New Insights on Complement Inhibitor CD59 in Mouse Laser-Induced Choroidal Neovascularization: Mislocalization After Injury and Targeted Delivery for Protein Replacement

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Abstract

Purpose: The membrane attack complex (MAC) in choriocapillaris (CC) and retinal pigment epithelium (RPE) increase with age and disease (age-related macular degeneration). MAC assembly can be inhibited by CD59, a membrane-bound regulator. Here we further investigated the role of CD59 in murine choroidal neovascularization (CNV), a model involving both CC and RPE, and tested whether CR2-CD59, a soluble targeted form of CD59, provides protection.

Methods: Laser-induced CNV was generated in wild type and CD59a-deficient mice (CD59^{-/-}). CNV size was measured by optical coherence tomography, and CR2-CD59 was injected intraperitoneally. Endogenous CD59 localization and MAC deposition were identified by immunohistochemistry and quantified by confocal microscopy. Cell-type-specific responses to MAC were examined in retinal pigment epithelial cells (ARPE-19) and microvascular endothelial cells (HMEC-1).

Results: CD59 levels were severely reduced and protein was mislocalized in the RPE surrounding the lesion. CNV lesion size and subretinal fluid accumulation were exacerbated in CD59^{-/-} when compared with those in WT mice, and an increase in MAC deposition was noted. In contrast, CR2-CD59 significantly reduced both structural features of CNV severity. *In vitro*, MAC inhibition in ARPE-19 cells prevented barrier function loss and accelerated wound healing and cell adhesion, whereas in HMEC-1 cells, CR2-CD59 decelerated wound healing and cell adhesion.

Conclusion: These data further support the importance of CD59 in controlling ocular injury responses and indicate that pharmacological inhibition of the MAC with CR2-CD59 may be a viable therapeutic approach for reducing complement-mediated ocular pathology.

Keywords: terminal complement pathway, membrane attack complex, CD59, CR2-CD59, choroidal neovascularization, wound healing

Introduction

AGE-RELATED MACULAR DEGENERATION (AMD) is the leading cause of blindness among the elderly in developed nations, with more than 1.7 million Americans suffering from the advanced form, and as many as 15 million people in the United States being affected by this disease overall,^{1,2} resulting in a loss of vision in the central portion of the eye. Despite the large number of those affected by both forms of this disease, therapies are largely limited to the prevention of further vision loss through the use of antioxidant supplementation as well as intraocular injections

of antivascular endothelial growth factor (VEGF) therapeutics to block or reverse angiogenesis of new blood vessels entering the eye (NIH Publication No. 03-2294).

In recent years, however, the complement system has been implicated as an important avenue of research and drug design with the discovery of complement gene polymorphisms that play a role in AMD development and progression.^{3, 4}

The complement system, which is part of the adaptive and innate immune system, is initiated through 3 separate pathways: the classical, the lectin, and the alternative pathway. Although these 3 pathways are activated independently of one another, they converge at the formation of a C3 convertase,

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C4bC2a (classical and lectin pathway C3 convertase), and C3bBb (alternative pathway C3 convertase), which then trigger subsequent formation of the common terminal pathway. The final step is the formation of the C5b-9 complex, also referred to as the membrane attack complex (MAC). Upon binding of multiple C9 components to the terminal components C5b, C6, C7, and C8 assembled as C5b-8, the MAC forms a pore in the cell membrane. Pore formation can be transient, leading to changes in cell signaling,⁵ or persistent, mediating cell lysis.^{6,7}

To keep the complement system in check, various complement regulatory proteins exist that can inhibit the cascade at different steps, both soluble and membrane bound. CD59, a membrane-bound complement pathway regulator, acts at the level of the MAC. This 18–21 kDa glycoprotein is able to inhibit MAC pore formation by preventing C9 from binding to the C5b-8 complex.^{8–11} CD59 is widely expressed and is attached to cell membranes through a glycosphosphatidylinositol anchor.¹²

CD59 localization has been investigated in cultured retinal pigment epithelium (RPE) cells as well as RPE cells in intact human retinas. As we previously reported, membrane localization of CD59 has been found both on the apical and basolateral side of the ARPE-19 cell line.¹³ Although both donor control and AMD eyes exhibit some RPE cell labeling for CD59 using immunohistochemistry, stronger labeling was observed in early stage AMD eyes, in particular on the apical side, with weaker labeling associated with RPE overlying drusen, as well as in geographic atrophy.¹⁴ Correspondingly, AMD severity has been found to correlate with MAC formation and loss of RPE cells.¹⁵

