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Research paper

ANXA1$_{Ac2–26}$ peptide, a possible therapeutic approach in inflammatory ocular diseases

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ABSTRACT

The eye is immunologically privileged when inflammatory responses are suppressed. One component responsible for the suppression of inflammatory responses is the blood retinal barrier, which comprises the retinal pigment epithelium. The destruction of this barrier initiates inflammation, which can affect any part of the eye. Therefore, inflammatory response is controlled by the action of anti-inflammatory mediators, among these mediators, annexin A1 (ANXA1) protein acts as a modulator of inflammation. In this study we aimed to improve the knowledge of this area by investigating how a peptide of the ANXA1 protein (ANXA1$_{Ac2–26}$) modulates the morphology, proliferation, migration and expression of genes and proteins in human retinal pigment epithelium cells (ARPE-19). Determining how signaling pathways (NF-κB and UBC) are modulated by the ANXA1$_{Ac2–26}$ peptide could be important for understanding the inflammatory process. ARPE-19 cells were activated by bacterial lipopolysaccharide endotoxin (LPS) and treated with ANXA1$_{Ac2–26}$ peptide, in a concentration of 1.7 μM and 33.8 μM. We observed that the LPS activation diminished the levels of endogenous ANXA1 after 2 h and 24 h and ANXA1$_{Ac2–26}$ peptide decreased the proliferation and re-establishes the migration of ARPE-19 cells. After using a hybridization approach, 80 differentially expressed genes were found. Five of these genes were selected (*LRAT*, *CTGF*, *MAP1B*, *ALDH1A3* and *SETD7*) and all were down-regulated after treatment with the peptide. The genes *CTGF* and *LRAT* would be considered as potential molecular markers of ophthalmologic inflammation. The expression of pro-inflammatory cytokines was also decreased after the treatment, indicating the efficiency of the anti-inflammatory peptide at high concentrations, since the reduction in the levels of these mediators were observed after the treatment with ANXA1$_{Ac2–26}$ peptide at 33.8 μM. Our results suggest that the retinal pigment epithelial cells are a potential target of the ANXA1 protein and point to possible applications of the ANXA1$_{Ac2–26}$ peptide as an innovative therapy for the treatment of ocular inflammation.

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Abbreviations: ACTB, Beta-actin; ALDH1A3, Aldehyde Dehydrogenase 1 Family, member A3; ANX, Annexin; ANXA1, Annexin A1; ANXA1$_{Ac2–26}$, peptide of the ANXA1; ARPE-19, human retinal pigment epithelium cells; BRB, blood retinal barrier; CTGF, Connective Tissue Growth Factor; DMEM, Dulbecco Minimum Essential Medium; DTT, DL-Dithiothreito; ECL, Electrochemical luminescence; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HCL, hydrochloric acid; HIF1A, hypoxia inducible factor 1, alpha subunit; HRP, horseradish peroxidase; IL, interleukin; IgG, Immunoglobulin; LPS, bacterial lipopolysaccharide endotoxin; LRAT, Lecithin Retinol Acyltransferase (phosphatidylcholine-retinol *O*-acyltransferase); MAP1B, Microtubule-Associated Protein 1B; MAPK, Mitogen-Activated Protein kinase; *Mbo*I, enzyme gene from Moraxella bovis; $MgCL_2$, magnesium chloride; MYC, v-myc avian myelocytomatosis viral oncogene; NLRs, NOD-like receptors (NLRs); NF-kB, factor nuclear kappa B; PLA2, phospholipase A2; RaSH, rapid subtraction hybridization; RLRs, RIG-I-like receptors; RPE, retinal pigment epithelium; SETD7, SET domain-containing (lysine methyltransferase) 7; SMAD2, SMAD family member 2; TLRs, Toll-like receptors; UBC, Ubiquitin C.

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1. Introduction

The eye is an immunologically privileged organ where inflammatory responses are suppressed and many factors are responsible for this suppression, including the presence of the blood retinal barrier (BRB) (Cai and Brandt, 2008). The retinal pigment epithelium (RPE) is a major component of the BRB that controls the nutrient flow to photoreceptors (Leung et al., 2009) and makes an essential contribution to ocular immune privilege (Streilein, 2003).

Inflammation is the main contributing factor to the functional impairment of the RPE (Leung et al., 2009). The epithelium is involved in various metabolic functions and supports the sensory retina; thus, inflammation of this area may lead to the appearance of ophthalmologic diseases (Hamilton and Leach, 2011; Steinberg, 1985; Strauss, 2005). The first line of defense against pathogens is mediated by receptors, which recognize the invaders. These receptors may initiate the inflammatory pathway of NF-kB and mitogen-activated protein kinase (MAPK) to trigger the expression of pro-inflammatory cytokines and chemokines (Corn and Vucic, 2014).

Bacterial lipopolysaccharide (LPS) can stimulate the RPE cells at concentrations of 100 ng/mL and 1 mg/mL to produce an inflammatory response (Elner et al., 2003; Garcia-Cabanes et al., 2001; Girol et al., 2013; Koga et al., 2003; Zanon et al., 2015) and in the retina, LPS increases the concentration of pro-inflammatory cytokines (Wang et al., 2005; Yang et al., 2007). RPE cells express diverse LPS receptors, including CD14 and TLR4, which play an important role in defense against a variety of ocular pathologies (Elner et al., 2005; Kindzelskii et al., 2004; Paimela et al., 2007).

Inflammation is a major contributing factor to many blinding disorders (Leung et al., 2009). Corticosteroids are frequently administered to treat this intraocular process, nevertheless, there is the risk of side effects (Jonas et al., 2003; McGhee et al., 2002; Yoshikawa et al., 1995). Corticosteroids induce the expression of the protein annexin A1 (ANXA1), which is one of the endogenous anti-inflammatory mediators (Blackwell et al., 1980; Flower, 1988). Anti-inflammatory agents block specific pathways related to inflammation (Serhan et al., 2008), these molecules are different from pro-resolving agents, which present pro-resolving properties together with anti-inflammatory actions (Sugimoto et al., 2016). The protein Annexin A1 (ANXA1) is one of these inflammatory pro-resolving mediators (Blume et al., 2011), and has been investigated as an anti-inflammatory therapeutic agent.

The annexins (ANX) comprise a well conserved super-family of proteins that are structurally related and characterized by a homologous C-terminal domain that is responsible for the properties of calcium and phospholipids binds (Yazid et al., 2010). The N-terminal domain, which is unique in length and sequence for each family member, includes potential phosphorylation, glycosylation and peptidase activity sites (Gerke and Moss, 2002). The ANXA1 anti-inflammatory and anti-proliferative actions have been shown in in vivo and in vitro studies that used administration of exogenous ANXA1 or its mimetic peptide of the N-terminal domain (Ac2–26 and Ac-AMVSEFLKQAWFIENEEQEYVQTVK) (Gastardelo et al., 2014, 2009; Gimenes et al., 2015; Girol et al., 2013; Prates et al., 2015).

