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ORIGINAL ARTICLE

Anti-inflammatory effect of galectin-1 in a murine model of atopic dermatitis

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Abstract
Atopic dermatitis (AD) is caused by both dysregulated immune responses and an impaired skin barrier. Although beta-galactoside-binding protein galectin-1 (Gal-1) has immunomodulatory effects in several inflammatory disorders, therapeutic strategies based on its anti-inflammatory properties have not been explored in AD. Thus, we evaluate pharmacological treatment with Gal-1 in the progression of an ovalbumin (OVA)-induced AD-like skin lesions. The skin of OVA-immunized male BALB/c mice was challenged with drops containing OVA on days 11, 14–18 and 21–24. Additionally, in the last week, a subset of animals was treated intraperitoneally with recombinant Gal-1 (rGal-1) or dexamethasone (Dex). Treatment with rGal-1 decreased the clinical signs of dermatitis in BALB/c mice and diminished local eotaxin and IFN-γ levels. The treatment also suppressed the infiltration of eosinophils and mast cells, which was verified by reduced expression of mouse mast cell protease 6 (mMCP6) and eosinophil peroxidase (EPX). These localized effects are associated with extracellular signal-regulated kinase (ERK) activation and downregulation of endogenous Gal-1. The inhibition of disease progression induced by rGal-1 was also correlated with reduced plasma IL-17 levels. Our results demonstrate that rGal-1 is an effective treatment for allergic skin inflammation in AD and may impact the development of novel strategies for skin inflammatory diseases.

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Key messages

- Pharmacological treatment with rGal-1 reduces clinical signs of atopic dermatitis.
- Systemic treatment with rGal-1 inhibits eosinophil and mast cell influx in the skin of AD animals.
- rGal-1 reduced local eotaxin levels and systemic IL-17 levels.
- The inhibition of disease progression induced by rGal-1 was correlated with upregulation of phosphorylated ERK.

Keywords Galectin-1 · Skin inflammation · Ovalbumin · Eosinophil · Mast cell · ERK

Introduction

Atopic dermatitis (AD), also known atopic eczema, represents the most common chronic inflammatory skin disease, characterized by itchy, red, swollen and cracked skin [1, 2]. AD etiology is multifactorial, and there is evidence that genetic predisposition and family history for atopic diseases, such as bronchitis, asthma and rhinitis, influence AD development.

The pathogenesis of AD is attributed to an imbalance in the adaptive immune system, including dysfunction in T helper (Th) cells and increased IgE production [1–3]. Cytokines and chemokines, such as interleukin (IL)-4, IL-5, IL-13, eotaxins, C-C motif chemokine ligand (CCL) 17, CCL18 and CCL22, produced by Th2 cells and dendritic cells stimulate the infiltration of mast cells and eosinophils into the skin. Th2 and Th17 lymphocytes predominate in patients with AD, but Th1 cells also contribute to the pathogenesis. Cytokines produced by Th2 and Th17 cells (IL-4/IL-13 and IL-17/IL-22, respectively) inhibit terminal differentiation of the epidermis and contribute to breakdown of the epithelial barrier in patients with AD.

There are a number of treatments for AD which include emollients, dexamethasone, topical glucocorticoids, calcineurin inhibitors, phototherapies and immunosuppressant such as cyclosporine A, which are effective in reducing inflammation but cause adverse effects [1, 2]. Thus, the discovery of new pharmacological agents that have high efficacy in controlling the inflammatory response with fewer side effects is critical.

Galectin-1 (Gal-1) is a 14.5-kDa β-galactoside-binding mammalian lectin with anti-inflammatory properties demonstrated in vitro and in vivo experimental models. In vitro, incubation with recombinant Gal-1 (rGal-1) inhibits the migration of human neutrophils [4] and lymphocytes [5] through endothelial cells following IL-8 or TNF-α stimulation, respectively. In vivo, rGal-1 administration is associated with inhibition of neutrophil extravasation into the peritoneal cavity after 4 h of carrageenan- [6], zymosan- [7], or IL-1β-induced peritonitis in rodents [4]. The anti-migratory effect of Gal-1 is also associated with the modulation of L-selectin and β2-integrin expression on the surface of leukocytes [5, 7, 8], but not E-selectin, ICAM-1 and VCAM-1 on endothelial cells [5]. In a mouse model of ovalbumin-induced allergic conjunctivitis, we have recently shown that rGal-1 inhibits IL-4, IL-13 and eotaxin levels in lymph nodes and is associated with reduced clinical signs of disease and decreased plasma IgE anti-ovalbumin levels [9]. However, the mechanisms by which Gal-1 modulates cellular responses in allergic inflammatory processes are not still completely determined, especially in the skin.

In addition to these immunomodulatory roles, the signal transduction events that lead to Gal-1 induced neutrophil and endothelial cell migration and T-cell death include the activation of mitogen-activated protein kinases (MAPKs) [10, 11]. MAPKs, well-conserved signaling pathways, include 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 [12]. This kinase family has been implicated in allergic responses, particularly in asthma, contributing to leukocyte recruitment, mast cell activation, pro-inflammatory cytokine production and differentiation of Th2 and Th17 lymphocytes [13, 14].

Given the known anti-inflammatory effects of Gal-1 and the current limitations in the treatment of AD, we evaluated the mechanism of action of this protein in an experimental model of ovalbumin-induced AD in mice.

Material and methods

Animals

Male BALB/c mice, weighing 20–25 g, were randomly distributed into five groups (n = 6 animals/group). The animals were housed in a 12-h light-dark cycle and were allowed food and water ad libitum. All animal procedures were approved by the Ethics Committee in Animal Experimentation of the Federal University of São Paulo—UNIFESP (CEP 1906060115/2015).

