Anti-Inflammatory Mechanisms of the Annexin A1 Protein and Its Mimetic Peptide Ac2-26 in Models of Ocular Inflammation In Vivo and In Vitro

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The eye is an immunologically privileged organ in which inflammatory responses are suppressed by many factors, including the retinal pigmented epithelium (RPE), the main component of the blood–retinal barrier (1, 2). However, some inflammatory processes can cause damage to ocular tissues, such as uveitis, an important cause of blindness worldwide (3). This ocular condition is prevalent in young adults and may be caused by injury or bacterial and parasitic infection or as a consequence of other systemic diseases, such as autoimmune disorders (4–7).

The animal model of endotoxin-induced uveitis (EIU) (8) has been used by several researchers to understand the pathogenesis of uveitis (4, 9). In this model, LPS binds to TLR4 on eye cells (10–12) and stimulates the synthesis and release of proinflammatory chemical mediators, such as NO, platelet-activating factor, TNF-α, IL-1β, and other cytokines (9, 10). The most commonly activated pathway in this model is that of NF-κB, which is followed by its translocation from the cytoplasm to the nucleus (9, 13). The subsequent increased expression of inflammatory mediators exacerbates the development of uveitis by breaking down the blood–ocular barrier, which leads to edema formation and contributes to leukocyte influx (9, 13, 14).

Current pharmacological treatments for uveitis include corticosteroids and chemotherapeutic agents, but the side effects of these drugs, such as increased ocular pressure and cytotoxicity, limit their usage and highlight the need for new therapeutic approaches (4, 5, 15). Among the anti-inflammatory mediators, the endogenous protein annexin A1 (AnxA1), the first characterized member of the annexin superfamily, acts to limit the development of acute inflammatory processes (16–18) and, therefore, may represent an important mediator in ocular inflammation.

Structurally, the annexins contain a small N-terminal region, which varies in length and composition, and a central domain consisting of four to eight repetitions of a highly conserved amino acid sequence. The N-terminal domain is unique for each member of the superfamil, and several investigations characterized this region as a promoter of anti-inflammatory actions (16, 19, 20).
inhibit cell migration (16, 22). Some studies suggest that the regulatory effects of AnxA1 on leukocyte transendothelial migration may be mediated by formyl peptide receptors (fpr) (17, 23) because these effects are blocked by Boc1 and Boc2 antagonists, nonselective fpr antagonists (24, 25).

Supporting this, we demonstrated AnxA1 binding to the fpr2 receptor in neutrophils collected from acute peritonitis exudate in mice, suggesting that the anti-inflammatory action of AnxA1 is mediated by the fpr2 receptor (24). Moreover, endogenous AnxA1’s access to membrane receptors appears to depend on phosphorylation processes that allow translocation of the protein from the cytoplasm to the cell surface (20, 22).

Although the anti-inflammatory activities of AnxA1 and its mimetic peptides, including Ac2-26, have been explored in several in vivo and in vitro investigations (16, 18–20, 26), the role of exogenous AnxA1 in ocular inflammatory processes has not been elucidated, and there are no studies on the administration of Ac2-26 in ocular tissues. Given the common side effects of the current therapies used to treat uveitis (4, 5, 15), we evaluated the effects of endogenous and exogenous AnxA1 protein in rodent ocular tissues in EIU and in an in vitro LPS-inflamed RPE human cell system and further evaluated AnxA1’s mechanism of action. These studies shed light on the genesis of ocular inflammatory disorders and may lead to new therapies for the treatment of these diseases, especially uveitis.

Materials and Methods

In vivo studies

Animals. Male Wistar rats weighing 150–200 g were randomly distributed into six groups (n = 10/group). We also used male mice (25 g body weight) divided into two groups of wild-type (WT) animals and two groups of AnxA1 knockout (AnxA1−/−) mice (27) (n = 3/group). The animals were housed with a 12-h light–dark cycle and were allowed food and water ad libitum. All of the experiments were conducted in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Ethics Committee in Animal Experimentation of São José do Rio Preto Medical School (No. 0467/2009).

Experimental model of uveitis and treatment protocols. For EIU development, the rats were inoculated into the right paw with 1 mg/kg Escherichia coli LPS (serotype 0127:B8; Sigma Chemical, Poole, Dorset, U.K.) diluted in 0.1 ml PBS (21). The animals were kept under these conditions for 24 h (n = 10) or 48 h (n = 10).

The therapeutic efficacy of the Ac2-26 N-terminal peptide of AnxA1 (Ac-AMYVESELQWAQFIENEEQYVQTVK) (28) was tested in two administration of 1 mg/kg peptide. For the topical treatment, the peptide fpr2 receptor, another EIU 24h group (29) was tested in two lute buffer 0.1 M (pH 7.4) for 24 h at 4°C. The therapeutic efficacy of the Ac2-26 N-terminal peptide of AnxA1 (Ac2-26) in the treatment of EIU and an in vitro LPS-inflamed RPE human cell system and further evaluated AnxA1’s mechanism of action. These studies shed light on the genesis of ocular inflammatory disorders and may lead to new therapies for the treatment of these diseases, especially uveitis.

Quantitative analysis of leukocytes in AqH. Neutrophils were quantified in a Neubauer chamber (Laboroptik; Friedrichsdorf, Hessen, Germany).

Histopathological analysis. The intact right eyes of the rats (n = 7) were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, sodium cacodylate buffer 0.1 M (pH 7.4) for 24 h at 4°C, dehydrated by graded methanol, and embedded in LR Gold resin (London Resin; Reading, Berkshire, U.K.) (19). After collecting the AqH, the left eyes (n = 7) were fixed in 10% formalin, dehydrated in graded alcohol, and embedded in paraffin for immunohistochemical analysis. Both eyes of mice from all groups (n = 3/group) were embedded in paraffin.

Infiltrating neutrophils in the anterior and posterior eye segments of LR Gold-embedded material were evaluated in 1-µm tissue sections in a blind fashion and counted using a high-power objective (40×) on an Axioskop 2-Mot Plus Plus Zeiss microscope (Carl Zeiss, Jena, Germany). The number of neutrophils in 10 sections/eye, from different animals, was averaged and recorded. The same method of quantification was used to analyze the number of neutrophils in the mice eyes embedded in paraffin. Values are reported as the mean (± SEM) number of cells/mm².