There is a wide variety of literature on the pharmacological effects of ANXA1 and its mimetic peptides on inflammatory processes in vitro (D'Acquisto et al., 2008; Gastardelo et al., 2014; Prates et al., 2015) and in vivo (de Paula-Silva et al., 2016; Facio et al., 2011; Gastardelo et al., 2009; Gimenes et al., 2015; Machado et al., 2016). However, few studies have tracked their actions on ocular inflammation (Girol et al., 2013). The purpose of the ANXA1 treatments was to maintain the anti-inflammatory properties and to avoid the side effects of glucocorticoids (Perretti and Gavins, 2003). Identification of the ANXA1 mechanism of action, along with the development of new therapeutic agents with the potential to mimic specific endogenous pathways, has increased the development of drug discovery programs (Gavins and Hickey, 2012).

For decades, scientists developed knowledge to prevent vision loss or restore vision in patients affected by retinal degeneration through drug therapy, gene enhancement or cell transplantation (Wiley et al., 2015). In this study we aimed to improve the knowledge of this area by investigating how a peptide of the ANXA1 protein modulates the morphology, proliferation, migration and expression of genes and proteins in ARPE-19 cells. Determining how signaling pathways, NF-κB and Ubiquitin C (UBC), are modulated by the $ANXA1_{Ac2–26}$ peptide and how this could be important for understanding the inflammatory process.

2. Materials and methods

2.1. ARPE-19 culture conditions

ARPE-19 cells (derived from human RPE; American Type Culture Collection) were grown in culture dishes, in a mixture of DMEM and HamF-12 (1:1) (Cultilab, Campinas, Brazil) (Dunn et al., 1996) supplemented with 10% FBS, 200 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin (Invitrogen) and were incubated at 37 °C and 5% CO_2. The morphology was observed by inverted microscopy.

2.2. Pharmacological treatments

ELISA and Western blotting assays were carried out to optimize the duration of inflammation activation by LPS for use in the following two groups: a) control and b) bacterial lipopolysaccharide endotoxin (LPS, *Escherichia coli*, serotype 0127: B8, Sigma Chemical Co. Poole, Dorset, UK); a concentration of 10 μg/mL (Paimela et al., 2007) was used for periods of 1, 2, 4, 24 and 48 h.

ARPE-19 cells were subdivided into three groups: a) control; b) activation by 10 μg/mL LPS (Paimela et al., 2007) for 24 h (Girol et al., 2013); and c) activation by LPS and treatment with two concentrations of the $ANXA1_{Ac2–26}$ peptide (Ac-MVSEFLKQAWFIENEEQEYVQTVK) of the ANXA1 protein (Raynal and Pollard, 1994). Treatment concentrations were chosen based on the results previously obtained by our research group: 1.7 μM (Rodrigues-Lisoni et al., 2006), was used in the growth curve, migration, rapid subtraction hybridization, quantitative PCR and multiplex assays, and 33.8 μM (Girol et al., 2013) was used in the quantitative PCR and multiplex assays.

2.3. ELISA assay

The evaluation of cytokines levels in the supernatant of ARPE-19 cells was performed using the immunoassay kit for IL-6 and IL-8 (R&D, Minneapolis, MN, USA) using the conditions described above. The quantification of cytokine levels was determined by an optical density reader (Molecular Devices Sunnyvale, CA) using standard curves according to the manufacturer's instructions. The results were shown as the mean ± standard error of the cytokine levels in pg/mL.

2.4. Western blotting

ARPE-19 cells were cultivated in75 cm^2 culture flasks, in accordance with the description above. The cells were collected in 1 mL of lyses buffer (1 complete mini tablet of EDTA-free protease inhibitor [Roche Applied Science, Mannheim, Germany], 50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X), subjected to centrifugation, and the supernatant was collected. Aliquots of these samples were subjected to the Bradford assay for quantification of proteins by an optical density reader (Molecular Devices Sunnyvale, CA). Equal amounts of protein (30 μg) were separated by gel electrophoresis according to the protocol in a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, USA) and transferred to nitrocellulose membranes (Hi-Bond C; Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with 5% powdered milk diluted in TBS-T (Bio-Rad Reagent, Hercules, USA). Then, the membranes were incubated overnight with

anti-ANXA1 (rabbit polyclonal IgG, 1:1000,Zymed Laboratories, San Francisco, CA, USA) and *anti*-α-tubulin (mouse monoclonal IgG, 1:1000, Zymed Laboratories, San Francisco, CA, USA). The following day, the membranes were incubated with anti-rabbit IgG (1:2000) conjugated with horseradish peroxidase (HRP) (Serotec, Oxford, UK). The HRP reaction product was visualized on photographic film (Hyperfilme, Amersham, Little Chalfont, UK) after application of the chemiluminescent ECL kit (Amersham, Little Chalfont, UK). The densitometry levels of ANXA1 were quantified using the Axioskop 2 software. As a control of the reaction, tubulin was detected by mouse monoclonal IgG antibody, 1:1000 (Zymed Laboratories, San Francisco, CA, USA).

2.5. Growth curve

ARPE-19 cells were seeded in triplicate in plastic 6-well plates in accordance with the description above. At 24 h later when the cells adhered, ARPE-19 cultures were incubated with serum free medium. After an additional 24 h, the cells were activated using LPS. The control group was maintained without any pharmacological manipulation. After 24 h of induction, one group was treated with the $ANXA1_{Ac2-26}$ peptide for 1, 2, 4, 24, 48 and 72 h (Gastardelo et al., 2014).

The cytotoxic effect of the peptide with regard to the proliferation and viability of ARPE-19 cells was investigated in all experimental conditions. The cells were harvested and quantified using the Countess® Automated Cell Counter (Invitrogen®) and Trypan Blue. Significant differences between the groups were determined by one-way analysis of variance (one-way ANOVA) and groups that were significantly different were further analyzed using the Bonferroni test. The same experiment was repeated three times.

2.6. Cell migration assay

ARPE-19 cells were seeded in triplicate in 24-well plates and processed using the growth curve assay. After treatment with the $ANXA1_{Ac2-26}$ peptide, grooves were made in all wells using a 10 μL pipette tip. Analyses were performed at 0, 8, 24, 48 and 72 h (these times were chosen according to standard laboratory procedures). The migration was monitored using images that were taken, and the number of cells was measured from three different microscope areas. Significant differences between the groups were determined by *t*-test. The same experiment was repeated twice.

Based on statistical analysis, treatment for 72 h was chosen as the time for the subsequent techniques: rapid subtraction hybridization, quantitative PCR and multiplex assay.

2.7. Rapid subtraction hybridization (RaSH)

The RaSH technique (Franco-Salla et al., 2016; Jiang et al., 2000; Prates et al., 2015; Rodrigues-Lisoni et al., 2006) was performed with two subtractions (Sub). The first was termed "Sub A", in which the ARPE-19 cells activated by LPS were referred to as *tester*, and the cells stimulated and treated with $ANXA1_{Ac2-26}$ were termed *driver*. In the second subtraction, "Sub B", the opposite test was performed.