Allergic dermatitis model and treatment protocols

To develop a mouse model of AD using skin sensitization, BALB/c mice were immunized on days 0 and 7 with a subcutaneous injection of 5 μg of ovalbumin (OVA; grade V; Sigma-Aldrich, MO, USA) and 10 mg/mL of aluminium hydroxide adjuvant diluted in 200 μL of sterile saline according to previous studies [9, 15] with modifications. On day 11, animals were shaved and the hair removed from the whole back. The skin of mice was challenged with drops containing 250 μg of OVA diluted in 50 μL of JOHNSON'S® baby oil on days 11, 14–18 and 21–24. In the last week (days 21–24), mice were treated with recombinant Gal-1 protein (rGal-1; Peprotech EC Ltd., London, UK) 3 μg/animal, or dexamethasone (Dex) (1 mg/kg, Sigma-Aldrich) intraperitoneal, diluted in 0.1 mL of sterile saline 15 min prior OVA challenge. rGal-1 and Dex doses were scaled up from the anti-inflammatory doses described previously [6, 9]. Control groups were constituted by Naïve (mice were not subjected to any procedures related to the AD model) and Sham (mice that received sterile saline and JOHNSON'S® baby oil alone). Twenty-four hours after the last OVA challenge, mice were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) followed by cardiac puncture using a syringe with 3.8% sodium citrate to obtain blood. Animals were then euthanized and the skin collected.

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 lymphocytes, neutrophils, monocytes and eosinophils. Data were reported as the mean ± SEM of the number of cells × 10^5/mL.

Analysis of IgE anti-ovalbumin and Gal-1 levels

Blood from various experimental conditions was centrifuged at 600×g for 10 min to collect the plasma and determine the IgE anti-OVA levels by ELISA. The concentration of IgE anti-OVA was measured using a commercially available mouse IgE anti-OVA immunoassay kit (Cayman Chemical Co., MI, USA) in accordance with the manufacturer's instructions. Gal-1 levels in skin homogenates were determined using a commercially available kit (RayBiotech, GA, USA), in accordance with the manufacturer's guidelines. All experiments were conducted in duplicate, and the data expressed as the mean ± SEM protein (ng/mL).

Skin thickness, histopathology and quantification of inflammatory cells

Skins were fixed in 4% paraformaldehyde for 24 h, washed in tap water, dehydrated in a decreasing ethanol series and embedded in paraffin. Sections of 3 μm were obtained in a Leica RM2155 microtome (Leica Microsystems, Nussloch, Germany) and subsequently stained with haematoxylin-eosin or toluidine blue 0.5% for histopathology and inflammatory cell quantification. Eosinophils, neutrophils and mast cells were quantified using a 40× objective on an Axio Scope A1 Zeiss microscope (Carls Zeiss, Jena, Germany). Three semi-serial sections of skin were analysed per animal, and the area was determined using AxioVision software (Carl Zeiss). Values are expressed as the mean ± SEM cells per square millimeter.

A 10× objective was used to measure skin thickness, with lines perpendicular to the keratin layer of epidermis up to the hypodermis. For each animal, three measurements of the epidermis + dermis were taken at random intervals using AxioVision software (Carl Zeiss). Values are shown as mean ± SEM of the thickness (mm).

Immunohistochemistry

Endogenous Gal-1 staining was performed in 3-μm sections of paraffin-embedded skin. 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 a rabbit polyclonal anti-Gal-1 antibody (Santa Cruz Biotechnology, CA, USA), diluted 1:200 in PBS 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 colour was developed using DAB substrate (Invitrogen). The sections were counterstained with haematoxylin.

Densitometry analysis of Gal-1 immunostaining was performed in the epidermis and dermis (n = 6 animals/group). The values were obtained as arbitrary units (a.u.) between 0 and 255 using AxioVision software on an Axioskop 2 mot plus Zeiss microscope (Carl Zeiss, Jena, Germany). The data are expressed as the mean ± SEM of a. u.

Western blot analysis

Skins 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 and PhosSTOP tablets (Roche Applied Science, Mannheim, Germany). Subsequently, samples were centrifuged at 10,000×g for 20 min at 4 °C to obtain organ supernatants. Protein levels were determined by the Bradford assay and normalized prior to boiling in Laemmli buffer (Bio-Rad Laboratories, USA). Pooled protein extracts (30 μg per lane) of skin (n = 3 animals per group) from indicated experimental conditions were loaded onto a 12% sodium dodecyl sulphate-polyacrylamide gel for electrophoresis together with appropriate molecular weight markers (Bio-Rad Life Science, USA) 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) was used to verify protein transfer. Membranes were incubated for 15 min in 5% BSA in Tris-buffered saline (TBS) prior to incubation with antibodies. Primary antibodies used herein, rabbit polyclonal antibody anti-ERK1/2 (1:5000); mouse monoclonal anti-phosphorylated (p)-ERK½ (1:2000) (Cell Signaling, Danvers, USA); goat polyclonal anti-mouse mast cell protease 6 (mMCP6; 1:5000) (R&D Systems, Minneapolis, USA); anti-eosinophil peroxidase (EPX; 1:200) (Santa Cruz Biotechnology); rabbit polyclonal anti-integrin αM (CD11b; 1:200) (Santa Cruz Biotechnology); anti-GAPDH (1:5000) (Sigma-Aldrich); and rat monoclonal anti-CD4 (1:500) (Rheabiotech-Imuny, Campinas, Brazil), all diluted in TBS with 0.1% Tween 20. Post primary antibody incubation, membranes were washed for 15 min with TBS and subsequently incubated for 60 min at room temperature with the appropriate secondary antibodies. Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG, goat anti-rat, rabbit anti-goat (1:2000) (Thermo Fisher Scientific Inc., MI, USA) or goat anti-mouse (1:2000) (Millipore Corporation, CA, USA). Finally, membranes were washed for 15 min with TBS, and immunoreactive proteins were detected (Westar Nova 2.0 chemiluminescent substrate kit; Cyanagen, Bologna, Italy) using a GeneGnome5 chemiluminescence detection system (SynGene, Cambridge, UK). Proteins were imaged and quantified using the software GeneTools (SynGene) to determine the relative expression of indicated proteins (a.u.).

Analysis of cytokines and chemokines

Multiplex analysis was performed with 25 μL of the skin supernatants and plasma using the MILLIPLEX MAP mouse cytokine/chemokine panel (MT17MAG47K-PX25; Millipore Corporation, USA) and MAGPIX® Multiplexing Instrument (Millipore) according to the manufacturer's instructions. Eight analytes were measured: IL-4, IL-10, IL-13, IL-17A, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), eotaxin and RANTES (Regulated upon Activation Normal T cell Expressed and presumably secreted). The concentration of analytes was determined by MAGPIX Xponent software (Millipore Corporation, MA, USA), and the results are reported as the mean ± SEM of analytes (pg/mL).

