A Novel Role of Complement in Retinal Degeneration

Minzhong Yu,[1] *Weilin Zou,*[2] *Neal S. Peachey,*[1,3,4] *Thomas M. McIntyre,*[2,5] *and Jinbo Liu*[2,5]

Purpose. The association of single nucleotide polymorphisms of components of the complement alternative pathway with the risk of age-related macular degeneration (AMD) indicates that complement signaling plays an important role in retinal physiology. How genetic variation leads to retinal degeneration is unknown. It has been assumed that complement activation augments immune responses, which in turn initiate AMD pathogenesis. To better understand the relationship between complement and the outer retina, we examined mice lacking the main complement component C3 and the receptors for complement activation fragments C3a (C3aR) and/or C5a (C5aR).

Methods. Complement mutant mice were studied along with wild-type (WT) littermates from 6 weeks to 14 months of age. Strobe flash electroretinography (ERG) was used to examine outer retinal function and a dc-ERG technique was used to measure ERG components generated by the retinal pigment epithelium. Retinas were examined by histology, immunohistochemistry, and biochemistry.

Results. Mice lacking C3aR and/or C5aR developed early onset and progressive retinal degeneration, accompanied by cleaved caspase-3 upregulation. Genetic deletion of C3aR and/or C5aR led to cell-specific defects that matched the cellular localization of these receptors in the WT retina. Compared to WT, $C3aR^{-/-}$ and $C3aR^{-/-}C5aR^{-/-}$ mice showed increased retinal dysfunction upon light exposure. $C3aR^{-/-}C5aR^{-/-}$ mice immunized with 4-hydroxynonenal-adducted protein developed severe retinal impairment unrelated to immune response.

Conclusions. C3aR- and C5aR-mediated signaling was necessary to maintain normal retinal function and structure. These receptors may be important biomarkers for predicting retinal degeneration including AMD. (*Invest Ophthalmol Vis Sci.* 2012; 53:7684–7692) DOI:10.1167/iovs.12-10069

Degeneration of photoreceptors underlies vision loss in early onset disorders such as retinitis pigmentosa (RP), cone-rod dystrophy, and Lebers congenital amaurosis, as well as in late onset conditions such as age-related macular degeneration (AMD). These retinal disorders affect more than 200,000 US children, 2 million US elderly, and more than 50 million people worldwide.[1,2] A breakthrough finding in understanding AMD pathophysiology was the observation that single nucleotide polymorphisms (SNPs) of the complement alternative pathway components factor H, factor B, and complement C3 strongly associate with the risk of AMD development.[3–9] Factor H is an inhibitor of complement activation, while factor B and C3 are essential components of complement activation. How variation in these genes is involved in the pathology of AMD remains unknown, although a current working hypothesis is that complement activation leads to AMD through an inflammatory mechanism.[10–12]

There are three pathways of complement activation, the classical, alternative, and lectin pathways. The main components of the alternative pathway include factor D, factor B, factor H, C3, and C5. The alternative pathway is initiated by spontaneous C3 hydrolysis C3(H_2O), whose function is equal to that of C3b. C3(H_2O) binds factor B so factor B is cleaved by factor D to form a C3 convertase, C3bBb. C3 convertases cleave C3 into C3a and C3b. A C3 convertase also binds C3b to form a C5 convertase (C3bBbC3b), which cleaves C5 to C5a and C5b. The coagulation/fibrinolysis proteases may also act as C3 and C5 convertases to produce C3a and C5a.[13] Fragments C3a and C5a activate complement signaling by binding the C3a receptor (C3aR) or the C5a receptor (C5aR), respectively.[14,15] C3 is a central component of all complement activation pathways. Outside of the retina, increased activation of C3aR or C5aR has been implicated in autoimmune diseases, transplant rejection, and inflammatory diseases.[16–19] Presuming an analogous role in AMD pathogenesis, multiple groups are exploring the possibility that complement inhibition may be effective for preventing or slowing AMD progression.[12,20–22] Several findings, however, argue against this strategy. For example, mice lacking C3a and/or C5a signaling have abnormal susceptibility to neuroexcitotoxicity,[23] abnormal neurogenesis,[24] abnormal differentiation and migration of neural progenitor cells,[25] abnormal liver cell survival/regeneration,[26] and abnormal remyelination.[27] Furthermore, the pathology associated with a model of retinopathy of prematurity was exacerbated in mice lacking either C3 or C5aR.[28] To date, the retinas of mice lacking C3aR or C5aR have not been examined. Here we report that $C3aR^{-/-}$ and/or $C5aR^{-/-}$ mice, as well as $C3^{-/-}$ mice, developed progressive retinal degeneration and retinal dysfunction that matched the cellular distribution of these proteins in the retina. These results established a requirement for the alternative complement pathway in normal maintenance of the outer retina.

Materials and Methods

Animals

Mice were obtained from the Jackson Laboratory (Bar Harbor, ME). $C3aR^{-/-}$ mice at BALB/cJ background (stock number: 005712) and $C5aR^{-/-}$ (stock number: 005676; at unknown generations of C57BL/6J background) were backcrossed to C57BL/6J wild-type (WT) mice (stock number: 000664) for eight generations at the Cleveland Clinic

From the [1]Department of Ophthalmic Research, Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, Ohio; the [2]Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio; [3]Research Service, Cleveland VA Medical Center, Cleveland, Ohio; and the Departments of [4]Ophthalmology and [5]Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio.

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Corresponding author: Jinbo Liu, Department of Cell Biology, Lerner Research Institute, The Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195; Liuj6@ccf.org.