Aliquots of 20 μg of total RNA were used for double-stranded complementary DNA (cDNA) synthesis. The first cDNA strand was obtained by reverse transcription, 50 mM oligo (dT) and 10 mM dNTPs at 65 °C for 5 min. The samples were placed on ice and 0.1 mM DTT, 40 U/μL RNAout, 5× Superscript III Buffer [250 mM Tris–HCL (pH 8.3), 375 mM KCL and 15 mM MgCL2] and 3 μL of Superscript III reverse transcriptase (RT) (200 U/μL) were added. The mixture was incubated at 42 °C for 60 min and then at 70 °C for 15 min. RNase H (2 U/μL)was added and the reaction was incubated at 37 °C for 20 min. Segments of β-actin cDNA were amplified to verify the quality of these reactions.

The second strand of cDNA was synthesized in reactions containing 5× Second Strand Buffer, 10 mM dNTPs, DNA ligase (10 U/μL) and DNA Pol I (10 U/μL).The samples were incubated for 2 h at 16 °C before T4 DNA Pol (5 U/μL) was added, and the sample was incubated for an additional 5 min. The action of T4 DNA Pol was inhibited by the addition of 0.5 M EDTA. Phenol:chloroform extraction was used to purify the samples, and the pellet was resuspended in 45 μL of water. The cDNA was used in amplification reactions for the segments of β-actin and it was digested with *Mbo*I enzyme (10 U/μL) and incubated for 1 h at 37 °C. Finally, >1 μL of this enzyme was added to the reaction and incubated overnight at 37 °C.

The cDNA was then supplemented with molecular adaptors XDPN-14 5′-CTGATCACTCGAGA and XDPN-12 5′-GATCTCTCGAGT (Sigma Chemical, final concentration 10 mM), 10× T4 DNA ligase buffer (Invitrogen©) and water, heated at 55 °C for 1 min, and cooled to 14 °C within 1 h. The cDNA received 9 U of T4 DNA ligase (3 U/μL) and ligation was carried out overnight at 14 °C. After phenol/chloroform extraction and ethanol/glycogen precipitation, the mixtures were diluted to 100 μL with TE buffer (10 mM Tris/1 mM EDTA). 40 μL of the mixtures were used for PCR amplification.

The PCR mixtures were set up using 10 μM XPDN-18 5′-CTGATCACTCGAGAGATC, 0.4 mM dNTPs, 10 × PCR buffer, 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). Thermocycler conditions were 1 cycle at 72 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 3 min. A total of 10 μg of purified PCR product (tester) was digested with 10 U/μL *Xho*I (Invitrogen) for 6 h at 37 °C, followed by phenol/chloroform extraction and ethanol precipitation.

One-hundred nanograms of the tester cDNA was mixed with 5 μg of the driver cDNA in hybridization solution (0.5 M NaCl, 50 mM Tris/HCl, 2% SDS and 40% formamide) and, after heating at 95 °C for 5 min, was incubated at 42 °C for 48 h. After extraction and precipitation, the hybridization mixture (1 μg) was linked with the *Xho*I-digested pZero plasmid and transformed into competent bacteria. Bacterial colonies were picked and used as the DNA template for PCR. Clones were sequenced using an automated DNA sequencer, and homologous sequences were identified using the BLAST program. Gene ontology (GO) annotation was used for the functional classification of up- and down-regulated genes using terms from the Gene Ontology database.

IPA (Ingenuity Systems©) software was used to connect the differentially expressed genes with biological functions, to relate the process in which these genes are involved and to recognize the pathways of interaction between them.

2.8. Quantitative PCR – RT-qPCR

The gene expression levels were measured in ARPE-19 cells plated in 75 cm^2 culture flasks, and the experiment was conducted according to the description above. Following treatment with DNase, RNA extraction was performed with TRIzol, according to the manufacturer's instructions (Invitrogen). cDNA synthesis was performed using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Five differentially expressed genes were selected for validation by quantitative real time PCR experiments, according to their direct or indirect involvement in inflammation and eye metabolism. The genes are: Connective Tissue Growth Factor (*CTGF*), Lecithin Retinol Acyltransferase (phosphatidylcholine-retinol *O*-acyltransferase) (*LRAT*), Microtubule-Associated Protein 1B (*MAP1B*), Aldehyde Dehydrogenase 1 Family, member A3 (*ALDH1A3*) and SET domain-containing (lysine methyltransferase) 7 (*SETD7*).

The primers were manually designed using the following criteria: 19–23 bp length, 30–70% GC content and a short amplicon size (90–110 bp) (Table 1). Real time PCR was performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction mixture consisted of a 20 μL volume solution containing 100–500 ng of Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of each primer and 100 ng cDNA. The PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 1
Primers for the genes analyzed by real time PCR.

Oligonucleotide	Sequence
LRAT right	5′ AGATGCCATAGTGGGTCAGG 3′
LRAT left	5′ CGAAGACAAAGGGAGGAACA 3′
CTGF right	5′ TGGAGATTTTGGGAGTACGG 3′
CTGF left	5′ GCAGGCTAGAGAAGCAGAGC 3′
MAP1B right	5′ CAGGATGGGTGGTGCTTAGT 3′
MAP1B left	5′ AGTCCGGCTCTTTCTCCTTC 3′
ALDH1A3 right	5′ GTCCGATGTTTGAGGAAGGA 3′
ALDH1A3 left	5′ GAATACGCTTTGGCCGAATA 3′
SETD7 right	5′ ACATACGTGCCCTGGAGAAC 3′
SETD7 left	5′ GCACCCTGGAGGGGTATTAT 3′

Melting curve analysis was performed for each gene to check the specificity and identity of the RT-PCR products.

For each primer set, PCR efficiency (linear equation: y = slope + intercept) was measured in triplicate for serial dilutions of the same cDNA sample. The PCR efficiency (E) was calculated by the formula $E = [10^{(-1/\text{slope})}]$ and ranged from 1.96 to 2.02 in the different assays.

Two control genes (*GAPDH* and *ACTB*) were used as internal standards. The relative expression ratio (fold change) of the target genes was calculated according to Pfaffl (2001).

2.9. Multiplex assay

The cytokine levels were quantified from the supernatants of ARPE-19 cells, and the experiment was conducted according to the description in Section 2.2. The MILLIPLEX MAP HCYTOMAG 60K kit and Magpix Luminex xMAP equipment (Millipore Corporation, Billerica, MA, USA) were used according to the manufacturer's instructions. The metabolites analyzed included interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 1 beta (IL-1β),regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein (MCP-1), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin 10 (IL-10), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). The assays were performed in quintuplicate to confirm the results. Metabolite concentrations were determined using Magpix Xponent (Millipore Corporation, Billerica, MA, USA) software.

