Article

Augmenting Endogenous Levels of Retinal Annexin A1 Suppresses Uveitis in Mice

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Purpose: The purpose of this study was to examine the expression of the anti-inflammatory protein Annexin A1 (AnxA1) in mice and human retinae during uveitis and to determine whether local administration of human recombinant AnxA1 (hrAnxA1) can suppress uveitis in mice.

Methods: Retinal sections from mice (healthy normal and uveitis) and postmortem human (no history of eye disease ($n = 5$) and uveitis ($n = 7$)) were stained for AnxA1 expression and imaged by immunofluorescence microscopy. AnxA1 cellular expression was determined by colabeling with CD45, glial fibrillary acidic protein (GFAP), and Iba-1 cells, with additional staining of AnxA1 receptors formyl peptide receptor 1 (FPR1) and FPRL1/FPR2. Mice with acute endotoxin-induced uveitis and chronic experimental autoimmune uveitis were treated locally by intravitreal injection with hrAnxA1, and disease was assessed by clinical scoring and quantification of leukocyte infiltrate via flow cytometry.

Results: Constitutive expression of AnxA1 was observed in both healthy mouse and human retinae, and its expression increased during uveitis compared to healthy controls. AnxA1 colocalizes predominantly with CD45$^+$ cells, GFAP$^+$ macroglia, and to a lesser extent, Iba-1$^+$ myeloid cells. We also demonstrate that local treatment with hrAnxA1 attenuates the severity of uveitis in mice.

Conclusions: These data indicate that locally expressed AnxA1 is elevated in the retina during intraocular inflammation. We demonstrate that local administration of hrAnxA1 to augment levels results in suppression of uveitis in mice.

Translational Relevance: Our data suggest that elevated expression of retinal AnxA1 in human uveitis may be immunoregulatory and that local supplementation with hrAnxA1 may provide a potential novel treatment for inflammatory eye diseases such as noninfectious uveitis.

Introduction

Annexin A1 (AnxA1) plays many roles in cell physiology and is both highly conserved across species and widely distributed across many cell types and biological fluids.[1] AnxA1 binds to membrane acidic phospholipids in the presence of Ca^{2+}, playing important intracellular membrane/actin–cytoskeletal-associated roles such as granule fusion and exocytosis, as well as extracellular and secreted roles such as marking apoptotic neurons for phagocyto-sis,[2] enhancing islet insulin secretion,[3] modulating acute and chronic inflammation,[4] and exhibiting altered expression linked to tumor progression.[5] AnxA1 contributes to immune regulation by acting in part as a key mediator of glucocorticoid action in cells of the innate immune system.[6] AnxA1 acts in the anti-inflammatory/proresolution arm of the innate response by moderating leukocyte adhesion and migration, cytokine production, and histamine release, promoting neutrophil apoptosis and stimulating their removal by macrophages (for reviews see

Refs. 7–9). AnxA1 is also expressed at low levels by T cells,[10] and we previously reported that AnxA1-deficient mice possess an increased Th17 autoimmune T-cell response that can drive experimental autoimmune uveitis (EAU) in mice.[11] Concomitantly, we reported that systemic administration of human recombinant AnxA1 (hrAnxA1) could ameliorate EAU in mice by reducing STAT3 phosphorylation and subsequently limiting the peripheral generation of pathogenic Th17 cells.[11] The potential, then, of AnxA1 to act upon both innate and adaptive arms of the immune response makes it an attractive therapeutic candidate for human autoimmune diseases such as uveitis, where macrophages, neutrophils, and T cells are known to participate in retinal damage.[12–14] Sight-threatening noninfectious uveitis is a heterogeneous group of disorders characterized by intraocular inflammation and is thought to effect approximately 4 million people globally.[15] Current systemic treatments, including corticosteroids, T-cell–targeting agents such as cyclosporine, and more recently TNF antagonists, may induce disease remission, but they remain limited by the accrual of significant side effects with the requirement of chronic use that leads to loss of efficacy.[16,17] Therefore, there remains an unmet need for new uveitis therapies and a need for noncorticosteroid local therapies.[18] While systemic administration of hrAnxA1 and its mimetic peptides have proven successful in suppressing multiple preclinical models of human inflammatory disease,[4] the efficacy of local administration of hrAnxA1 for the treatment of intraocular inflammation has not been fully explored. In this study, we assessed the extent of constitutive AnxA1 expression in the eye and showed that AnxA1 levels are significantly elevated in the retina during uveitis in both mice and patients compared to healthy controls. In inflamed human retinae, we detect AnxA1 in infiltrating CD45+ leukocytes, glial fibrillary acidic protein+ (GFAP+) macroglia, and in Iba-1+ myeloid cells. Furthermore, we showed the presence of two receptors of AnxA1, formyl peptide receptors 1 (FPR1), and FPRL1/FPR2 in human retinal tissue. To investigate the potential efficacy of local administration of AnxA1 in ocular inflammation, we administered hrAnxA1 by intravitreal injection in mice with uveitis and observed reduced retinal damage and a reduced burden of leukocyte infiltration. Taken together, these data support that intraocular administration of exogenous hrAnxA1 in patients with uveitis may have an impact on both infiltrating leukocytes and

the retinal tissue itself, corroborating this approach as a therapy for the treatment of noninfectious posterior uveitis.

Results

AnxA1 in the Mouse Retina

First we used immunohistochemistry to assess the basal level of endogenous AnxA1 in the healthy C57BL/6 mouse retina, confirming a previous report[19] that it is most abundant in the ganglion cell and nerve fiber layers, with some staining observed in the RPE (Fig. 1A). Western blot analysis revealed full-length 37-kDa AnxA1 protein from both lysed and nonlysed whole eyes, suggesting the presence of soluble secreted AnxA1 within the eye (Supplemental Fig. 1). We then assessed the levels of AnxA1 in the retina at peak of EAU disease in C57BL/6 mice (d26) and observed a considerable increase in AnxA1 staining throughout the retina (Fig. 1B). AnxA1 levels appeared to increase within the ganglion cell layer (GCL) and RPE compared with basal levels, and staining was now observed in the outer nuclear layer (ONL) and photoreceptor layer (PRL) (Fig. 1B). Quantification of AnxA1 staining confirmed our observations, showing a significant increase in the levels of AnxA1 in the retina during C57BL/6 EAU (Fig. 1C).