Statistical analysis

The data were analysed using GraphPad Prism 5.0 software. Results were confirmed to follow a normal distribution using

the Kolmogorov-Smirnov test of normality with Dallal-Wilkinson-Lillie for corrected P value. Data that passed the normality assumption was analysed using ANOVA with the Bonferroni post hoc test. Data that failed the normality assumption were analysed using the nonparametric Kruskal-Wallis followed by the Dunn post-test. Differences were considered statistically significant at a value of $P < 0.05$.

Results

Pharmacological treatment with rGal-1 reduces clinical signs of AD

Clinical assessment of AD and skin thickness measurements were performed by macroscopic and histological analysis of mouse skins on the final day of the experimental protocol (day 24). The AD group exhibited skin with erythema, erosion and dryness associated with a marked epidermal hyperplasia and increase in skin thickness compared to the control groups (Naive and Sham) (Fig. 1). Intraperitoneal treatment with rGal-1 or Dex reduced this AD clinical response, leaving skins with a similar appearance to control animals. Furthermore, rGal-1 or Dex-treated animals displayed a diminished skin thickness and mild epidermal hyperplasia in relation to the AD group (Fig. 1).

rGal-1 regulates systemic immune responses

We first assessed the systemic immune response by measuring OVA-specific plasma IgE levels. Epicutaneous serial OVA administration significantly increased the plasma anti-OVA IgE concentration in all AD groups (AD: 231 ± 28; rGal-1: 257 ± 45; Dex: 205 ± 28 ng/mL) in relation to control groups ($P < 0.001$), supporting the efficacy of our experimental model.

Next, we evaluated the leukocyte recruitment from the blood due to the allergic response induced by epicutaneous challenge with OVA. Blood eosinophilia was exhibited by the AD-untreated and rGal-1 or Dex-treated groups at 24 h (Fig. 2a). Furthermore, AD induced lymphocytosis and monocytosis that were abrogated by the rGal-1 treatment (Fig. 2c, d) whereas Dex induced only a reduction in the monocyte numbers. No differences were detected in the blood neutrophil numbers (Fig. 2b).

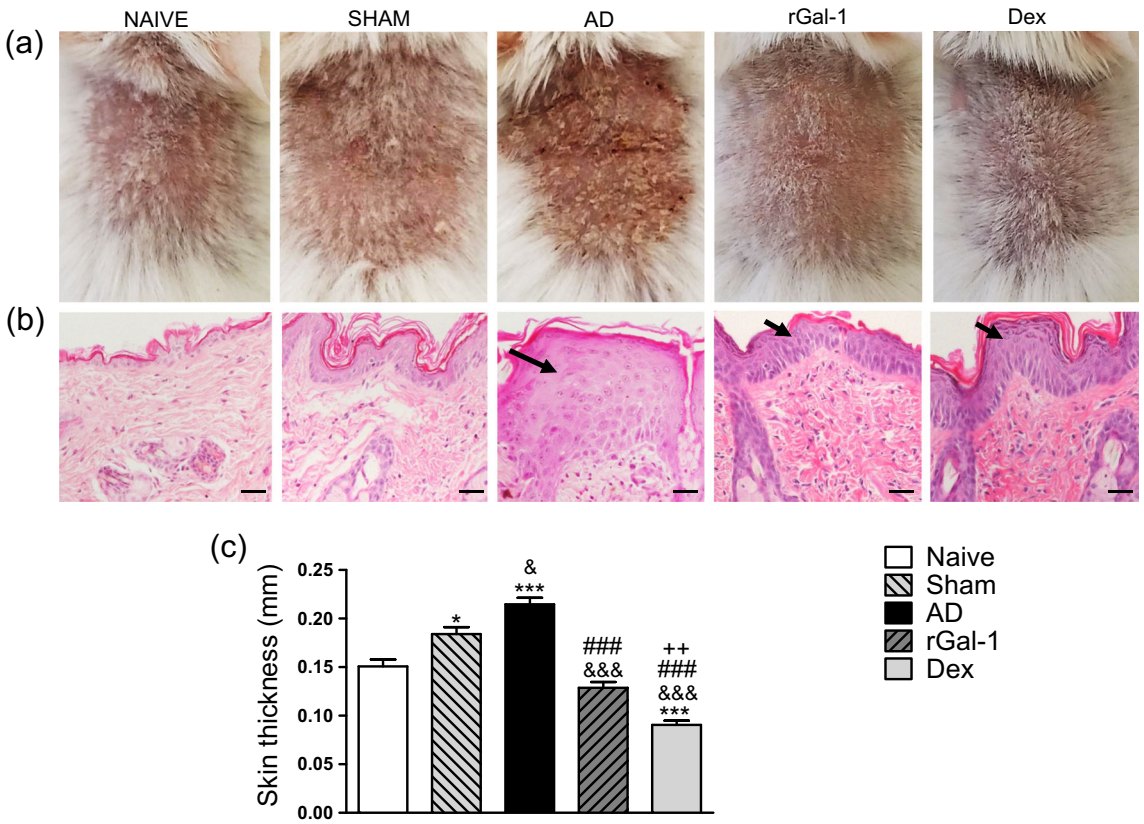


Fig. 1 Effect of rGal-1 administration on skin in the AD model. Macroscopic and histological analyses of skin lesions were taken on the final day of the experiment (day 24). **a** AD skin exhibited erythema, erosion and dryness whereas rGal-1 and Dex treatments abrogated this effect showing a similar skin appearance as controls (Naïve and Sham). **b** Histologically, control animals showed normal epidermis with continuous basal layer, 1–2 cell thick spinous layer and thin stratum corneum. AD results in marked hyperplasia in the epidermis (*arrow*) with prominent thickened stratum spinosum (*arrow*). Note mild epidermal hyperplasia (*short arrows*) after rGal-1 and Dex treatments. *Stain*: haematoxylin-eosin. *Bars*: 50 μm. **c** Skin thickness. Data represents mean ± SEM of skin thickness (mm) (n = 6/group). $^{*}P < 0.05$; $^{***}P < 0.001$ vs Naive; $^{\&}P < 0.05$; $^{\&\&\&}P < 0.001$ vs Sham; $^{\#\#\#}P < 0.001$ vs AD, $^{++}P < 0.01$ vs rGal-1 (ANOVA, Bonferroni post-test)

