

# Beneficial effect of annexin A1 in a model of experimental allergic conjunctivitis



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## ABSTRACT

Annexin A1 (ANXA1), a 37 kDa glucocorticoid-regulated protein, is a potent anti-inflammatory mediator effective in terminating acute inflammatory response, and its role in allergic settings has been poorly studied. The aim of this investigation was to evaluate the mechanism of action of ANXA1 in intraocular inflammation using a classical model of ovalbumin (OVA)-induced allergic conjunctivitis (AC). OVA-immunised Balb/c mice, wild-type (WT) and ANXA1-deficient (*Anxa1*<sup>−/−</sup>), were challenged with eye drops containing OVA on days 14–16 with a subset of WT animals pretreated intraperitoneally with the peptide Ac<sub>2-26</sub> (N-terminal region of ANXA1) or dexamethasone (DEX). After 24 h of the last ocular challenge, WT mice treated with Ac<sub>2-26</sub> and DEX had significantly reduced clinical signs of conjunctivitis (chemosis, conjunctival hyperaemia, lid oedema and tearing), plasma IgE levels, leukocyte (eosinophil and neutrophil) influx and mast cell degranulation in the conjunctiva compared to WT controls. These anti-inflammatory effects of DEX were associated with high endogenous levels of ANXA1 in the ocular tissues as detected by immunohistochemistry. Additionally, Ac<sub>2-26</sub> administration was effective to reduce IL-2, IL-4, IL-10, IL-13, eotaxin and RANTES in the eye and lymph nodes compared to untreated WT animals. The lack of ANXA1 produced an exacerbated allergic response as detected by the density of the inflammatory cell influx to the conjunctiva and the cytokine/chemokine release. These different effects observed for Ac<sub>2-26</sub> were correlated with diminished level of activated ERK at 24 h in the ocular tissues compared to untreated OVA group. Our findings demonstrate the protective effect of ANXA1 during the inflammatory allergic response suggesting this protein as a potential target for new ocular inflammation therapies.

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## 1. Introduction

Allergic conjunctivitis (AC) is defined as an inflammatory response initiated by type I (IgE-dependent and mast cell mediated) and/or type IV (mediated by T cells) hypersensitivity reactions following exposure to an allergen (Friedlaender, 2011; Marback et al., 2007). Binding of allergen to IgE cross-links its receptors on the surface of mast cells resulting in their degranulation and the release of inflammatory mediators, which may lead vasodilatation,

increased vascular permeability and the infiltration of inflammatory cells into the conjunctiva. This process triggers the acute phase of the disease characterised by clinical manifestations and bilateral hyperaemia, chemosis, lid oedema and pruritus (Stern et al., 2005a,b; Bilkhu et al., 2012).

The pharmacological treatment of intraocular inflammatory conditions, especially allergic conjunctivitis, includes antihistamines, mast cell membrane stabilisers and corticosteroids which are used in the more severe forms of AC and have a greater risk of adverse effects such as increased intraocular pressure and cataractogenesis (McGhee et al., 2002; Friedlaender, 2011). In this respect, the discovery of new pharmacological agents that have high efficacy in controlling the inflammatory response with fewer side effects is critical.

As a potential anti-inflammatory agent we highlight the annexin

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A1 (ANXA1), a 37 kDa protein that downregulates the inflammatory response in experimental models of acute (Gastardelo et al., 2009; Girol et al., 2013), chronic (Oliani et al., 2008; Dalli et al., 2010) and systemic (Damazo et al., 2005) inflammation and is expressed in different immune cells, particularly mast cells and neutrophils (Oliani et al., 2000, 2001; Gil et al., 2006). However, the relationship between ANXA1 and ocular inflammation has not been well studied.

Studies performed in our laboratory using experimental models of endotoxin-induced uveitis (da Silva et al., 2011; Girol et al., 2013) and ocular toxoplasmosis (Mimura et al., 2012), both with predominant Th1 cytokine profile, revealed a modulation of endogenous expression of ANXA1 in inflammatory cells and retinal pigment epithelium and indicated that this protein, as well as its mimetic peptide Ac<sub>2-26</sub>, is a key mediator in the homeostasis of inflammatory and infectious ocular processes.

In asthma, a Th2 cytokine profile disease, decreased levels of ANXA1 protein, but not mRNA, has been described in the lungs, which is likely due to protein cleavage to the 33 kDa isoform (Chung et al., 2004). In the bronchoalveolar fluid, the anti-allergic activity of Ac<sub>2-26</sub> was associated with the inhibition of mast cell degranulation, histamine release and the influx of neutrophils and eosinophils into the lung, but not with altered levels of serum IgE and eosinophil chemotaxis in vitro (Bandeira-Melo et al., 2005; Wang et al., 2011).

Given these considerations and the common side effects of the current therapies used to treat conjunctivitis and other eye inflammatory processes (Read et al., 2006; Rosenbaum, 2010), we evaluated the mechanism of action of endogenous and exogenous ANXA1 protein in ovalbumin-induced conjunctivitis in WT and AnxA1<sup>-/-</sup> mice. These studies shed light on the genesis of ocular inflammatory disorders and may lead to new therapies for the treatment of ocular allergy.

## 2. Materials and methods

### 2.1. Animals

Male wild-type (WT) and AnxA1-knockout (AnxA1<sup>-/-</sup>) BALB/c mice (Hannon et al., 2003), weighing 20–25 g, were randomly distributed into 6 groups (n = 5/group). The animals were housed in a 12 h light–dark cycle and were allowed food and water ad libitum. All experimental procedures were submitted and approved by the Ethics Committee in Animal Experimentation of the Federal University of São Paulo – UNIFESP (No. 0076/12), and by the Ethics Committee on Animal Use of the School of Pharmaceutical Sciences, USP (No. CEUA/FCF/285) and the Internal Biosafety Commission (CIBio).

### 2.2. Experimental model of allergic conjunctivitis and treatment protocols

WT and AnxA1<sup>-/-</sup> mice (n = 5/group experimental) were immunised 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 (ALUM) diluted in 200 µL of sterile saline according to models from literature (Shoji et al., 2005; Chung et al., 2009) 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), animals 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 ANXA1, WT sensitised mice were pretreated i.p. on days 14, 15 and 16 with its mimetic peptide Ac<sub>2-26</sub> (1.0 mg/kg; Ac-

AMVSEFLKQAWFIENEEQEYVQTVK - Invitrogen, São Paulo, Brazil) (Girol et al., 2013) or dexamethasone (1.0 mg/kg, Sigma–Aldrich) (Damazo et al., 2006), diluted in 0.1 ml of sterile saline, 15 min before the instillation of OVA.

