



Immunopharmacology and Inflammation

Involvement of the annexin A1-Fpr anti-inflammatory system in the ocular allergy



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ABSTRACT

Annexin A1 (ANXA1)-formyl peptide receptor (Fpr) system is potent effective mediators in the control of the inflammatory response. In this study, we evaluate the potential involvement of the Fpr family in the protective effect of the mimetic peptide of ANXA1 (ANXA1_{2–26}) using an experimental allergic conjunctivitis (AC) model in mice. Ovalbumin (OVA)/Alum-immunized wild-type (WT) and ANXA1-null (ANXA1^{−/−}) Balb/c mice (days 0 and 7) were challenged by eye drops containing OVA on days 14–16, and two groups received ANXA1_{2–26} alone or with Fpr antagonist Boc2 intraperitoneally during challenged days. As expected, plasma IgE anti-OVA levels increased significantly in the OVA-immunized WT and ANXA1^{−/−} mice, supporting the efficacy of AC model. AC increased Fpr1 and Fpr2 levels in the conjunctiva and the lack of endogenous ANXA1 exacerbated Fpr2 expression only. In contrast, administering ANXA1_{2–26} in the WT mice diminished Fpr2 levels in the conjunctiva, and the effect was reverted by Boc2. Ultrastructural analysis showed the co-localization of Fpr2 and ANXA1 in the plasma membrane of mast cells (MCs), eosinophils and neutrophils, supporting this system as being operative in the AC. Boc2 abrogated the ANXA1_{2–26} effect by increasing the MC degranulation and the eosinophil influx in the conjunctiva, and these findings were supported by peroxidase eosinophil, eotaxin and MC protease levels. Additionally, the ANXA1_{2–26}-Fpr system in the AC was associated with the activation of ERK and JNK. Collectively, the data provided in vivo supports the anti-allergic effects of the ANXA1-Fpr system and may serve as a therapeutic target in this ocular disorder.

1. Introduction

Annexin A1 (ANXA1) is a 37 kDa protein that belongs to a superfamily of proteins that bind to phospholipids in a calcium-dependent manner (Rescher and Gerke, 2004). Since the ANXA1 is an endogenous mediator of the anti-inflammatory actions of glucocorticoids (Flower, 1988) and their selective receptor agonist mapracorat (Baiula et al., 2011; Shafiee et al., 2011), its activity and protective role have been explored in several experimental models of acute, chronic and systemic inflammation (Damazo et al., 2005; Gavins et al., 2007; Girol et al., 2013; Stuqui et al., 2015). Administering the ANXA1 or its derived-mimetic peptide ANXA1_{2–26} lead to the activation of their G protein-coupled transmembrane receptors, the formyl peptide receptors (Fpr), which are more abundant in adherent leukocytes, and promotes cellular activation and intracellular signaling, with the detachment of leukocytes from endothelial cells (Gavins et al., 2003).

However, in allergic inflammation, the role of the ANXA1 protein and its relationship with Fpr have not been explored, but they have been demonstrated independently in the experimental models of ovalbumin (OVA)-induced asthma. One of these studies showed the anti-allergic activity of the ANXA1_{2–26} peptide through the inhibition of mast cell degranulation, histamine release in tissues, and neutrophil and eosinophil accumulation in bronchoalveolar fluid (BAL); however, no changes in the serum IgE levels and the eosinophil chemotaxis in vitro were detected (Bandeira-Melo et al., 2005; Wang et al., 2011). The administration of the permeable ANXA1 protein (Tat-ANXA1) significantly decreased serum IgE and the BAL Th2 cytokine levels (IL-4, IL-5 and IL-13), and inhibited the phosphorylation of ERK, p38 and SAPK/JNK showing the important mechanisms of ANXA1 action to attenuate the pathogenesis of the disease (Lee et al., 2012). In contrast, Fpr2 knockout mice showed a decrease in the recruitment of dendritic cells in the respiratory mucosa and in the secondary lymphoid organs,

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as well as in the production of Th2 and IgE cytokines, suggesting a critical role for this receptor in the progression of the allergic respiratory inflammation and immune response (Chen et al., 2010).

Our research group demonstrated in an OVA-induced AC in mice that the administration of ANXA1_{2–26} negatively regulates the allergic response through inhibiting clinical signs, and releasing Th2 cytokines (IL-4 and IL-13) and chemokines, indicating this protein as a potential target for new therapeutic alternatives in ocular inflammation (Gimenes et al., 2015).

Considering that ANXA1 allows via Fpr to control the inflammatory response and that this relationship has not yet been explored in allergies, this study evaluate the ANXA1-Fpr system by using an experimental AC model induced by OVA in mice.

2. Materials and methods

2.1. Animals

Male wild-type (WT) and ANXA1-knockout (Anx1^{-/-}) BALB/c mice (Hannon et al., 2003), weighing 20–25 g, 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 (Protocols n° 0076/12; 1975251115), by the Ethics Committee on Animal Use of the School of Pharmaceutical Sciences, USP (No. CEUA/FCF/285) and by the Internal Biosafety Commission (CIBio).