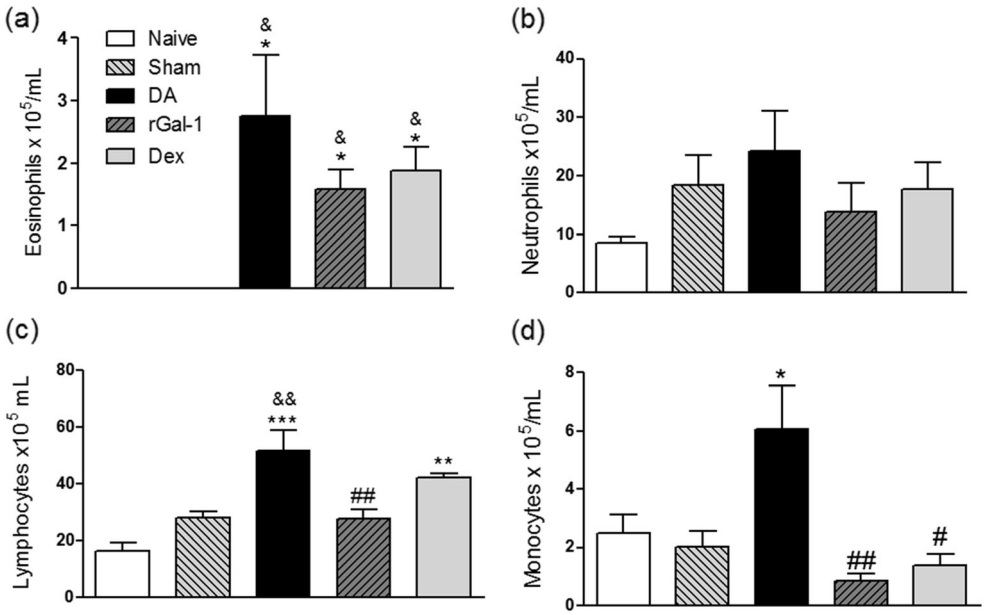


Fig. 2 Effect of rGal-1 on systemic immune responses in the AD model. **a–d** Quantification of blood eosinophils, neutrophils, lymphocytes and monocytes. Data represent the mean ± SEM of the number of leukocytes × 10^5/mL in mice under different experimental conditions ($n = 6$/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Naive; $^{\&}P < 0.05$; $^{\&\&}P < 0.01$ vs Sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs AD (ANOVA, Bonferroni post-test)

Levels of the chemokines and cytokines belonging to Th1, Th2 and Th17 pathways were analysed by multiplex assay in plasma samples of all experimental groups. Both rGal-1 and Dex treatments markedly reduced plasma IL-17A levels compared to the AD group (Fig. 3g), while rGal-1 alone diminished the plasma eotaxin concentration (Fig. 3a). However, multiplex immunoassays revealed no significant difference in the plasma levels of RANTES, IL-4, IL-13, IL-10 or IFN-γ between the untreated AD and the Dex- and rGal-1-treated AD groups (Fig. 3b–f).

Effect of rGal-1 on the inflammatory response of the skin and the ERK pathway

Histopathological analysis of control skins revealed a normal morphology (Fig. 4a, e). In contrast, sensitized and OVA-challenged mice exhibited an inflammatory response characterized by significant infiltration of inflammatory cells in the dermis, particularly eosinophils and degranulated mast cells (Fig. 4b, f). Treatment with rGal-1 and Dex resulted in fewer inflammatory cells in the dermis compared to the untreated AD group (Fig. 4c, d, g, h).

Inflammatory cell counts confirmed the histological observations with significant increase of eosinophils, neutrophils and mast cells in AD group compared to the controls (Fig. 4i). Conversely, rGal-1 and Dex administration produced a significant decrease in eosinophil and neutrophil influx (Fig. 4i).

The presence of inflammatory cells in the mouse skins was also confirmed by immunoblot analysis. T cell influx in AD groups (treated and untreated) was evidenced by CD4 expression which demonstrated mild increased levels in the rGal-1- and Dex-treated groups (Fig. 4j). On the other hand, both treated groups exhibited reduced levels of endogenous adhesion molecule CD11b, EPX and mMCP6 compared to AD group which corroborates histological data. In the same way, diminished levels of eotaxin, RANTES and IFN-γ were detected in skin homogenates from both treated groups, but only Dex treatment caused a significant reduction compared to AD group (Fig. 5a–c). On the other hand, rGal-1 increased TNF-α and IL-10 levels (Fig. 5d, g). IL-4 and IL-13 levels showed no differences between AD and rGal-1 and Dex-treated groups (Fig. 5e, f).

To understand the downstream systemic molecular signalling pathways involved regulated by rGal-1 treatment in AD, we analysed the phosphorylation levels of ERK by Western blot using pooled extracts of skins ($n = 3$ animals per group). rGal-1 and Dex treatments increased p-ERK levels compared with the nontreated AD group (Fig. 5h), suggesting both treatments function via a similar mechanism.

AD induces increased expression of Gal-1 protein

To further characterize the Gal-1 expression in mouse skin during the AD inflammatory response, we evaluated Gal-1 protein levels by immunohistochemistry.