AnxA1 in the Human Retina

We next assessed the levels of AnxA1 in the human retina from both healthy controls and patients with noninfectious uveitis. The pattern of staining was similar to that observed in the mouse eye. AnxA1 was found predominantly in the ganglion and nerve fiber layers (Fig. 2A), with some staining observed in the inner nuclear layer and some perivascular staining around superficial blood vessels that could be retinal endothelium.[20] Quantification of AnxA1 staining throughout the retina revealed a significant increase in the levels of AnxA1 in the retina from noninfectious uveitis patients compared to that from healthy controls (Fig. 2B, C).

AnxA1 Levels Correlate with Inflammatory Markers in Human Uveitis Retinae

We further analyzed healthy retinae and tissue from uveitis patients to assess changes in the level and distribution of AnxA1 during ocular inflammation.
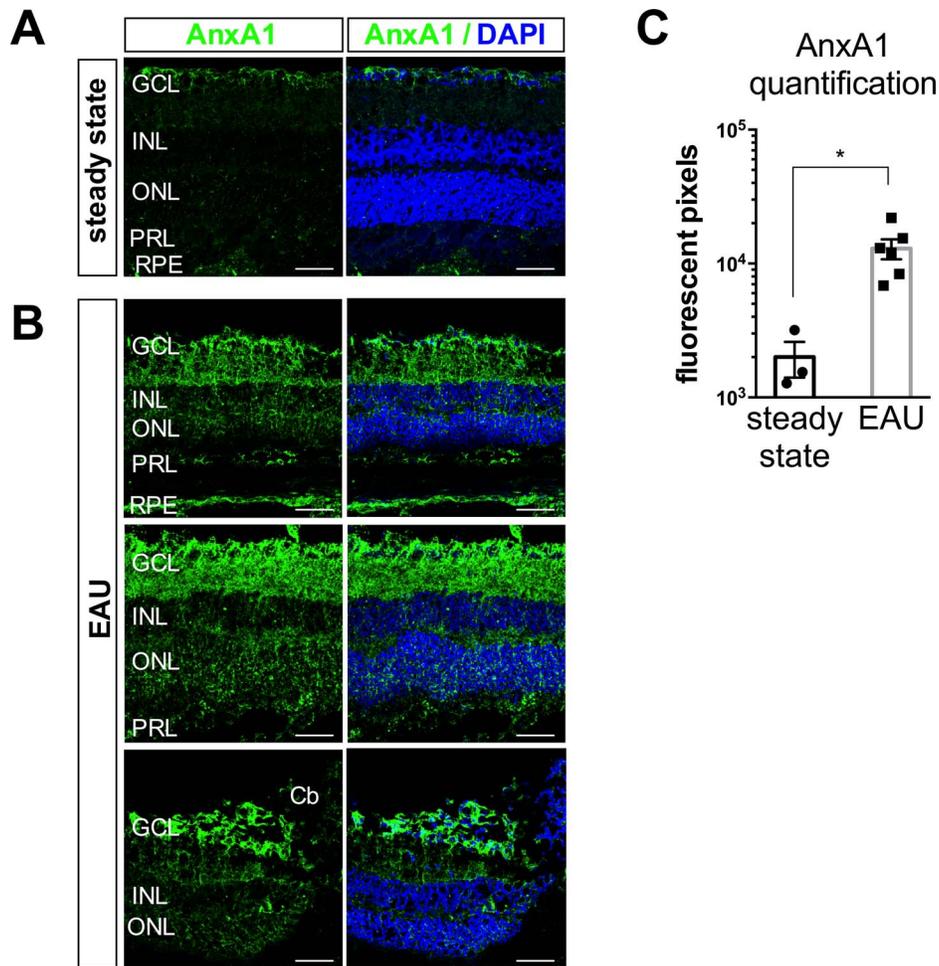
Figure 1. Level of endogenous AnxA1 in the C57BL/6 mouse retina is elevated during uveitis (EAU). AnxA1 was stained by immunohistochemistry (with Triton X-100) on retinal sections cut from eyes of C57BL/6 naïve steady-state mice ($n = 3$) (A) and those from peak EAU mice ($n = 6$) (B). INL, inner nuclear layer; Cb, ciliary body. (C) Quantification of retinal AnxA1 staining compared between steady-state and peak EAU. Each data point represents the mean of three random sections per eye $\pm$ SEM, *$P < 0.05$ Student's *t*-test. *Scale bars*: 30 μm.