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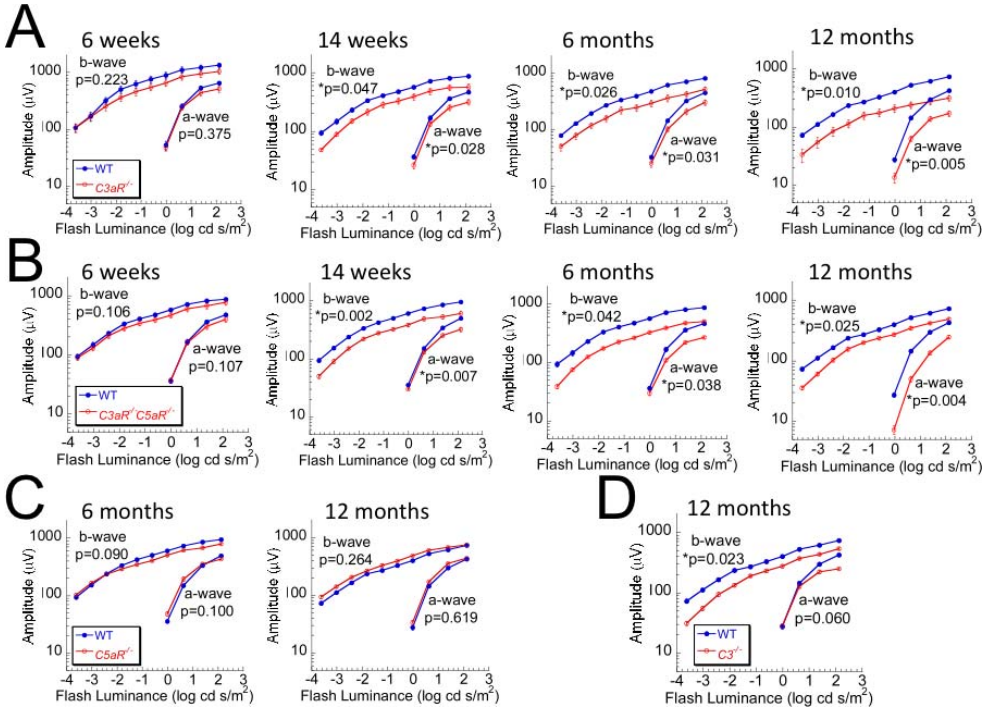

Figure 1. ERG measures of outer retinal function in complement mutant mice. ERGs were recorded in (**A**) *C3aR*$^{-/-}$, (**B**) *C3aR*$^{-/-}$*C5aR*$^{-/-}$, (**C**) *C5aR*$^{-/-}$, (**D**) *C3*$^{-/-}$, and their WT littermates at 6 weeks to 12 months of age. *Data points* indicate average (±SEM) of up to 14 mice. *$P < 0.05$.

Foundation. Back-crossed eight-generation *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice were then intercrossed to generate *C3aR*$^{-/-}$*C5aR*$^{-/-}$ double knockouts. *C3*$^{-/-}$ mice (stock number: 003641) were obtained on a C57BL/6J background. All of the mice in this study were on C57BL/6J background unless indicated specifically. The discovery[29] that many mouse lines carry *Crb1*^{rd8} caused us to evaluate the mice studied here. We screened the available samples (14 *C3aR*$^{-/-}$, nine *C5aR*$^{-/-}$, 15 *C3aR*$^{-/-}$*C5aR*$^{-/-}$, two *C3*$^{-/-}$) for the *Crb1*^{rd8} allele. All mice were WT except for a single mutant that was *Crb1*$^{+/rd8}$ heterozygous. All animal studies were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation. The animals were treated in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Materials and Reagents

4-Hydroxynonenal (4-HNE) was from Alpha Diagnosis International Inc. (San Antonio, TX). Mouse serum albumin (MSA) was from Sigma (St. Louis, MO). Cleaved caspase 3 detection kits were obtained from Cell Signaling Technology (Danvers, MA). Antibodies: NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), p65: polyclonal rabbit (Cell Signaling Technology), phospho-IKKα/β, IkBα, and phospho-p65: polyclonal rabbit (Cell Signaling Technology), C3aR: monoclonal mouse (Santa Cruz Biotechnology, Santa Cruz, CA), C5aR: monoclonal mouse (Santa Cruz Biotechnology), Interleukin-17 (IL-17): monoclonal mouse (R&D Systems, Minneapolis, MN), PKCα: monoclonal mouse (Santa Cruz Biotechnology), Calbindin D-28: polyclonal rabbit (Millipore, Billerica, MA). PKCα was used to label rod bipolar cells[30] and Calbindin D-28 was used to label horizontal and amacrine cells.[31]

Electroretinography

After overnight dark adaptation and pupil dilation (1% tropicamine; 2.5% phenylephrine HCl), electroretinograms (ERGs) were recorded from the corneal surface of anesthetized mice (ketamine 80 mg/kg; xylazine 16 mg/kg) using published procedures[32–34] to examine function of the outer retina and retinal pigment epithelium (RPE).

Anatomical Analysis

Immediately after sacrifice, one eye was embedded in ornithine carbamoyltransferase freezing medium, flash frozen on powdered dry ice, and immediately transferred to a –80°C freezer. The other eye was fixed in 4% paraformaldehyde for 4 hours after the cornea and lens were removed. The eye cup was immersed through a graded series of sucrose solutions: 10% for 1 hour, 20% for 1 hour, and 30% overnight. 5-μm sections were cut on a cryostat. For immunofluorescence staining, retinal sections were incubated with primary antibodies (1:100) overnight at 4°C, in FITC-labeled secondary antibodies (1:1000) for 1 hour, and were then washed and mounted using mounting medium (VECTASHIELD; Vector Laboratories, Burlingame, CA). For electron microscopy sample preparation, ultrathin sections (85 nm) and electron micrographs were performed by the image core in the Cleveland Clinic Foundation. All images were taken along the horizontal meridian, approximately 200 μm from the edge of the optic nerve head.

Light Exposure

After pupil dilation (2.5% phenylephrine HCl; 1% cyclopentolate HCl; 1% mydriacyl), mice were exposed for 8 hours to white light of 7500 lux. ERGs were recorded on day 6 after light exposure, after which eyes were extracted and prepared for anatomical analysis.