With these recent findings linking MAC formation and CD59 expression to AMD severity,^{14,15} soluble forms of CD59 (sCD59) are currently being investigated as a possible therapeutic approach to treating AMD progression. Importantly, in hemolysis assays, the nonlipid-bound form shows no activity, whereas complete inhibition could be obtained with the lipid-bound form of sCD59.¹⁶ Hence, approaches have focused on aiding membrane targeting, as well as increasing half-life and concentration at the site of pathology.^{17–21}

To directly target compounds to sites of complement activation, Tomlinson and colleagues have utilized the complement receptor 2 (CR2) opsonin binding domain for the generation of CR2–complement inhibitor fusion proteins.^{22,23} These CR2-based inhibitors have been shown to be effective in many different tissues, including spinal cord²⁴ and the brain,²⁵ and colocalization between CR2–fusion proteins and opsonins has been documented. Injury-specific targeting has been shown with all the CR2–fusion proteins made to date (CR2–fH,²⁶ CR2–Crry,²⁷ and CR2–CD59²⁸), with labeled CR2–fusion protein accumulating only in the diseased tissues and not in unaffected tissues.

In the eye, we have shown previously that the CR2 domain can promote targeting of the inhibitory domain of complement factor H (CFH) (CR2–fH) to choroidal neovascularization (CNV) lesions and significantly reduce their size.²⁶ Finally, using both immunohistochemistry²⁶ and whole animal imaging,²⁵ CR2-targeted inhibitors were shown to be present in the lesion sites for prolonged periods of time (brain: calculated tissue half-life of 48.5 h; CNV lesions: calculated tissue half-life of >24 h). Taken together, these inhibitors are accumulating in sites of injury at sites of complement activation, and are retained for extended periods of time. Recently, a corresponding CR2–CD59–fusion

protein has been developed and functionality established in hepatic injury.²⁸

Considering the given data, we examined whether CD59 expression and localization in the RPE are altered by pathology in mice similarly to that reported in humans. We also tested whether replenishing CD59 levels using CR2-mediated targeting can reduce CNV progression. Finally, cell-specific responses of RPE and endothelial cells to CR2–CD59-mediated MAC inhibition were investigated.

Methods

Animals

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University Animal Care and Use Committee. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). CD59a-deficient mice (CD59^{−/−}) provided by Dr. B. Paul Morgan (Cardiff University, United Kingdom) were generated on the C57BL/6 background as previously described and confirmed by genotyping.²⁹

CNV lesions were produced on day 0 using 3–5-month-old mice. Mice were first anesthetized by intraperitoneal injection (xylazine and ketamine, 20 and 80 mg/kg, respectively), and pupils were dilated (2.5% phenylephrine HCL and 1% atropine sulfate). Using a coverslip as a contact lens, 4 laser spots were generated around the optic nerve of each eye by argon laser photocoagulation (532 nm, 100 μm spot size, 0.1 s duration, 100 mW).²⁶ Bruch's membrane rupture was determined after bubble formation at the site of laser burn.³⁰

Wild type mice were injected by intraperitoneal injection with a volume of 200 μL of phosphate-buffered saline (PBS, 1 ×) or CR2–CD59 (100 μg in PBS), immediately after (day 0) laser-induced photocoagulation as well as on days 2 and 4. Systemic administration was chosen, because the CR2-targeted inhibitors successfully target to sites of complement inhibition,³¹ avoiding (repeated) perturbations of the eye; intravitreal injections have been shown to have widespread effects on the retina.³² Imaging of the mouse eye was performed on day 5 before the mouse was sacrificed and tissue collected on day 6. Mice treated with CR2–fH were injected intravenously (250 μg in PBS) on day 3 after laser-induced CNV and eyes were collected after 24 h.

Complement inhibitors

The CR2–fH (72 kD molecular weight),²⁹ CR2–Crry (70 kD),²³ and CR2–CD59 (36 kD)²⁸ constructs were generated as previously described. In brief, the murine CR2 sequence encoding the 4 N-terminal short consensus repeat units was joined to the sequence encoding the inhibitory domain of CFH, the mouse soluble Crry, or the extracellular region of mouse CD59a. The sequence encoding the fusion protein was cloned in the PBM vector²² and used for stable transfection in CHO cells. Protein expression was detected using ELISA, and recombinant protein was purified using affinity chromatography.²²

Immunohistochemistry

Eyes were collected and anterior chamber, lens, and retina were removed. Eyecups were fixed in 4% paraformaldehyde

at 4°C overnight. Immunohistochemistry was performed on either flatmount preparations or frozen sections.