Costaining with CD45 revealed the presence of infiltrating leukocytes both in the vitreous and throughout retinal layers. All CD45$^+$ cells observed expressed AnxA1 (Fig. 3). Immunostaining using AnxA1 and GFAP-specific antibodies revealed Anx-A1$^+$ macroglia in the ganglion/nerve fiber layer in healthy retinae that are likely to be retinal astrocytes and Müller glia (Fig. 4). In uveitis tissue, a marked increase in GFAP staining throughout the GCL, inner plexiform layer (IPL), INL, and ONL was observed compared to that in healthy tissue. The increased GFAP in the GCL and IPL largely colocalized with AnxA1 staining, again indicating that activated macroglia express AnxA1 (Fig. 4). However the colocalization of AnxA1 with GFAP in the INL and ONL appeared variable across eyes from uveitis patients (Fig. 4). While the number of cells coexpressing AnxA1 and GFAP was evidently increased in uveitis tissue compared to healthy controls, the proportion of GFAP$^+$ cells coexpressing AnxA1 was qualitatively decreased in disease. This suggests a differential expression of AnxA1 by macroglia subtypes that warrants future investigation. Similarly, expression of Iba-1, a myeloid marker of microglia and macrophages, was elevated in uveitis tissue compared to healthy tissue (Fig. 5A). While Iba-1 staining also increased in uveitis tissue (Fig. 5B), the proportion of Iba-1$^+$ cells coexpressing AnxA1 remained constant at approximately 60% (Fig. 5C) (healthy mean 60.01 $\pm$ 9.181, $n = 4$; uveitis mean 53.57 $\pm$ 10.79, $n = 3$).
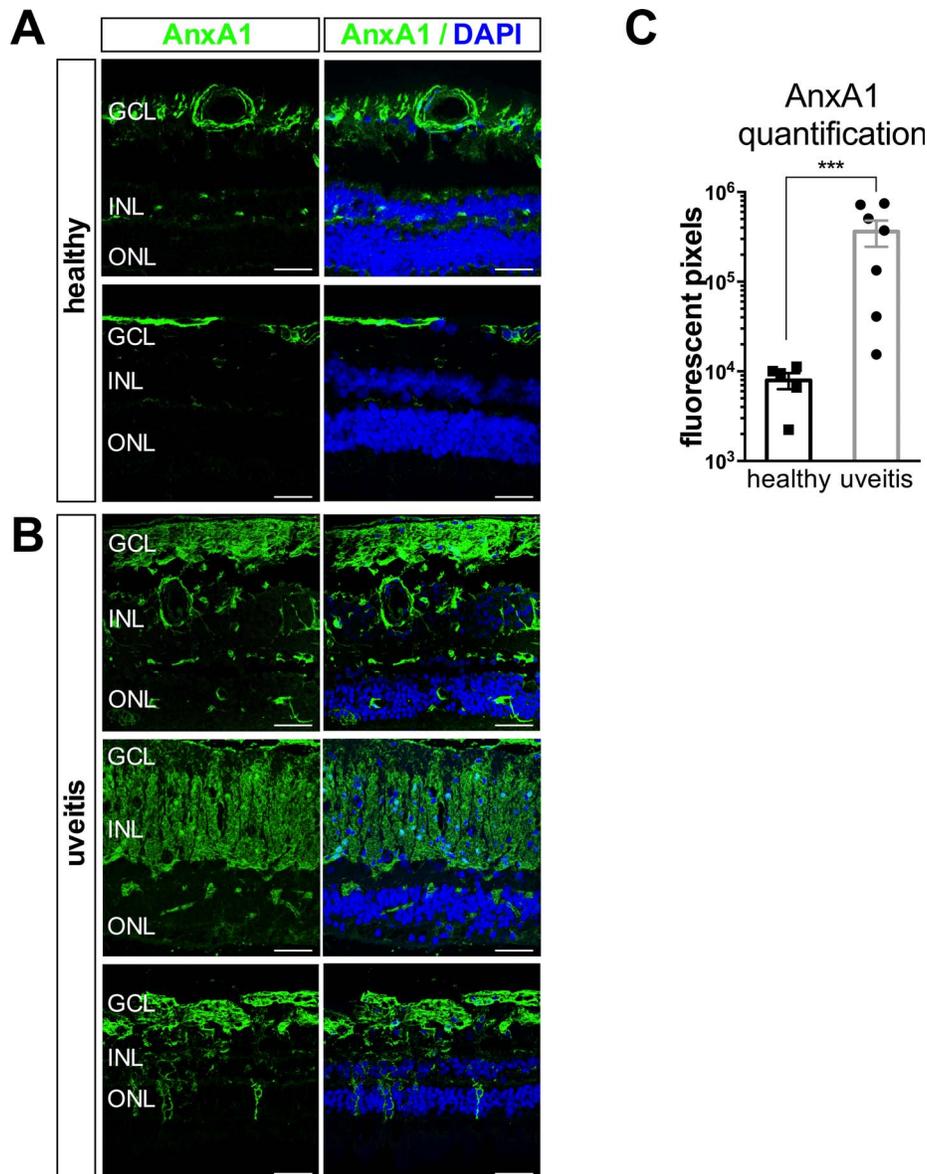
Figure 2. Level of endogenous AnxA1 in the human retina is elevated during uveitis. AnxA1 was stained by immunohistochemistry (with Triton X-100) on retinal sections cut from eyes of healthy donors (A) or uveitis patients (B). (C) Quantification of retinal AnxA1 staining compared between healthy and uveitis patients; each data point represents the mean of three random sections per eye $\pm$ SEM, ***$P < 0.001$ Mann-Whitney U test. *Scale bars*: 50 μm.

Local Administration of hrAnxA1 Attenuates Acute and Chronic Uveitis in Mice

Human recombinant AnxA1 shares approximately 88% amino acid sequence identity with rodent AnxA1.[21] Both full-length hrAnxA1 and the AnxA1 N-terminal peptide (Ac$_{2-26}$) have been administered as a pharmacologic treatment for inflammation in several mouse models.[22] We therefore used hrAnxA1 to assess the anti-inflammatory role of AnxA1 in the eye and to assess the therapeutic potential of hrAnxA1 in uveitis.

Firstly, single administration of hrAnxA1 by intravitreal injection in lipopolysaccharide (LPS)-induced endotoxin-induced uveitis (EIU) in C57BL/6 mice showed a dose-response reduction of neutrophils infiltrating into treated eyes compared to PBS-treated eyes at peak EIU (18 hours). Neutrophils were significantly reduced at the 500-ng dose (Fig. 6A). We then examined whether local injection of hrAnxA1 could suppress the rapid antigen-specific T-cell–mediated disease observed in IRBP peptide–induced EAU in B10.RIII mice and exploited the reproducible and
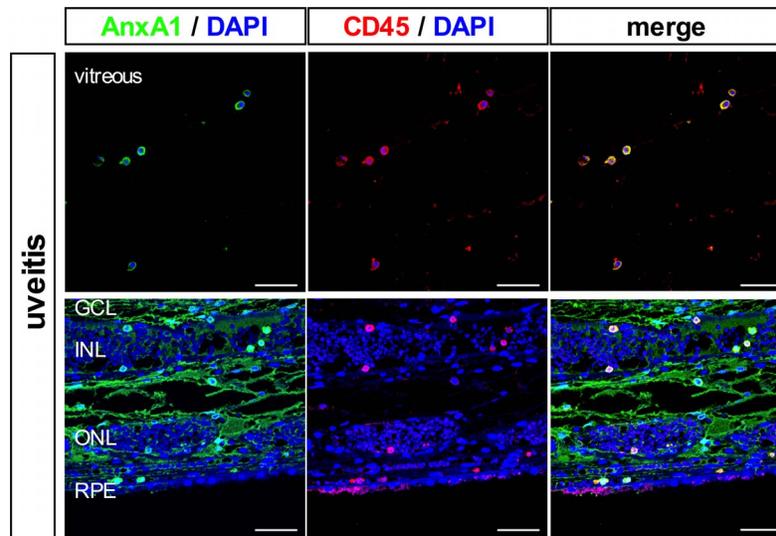
Figure 3. Colocalization of AnxA1 with CD45^{+} leukocytes in human uveitis retinae and vitreous. AnxA1 and CD45 were stained by immunohistochemistry on retinal sections cut from eyes of uveitis patients. *Scale bars*: 50 µm.