HNE-MSA Immunization

We made 4-HNE-conjugated MSA (HNE-MSA) following a published method.[35] 4-HNE modification of MSA was confirmed by the Mass Spectrometry Core II of the Cleveland Clinic. *C3aR*$^{-/-}$*C5aR*$^{-/-}$ and WT littermates were immunized subcutaneously with 200 μg of

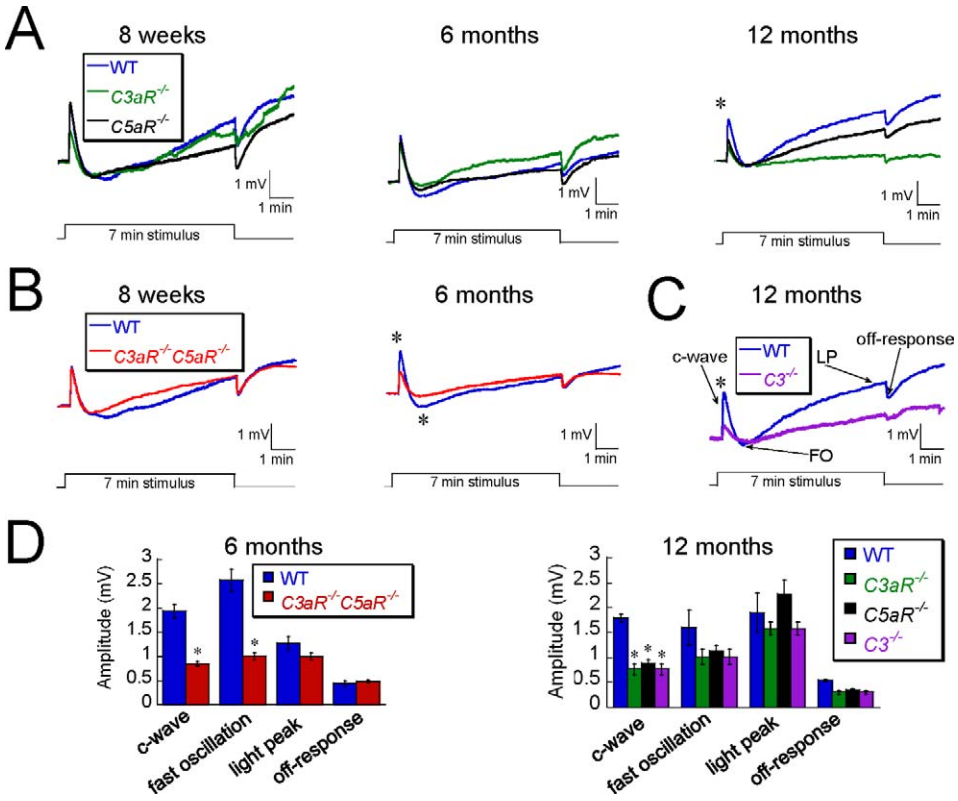

Figure 2. ERG measures of RPE function in complement mutant mice. DC ERG obtained from (**A**) *C3aR*$^{-/-}$, *C5aR*$^{-/-}$, (**B**) *C3aR*$^{-/-}$*C5aR*$^{-/-}$, (**C**) *C3*$^{-/-}$, and their WT littermates at 8 weeks to 12 months of age. Waveforms represent the grand average response of 6 to 10 mice. (**D**) Average (±SEM) amplitude of major components of DC-ERG (c-wave, fast-oscillation [FO], light peak [LP], off-response) in mutant mice and WT littermates. $^{*}P < 0.05$.

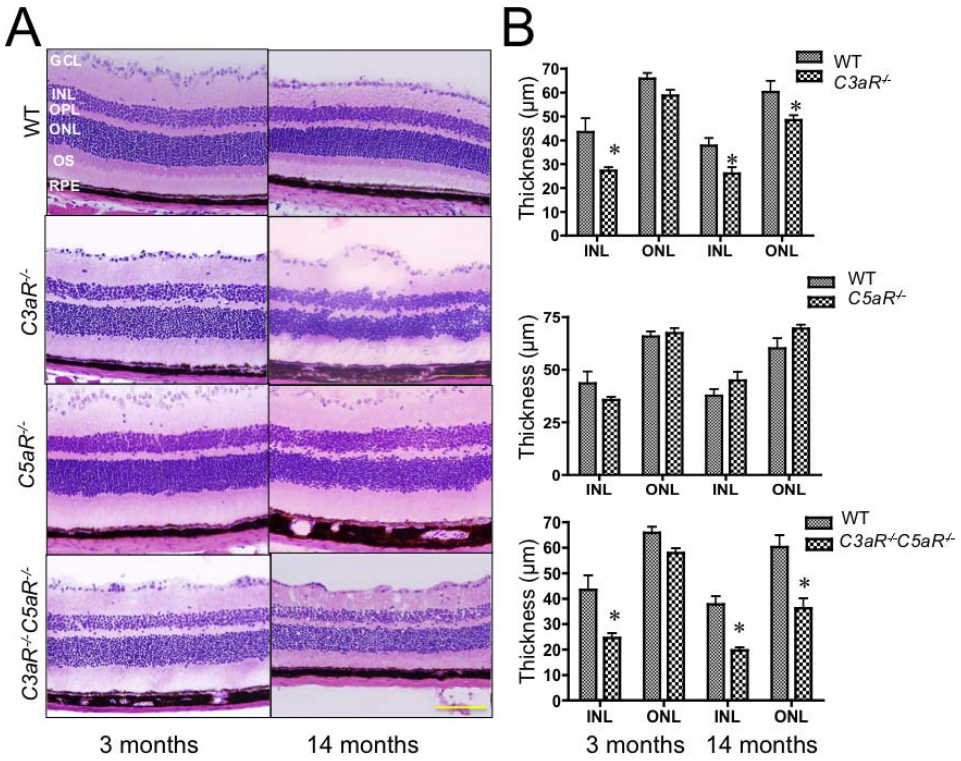

Figure 3. Anatomical analysis of complement mutant mice. (**A**) H&E staining of retinal cross-sections obtained from *C3aR*$^{-/-}$, *C5aR*$^{-/-}$, *C3aR*$^{-/-}$*C5aR*$^{-/-}$, and WT littermates. Images are representative of at least 10 mice from each group. All sections of photos were taken at the central areas (200 µm from the optic nerve head). Magnification: ×40; *scale bar* indicates 100 µm. (**B**) Thickness of ONL and INL. *Bars* indicate average (±SEM) of at least 10 mice per group. $^{*}P < 0.05$.