Twenty-four hours after the last OVA challenge, mice were anaesthetised to obtain blood through cardiac puncture using a syringe with 10% EDTA for analysis of IgE levels and leukocyte quantification. The mice were then sacrificed and the eyes and cervical lymph nodes were collected.

### 2.3. Clinical score

After 20 min of OVA challenge, mice were examined on days 14, 15 and 16 in order to clinically verify the occurrence and severity of conjunctivitis. Four clinical signs were observed in a blind fashion: chemosis, conjunctival hyperaemia, lid oedema and tearing. Scoring similar to that described by Magone et al. (1998) 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 ranging from 0 to 12+, and the data were expressed as the mean ± standard error of the mean (SEM) for each group.

### 2.4. 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 and eosinophils. Data were reported as the mean ± SEM of the number of cells × 10<sup>5</sup>/mL.

### 2.5. IgE levels

Whole blood was centrifuged at 1500 rpm for 10 min to collect the plasma and determine the total IgE levels by ELISA at different experimental conditions. The concentration of IgE was measured using a commercially available mouse IgE immunoassay kit (Bio-Legend, San Diego, USA) with levels estimated according to the manufacturer's instructions. All estimations were made in duplicate and the data expressed as the mean ± SEM.

### 2.6. Histopathological analysis and quantification of inflammatory cells in ocular tissues

The right eyes were fixed in 10% buffered formalin for 24 h, 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 GmbH, Wetzlar, Germany) and subsequently stained with haematoxylin–eosin or toluidine blue 0.5% (Taab Laboratories, UK) for histopathological analysis.

Inflammatory cells eosinophils, neutrophils and mast cells were quantified using a high power objective (63×) on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany). Three semi-serial sections of conjunctiva were analysed 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 mm<sup>2</sup>.

### 2.7. Immunohistochemistry analysis

The detection of endogenous ANXA1 in WT ocular tissues was conducted in 4 µm 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-AnxA1 (1:2000) (Zymed Laboratories, Cambridge, U.K.), diluted in 1% BSA. After washing, sections were incubated with a secondary biotinylated antibody (LAB-SA Detection kit, Invitrogen, Paisley, U.K.). Positive staining was detected using a peroxidase-conjugated streptavidin complex and colour was developed using DAB substrate (Invitrogen Paisley, U.K.). The sections were counterstained with haematoxylin.

Densitometric analyses for the ANXA1 immunostaining were performed in two slides from each eye ( $n = 5$  animals/group), and 30 points were analysed in six fields of cornea and bulbar conjunctiva for an average related to the intensity of immunoreactivity (Girol et al., 2013). The values were obtained as arbitrary units (a.u.) between 0 and 255 using AxioVision software on an Axioskop 2-Mot Plus Zeiss microscope (Carls Zeiss). The data were expressed as the mean  $\pm$  SEM of arbitrary units.

## 2.8. Analysis of cytokines and chemokines

Left eyes and 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). The samples were centrifuged at 12,000 rpm for 20 min at 4 °C to obtain organ supernatants. For multiplex analysis, 25  $\mu$ l of the eye and lymph node supernatants were employed using the MILLIPLEX MAP mouse cytokine/chemokine panel (MCYTMAg-70K-PX32; Millipore Corporation, USA) and MAGPIX® Multiplexing Instrument (Millipore) according to the manufacturer's instructions. Seven analytes were studied: IL-2, IL-4, IL-10, IL-13, interferon gamma (IFN- $\gamma$ ), eotaxin, and Regulated upon Activation Normal T cell Expressed and presumably Secreted (RANTES). The concentration of analytes was determined by MAGPIX Xponent software (Millipore Corporation, Billerica, MA, USA), and the results are reported as the mean  $\pm$  SEM.

## 2.9. 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, Mannheim, Germany). Protein levels were determined by Bradford assay and equalised prior to boiling in Laemmli buffer (Bio-Rad Laboratories, USA). To detect ANXA1 and mitogen-activated protein kinases (MAPKs), protein extracts (40  $\mu$ g per lane) were loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis together with appropriate molecular weight markers (Bio-Rad Life Science, USA) and transferred to ECL Hybond nitrocellulose membrane. Reversible protein staining of the membranes with 0.1% Ponceau-S in 5% acetic acid (Santa Cruz Biotechnology) was used to verify even protein transfer. Membranes were incubated 15 min in 5% BSA in Tris-buffered saline (TBS) before the addition of rabbit polyclonal antibodies anti-ANXA1, anti-ERK2, anti-phosphorylated (p)-ERK 1/2, anti-JNK1, anti-p-JNK, anti-p38, anti-p-p38, and anti- $\alpha$ -Tubulin, all diluted 1:200 in TBS with 0.1% Tween 20 (antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, and BioLegend, San Diego, CA, USA). This was followed by 15 min of washing with TBS and incubation for 60 min 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 min 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 1.440 (HIH, Bethesda, MD) to determine relative

expression (arbitrary units, a.u.) of phosphorylated/total MAPKs.

## 2.10. Statistical analysis

The data were analysed using one-way ANOVA followed by Bonferroni t-tests using GraphPad Prism 5.0 software. Nonparametric analysis of the scores assigned to the conjunctival symptoms was done using the Mann–Whitney test. In all cases, a probability value  $<0.05$  was considered significant.

## 3. Results

### 3.1. *Ac*<sub>2-26</sub> treatment reduces the clinical signs of conjunctivitis and total IgE production

Four clinical signs of conjunctivitis (chemosis, conjunctival hyperaemia, lid oedema and tearing) were evaluated in WT and AnxA1<sup>−/−</sup> mice after 20 min of OVA challenge. WT and AnxA1<sup>−/−</sup> animals from the allergic conjunctivitis group (OVA) presented moderate to severe clinical signs compared to the respective controls (SHAM) (Fig. 1A, B, E and F). Alternately, *Ac*<sub>2-26</sub> and DEX pharmacological treatments reduced these clinical signs (Fig. 1C and D), indicating their anti-inflammatory effects in the eyes. Score data confirmed these observations and demonstrated that these treatments were effective against the clinical signs of conjunctivitis with a significant decrease on the 16th day (Fig. 1G).

The effectiveness of our experimental model was confirmed by the significant increase in IgE levels in WT animals (OVA), exacerbated in the AnxA1<sup>−/−</sup> mice (OVA) compared to their correspondent SHAM groups. As expected, the pharmacological treatments (*Ac*<sub>2-26</sub> and DEX) of WT mice significantly decreased IgE (Fig. 1H).