2.2. Experimental model of allergic conjunctivitis and treatment protocols

WT and Anx1^{-/-} mice (n = 5/group) were immunized on days 0 and 7 with a subcutaneous injection of 5 µg of ovalbumin (OVA, grade V) and 15 mg/ml of aluminium hydroxide adjuvant (ALUM) diluted in 200 µl of sterile saline as reported previously (Andrade et al., 2018; Gimenes et al., 2015; Mello et al., 2015). On days 14, 15 and 16 after an intraperitoneal (i.p.) injection of anesthesia with ketamine (100 mg/kg, Ceva Saúde Ltda., São Paulo, Brazil) and xylazine (20 mg/kg, Syntec, São Paulo, Brazil), animals received the direct instillation of 250 µg of OVA in 10 µl of sterile saline onto the conjunctival sac. Also, part of the WT OVA-sensitized mice were pretreated i.p. on days 14, 15 and 16 with 1.0 mg/kg of ANXA1 mimetic peptide (ANXA1_{2–26}; Ac-AMVSEFLKQAWFIENEEQEYVQTVK - Invitrogen, São Paulo, Brazil) or with ANXA1_{2–26} + Fpr antagonist (Boc2; N-t-butyloxycarbonyl-Phe-Phe-Dleu-Dleu-Phe; ICN Pharmaceuticals, Basingstoke, UK), 10 µg/animal (Smith et al., 2015) diluted in 0.1 ml of sterile saline, 15 min before the instillation of OVA. Control animals received sterile saline alone.

Twenty-four h after the last OVA challenge, mice were anaesthetized and blood was obtained via cardiac puncture using a syringe with 10% EDTA for the analysis of IgE levels and leukocyte quantification. The mice were then euthanized, and the eyes, eyelids and peritoneal wash were collected.

2.3. Analysis of IgE anti-ovalbumin levels

Blood samples from all experimental groups were centrifuged at 600 × g for 10 min to collect the plasma and determine the total IgE anti-OVA levels by ELISA. The concentration of IgE anti-ovalbumin was measured using commercially available mouse IgE anti-OVA immunoassay kits (Cayman Chemical Co., Ann Arbor, MI, USA, respectively), in accordance with the manufacturer's guidelines. All experiments were conducted in duplicate and the data were expressed as the mean ± S.E.M. of protein (ng/ml).

2.4. Immunohistochemistry

The detection of Fpr1 and Fpr2 expression was conducted in 4 µm sections of paraffin-embedded WT and Anx1^{-/-} palpebral conjunctivas.

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-Fpr1 or anti-Fpr2 (1:4000 and 1:200, respectively) (Abcam, 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 the colour was developed using a DAB substrate (Invitrogen Paisley, U.K.). Then, the sections were counterstained with hematoxylin.

Densitometric analyses for the Fpr1 and Fpr2 immunostaining were performed in two slides from each eye (n ≈ 5 animals/group), and 30 points were analysed in six fields of conjunctiva for an average related to the intensity of immunoreactivity (Gimenes et al., 2015; 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, Jena, Germany), and the data were expressed as the mean ± S.E.M. of arbitrary units.

2.5. Ultrastructural Immunocytochemical Analysis

To detect the co-localization of endogenous ANXA1 protein with the Fpr2 in inflammatory cells, peritoneal wash was collected and centrifuged at 600 × g for 15 min and cells were resuspended and fixed in a 4% paraformaldehyde, 0.5% glutaraldehyde and 0.1% sodium cacodylate buffer (pH 7.4) for 24 h at 4 °C. Peritoneal cells were washed in sodium cacodylate, dehydrated through a graded series of methanol washes and embedded in LR Gold (Sigma-Aldrich, St. Louis, MO, USA). Ultrathin sections (~90 nm) of peritoneal cells were placed on nickel grids and incubated sequentially with the following reagents at room temperature: i) 0.1 mol/l PBS containing 1% egg albumin (PBEA); ii) 5% PBEA for 30 min; iii) sheep polyclonal antibody LPCS1 raised against ANXA1 (Oliani et al., 2001) and rabbit polyclonal antibody Fpr2 (1:200 and 1:100, respectively, in PBEA 1%; Santa Cruz Biotechnology, CA, USA) for 2 h; and iv) three washes (5 min each) in 1% PBEA. To detect ANXA1, a donkey anti-sheep IgG antibody (1:50 in PBEA) conjugated to 10-nm colloidal gold (British Biocell, Cardiff, UK) was added. To detect Fpr2, a goat anti-rabbit IgG antibody (1:50 in PBEA) conjugated to 15-nm colloidal gold (British Biocell) was added. After 1 h, the sections were washed extensively with 1% PBEA and then with distilled water. These sections were stained with uranyl acetate and lead citrate and examined using a ZEISS EM900 electron microscope (Carls Zeiss).

2.6. Histopathological analysis and quantification of inflammatory cells in palpebral conjunctiva

Eye lids were fixed in 4% paraformaldehyde for 24 h, washed in tap water, dehydrated in an increasing ethanol series, and embedded in paraffin. Then, sections of 4 µm were obtained using a Leica RM2155 microtome (Leica Microsystems, Nussloch, Germany) and subsequently stained with haematoxylin-eosin or 0.5% toluidine blue for histopathology and inflammatory cell quantification. Eosinophils and mast cells were quantified using a 40x objective on an Axio Scope A1 Zeiss microscope (Carls Zeiss). Three semi-serial sections of palpebral conjunctiva were analysed per animal, and their areas were determined using AxioVision software (Carls Zeiss). Values are expressed as the mean ± S.E.M. of cells per mm².

2.7. Western blot analysis

Both eyes 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 Bradford assay and normalized prior to boiling in Laemmli buffer (Bio-Rad Laboratories, USA). Then, pooled protein extracts (30 µg per lane) of eyes (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. Also, 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 were as follows: goat polyclonal anti-mouse mast cell protease 6 (mMCP6; 1:5000) (R&D Systems, Minneapolis, EUA), rabbit anti-Fpr2 (1:200) and goat anti-eosinophil peroxidase (EPX; 1:200) (Santa Cruz Biotechnology), mouse anti-phosphorylated (p)-ERK1/2, anti-p-JNK and anti-p-p38 (1:2000) (Cell Signaling, Danvers, MA, EUA) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000) (Sigma-Aldrich), 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-mouse (1:5000; Millipore Corporation, Temecula, CA, USA), goat anti-rabbit IgG or rabbit anti-goat (1:2000) (Thermo Fisher Scientific Inc., MI, 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 (arbitrary units, a.u.).

