

Retinal Macrophages Synthesize C3 and Activate Complement in AMD and in Models of Focal Retinal Degeneration

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PURPOSE. Complement system dysregulation is strongly linked to the progression of age-related macular degeneration (AMD). Deposition of complement including C3 within the lesions in atrophic AMD is thought to contribute to lesion growth, although the contribution of local cellular sources remains unclear. We investigated the role of retinal microglia and macrophages in complement activation within atrophic lesions, in AMD and in models of focal retinal degeneration.

METHODS. Human AMD donor retinas were labeled for C3 expression via in situ hybridization. Rats were subject to photo-oxidative damage, and lesion expansion was tracked over a 2-month period using optical coherence tomography (OCT). Three strategies were used to determine the contribution of local and systemic C3 in mice: total C3 genetic ablation, local C3 inhibition using intravitreally injected small interfering RNA (siRNA), and depletion of serum C3 using cobra venom factor.

RESULTS. Retinal C3 was expressed by microglia/macrophages located in the outer retina in AMD eyes. In rodent photo-oxidative damage, C3-expressing microglia/macrophages and complement activation were located in regions of lesion expansion in the outer retina over 2 months. Total genetic ablation of C3 ameliorated degeneration and complement activation in retinas following damage, although systemic depletion of serum complement had no effect. In contrast, local suppression of C3 expression using siRNA inhibited complement activation and deposition, and reduced cell death.

CONCLUSIONS. These findings implicate C3, produced locally by retinal microglia/macrophages, as contributing causally to retinal degeneration. Consequently, this suggests that C3-targeted gene therapy may prove valuable in slowing the progression of AMD.

Keywords: age-related macular degeneration, inflammation, macrophages, retinal degeneration, complement

The complement system comprises a cascade of proteins that are cleaved sequentially to initiate the destruction of a pathogen, foreign body, or cell debris.^{1,2} Prolonged cleavage of C3 may lead to membrane attack complex (MAC) assembly and cytolysis.^{1,2} Complement activation is under tight control, including regulation by complement factor H (CFH), a critical inhibitor. However, in neurodegenerative diseases, including age-related macular degeneration (AMD), regulation of complement activation is compromised, contributing to disease onset and progression (reviewed in Refs. 3–5).

AMD affects photoreceptors and the retinal pigment epithelium (RPE) of the macula, which mediates high acuity vision. Multiple factors contribute to AMD pathogenesis (reviewed in

Ref. 6); however, the Y402H mutation in *CFH* is the most highly associated risk factor.^{7,8} Other variants in *C3*, *CFB*, and *C2* genes are associated with AMD susceptibility (reviewed in Ref. 5), with complement gene variants in the population estimated to account for ~70% of the risk for developing AMD.⁵

Poor understanding of the cellular events that initiate and feed complement activation in the retina has been a significant obstacle in development of innovative approaches in the management of AMD.⁴ Immunohistochemistry analyses have revealed that by-products of C3 catabolism are deposited in affected areas of RPE/Bruch's membrane, including drusen deposits, from AMD patients.^{7,9–14} This deposition, however, depends upon the availability of C3 within the retinal



microenvironment, and the sources of C3—systemic circulation or local retinal mediators—are unclear. Whole-transcriptome analyses of AMD donor eyes show that C3 mRNA is significantly increased in neural retina, but not in RPE/choroid,¹⁵ suggesting that cells in the neural retina may be critically engaged in AMD pathogenesis.

In this study, we seek to identify the retinal modulators of complement in AMD and uncover their significance in the pathogenic activation of the cascade in the retina. We identified retinal macrophages as the local producer of C3 in both early and advanced forms of AMD. Using a model of outer-retinal degeneration and inflammation in rodents and optical coherence tomography (OCT), we show that C3-expressing macrophages are associated with the expansion of atrophic lesions. Crucially, we demonstrate that intravitreal delivery of small interfering RNA (siRNA) suppresses this local expression of C3 and ameliorates retinal complement activation and degeneration; on the other hand, inhibition of serum-derived C3 has no effect on retinal complement levels or degeneration. Taken together, these findings inform our understanding of the role of complement in AMD, pinpointing macrophages as the key source of C3 that drives deleterious complement activation, and providing an indication for locally targeted gene therapy to suppress complement activation in retinal degeneration.

METHODS

Experimental Animals

All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The study was approved by the Australian National University Animal Experimentation Ethics Committee (Application ID: 2014/56). Sprague-Dawley (SD) albino rats aged 120 to 150 postnatal days were used in this study. Additionally, C57BL/6J mice and C3-knockout (KO) mice (C3^{-/-}, 129S4-C3tm1Crr/J), both aged 60 to 80 days, were obtained from the Australian Phenomics Facility. For the C3 KO experiments, the KO mice were compared to isogenic C57BL/6J littermates. All animals were housed and maintained under standard laboratory conditions in 12:12-hour light to dark cycle of 5 lux (dim-reared). Age-matched dim-reared animals with no photo-oxidative damage (PD) were used as controls.

PD and Tissue Collection

SD rats were placed into transparent Perspex open-top boxes and exposed to 1000 lux white light for 24 hours (COLDF2, 2x36W, IHF, Thorn Lighting, Spennymoor, UK), with free access to food and water.¹⁶ Following PD, animals were euthanized with an overdose of barbiturate (60 mg/kg Valbarb; Virbac, NSW, Australia) for tissue collection (0 days) or were held for 3, 7, 14, or 56 days under dim lighting (5 lux), then euthanized.

C57BL/6J and C3^{-/-} mice were housed in Perspex boxes coated with a reflective interior, and exposed to 100 Klux of natural white LED for 1, 3, 5, and 7 days, with free access to food and water.¹⁷ Each animal was administered with pupil dilator eye-drops twice daily during PD (Atropine Sulphate 1% w/v eye-drops; Bausch and Lomb, NSW, Australia). Following PD, electroretinography (ERG) was used to measure mouse retinal function in response to full-field flash stimuli under scotopic conditions in dim-reared control and 7-day damaged mice as described previously.¹⁷ Animals were euthanized with CO₂ prior to tissue collection. Eyes were collected and processed for cryosections or RNA extraction, as previously described in our publications.^{17,18}

Optical Coherence Tomography

Cross-sectional and fundus images of live rat retinas were undertaken using a Spectralis HRA+OCT device (Heidelberg Engineering, Heidelberg, Germany). Animals were anesthetized with an intraperitoneal injection of Ketamine (100 mg/kg; Troy Laboratories, NSW, Australia), and Xylazil (12 mg/kg; Troy Laboratories). Following anesthesia, a pupil dilator was administered to both eyes (Tropicamide 0.5% w/v eye-drops; Bausch and Lomb).

Animals were restrained on a custom-made platform attached to the imaging device, adapted for rat eyes according to manufacturer's specifications. To maintain corneal hydration and improve OCT image quality to the manufacturer's minimum standard (25 dB), hypromellose 0.3% eye-drops (GenTeal; Novartis, NSW, Australia) were administered and a rodent contact lens was placed on the eye (PMMA lenses, radius of curvature of the central optic zone of 2.70 mm and diameter of 5.20 mm; Cantor + Nissel, Brackley, UK).¹⁹ Fundus and cross-sectional images were taken from 0 to 3 mm superior to the optic nerve (ON), and 1 to 2 mm inferior to the ON. Eye gel (GenTeal; Novartis) was administered to both eyes for recovery.

Treatment of Mice With Cobra Venom Factor (CVF)

To achieve systemic complement depletion, CVF (Quidel, San Diego, CA, USA) was dissolved in endotoxin-free PBS to a final concentration of 0.1 µg/µL. C57BL/6J mice were administered with 25 µg CVF intraperitoneally prior to 7 days of PD, and on days 3 and 5 during damage to sustain complement depletion. To confirm an inhibition of complement activity, peripheral blood was collected at 7 days for a hemolytic assay. The serum of CVF-treated mice was extracted and incubated with sheep red blood cells (Applied Biological Products Management, SA, Australia) presensitized with hemolysin (Sigma-Aldrich Corp., St. Louis, MO, USA) as described previously.²⁰ The absorbance at 540 nm was read using an Infinite-200-PRO plate spectrometer (Tecan, Mannedorf, Switzerland), and the percentage of hemolysis was calculated and compared to serum from PBS-injected mice as a positive control, and C3^{-/-} serum as a negative control.