Immunohistochemical studies. AnxA1, serine-phosphorylated AnxA1 (AnxA1-S²-P), tyrosine-phosphorylated AnxA1 (AnxA1-Y²-P), fpr2 receptor, and cyclooxygenase-2 (COX-2) in ocular tissues were assessed in 5-µm sections of the paraffin-embedded eyes. After an Ag-retrieval step using buffer (pH 6), the endogenous peroxide activity was blocked, and the sections were incubated overnight at 4°C with the primary rabbit polyclonal Abs anti-AnxA1 (1:2000) (Zymed Laboratories, Cambridge, U.K.), anti–AnxA1-S²-P (20, 22, 30), anti–AnxA1-Y²-P (1:1000) (30), anti-fpr2 (1:2000) (24, 26), and anti-COX-2 (1:1000) (Invitrogen, Paisley, U.K.) diluted in 1% BSA (Fisher Scientific, Loughborough, U.K.) (1% BSA for washing, sections incubated in 0.1% BSA) (Dako, Cambridge, U.K.). Positive staining was detected using a peroxide-conjugated streptavidin complex, and color was developed using DAB substrate (Dako). The sections were counterstained with hematoxylin.

For densitometric analyses, three slides from each animal were used, and 15 points were analyzed in three fields of cornea, iris, ciliary processes, and retina for an average related to the intensity of immunoreactivity. The values were obtained as arbitrary units (a.u.).

In vitro studies

Cell culture and treatment protocols. ARPE-19 cells (derived from human RPE; American Type Culture Collection) were grown in a mixture (1:1) of DMEM and Ham F-12 (Cultilab, Campinas, Brazil) (31) supplemented with 10% FBS, 200 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin (Invitrogen).

To determine the optimal length of treatment with the peptide Ac2-26, ARPE-19 cells were cultivated initially in complete medium, as described earlier, and incubated with 10 µg/ml LPS (type Escherichia coli, serotype 0127:B8, Sigma Chemical) for 1, 2, 4, 24, or 48 h. The LPS concentration was chosen based on a study that used the same cell line (32). To evaluate the anti-inflammatory effect of AnxA1, 0.001, 0.005, 0.01, and 0.1 mg/ml Ac2-26 was tested in the activated cells (five independent experiments/ group).

Subsequently, the supernatants were collected to measure the levels of proinflammatory cytokines IL-6 and IL-8. The results revealed that only 0.1 mg/ml peptide was effective in significantly reducing their levels (Supplemental Fig. 1) compared with cells activated by LPS. After determining the best treatment protocol, we established the experimental design. The cells were subjected to the following test conditions: growth on complete medium (control), activated with 10 µg/ml LPS, activated by LPS and treated with 0.1 mg/ml Ac2-26 (LPS/Ac2-26), and treated with Ac2-26 alone (Ac2-26). The cytotoxic effect of the peptide Ac2-26 with regard to the proliferation and viability of ARPE-19 cells was investigated in all experimental groups at 1, 2, 4, 24, 48, and 72 h. The cells were trypsinized and quantified using a Countess Automated Cell Counter (Invitrogen). The results showed no significant difference in the rate of cell proliferation or cell viability among the cell groups studied (Supplemental Fig. 2) compared with their respective controls. Cellular morphology was evaluated using an inverted microscope (CKX41; Olympus). The experimental procedures were conducted in accordance with the rules of the Research Ethics Committee of Federal University of São Paulo Hospital (No. 193009).

Immunofluorescence analysis. For COX-2 detection, ARPE-19 cells were grown on coverslips, fixed in paraformaldehyde 4% for 24 h, washed in PBS, Tween 20 (0.4%), blocked with BSA 1% diluted in 3% normal goat serum, and incubated with polyclonal rabbit anti-COX-2 Ab (Abcam, Cambridge, U.K.) (1:200 in goat normal serum 1.5%). After washing, the cells were incubated with fluorescent Ab anti-rabbit IgG, FITC (Serotec, Oxford, U.K.) (1:100 in goat normal serum 1.5%) for 1 h. The slides were mounted with a solution containing glycerol and PBS (1:1). Goat normal serum was used in the secondary control. The cells were analyzed using a filter with a wavelength of 546 nm on an Axioskop 2-Mot Plus Zeiss microscope, and the enzyme was quantified by densitometry.

Quantitative analysis of NF-κB. To evaluate the translocation of transcription factor NF-κB from the cytoplasm to the nucleus, nuclear extracts were collected by a lysis buffer 0.1% Triton X-100, 1% NP-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. The nuclear extracts were collected by centrifugation at 10,000 g at 4°C for 10 min.

Western blots were performed with standardized conditions to detect phosphorylated and total NF-κB p65. No relevant differences were observed in signal intensity of NF-κB p65 in all tested conditions.
from all experimental groups were prepared at 4 and 24 h (five independent experiments/group) in accordance with the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). The protein concentration was determined using the Bradford assay. For the quantification of NF-κB, a commercial high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) was used.

Gene-expression determination of IL-6, IL-8, and COX-2 in ARPE-19 cells. Expression of mRNA for IL-6, IL-8, and COX-2 was determined by PCR. The total RNA of LPS-activated cells for 4 h and either not treated (LPS) or treated with Ac2-26 (LPS/Ac2-26) (five independent experiments/group) was isolated with TRIzol reagent (Invitrogen), according to the manufacturer's specifications. The cDNA was obtained from 2 μg total RNA, using a commercial High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), and stored at −20°C.

To perform PCR, 2.5 mg/ml cDNA was incubated with 2.5 U Taq DNA polymerase, 0.4 ml 3'- and 5'-specific primers, and 200 mM deoxyribonucleotide triphosphates mix in thermophilic 5× buffer (pH 8.5) for DNA polymerase containing 1.5 mM MgCl2. The following sequences of primers were used: GAPDH, 5'-GGCATGGAACCTGGTCTAGC-3' (sense) and 5'-GGGATGACTGATTGGTCG-3' (antisense); IL-6, 5'-GCCGCTCACCCACAGACA-3' (sense) and 5'-CGCTGGAAGATGTACGG-GAT-3' (antisense); IL-8, 5'-CTGGGCCGGCTGCCTCTT-3' (sense) and 5'-TTAGACCTCCTCTGGCAGAATG-3' (antisense); and COX-2, 5'-CCTCCTGCTTGCGCTTGTA-3' (sense) and 5'-ACACATCTATTGTAGT-CAGGAAAGCT-3' (antisense). The reagents used for the synthesis of cDNA were obtained from Applied Biosystems, and those for PCR were obtained from Promega (Madison, WI).

