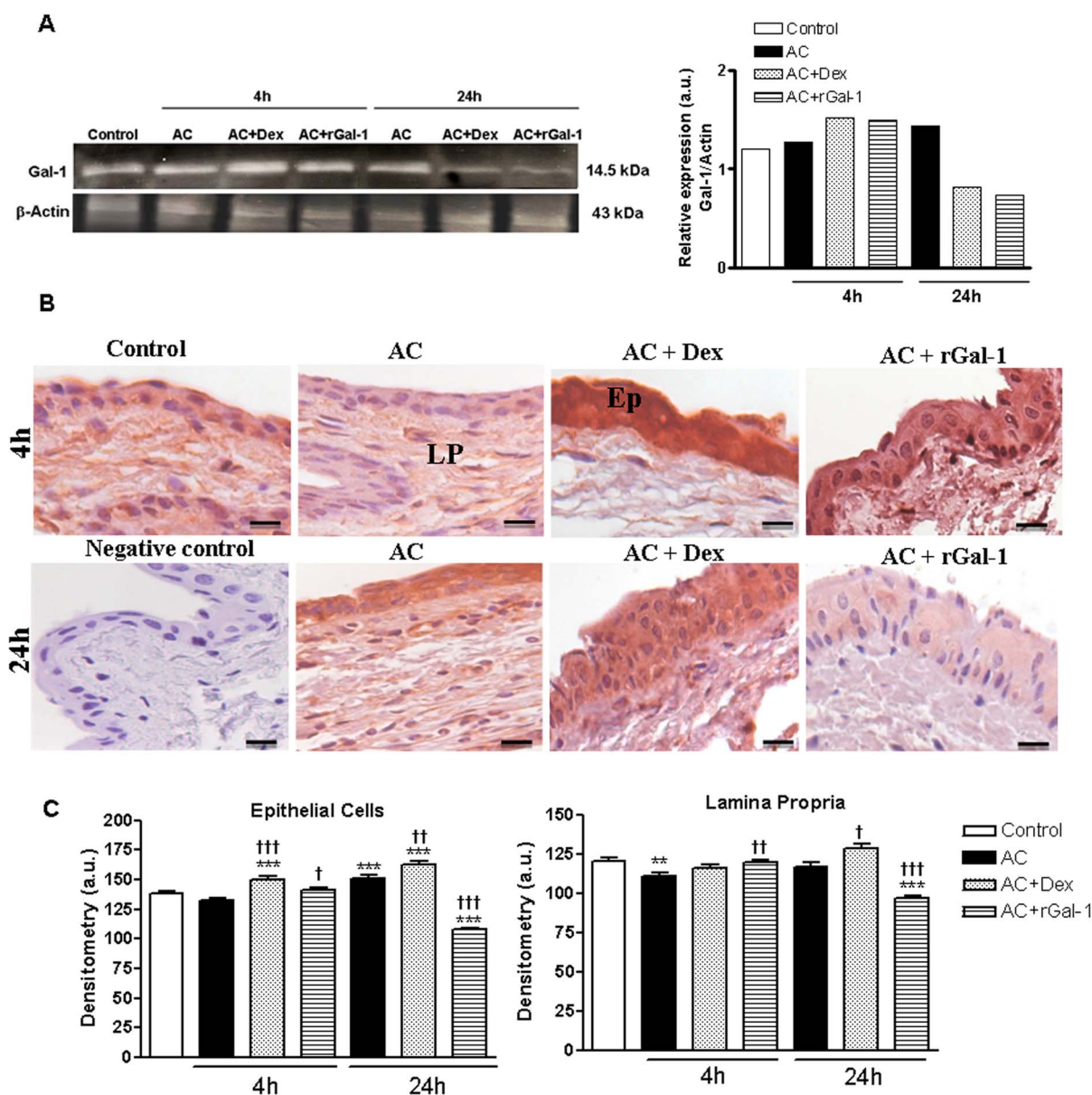


FIGURE 5. Expression of Gal-1 in the ocular tissues. (A) Gal-1 endogenous levels were determined in the pooled extracts of mouse eyes ($n = 5$ animals/group) from the control and the allergic conjunctivitis (AC) groups, treated or not with Dex (AC+Dex) or rGal-1 (AC+rGal-1), at 4 and 24 hours. Immunoblot analysis revealed high Gal-1 expression in the Dex- and rGal-1-treated groups at 4 hours and in the nontreated AC group at 24 hours. Immunoreactive bands were semiquantified by densitometry and expressed as arbitrary units relative to β -actin. The data illustrate one representative of two independent experiments. (B) Expression of Gal-1 in the bulbar conjunctiva. Gal-1 immunoreactivity was detected in the epithelial cells (Ep) and lamina propria (LP). No immunoreaction was detected in the sample used as a negative control. Counterstain: Hematoxylin. Scale bars: 10 μ m. (C) Densitometric analysis of Gal-1 expression in the epithelial cells and lamina propria. Data (AU) represent mean $\pm$ SEM of Gal-1 immunoreactivity. ** $P < 0.01$ and *** $P < 0.001$ versus control; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus AC group at the corresponding experimental time.