Intravitreal Injections of siRNA

In vivo RNA interference was performed using C3 siRNA (s63165; Thermo Fisher Scientific, Waltham, MA, USA) and negative control siRNA (12935300, Stealth RNAi Med GC; Thermo Fisher Scientific), which were encapsulated using a cationic liposome-based formulation (Invivofectamine 3.0 Reagent; Thermo Fisher Scientific) as per the manufacturer's instructions. To purify and increase the concentration of the siRNA formulation to a final concentration of 0.3 µg/µL in endotoxin-free PBS, the samples were spun at 4000g through an Amicon Ultra-4 Centrifugal Filter Unit (Merck Millipore, Billerica, MA, USA). Intravitreal injections were performed as described in our previous publication²¹; 1 µL C3 siRNA or negative control siRNA formulation was injected into both eyes of each C57BL/6J mouse on day 4 of PD. Animals were recovered, and then returned to PD for the remainder of the 7-day time course. Mouse livers were collected 3 days after the siRNA injections for analysis of systemic C3.

Immunohistochemistry and Quantification of Outer Nuclear Layer (ONL) Thickness and Microglia/Macrophages in Cryosections

All histologic and immunofluorescent analyses utilized retinal cryosections that were cut along the parasagittal plane (supero-inferior), described previously.^{22,23} These sections also includ-

TABLE 1. Primary Antibodies Used for Immunohistochemistry

Primary Antibody	Dilution	Catalogue Number	Company
Rabbit α -IBA1	1:500	019-19741	Wako, Osaka, Japan
Goat α -C3d	1:100	AF2655	R&D Systems, MN, USA
Rabbit α -C3	1:50	AB118877	Abcam, Cambridge, UK
Rabbit α -Rhodopsin	1:500	AB9279	Merck Millipore, MA, USA
Rabbit α -GFAP	1:500	Z0334	Dako, Glostrup, Denmark

ed ON head, in order to maintain regional consistency between replicates and groups.

To detect and localize specific proteins in the retina, immunohistochemistry was performed using primary antibodies (Table 1) as described in our previous protocols with minor modifications.^{18,24} Mouse cryosections were immunolabeled with the α -C3 antibody instead of α -C3d, as it achieved more optimal staining. Fluorescence in retinal sections was visualized under a laser-scanning A1⁺ confocal microscope (Nikon, Tokyo, Japan), and images were acquired using the NIS-Elements AR software (Nikon). Images were processed using Photoshop CS6 software (Adobe Systems, San Jose, CA, USA). Immunolabeled IBA1⁺ cells in the outer retina (ONL-RPE) were counted across each whole retinal section and then averaged for each group, consistent with our prior methods.^{17,23} For quantification, only the cell bodies of the IBA1⁺ cells were counted so as to prevent the occasional ambiguity of IBA1⁺ processes from skewing the data set.

To assess ONL thinning on cryosections following PD, the sections were stained with Toluidine Blue and then quantified in accordance with our previous methodology.¹⁷ Briefly, number of rows of photoreceptor nuclei was quantified within the lesion of the superior retina, and counts were subsequently averaged for each experimental group.

In Situ Hybridization on Human and Rat Retinas

To localize *C3* mRNA transcripts in retinal cryosections, *C3* was cloned from PCR products derived from human (460 bp amplicon) and rat (483 bp amplicon) retinal cDNA. These cloned templates were then synthesized into a digoxigenin (DIG)-labeled riboprobe that was specific to human or rat *C3* mRNA, according to our published methodology.¹⁸

In situ hybridization (ISH) was performed on cryosections from either human AMD donor tissue or rat retinas subjected to PD. AMD tissues had been extensively categorized and processed for cryosectioning in an earlier investigation.²⁵ In brief, human eyes were collected with informed consent through the Lions NSW Eye Bank, Sydney, Australia, with ethical approval from the Human Research Ethics Committee of the University of Sydney and The Australian National University. Grading for the eyes ranged from normal to early- or late-AMD, and was assigned by a team of experienced graders according to published pathologic criteria.²⁶

ISH was conducted using our established protocol.²⁷ Both rat and human *C3* riboprobes were hybridized overnight at 57°C and then washed in saline sodium citrate (pH 7.4) at 60°C. The bound probe was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), and sections were double-labeled using IBA1 immunohistochemistry.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

RNA extraction and purification was performed on retinas using a combination of TRIzol reagent (Thermo Fisher Scientific) and an RNAqueous Total RNA Isolation Kit (Thermo

TABLE 2. Taqman Hydrolysis Probes Used for qPCR

Gene Symbol	Gene Name	Catalogue Number	Entrez Gene ID
<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	Rn99999916_s1 Mm99999915_g1	24383 14433
<i>Actb</i>	Actin, beta	Mm01205647_g1	11461
<i>C1s</i>	Complement component 1, s subcomponent	Rn00594278_m1 Mm01625167_g1	192262 50908
<i>C2</i>	Complement component 2	Rn00597176_m1 Mm00442726_m1	24231 12263
<i>C3</i>	Complement component 3	Mm00437858_m1	12266
<i>C4a</i>	Complement component 4A (Rodgers blood group)	Rn00709527_m1 Mm01132415_g1	24233 625018
<i>Cfb</i>	Complement factor B	Rn01526084_g1 Mm00433918_g1	294257 14962
<i>Cfd</i>	Complement factor D (adipsin)	Rn01535436_g1	54249
<i>Cfb</i>	Complement factor H	Rn00590326_m1 Mm01299248_m1	155012 12628
<i>Cfi</i>	Complement factor I	Rn00577333_m1	79126
<i>Serping1</i>	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	Rn01485600_m1	295703
<i>Cntf</i>	Ciliary neurotrophic factor	Mm00446373_m1	12803
<i>Fgf2</i>	Fibroblast growth factor 2	Mm01285715_m1	14173
<i>Rho</i>	Rhodopsin	Mm00520345_m1	212541

Fisher Scientific) as described in our previous publication.²⁸ cDNA was prepared from 500 ng of each RNA sample using a Tetro cDNA Synthesis Kit (Bioline Reagents, London, UK) according to the manufacturer's protocol.

Gene expression changes were measured via qPCR using Taqman hydrolysis probes (Table 2) and Taqman Gene Expression Master Mix (Thermo Fisher Scientific). Each qPCR was run using a QuantStudio 12K Flex instrument (Applied Biosystems, Foster City, CA, USA) at the Biomolecular Resource Facility (The John Curtin School of Medical Research, The Australian National University, Canberra, Australian Capital Territory, Australia). Analysis was performed using the comparative cycle threshold method ($\Delta\Delta C_T$), which was normalized to the expression of *Gapdh* and *Actb* reference genes, as established previously.^{29,30}

OCT and Fundus Images

OCT and fundus images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Retinal thickness and ONL depth were measured in five OCT transects (superior and inferior to the ON) spaced at 1-mm intervals, with five points sampled across each image. ONL thickness ratios were calculated as the ONL thickness relative to the distance between the outer and inner limiting membranes. To detect fundus lesions, images of the area of interest (2–3 mm superior to the ON) were converted to grayscale, and contrast was optimized using the “curves” function in Photoshop CS6 using a standard approach. Images were then converted to bitmaps using controlled parameters. ImageJ was used to measure the lesion size manually (arbitrary units).