Gal-1 expression was detected in both the epidermis and dermis under all experimental conditions, consistent with previous studies [16, 17] suggesting that the epithelium is a potential source of this lectin. Twenty-four hours after the last epicutaneous OVA challenge, extracellular matrix of the dermis from the AD group displayed intense immunostaining for Gal-1 compared with controls (Naïve and Sham) (Fig. 6a). rGal-1- and Dex-treated AD groups produced lower Gal-1 immunostaining in the epidermis and dermis compared with the untreated AD group. No immunostaining was detected in the negative control section (Fig. 6b). The histologic findings were supported by densitometry analyses of Gal-1 expression in the epidermis and dermis (Fig. 6c, d). Similarly, ELISA data

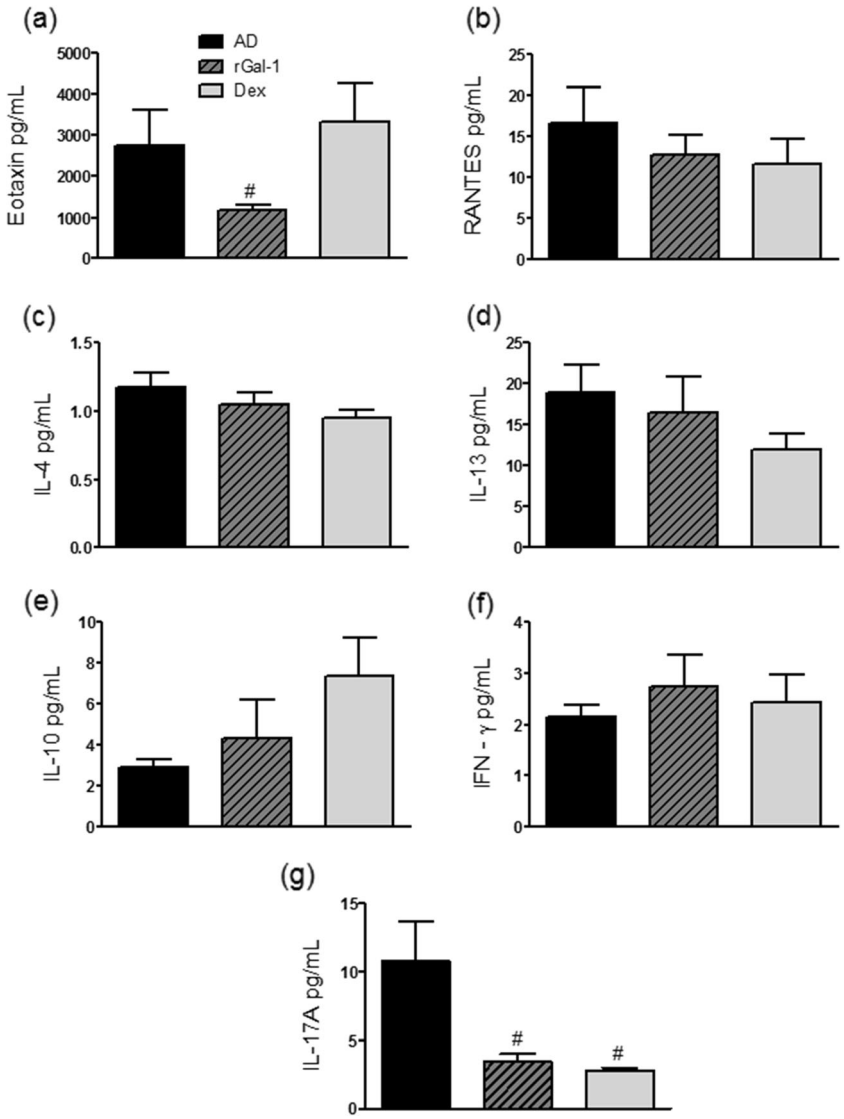


Fig. 3 Effect of rGal-1 on the chemokine/cytokine systemic release. **a–g** Multiplex immunoassay of eotaxin (**a**), RANTES (**b**), IL-4 (**c**), IL-13 (**d**), IL-10 (**e**), IFN-γ (**f**) and IL-17A (**g**) in plasma. Values are expressed as the mean ± SEM of the chemokines/cytokines (pg/mL; $n = 6$/group). $^{\#}P < 0.05$ vs AD (Kruskal-Wallis, Dunn post-test)

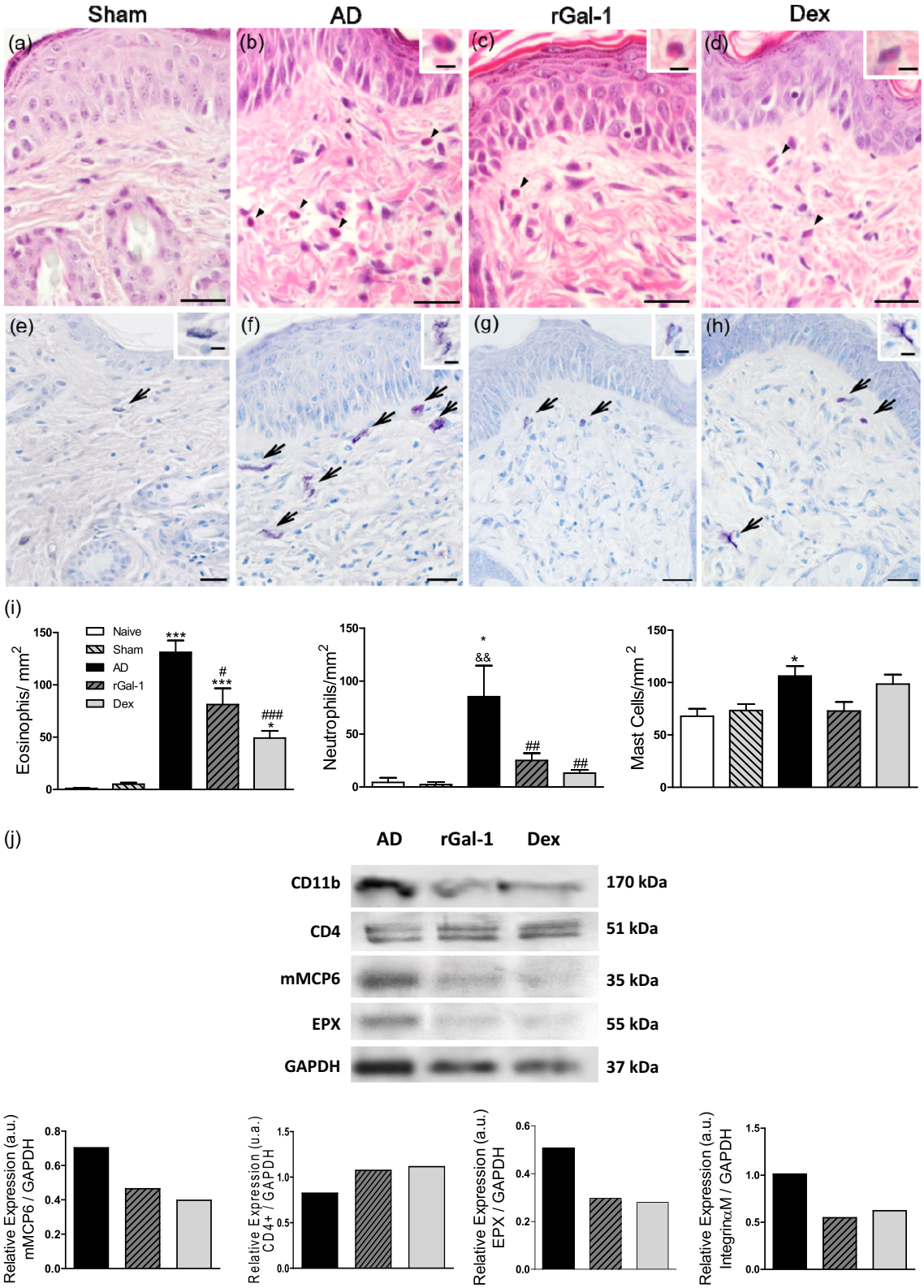
from skin homogenates showed that rGal-1- and Dex-treated samples exhibited decreased endogenous Gal-1 levels compared to AD samples (Fig. 6e).