validated readouts of clinical assessment score and flow cytometric assessment of retinal infiltrate previously reported.[23–26] Following clinical confirmation of disease at day 9 by fundus examination that revealed optic disc swelling (Fig. 6B), mice received 500 ng/hour AnxA1 by intravitreal injection in one eye and an injection of PBS in the contralateral eye. Analysis undertaken at peak disease (day 12) by fundus examination and flow cytometry for quantification of infiltrating leukocyte subsets revealed suppression of clinical disease signs (Fig. 6C) and reduced leukocyte burden in the hrAnxA1-treated eyes compared to controls (Fig. 6D). Analysis of leukocyte subsets revealed significant suppression of total myeloid CD11b^{+} cells, specifically Ly6G^{+} neutrophils (Fig. 6D).

The AnxA1 Target Receptors FPR1 and FPRL1/FPR2 Are Expressed in Human Retinae

Since administration of hrAnxA1 suppresses uveitis in mice, elevated expression of endogenous AnxA1in the retina following inflammation could be an attempt at immune regulation by the immunologically privileged retina. It has been previously established that CD45^{+} leukocytes express FPRs that act to mediate signaling by secreted AnxA1.[27] In neutrophils, AnxA1 signaling acts to inhibit their extravasation into inflamed tissues and downregulate their proinflammatory cytokine production.[28] While infiltrating leukocytes may be the target of elevated levels of AnxA1 in the retina during inflammation, it is not known if retinal cells have the capacity to respond to AnxA1. Given the marked increase in AnxA1 expression in

both the inflamed mouse and human retina, we sought to determine whether FRPs are expressed in the human eye. Leukocytes are known to express FPRs,[27,29] but FPR expression has yet to be examined in the human retina. Staining of retinal sections revealed the presence of both FPR1 and FPRL1/FPR2 in healthy (Fig. 7A) and uveitis retinae (Fig. 7B). FPR1-positive staining was observed in the GCL, ONL, and photoreceptors (Fig. 7A and B). FPRL1/FPR2-positive staining was observed in the GCL, IPL, and photoreceptors (Fig. 7A and B). While the pattern of staining was similar for both FPRs in healthy and uveitis tissue, increased staining was observed in some uveitic eyes, particularly in the iris, a location where infiltrating leukocytes are known to enter the eye from the periphery (Fig. 7B).[30]