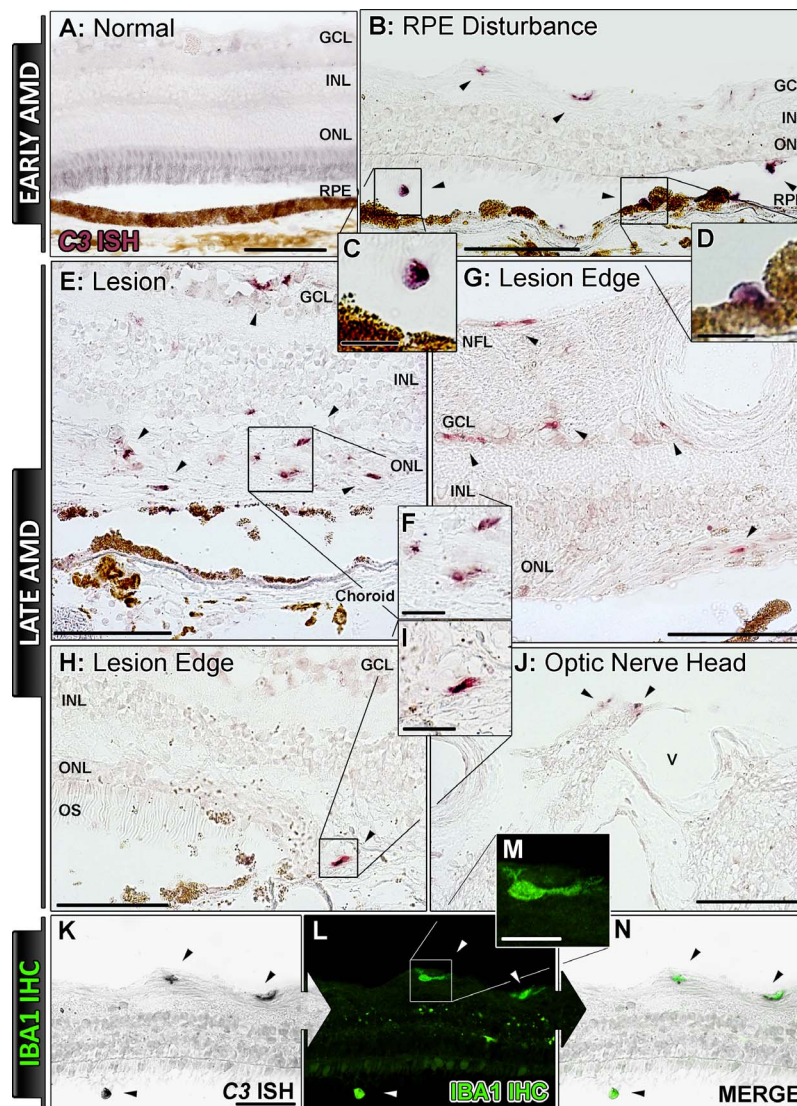


FIGURE 1. *C3* expression in human AMD retinas. (A) No *C3*-expressing cells were detected in the normal retina. (B–D) Subretinal *C3*-expressing cells were present in early AMD retinas exhibiting RPE disturbance. *C3* mRNA expression was detected near the inner retinal vasculature. (E–J) In late AMD retinas, *C3*-expressing cells were detected within the lesion (E, F), at the lesion edges (G–I), and at the ON head (J). These *C3*-expressing cells were identified as IBA1⁺ microglia/macrophages. Representative images derived from *N* = 2 to 3 per group. GCL, ganglion cell layer; INL, inner nuclear layer; OS, outer segments; V, vasculature. Scale bars represent 100 μ m in A, B, E, G, H, J; 50 μ m in K; 10 μ m in C, D, F, I, M.

Statistics

All graphing and statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA, USA). Significant trends in time-course data sets were ascertained using the 1-way or 2-way analysis of variance (ANOVA) to determine statistical significance ($P < 0.05$); Sidak's or Tukey's post hoc tests were applied where multiple statistical comparisons were desired, while Fisher's uncorrected least significant difference (LSD) was used for instances of single comparisons. Student's *t*-test was utilized for other single comparisons, where noted.

RESULTS

Localization of *C3* mRNA in Human Donor Retinas: A Role for Macrophages

We investigated the localization of *C3* mRNA in normal aged human donor retinas and age-matched AMD-affected retinas

using ISH (Fig. 1). We detected minimal *C3* mRNA expression in normal retinas, and little to none within RPE cells or choroid (Fig. 1A). However, in early AMD retinas, *C3*-expressing cells were detected in the nerve fiber layer (NFL), superficial retinal vasculature, and subretinal space in regions adjacent to RPE disturbance (Figs. 1B–D). We detected numerous *C3*-expressing cells in regions of advanced scarring (Figs. 1E, 1F), at lesion edges (Figs. 1G–I), and near the ON head (Fig. 1J). In contrast, barely any labeling was detected in the choroid of these lesioned areas (Fig. 1E). Counter immunolabeling indicated that most of the *C3*-expressing cells were immunopositive for the macrophage/microglia marker IBA1 (Figs. 1K–N).

Complement Activation and Macrophage Recruitment Within Rat Retinal Lesions

Progression of lesion expansion in rat retinas following PD was tracked in vivo using OCT at 0, 3, 7, 14, and 56 days (Fig. 2). Measurements of ONL thickness were taken from cross-

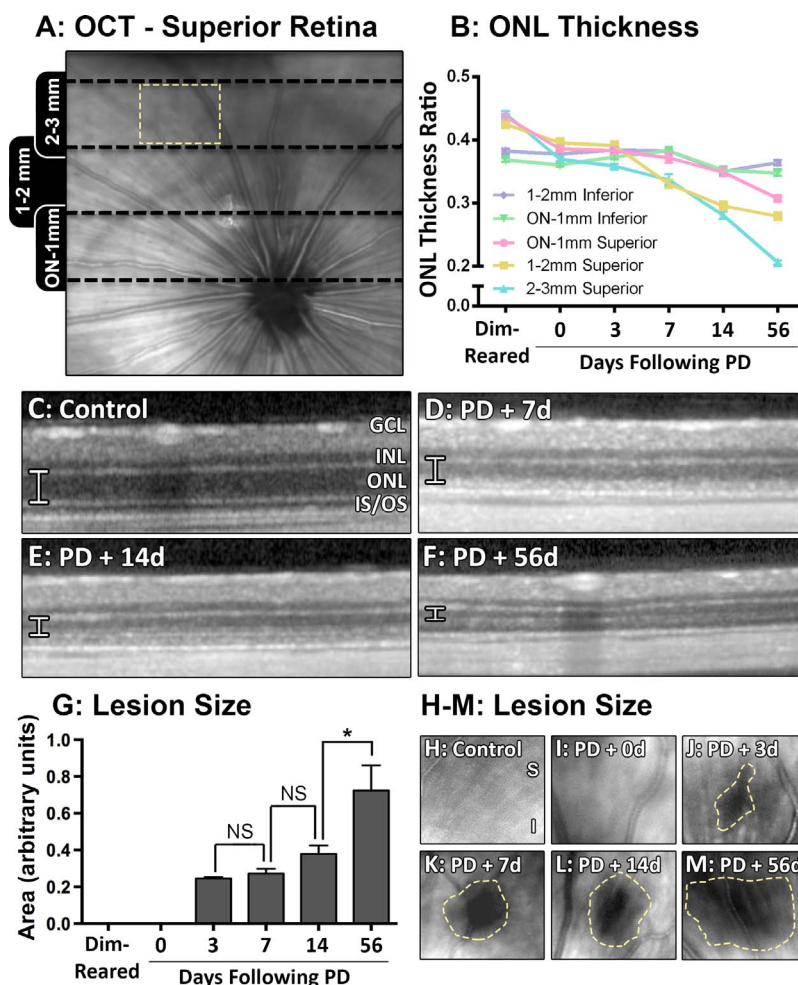


FIGURE 2. ONL thickness ratio changes following PD in rats. (A) The regions of the superior retina imaged using OCT were from the ON to 1 mm, 1 to 2 mm, and 2 to 3 mm above the ON. (B) The ONL thickness ratio from ON-1 mm (superior) and ON-2 mm (inferior) showed minimal change over the time course. The trend indicates a decrease in ONL thickness at 1 to 3 mm superior from 0 days onward ($P < 0.05$). A significant decrease in ONL thickness was observed at 2 to 3 mm (superior) up to 56 days ($P < 0.05$). (C–F) Representative OCT images of the control (C), in comparison to days 7 to 56 (D–F) at 2 to 3 mm (superior) illustrate substantial ONL thinning over the time course. (G) The area (arbitrary units) of the retinal lesion as observed on fundus images from days 3 to 56 was quantified using bitmap analysis of the lesion area in ImageJ. A gradual increase in lesion size over time was observed, which was significant from 14 to 56 days ($P < 0.05$). (H–M) Representative lesion area fundus images from 2 to 3 mm superior to the ON (dashed box in A). No lesion was detected in control (H) and 0 day (I) images. A retinal lesion was observed in fundus images from 3 days (J), which gradually increased in size at 7 days (K), 14 days (L), and 56 days (M). Statistical analysis was determined using 1-way ANOVA with Tukey's post hoc test ($P < 0.05$). GCL, ganglion cell layer; INL, inner nuclear layer; IS/OS, inner and outer segments; S, superior; I, inferior. $N = 4$ animals per group.