2.8. Analysis of cytokines and chemokines

For multiplex analysis, 25 µl of the eye supernatants were employed using the MILLIPLEX MAP mouse cytokine/chemokine panel (MCYTMA670K-PX32; Millipore Corporation) and MAGPIX® Multiplexing Instrument (Millipore Corporation) according to the manufacturer's instructions. Then, four analytes were studied: Tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), 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), and the results are reported as the mean ± S.E.M.

2.9. Statistical analysis

The data were analysed using one-way ANOVA followed by Bonferroni *t*-tests between corresponding time points using GraphPad Prism 6.0 software. Non-parametric analysis of the scores assigned to the conjunctival symptoms was performed using the Mann-Whitney test. In all cases, a probability value < 0.05 was considered significant.

3. Results

3.1. Lack of endogenous ANXA1 exacerbates Fpr2 expression, but not Fpr1, in the AC model

After 24 h of the last OVA challenge, anti-OVA IgE levels were significantly increased in the plasma of WT (90.7 ± 7.1 ng/ml) and ANXA1^{-/-} (63.3 ± 21.9) animals from the AC group compared to the respective SHAM controls (WT, 1.0 ± 0.5 and ANXA1^{-/-}, 2.9 ± 1.5 ; $P < 0.05$ vs. AC).

Once the effectiveness of AC model was confirmed, the Fpr1 and Fpr2 expressions in the conjunctiva were addressed by immunohistochemistry. Both receptors were detected in conjunctival epithelial cells in all experimental conditions (Fig. 1A), and AC was

associated with intense expression of both Fprs in this tissue compared with control (Fig. 1A and B). Furthermore, the lack of ANXA1 protein in the OVA-sensitized mice produced a marked increase in the Fpr2 expression, but not Fpr1, compared to WT AC (Fig. 1A and B). Similarly, the eye homogenates from AC ANXA1^{-/-} group also exhibited elevated levels of Fpr2 compared to AC WT (Fig. 1C).

3.2. ANXA1₂₋₂₆ anti-inflammatory effect is blocked by Boc2 administration

To confirm the importance of the ANXA1-Fpr system in the ocular allergy, the AC model was performed with WT Balb/c mice, including AC groups treated with ANXA1 mimetic peptide (ANXA1₂₋₂₆) or with Boc2 + ANXA1₂₋₂₆. As expected, after 24 h of the last OVA challenge, increased plasma anti-IgE OVA levels were detected in the OVA-sensitized mice (AC: 67.8 ± 7.7 ng/ml; ANXA1₂₋₂₆: 76.6 ± 9.0 and Boc2 + ANXA1₂₋₂₆: 44.3 ± 4.1) compared with the Sham group (4.0 ± 0.6 , $P < 0.01$ vs. AC group treated or not).

In addition, in the palpebral conjunctivae of OVA-challenged mice, an intense influx of eosinophils was detected, especially in the AC and Boc2 + ANXA1₂₋₂₆ groups (Fig. 2A). The quantitative analysis of these cells confirmed histological findings, showing a significant increase of eosinophils in the AC and Boc2 + ANXA1₂₋₂₆ groups compared to the group SHAM (Fig. 2B), which is an effect not detected in the group treated with the ANXA1₂₋₂₆ alone.

The presences of mast cells with metachromatic cytoplasmic granules were detected in the conjunctivae lamina propria (Fig. 2C). Also, the analysis of intact and degranulated mast cells showed a greater proportion of intact cells (69%) compared to degranulated cells (31%) in the SHAM group, whereas in the AC group, an increase in the proportion of degranulated cells (39%) was shown in relation to the group SHAM. Treatment with ANXA1₂₋₂₆ increased the proportion of intact cells (75%) compared to the AC group (61%). However, treatment with Boc2 + ANXA1₂₋₂₆ presented a significant increase in the proportion of degranulated mast cells (57%) and a decrease in intact cells in relation to the SHAM and ANXA1₂₋₂₆ groups (Fig. 2C and D).

These histological and quantitative observations of mast cells and eosinophils were corroborated by the expression of their mediators in eye homogenates. Strong immunoreactivity for mouse mast cell protease-6 (mMCP6) was detected in the group treated with ANXA1₂₋₂₆ compared to the AC and Boc2 + ANXA1₂₋₂₆ groups (Fig. 2E and F). Considering this protease a pre-formed mediator of mast cells,¹⁸ its poor immunoreactivity induced by the Fpr antagonist is in agreement with the higher proportion of degranulated cells in this group. In contrast, the expression of eosinophil peroxidase (EPX) presented strong immunoreactivity in the Boc2 + ANXA1₂₋₂₆ group in relation to the other experimental groups, indicating a greater influx of these cells in the eye (Fig. 2E and G).

3.3. ANXA1₂₋₂₆ downregulates Fpr2 levels in the conjunctiva

Fpr2 expression was detected in the epithelium from the palpebral conjunctiva of all groups. In addition, AC was associated with intense expression of this receptor in comparison to control, whereas treatment with ANXA1₂₋₂₆ caused a decrease in its immunoreactivity. The treatment with Boc2 + ANXA1₂₋₂₆ reversed the poor immunoreactivity caused by ANXA1₂₋₂₆ alone (Fig. 3A). Lastly, no immunostaining was detected in the samples used as negative control (Fig. 3B).