### 3.2. The *in vivo* administration of *Ac*<sub>2-26</sub> inhibits the allergic inflammatory response in the conjunctiva, which is exacerbated in AnxA1<sup>−/−</sup> animals

Initially, the allergic inflammatory response was investigated by the quantification of blood leukocytes in the different experimental groups (Table 1). The significant increase in eosinophil numbers observed in WT animals from OVA group was exacerbated in AnxA1<sup>−/−</sup> mice. The administration of *Ac*<sub>2-26</sub> significantly reduced eosinophils, whereas DEX had no demonstrable effect in relation to the OVA WT animals. Additionally, SHAM AnxA1<sup>−/−</sup> animals presented the significant increase of neutrophils compared to SHAM WT mice (Table 1).

In the bulbar conjunctiva, 24 h after the last OVA challenge, both genotypes of mice showed intense inflammatory responses characterised by the influx of neutrophils and eosinophils into the lamina propria compared to their correspondent controls (Fig. 2A, B, E, F, M and N). The pharmacological treatments (DEX and *Ac*<sub>2-26</sub>) were effective in reducing the migration of these inflammatory cells (Fig. 2C, D, M and N).

We also analysed mast cells (Fig. 2G–L), whose biological mediators are essential for triggering the clinical signs of conjunctivitis. No significant differences in the number of mast cells were noted in the WT animals (SHAM, OVA and DEX) whereas elevated number of these cells was detected in *Ac*<sub>2-26</sub> treated animals. Similarly, SHAM AnxA1<sup>−/−</sup> mice demonstrated increased number of mast cells in the lamina propria of the conjunctiva compared to SHAM WT (Fig. 2O). The histopathological analysis revealed the presence of intact metachromatic cells in SHAM WT (85%) and AnxA1<sup>−/−</sup> (75%) mice (Fig. 2G and K). OVA WT animals had increased degranulated mast cells (40%) (Fig. 2H). In the pharmacological treated-groups intact mast cells were noted (Fig. 2I and J),

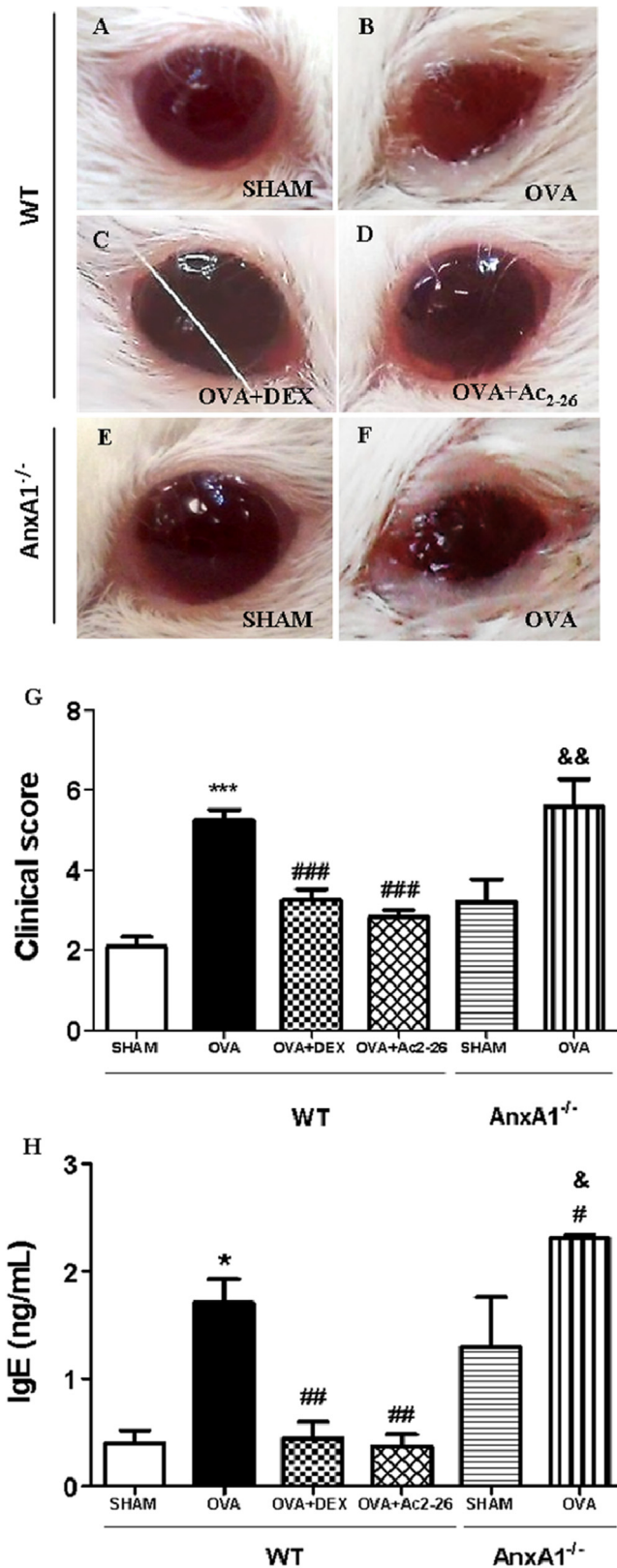


**Fig. 1.** Clinical score of allergic conjunctivitis and IgE levels. WT and AnxA1<sup>-/-</sup> mice were challenged with OVA (AC) or sterile saline (SHAM) and clinical appearances evaluated after 20 min in the 16th day. SHAM mice (A, E), OVA group (B, F) exhibited chemosis, conjunctival hyperaemia, lid oedema and tearing, which were reduced after DEX (C) and Ac<sub>2-26</sub> (D) treatments. Clinical score (G) and total IgE levels in the plasma (H). Data represent the mean ± SEM (n = 5 animals/group).

and a reduced proportion of degranulated cells was observed in the peptide Ac<sub>2-26</sub> treated group (27%) compared to the OVA WT (40%), an effect not seen after DEX treatment (40%).

### 3.3. ANXA1 expression is upregulated by DEX treatment in the ocular tissues during ocular allergy

Immunohistochemistry demonstrated that ocular tissues of WT mice were positive for endogenous ANXA1 in all experimental conditions. Confirming our previous studies in the murine eyes (Girol et al., 2013; Mimura et al., 2012) ANXA1 immunoreactivity was detected in the anterior and posterior eye segments, with particular epithelial localization in the conjunctiva, cornea, ciliary body and iris, as well as in the retina and migrated inflammatory cells, especially neutrophils (data not shown).