Procedures in vivo and in vitro

Quantitative analysis of chemical mediators in supernatants of ocular tissues and ARPE-19 cells. The intact eyes (right and left) from rats of all experimental groups were prepared at 4 and 24 h (five independent experiments/group) and treated with Ac2-26 (LPS/Ac2-26) (five independent experiments/group) or with Ac2-26 (LPS/Ac2-26) (five independent experiments/group) treated with Ac2-26 (LPS/Ac2-26) (five independent experiments/group). The total RNA of LPS-activated cells for 4 h and either not treated (LPS) or treated with Ac2-26 (LPS/Ac2-26) (five independent experiments/group) was isolated with TRIzol reagent (Invitrogen), according to the manufacturer's specifications. The cDNA was obtained from 2 μg total RNA, using a commercial High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), and stored at −20°C.

Western blotting analysis of ocular tissue supernatants and ARPE-19 cells. The expression of AnxA1 and fpr2 were verified in the supernatants obtained from all rat groups studied, as well as in ARPE-19 cell groups. The cells from different treatments (control, LPS, LPS/Ac2-26, and Ac2-26) were collected; washed with 5 ml cold PBS; lysed on ice for 15 min with 1 ml a pH 7.4 solution containing one complete mini EDTA-free protease inhibitor mixture tablet (Roche Applied Science, Mannheim, Germany), Tris-HCl 50 mM, NaCl 150 mM, and 1% Triton-X; placed in microtubes; and centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentration in the supernatant was measured using a Bradford assay (Bio-Rad, Hercules, CA).

Equal amounts of rat samples (40 μg) and ARPE-19 cell samples (30 μg) and molecular weight markers were separated by electrophoresis on 15% polyacrylamide gels, using the Mini-PROTEAN Tetra Cell system (Bio-Rad, Hercules, CA), and transferred to nitrocellulose membranes (Hy Bond-C; Amersham Biosciences, Little Chalfont, U.K.). AnxA1 was detected in rat and ARPE-19 cell samples using the rabbit polyclonal Ab anti-AnxA1 (1:1000; Zymed Laboratories, San Francisco, CA), whereas fpr2 was detected in rat samples using rabbit polyclonal Abs anti-fpr2 (1:1000) (24, 26) and in ARPE-19 cells using anti-fpr2 (1:500; Abcam, Cambridge, MA). The signal was amplified using the secondary Ab HRP-

**FIGURE 1.** Histopathological analysis of ocular tissues in EIU. (A–C) Absence of leukocytes in control ocular tissues. Influx of neutrophils (arrows) in the iris, ciliary processes, and retina 24 h after uveitis induction by LPS (EIU 24h) (D–F) and after Boc2 administration (EIU/Ac2-26/Boc2) (M–O). Decreased cell extravasation after systemic (G–I) and topical (J–L) treatments with peptide Ac2-26. Sections: 1 μm. Scale bars, 10 μm. Quantitative analysis of neutrophils in AqH (P) and ocular tissues (Q). The data represent the mean (± SEM) number of neutrophils/105 ml and neutrophils/mm2 in the AqH and ocular tissues, respectively (n = 7 animals/group). ***p < 0.001, *p < 0.05 versus control; †††p < 0.001, ††p < 0.01, versus EIU 48h; ‡‡‡p < 0.001, ‡‡p < 0.01, versus EIU 24h; ††††p < 0.001, †††p < 0.01, ††p < 0.05, versus EIU 48h; †††p < 0.001, versus EIU/Ac2-26 systemic; ††††p < 0.001, †††p < 0.01, versus EIU/Ac2-26 topical.
conjugated anti-rabbit IgG (1:2000; Serotec), and the reaction product was visualized using photographic Hyperfilm after application of the ECL chemiluminescent kit (both from Amersham). Tubulin was detected as reaction control with the mouse mAb IgG (1:1000; Zymed Laboratories). AnxA1 and fpr2 densitometry was performed using Axioskop 2 software.

Statistical analysis
Data were analyzed using GraphPad software version 4.00. One-way ANOVA, followed by the Bonferroni post hoc test for multiple comparisons, was performed to evaluate the mean ± SEM to compare changes in neutrophil numbers, densitometric analysis, and ELISA and PCR values. The p values < 0.05 were considered statistically significant.

Results
Ac2-26 inhibition of neutrophil influx into AqH and ocular tissues in EIU
The eyes collected from control rats did not exhibit transmigrated leukocytes in the anterior (Fig. 1A, 1B) or posterior (Fig. 1C) segments. In contrast, 24 h after intraocular injection of LPS (EIU 24h), we observed an influx of leukocytes, especially neutrophils. These cells were detected primarily in the anterior ocular segment, transmigrated into the AqH, and in the stroma of the iris, ciliary body, and ciliary processes (Fig. 1D, 1E). There were also changes in the posterior ocular segment, with transmigration of leukocytes into the vitreous and retina, especially in the inner plexiform layer (Fig. 1F) (21). A decrease in the number of leukocytes migrating into the AqH was observed after systemic (EIU/Ac2-26 systemic) (15.83 ± 1.30, p < 0.001) or topical (EIU/Ac2-26 topic) (19.00 ± 3.02, p < 0.001) pharmacological intervention (Fig. 1P) compared with EIU 24h animals (55.67 ± 5.67). We also observed reductions in neutrophil extravasation in the ocular tissues collected from EIU/Ac2-26 systemic–treated rats (204 ± 37.79, p < 0.001) (Fig. 1G–I, 1Q) or EIU/Ac2-26 topic–treated rats (313 ± 39.10, p < 0.001) (Fig. 1J–L, 1Q) compared with EIU 24h animals (747.8 ± 57.39). The efficacy of EIU/Ac2-26 treatment was more pronounced after systemic administration.