Densitometric analysis confirmed the histological observations, showing a significant increase of Fpr2 expression in the epithelium in the AC group in relation to the control. Pharmacological treatment with ANXA1₂₋₂₆ negatively regulates the expression of this receptor in this experimental model, and when adding the antagonist, Boc2, the expression of Fpr2 undergoes a significant increase over that treated with ANXA1₂₋₂₆ alone (Fig. 3C).

In addition, ultrastructural studies of WT peritoneal cells using postembedding immunogold labelling showed co-localization of

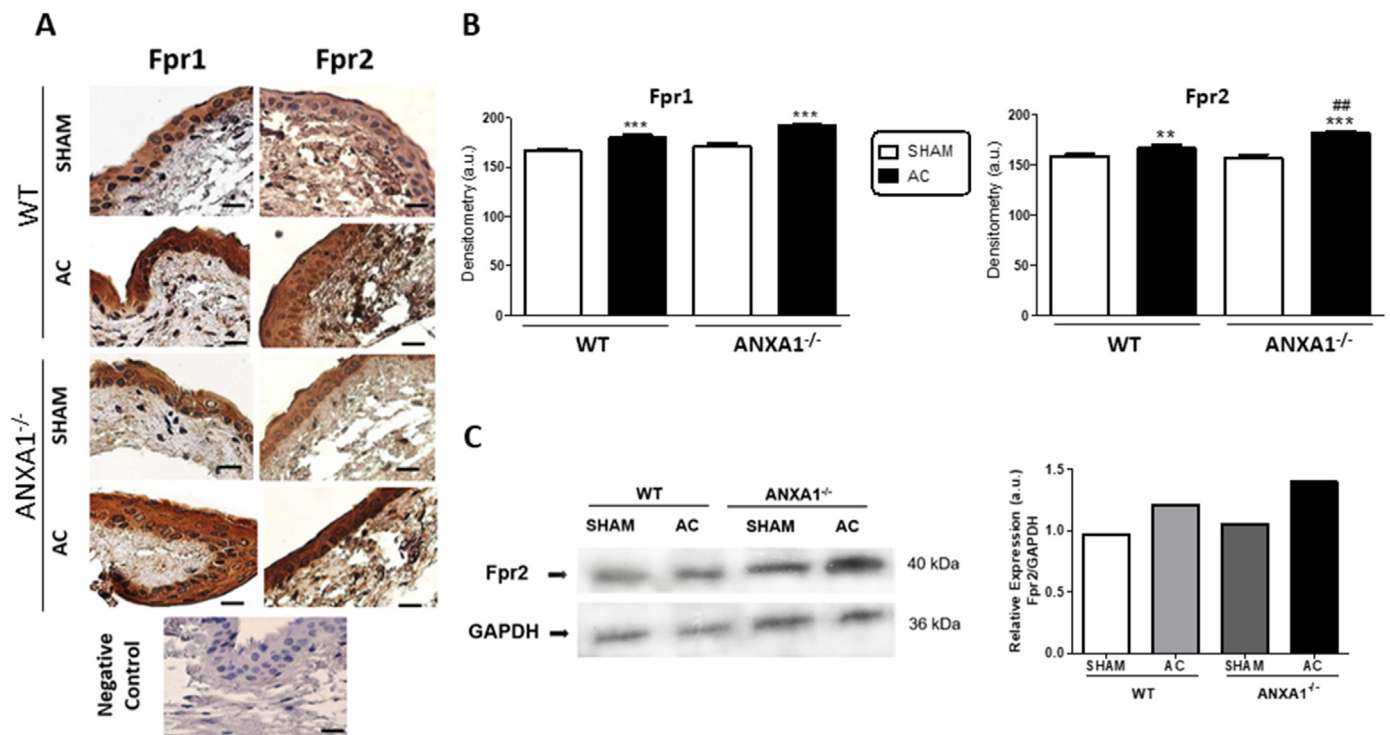


Fig. 1. Effect of lack of endogenous ANXA1 in the Fpr1 and Fpr2 expression in conjunctiva. (A) Epithelial cells from AC groups displayed intense Fpr1 and Fpr2 immunoreactivity compared with their respective control groups. Absence of immunoreactivity in the negative control sample. Counterstain: Haematoxylin. Bars: 10 μ m. (B) Densitometric analysis of Fpr expression in the epithelium. A marked increase in the Fpr2 expression was detected in the epithelium from OVA-sensitized ANXA1^{-/-} mice compared to WT. Data (arbitrary units, a. u.) represent the mean $\pm$ S.E.M. of Fpr levels ($n = 5$ /group). AC, allergic conjunctivitis. ** $P < 0.01$, *** $P < 0.001$ vs. SHAM of respective genotype; ## $P < 0.01$ vs. WT AC. (C) Representative immunoblot shows the levels of Fpr2 (40 kDa) in the pooled extracts of WT and ANXA1^{-/-} eyes ($n = 3$ animals per group). Equal loading was confirmed with anti-GAPDH (36 kDa). Immunoreactive bands for proteins were semi-quantified by densitometry and are expressed as arbitrary units (a.u.) of the ratio of Fpr2/GAPDH (data represent one illustrative blot from two independent experiments).

ANXA1 and Fpr2 in the mast cells, neutrophils and eosinophils (Fig. 3D–F). In these cells gold particles associated with ANXA1 and Fpr2 were mainly detected in the plasma membrane and, to a lesser extent, throughout the cytosol.