Discussion

In this study, we evaluated the effect of pharmacological treatment with recombinant Gal-1 (rGal-1) on OVA-induced AD model in mice. Using macroscopic, histological, biochemical and molecular analyses, we showed that systemic rGal-1 treatment was as effective as dexamethasone (Dex), in the regulation of the allergic inflammatory response in skin.

The development of AD was confirmed through the detection of clinical signs such as erythema and dryness, increased skin thickness and plasma anti-OVA IgE levels compared to control animals. Histological analysis revealed epidermal hyperplasia and a marked influx of eosinophils and mast cells in the dermis, which were confirmed by increased EPX and mMCP-6 protein levels in skin

Fig. 4 Histopathology of the skin. **a**, **e** Control (Sham and Naive). **b**, **f** AD characterized by significant infiltration of eosinophils (*arrowheads*; *inset*) and degranulated mast cells (*arrows*; *inset*) into the dermis. Pharmacological treatment with rGal-1 (**c**, **g**) and Dex (**d**, **h**) resulted in lesser infiltration of inflammatory cells. *Stain*: haematoxylin-eosin (**a–d**) and toluidine blue (**e–h**). *Bars*: 50 μm (**a–h**) and 10 μm (*insets*). **i** Quantitative analysis of eosinophils, neutrophils and mast cells in the skin. Data represent the mean ± SEM of the cell number per square millimeter ($n = 6$ animals/group). $^{*}P < 0.05$, $^{***}P < 0.001$ vs control groups (naive and sham); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs AD (ANOVA, Bonferroni post-test). **j** Western blot analysis to measure CD11b, CD4, mMCP6 and EPX levels in the pooled extracts of mouse skins ($n = 3$ animals/group) from mice with AD untreated or treated with rGal-1 or Dex. GAPDH was used as a protein loading control. Immunoreactive bands for proteins were semi-quantified by densitometry and are expressed as arbitrary units (a.u.) of the ratio of CD11b, CD4, mMCP6 or EXP/GAPDH (data represent one illustrative blot from two independent experiments)



homogenates. Further, blood leukocyte counts revealed eosinophilia, lymphocytosis and monocytosis. These results suggest that external exposure to an allergen can induce localized allergic inflammation in the skin and a systemic sensitization to specific allergen as demonstrated by previous AD models induced by OVA, 2,4-dinitrofluorobenzene, 2,4-dinitrochlorobenzene and oxazolone [15, 18, 19].