sectional images along three horizontal transects in the superior retina (Fig. 2A), and two in the inferior retina. Graphical representation of those measurements showed a gradual ONL thinning in the superior retina between 0 and 56 days, which was most prominent at 2 to 3 mm superior to the ON head (Fig. 2B). The data show a significant lesion detectable by 7 days ($P < 0.05$, Figs. 2B, 2D), which continued to develop further over the time course. The thinnest ONL was detected in the 2- to 3-mm transect at 56 days ($P < 0.05$, Figs. 2B, 2F). A retinal lesion (2–3 mm superior to the ON) was detected in fundus images from 3 days onward (Figs. 2G–M). The lesion size steadily increased from 3 to 14 days, and doubled from 14 to 56 days ($P < 0.05$, Figs. 2G–M).

Expression of a suite of complement components (*C1s*, *C2*, *C3*, and *C4a*) and regulators (*Cfb*, *Cfd*, *Serping1*, *Cfb*, *Cfi*) was analyzed across the time course in whole rat retinas (Fig. 3). All complement genes showed a significant trend in upregulation across the time course ($P < 0.05$), with *C1s*, *C3*, and *C4a* remaining significantly increased at 56 days postdamage

compared to dim-reared controls ($P < 0.05$, Fig. 3B). While the complement regulators *Cfb*, *Cfd*, *Serping1*, *Cfb*, and *Cfi* were all highly upregulated early in the time course (0–7 days, Fig. 3C), none were found to show any significant change by 56 days, compared to controls (Fig. 3D, $P > 0.05$).

Following PD, there was an incursion of IBA1⁺ microglia and macrophages into the ONL and subretinal space (Fig. 4). This persisted within the lesion up to 56 days (Fig. 4A). Counts of IBA1⁺ cells in the ONL and subretinal space showed a ~7-fold increase between 0 and 7 days (Figs. 4A, 4C), which decreased to approximately half the number by 14 days, but remained significantly higher than control animals in the outer retina at 56 days ($P < 0.05$, Figs. 4A, 4D, 4E).

ISH to localize expression of *C3* mRNA at 7, 14, and 56 days postexposure was consistent with previous findings in this model at 7 days,¹⁸ showing *C3* mRNA localization in the neural retina and subretinal space (Figs. 5A–F). We detected accumulations of *C3*-expressing macrophages predominantly clustered amongst the remnants of the ONL and at the lesion

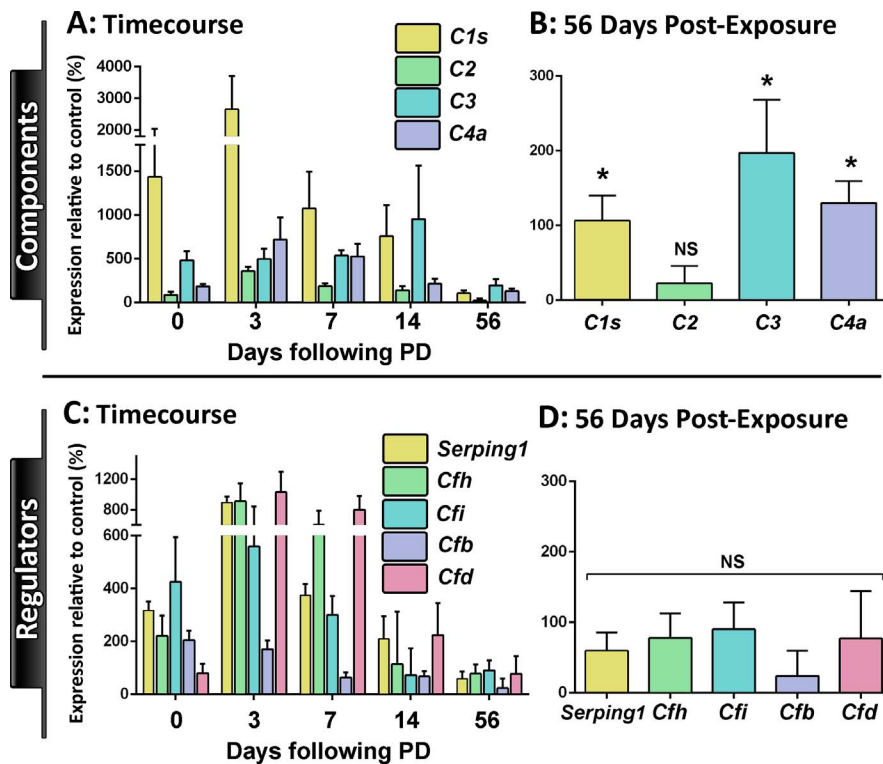


FIGURE 3. Complement gene expression in rat retinas following PD. All genes investigated were upregulated until 56 days postdamage compared to dim-reared controls. (A) Complement components *C1s*, *C2*, and *C4a* reached peak expression at 3 days. Expression of *C3* was highest at 0 to 7 days. (B) *C1s*, *C3*, and *C4a* were still significantly increased at 56 days compared to controls ($P < 0.05$). (C) Expression of the complement regulator gene *Cfb* was highest at 0 days, whereas *Cfd* expression peaked at 3 days. *Serping1*, *Cfb*, *Cfi* expression all peaked at 3 days. (D) *Serping1*, *Cfb*, *Cfi*, *Cfb*, and *Cfd* showed no statistically significant change after 56 days postexposure ($P > 0.05$). The trend in expression was determined using 1-way ANOVA (A, C, $P < 0.05$), and Student's *t*-test was used to compare controls and 56 days (B–D). $N = 4$ animals per group.

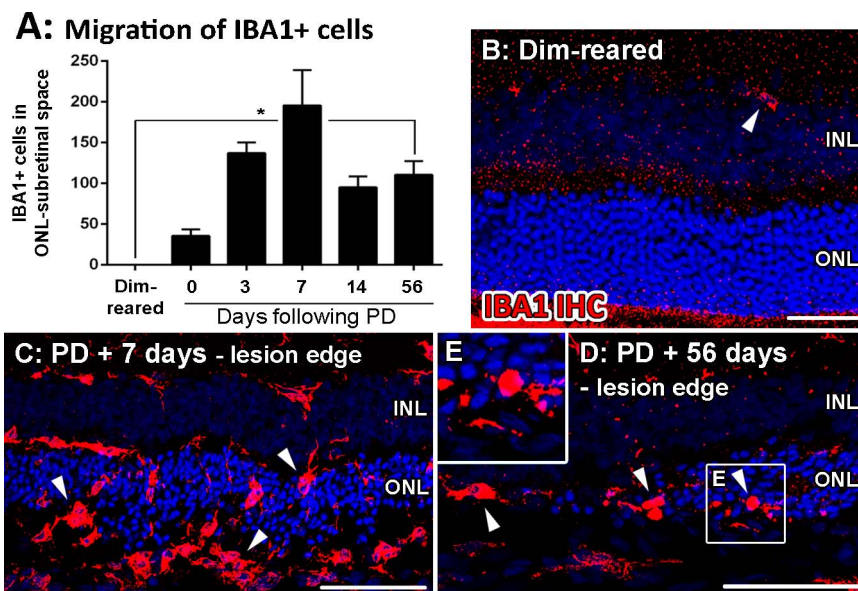


FIGURE 4. Infiltration of IBA1⁺ microglia/macrophages into the outer rat retina following PD. (A) In dim-reared controls, there were no IBA1⁺ cells in the outer retina (ONL-RPE). Upon damage, an increase in IBA1⁺ cell numbers in the outer retina reached a peak at 7 days, and remained significantly increased at 56 days ($P < 0.05$). (B) IBA1⁺ cells were detected only in the inner retinas of controls. (C) IBA1⁺ cell numbers in the outer retina and subretinal space peaked at 7 days at the lesion edge. (D, E) At 56 days, there were significant numbers of IBA1⁺ cells in the outer retina at the lesion edges, even though very few photoreceptor cell nuclei remained. Statistical significance was determined using a 1-way ANOVA. INL, inner nuclear layer. $N = 4$ animals per group. Scale bars represent 50 μ m.