3.4. Effects of ANXA1_{2–26} and Boc2 in the cytokine/chemokine production and in the MAPK pathway

The pattern of Th1 (IFN- γ), Th2 (IL-13) and chemokine (eotaxin and RANTES) levels were evaluated in eye homogenates after the last challenge with OVA to verify the local allergic inflammatory response.

The significant increase of eotaxin was detected in animals treated with Boc2 + ANXA1_{2–26} compared to control animals (Fig. 4A), but no significant changes were detected in the levels of RANTES, IFN- γ and TNF- α among the different groups (Fig. 4B–D). However, a higher level of all cytokines was in the AC group compared to the SHAM group, and it decreased after ANXA1_{2–26} treatment and increased again when Boc2 was added.

In addition, strong immunoreactivity for phosphorylated ERK (pERK) was exhibited in the AC group in relation to the other groups. In contrast, treatment with Boc2 + ANXA1_{2–26} exhibited decreased levels of the pJNK and pERK in relation to the other groups, while p-p38 showed increased levels (Fig. 4E–H).

4. Discussion

The pharmacological effects of the anti-inflammatory protein ANXA1 and its mimetic peptide ANXA1_{2–26} have been demonstrated in several inflammatory models (Gavins et al., 2003; Girol et al., 2013; Olani et al., 2001; Perretti et al., 2017; Stوقي et al., 2015), but few studies have monitored their actions on inflammatory allergic processes

and their relationship with Fpr receptors, particularly in the eyes. Therefore, to evaluate the Fpr expression and its relation to the ANXA1 protein in intraocular inflammation, a classic experimental model of OVA-induced allergic conjunctivitis (AC) was used (Andrade et al., 2018; Chung et al., 2009; Gimenes et al., 2015; Mello et al., 2015; Shoji et al., 2005).

The first part of the study showed increased levels of Fpr1 and Fpr2 in the conjunctiva during AC. In the lack of ANXA1 protein, Fpr2 levels, but not Fpr1, were exacerbated in the conjunctiva from AC mice, as well as, in the eye homogenates. In addition, as demonstrated in previous studies, ANXA1^{-/-} mice with AC exhibited a marked inflammatory response compared to the WT AC group characterized by neutrophilia and eosinophilia in the conjunctiva (Gimenes et al., 2015). Altogether, these findings indicate that the ANXA1-Fpr system is operative in these inflammatory cells during AC response.

To test the role of the ANXA1-Fpr system in the AC model, the next step of this study evaluated the pharmacological treatments with the mimetic peptide of ANXA1_{2–26} isolated and associated with the Fpr-nonspecific antagonist (Boc2) in the influx of eosinophils in the palpebral conjunctivae. As expected, AC group exhibited intense eosinophilia in the conjunctiva, abrogated by the pharmacological treatment with ANXA1_{2–26} alone. In contrast, the association of Boc2 + ANXA1_{2–26} produced eosinophilia as detected in the untreated AC group. In fact, the anti-migratory action of the ANXA1_{2–26} on eosinophils was demonstrated in experimental models of OVA-induced allergy such as pleuritic (Bandeira-Melo et al., 2005), asthma (Wang et al., 2011) and conjunctivitis (Gimenes et al., 2015). In addition, eosinophils express Fpr receptors that are related to their migratory activity (Devosse et al., 2009; Svensson et al., 2009). The ultrastructural analysis confirmed these findings through the co-localization of ANXA1 and Fpr2 in eosinophils, as well as in neutrophils and mast cells. Hughes et al. recently