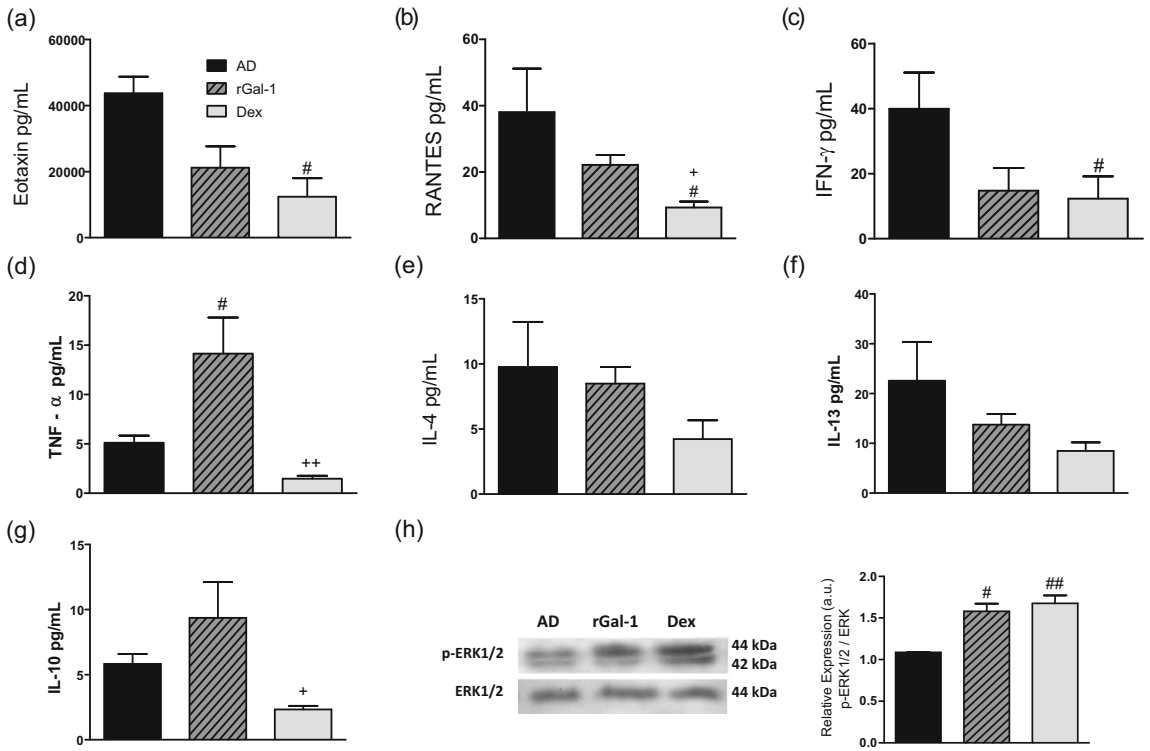


Fig. 5 Effect of rGal-1 administration on local immune response in the AD model. **a–c** Multiplex immunoassay of skin homogenates to detect eotaxin (**a**), RANTES (**b**), IFN-γ (**c**), TNF-α (**d**), IL-4 (**e**), IL-13 (**f**) and IL-10 (**g**). Values are expressed as the mean ± SEM of the chemokines/cytokine (pg/mL; $n = 6$/group). $^{\#}P < 0.05$ vs AD; $^{+}P < 0.05$, $^{++}P < 0.05$ vs rGal-1. **h** Western blot analysis to measure phosphorylated and total ERK levels in the pooled extracts of mouse skins ($n = 3$ animals/group) from mice with AD untreated or treated with rGal-1 or Dex. Immunoreactive bands for proteins were semi-quantified by densitometry and are expressed as arbitrary units (a.u.) of the ratio of p-ERK/total ERK (data represent one illustrative blot from 2 to 4 independent experiments). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (Kruskal-Wallis, Dunn post-test)

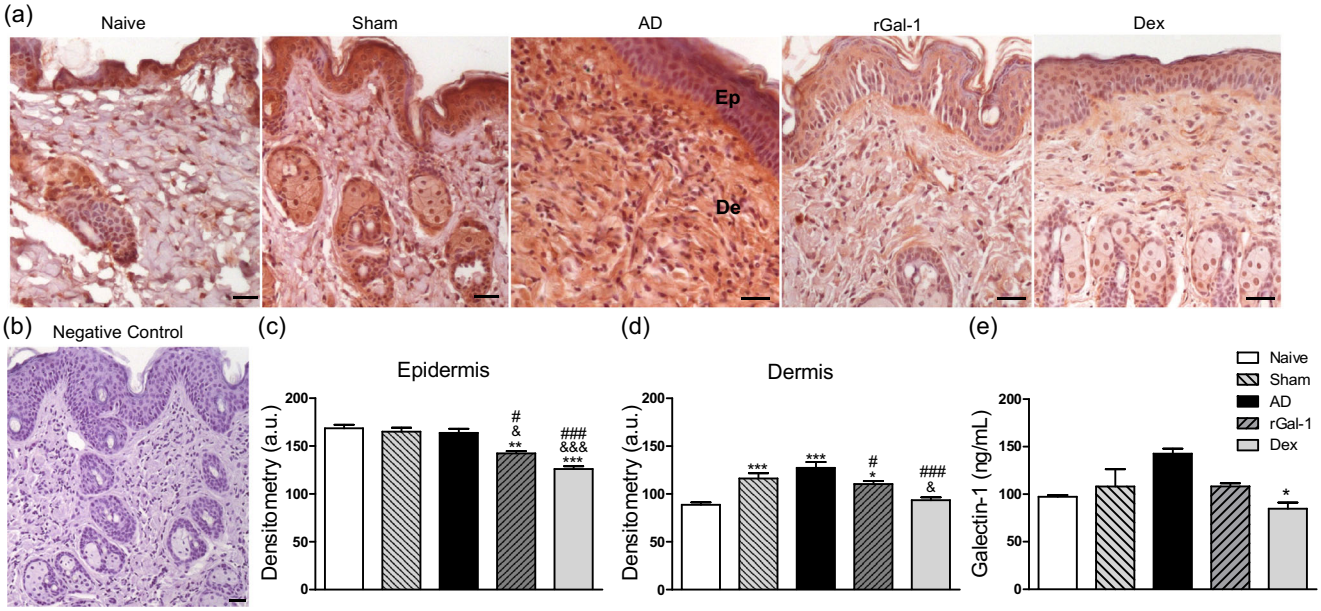


Fig. 6 Expression of Gal-1 in the skin. **a** Dermis (De) from AD animals showed strong Gal-1 immunostaining compared to the control skins (Naïve and Sham). rGal-1- and Dex-treated groups displayed lower endogenous Gal-1 immunoreactivity in the epidermis (Ep) and dermis (De) compared to the AD group. **b** No staining was detected in the sample used as a negative control. *Counterstain*: haematoxylin. *Bars*: 50 μm. **c**, **d** Densitometric analysis of Gal-1 expression in the epidermis and dermis. **e** Immunoassay for Gal-1 detection in skin homogenates. Data represent mean ± SEM of Gal-1 expression (a.u.; $n = 6$ animals/group). $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ vs Naive; $^{\&}P < 0.05$; $^{\&\&\&}P < 0.001$ vs Sham; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs AD (ANOVA, Bonferroni post-test)

Intraperitoneal administration of rGal-1, as well as Dex, decreased the clinical signs and skin thickness associated with AD, but did not reduce IgE levels 24 h after the last epicutaneous OVA challenge. Our group has previously shown that systemic rGal-1 and Dex treatments reduced the clinical signs of OVA-induced allergic conjunctivitis in mice; the effect was associated with a significant decrease in the IgE, IL-4 and IL-13 levels 4 h after the final OVA challenge [9]. Downregulation of IgE and Th2 cytokine levels by rGal-1 and Dex in the ocular allergy was time dependent once no difference between treated and nontreated groups was detected at 24 h.

Systemic administration of rGal-1 also markedly reduced plasma IL-17A levels compared to the untreated AD group, an important cytokine biomarker for AD patients [20, 21]. In a previous mouse model, splenocytes isolated from OVA-induced AD BALB/c mice had significantly increased levels of IL-4, IL-5, IFN-γ and IL-17 after 72 h of OVA stimulation in vitro, whereas unstimulated cells were not significantly different than control animals [22]. Furthermore, in oxazolone-induced AD IL-17-null mice exhibit reduction of skin edema, eosinophilia, IL-4 and IL-13 production in skin and lymph nodes relative to wild-type animals [19, 23]. The lack of IL-17 expression also ameliorated the skin-barrier dysfunction as evidenced by decreased in transepidermal water loss [19]. The results indicate that the IL-17 gene may play a role in modulating immune dysregulation and affecting skin barrier in AD mouse models.