Given that the bulbar conjunctiva is the target organ of our experimental model and that it has an intimate contact with the cornea (Chen et al., 2014; Fukuda and Nishida, 2010), we then evaluated the modulation of endogenous ANXA1 protein in these compartments. Similar patterns of ANXA1 immunostaining were detected in the conjunctiva and cornea between control and OVA group (Fig. 3A and D). In the DEX-treated group, both compartments (conjunctiva and cornea) presented strong immunostaining of ANXA1 (Fig. 3E and F) compared to the OVA group. In contrast, treatment with the mimetic peptide Ac<sub>2-26</sub> resulted in lower ANXA1 immunoreactivity in these compartments (Fig. 3G and H). No immunostaining was detected in the negative control sections (Fig. 3I and J). The histological findings were supported by densitometric analyses of ANXA1 expression in the epithelial cells in the conjunctiva and cornea (Fig. 3K and L). The data obtained through immunoblots confirmed the downregulation of ANXA1 protein on mouse ocular tissues after Ac<sub>2-26</sub> treatment (Fig. 3M).

### 3.4. Ac<sub>2-26</sub> downregulates cytokines and chemokines during allergic conjunctivitis response

The allergic response in mice was characterised by changes in two cytokine profiles (Th1 and Th2) and chemokines in the different experimental conditions. After 24 h of OVA challenge, WT animals significantly increased IL-2 in the eyes compared with SHAM WT (Fig. 4A), an effect that was reversed by DEX and Ac<sub>2-26</sub> treatments. The peptide also presented an antagonistic action on the IFN-γ (Th1) production, reducing its levels in the eye (Fig. 4B) and increasing it in the lymph nodes (Fig. 4G). OVA AnxA1<sup>-/-</sup> mice demonstrated high levels of IL-2 and IFN-γ in lymph nodes (Fig. 4F and G) compared to SHAM AnxA1<sup>-/-</sup> and OVA WT animals. Reduced levels of IL-2 were observed in the eyes (Fig. 4A) of OVA AnxA1<sup>-/-</sup> animals compared to WT under the same experimental condition.

In the Th2 profile, a significant increase of IL-4 was noted in lymph nodes (Fig. 4H) compared with SHAM WT, an effect that was reversed by Ac<sub>2-26</sub> treatment. This treatment also decreased IL-10 in eyes (Fig. 4D) and IL-13 in lymph nodes (Fig. 4J). The lack of endogenous ANXA1 produced an exacerbated humoral response in OVA, increasing the levels of IL-4 (eyes; Fig. 4C), IL-10 (eyes and lymph nodes; Fig. 4D and I), IL-13 (lymph nodes; Fig. 4J) and reduced IL-4 (lymph nodes; Fig. 4H) levels compared to the OVA WT animals. Additionally, SHAM AnxA1<sup>-/-</sup> animals presented changes in relation to the SHAM WT group, especially in the eyes with increased levels of IL-10 and IL-13 (Fig. 4D and E, respectively).

\*p < 0.05 and \*\*\*p < 0.001 vs. WT (SHAM), #p < 0.05, ##p < 0.01 and ###p < 0.01 vs. WT (OVA), &p < 0.05 and &&p < 0.01 vs. AnxA1<sup>-/-</sup> (SHAM).

**Table 1**  
Quantification of blood eosinophils and neutrophils in WT and AnxA1<sup>-/-</sup> animals.

| Groups                    | Cells × 10 <sup>5</sup> /mL                  |               |
|---------------------------|----------------------------------------------|---------------|
|                           | Eosinophils                                  | Neutrophils   |
| SHAM                      | 0.0 ± 0.0                                    | 8.16 ± 2.00   |
| OVA                       | 1.01 ± 0.23**                                | 8.39 ± 1.28   |
| OVA + DEX                 | 0.91 ± 0.13                                  | 5.74 ± 1.93   |
| OVA + Ac <sub>2-26</sub>  | 0.10 ± 0.10 <sup>#</sup>                     | 5.85 ± 0.94   |
| SHAM AnxA1 <sup>-/-</sup> | 0.25 ± 0.10                                  | 19.0 ± 1.90** |
| OVA AnxA1 <sup>-/-</sup>  | 3.75 ± 0.430 <sup>###, &amp;&amp;&amp;</sup> | 16.6 ± 1.79   |

The data represent the mean ± SEM of the number of leukocytes × 10<sup>5</sup>/mL in WT and AnxA1<sup>-/-</sup> mice at different experimental conditions (n = 5 mice/group); SHAM control; OVA (allergic conjunctivitis), treated or not with DEX or Ac<sub>2-26</sub> mimetic peptide; \*\*p < 0.01 vs. WT (SHAM); <sup>#</sup>p < 0.05 and <sup>###</sup>p < 0.001 vs. WT (OVA); &&&p < 0.001 vs. AnxA1<sup>-/-</sup> (SHAM).

demonstrated reduced eotaxin and RANTES levels (Fig. 5A and B) compared to respective WT experimental condition.

### 3.5. Allergic conjunctivitis induce ERK (extracellular signal-regulated kinase) activation that is regulated by Ac<sub>2-26</sub> treatment

To understand the downstream molecular signalling pathways involved in the effect of ANXA1 treatment in AC, we performed western blot analysis to assess the phosphorylation of ERK (extracellular signal-regulated kinase), JNK (c-jun N-terminal kinase) and p38 MAPKs by using pooled extracts of eyes at different experimental conditions (n = 5 animals per group). In the 24 h, OVA-induced elevated p-ERK expression that was reduced by DEX and Ac<sub>2-26</sub> treatments (Fig. 6). The genotypic differences demonstrated