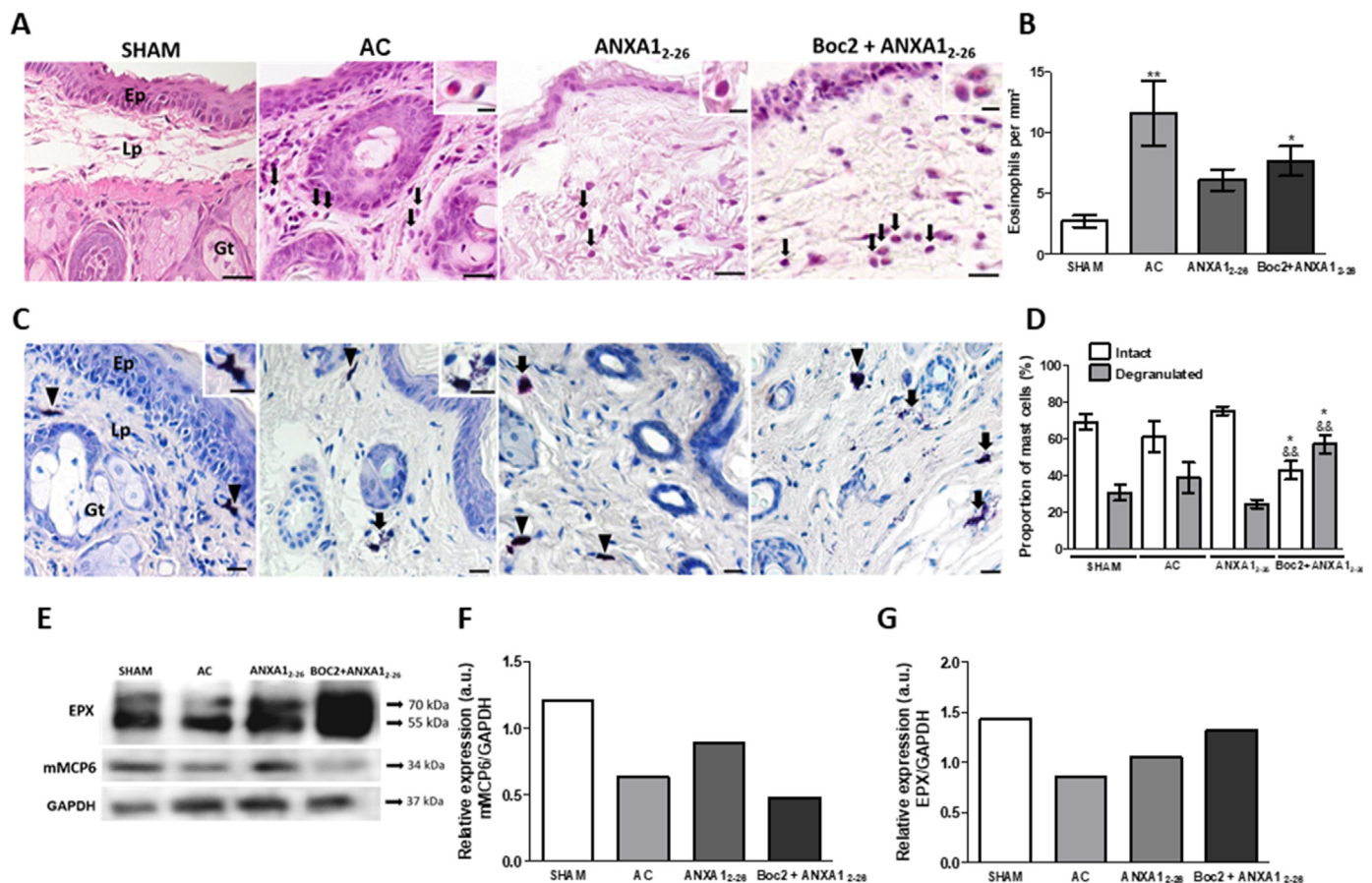


Fig. 2. Inflammatory response in AC model. Intense influx of eosinophils (A; arrows) and mast cells (C; arrows/ arrowheads) in the lamina propria (Lp) from AC and Boc2 + ANXA1₂₋₂₆ groups compared to SHAM and ANXA1₂₋₂₆. Insets: detail of eosinophils and mast cells. (C) Intact (arrowheads) and degranulated (arrows) mast cells with metachromatic cytoplasmic granules. Epithelium, Ep. Tarsal gland, Gt. Stain: haematoxylin-eosin (A); toluidine blue (C). Bars: 50 μ m (A); 10 μ m (C; insets). (B, D) Quantification of eosinophils and mast cells. Data represent mean $\pm$ S.E.M. of the number of cells/mm² and proportion (%) of degranulated mast cells percentage (n = 5/group). * $P < 0.05$; ** $P < 0.01$ vs. SHAM; ^{&&} $P < 0.01$ vs. ANXA1₂₋₂₆. (E) Western blot analysis to measure EPX and mMCP6 levels in the pooled extracts of eyes (n = 3 animals/group) from SHAM, AC, ANXA1₂₋₂₆ and Boc2 + ANXA1₂₋₂₆. 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 EPX or mMCP6/GAPDH (data represent one illustrative blot from two independent experiments).

showed that mast cells have a protective role in the regulation of extravasated neutrophils by the release of ANXA1 which then acts on neutrophil Fpr2 (Hughes et al., 2017). Then, it is reasonable to suppose that ANXA1-Fpr system acts to control the migration of leukocytes during AC, opening new therapeutic possibilities for inflammatory allergic diseases.

This study also evaluated the effect of pharmacological treatments with ANXA1₂₋₂₆ and Boc2 on the mast cell density in the palpebral conjunctiva. Histological and quantitative analyzes of mast cells in the conjunctiva revealed a higher proportion of degranulated cells (39%) in the AC group in relation to the control (31%). Treatment with ANXA1₂₋₂₆ reduced the proportion of degranulated cells (25%) and increased intact cells (75%) compared to the AC group (61%), corroborating previous studies (Gimenes et al., 2015). Similarly, in OVA-induced pleurisy in mice, pretreatment with ANXA1 significantly increased the number of intact mast cells in the pleural fluid and, in vitro, decreased histamine release (Bandeira-Melo et al., 2005). In addition, ANXA1^{-/-} mast cells, activated or not by anti-IgE antibodies, are more susceptible to degranulation and release of histamine and prostaglandins than WT cells, in response to different stimuli as zymosan, 48/80 and nedocromil (Yazid et al., 2010, 2013).

Besides, in this study the ANXA1₂₋₂₆ effect on mast cell degranulation was reversed after combined treatment with Boc2, significantly increasing the proportion of degranulated mast cells (57%).

These findings were corroborated by the reduced levels of protease 6, a mast cell pre-stored mediator (Dai and Korthuis, 2011), in the eye homogenates. In vitro studies showed that administering the Fpr2 antagonist, WRW4, inhibited degranulation of mast cells after activation by the NRC-04 peptide of pleuridin (Pundir et al., 2014). Altogether, the different studies pointed a role of ANXA1 in the regulation of mast cell activation through Fpr receptors.

Immunohistochemical and densitometric analyzes of the conjunctiva revealed increased expression of Fpr2 in AC group compared to the SHAM group. Similarly, in lipopolysaccharide-induced uveitis in mice, an inflammatory model with a type 1 T helper cell (Th1) cytokine profile, epithelial cells from ocular tissues increased the expression of Fpr2 (Girol et al., 2013). Also, the anti-inflammatory effect of ANXA1₂₋₂₆ was associated with increased Fpr2 in these cells and the addition of Boc2 significantly decreased their levels. In the proposed AC model, for a disease with a characteristic Th2 cytokine profile, the ANXA1₂₋₂₆ decreased the levels of Fpr2 in epithelial cells of the conjunctiva in relation to the AC group, whereas Boc2 caused an increase in relation to the group treated with the peptide only.