It is important to note that Gal-1 plays an essential role in modulating adaptive immune responses by altering the phenotype of T cells. In this regard, human skin-resident T cell cultures incubated with a mouse Gal-1 human Ig chimera (Gal-1hFc), and stimulated with phorbol 12-myristate-13-acetate (PMA)/ionomycin and brefeldin A, increased IL-4$^+$ and IL-10$^+$ lymphocyte populations and decreased IL-17$^+$ population [24]. Reinforcing this regulatory aspect of Gal-1 on Th cells, another study found that lymphocytes isolated from Gal-1-null mice with chicken collagen II (CII)-induced arthritis significantly upregulated IL-17 and IL-22 production in vitro after 48 h of CII stimulation when compared to wild-type cells [25]. Furthermore, CD4$^+$ T cells under Gal-1 treatment increased the secretion of Th2 cytokines and their expansion to CD4$^+$FOXP3$^+$ Treg cells [26, 27]. In our AD model, rGal-1 treatment increased plasma and skin levels of IL-10, as well as CD4 expression, in relation to untreated group. Taken together, our data suggest that Gal-1 plays a functional role in the AD regulation through favouring Treg cell influx and downregulation of IL-17 production.

In addition to regulating Th2/Th17, Gal-1 was able to downregulate Th1 cytokine (IL-1β, IFN-γ, TNF-α) production, as demonstrated in previous studies using experimental models of arthritis [28], colitis [29] and uveitis [30]. The addition of Gal-1 to human monocyte-derived dendritic cell-T cell cocultures from psoriasis patients has also been shown to reduce IFN-γ production. This could be reversed by the addition of lactose [31]. Furthermore, it was shown that IFN-γ/mast cell axis is involved in the pathology of chronic allergic inflammation of the airways in mice [32]. In the study, IFN-γ significantly increased the release of histamine, IL-6 and IL-13 by IgE + specific antigen-stimulated bone marrow-derived cultured mast cells in vitro, after 1 or 24 h of challenge, whereas treatment of the cells with IFN-γ alone had no effect. In support of this finding, in our AD model, diminished levels of IFN-γ were detected in the skin of rGal-1-treated mice. This reduction correlated with reduced levels of mMCP6 when compared to the untreated AD group, suggesting that the exogenous action of Gal-1 is essential to regulate the activation of mast cells and the initiation of clinical signs of AD.

Another important local effect of rGal-1 discovered in our AD model is the inhibitory role on eosinophil and neutrophil migration, which was validated by the downregulation of EPX, adhesion molecule CD11b and chemokines eotaxin and RANTES. Similar findings were detected in a OVA-induced allergic conjunctivitis in BALB/c mice, in which pharmacological pretreatment with rGal-1 reduced eotaxin and RANTES levels in the lymph nodes compared with the untreated AC group after 24 h of last challenge [9]. It was recently shown that the Gal-1-null mice presented increased OVA-induced airway inflammation demonstrated by increased number of eosinophils and lymphocytes in bronchoalveolar fluid and lung and higher airway resistance relative to WT OVA-challenged mice [33].

Our immunohistochemistry studies demonstrated that AD elevated levels of Gal-1 in the dermis in relation to the control groups. On the other hand, exogenous administration of rGal-1 downregulated the expression of endogenous Gal-1 protein in the skin, likely as a mechanism of negative feedback. Exposure to the allergen OVA resulted in increased Gal-1 expression in the lungs, eyes and conjunctiva due to the recruitment of Gal-1-expressing inflammatory cells, including eosinophils [9, 33]. Expression of Gal-1 has been demonstrated in the nucleus, cytosol and cell membranes as well as in the extracellular matrix of tissues despite the absence of a secretion signal sequence [4, 7, 9]. Extracellularly, Gal-1 acted as a nonpermissive substrate with respect to processes relevant for eosinophil migration [34]. Similarly, eosinophils exposed to lower concentrations of rGal-1 (0.1–0.25 μM) exhibited increased adhesion to recombinant murine to vascular cell adhesion molecule-1 (VCAM-1) in a dose-dependent manner compared with untreated cells under static conditions. However, despite the increased adhesion, eosinophils treated with rGal-1 demonstrated significantly reduced migration toward eotaxin-1 in in vitro chemotaxis assays [33]. In the same study, in vitro administration of rGal-1 exerted divergent effects on eosinophils that were N-glycan and dose dependent. At low concentrations (≤0.25 μM), rGal-1 inhibited ERK1/2 activation and eotaxin-1-induced migration of eosinophils

while at high concentration (≥ 1 μM), induced ERK1/2-dependent apoptosis of eosinophils and decreased expression of adhesion molecules CD49d and CCR3. In fact, ERK1/2-dependent pathway has also been shown to be critically involved in the regulatory effects of galectin-1 on monocyte activation and T cell viability [35, 36].

In line with these previous reports, we herein found in our AD model localized (skin) activation of ERK1/2, plus markedly increased TNF-α levels, following rGal-1 treatment which was not associated with increased inflammatory response. Moreover, recent findings indicate that Gal-1-exposed intestinal epithelial cells (IECs) in the presence of proinflammatory cytokines, such as TNF, induced caspase-dependent intrinsic pathway of apoptosis [37]. Furthermore, IECs exposed to Gal-1 in the presence of TNF augmented the secretion of epidermal growth fator (EGF), thymic stromal lymphopoietin (TSLP), IL-10 and TGF-β1. In the same way, Gal-1 coincubated with IL-13-induced upregulation of IL-10, TGF-β1 and IL-25 secretion in IECs. These findings support that Gal-1 can regulate cellular response in an either Th2 or Th1/Th17 proinflammatory microenvironment, as well as, through activation of the ERK pathway in the AD.

Overall, the data presented herein identify Gal-1 as a potential therapeutic agent to regulate AD, as it modulates specific phases of the inflammatory process with clear outcomes under in vivo conditions. We therefore propose that strategies aimed at potentiating the Gal-1 pathway may provide novel pharmacological approaches to the downregulation of the allergic inflammatory response in the skin.

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Conflict of interest The authors declare no conflict of interest.

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