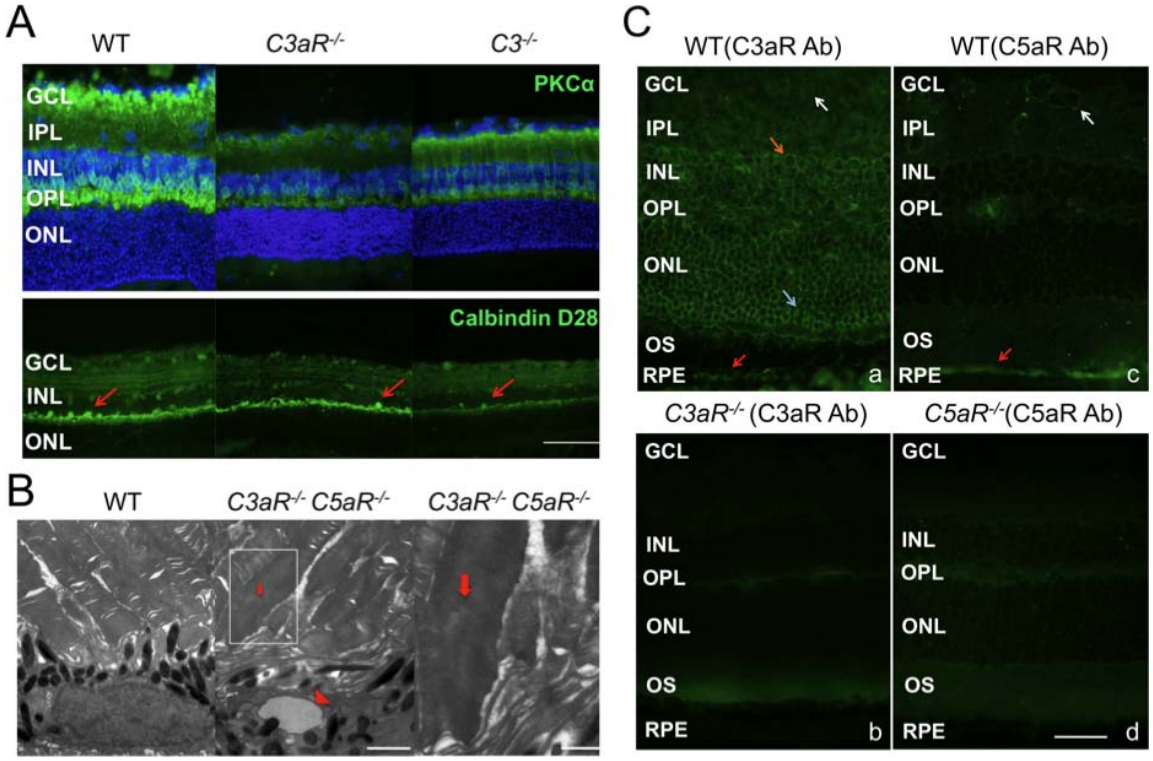

Figure 4. Immunohistochemical analysis. (**A**) Staining of retinal sections of 14-month-old WT, *C3aR*$^{-/-}$, and *C3*$^{-/-}$ mice for rod bipolar cells (PKCα antibody; *upper panels*; magnification: ×60) or for horizontal and amacrine cells (Calbindin D-28 antibody; *lower panels*; magnification: ×40; *scale bar* indicates 100 μm). Images are representative of at least five mice from each group. All sections of photos were taken at the central areas (200 μm from the optic nerve head). (**B**) Electron microscopy of RPE-outer segment interface in 6-month-old WT (*left*) and *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice (*middle* and *right*). *Triangle* indicates the apoptotic RPE nucleus (*middle*), magnification: ×7100; *scale bar* indicates 2 μm for *left* and *middle* images. *Arrows* indicate swollen outer segment (*right*; magnification: ×19,500; *scale bar* indicates 1 μm). (**C**) Distribution of C3aR and C5aR in retina. C3aR antibody labeling of WT (**a**) showed C3aR expression in INL (*orange arrow*), ONL (*light blue arrow*), RPE (*red arrow*), and ganglion cell layer (*white arrow*), but was missing in the *C3aR*$^{-/-}$ retina (**b**). C5aR antibody labeling of WT retina (**c**) showed C5aR expression in RPE (*red arrow*) and ganglion cells (*white arrow*), which was missing in *C5aR*$^{-/-}$ retina (**d**). Magnification: ×60; *scale bar* indicates 50 μm.

HNE-MSA, or MSA as control, in complete Freund's adjuvant at day 1, and in incomplete Freund's adjuvant at days 10 and 50. Two months later after first immunization, ERGs were recorded for those immunized mice, after which eyes were extracted and prepared for anatomic analysis.

Retinal Cell Isolation and Analysis

Retinal cells were isolated using a published procedure.[36] Briefly, each mouse eyeball was enucleated and placed in sterile culture plates with sterile 1× PBS. All subsequent procedures were conducted in a sterile tissue culture hood. Each eyeball was cut from the middle of the cornea. After the lens was removed, the retina and RPE were isolated. To release cells, retinas were incubated in Ringer buffer containing 10 to 20 U/mL papain and 0.1 mg/mL cysteine for 30 minutes at 30°C; 11-*cis* retinal was not added. After rinsing with Ringer buffer, the retinas were triturated by a glass pipette. The retinal cells were counted by trypan blue, and few dead cells were seen. Neural retinal cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (0% FCS) with 5 μg/mL HNE-MSA for 4 hours to detect apoptotic cells by Annexin V antibody staining/flow-cytometry. Another portion of retinal cells was grown on sterile cover slips overnight to seed cells and the next day exposed under light (FLUOREX 27W; Lights of America, Orange, CA) (fluorescent linear bulb at 10 cm distance) for 12 hours for p65 staining.

ELISPOT Assay

Splenocytes (6×10^5) from HNE-MSA immunized mice were incubated for 24 hours in IL-17 monoclonal antibody (R&D Systems)-coated and PBS-1% BSA-blocked 96-well MultiScreen HTS filter plates for the enzyme-linked immunosorbent spot (ELISPOT) (Whatman, Piscataway, NJ) with 0.5 μg/mL HNE-MSA. Spots were developed and quantitated with a CTL ImmunoSpot Analyzer (Cellular Technology, Shaker Heights, OH).

Mass Spectrometry

Mouse plasma was collected and frozen immediately. Mass spectrometric analyses for phospholipids were performed on-line using electrospray ionization tandem mass spectrometry in the positive ion mode with multiple reaction monitoring using the molecular cation $[MH]^+$ and the *m/z* 184 daughter phosphocholine ion. For free fatty acid analysis, negative-ion mode with multiple reaction monitoring of parent and individual daughter ions of oxidized and unoxidized free fatty acids used the *m/z* transitions.