For flatmount preparations, eyecups were washed and incubated in anti-C5b-9 (1:200 of 5 mg/mL solution; Abcam, Cambridge, MA) or anti-CD102 (1:200; BD Bioscience, San Diego, CA) in blocking solution (3% bovine serum albumin, 10% normal goat serum, and 0.4% Triton X in Tris-buffered saline) overnight at 4°C before being incubated again overnight at 4°C with the appropriate fluorescent-labeled secondary antibody (Invitrogen, Grand Island, NY). Tissues were extensively washed before flatmount preparation, in which 4 relaxing cuts were made to flatten the eyecup. Eyecups were then coverslipped with mounting medium (Fluoromount; Southern Biotechnology Associates, Inc., Birmingham, AL).

For sections, tissues were cryoprotected in 30% sucrose, frozen in TissueTek O.C.T. (Fisher Scientific, Waltham, MA), and cut into 14 µm cryostat sections. After the slides were washed in PBS, they were blocked with 10% normal goat serum and 3% bovine serum albumin (in PBS containing 0.4% Triton X). Tissues were incubated overnight in blocking solution containing the antibodies of interest, CD59 (1:300; Abcam) and Crry (1:300; BD Bioscience), followed by incubation with the appropriate fluorescent-labeled secondary antibody for 2 h (1:500; Molecular Probes, Carlsbad, CA). Sections were mounted as already described. Control experiments included omission of primary antibody.

Microscopy

Eyecups and sections were analyzed by confocal microscopy (40× oil lens, Leica TCS SP2 AOBS; Leica Bannockburn) obtaining fluorescent measurements at 1 µm intervals. The same laser intensity was used for all paired experiments, and the entire depth of the lesion was determined using a Z-stack of images. Images were imported into Adobe Photoshop software (San Jose, CA) for further analysis as well as presentation. For flatmount preparations, the overall fluorescence for each slice was determined to obtain the intensity mean amplitude of each lesion, and the background was subtracted. Data are expressed as mean ± standard error of the mean (SEM) per lesion. Costaining of anti-C5b-9 and anti-CD102 was imaged using fluorescence microscopy (Zeiss) equipped with a digital black-and-white camera using a fixed exposure time per experiment.

Dot blot analysis

Mouse retina, RPE/choroid, and spleen protein were extracted by first solubilizing the tissue in RIPA buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, and 0.1% sodium deoxycholate; Thermo Fisher Scientific, Waltham, MA, USA) in protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After centrifugation, the lysate was collected and the protein samples (25 µL) were loaded directly in each well of a 96-well plate. Using a vacuum transfer through the Bio-Dot[®] Microfiltration Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA), samples were transferred onto a nitrocellulose membrane.

The dotted membrane was rinsed with tris-buffered saline and Tween-20 (TBST) wash buffer before being blocked with 5% nonfat milk/TBST buffer for 2 h at room temperature to remove nonspecific binding. Incubation with primary antibodies in 5% nonfat milk-TBST buffer (1:1,000)

against CR2 primary antibody (10 µg/mL; rat antimouse CD21, clone 7G6) was performed overnight. The immunoreactive proteins were observed with horseradish peroxidase-conjugated secondary antibodies (anti-Rat; Santa Cruz Biotechnology, Inc., Dallas TX) followed by incubation with Clarity[™] Western ECL Blotting Substrate (Bio-Rad Laboratories, Inc.) and chemiluminescent detection.

CNV lesion size assessment

On day 5 postlaser treatment, optical coherence tomography (OCT) was conducted using an SD-OCT BiopTigen[®] Spectral Domain Ophthalmic Imaging System (BiopTigen, Inc., Durham, NC). OCT rather than confocal microscopy was chosen, because Giani et al.³³ have convincingly shown that laser-induced CNV in mouse can be analyzed by OCT; we have, in unpublished data, confirmed a linear correlation between CNV sizes obtained by OCT and immunohistochemistry using the same animals. Before imaging, mice were anesthetized and eyes were hydrated with normal saline.