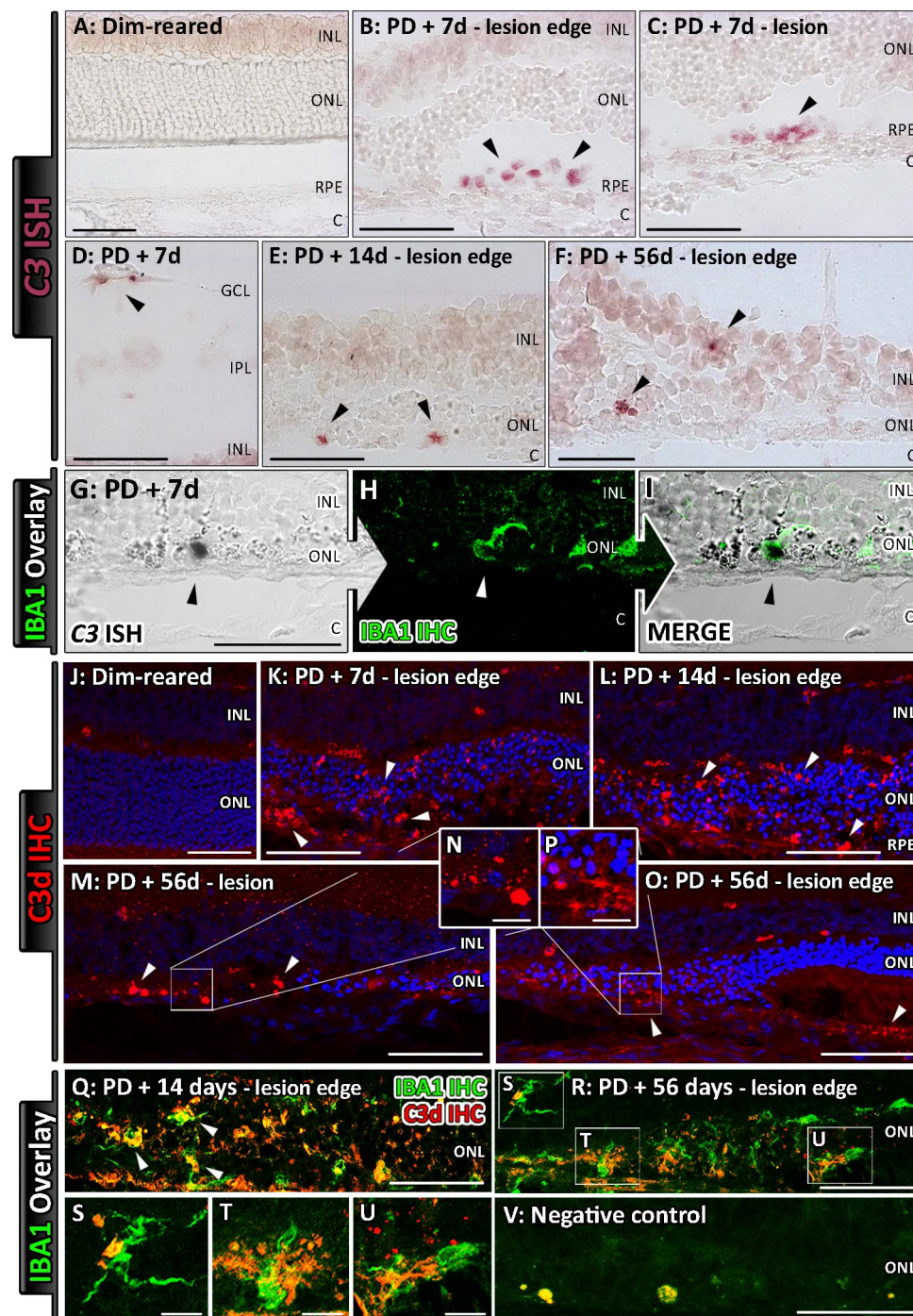


FIGURE 5. Infiltration of *C3*-expressing cells and *C3d* protein deposition in the outer rat retina following PD. (A) There was no outer-retinal *C3* expression in dim-reared controls. (B–D) At 7 days postdamage, *C3*-expressing cells were detected within the lesion and at the lesion edges in the outer retina (B, C), as well as near the inner retinal vasculature (D). (E) *C3*-expressing cells were present in the outer retina at 14 days. (F) At 56 days, *C3*-expressing cells were still present at the lesion edges. (G–I) These *C3*-expressing cells were identified as IBA1⁺ microglia/macrophages. (J) Low levels of *C3d* were detected in the inner retinal vessels of controls. (K) At 7 days postdamage, *C3d* deposition was evident throughout the outer retina (ONL and subretinal space) at the lesion edges. (L) *C3d* labeling peaked at 14 days, with large amounts of protein deposition detected at the lesion edges. (M–P) At 56 days, there was still *C3d* labeling present within the lesion (M, N) and at the lesion edges (O, P). (Q–U) At 14 days (Q) and 56 days (R–U), there was some co-localization of the *C3d* protein with IBA1⁺ microglia/macrophages at the lesion edges. (V) The negative control (no primary antibodies) showed only background staining, with some visible autofluorescence of debris near the outer retina. Representative images derived from *N* = 4 animals per group. INL, inner nuclear layer; C, choroid; IPL, inner plexiform layer. Scale bars represent 50 μ m in A–M, O, Q, R, V; 10 μ m in N, P, S–U.

edges, and near the inner retinal vasculature up to 56 days (Figs. 5A–F). Co-localization with IBA1⁺ immunolabeling (Figs. 5G–I) suggests the identity of these C3-expressing cells as macrophages. We did not observe C3 expression by other cell types in retina, including RPE cells, consistent with our previous reports.^{18,30}

We used immunoreactivity for C3d, a by-product of C3 cleavage, to visualize the distribution of C3 following PD (Figs. 5J–V). C3d labeling was absent in dim-reared controls (Fig. 5J). However, C3d immunoreactive deposits were prominent at 7 and 14 days postdamage in the ONL, amongst the photoreceptor segments and in the subretinal space, particularly at the lesion edges (Figs. 5K, 5L). These features persisted up to 56 days (Figs. 5M–P). Double immunolabeling for IBA1 and C3d showed a close association of macrophages with C3d deposits situated within the lesion (Figs. 5Q–V).

Suppression of Local C3 Inhibits Retinal Atrophy—Mouse Studies

To better understand the role of locally expressed C3 in complement deposition in the subretinal space, and its role in retinal atrophy, we used three different strategies to inhibit C3 in a model of retinal degeneration¹⁷: C3 KO (C3^{−/−}), local inhibition of C3 using siRNA, and systemic C3 depletion using CVF.

C3 Knockouts. Previous reports indicate that complete ablation of C3 reduces the ERG function and outer-retinal integrity in 12-month-old animals.³¹ For this reason, we carried out experiments on P60–80 mice, in which no functional or histologic deviations were observed.^{31,32} Dim-reared C3^{−/−} animals were assessed for any functional or histopathological changes compared to wild types (Wt). These comparisons showed no reduction in ERG amplitudes, changes in rhodopsin (*Rhod*) expression, or upregulation of the stress/neuroprotective factors GFAP, *Fgf-2*, or *Cntf* (Supplementary Fig. S1).

C3 gene expression and immunoreactivity for C3 were confirmed as absent in C3^{−/−} animals and in dim-reared Wt mice (Figs. 6A, 6B). Increased C3 expression in Wt retinas was evident after 1 day of PD and was upregulated across the exposure time course, with peak expression on day 5 ($P < 0.05$, Fig. 6A). Immunoreactivity for C3 deposits in the ONL and subretinal space of Wt retinas was evident at 5 and 7 days (Figs. 6C–E), and absent in C3^{−/−} retinas over the same period (Fig. 6F). Over the time course of PD, the ONL was better preserved in C3^{−/−} mice compared to Wt (Figs. 6G–M); at 7 days ONL thickness was significantly greater in the C3^{−/−} mice compared to Wt ($P < 0.05$, Figs. 6G, 6J, 6M).