Statistics

ERG measures were evaluated with a two-way ANOVA. The power analysis was conducted by the F-test of one-way ANOVA, where we considered numbers as outcome and groups as the factor. $P < 0.05$ was considered statistically significant. All other comparisons between

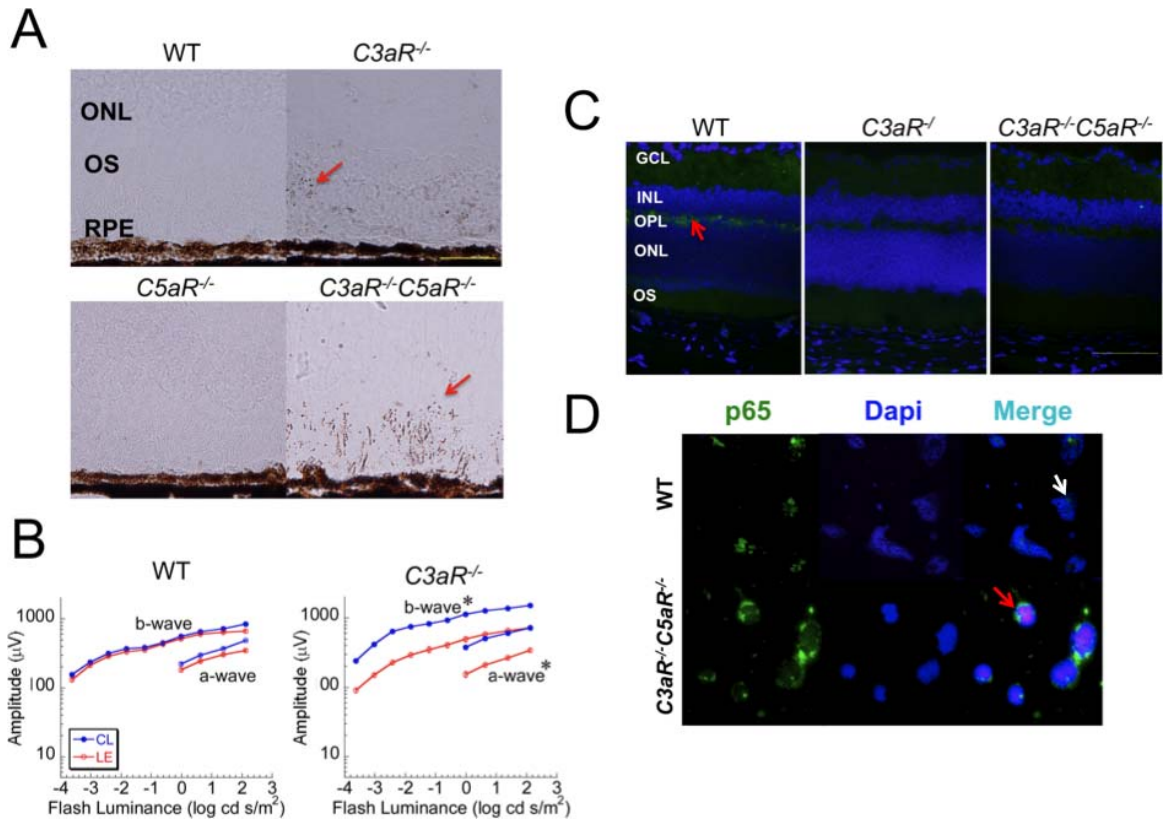

Figure 5. Light exposure. (**A**) Retinal sections of 14-month-old WT, *C3aR*$^{-/-}$, *C5aR*$^{-/-}$, and *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice probed for cleaved casepase-3, which appears as *brown spots* (*pointed by arrows*). *Scale bar* indicates 50 μm. (**B**) ERG studies 6 days following 8-hour light exposure of 6-week-old *C3aR*$^{-/-}$*C5aR*$^{-/-}$ and WT mice (at Balb/c background). *Data points* indicate the average (±SEM) of five mice per group at each condition. $^{*}P < 0.05$. (**C**) Retinal cross-sections made following ERG analysis and reacted with phospho-IKKα/β. Bright green staining (*pointed by an arrow*) was detected in the OPL of WT littermates but not *C3aR*$^{-/-}$ or *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice. Pictures represent two independent experiments with the same results, magnification: ×60; *scale bar* indicates 500 μm. (**D**) Antibody staining for p65 nuclear translocation of isolated neural retinal cells obtained from WT (*top*) or *C3aR*$^{-/-}$*C5aR*$^{-/-}$ (*bottom*) mice after 12-hour light exposure. The *white arrow* points to p65 (*green*) in the nucleus of WT retinal cells and the *red arrow* points to the p65 (*green*) in the cytoplasm of *C3aR*$^{-/-}$*C5aR*$^{-/-}$ retinal cells.

WT and mutant data were made using two-tailed, unpaired Student's *t*-tests.

Results

Outer Retinal Function Is Impaired in Mice Lacking C3aR and/or C5aR

We used ERGs to compare outer retinal function of WT and complement mutant mice at ages ranging from 6 weeks to 12 months. Strobe flash ERGs were used to evaluate function of photoreceptors (a-wave) and depolarizing bipolar cells (b-wave). At 6 weeks of age, there was no significant difference between ERG a- and b-wave amplitudes of WT and *C3aR*$^{-/-}$ or *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice (Figs. 1A, 1B). However, by 14 weeks of age, ERG a- and b-waves of *C3aR*$^{-/-}$ and *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice were significantly reduced, and this reduction progressed with increasing age (Figs. 1A, 1B). In comparison, there was no difference between the a- or b-waves of WT and *C5aR*$^{-/-}$ mice, even at 12 months of age (Fig. 1C). ERG b-waves of *C3*$^{-/-}$ mice were also significantly decreased at 12 months old (Fig. 1D), but not at younger ages.