Discussion

Our examination of AnxA1 expression in the eye has revealed the association of elevated levels of AnxA1 protein with inflammation in both mouse and human retinal tissue. This supports the relevance of using mouse models of uveitis to study AnxA1 biology in inflammatory disease. AnxA1 is known to participate in proresolution pathways of inflammation,[4] and we therefore postulate that elevated AnxA1 expression in the retina is an attempted regulatory response to ocular inflammation and infiltrating leukocytes. This notion is further supported by the suppression of disease in mice with uveitis following local administration of hrAnxA1.
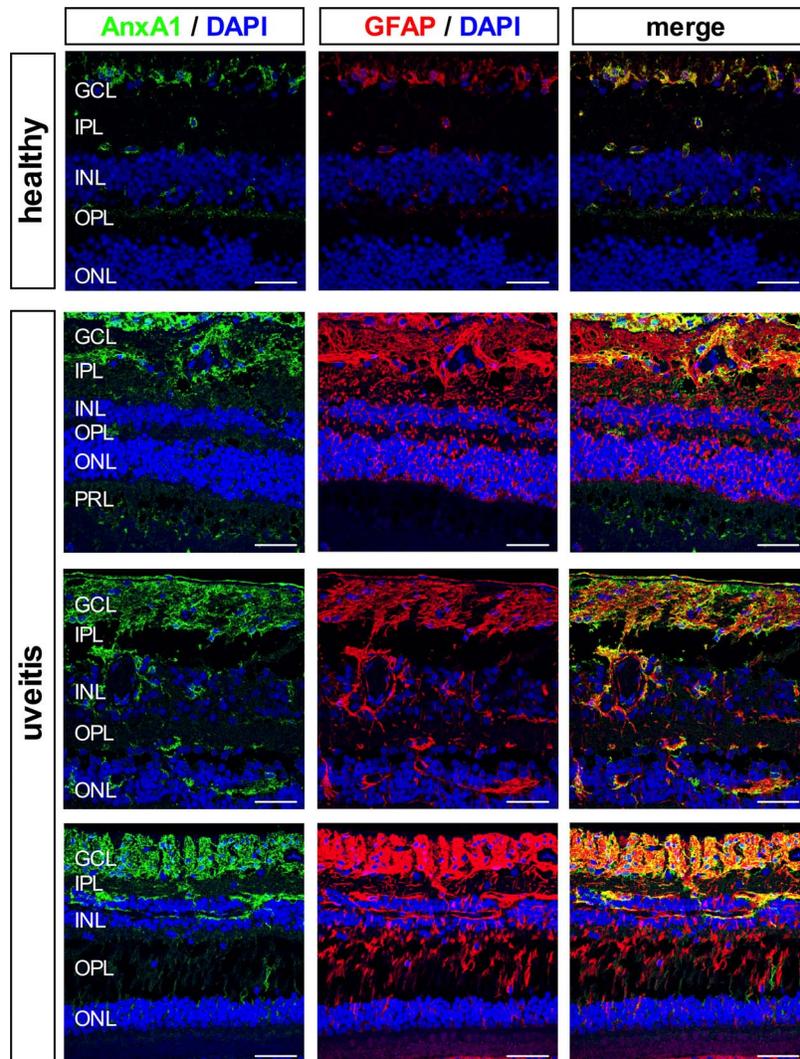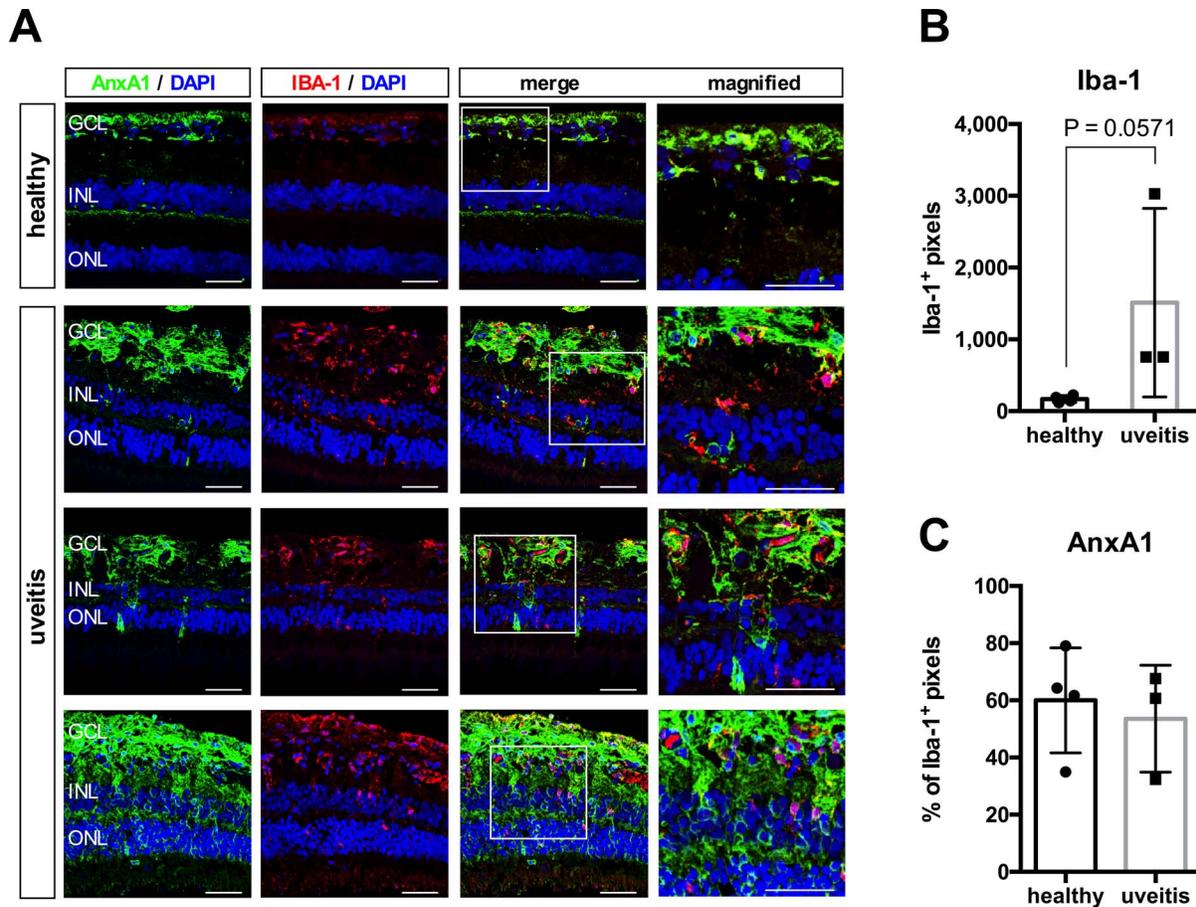
Figure 4. Colocalization of AnxA1 with GFAP in human retinae. AnxA1 and GFAP were stained by immunohistochemistry (with Triton X-100) on retinal sections cut from healthy donor eyes and uveitis patients. *Scale bars*: 50 μm.

Study of AnxA1 in the brain has revealed an important role of AnxA1 in regulating blood–brain barrier function through endothelium tight-junction integrity,[31] and AnxA1 is also thought to carry out possible neuroprotective roles.[32,33] AnxA1 expression in the human CNS is almost exclusive to microglia,[2] hence we examined expression of AnxA1 in retinal microglia. In the retina, however, elevated levels of AnxA1 were found in GFAP+ macroglia (astrocytes and Müller glia), but only in a proportion of Iba-1+ myeloid cells. The upregulation of AnxA1 in astrocytes and microglia has been reported in rats during EAE,[34,35] similar to our EAU data, and it has also been reported that microglia but not astrocytes secrete AnxA1,[36] perhaps explaining why supplementation of endogenous AnxA1 with hrAnxA1 can effectively suppress uveitis in mice. Of note, our data show similarities with reports showing elevated expression of AnxA1 in brains of Alzheimer's,[37] multiple sclerosis,[38] and Parkinson's disease[39] patients, suggesting conserved roles of AnxA1 across neural tissue. The potential impact of AnxA1 modulation for ocular diseases (other than uveitis) that exhibit an inflammatory component is also of note, with reports of the ability of exogenous hrAnxA1 to modulate mast cells[40,41] and suppress allergic conjunctivitis in mice[42] and the association of the inactive form of AnxA1 in tears from active vernal keratoconjunctivitis patients compared to controls.[43]

AnxA1 is known to mediate some aspects of glucocorticoid-mediated immune suppression,[44] and

Figure 5. Colocalization of AnxA1 with Iba-1 in human retinae. (A) AnxA1 and Iba-1 were stained by immunohistochemistry (with Triton X-100) on retinal sections cut from healthy donor eyes and uveitis patients. *White box* indicates magnified region; the *white boxes* show magnified region (B) quantification of Iba-1 pixels in healthy compared to uveitis eyes. Each data point represents the mean of three random sections per eye ± SEM; Mann-Whitney *U* test. (C) Quantification of the colocalization of AnxA1 signal with Iba-1 in healthy compared to uveitis eyes; each data point represents the mean of three random sections per eye ± SEM. *Scale bars*: 50 μm.