The a-wave and b-wave responses of the ERG reflects the differences in retinal morphology of Wt and C3^{−/−} animals described above, where the ERG a-wave and b-wave intensity response characteristics between groups was significantly different ($P < 0.05$, Figs. 6N, 6O). C3^{−/−} mice had higher a- and b-wave responses compared to Wt mice (Figs. 6N, 6O); this difference was most pronounced at the highest flash intensity ($P < 0.05$, Fig. 6P).

Local C3 Inhibition Using siRNA. C3 siRNA significantly reduced C3 gene expression in retinas at 7 days PD ($P < 0.05$, Fig. 7A). Lower levels of C3 protein deposits were observed in C3 siRNA-injected retinas compared with those injected with negative control siRNA (Figs. 7B, 7C). In contrast, liver expression of C3 was not affected by intravitreal injection of C3 siRNA, indicating that siRNA injected into the eye was largely localized there (Fig. 7D).

Quantitative analysis of retinas postdamage showed that the photoreceptor population was protected against cell death in C3 siRNA-injected retinas compared with negative control siRNA (Fig. 7E). Comparison of retinal thickness shows that the

ONL is significantly thicker ($P < 0.05$) in C3 siRNA-injected animals compared with negative control siRNA (Fig. 7E). Furthermore, ERG analyses showed that the mean a-wave and b-wave amplitudes of the ERG were significantly greater in C3 siRNA-treated animals compared to negative siRNA controls, which was most pronounced at the highest flash intensity ($P < 0.05$, Fig. 6H). The ERG a-wave and b-wave intensity response characteristics between groups were significant ($P < 0.05$, Figs. 7F, 7G).

Systemic C3 Depletion Using CVF. To understand the possible role of serum complement components in the retina, we systemically depleted serum complement activity in Wt mice, using CVF. A hemolysis assay using serum from C3^{−/−} animals, and animals injected with CVF or PBS, indicated that at 7 days damage, serum complement activity was depleted by CVF to a comparable level to C3^{−/−} mouse serum, but not in PBS-injected animals, indicating that systemic complement was inhibited by CVF ($P < 0.05$, Fig. 8A).

To understand the impact of CVF on complement gene expression in the retina, we compared levels of C3, *C1s*, *C2*, *C4a*, *Cfb*, and *Cfb* at 7 days in animals injected with CVF, with PBS-injected controls. The elimination of systemic complement by CVF did not significantly alter the retinal expression of those complement components or regulatory genes (Fig. 8B). In addition, no difference was observed in retinal C3 deposition between CVF- and PBS-injected animals at 7 days (Figs. 8C, 8D).

Analysis of retinal morphology showed that depletion of systemic complement does not protect against retinal degeneration. No difference in ONL thickness in CVF-injected mice compared with PBS-injected mice was observed (Fig. 8E). Similarly, there were no significant differences in mean a-wave or b-wave amplitudes in CVF- and PBS-injected animals after 7 days damage, and no significant difference in the ERG intensity response characteristics between groups (Figs. 8F–H).

DISCUSSION

This is the first study to demonstrate the key importance of locally expressed sources of complement in driving complement-induced retinal atrophy. First, we show that C3 expression by macrophages in the human retina is closely linked to retinal atrophy in AMD. Second, we demonstrate in vivo the emergence and progression of a focal atrophic lesion in the rat retina over a period of 56 days, and the modulation of expression of a range of complement-related genes, including the persistent elevation of C3 and its expression by macrophages over the time course. Third, using C3^{−/−} mice, knockdown and depletion strategies we demonstrate conclusively that C3 expressed in the retina, but not serum C3, plays a decisive role in complement propagation in retinal degeneration. Taken together, these results suggest that a therapeutic approach targeting C3 could slow the progression of many neurodegenerative diseases including AMD.

Role of Complement in Onset of Retinal Atrophy

Complement dysregulation has been highly implicated in the progression of AMD.⁵ Consistent with other similar findings that complement overactivation contributes to retinal degenerations,^{33–38} our previous studies show an upregulation of complement genes *C1s*, *C2*, *C3*, and *C4a* in retinal degenerations.^{17,18,30,39} This study extends those collective findings showing that *C1s*, *C2*, *C3*, and *C4a* are upregulated during progressive lesion expansion, and that accumulation of C3-expressing macrophages at the lesion edges is a constant feature of lesion progression. This complement gene expres-