We used dc-ERGs to document abnormal function of the RPE, whose activity is reflected in four major components (c-wave, fast oscillation, light peak, and off-response). At 8 weeks of age, we saw no significant difference in ERG potentials generated by the RPE between WT and complement mutant mice (Figs. 2A, 2B). At 12 months of age, however, the amplitude of the c-wave was significantly reduced in *C3aR*$^{-/-}$, *C5aR*$^{-/-}$, and *C3*$^{-/-}$ mice (Figs. 2A, 2C, 2D). No other component of the dc-ERG was significantly different between WT and *C3aR*$^{-/-}$, *C5aR*$^{-/-}$, or *C3*$^{-/-}$ mice. The amplitudes of the c-wave and the fast oscillation were reduced at an earlier time point (6 months) in *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice (Figs. 2B, 2D).

Progressive Retinal Cell Loss in Mice Lacking C3aR and/or C5aR

Retinal structure was examined at ages where ERG studies were conducted. No significant changes in outer nuclear layer (ONL) thickness were noted at 3 months of age, but the ONL was disorganized at this age in *C3aR*$^{-/-}$ and *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice (Figs. 3A, 3B). At older ages, we noted ONL thinning and disorganization in mice lacking C3aR (either *C3aR*$^{-/-}$ or *C3aR*$^{-/-}$*C5aR*$^{-/-}$) but not C5aR. The inner nuclear layer (INL) of *C3aR*$^{-/-}$ or *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice was significantly thinner than WT littermates at 3 and 14 months of age (Figs. 3A, 3B). Because rod bipolar cells are a main component of the INL, we used PKCα immunolabeling to identify this cell type. In comparison to WT (60 ± 12; $n = 5$), the average (±SD)/200-μm field density of rod bipolar cells was significantly decreased at 3 months and declined further at 14 months in *C3aR*$^{-/-}$ (23

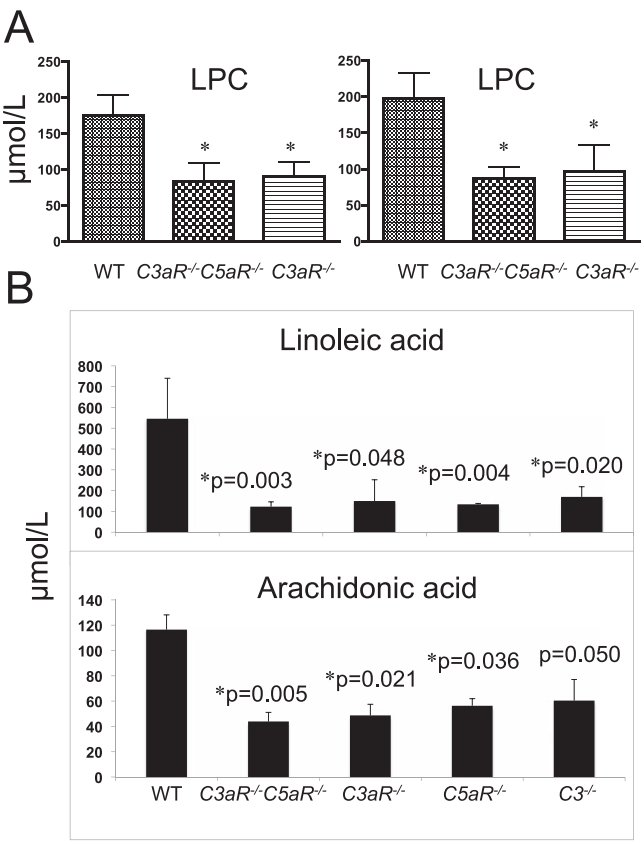

Figure 6. Mass spectrometric analysis of mouse plasma for phospholipids and free fatty acids. (**A**) LPC levels in mice at 2 to 3 months (*left*) or 14 months (*right*) of age. (**B**) Levels of LA and AA in 14-month-old WT and complement mutant mice. *Bars* indicate average (±SEM) of 8 to 10 mice. $^*P < 0.05$. Figures are a representative of three independent experiments with equal numbers of males and females.

± 7; $n = 8$) and $C3aR^{-/-}C5aR^{-/-}$ (20 ± 5; $n = 5$) retinas (Fig. 4A). The number of rod bipolar cells was also decreased in 14 month old $C3^{-/-}$ mice (31 ± 10; $n = 5$) (Fig. 4A), but not in mice lacking only C5aR (65 ± 11; $n = 5$). We used calbindin to immunolabel horizontal cells. In comparison to WT (11 ± 3; $n = 5$), the average (±SD)/300-µm field density of horizontal cells was decreased in $C3aR^{-/-}$ (3 ± 2; $n = 8$), $C3aR^{-/-}C5aR^{-/-}$(3 ± 2; $n = 5$) and $C3^{-/-}$ (4 ± 3; $n = 5$) mice (Fig. 4A), but not in mice lacking only C5aR (12 ± 4; $n = 5$). Electron microscopy showed that RPE cell nuclei were shrunken or apoptotic and the normal structure of outer segment discs was lost in $C3aR^{-/-}C5aR^{-/-}$ mice at 6 months (Fig. 4B).

We used antibodies to define the location of C3aR and C5aR. Consistent with a recent report,[37] C3aR is expressed in multiple layers of the WT retina (INL, ONL, RPE, ganglion cell layer) and is missing in the $C3aR^{-/-}$ retina (Fig. 4C). C5aR is expressed in RPE and ganglion cells of the WT retina, but not in the $C5aR^{-/-}$ retina (Fig. 4C). These distributions match the functional and anatomical changes in these mutant mice, indicating that C3aR and C5aR play critical and cell-specific roles for the maintenance of neural retina and RPE cells.

Increased Activated Caspase-3 and Decreased NF-κB Activation in the Absence of C3aR or C3aR/C5aR

We studied cellular apoptosis to examine the mechanism underlying cell loss and dysfunction in $C3aR^{-/-}$ and $C3aR^{-/-}C5aR^{-/-}$ retinas. Levels of apoptotic cleaved caspase-3 were elevated in $C3aR^{-/-}$ and $C3aR^{-/-}C5aR^{-/-}$ retinas at 3 months and increased further at 14 months (Fig. 5A).