3. Results

3.1. Inflammatory mediators and annexin A1protein expression in ARPE-19 cells

The presence of IL-6 and IL-8 cytokines was measured in the supernatant of ARPE-19 cells by ELISA assay. Cells activated with LPS showed a significant increase in the release of IL-6 (Fig. 1A) and IL-8 (Fig. 1B)

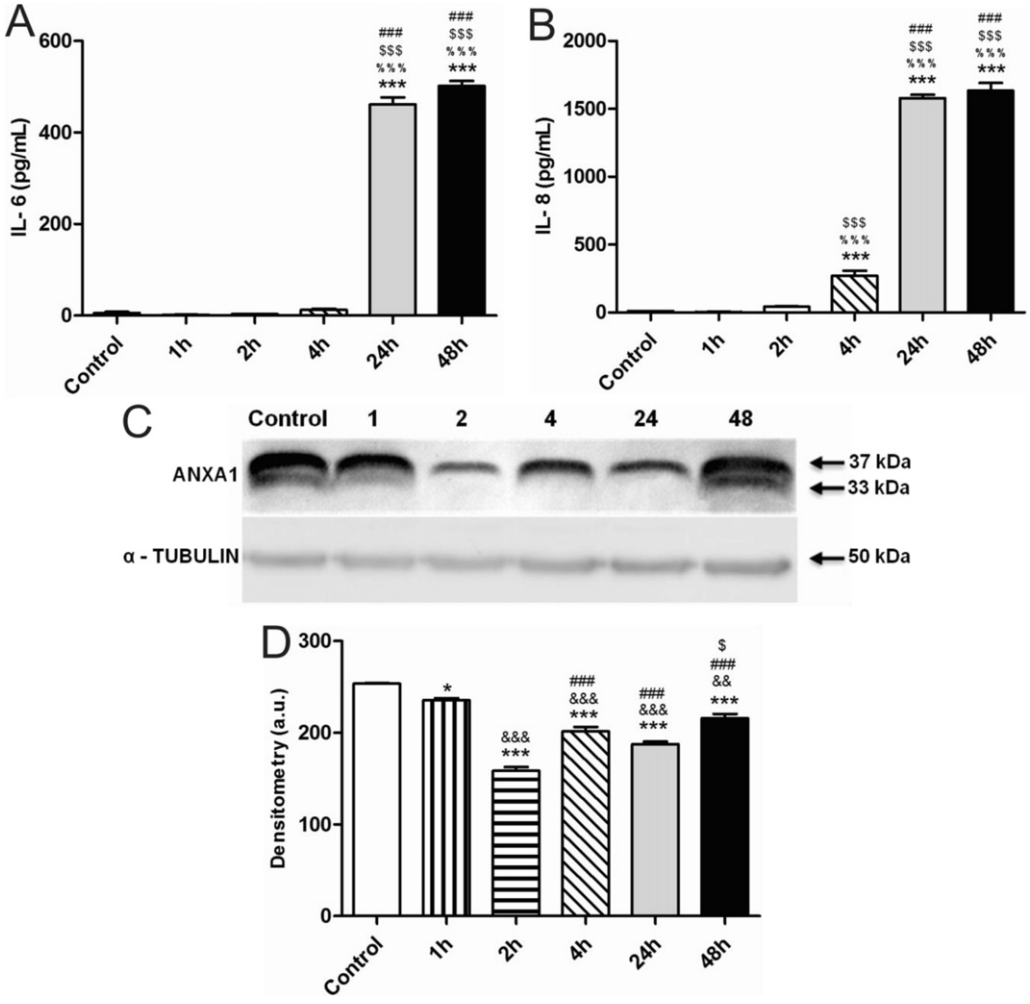

Fig. 1. Analysis of pro-inflammatory mediators and ANXA1 expression in ARPE-19 cells after activation by LPS. Dosages of [A] IL-6 and [B] IL-8 were monitored by ELISA; [C] Representative Western blotting illustrating the intact (37 kDa) and N-terminal cleaved (33 kDa) forms of ANXA1 and [D] Densitometry analyses of endogenous ANXA1 protein. The times used were 1, 2, 4, 24 and 48 h. The results were obtained as mean ± standard error of the cytokine levels in pg/mL (ELISA) with five independent experiments and, the index densitometry of the bands (Western blotting) with three independent experiments. 1 symbol = $p < 0.05$; 2 symbols = $p < 0.01$; 3 symbols = $p < 0.001$ [A] and [B] * vs. control; % vs. 1 h; $ vs. 2 h; # vs. 4 h; [D] * vs. control; & vs. control; # vs. 2 h; $ vs. 24 h (ANOVA followed by Bonferroni's test).

cytokines after 24 h compared to the control group. High levels of cytokines were maintained at 48 h. The expression of ANXA1 in these cells under control conditions and after the activation with LPS by Western blotting was determined (Fig. 1C). The immunoreactivity of ANXA1 was detected in cells under all experimental conditions, and low levels of intact protein (37 kDa) were observed at 2 and 24 h compared to those of the controls at 1, 4 and 48 h after induction (Fig. 1D). Tubulin showed similar immunoreactivity in all experimental conditions (Fig. 1C). Therefore, the time of 24 h was chosen for the induction of inflammation by LPS.

3.2. Migration and proliferation in ARPE-19 cells

The cellular morphology is characterized by a monolayer of elongated cells ordered in hexagonal form in the control group. There was no change after activation by LPS or treatment with the anti-inflammatory peptide $ANXA1_{Ac2-26}$ (Figs. 2A–C). ARPE-19 cells showed a progressive growth between 1 and 72 h. The three groups did not exhibit an intense difference in growth (Fig. 2D). Nonetheless, there was a significant difference after 72 h, and proliferation was diminished in the cells treated with the $ANXA1_{Ac2-26}$ in comparison to the control group. The cell migration was influence by the treatment. ARPE-19 activated with LPS exhibited a decrease in migration and an increase in migration after $ANXA1_{Ac2-26}$ treatment (Fig. 2E). After 24 to 72 h of LPS activation, migration was affected in these cells, but treatment with $ANXA1_{Ac2-26}$ re-establishes the migration similar to that of the control group. Thus, the assays showed that the period of 72 h was the most statistically significant ($P \leq 0.05$).

3.3. Genes identified using the RaSH approach

RaSH was performed on ARPE-19 cells from the group activated by LPS and those stimulated and treated with $ANXA1_{Ac2-26}$ peptide for 72 h. A total of 142 clones were isolated and sequenced. By screening these sequences through GenBank (*Blast*), followed by rigorous classification and selection, 80 differentially expressed genes were identified. The research acquired two libraries. Twenty-one genes of the "Sub A" library exhibited changes in expression levels in response to LPS activation, whereas 59 genes of the "Sub B" library exhibited changes in expression with $ANXA1_{Ac2-26}$ treatment. Therefore, it was expected that the gene expression of one library would be lower than that of the other one. The gene libraries and their different functions and processes are described in the Gene Ontology (GO) databases.

The 80 differentially expressed genes were further analyzed through IPA (Ingenuity Systems©). Genes in the "Sub A" library were found to be associated with cellular organization, cell cycle and DNA repair and replication. Genes in the "Sub B" library were found to be associated with immunological and inflammatory disease, cell cycle and cellular development. Moreover, some of these genes were chosen because they form a network of important interactions with genes such as *MYC*, *SMAD2* and *UBC* in the "Sub A" library (Fig. 3A and Supplement 1A) and *HIF1A* and *NF-kB* in the "Sub B" library (Fig. 3B and Supplement 1B).