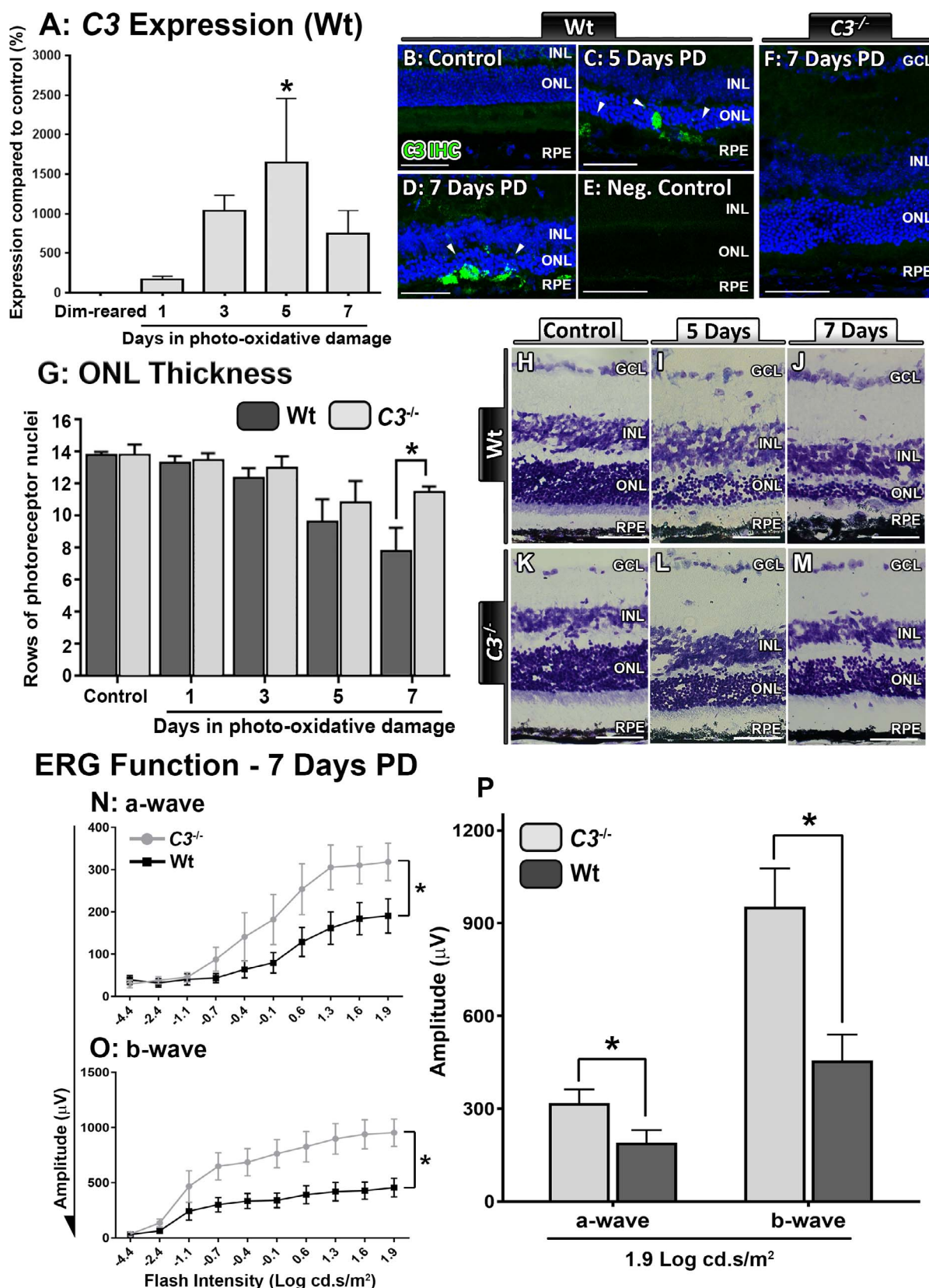


FIGURE 6. C3 expression in C57BL/6J Wt and C3^{-/-} mouse retinas. (A) C3 gene expression following PD increased to a peak at 5 days ($P < 0.05$ compared to control, $N = 8$ animals). (B–F) C3 protein deposition was evident at 5 and 7 days in Wt retinas (C, D). C3 detection was absent in C3^{-/-} retinas at 7 days (F), which was comparable to Wt controls (B). (G) ONL thinning was significant in Wt retinas compared to C3^{-/-} retinas at 7 days ($P < 0.05$, $N = 6$ animals). (H–M) Toluidine blue staining of Wt and C3^{-/-} retinas show no ONL disturbance in the controls (H, K), and progressive thinning at 5 days (I, L) and 7 days (J, M), with C3^{-/-} retinas having a thicker ONL than Wt retinas. (N–P) C3^{-/-} retinas at 7 days demonstrated a significantly higher a-wave (N) and b-wave (O) compared to Wt retinas ($P < 0.05$, $N = 10$ animals), which was most pronounced at 1.9 Log cd.s/m² (P, $P < 0.05$, $N = 10$ animals). Statistical significance was determined using 1-way (A) or 2-way ANOVA (G, Sidak's post hoc test; P, uncorrected Fisher's LSD for the 1.9 Log cd.s/m² comparison). GCL, ganglion cell layer; INL, inner nuclear layer. Scale bars represent 50 μm.

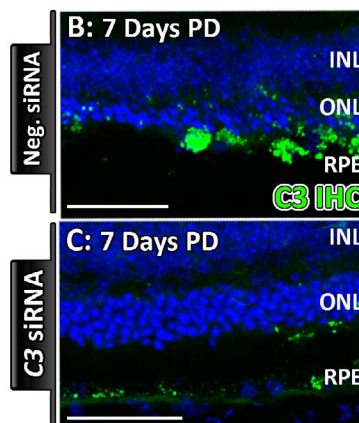
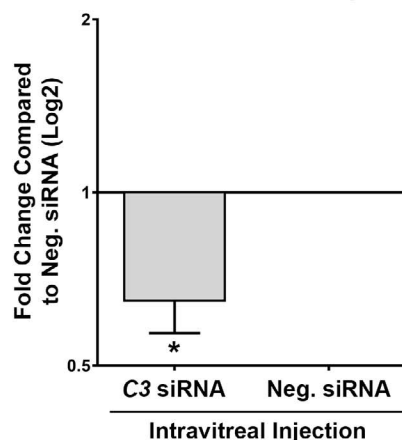
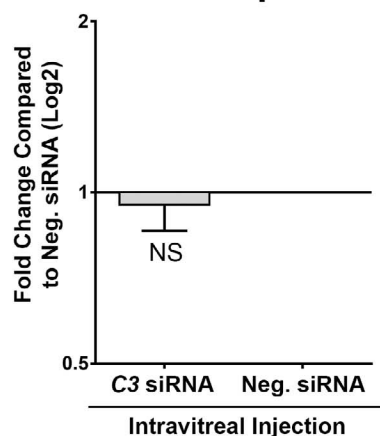
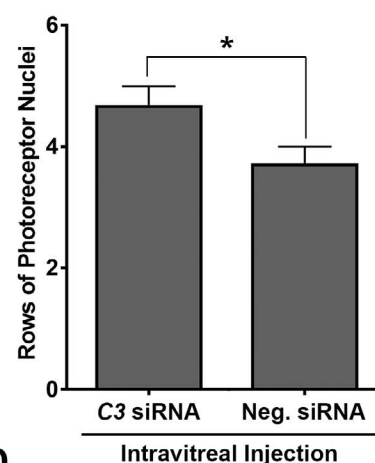
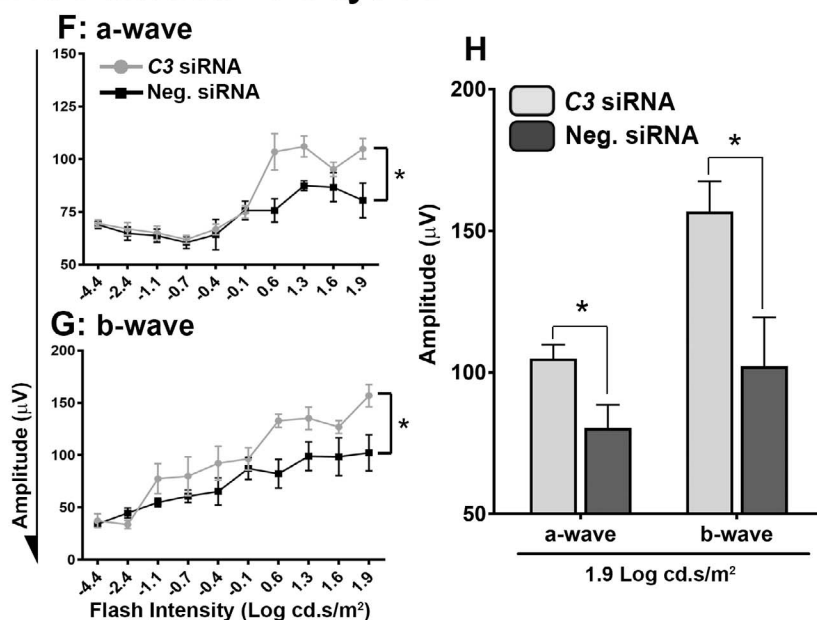
A: Retinal C3 - 7 Days PD**D: Liver C3 Expression****E: ONL - 7 Days PD****ERG Function - 7 Days PD**

FIGURE 7. Inhibition of retinal C3 expression using C3 siRNA and 7 days of PD. (A) Retinal C3 gene expression was significantly decreased following an intravitreal injection of C3 siRNA compared to the negative siRNA controls ($P < 0.05$, $N = 11$ animals). (B, C) There was a reduction in C3 protein deposition in the C3 siRNA-injected retinas compared to negative siRNA controls. (D) Liver C3 gene expression was not affected at 3 days after the C3 siRNA intravitreal injection ($N = 3$ animals). (E) There was a significant reduction in photoreceptor loss in C3 siRNA-injected retinas compared to negative siRNA controls ($P < 0.05$, $N = 13$ animals). (F–H) C3 siRNA retinas displayed a larger a-wave (F) and b-wave (G) compared to negative siRNA controls, which was most pronounced at 1.9 Log cd.s/m² (H), indicating a significantly improved retinal function ($P < 0.05$, $N = 4$ animals). Statistical significance was determined by an unpaired Student's *t*-test (A, D, E) or a 2-way ANOVA (F–G, uncorrected Fisher's LSD for the 1.9 Log cd.s/m² comparison). INL, inner nuclear layer. Scale bars represent 50 μ m.