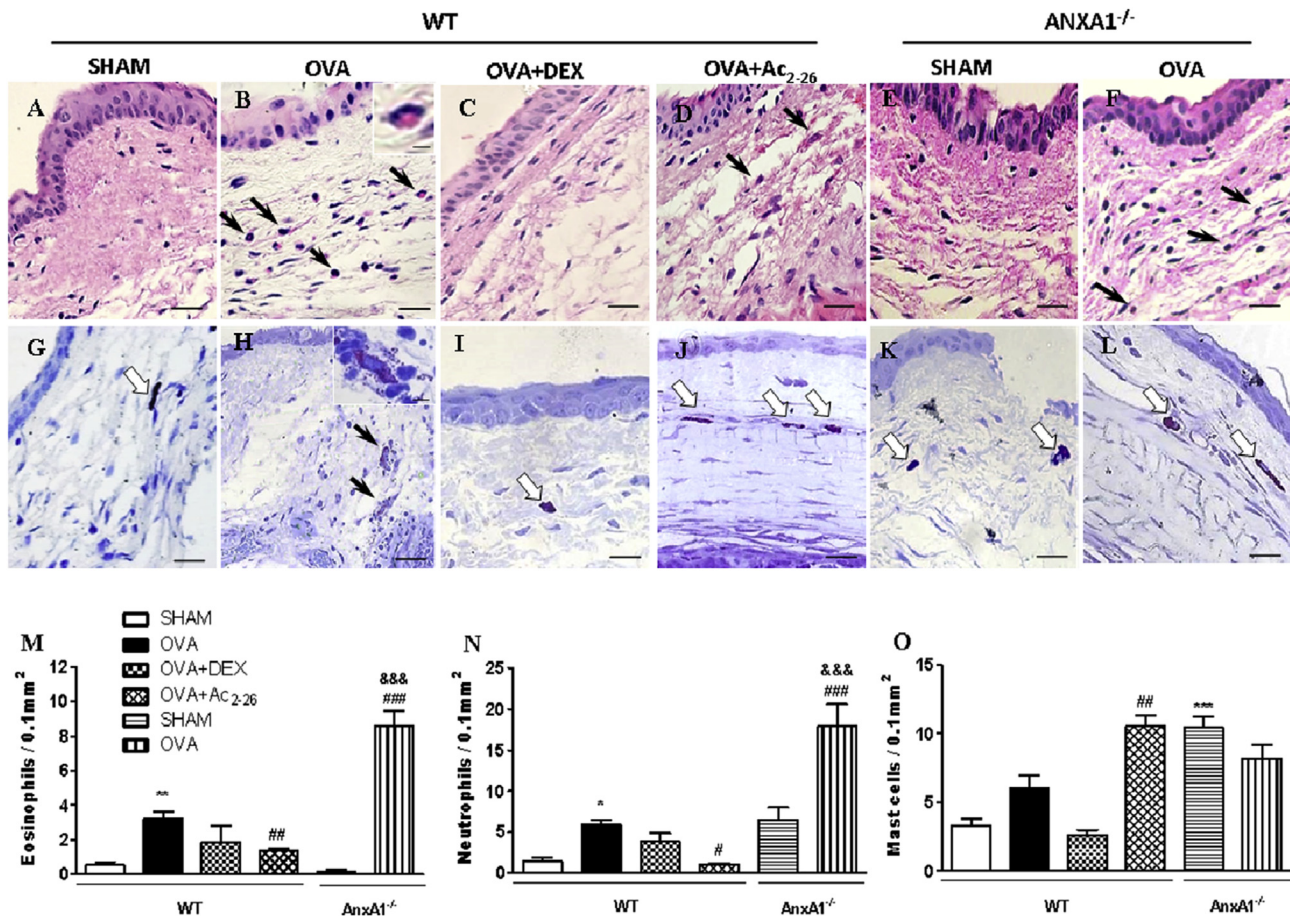


**Fig. 2.** Histopathological analysis in bulbar conjunctiva of the WT and AnxA1<sup>-/-</sup> mice. After 24 h of last OVA challenge, influx of eosinophils and neutrophils (arrows) in WT (B, inset) and AnxA1<sup>-/-</sup> (F) OVA group, absence in control (A, E) and decreased in DEX (C) and Ac<sub>2-26</sub> (D) treatments. A high proportion of degranulated mast cells (black arrows) were detected in the OVA WT mice (H, inset), whereas intact metachromatic cells (white arrows) were observed in the SHAM (G, K), DEX (I) and Ac<sub>2-26</sub> (J), and OVA AnxA1<sup>-/-</sup> mice (L). Stain: haematoxylin–eosin (A–F) and toluidine blue (G–L). Scale Bars: 10 µm; B and H insets: 5 µm. Quantitative analysis of eosinophils (M), neutrophils (N) and mast cells (O). Data represent the mean ± SEM of the number of cells per 0.1 mm<sup>2</sup> (n = 5 animals/group). \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 vs. WT (SHAM); <sup>#</sup>p < 0.05; <sup>##</sup>p < 0.01 and <sup>###</sup>p < 0.001 vs. WT (OVA); &&&p < 0.001 vs. AnxA1<sup>-/-</sup> (SHAM).

Finally, we evaluated chemokines and found significant increases in eotaxin (eyes and lymph nodes; Fig. 5A and C) and RANTES (lymph nodes; Fig. 5D) of OVA WT mice (Fig. 5A and C) compared to the control. Pharmacological treatments were effective in the reduction of these chemokines in the two organs analysed (Fig. 5). OVA AnxA1<sup>-/-</sup> mice exhibited elevated levels of eotaxin in the lymph nodes (Fig. 5D) in relation to SHAM AnxA1<sup>-/-</sup>. Changes in chemokines related to genotypic differences among animals were detected in the eyes. SHAM and OVA AnxA1<sup>-/-</sup> mice

increased levels of ERK in SHAM and OVA ANXA1<sup>-/-</sup> mice compared to the respective WT groups (Fig. 6). The levels of JNK and p38 showed no significant changes between untreated and treated-OVA groups (data not shown).

## 4. Discussion

Despite the availability of antihistamines, mast cell membrane stabilisers and corticosteroids to target the clinical symptoms of AC,