Five differentially expressed genes, *CTGF*, *LRAT*, *MAP1B*, *ALDH1A3* and *SETD7*, were selected for validation by quantitative real time PCR experiments (Table 1) because of their direct or indirect involvement in the visual cycle, nervous and visual system development and function, and ophthalmic and inflammatory disease.

3.4. Real-time PCR validation of differentially expressed genes

The RaSH analysis indicated the down-regulation of two genes (*CTGF* and *LRAT*) and the up-regulation of three genes (*MAP1B*, *ALDH1A3* and *SETD7*) in ARPE-19 cells treated with the peptide. However, the quantitative PCR experiment with 1.7 μM of $ANXA1_{Ac2-26}$ showed oscillation in the gene expression without statistically significant alterations (Fig. 4A). Nevertheless, the experiment using 33.8 μM showed significantly reduced expression of the five selected genes (Fig. 4B).

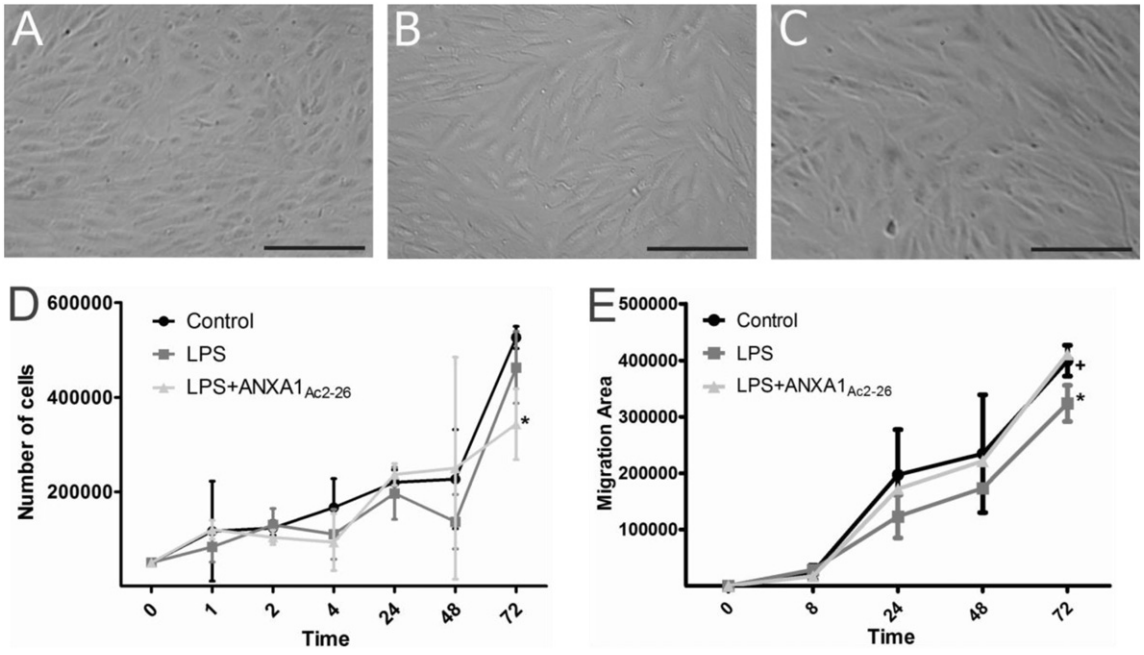

Fig. 2. Morphological analysis and effects of treatment with $ANXA1_{Ac2-26}$ peptide on the proliferation and migration of ARPE-19 cells. The cell morphology was observed by inverted microscopy in the [A] control group, that is characterized by a monolayer of elongated cells ordered in hexagonal form, was not altered in the groups [B] activated by LPS [10 μg/mL] and [C]activated by LPS and treated with$ANXA1_{Ac2-26}$ [1.7 μM]. [D] Cell growth showed a progressive growth between 0 and 72 h, and did not differ between the experimental groups. [E] Cell migration decreased after activation by LPS and increased after administration of the $ANXA1_{Ac2-26}$ peptide. ARPE-19cells were seeded in completeDMEM:F-12 medium at a concentration of 5×10^4 per well (6 well plate) and activated by LPS [10 μg/mL] and treated with $ANXA1_{Ac2-26}$ [1.7 μM]. Assays were performed with three independent experiments. Bar: 100 μm. [D] Graph with x = time (h) and y = number of cells. $^*p < 0.05$ LPS + $ANXA1_{Ac2-26}$ vs. Control. [E] Graph with x = time (h) and y = migration area (mm^2). $^*p < 0.05$ LPS vs. control; $+p < 0.05$ LPS vs. LPS + $ANXA1_{Ac2-26}$.