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

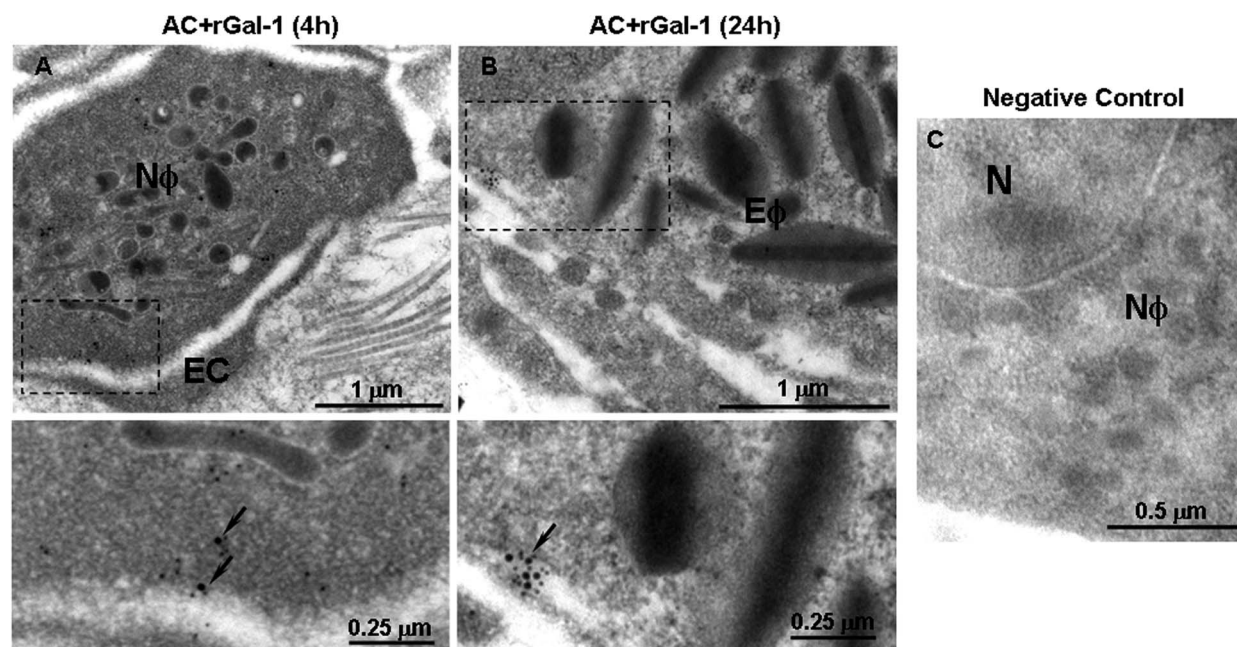


FIGURE 6. Ultrastructural colocalization of Gal-1 and β 2-integrin immunogold particles in neutrophil (A) and eosinophil (B) on bulbar conjunctiva of rGal-1-treated mice (24 hours). Colocalized particles (arrows) of Gal-1 and β 2-integrin (10- and 15-nm colloidal gold particles, respectively) are highlighted in the squared areas from inflammatory cells and detected on the plasma membranes of neutrophil (N ϕ) interacting with endothelial cell (EC) and eosinophil (E ϕ). (C) The absence of gold labelling for Gal-1 and β 2-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.^{36,37} Inhibitory effects of Dex in RANTES and eotaxin levels were also described in *in vitro* studies using ionomycin-stimulated human mast cells³⁸ and IL-4+TNF- α -stimulated human conjunctival epithelial cells.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 acute¹⁰ and systemic⁴⁻⁶ inflammation. In ocular inflammation, this protective effect of Gal-1 was demonstrated in experimental models of Th1 responses, such as uveitis⁵ and herpetic stromal keratitis.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 β 2-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.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,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*^{9,10} and *in vitro*^{7,11} models of acute inflammation. As observed for eosinophils, rGal-1 induced high endogenous levels of Gal-1 and β 2-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.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 ($\sim$ 2.7–27.5 nM) but is capable of inhibiting the capture and rolling of these cells at the endothelium.¹¹ However, at high concentrations ($\sim$ 275 nM), Gal-1 increases the adherence of neutrophils stimulated by PAF to the endothelium¹¹ and stimulates the chemotaxis of these cells.¹³ *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 mesentery, with a significant decrease in the expression of CD11b at 4 hours (early acute inflammation) after zymosan-induced peritonitis.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 untreated