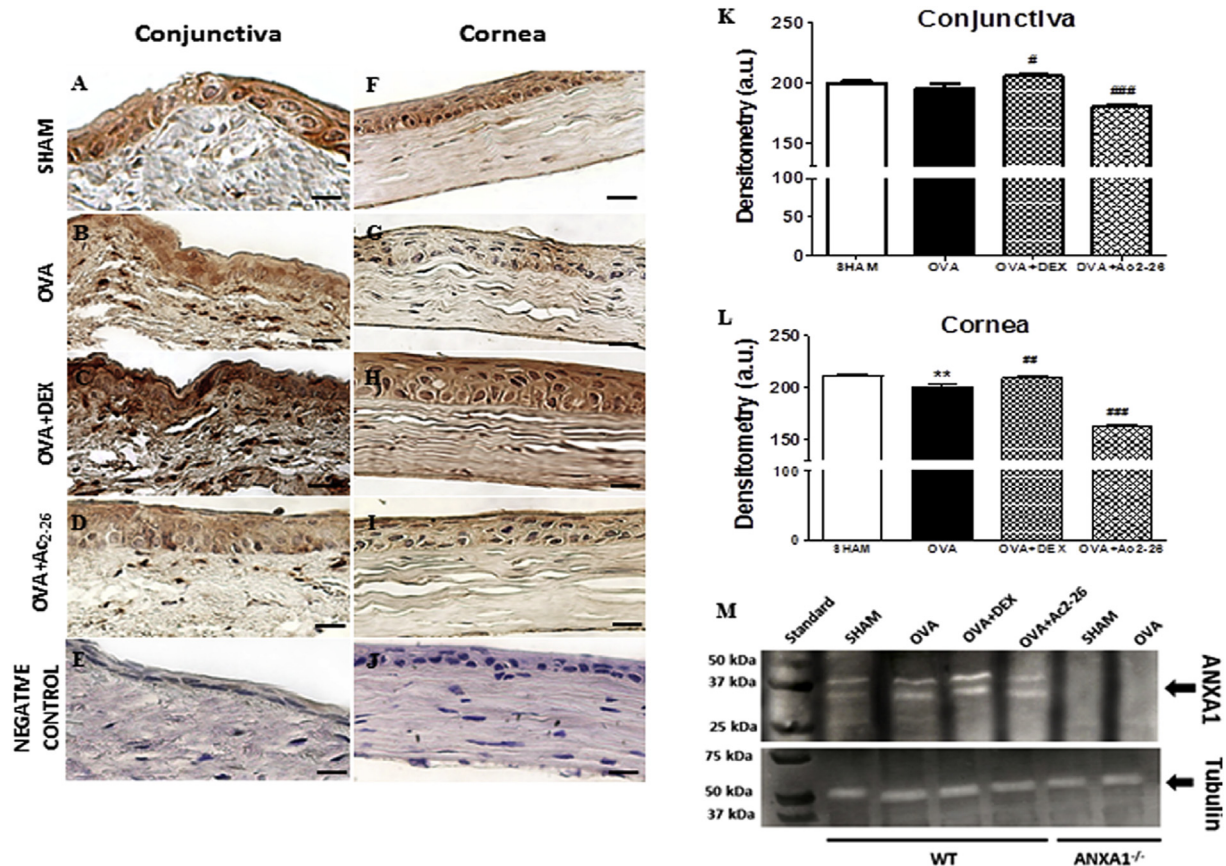


**Fig. 3. Expression of ANXA1 in conjunctiva and cornea of WT mice.** Ac<sub>2-26</sub> treatment induced low ANXA1 immunoreactivity in the conjunctiva (D) and cornea (I) compared with SHAM (A, F), OVA (B, G) and DEX-treated (C, H) groups. Negative control sections (E, J). Counterstain: haematoxylin. Scales Bars: 10  $\mu$ m. Densitometric analysis of epithelial cells immunostained for ANXA1 in the conjunctiva (K) and cornea (L). Values (arbitrary units, a.u.) represent the mean  $\pm$  SEM (n = 5 animals/group). \*\**p* < 0.01 vs. WT (SHAM); #*p* < 0.05, ##*p* < 0.01 and ###*p* < 0.001 vs. WT (AC). (M) Representative immunoblots show the levels of intact (37 kDa) and N-terminal-cleaved (33 kDa) forms of ANXA1 in the pooled extracts of WT eyes (n = 5 animals per group). No immunoreactive bands were detected in the AnxA1<sup>-/-</sup> ocular tissues. Equal loading was confirmed with anti- $\alpha$ -Tubulin (55 kDa).

the greater risk of adverse effects provided by corticosteroids (McGhee et al., 2002; Friedlaender, 2011) prompted us to investigate the action of Ac<sub>2-26</sub> in intraocular inflammation using a classical model of OVA-induced AC in WT and AnxA1<sup>-/-</sup> mice (Shoji et al., 2005; Chung et al., 2009).

The effectiveness of AC model was confirmed by the detection of clinical signs of conjunctivitis (intense hyperaemia, lid oedema and tearing) in the eyes (WT and AnxA1<sup>-/-</sup> mice) and significant increase in the plasma IgE levels compared to their respective controls, and in agreement with the literature (Shoji et al., 2005; Chung et al., 2009; Ng et al., 2011). Both pharmacological treatments (DEX and Ac<sub>2-26</sub>) significantly decreased the clinical signs of conjunctivitis, and the total IgE 24 h from the last OVA challenge. Similar results were obtained in an OVA-induced asthma model, in which IgE serum levels were reduced in mice after treatment with peptide Ac<sub>2-26</sub> (Wang et al., 2011).

The allergic inflammatory response in the conjunctiva of OVA mice was associated with a significant increase in eosinophils and neutrophils, and was exacerbated in the AnxA1<sup>-/-</sup> animals, compared to controls (WT and AnxA1<sup>-/-</sup>). This effect was abrogated by Ac<sub>2-26</sub> and DEX treatments, corroborated by eotaxin and RANTES decreased levels, and in agreement with previous results using AC (Sohn et al., 2003; Shoji et al., 2005) and asthma (Bandeira-Melo et al., 2005; Wang et al., 2011) models in rodents. The exacerbation of allergic responses in AnxA1<sup>-/-</sup> mice was also demonstrated in OVA-induced-asthma model as evidenced by airway hyperresponsiveness, higher influx of leukocytes in

bronchoalveolar lavage and lung compared to WT (Ng et al., 2011). Additionally, this anti-migratory effect of DEX-treated OVA group was associated with high endogenous levels of ANXA1 in the ocular tissues, as detected by immunohistochemistry and western blotting analysis. Altogether, these results demonstrate a critical regulatory role for endogenous ANXA1 in the development of allergic inflammatory response.

Furthermore, the administration of Ac<sub>2-26</sub> significantly increased the number of mast cells and decreased the proportion of degranulated cells (27%) compared to OVA WT animals (40%), suggesting a negative effect of peptide in mast cell activation. Similarly, the pretreatment of rats with the peptide Ac<sub>2-26</sub> in an OVA-induced pleuritis model significantly increased intact mast cell numbers in the pleural fluid, and, in vitro, decreased the release of histamine (Bandeira-Melo et al., 2005). Corroborating these findings, in vitro studies revealed that pre-treatment with human recombinant ANXA1 inhibited histamine/prostaglandin release of IgE/anti-IgE-challenged human mast cells, as well as detected by cromoglycate, nedocromil and DEX (Yazid et al., 2013). Additionally, nedocromil was inactive in inhibiting the IgE cross-linking or 48/80-induced degranulation of murine bone marrow derived-mast cells from ANXA1<sup>-/-</sup> mice confirming an important role of this protein in mast cell mediator release. Thus, in our in vivo model, the high incidence of mast cell in the peptide-treated group was due by its capacity to prevent cell degranulation and then, facilitate the mast cell identification in the sections.