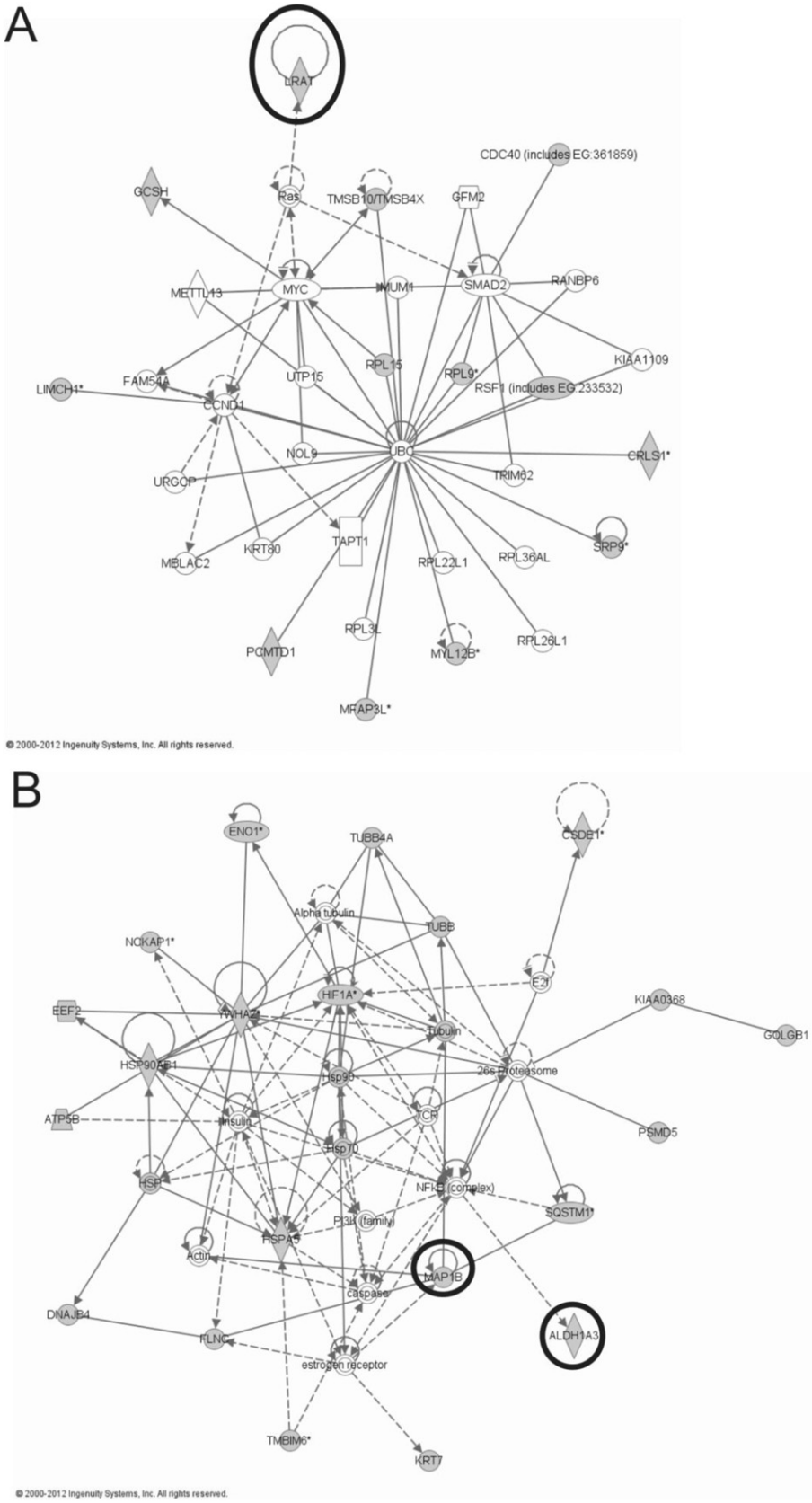

Fig. 3. Differentially expressed genes were analyzed using a manually curate structured network tool (Ingenuity Pathway Analysis). [A] Subtraction A (Sub A), after LPS activation, *LRAT* gene network showed associated functions related to tumorigenesis, apoptosis, cell proliferation and necrosis. [B] Subtraction B (Sub B), after treatment with $ANXA1_{Ac2–26}$ peptide, Gene network showed associated functions related to cancer, inflammatory diseases, cell cycle, senescence, death and cell replication. White nodes: genes identified by IPA; grey nodes: genes identified by IPA and RaSH; black node: identified by IPA and validated by Real Time PCR.

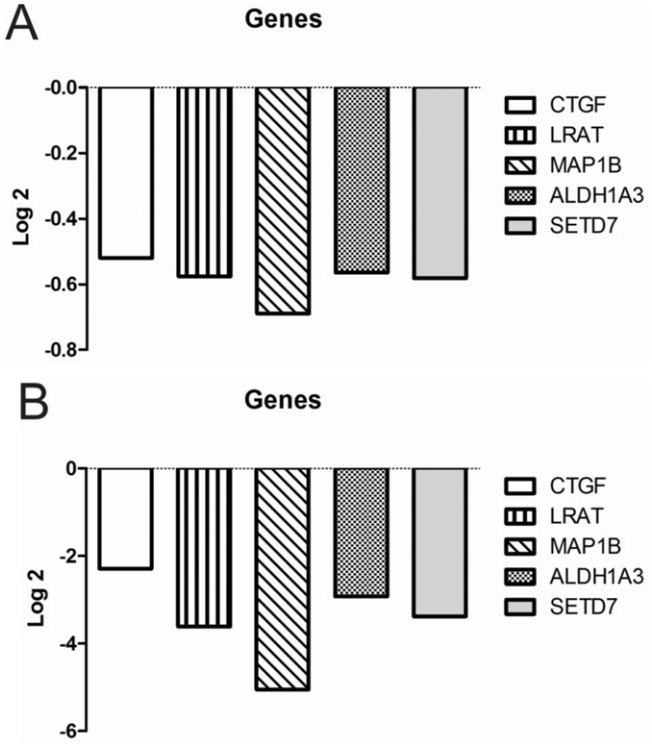

Fig. 4. Gene expression by real time PCR. [A] The genes showed oscillation in expression without statistically significant alterations in cells treated with $ANXA1_{Ac2-26}$ peptide [1.7 μM] compared with cells activated by LPS. [B] Significantly reduced expression of the *CTGF*, *LRAT*, *MAP1B*, *ALDH1A3* and *SETD7* gene in cells treated with $ANXA1_{Ac2-26}$ peptide [33.8 μM] compared with cells activated by LPS. ARPE-19 cells were seeded in complete DMEM:F-12 medium at a concentration of 1×10^6 per flask, activated by LPS [10 μg/mL] and treated with $ANXA1_{Ac2-26}$ peptide. Assays were performed with three independent experiments. Data represent logarithm base 2 and relative expression ≥ 1.0 and ≤ −1.0 was statistically significant.

3.5. Metabolite analysis in the supernatant of ARPE-19 cells by multiplex assay

The LPS activation increased the concentration of IL-6, IL-8, IL-1β, Rantes, MCP-1, TNF-α, INF-γ and VEGF (Figs. 5 and 6) in the supernatant obtained from ARPE-19 cells. The treatment with 1.7 μM $ANXA1_{Ac2-26}$ for 72 h (Fig. 5) did not reduce the levels of the inflammatory mediators. Nonetheless, the treatment with 33.8 μM (Fig. 6) significantly diminished the levels of IL-6, IL-8, IL-1β, Rantes, MCP-1, TNF-α, IFN-γ and VEGF, indicating the efficiency of the anti-inflammatory peptide at high concentrations.

4. Discussion

The inflammation is triggered through the pathogen recognition receptors (PRRs) and the activation of diverse pathways, which induce the secretion of pro-inflammatory molecules (Kauppinen et al., 2016; Kumar et al., 2011), it could be related to the development of ocular pathogenesis. Our in vitro analysis showed that the $ANXA1_{Ac2-26}$ may play an important role in gene cascades and a regulatory role in ocular inflammation. The protective role of $ANXA1_{Ac2-26}$ peptide was observed in retinal ganglion cell line (RGC-5), where the treatment decreased the expression of caspase 3 and bax, while increased the expression of bcl2 in cell lysates (Shao et al., 2012). Thus, our data demonstrate the importance of RPE cells as a potential target for gene activities involving the action of ANXA1, and its applications should be considered as a possible therapeutic approach in inflammatory ocular diseases.

In this study, we observed that after the treatment, the morphology was not modified, cells proliferation decreased, and cell migration increased. The constitutive over-activation of ERK1/2 may directly interfere with the anti-proliferative effects of ANXA1 on cells; thus, this characteristic may be partially responsible for growth arrest (Prates et al., 2015). The presence of the protein ANXA1 at different concentrations promotes cell migration, which suggests that the presence of the protein modulates cell invasiveness (Kang et al., 2012). Together, these data show us that the protein may influence different processes in inflammatory environments to restore homeostasis.

The analysis of the inflammatory mediators after activation with LPS showed an increase in the release of TNF-α, IL-10, IL-8, IL-6 and MCP-1, which are involved with inflammation. Several investigations indicate that in ocular inflammation, pro-inflammatory cytokines are mainly produced by the inflammatory endothelial cells and the RPE (Ooi et al., 2006; van Laar and van Hagen, 2006; Zenkel et al., 2010). Other studies have also demonstrated the release of some inflammatory mediators, such as IL-6 and IL-8, in RPE cells activated by LPS (Girol et al., 2013; Leung et al., 2009; Paimela et al., 2007; Zanon et al., 2015). The treatment with 33.8 μM of $ANXA1_{Ac2-26}$ peptide decreased the release of these mediators, it was also seen in our research group, where the ANXA1 had modified the expression of pro- and anti-inflammatory cytokines (Damazo et al., 2011; Girol et al., 2013). Our data show that $ANXA1_{Ac2-26}$ reduces the release of inflammatory mediators by epithelial cells, consequently preventing the development of inflammation.