The reduced number of transmigrated cells in the pharmacologically treated groups was similar to that observed in EIU 48h animals, which achieved a temporal remission in the inflammatory process. The numbers of neutrophils were significantly reduced in the AqH (19.75 ± 3.79, p < 0.001) (Fig. 1P) and tissues (192.7 ± 40.38, p < 0.001) (Fig. 1Q) at this time point compared with the same ocular compartments 24 h after inflammation induction.

Simultaneously treating animals with Ac2-26 and Boc2 (EIU/Ac2-26/Boc2) reversed the number of neutrophils migrating into the AqH (47.67 ± 4.47) (Fig. 1P) and ocular tissues (623.9 ± 45.77) (Fig. 1M–O, 1Q), showing that treatment with an fpr antagonist prevented the inhibitory effect of Ac2-26 on neutrophil extravasation and restored these values to those of EIU 24h animals (Fig. 1P, 1Q).

In vivo administration of Ac2-26 reduces protein leak and chemical mediator release during EIU
The total protein levels were increased in the EIU 24h animals (p < 0.001) compared with the control group. The protein levels were lower in the EIU 48h group (p < 0.001) than in the EIU 24h group, showing resolution of the inflammatory process. In addition, reduced levels of protein were detected in EIU 24h animals treated either systemically (p < 0.001) or topically (p < 0.05) with Ac2-26 compared with nontreated EIU 24h rats (Fig. 2A). In contrast, in the EIU 24h animals treated simultaneously with Ac2-26 and Boc2, the total protein concentration was similar to that in the nontreated EIU 24h animals (Fig. 2A), showing that Boc2 treatment reversed the inhibitory effects of Ac2-26 on enhanced vascular permeability in the ocular tissue.

The role of chemical mediators in EIU and the effects of Ac2-26 treatment were analyzed by measuring the cytokines IL-1β, IL-6,
and TNF-α, as well as NO, in the ocular tissue. The results indicated low levels of cytokines and NO in eyes of control rats. As expected, the levels of these mediators were increased significantly in the eyes of EIU 24h animals \((p < 0.001)\) (Fig. 2B–E). When Ac2-26 was administered, systemically or topically, we observed decreased levels of IL-1β, TNF-α, and NO for both treatments and decreased levels of IL-6 in systemically treated animals compared with the nontreated EIU 24h animals. Similar reductions in cytokine secretion and NO production were observed in the EIU 48h group, showing temporal resolution of the inflammatory process (Fig. 2B–E).

When Ac2-26 was administered, systemically or topically, we observed decreased levels of IL-1β, TNF-α, and NO for both treatments and decreased levels of IL-6 in systemically treated animals compared with the nontreated EIU 24h animals. Similar reductions in cytokine secretion and NO production were observed in the EIU 48h group, showing temporal resolution of the inflammatory process (Fig. 2B–E). In contrast, animals from the EIU 24h group treated simultaneously with Ac2-26 and Boc2 showed levels of chemical mediators equivalent to the nontreated EIU 24h animals, with a significant increase in IL-1β \((p < 0.001)\), IL-6 and TNF-α \((p < 0.001)\), and NO \((p < 0.001)\) compared with the naive group (Fig. 2B–E).

**COX-2 expression during EIU is inhibited after Ac2-26 treatment but exacerbated in the presence of Boc2 and in AnxA1−/− animals**

We observed COX-2 expression only in the retina of control rats (Fig. 3A–C), where it was reported to be constitutively expressed (33). In the EIU 24h animals, we detected COX-2 expression in the anterior and posterior ocular segments (Fig. 3D–F). Densitometric analysis corroborated the increased expression of COX-2 in the EIU 24h and 48h animals \((p < 0.001)\) compared with the control animals (Fig. 3Q), as well as the decreased COX-2 expression in the EIU 24h animals after systemic \((p < 0.001, \text{Fig. } 3G–I)\) or topical \((p < 0.001, \text{Fig. } 3J–L)\) pharmacological treatment with respect to the EIU 24h group. The assays also showed reduced COX-2 expression in the EIU/Ac2-26 systemically treated animals compared with the EIU 48h group \((p < 0.001)\). Increased expression of the enzyme was observed in the ocular tissues of EIU/Ac2-26 topic–treated animals compared with the EIU/Ac2-26/systemic group \((p < 0.05)\).

The EIU/Ac2-26/Boc2 group showed higher expression of COX-2 compared with the EIU 24h/Ac2-26–treated animals (Fig. 3M–O), with a significant increase \((p < 0.001)\) in the enzyme levels (Fig. 3Q). No expression was detected in the reaction control (Fig. 3P). In addition, the expression of COX-2 in mice was similar to that observed in rat eyes, with increased expression in inflamed ocular tissues from WT animals after 24 h of EIU (WT EIU 24h, \(p < 0.001\), Fig. 4D–F, 4O) compared with WT naive mice (Fig. 4A–C, 4O). Increased COX-2 expression was also observed in the inflamed eyes of AnxA1−/− mice (AnxA1−/− EIU 24h, \(p < 0.001\), Fig. 4J–L, 4O) relative to naive AnxA1−/− mice (Fig. 4G–I, 4O). Densitometric analysis also revealed exacerbated inflammation in

![FIGURE 3.](http://www.jimmunol.org/) Expression of COX-2 in ocular tissues in EIU. In control eyes, there was no immunostaining of the epithelia of the cornea (A) or ciliary processes (B) and weak immunostaining of the retina (C). Epithelial and neuronal cells showing intense immunoreactivity for COX-2 in the cornea (D), ciliary processes (E), and retina (F) of animals 24 h after LPS administration. The systemic (G–I) and topical (J–L) treatment with the Ac2-26 peptide led to decreased expression in epithelial and neuronal cells, but intense immunostaining is observed after Ac2-26 plus Boc2 administration (M–O). The insets show higher magnifications of the RPE. (P) Absence of immunoreactivity in the reaction control. Sections: 5 μm. Counterstaining: hematoxylin. Scale bars, 10 μm. (Q) Densitometric analysis of COX-2. Data indicate the mean (± SEM) densitometric index (a.u.) \((n = 7\) animals/group). ***\(p < 0.001\), *\(p < 0.05\), versus control; \(11^*p < 0.001\), versus EIU 24h; \(11^*p < 0.001\), \(11^*p < 0.01\), versus EIU 48h; \(11^*p < 0.001\), \(^3p < 0.001\), ‡‡\(p < 0.05\), versus EIU/Ac2-26 systemic; xxx\(p < 0.001\), versus EIU/Ac2-6 topic.
the ocular tissues of AnxA1−/− EIU 24h mice (p < 0.01) compared with WT EIU 24h mice (Fig. 4O).