AC is classically exemplified as a Th2 disease due to the

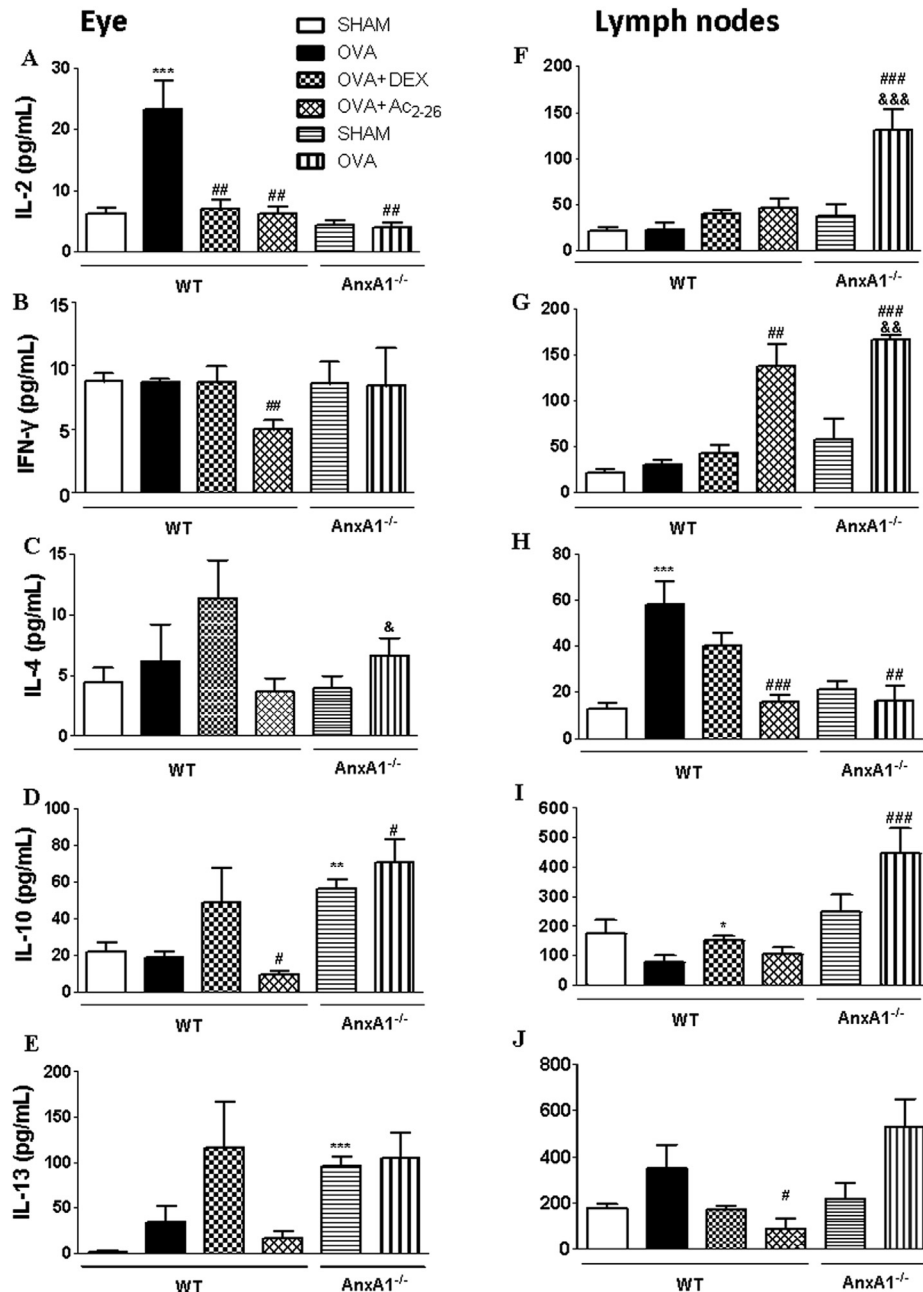


**Fig. 4.** Effect of ANXA1 in the cytokine levels in eyes and lymph nodes of WT and AnxA1<sup>-/-</sup> mice. IL-2 (A, F), IFN-γ (B, G), IL-4 (C, H), IL-10 (D, I) and IL-13 (E, J). Data represent the mean ± SEM of cytokine dosage (pg/ml) (n = 5 animals/group). \*p < 0.05 and \*\*p < 0.01 and \*\*\*p < 0.001 vs. WT (SHAM), #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. WT (OVA); &p < 0.05, &&p < 0.01 and &&&p < 0.001 vs. AnxA1<sup>-/-</sup> (SHAM).

abundant systemic levels of IgE and IL-4, IL-5 and IL-13 (Galli and Tsai, 2012). Alternately, counterbalance in the production of Th1 cytokines (IFN-γ, IL-2 and IL-12) is crucial to the regulation of allergic responses (Theofilopoulos et al., 2001; Gor et al., 2003). The tears of patients with seasonal conjunctivitis, vernal and atopic keratoconjunctivitis presented high levels of Th1/Th2 cytokines such as IL-1 β, IL-2, IL-5, IL-6, IL-12, IL-13 and IFN-γ (Leonardi et al., 2006). Recent investigations support those findings revealing increased levels of IL-2 and IFN-γ in the tears of patients with delayed keratoconjunctival response provoked by nasal tests using different allergens (Pelikan, 2013, 2014). Considering this aspect, the next step of our study was to analyse the levels of Th1 and Th2 cytokines in the eye and lymph nodes of WT and AnxA1<sup>-/-</sup> mice at different experimental conditions to determine the pathways of

ANXA1 actions.

OVA challenge significantly increased the levels of IL-2 in the eyes and IL-4 in the lymph nodes in the OVA WT group compared to SHAM. The lack of endogenous ANXA1 in the OVA group exacerbated the humoral response by increasing Th2 cytokine levels in the eyes (IL-4 and IL-10) and lymph nodes (IL-10 and IL-13). We also observed increased levels of IL-2 and IFN-γ in the lymph nodes, compared to AnxA1<sup>-/-</sup> SHAM. High amounts of Th2 cytokine (IL-4, IL-5, IL-6, IL-10 and IL-13) mRNA and protein in lymph nodes have also been described in mice with AC induced by ragweed pollen (Zheng et al., 2010; Li et al., 2011), OVA (Chung et al., 2009) and mite (Giavina-Bianchi et al., 2008), emphasising the importance of these cytokines in the induction of allergic responses. In addition to the capacity of IL-10 to inhibit mast cell degranulation (Bundoc and