Excessive light will induce retinal degeneration.[38] If C3aR and C5aR normally function to ameliorate light-induced retinal damage, the effect should be more pronounced in mice lacking these proteins. In comparison to WT, after an 8-hour light exposure $C3aR^{-/-}$ mice showed a significantly greater loss in retinal function in both C57BL/6J and BALC/cJ backgrounds. In comparison to WT, $C3aR^{-/-}$ mice in BALB/cJ background showed a greater decrease in retinal function (Fig. 5B). Histologic studies showed the same findings in both C57BL/6J and BALB/cJ mice. We used four NF-κB pathway antibodies that work for immunohistochemistry to examine cellular stimulation in retinas removed seven days after 8-hour light exposure. We found that level of phospho-IKKα/β (not IkBα, p65, and phospho-p65) was significantly increased in the WT OPL but not in the OPL of $C3aR^{-/-}$ or $C3aR^{-/-}C5aR^{-/-}$ mice (Fig. 5C).

In isolated retinal cells, light exposure of WT and $C3aR^{-/-}C5aR^{-/-}$ retinal cells was carried out in parallel in six-well plates. We observed p65-nuclear translocation in almost all WT but not in $C3aR^{-/-}C5aR^{-/-}$ cells after the 12 hour-light stimulation (Fig. 5D). These results indicate that C3aR could play an important role in protecting retinal cells from light-induced retinal degeneration through the NF-κB pathway.

Plasma Lysophosphatidylcholine or Free Polyunsaturated Fatty Acids Are Significantly Decreased in the Absence of Complement Proteins

Light and aging produce reactive oxygen species, which attack phospholipids, polyunsaturated fatty acids (PUFAs), proteins, and DNA to cause lipid peroxidation, fatty acid oxidation, protein modification, and DNA breakage, respectively. Phospholipids and PUFAs are essential components of cellular membranes and are also the primary targets of oxidative attack. In comparison to WT littermates, the levels of lysophosphatidylcholine (LPC) were significantly reduced in plasma of $C3aR^{-/-}$ and $C3aR^{-/-}C5aR^{-/-}$ mice at 3 and 14 months of age (Fig. 6A). LPC may be important not only for ganglion cell survival[39] but also for INL and ONL cell survival and regeneration. We found that the free PUFAs linoleic acid (LA) and arachidonic acid (AA) were significantly decreased in all complement mutant mice at 14 months of age (Fig. 6B), but not in mice aged 2 to 3 months, perhaps reflecting an age-related increase in oxidative stress in the receptor mutant retina.

Retinal Dysfunction in C3aR/C5aR Deficiency Is Independent of Immune Responses

In comparison to WT, after immunization with HNE-MSA, $C3aR^{-/-}C5aR^{-/-}$ mice developed a more severe reduction in ERG amplitude (Fig. 7A). These changes are unlikely to reflect an immune-mediated response because this pathway is truncated in $C3aR^{-/-}C5aR^{-/-}$ mice. Annexin V, indicative of cell apoptosis, was elevated in HNE-MSA immunized $C3aR^{-/-}C5aR^{-/-}$ retina (Fig. 7B). The impaired retinal function was independent of T-cell activation as IL17-producing cells were markedly diminished in the HNE-MSA immunized $C3aR^{-/-}C5aR^{-/-}$ mice (Fig. 7C, left panel). Consistent with this observation, incubation of retinal cells with 5 µg/mL of HNE-MSA showed greater cell death in $C3aR^{-/-}C5aR^{-/-}$ than in WT retinas (Fig. 7C, right panel). In summary, when mice were immunized with 4-HNE-adducted protein, $C3aR^{-/-}C5aR^{-/-}$ showed a more severe phenotype than WT and this phenotype