The RaSH technique generated 80 differentially expressed genes, showing that ANXA1 may have a regulatory effect on gene expression after activation with LPS and treatment with $ANXA1_{Ac2-26}$ peptide. Genes selected for validation were *LRAT*, *CTGF*, *MAP1B*, *SETD7* and *ALDH1A3*, which would indicate involvement with the important functions associated with ocular disease, retinal fibrosis syndrome, nervous system functions and development, eye system function and development, and inflammatory disease.

The treatment with the $ANXA1_{Ac2-26}$ peptide revealed a reduction in the expression of all the analyzed genes, confirming the down-regulation of the *CTGF* and *LRAT* genes found in RaSH. Furthermore, a significant reduction in expression occurred at the concentration of 33.8 μM, indicating that the mimetic peptide is probably more effective at higher concentrations in these cells. Thus, the *CTGF* and *LRAT* genes would be considered as potential molecular markers of ophthalmologic inflammation, while *MAP1B*, *ALDH1A3* and *SETD7* genes require further testing.

The *CTGF* gene is correlated with different degrees of retinal fibrosis syndrome that depend on expression levels (Chen et al., 2012). It was demonstrated that its expression at high levels was present in age-related macular degeneration, where it was related to it the production of matrix proteins through the ERK signaling pathway ($p42/p44^{MAPK}$) and $p38^{MAPK}$ (Nagai et al., 2009). The treatment with the peptide decreased the expression of this gene, indicating that ANXA1 may alter its expression through the NF-κB signaling pathway, supported by the fact that these genes are related by the IPA system. The down-regulation of *CTGF* may be an alternative to homeostasis restoration. On the other hand, it was shown that a time-dependent increase in the levels of *CTGF* was concomitant with the retinal vascularization development in neonatal mice (Chintala et al., 2012). The expression of this gene may be important for the development and remodeling of retinal vessels, explaining its increase after LPS induction, and decreased, after the treatment with 33.8 μM, as an attempt to restore homeostasis.

LRAT is important in the retinoid regeneration process, known as the visual cycle, where it esterifies all-*trans*-Retinol to all-*trans*-Retinyl Ester (Mondal et al., 2000; Muñiz et al., 2014). This protein is expressed in diverse fetal and adult tissues, including the RPE (Mondal et al., 2000; Ruiz and Bok, 2010). Mutations in this gene are associated with the early onset of severe retinal dystrophy (Tang et al., 2013; Trevino et al., 2005) and are also associated with progressive retinal atrophy (Dev Borman et al., 2012). The down-regulation of this gene, which is related to the UBC pathway, as shown by the IPA system, may be related to the inflammatory environment, perhaps when there is impairment in the visual system or to help activate the inflammatory signaling cascades.

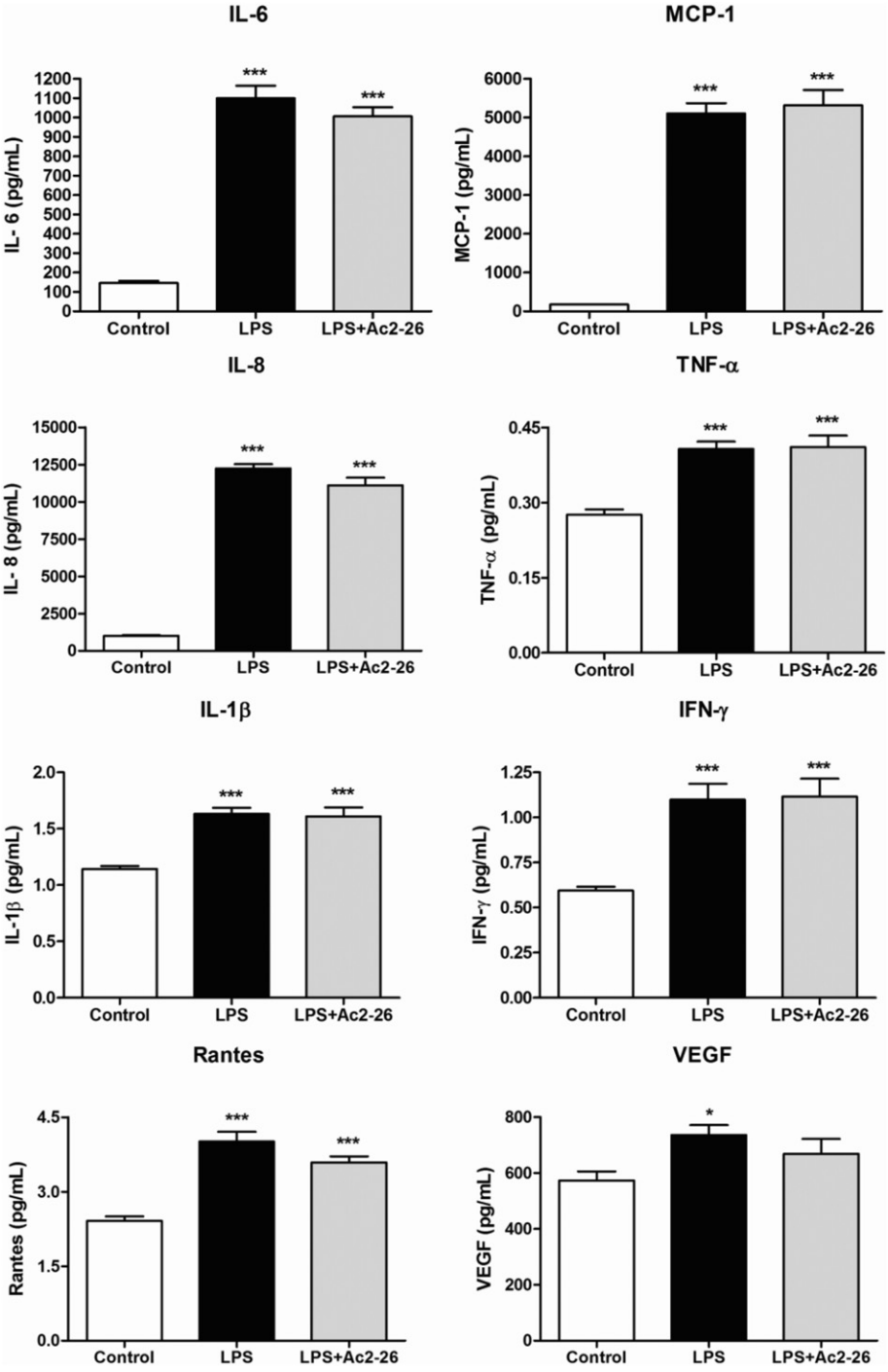

Fig. 5. Effect of $ANXA1_{Ac2-26}$ [1.7 μM] peptide on the expression of pro- and anti-inflammatory metabolites. Analysis of IL-6, IL-8, IL-1β, Rantes, MCP-1, TNF-α, IFN-γ and VEGF, were monitored after the treatment for 72 h. ARPE-19 cells were seeded in complete DMEM:F-12 medium at a concentration of 5×10^4 per well (6-well plate), activated by LPS [10 μg/mL] and treated with the $ANXA1_{Ac2-26}$ peptide. Assays were performed with three independent experiments. Data represent mean ± standard error. Metabolite concentrations were determined using Magpix Xponent software. $^*p < 0.05$ LPS and LPS + $ANXA1_{Ac2-26}$ vs. control.