glucocorticoid steroids are the current frontline treatment for noninfectious uveitis patients.[45] The attractiveness of locally administered hrAnxA1 as a therapeutic lies in the possibility of delivering the benefits of glucocorticoid steroids without the side effects,[46] although this exact mechanism is yet to be determined and will require future work to identify the specific retinal cell types expressing FPRL1/FPR2 receptors for AnxA1 and a characterization of their response to exogenous hrAnxA1. Our data confirm and extend a previous report where elevated AnxA1 expression was observed in rodent EIU, and topical application of an AnxA1 mimetic peptide suppressed EIU infiltration through the FPRL1/FPR2 receptor.[19] Our data compliment those reported by Girol and colleagues[19] by confirming the relevance of AnxA1 in human retinae and by revealing successful treatment of the T-cell–driven EAU mouse model

using hrAnxA1. We also report, we believe for the first time, the expression of the target receptors, FPR1 and FPRL1/FPR2, receptors of AnxA1 in human retinae. FPRL1/FPR2 is known to mediate signals from AnxA1, lipoxin A(4), and serum amyloid A to regulate inflammation.[27] Retinal expression of FPRL1/FPR2 in astrocytes, microglia, and Müller glia may allow hrAnxA1 to modulate the retinal tissue itself (in addition to infiltrating leukocytes), perhaps via downregulation of chemokine production, and thus may explain the potent local effects of hrAnxA1 we observed in mouse models of uveitis. While FPR1 has mainly been studied in neutrophil migration, it is known to be expressed by non-hematopoietic cells with as yet unknown biological function,[47] and hence the significance of expression in the human retina is unknown. While our data are encouraging with respect to the therapeutic potential of hrAnxA1 for
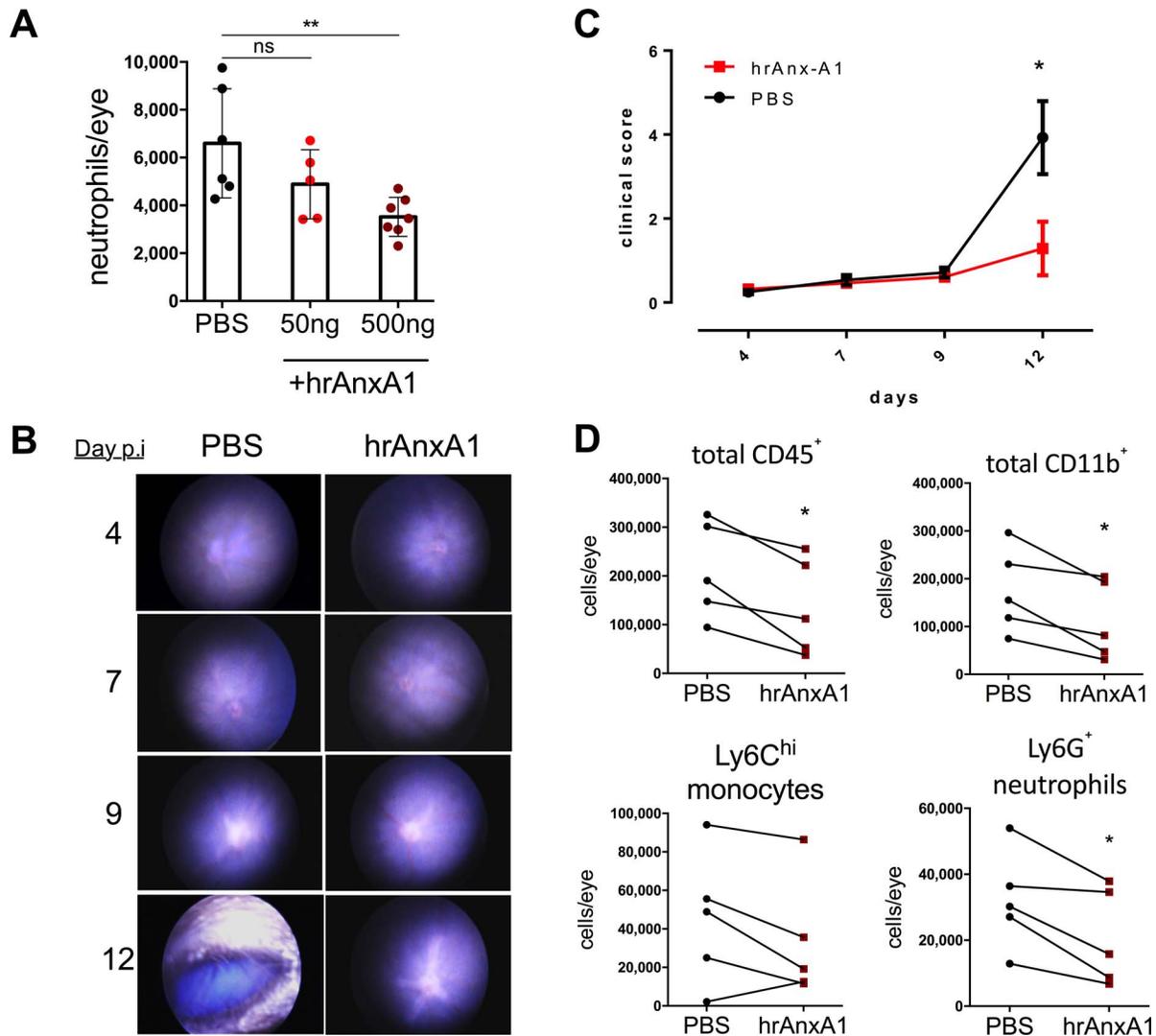
Figure 6. Local administration of hrAnx-A1 suppresses uveitis in mice. (A) Quantification by flow cytometry of neutrophils (CD45$^+$CD11b$^+$Ly6G$^+$) infiltrating the eye at peak EIU disease (18 hours post induction) following administration of 2 μL PBS vehicle or hrAnxA1 (50 ng and 500 ng, respectively) by intravitreal injection. Data representative of two independent experiments, $n = 5$–7 mice $\pm$ SEM, **$P < 0.05$ Kruskal-Wallis multiple comparison test; ns, not significant. (B) Representative fundus images of EAU mice day 4–12 post induction having been treated at day 10 with a single 2-μL injection of PBS vehicle in one eye or 500 ng hrAnx-A1 in the contralateral eye. (C) Clinical scoring of fundus imaging of B10.RIII EAU mice at peak disease (day 12). Data presented as mean $\pm$ SEM, $n = 5$ mice/group; *$P < 0.05$ paired t-test. (D) Graphs showing quantification by flow cytometry of total CD45$^+$ leukocytes, total CD11b$^+$ counts, CD11b$^+$Ly6C$^+$ (monocytes/macrophages), and C11b$^+$Ly6G$^+$ (neutrophils) from individual eyes of EAU mice treated with PBS vehicle and 500 ng AnxA1. Data presented as mean $\pm$ SEM, $n = 5$ mice/group, *$P < 0.05$ paired t-test.