Endogenous AnxA1 modulates inflammation in ocular tissues

AnxA1 expression was observed in the epithelia of the cornea, iris, ciliary processes, and retina of control rats (Fig. 5A–D). There was an increase in the AnxA1 expression in these regions in the EIU 24h group (p < 0.001, Figs. 5E–H, 6A, 6B, 6M) compared with the control group. However, we observed reduced expression of AnxA1 in the EIU 48h group (p < 0.01, Figs. 5I–L, 6M) and in EIU 24h animals treated with Ac2-26 peptide administered either systemically (p < 0.001, Figs. 5M–P, 6C–F, 6M) or topically (p < 0.001, Figs. 5Q–T, 6M). The ocular tissues of the EIU/Ac2-26 systemic animals showed decreased immunostaining densities compared with the control (p < 0.05), EIU 48h (p < 0.001), and EIU/Ac2-26 topic–treated (p < 0.001) groups (Fig. 6M). Expression in the EIU/Ac2-26/Boc2 group was similar to that of EIU 24h animals, with a significant increase in AnxA1 expression relative to the other groups (Figs. 5U–W, 6M). The immunolabeling specificity was confirmed using the reaction control (Fig. 5X).

AnxA1−/− mice were used to confirm the role of endogenously expressed AnxA1 on EIU. As expected, we did not observe im-

FIGURE 4. COX-2 overexpression and exacerbated neutrophil migration in ocular tissues of AnxA1-knockout mice in EIU. Absence of immunoreactivity in the epithelia of the cornea and ciliary processes and weak immunostaining in the retina of control eyes from WT (A–C) and AnxA1-knockout (AnxA1−/−) (G-I) mice. Intense expression in the cornea, ciliary processes, and retina 24 h after LPS administration in the eyes of WT (D–F) and AnxA1−/− (J–L) animals. (M) Absence of immunoreactivity for AnxA1 in AnxA1−/− mice after 24 h of induced uveitis (AnxA1−/− EIU 24h) as a negative control to AnxA1, indicating the Ab specificity. Sections: 5 μm. Counterstaining: hematoxylin. Scale bars, 10 μm. Quantitative analysis of neutrophils (N) and densitometric analysis of COX-2 (O) in ocular tissues. The data represent the mean (± SEM) neutrophils/mm² and densitometric index (a.u.) in the ocular tissues (n = 3 animals/group). ***p < 0.001, *p < 0.5, versus WT naive; †††p < 0.001, versus AnxA1−/− naive; ‡‡p < 0.01, versus WT EIU 24h.
The Fpr2 receptor was expressed in the same ocular tissues in which AnxA1 was observed (Fig. 6G–L), and pharmacological treatment increased the expression of Fpr2 (Fig. 6I–L). The expression of AnxA1 was observed (Fig. 6G–L), and pharmacological treatment increased the expression of AnxA1 (Fig. 6G–L). The expression of AnxA1 was higher in animals treated with the peptide (p < 0.001), compared with naive mice (Fig. 4N). A more marked transmigration was observed in AnxA1−/− EIU 24h mice (p < 0.01) in relation to WT EIU 24h mice (Fig. 4N). Furthermore, as described previously, the expression of COX-2 was exacerbated in AnxA1−/− animals (Fig. 4O).

AnxA1 phosphorylation during EIU is phosphorylated

Phosphorylation is an important posttranslational modification that mediates the translocation of AnxA1 from the cytoplasm to the cell surface (20). Immunohistochemical analysis revealed the presence of AnxA1-S27-P04 in the anterior and posterior ocular segments (Supplemental Fig. 3), perfectly overlapping with the distribution of AnxA1 detected with an Ab directed against all AnxA1 forms. In contrast, no AnxA1-Y21-P04 was detected in the ocular tissues of any of the groups (Supplemental Fig. 3). The immunolabeling specificity was confirmed using the reaction control.

Densitometric analysis of AnxA1-S27-P04 expression (Fig. 6N) was similar to that observed for total AnxA1 from the cytoplasm to the cell surface (20). Immunohistochemical analysis revealed the presence of AnxA1-S27-P04 in the anterior and posterior ocular segments (Supplemental Fig. 3), perfectly overlapping with the distribution of AnxA1 detected with an Ab directed against all AnxA1 forms. In contrast, no AnxA1-Y21-P04 was detected in the ocular tissues of any of the groups (Supplemental Fig. 3). The immunolabeling specificity was confirmed using the reaction control.

The expression of AnxA1 in AnxA1−/− mice (Fig. 4M). The quantification of neutrophils in ocular tissues of mice showed an increase in these cells in EIU animals, both WT and AnxA1−/−, compared with naive mice. Immunohistochemical analysis revealed the presence of AnxA1-S27-P04 in the anterior and posterior ocular segments (Supplemental Fig. 3), perfectly overlapping with the distribution of AnxA1 detected with an Ab directed against all AnxA1 forms. In contrast, no AnxA1-Y21-P04 was detected in the ocular tissues of any of the groups (Supplemental Fig. 3). The immunolabeling specificity was confirmed using the reaction control.

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In addition, to correlate the in vivo findings with other quantitative assays, we determined the expression of AnxA1 and Fpr2 in ARPE-19 cells by Western blotting in control cells, cells activated with LPS 24 h without treatment (LPS) or with Ac2-26 treatment (LPS.Ac2-26), and cells treated only with Ac2-26 (Ac2-26). The different experimental conditions did not affect cellular proliferation or viability (Supplemental Fig. 2), and AnxA1 and Fpr2 expression was detectable in all groups (Fig. 7D).