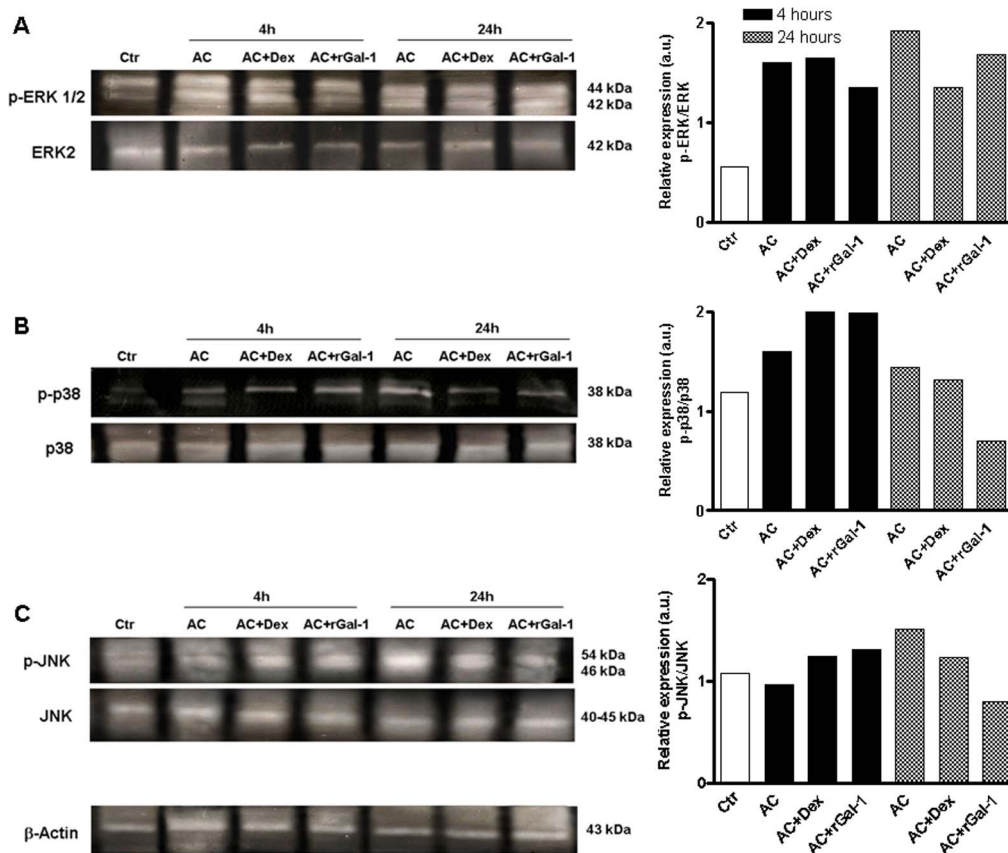


FIGURE 7. Effect of rGal-1 on the activation of MAPK in mouse eyes. Representative immunoblots demonstrate the levels of (A) ERK2 and p-ERK1/2, (B) p38 and p-p38, and (C) JNK1 and p-JNK in the pooled extracts of mouse eyes ($n = 5$ animals/group) from control (Ctr) mice and mice with allergic conjunctivitis (AC) treated or not with Dex (AC+Dex) or rGal-1 (AC+rGal-1) at 4 and 24 hours. Immunoreactive bands for MAPKs were semiquantified by densitometry and are expressed as arbitrary units of the ratio of p-MAPK/total MAPK (data illustrate one representative of two independent experiments). β -actin was used as loading control.

group.¹⁰ 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 (Fc γ RI) 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 compart-

ments 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 betamethasone), 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, β 2-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.

Acknowledgments

Supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil (2011/21845-3) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil (476136/2011-3 and 302768/2010-6).

The authors thank Kátia de Vasconcelos and Licurgo de Carvalho from the Departamento de Morfologia e Genética, UNIFESP, for their technical assistance in the Western blotting analysis.

Disclosure: **C.B. Mello**, None; **L. Ramos**, None; **A.D. Gimenes**, None; **T.R.M. Andrade**, None; **S.M. Oliani**, None; **C.D. Gil**, None