The studied genes in this work showed differential expression in ARPE-19 cells in an $ANXA1_{Ac2-26}$ treatment-dependent manner. The peptide altered cell proliferation and migration, and gene and protein expression. Few studies have evaluated the effect of $ANXA1_{Ac2-26}$ in the expression of the selected genes, and future molecular studies are needed to show the mechanisms of action of this anti-inflammatory protein. Other experiments will be needed to determine whether the other genes, obtained from the RaSH technique, are causally related or associated with changes induced by the treatment.

5. Conclusion

Our findings show that ANXA1 is involved in the signaling cascades of inflammatory processes, NF-κB and UBC, which decrease cell proliferation and increase migration, modulating *CTGF* and *LRAT* gene expression in RPE cells. Therefore, our results indicate that RPE cells are potential targets for the gene activity of the $ANXA1_{Ac2-26}$ peptide, and their possible use for innovative therapies in the treatment of ocular inflammations should be considered.

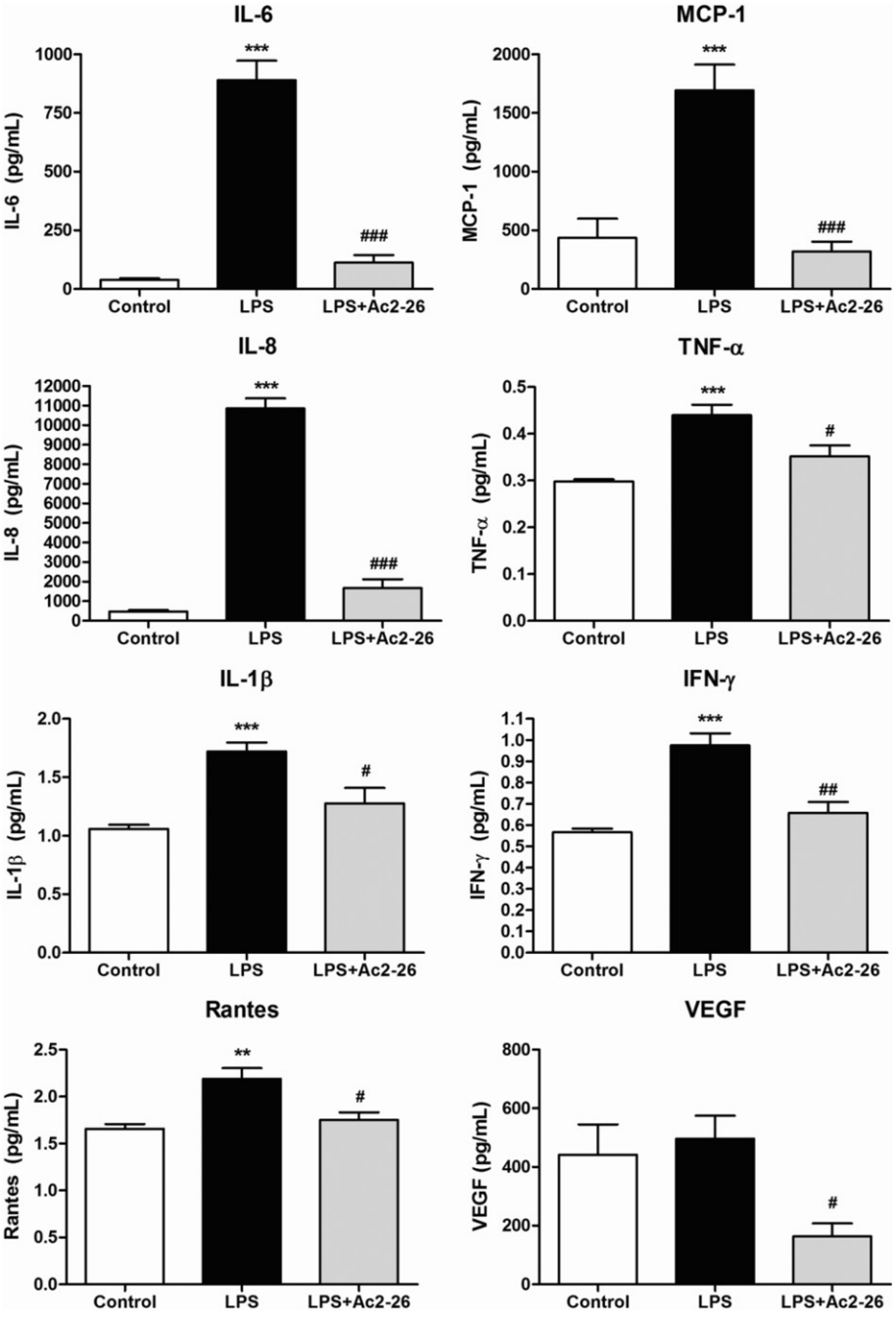

Fig. 6. Effect of $ANXA1_{Ac2-26}$ [33.8 μM] peptide on the expression of pro- and anti-inflammatory metabolites. Analysis of IL-6, IL-8, IL-1β, Rantes, MCP-1, TNF-α, IFN-γ and VEGF, were monitored after treatment for 72 h. ARPE-19 cells were seeded in complete DMEM:F-12 medium at a concentration of 5×10^4 per well (6-well plate), activated by LPS [10 μg/mL] and treated with the $ANXA1_{Ac2-26}$ peptide. Assays were performed with three independent experiments. Data represent mean ± standard error. Metabolite concentrations were determined using Magpix Xponent software. $^*p < 0.05$ LPS and LPS + $ANXA1_{Ac2-26}$ vs. control; $\#p < 0.05$ LPS + $ANXA1_{Ac2-26}$ vs. LPS.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2017.02.032.

Author contributions statement

LTC carried out the cell culture experiments, RaSH assay, real-time PCR assay, assisted with the ELISA and Western blotting assays, performed the statistical analysis, and drafted the manuscript. NMS assisted with the cell culture experiments. KKOM carried out the ELISA and Western blotting assay. LMS and AML carried out the Migration assay. ARDS and WASJr assisted with the RaSH assay. BRC assisted with the real-time PCR assay and Ingenuity® Systems analysis. EHT and SMO were involved in drafting the manuscript and revising it for important intellectual content. FCR-L conceived the study, participated in its design and coordination, and helped draft the manuscript. All of the authors read and approved the final manuscript.

Disclosures

The authors have declared that no competing interests exist.

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