The analyzes demonstrated a significant increase in eotaxin levels only in the AC group treated with Boc2 + ANXA1₂₋₂₆ compared to the control group, corroborating the influx of eosinophils in the palpebral conjunctiva detected in this group. Investigations using the same experimental model of conjunctivitis detected a significant increase of

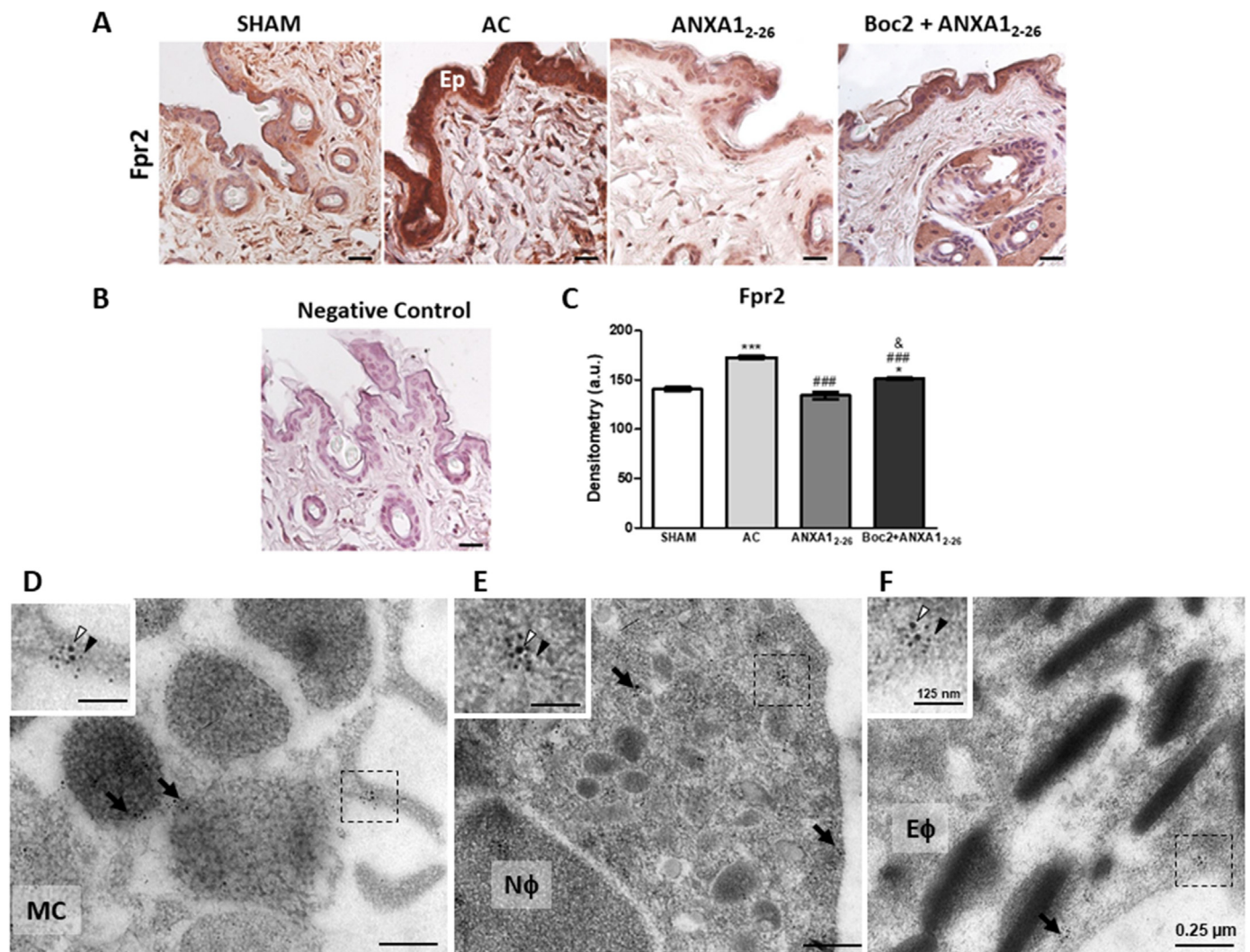


Fig. 3. Fpr2 levels in the palpebral conjunctiva. (A) AC exhibited strong immunoreactivity for Fpr2 in the epithelium (Ep) compared to SHAM. ANXA1₂₋₂₆ treatment reduced Fpr2 levels, an effect abrogated by Boc2 administration (Boc2+ANXA1₂₋₂₆). (B) No immunostaining was detected in the negative control sample. Counterstain: Haematoxylin. Bars: 20 μ m. (C) Densitometric analysis of Fpr2 expression in the epithelium. Data (arbitrary units, a.u.) represent the mean $\pm$ S.E.M. of Fpr2 levels (n = 5/group). * $P < 0.05$, *** $P < 0.001$ vs. SHAM; ### $P < 0.001$ vs. AC; & $P < 0.05$ vs. ANXA1₂₋₂₆. (D) Ultrastructural colocalization of ANXA1 and Fpr2 immunogold particles in mast cell (MC), neutrophil (N ϕ) and eosinophil (E ϕ). Colocalized 10- and 15-nm colloidal gold particles of ANXA1 (black arrowheads) and Fpr2 (white arrowheads), respectively, are highlighted in the squared areas from inflammatory cells and detected on their cytosol/plasma membranes.

eotaxin, RANTES IL-4 and IL-13 in the lymph nodes, at times 4 and 24 h after the last OVA challenge (AC group), while no alterations were detected in the eye homogenates (Gimenes et al., 2015; Mello et al., 2015). This study observed a tendency to reduce levels of IL-13, IFN- γ and RANTES in the group treated with ANXA1₂₋₂₆ compared to the AC group, showing levels similar to the animals treated with Boc2+ANXA1₂₋₂₆. In studies using the OVA-induced asthma model in mice, the treatment with mimetic derivatives of the ANXA1, ANXA1₂₋₂₆ and Tat-ANXA1 protein reduced IL-4, IL-5, IL-13 and eotaxin in the bronchoalveolar lavage (proteins) and lung biopsies (mRNA) (Lee et al., 2012; Wang et al., 2011). These data indicate a protective role of ANXA1 in allergic inflammation by regulating the secretion of Th2 cytokines.