AnxA1 and Fpr2 expression in ocular supernatants and LPS-activated ARPE-19 cells corroborate the tissue expression in EIU

To confirm the data obtained from immunohistochemical studies, we performed Western blotting analysis of AnxA1 and Fpr2 expression in the supernatants from rat ocular tissues after maceration (Fig. 7A). Increased expression of intact and N-terminal–cleaved AnxA1 was verified in EIU 24h, EIU.Ac2-26 systemic, EIU.Ac2-26 topical, and EIU.Ac2-26/Boc2 groups (p < 0.001) relative to control and EIU 48h animals (Fig. 7B). The expression of Fpr2 was augmented after treatment with Ac2-26 in the EIU.Ac2-26 systemic animals compared with controls (p < 0.001, Fig. 7C). Boc2 administration strongly reduced the receptor levels (Fig. 7A, 7C).
Densitometric analysis revealed that LPS treatment and Ac2-26 administration significantly reduced the levels of intact and N-terminal–cleaved AnxA1 (p < 0.001) in comparison with control cells. Simultaneous incubation with the peptide and LPS led to reduced expression of both AnxA1 forms compared with cells treated only with LPS (Fig. 7E).

Also, we observed that ARPE-19 cells express the fpr2 receptor (Fig. 7F), and the receptor was upregulated in groups treated with LPS, Ac2-26, or LPS plus Ac2-26 (p < 0.001) compared with control cells. Higher receptor expression levels were observed in LPS/Ac2-26 (p < 0.05) and Ac2-26 (p < 0.001) groups relative to the LPS-treated group. Higher receptor expression was also observed in the Ac2-26 group (p < 0.001) relative to the LPS/Ac2-26 group (Fig. 7F).

**FIGURE 6.** Expression of AnxA1 and fpr2 in rat ocular tissues. Immunoreactivity for AnxA1 (A, B) and fpr2 (G, H) in the anterior eye segment 24 h after administration of LPS (EIU 24h). After systemic treatment with Ac2-26 (EIU/Ac2-26 systemic), decreased expression of AnxA1 was observed in the anterior (C, D) and posterior (E, F) ocular segments, and intense immunostaining was observed for fpr2 (I–L), primarily in the periphery of the ciliary processes epithelium and on the surface of endothelial cells (arrows). Sections: 5 μm. Counterstaining: hematoxylin. The dashed boxes indicate the magnified areas in those panels. Scale bars, 10 μm. Densitometric analysis of AnxA1 (M), AnxA1-S27-PO4 (N), and fpr2 (O). Data represent the mean (± SEM) densitometric index (a.u.) (n = 7 animals/group). **p < 0.001, *p < 0.01, *p < 0.05, versus control; †††p < 0.001, ††p < 0.01, †p < 0.05, versus EIU 24h; ††††p < 0.001, ††††p < 0.01, †††p < 0.05, versus EIU/Ac2-26 systemic; an**p < 0.01, versus EIU/Ac2-26 topical.

**LPS-induced cytokine secretion and cytoplasmic COX-2 expression by ARPE-19 cells are reduced by Ac2-26**

Because in vivo LPS-induced ocular inflammation was characterized by enhanced neutrophil infiltration, cytokine secretion, and COX-2 expression, which were reversed by peptide treatment, we investigated the role of the Ac2-26 peptide on the secretion of chemotactic cytokines and the cellular localization of COX-2 in LPS-stimulated ARPE-19 cells.

Because LPS induces RPE cells to secrete high levels of IL-6 and IL-8 (2), we checked the presence of these mediators in the cell supernatants under the different experimental conditions. We observed significant increases in the levels of IL-6 and IL-8 in the LPS groups (p < 0.001) relative to controls (Fig. 8A, 8B). However, treatment with the peptide significantly reduced the levels of these cytokines (p < 0.001). The Ac2-26 groups showed patterns similar to the untreated control groups for both of these cytokines.

Immunofluorescence analysis of COX-2 expression showed intense nuclear positivity in the control group (Fig. 8C1, 8D), whereas LPS treatment induced predominantly cytoplasmic positivity (p < 0.001, Fig. 8C2, 8D). There was no difference in the immunofluorescence intensity between the cytoplasm and nucleus in the LPS/Ac2-26 group (Fig. 8C3, 8D). This group showed increased cytoplasmic immunoreactivity compared with controls (p < 0.001) but reduced cytoplasmic expression in relation to the LPS-treated group (p < 0.001). Nuclear positivity in the LPS/Ac2-26 group was also decreased compared with the control group (p < 0.001). Cells treated with the peptide alone showed an expression pattern resembling that of the control group (Fig. 8C4, 8D).

**Molecular analysis of NF-κB in ARPE-19 cells**

To evaluate the anti-inflammatory mechanism of Ac2-26, we quantified NF-κB nuclear translocation in ARPE-19 cells. LPS treatments for 4 or 24 h significantly increased NF-κB translocation from the cytoplasm to the nucleus relative to the control group (Fig. 9). There was no significant difference between the LPS and LPS/Ac2-26 groups at either analyzed time point. Ac2-26–treated cells showed NF-κB levels coincident with those of control cells (Fig. 9).
Effects of AnxA1 on IL-6, IL-8, and COX-2 gene expression in ARPE-19 cells

To assess whether the reductions in IL-6, IL-8, and COX-2 expression in ARPE-19 cells were dependent on changes in protein synthesis, we quantified the mRNA levels of these proteins by PCR. We observed no significant differences in mRNA levels for IL-6 (Fig. 10A), IL-8 (Fig. 10B), or COX-2 (Fig. 10C) between the LPS and LPS/Ac2-26 groups. A model of all our results can be seen in Fig. 11.

Discussion

The severe side effects of the current treatments for uveitis challenge researchers to develop new therapeutic strategies (4, 5, 15).

Effects of AnxA1 on IL-6, IL-8, and COX-2 gene expression in ARPE-19 cells

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In this study, we developed in vivo and in vitro experimental models to evaluate AnxA1, a known anti-inflammatory protein (16–18) that has not been well studied in ocular tissue. Moreover, to our knowledge, this is the first study of the administration of the AnxA1 mimetic peptide in ocular tissues.

Our in vivo analyses found that the inflammatory stimulus induced by LPS caused the release of chemical mediators (TNF-α, IL-1β, IL-6, and NO), increased expression of the proinflammatory enzyme COX-2, breakdown of the blood–ocular barrier, and intense influx of leukocytes in EIU 24h rats (9, 14, 21). The inflammatory process regressed in a temporal manner, with decreased leukocyte extravasation and levels of proinflammatory mediators observed in EIU 48h animals (9).