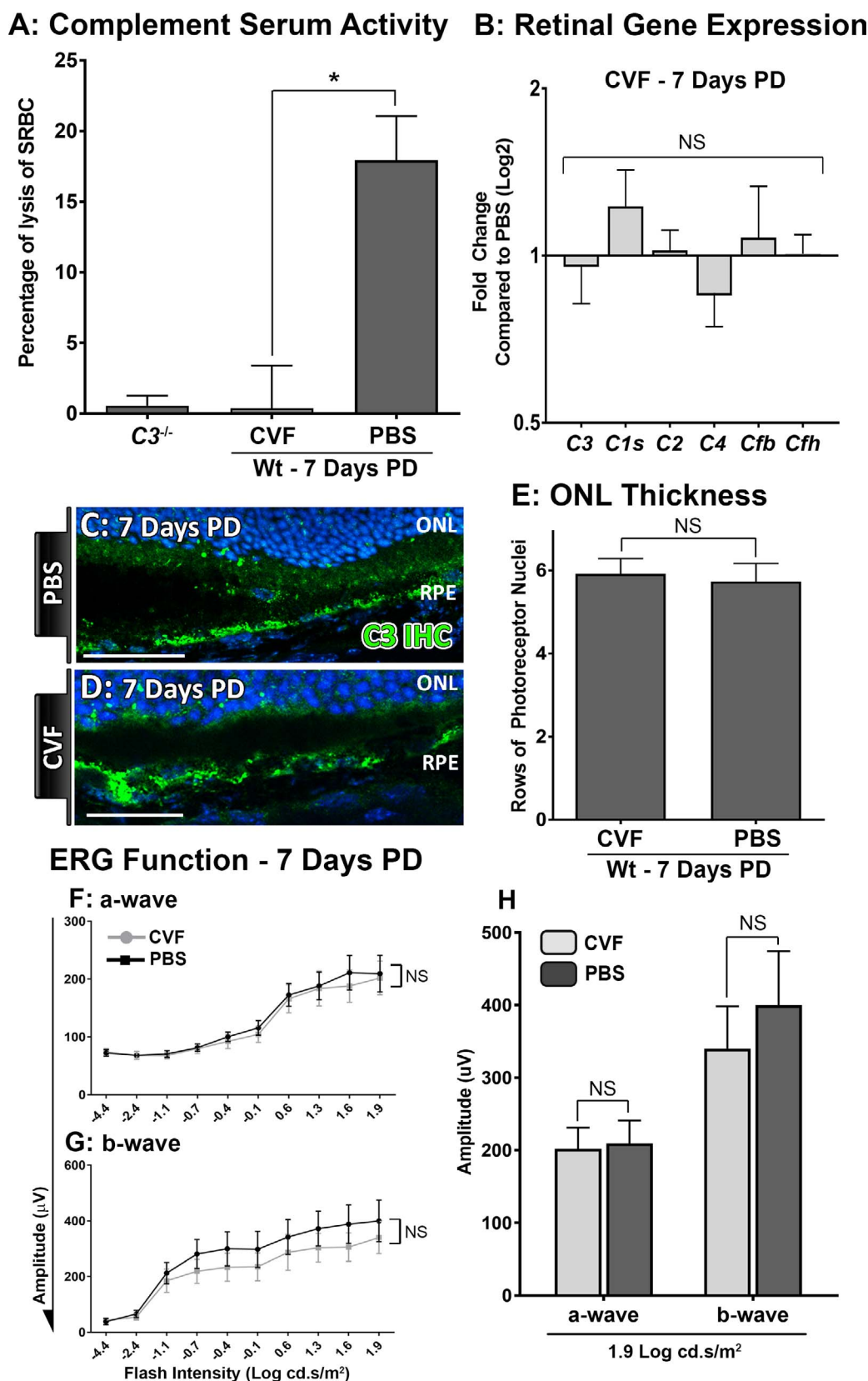


FIGURE 8. Depletion of systemic complement using CVF at 7 days of PD. (A) Complement activity in the serum was inhibited in CVF-injected animals compared to PBS controls as determined in a hemolytic assay using sheep red blood cells (SRBCs), where complement inhibition in CVF-injected animals was comparable to C3^{-/-} animals ($N = 4$ animals). (B) Retinal expression of complement genes (*C3*, *C1s*, *C2*, *C4a*, *Cfb*, *Cfh*) were not significantly affected by CVF depletion of systemic complement ($N = 9$ animals). (C, D) There was no difference in C3 protein deposition between CVF and PBS-injected animals. (E) No difference in ONL thickness was observed between CVF and PBS-injected animals ($N = 6$ animals). (F–H) Depletion of systemic complement did not decrease retinal function, as there was no significant difference in the a-wave (F) or b-wave (G) between CVF and PBS groups ($N = 5$ animals). Statistical significance was determined by an unpaired Student's *t*-test (A, B, E) or a 2-way ANOVA (F, G, uncorrected Fisher's LSD for the 1.9 Log cd.s/m² comparison). Scale bars represent 50 μ m.

sion profile is consistent with a broad scope of literature, which has genetically and histologically linked focal retinal atrophy, including AMD pathogenesis, to irregularities in the complement system.^{7,10–15,40} In particular, sustained upregulation of *C1s*, *C3*, and *C4a* demonstrated in this study is consistent with findings from systems-level analysis of AMD neural retinas.¹⁵

C3 propagation is strongly implicated in AMD through the association of the Y402H variant of *CFH*.^{7,8} We pursued the effects of disrupting local and systemic C3 using a triad of C3 inhibition strategies. The results show that the retina is protected from atrophy in both ablation and local knockdown of C3, but not from systemic CVF depletion of C3. This demonstrates that only locally expressed C3—not serum C3—is linked to retinal atrophy in this study. Serum complement components are constitutively produced and replenished by the liver,⁴¹ and it has been reported that C3 levels are elevated in the peripheral blood of AMD patients.⁴² Because complement components from serum would not be able to cross the intact blood-retinal barrier (BRB) due to their large size, it is unlikely that they could participate in the onset of retinal atrophy. However, as retinal atrophy progresses and there is breakdown of the outer BRB, there are no barriers to the permeation of serum constituents and this could contribute to further progression of atrophy.

Local C3-Expressing Macrophages As a Therapeutic Target for Reducing Retinal Atrophy

Macrophage infiltration of the subretinal space is a feature of AMD histopathology,^{43–45} including at the lesion edges in geographic atrophy,⁴³ and their pathogenic involvement in the disease is well-supported through findings in both laser-induced neovascularization^{46,47} and PD^{17,44,48} models. In the present study, we show that C3 is expressed only by retinal and subretinal macrophages, and not by RPE cells in AMD-affected retinas. Our current and previous findings^{18,30} are consistent with a transcriptome-wide analysis, combining over 60 donor samples covering all forms of AMD, which demonstrated C3 upregulation in neural retina, but not RPE/choroid.¹⁵

Despite this we do not suggest that the RPE/choroid interface plays no role in modulating complement, as these tissues are known to express a range of other complement constituents, and the MAC is abundant in the choriocapillaris in early AMD.⁴⁹ RPE cells have been reported in some studies as a local source of C3 in mouse retina in homeostasis or resulting from retinal degenerations.^{50–52} These studies relied upon PCR of combined extracts of RPE/choroid, or used cultured RPE. However, no study has confirmed that RPE expresses complement genes in the retinal environment in situ.

We surmise that complement activation and deposition in the outer retina is dependent on the summative effects of a range of mediators and regulators, and that accumulation of subretinal macrophages is a key component from the perspective of C3 synthesis and deposition. We identify retinal C3-expressing macrophages as a novel therapeutic target for mitigating the damaging effects of complement in retinal atrophy, and highlight a potential role for C3-targeted gene therapies.

An outstanding question is whether the C3-expressing macrophages associated with retinal atrophy derive from the pool of resident microglia, or recruited bone marrow monocytes, or both. O'Koren et al.⁵³ have shown, using genetic lineage tracing, that subretinal macrophages are predominantly resident microglia. On the other hand, their localization of some C3-expressing macrophages adjacent to the superficial retinal vasculature and ON suggest recruitment

pattern of bone marrow-derived macrophages,⁵⁴ suggesting that the C3-expressing population may also comprise nonresident macrophages.

CONCLUSIONS

Through combined observations in human donor tissue and models of retinal degeneration, our study illustrates a novel role of macrophages in priming the pathogenic activation of complement via their secretion of C3. We emphasize that this key contribution of subretinal macrophages does not preclude important roles for the RPE/choroid interface itself in influencing complement activation. However, our findings do reveal that the orchestration of complement-mediated pathology in AMD involves more elements than surmised previously,⁵ of which subretinal macrophages are a pivotal element. In demonstrating the efficacy of intravitreal C3 siRNA in mediating complement-induced retinal atrophy while also negating a contribution of systemic C3, we provide vital proof-of-principle support for locally administered gene therapy to target complement-induced retinal atrophy. This work lays a foundation for testing advanced gene-editing approaches, such as CRISPR/Cas9 technology, with the potential to revolutionize the therapeutic landscape for complement-mediated retinal degenerations including AMD.

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