Another aspect investigated in this study was the potential role of ANXA1₂₋₂₆ and Boc2 in MAPK signaling cascades by the western blot technique in macerated eyes. Treatment with ANXA1₂₋₂₆ resulted in decreased ERK expression compared to the AC group, confirming previous data (Gimenes et al., 2015). In an OVA-induced asthma model, treatment with Tat-ANXA1 inhibited the phosphorylation of MAPKs in

a dose-dependent manner in lung biopsies compared to the untreated group (Lee et al., 2012). Furthermore, previous studies have shown that inhibition of the signaling cascade via ERK has therapeutic potential for treating allergic inflammation induced in a murine model of asthma (Duan et al., 2004).

In contrast, treatment with Boc2 antagonist associated with ANXA1₂₋₂₆ inhibited the phosphorylation of pERK and pJNK kinases but activated p-p38. In addition to Boc2, cyclosporin also proved to be an efficient Fpr receptor antagonist in human neutrophils, inhibiting ERK and AKT phosphorylation after stimulation by n-formyl-methionyl-leucyl-phenylalanine or fMLP (Yan et al., 2006), as well as ERK and p38 after stimulation by molecular patterns associated with mitochondrial damage (mitDAMPs) (Hazeldine et al., 2015). Consequently, these data indicate that the anti-inflammatory activity of the ANXA1 mimetic peptide in this experimental model is related to the regulation of the MAPKs signaling cascade, especially ERK, an independent effect of its binding to Fpr receptors.

In conclusion, the data indicate that the ANXA1/Fpr system acts to modulate the anti-inflammatory and antiallergic response in the AC

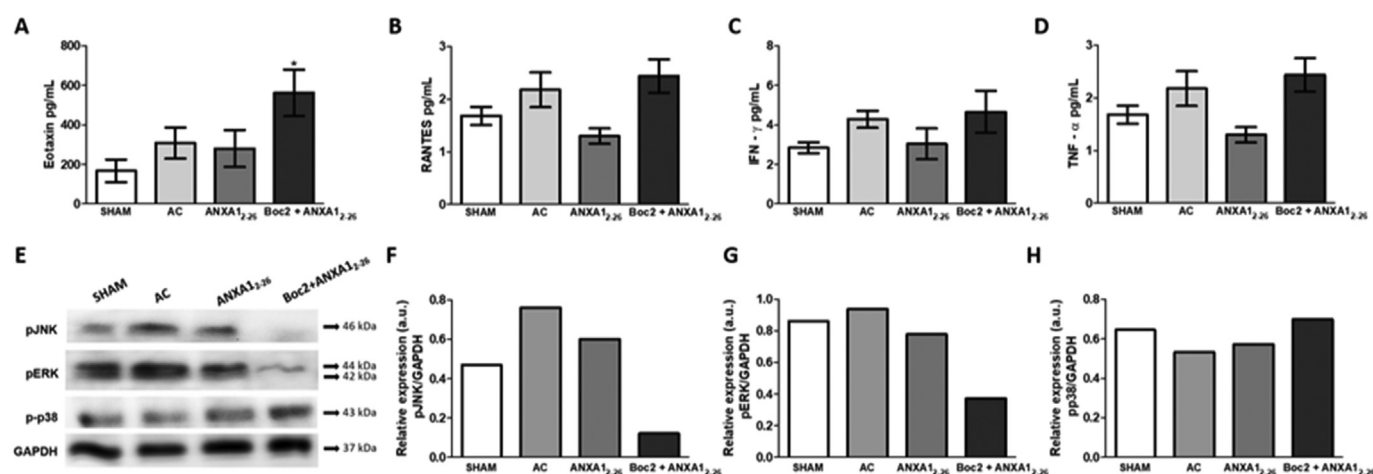


Fig. 4. Effect of ANXA1 and Boc2 administration on the local immune response in the AC model. Multiplex immunoassay of eye homogenates to detect eotaxin (A), RANTES (B), IFN-γ (C) and TNF-α (D). Values are expressed as the mean $\pm$ S.E.M. of the chemokines/cytokine (pg/mL; n = 5/group). * $P < 0.05$ vs. SHAM. (E) Western blot analysis to measure phosphorylated MAPK (pJNK, pERK and p-p38) levels in the pooled extracts of mouse eyes (n = 3 animals/group). Immunoreactive bands for proteins were semi-quantified by densitometry and are expressed as arbitrary units (a.u.) of the ratio of p-MAPK/GAPDH (data represents one illustrative blot from 2 independent experiments).

model induced by OVA, constituting an important therapeutic target in ocular inflammation.

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Conflict of interest

The authors declare no conflict of interest in relation to the content of this article.

Author contributions

MPM: Formal analysis, Data curation, Investigation, Methodology, Writing - original draft; ADG: Investigation, Methodology, Writing - original draft; FECA: Methodology; SMO: Funding acquisition, Writing - review & editing; CDG: Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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