Using the BiopTigen[®] InVivoVue software, all SD-OCT images were acquired with rectangular volume scans set at 1.6 × 1.6 mm, consisting of 100 B scans (1,000 A scans per B scan). Cross-sectional area of the lesions was used to measure CNV size. As described by Giani et al., the *en face* fundus reconstruction tool of the BiopTigen[®] SD-OCT system was used to determine the center of the lesion by identifying the midline passing through the area of the RPE-Bruch's membrane rupture with the axial interval positioned at the level of the RPE-choroid complex.³³

Subretinal fluid accumulation (SRFA) was obtained by measuring the fluid area from the section of each lesion in which the peak accumulation was observed. SRFA as described by Giani et al. was defined as the hyporeflective spaces under the retina.³³ Vertical calipers were set at 0.100 mm at the site of each lesion, and ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <<http://rsb.info.nih.gov/ij/index.html>>) was used to measure the area around the hyporeflective spot produced in the fundus image. Based on the size of the individual pixels (1.6 × 1.6 µm), the lesion and fluid dome sizes were calculated.

Cell cultures

ARPE-19 cells, a human retinal epithelial cell line, were purchased from ATCC and expanded in Dulbecco's modified Eagle's medium F12 (Invitrogen) with 10% fetal bovine serum (FBS) and antibiotics as described before.¹³ HMEC-1 cells, a human microvascular endothelial cell line, were also purchased from ATCC and grown in MCDB131 medium (Gibco), supplemented with 5 ng/mL human recombinant epidermal growth factor, 1 µg/mL hydrocortisone, 10 mM glutamine, plus 10% FBS and antibiotics.

Transepithelial resistance assays

For barrier function assays, ARPE-19 cells were grown as mature monolayers on 6-well transwell inserts (Corning, 0.4 µm PET, 24 mm insert) in the presence of 5% FBS for 2–3 weeks.³⁴ For the final 2–3 days before the experiments, cells were changed to serum-free medium. Complement activation was induced as reported previously,¹³ exposing cells to 0.5 mM H₂O₂ in the presence of 10% normal human serum (NHS).

As we have shown previously that sublytic complement activation results in VEGF release, which, in turn, reduces barrier function,¹³ transepithelial resistance (TER) measurements are a convenient readout for the level of activity in the complement cascade. TER was determined by measuring the resistance across the monolayer with an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL). The value for cell monolayers was determined by subtracting the TER for filters without cells and percentage calculated using the starting value as reference.

In vitro wound healing assay

ARPE-19 and HMEC-1 cells were grown on ACEA electronic microtiter plates (E-Plates View 16 PET; ACEA Biosciences, San Diego, CA) to confluence for at least 2 days to form a stable monolayer. The wound was induced by making a scratch across the confluent monolayer using a 1 mL micropipette tip, and then the medium was replaced immediately by a fresh medium to remove loose cell debris. The fresh medium contained 5% NHS in the presence or absence of a complement inhibitor, CR2-CD59.

Wound healing properties were observed for the following 5 days, covering cell proliferation, migration, and adhesion (barrier formation) phase of the wound repair, which were monitored by the xCelligence RTCA DP (ACEA Biosciences; San Diego, CA), the real-time electronic cell impedance scoring system. The impedance detected in the bottom on the culture well was represented by cell index (CI), in which $CI = (\text{impedance at time point } n - \text{impedance}$

in the absence of cells)/(nominal impedance value). Two key features of the wound repair properties were quantified; the early phase slope depicting the gradual increase in CI value represents mostly cell proliferation, whereas the late-phase plateau of the CI reflects cell adhesions.

Statistics

Data are presented as means $\pm$ SEM. Single comparisons were analyzed by unpaired *t*-tests, with mean value differences considered significant at $P \leq 0.05$. Student *t*-test was used to analyze differences using the Holm-Sidak method with $\alpha = 5.0\%$ and $P < 0.01$.

Results

Localization of CD59 and Crry after CNV

Alterations in CD59 labeling have been reported for membrane-bound CD59 in response to AMD pathology in human RPE. Likewise, loss of polarity and membrane association has been shown for Crry, a mouse-specific C3 convertase inhibitor, in another barrier epithelium, the mouse kidney epithelium, in response to ischemia reperfusion injury.³⁵ We examined the distribution of CD59 in mouse RPE after laser-induced photocoagulation. As Crry is also expressed in the mouse RPE, we chose to examine its localization after injury to determine whether it demonstrates similar behavior in this tissue as it does in the kidney. As CNV induction destroys the RPE within the laser lesion and leads to oxidative stress in