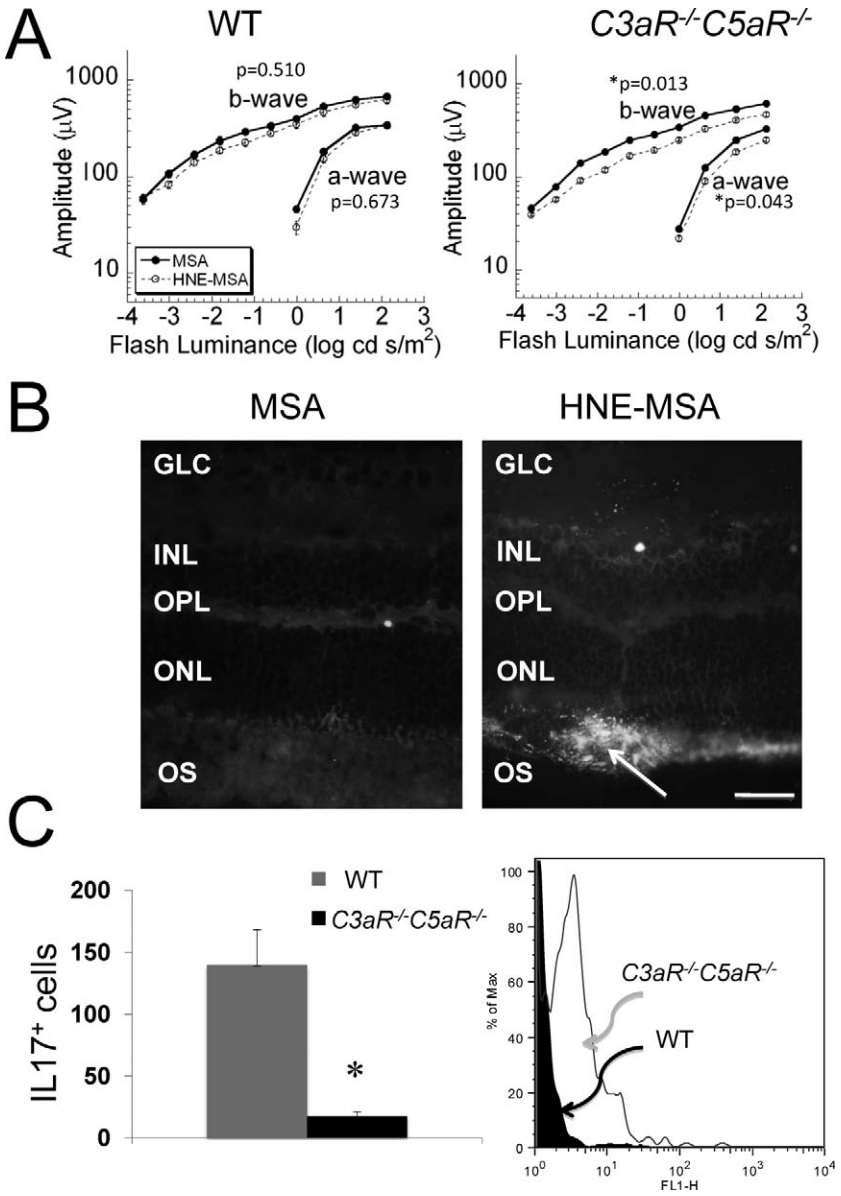

Figure 7. 4-HNE-MSA immunization. *C3aR*$^{-/-}$*C5aR*$^{-/-}$ and WT littermates were immunized at 8 weeks of age with 4-HNE-MSA or MSA. (**A**) The ERG results indicate no significant impact of immunization on WT mice (*left*), but a significant reduction in *C3aR*$^{-/-}$*C5aR*$^{-/-}$ animals. *Data points* indicate average (±SEM) of 5 WT or 7-8 *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice per group. *$P < 0.05$. (**B**) Retinal cross-sections of immunized *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice exposed to Annexin V antibody. Annexin V expression is increased in the ONL of HNE-MSA immunized *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice (*right panel arrow*) but not in MSA immunized *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice (*left*). Magnification: ×60; *scale bar* indicates 100 μm. (**C**) *Left*: Spleen cells were isolated from HNE-MSA immunized *C3aR*$^{-/-}$*C5aR*$^{-/-}$ or HNE-MSA immunized WT littermates and incubated with 0.5 μg of HNE-MSA for 24 hours. IL-17 producing cells were analyzed by ELISPOT assays (*left*). *$P < 0.05$. *Right*: Retinal cells isolated from *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice or WT littermates were incubated with 5 μg/mL of HNE-MSA for 4 hours, stained by Annexin V antibody, and then analyzed by flow cytometry (*right*). The *black peak* depicts the WT flow histogram and the *white peak* shows the flow histogram of *C3aR*$^{-/-}$*C5aR*$^{-/-}$ retinal cells. Data are representative of three independent experiments.

was not related to immune responses. The heightened sensitivity of *C3aR*$^{-/-}$*C5aR*$^{-/-}$ retinal cells to the fatty acid oxidation 4-HNE fragment was observed in vivo and in vitro, indicating that a decreased capacity to ameliorate oxidative damage may underlie the retinal changes observed in these animal models.

Discussion

A critical role for the alternative pathway of the complement system has been implicated in AMD pathogenesis based on the identification of complement components in the drusen of AMD patient eyes[40,41] and genetic studies in which AMD risk is related to a series of SNPs of factor H, factor B, and C3.[1–7] Complement is an essential component of the innate immune system, and is ubiquitously located on cell surfaces and in the circulation. How variation in these genes is involved in the pathology of AMD is unknown. The current interpretation holds that complement activation leads to AMD through an inflammatory mechanism, and has led to the development of treatment strategies based on complement inhibition.[42–44]

In this project, we examined mice with truncated complement activation due to mutations in the central complement component C3 or the receptors for one or both complement activation fragments. We report a progressive loss of photoreceptor, bipolar cells and horizontal cells in mice lacking C3aR, and RPE dysfunction in mice lacking C5aR or C3aR. These changes match the tissue distribution of these proteins.[37,45,46] The retinal dysfunction in *C3*$^{-/-}$ mice was variable at young ages, but became consistent and statistically significant in older mice. Overall, *C3*$^{-/-}$ and *C3aR*$^{-/-}$ mice displayed a similar phenotype, but the onset was later in the *C3*$^{-/-}$ mutant. Increased intrinsic apoptosis may play an important role in retinal cell loss in these models. The ERG reductions seen in *C3aR*$^{-/-}$ and *C3*$^{-/-}$ mice were consistent with a panretinal loss of retinal cells, which we confirmed by anatomic evaluation. Although this phenotype is most similar to that of human disorders like RP, no disease loci have been linked to the region near *C3* or *C3aR* (https://sph.uth.tmc.edu/Retnet/). However, low serum C3 levels were reported in RP patients.[47] Whether *C3* or *C3aR* mutations underlie some forms of RP remains to be determined.

Our results indicated that signaling through C3aR and C5aR was important for the normal retina to maintain their structure and their function. Both of these proteins are members of the class A subfamily 8 of G protein-coupled receptors (GPCRs). In general, GPCR signaling is important for cell growth, activity, and survival. GPCR signaling can vary widely across different cell types, and the role of C3aR and C5aR signaling in the retina remains to be clarified. In the brain, macrophages, dendritic cells, and neutrophils, C5a•C5aR activates MAPK, PI3K-Akt, NF-κB, and cAMP response element-binding signaling.[48–52] C3a•C3aR activates NF-κB in tubular epithelial cells,[53] nerve growth factor-induced nuclear factor of activated T-cells (NFAT) activation in human mast cells,[54] and transcription factors activator protein-1 in glial cells.[55] C3a•C3aR and C5a•C5aR both activate PI3K-Akt pathway in T cells.[18] Our results suggested that the NF-κB pathway may be involved in retinal repair and regeneration.

Oxidation of PUFA generates the chemically reactive 4-HNE fatty acid fragment, and light exposure increases 4-HNE-protein modification in the retina prior to cell apoptosis.[56] 4-HNE triggers mitochondrial apoptosis. Mice immunized against the protein adduct carboxyethylpyrrole developed focal RPE pathology and photoreceptor degeneration due to increased immune responses.[57] Because 4-HNE is found in the mouse retina, we expected *C3aR*$^{-/-}$*C5aR*$^{-/-}$ mice to have less retinal damage from suppressed immune responses following HNE-MSA immunization. Surprisingly, the impact was much greater in *C3aR*$^{-/-}$*C5aR*$^{-/-}$ than in WT mice. This result suggested that the retinal dysfunction did not result from complement-mediated immune responses because the immunized *C3aR*$^{-/-}$*C5aR*$^{-/-}$ had significantly suppressed immune responses compared to the immunized WT mice.

Our findings indicate a novel role of complement signaling in retinal maintenance. This result will have particular relevance to current therapeutic approaches and clinical trials based upon inhibition of complement activation.[11,42–44] Our observations indicate that retinal cells require a certain level of complement activity and raise questions concerning the long-term use of complement inhibition as a treatment for human disease.

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