The results obtained after systemic and topical EIU treatment with Ac2-26 revealed the anti-inflammatory activity of AnxA1 in ocular tissues. Both treatments, especially the systemic treatment, led to decreased leukocyte influx in ocular tissues and AqH and may be associated with the mimetic peptide’s ability to reduce the production of TNF-α, IL-1β, IL-6, and NO, as well as to inhibit COX-2 expression in ocular tissues during EIU. An exacerbated inflammatory response, characterized by increased neutrophil influx and COX-2 expression, was observed in AnxA1−/− mice; this supports the importance of AnxA1 in the resolution of ocular inflammation and shows that AnxA1’s actions on ocular tissue do not depend on animal species. These results are consistent with several investigations into the anti-inflammatory effects of endogenous or exogenous AnxA1, especially its N-terminal mimetic peptide, which demonstrated inhibited leukocyte recruitment to inflammatory sites (17, 18, 26, 27, 34).

AnxA1 expression also inhibits the release of cytokines in response to LPS in macrophages (34, 35) and in zymosan-induced peritonitis (27). Studies using AnxA1-knockout mice showed increased expression of COX-2 in macrophages (36), lung, thymus (37), cartilage, bone (38), and spinal cord (39).

After evaluating the anti-inflammatory activity of exogenous AnxA1, we studied the expression of the endogenous protein and its possible mechanism of action in our in vivo model. AnxA1 expression was observed in the epithelia of the cornea, iris, ciliary processes, and retina in all of the groups, and it occurred in the same regions where TLR4 expression is observed (10–12). In uveitis, cytokines and NO are produced mainly by inflammatory and endothelial cells, but these mediators can also be released by the epithelial cells of the cornea and RPE (40), consistent with the sites of AnxA1 expression.

There was increased AnxA1 immunostaining in all regions studied in the EIU 24h group, whereas in the Ac2-26–treated groups, especially those receiving systemic treatment, there was decreased expression of AnxA1 compared with the EIU 24h, EIU 48h, and control groups. Our results in ocular tissues are consistent with those found in the liver of mice, in which a marked increase in AnxA1 expression was observed after LPS endotoxemia (30, 41), followed by a reduction in AnxA1 expression in the later phases of inflammation (30). In this study, we also show the same pattern of AnxA1 expression in EIU in rats.

**FIGURE 9.** NF-κB in ARPE-19 cells after Ac2-26 treatment. Dosage of NF-κB on nuclear extract of cells at 4 h (A) and 24 h (B). Data represent the mean (± SEM) absorbance/mg protein (n = 5 independent experiments/group). ***p < 0.001, **p < 0.01 versus control; †††p < 0.001, versus LPS; ‡‡‡p < 0.001, versus LPS/Ac2-26.

**FIGURE 10.** Effect of Ac2-26 on gene expression of proinflammatory mediators in ARPE-19 cells. Relative mRNA expression of IL-6 (A), IL-8 (B), and COX-2 (C) was determined using PCR. Data represent the mean (± SEM) a.u. (n = 5 independent experiments/group).
Because the AnxA1 N-terminal domain presents sites for posttranslational modifications, including phosphorylation, glycosylation, and proteolysis (16, 18–20), and to better understand the mechanism of action of AnxA1 in ocular inflammation, we analyzed possible posttranslational modifications that the endogenous protein may undergo (20, 30). We found a specific AnxA1 posttranslational modification by immunohistochemical analysis, with AnxA1 phosphorylation on a serine residue rather than on a tyrosine, which suggests its activation by protein kinase C (22) and subsequent translocation to the cell membrane (20). Phosphorylated AnxA1 may act endogenously in epithelial and neuronal tissues, promoting the reduction of COX-2 expression (42).

As shown for AnxA1, AnxA1-S27-PO4 expression was more intense in EIU 24 h rats, with decreased immunoreactivity in the treated groups and in EIU 48 h animals, consistent with the late phases of ocular inflammation (21). In contrast with our results, other researchers (30) have observed increased expression of AnxA1-S27-PO4 in hepatocytes in the early phase of LPS-induced inflammation, while AnxA1-Y21-PO4 was observed in the resolution phase of inflammation. However, in our system, AnxA1 serine phosphorylation occurs in the resolution phase of disease.

After demonstrating the anti-inflammatory action of endogenous AnxA1 and its mimetic peptide in EIU, and because cell surface AnxA1 may act through the fpr (23–25), we verified the expression of the fpr2 receptor and conducted experiments using Boc2, an fpr antagonist (23). Our results demonstrated increased expression of fpr2 in the EIU 48 h and Ac2-26–treated groups, with preferential expression on the surface of the ciliary processes, epithelial cells, and endothelial cells. Interestingly, receptor expression was significantly decreased in the EIU/Ac2-26/Boc2 group compared with the other groups, suggesting fpr2 inactivation after binding to its antagonist (43).

To validate the data obtained from immunohistochemistry, the levels of AnxA1 and fpr2 were analyzed in the supernatants of rat ocular tissues after maceration. As expected, the expression of AnxA1 was lower in control and EIU 48 h groups and was increased in EIU 24 h and EIU/Ac2-26/Boc2 animals. However, unlike the data obtained in the tissues, we observed high levels of the protein in the peptide-treated groups. This apparent discrepancy may be explained by the different methods used. With the immunohistochemical studies, we could localize the expression of AnxA1 in tissues, where it was quantified by densitometry. Western blotting analysis showed high levels of the protein in the peptide-treated groups, as opposed to data obtained from the immunohistochemical and densitometric studies. This apparent discrepancy may be explained by the different approaches of the methodologies used. In immunohistochemical studies, we quantified the AnxA1 in the eye tissues, but it was not considered in the expression of the protein in inflammatory cells or in aqueous and vitreous humors. For Western blotting analysis, we used the macerated material obtained from breaking-up whole eyes and, hence, the protein expression in inflammatory cells and ocular humors could also be quantified. In contrast, the whole eye was used when obtaining the supernatants, which allowed the quantification of AnxA1 in all tissues in combination with inflammatory cells. These data also indicate the protein release into ocular chambers during inflammation. In a previous investigation (21), we showed increased expression of AnxA1 in AqH of rats pretreated with c48/80 and evaluated at 24 h after LPS-induced uveitis, pointing to mast cells as an important source of AnxA1.