treating uveitis, further work is required to examine the potential for (adverse) downstream AnxA1 signaling events following hrAnxA1 administration.

In summary, this study has shown AnxA1 is significantly elevated in mouse and human uveitic retinae. Furthermore, we have demonstrated effective treatment for both acute and chronic uveitis in mice by local administration of full-length hrAnxA1. This provides proof of concept for a novel therapeutic approach for the treatment of patients with noninfectious uveitis.

Methods

Animals

All mouse procedures were conducted under the regulation of the UK Home Office Animals (Scientific
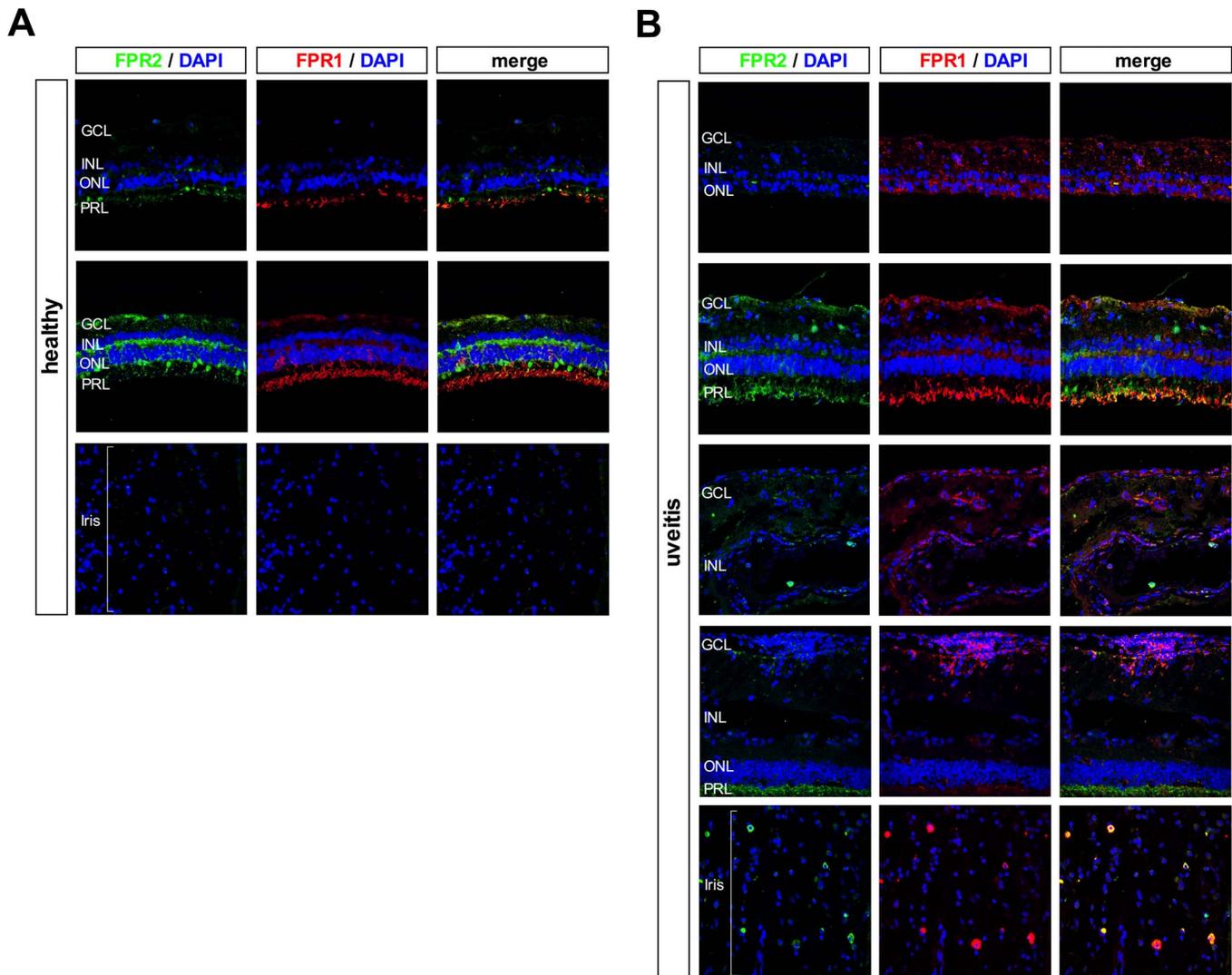
Figure 7. FRPs are expressed in human retinae. FPR1 and FPRL1/FPR2 were stained by immunohistochemistry on retinal sections cut from (A) healthy donor eyes and (B) uveitis patients. Due to the primary antibodies being raised in the same species, FPR1 was stained first alone, followed by its secondary antibody, followed by staining with the directly conjugated FPRL1/FPR2 AF647 antibody.

Procedures) Act 1986, with University College London Ethics Committee approval and in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmology and Vision Research. Wild-type C57BL/6J female mice aged between 6 and 10 weeks were used.

Human Ocular Tissue

Healthy adult (age 18–65 years) human postmortem eyes from anonymous donors collected by Moorfields Biobank following corneal removal for transplantation ($n = 5$) and noninfectious uveitis patient eyes (described in Supplementary Table S1)

collected by Moorfields Biobank ($n = 6$ post mortem and $n = 1$ enucleation) were fixed in 10% formalin and embedded in paraffin wax. Use of human tissue for this project was supported by NIHR funding and approved by the NHS Research Ethics Committee (10/H0106/57-14ETR41).