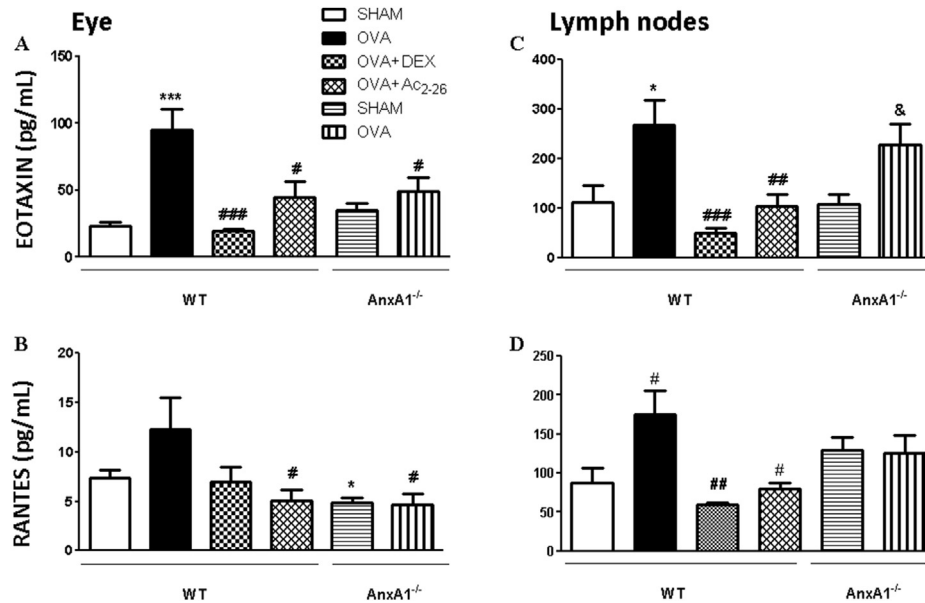


**Fig. 5.** Effect of ANXA1 in the chemokine levels in eyes and lymph nodes of WT and *AnxA1*<sup>-/-</sup> mice. Eotaxin (A, C) and RANTES (B, D). Data represent the mean  $\pm$  SEM of the chemokine dosage (pg/mL) ( $n = 5$  animals/group). \* $p < 0.05$  and \*\*\* $p < 0.001$  vs. WT (SHAM); # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$  vs. WT (OVA); & $p < 0.05$  vs. *AnxA1*<sup>-/-</sup> (SHAM).

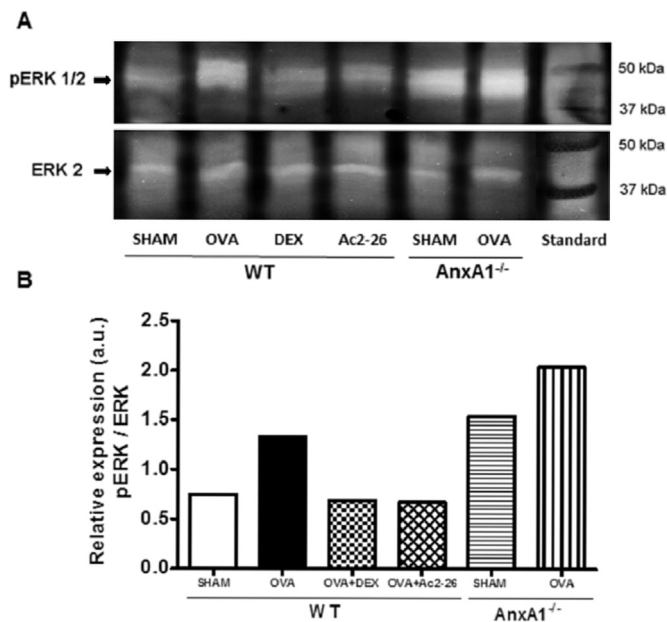


**Fig. 6.** Effect of ANXA1 in the activation of ERK in mice eyes. Representative immunoblots show the levels of ERK2 and p-ERK1/2 in the ocular tissues from SHAM (WT and *AnxA1*<sup>-/-</sup>), OVA (WT and *AnxA1*<sup>-/-</sup>) and WT treated or not with DEX or Ac2-26 (A). Immunoreactive bands for ERK were semiquantified by densitometry and expressed as arbitrary units (a.u.) of the ratio of p-ERK/total ERK (B) (data shown illustrates one representative of two independent experiments).

Keane-Myers, 2007) and suppress inflammatory responses by CD4<sup>+</sup> T cells (Skelsey et al., 2003), IL-10-deficient animals exhibited less severe inflammation with lower eosinophilia compared to WT mice in a pollen-induced AC model (Fukushima et al., 2007).

Our data provided evidence that pharmacological treatment with peptide Ac2-26 reduced significantly IL-4 and IL-13 levels in lymph nodes and IL-10 in the eyes. Regarding Th1 profile, ANXA1 exhibited an antagonistic effect for IFN- $\gamma$  decreasing its levels in eyes and increasing it in lymph nodes, whereas IL-2 was reduced in the eyes by both pharmacological treatments.

Similar results were obtained in studies using OVA-induced asthma in mice that presented reduced IL-4 and IL-5 after treatment with Ac2-26 and Tat-ANXA1, mimetic peptides of the ANXA1 protein (Wang et al., 2011; Lee et al., 2012). Furthermore, in vitro studies with cultured T lymphocytes of mice treated with human recombinant ANXA1 exhibited diminished levels of IL-10 (D'Acquisto et al., 2008). We reason that these data indicate the importance of the Th1/Th2 balance in the development of allergic inflammatory responses and suggest a protective role for ANXA1 in ocular allergy through the negative regulation of both cytokines profiles.

Therefore, we concluded this study by testing the potential of exogenous/endogenous ANXA1 and MAPK cascades in the AC model. MAPKs are well-conserved signalling pathways that include the JNK, ERK and p38 proteins, and have been implicated in allergic responses, particularly in asthma, contributing to leukocyte recruitment, mast cell activation, pro-inflammatory cytokine production, and Th2 differentiation (Pelaia et al., 2005; Arthur and Ley, 2013). The treatment of WT mice with Ac2-26 results in specific inhibition of the ERK pathway, while a lack of ANXA1 expression leads to prolonged AC activation of ERK. This inverse relationship between the amount of ANXA1 and the activity of ERK, following allergic inflammatory response, may be indicative of the anti-inflammatory effects of this protein. Our findings corroborates the studies with asthma rat model which treatment with Tat-ANXA1 effectively inhibited OVA-induced phosphorylation of MAPKs in dose-dependent fashion in lung biopsies (Lee et al., 2012).

Collectively, this study reveals the potential immunomodulatory activity of ANXA1 protein in ocular allergic inflammation. Treatment with Ac2-26 negatively regulates the development of clinical signs of conjunctivitis through the inhibition of the recruitment and the activation of immune cells (eosinophils, neutrophils and mast cells) in the tissues, and release of key cytokines and chemokines in the allergic response. ANXA1 deficiency exacerbates the inflammatory response in the AC model, giving support to an important regulatory role for the endogenous protein in this disease. Thus, ANXA1 represents a potential target for the development of therapeutic approaches in ocular inflammation.

## Conflicts of interest

The authors declare no conflict of interest.

## Disclosure

S. M. Oliani and C. D. Gil share senior authorship of this manuscript.

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