With regard to the Western blotting analysis of fpr2, the data confirm the observations from immunohistochemistry and showed increased expression of the receptor in the systemic–treated animals and reduced expression of fpr2 after administration of Boc2, which corroborates the idea of inactivation of the receptor by the antagonist (43).

In the EIU/Ac2-26/Boc2 group, the fpr antagonist abrogated the anti-inflammatory effects of the peptide, and this group was characterized by extravasated neutrophils, elevated levels of proinflammatory mediators, and intense expression of COX-2 in ocular tissues. These findings support the idea that AnxA1 and Ac2-26 act through the fpr. Similar results were observed in a murine experimental peritonitis model, in which the anti-inflammatory effects of the Ac2-26 peptide were reduced in the presence of the antagonist Boc2 (25). In another experiment using the same model, leukocyte influx was inhibited in the presence of the peptide in AnxA1- and fpr-knockout animals, but inflammation

![FIGURE 11. Schematic model of AnxA1 and Ac2-26 peptide mechanism of action in EIU. LPS, through TLR4 and NF-κB, triggers the production of COX-2, the release of cytokines (1), and phosphorylation of endogenous AnxA1 (2). The serine-phosphorylated AnxA1 may act endogenously to reduce the expression of COX-2 (3). This posttranslational modification also allows the translocation of AnxA1 to the cell surface, where it exerts anti-inflammatory actions, in an autocrine or paracrine manner, through the fpr2 receptor (4). The Ac2-26 peptide mimics the actions of AnxA1 after binding with fpr2 (5) and inhibits the influx of neutrophils (6).](http://www.jimmunol.org/)
was restored following Boc2 administration (24). Other studies demonstrated that the beneficial effects of Ac2-26 treatment for gastric ulcers were impaired in the early phases of ulcer healing in animals cotreated with Boc2 (44).

Based on the relevance of the phenotypic and functional characteristics of human ARPE-19 cells to inflammation (32) and to confirm the in vivo rodent data, we subsequently assessed AnxA1 expression in ARPE-19 cells activated by LPS and treated with Ac2-26.

Western blotting confirmed the expression of AnxA1 and fpr2 in ARPE-19 cells. The data showed lower levels of AnxA1 after LPS activation, which indicated that the protein is externalized during inflammation. Furthermore, mimetic peptide administration decreased AnxA1 levels, corroborating the in vivo findings. Other researchers (45) observed a reduction in AnxA1 expression in murine bone marrow–derived dendritic cells after 24 h of LPS stimulation, with increased AnxA1 levels found in the supernatant. Fpr2 expression in ARPE-19 cells was also consistent with the in vivo results and showed increased receptor levels in LPS-activated RPE cells, especially after the administration of Ac2-26.

An analysis of proinflammatory mediators showed increased levels of IL-6 and IL-8 in LPS-activated cells compared with control cells. These data were consistent with other studies, which also showed the release of IL-6 (2, 32) and IL-8 (2) after LPS activation of ARPE-19 cells. Moreover, peptide treatment was able to reduce the levels of IL-6 and IL-8 production compared with the LPS group, corroborating the in vivo results and showing a direct effect of Ac2-26 on eye cells. Those data suggested that the decreased number of neutrophils is linked to reduced levels of cytokines in the ocular tissue. In addition, cells treated with peptide alone exhibited the same expression pattern as did the control cells, indicating that AnxA1 does not induce the production of inflammatory mediators at the levels used.

Immunofluorescence analysis of COX-2 levels showed increased expression of this enzyme in the cytoplasm of ARPE-19 cells after LPS activation. However, ARPE-19 cells stimulated with LPS and treated with Ac2-26 showed decreased levels of COX-2 expression. A recent study (46) showed increased expression of COX-2 in astrocytes and microglial cells isolated from the cerebral cortex of rodents and exposed to LPS relative to control. Again, our in vitro results were in agreement with those found in EIU.

Because of the importance of understanding the anti-inflammatory mechanism of AnxA1 in ocular tissues, we conducted in vitro experiments to evaluate the AnxA1-signaling pathway. Our data showed that LPS increased the translocation of the transcription factor NF-κB to the nucleus, leading to the release of IL-6 and IL-8 into the cell culture supernatant and increased expression of COX-2 in the ARPE-19 cytoplasm. However, analysis of NF-κB after Ac2-26 treatment showed that the anti-inflammatory effects of AnxA1 are independent of NF-κB translocation and are consistent with results observed in RAW264-7 macrophages activated by LPS and treated with AnxA1 (47). Another study using the same macrophage cell line revealed reduced activity of the COX-2 pathway independent of NF-κB after phytotherapy (48).

Although our results indicated that AnxA1 did not alter NF-κB translocation, this subject is still controversial. Other studies showed that AnxA1 is capable of inhibiting NF-κB activity in the synovial cells of rats subjected to collagen-induced arthritis (49); in different lineages of human mammary (50), pancreatic, and colon cancer cells; and in mouse intestinal mucosa epithelial cells (51).

We used PCR to evaluate whether the reduced expression of IL-6, IL-8, and COX-2 in ARPE-19 cells following Ac2-26 treatment was due to pre- or posttranscriptional activity. Interestingly, the inhibitory effect on these inflammatory mediators is not dependent on gene-expression control, because the mRNA levels for the three studied mediators were not altered by Ac2-26 treatment. These data suggested that AnxA1 acts posttranscriptionally, perhaps by cleavage or degradation of the analyzed mediators, and somehow inhibits their externalization or expression. The reported ability of AnxA1 to reduce cytosolic phospholipase A2 activity and the release of arachidonic acid (42) indicate that AnxA1 can promote posttranscriptional inhibition.

Taken together, our results indicated the participation of AnxA1 and its mimetic peptide in resolving ocular inflammation both in vivo and in vitro and demonstrated the potential application of AnxA1 in ocular inflammatory conditions, especially uveitis. Moreover, our data allow us to report that the anti-inflammatory activities of AnxA1 occur after a specific serine-phosphorylation event. The phosphorylated AnxA1 may act endogenously to reduce the expression of COX-2, and it can also be translocated to the cell surface, where both the protein and its mimetic peptide can activate the fpr2 receptor, inhibiting the release of inflammatory mediators independently of the NF-κB–signaling pathway, possibly in a posttranscriptional manner (Fig. 11). However, further studies are needed to address this question.

Disclosures
The authors have no financial conflicts of interest.

References