References

- Friedlaender MH. Ocular allergy. *Curr Opin Allergy Clin Immunol*. 2011;11:477-482.
- Rosario N, Bielory L. Epidemiology of allergic conjunctivitis. *Curr Opin Allergy Clin Immunol*. 2011;11:471-476.
- Pacharn P, Vichyanond P. Immunomodulators for conjunctivitis. *Curr Opin Allergy Clin Immunol*. 2013;13:550-557.
- Rabinovich GA, Daly G, Dreja H, et al. Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med*. 1999;190:385-398.
- Toscano MA, Commodaro AG, Ilarregui JM, et al. Galectin-1 suppresses autoimmune retinal disease by promoting concomitant Th2- and T regulatory-mediated anti-inflammatory responses. *J Immunol*. 2006;176:6323-6332.
- Santucci L, Fiorucci S, Rubinstein N, et al. Galectin-1 suppresses experimental colitis in mice. *Gastroenterology*. 2003;124:1381-1394.
- La M, Cao TV, Cerchiaro G, et al. A novel biological activity for galectin-1: inhibition of leukocyte-endothelial cell interactions in experimental inflammation. *Am J Pathol*. 2003;163:1505-1515.
- Norling LV, Sampaio AL, Cooper D, Perretti M. Inhibitory control of endothelial galectin-1 on in vitro and in vivo lymphocyte trafficking. *FASEB J*. 2008;22:682-690.
- Gil CD, Cooper D, Rosignoli G, Perretti M, Oliani SM. Inflammation-induced modulation of cellular galectin-1 and -3 expression in a model of rat peritonitis. *Inflamm Res*. 2006;55:99-107.
- Gil CD, Gullo CE, Oliani SM. Effect of exogenous galectin-1 on leukocyte migration: modulation of cytokine levels and adhesion molecules. *Int J Clin Exp Pathol*. 2010;4:74-84.
- Cooper D, Norling LV, Perretti M. Novel insights into the inhibitory effects of Galectin-1 on neutrophil recruitment under flow. *J Leukoc Biol*. 2008;83:1459-1466.
- Hsieh SH, Ying NW, Wu MH, et al. Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene*. 2008;27:3746-3753.
- Auvynet C, Moreno S, Melchy E, et al. Galectin-1 promotes human neutrophil migration. *Glycobiology*. 2013;23:32-42.
- Brandt B, Abou-Eladab EF, Tiedge M, Walzel H. Role of the JNK/c-Jun/AP-1 signaling pathway in galectin-1-induced T-cell death. *Cell Death Dis*. 2010;1:e23.
- Masamune A, Satoh M, Hirabayashi J, Kasai K, Satoh K, Shimosegawa T. Galectin-1 induces chemokine production and proliferation in pancreatic stellate cells. *Am J Physiol Gastrointest Liver Physiol*. 2006;290:G729-G736.
- Chung LY, Tang SJ, Sun GH, et al. Galectin-1 promotes lung cancer progression and chemoresistance by upregulating p38 MAPK, ERK, and cyclooxygenase-2. *Clin Cancer Res*. 2012;18:4037-4047.
- Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol*. 2013;13:679-692.
- Pelaia G, Cuda G, Vatrella A, et al. Mitogen-activated protein kinases and asthma. *J Cell Physiol*. 2005;202:642-653.
- Read RW, Yu F, Accorinti M, et al. Evaluation of the effect on outcomes of the route of administration of corticosteroids in acute Vogt-Koyanagi-Harada disease. *Am J Ophthalmol*. 2006;142:119-124.
- Rosenbaum JT. Future for biological therapy for uveitis. *Curr Opin Ophthalmol*. 2010;21:473-477.
- Shoji J, Sakimoto T, Muromoto K, Inada N, Sawa M, Ra C. Comparison of topical dexamethasone and topical FK506 treatment for the experimental allergic conjunctivitis model in BALB/c mice. *Jpn J Ophthalmol*. 2005;49:205-210.
- Chung SH, Nam KH, Kweon MN. Staphylococcus aureus accelerates an experimental allergic conjunctivitis by Toll-like receptor 2-dependent manner. *Clin Immunol*. 2009;131:170-177.
- Damazo AS, Yona S, Flower RJ, Perretti M, Oliani SM. Spatial and temporal profiles for anti-inflammatory gene expression in leukocytes during a resolving model of peritonitis. *J Immunol*. 2006;176:4410-4418.
- Magone MT, Chan CC, Rizzo LV, Kozhich AT, Whitcup SM. A novel murine model of allergic conjunctivitis. *Clin Immunol Immunopathol*. 1998;87:75-84.
- Chung YW, Oh HY, Kim JY, Kim JH, Kim IY. Allergen-induced proteolytic cleavage of annexin-1 and activation of cytosolic phospholipase A2 in the lungs of a mouse model of asthma. *Proteomics*. 2004;4:3328-3334.
- Leonardi A, Curnow SJ, Zhan H, Calder VL. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy*. 2006;36:777-784.
- Fukushima A, Sumi T, Fukuda K, et al. Analysis of the interaction between IFN-gamma and IFN-gammaR in the effector phase of experimental murine allergic conjunctivitis. *Immunol Lett*. 2006;107:119-124.
- Giavina-Bianchi P, Kalil J, Rizzo LV. Development of an animal model for allergic conjunctivitis: influence of genetic factors and allergen concentration on immune response. *Acta Ophthalmol*. 2008;86:670-675.
- Li DQ, Zhang L, Pflugfelder SC, et al. Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4-dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways. *J Allergy Clin Immunol*. 2011;128:1318-1325, e1312.
- Fukuda K, Kuo CH, Morohoshi K, Liu FT, Ono SJ. The murine CCR3 receptor regulates both eosinophilia and hyperresponsiveness in IgE-mediated allergic conjunctivitis. *Br J Ophthalmol*. 2012;96:1132-1136.