EAU Induction

Six-week-old C57BL/6 mice or B10.RIII mice (as indicated in the Results and figure legends) were immunized in the flank with 500 µg RBP_{1-20} and 300 µg $RBP_{161-180}$, respectively, in PBS emulsified with complete Freund's adjuvant (Sigma-Aldrich Corp., Gillingham, UK) supplemented with 1.5 mg/mL

Mycobacterium tuberculosis complete H37 Ra (Difco Microbiology, Lawrence, KS) (1:1 vol/vol). The mice also received 1.5 µg *Bordetella pertussis* toxin (Sigma-Aldrich Corp.) intraperitoneally.

EIU Induction

Disease was induced in C57BL/6 mice following induction of general anesthesia using ketamine by administration of 1 ng LPS (lipopolysaccharides from *Escherichia coli* O55:B5; Sigma-Aldrich Corp.) dissolved in PBS by intravitreal injection of 2 µL using a microsurgical syringe and 38-gauge needle (Hamilton, Giarmata, Romania).

hrAnxA1 Administration

hrAnxA1 was diluted in PBS and delivered directly to the eye by intravitreal injection of 2 µL at the stated quantities (for the case of EAU) or diluted 1:1 with LPS (1 µg/mL) at the stated quantities and delivered by 2-µL intravitreal injection at EIU induction.

Fundus Imaging

The topical endoscopic fundus imaging (TEFI) system was used for routine in vivo imaging of mouse retinal fundus as previously described.[48] Clinical scoring of TEFI images was carried out using a modified scoring system that has been previously described.[48]

Immunohistochemistry and Fluorescent Imaging

Human ocular 3-µm sections were cut and dewaxed in histoclear (National Diagnostics, Atlanta, GA), rehydrated, and subjected to antigen retrieval using antigen unmasking solution (H-3300; Vector Labs, Peterborough, UK) with pressure cooker heating for 2 minutes after achieving rich full pressure (13 Pa), prior to blocking and staining. Mouse eyes were enucleated and fixed in 4% paraformaldehyde for 1 hour before freezing in OCT medium (Fisher Scientific, Loughborough, UK) and 16-µm cryosections cut. Sections were blocked in 5% donkey serum (with and without 0.2% Triton X-100, as indicated) for 1 hour prior to staining with rabbit anti-AnxA1 (ab196830; Abcam, Cambridge, UK), goat anti-AnxA1 (ab115770; Abcam), mouse anti-human GFAP (M0761; Dako, Santa Clara, CA), mouse anti-human CD45 (M0701, Dako), rabbit anti-Iba-1 (019-19741; Wako, Neuss, Germany), rabbit anti-FPRL1/FPR2 (NLS1878AF647; Novus Biologicals, Littleton, CO), rabbit anti-human FPR1

(NBP2-47452; Novus Biological), and the appropriate secondary antibodies: goat anti-rabbit IgG (H+L) Alexa Fluor 488 (A-11008; Life Technologies, Carlsbad, CA), donkey anti-goat IgG (H+L) Alexa Fluor 488 (A-11055; Life Technologies), goat anti-mouse IgG (H+L) Alexa Fluor 546 (A-11030; Life Technologies), goat anti-rabbit IgG (H+L) Alexa Fluor 546 (A-11010; Life Technologies). No primary antibody control stains were carried out for all secondary antibodies on mouse and human tissue (Supplemental Fig. 2). Sections were stained with DAPI (D1306; Life Technologies) with appropriate washing steps prior to imaging with a confocal microscope (DM5500Q; Leica, Wetzlar, Germany). Quantification of fluorescent pixels was carried out following file conversion to FCS files using FlowJo software (Treestar, Ashland, OR).

Flow Cytometry

Enucleated eyes were dissected in 100 µL cold Dulbecco's modified Eagle's medium. After incision at the limbus with a 29-gauge needle, a circumferential cut around the eye following the limbus was made. Iris was dissected, releasing anterior chamber infiltrating cells into the dissection media. The retina and vitreous were then removed carefully from the eye cup, leaving the sclera/retinal pigment epithelium/choroid intact. The dissection media containing anterior fluid, vitreous, and retina were then pipetted into a 1.5-mL Eppendorf tube and mechanically disrupted by rapping 10 times for a single-cell suspension, followed by centrifugation through a 70-µm cell strainer. The resulting single-cell suspensions were blocked with anti-mouse CD16/32 (2.4G2; eBioscience, Waltham, MA) for 5 minutes prior to surface staining with the following antibodies: αGr1-APC, αLy6C-PE, αCD11b-PerCP, αCD45-FITC, APC-efluor780 live dead dye. Absolute cell numbers were obtained as previously detailed[25] using a BD LSR Fortessa-x20 with FACSDiva software (version 8.1; BD Cytometry Systems, San Jose, CA). Single-stained beads (OneComp eBeads; eBioscience) of all fluorophores were used to generate compensation matrices, and fluorescence-minus-one controls were used for positive gating; data were analyzed using FlowJo software (Treestar).

Western Blot

Detection of AnxA1 was carried out on whole mouse eye homogenate. Enucleated mouse eyes were homogenized with a micropestle and mortar tube

(Eppendorf) in cold PBS and spun to remove cells or in RIPA buffer containing 1:100 freshly added protease inhibitor cocktail (Sigma-Aldrich Corp.), freeze-thawed, and spun to remove cellular material. Proteins were separated using an 8% SDS-PAGE gel and blotted to a transfer membrane (Immobilon-P; Millipore, Watford, UK), followed by detection with rabbit anti-AnxA1 (ab196830; Abcam), then by incubation with the secondary antibody swine anti–rabbit-HRP (P0399; Dako) and subsequent development with a detection kit (ECL Plus; Amersham, GE Healthcare, Amersham, UK).

Statistical Analysis

Data were analyzed for statistical significance using statistical analysis software (GraphPad Inc., La Jolla, CA).

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