31. Sohn J, Kim TI, Yoon YH, Kim JY, Kim SY. Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis. *J Clin Invest*. 2003;111:121-128.
32. Fukushima A, Ishida W, Ojima A, et al. Participation of CD11b and F4/80 molecules in the conjunctival eosinophilia of experimental allergic conjunctivitis. *Int Arch Allergy Immunol*. 2010;151:129-136.
33. Ono SJ, Lane K. Comparison of effects of alcaftadine and olopatadine on conjunctival epithelium and eosinophil recruitment in a murine model of allergic conjunctivitis. *Drug Des Devel Ther*. 2011;5:77-84.
34. Miyazaki D, Tominaga T, Yakura K, et al. Conjunctival mast cell as a mediator of eosinophilic response in ocular allergy. *Mol Vis*. 2008;14:1525-1532.
35. Rabinovich GA, Sotomayor CE, Riera CM, Bianco I, Correa SG. Evidence of a role for galectin-1 in acute inflammation. *Eur J Immunol*. 2000;30:1331-1339.
36. Ma C, Ma Z, Liao XL, Liu J, Fu Q, Ma S. Immunoregulatory effects of glycyrrhizic acid exerts anti-asthmatic effects via modulation of Th1/Th2 cytokines and enhancement of CD4(+)CD25(+)Foxp3+ regulatory T cells in ovalbumin-sensitized mice. *J Ethnopharmacol*. 2013;148:755-762.
37. Seo JW, Cho SC, Park SJ, et al. 1'-Acetoxychavicol acetate isolated from *Alpinia galanga* ameliorates ovalbumin-induced asthma in mice. *PLoS One*. 2013;8:e56447.
38. Baiula M, Spartà A, Bedini A, et al. Eosinophil as a cellular target of the ocular anti-allergic action of mapracorat, a novel selective glucocorticoid receptor agonist. *Mol Vis*. 2011;17:3208-3223.
39. Cavet ME, Volhejn S, Harrington KL, Zhang JZ. Anti-allergic effects of mapracorat, a novel selective glucocorticoid receptor agonist, in human conjunctival fibroblasts and epithelial cells. *Mol Vis*. 2013;19:1515-1525.
40. Rajasagi NK, Suryawanshi A, Schrawat S, et al. Galectin-1 reduces the severity of herpes simplex virus-induced ocular immunopathological lesions. *J Immunol*. 2012;188:4631-4643.
41. Delbrouck C, Doyen I, Belot N, et al. Galectin-1 is overexpressed in nasal polyps under budesonide and inhibits eosinophil migration. *Lab Invest*. 2002;82:147-158.
42. Ochkur SI, Kim JD, Protheroe CA, et al. The development of a sensitive and specific ELISA for mouse eosinophil peroxidase: assessment of eosinophil degranulation ex vivo and in models of human disease. *J Immunol Methods*. 2012;375:138-147.
43. Malik RK, Ghurye RR, Lawrence-Watt DJ, Stewart HJ. Galectin-1 stimulates monocyte chemotaxis via the p44/42 MAP kinase pathway and a pertussis toxin-sensitive pathway. *Glycobiology*. 2009;19:1402-1407.
44. Barrionuevo P, Beigier-Bompadre M, Ilarregui JM, et al. A novel function for galectin-1 at the crossroad of innate and adaptive immunity: galectin-1 regulates monocyte/macrophage physiology through a nonapoptotic ERK-dependent pathway. *J Immunol*. 2007;178:436-445.
45. Fautsch MP, Silva AO, Johnson DH. Carbohydrate binding proteins galectin-1 and galectin-3 in human trabecular meshwork. *Exp Eye Res*. 2003;77:11-16.
46. Sena AA, Provazzi PJ, Fernandes AM, Cury PM, Rahal P, Olini SM. Spatial expression of two anti-inflammatory mediators, annexin 1 and galectin-1, in nasal polyposis. *Clin Exp Allergy*. 2006;36:1260-1267.
47. Fernandes AM, Babeto E, Rahal P, Provazzi PJ, Hidalgo CA, Anselmo-Lima WT. Expression of genes that encode the annexin-1 and galectin-1 proteins in nasal polyposis and their modulation by glucocorticoid. *Braz J Otorhinolaryngol*. 2010;76:213-218.