FIG. 1. Loss of CD59 membrane localization after CNV. We examined the distribution of CD59 and Crry in mouse RPE after laser-induced photocoagulation of Bruch's membrane to induce choroidal neovascularization. (A, F) Here we show in 3-month-old control mice that CD59 and Crry localize to the basolateral side of the RPE. (B, G) However, within 3 days post-CNV induction, in the region adjacent to the lesion, CD59 is mislocalized to the apical side of the RPE, whereas Crry appears to be absent. (H, I, J) By day 7, Crry labeling has returned to the basolateral side of the RPE, whereas CD59 (C) remains mislocalized to the apical side. (D) By day 14 post-CNV, CD59 labeling is still present on the apical side, but some has returned to the basolateral side. (E) Finally by day 28, CD59 labeling has returned exclusively to the basolateral side of the RPE. (K) The cartoon is provided to summarize our findings. BRM, Bruch's membrane; CNV, choroidal neovascularization; POS, photoreceptor outer segments; RPE, retinal pigment epithelium.

the regions surrounding the lesion,³⁶ we focused on the regions adjacent to the lesions (perilesion area).

In 3-month-old control eyes, CD59 and Crry were localized to the basolateral side of the RPE (Fig. 1A, F). However, within 3 days post-CNV, in the perilesion area, CD59 was severely downregulated and mislocalized to the apical side of the RPE, whereas Crry appeared to be completely absent (Fig. 1B, G). By day 7, Crry labeling had returned to the basolateral side of the RPE, whereas CD59 remained mislocalized to the apical side (Fig. 1C, H). By day 14 post-CNV, CD59 labeling was still present on the apical side, but some had returned to the basolateral side (Fig. 1D). Finally by day 28, CD59 labeling had returned almost exclusively to the basolateral side of the RPE (Fig. 1E). Localization of CD59 and Crry in regions distant from the CNV lesions was found to be unaffected (data not shown).

Targeting of CR2-CD59 to injured ocular tissues after CNV

Targeting of CR2-CD59 to the RPE/choroid after laser-induced CNV was confirmed by dot blot analysis (Fig. 2). The presence of CR2-CD59 was confirmed in the RPE/choroid and retina fraction of mice treated with CR2-CD59 (100 µg, intraperitoneal injection [IP]) when compared with mice treated with PBS (1×). As expected, only residual binding was present in the spleen of CR2-CD59-treated mice or the RPE/choroid of PBS-treated mice. As intravenous administration of CR2-fH has previously been shown to target the RPE after laser-induced injury and reduce CNV lesion size,²⁶ CR2-fH

was used as a positive control. Like CR2-CD59, CR2-fH-treated mice also show presence of CR2-positive material in the RPE/choroid and retina, thereby confirming the ability of a CR2-targeted inhibitor to reach the eye through intraperitoneal and intravenous injections.

Modulation of CNV lesion size by CD59

To confirm that the loss of CD59 surrounding the CNV lesions contributes to pathology, we examined the effect of laser-induced photocoagulation in mice deficient in CD59 (CD59^{-/-}). SD-OCT images were taken on day 5, based on the findings by Giani et al., CNV size was maximal at this time point.³³ When compared with wild type mice, CD59^{-/-} mice had significantly larger CNV lesions (Fig. 3A, B). Quantitative analysis confirmed a 40% increase in CNV size (Fig. 3C) in CD59^{-/-} mice when compared with that in controls (CD59^{-/-}, 14,890 ± 626.1 µm²; WT, 8,897 ± 502.5 µm²; $P < 0.0001$). Likewise, SRFA was also determined to be significantly increased in CD59^{-/-} mice (Fig. 3H) when compared with that in WT mice (Fig. 3G) (CD59^{-/-}, 1,161 ± 337.0 µm²; WT 584.1 ± 73.68 µm²; $P < 0.01$; Fig. 3I).

As loss of CD59 could be observed in the perilesion area and CNV size was augmented in mice lacking CD59, we investigated the use of CR2-CD59, a novel targeted inhibitor that specifically targets CD59 to sites of complement activation, in the mouse CNV model. After laser-induced photocoagulation, wild type mice were given a 200 µL intraperitoneal injection with PBS (1×, control) or CR2-CD59 (100 µg) on days 0, 2, and 4.

Fundus imaging by SD-OCT on day 5 revealed a significant decrease in lesion size after CR2-CD59 treatment (Fig. 3E) when compared with that after PBS injection (Fig. 3D). Image analysis indicated a >30% decrease in lesion size ($P < 0.001$) in CR2-CD59-injected mice (7,163 ± 468.6 µm²) when compared with that in PBS-injected mice (10,400 ± 591.5 µm²; Fig. 3F). An even greater decrease was observed in fluid accumulation where CR2-CD59-injected mice (Fig. 3K) demonstrated a nearly 60% decrease in area when compared with that in PBS-injected mice (Fig. 3J) (CR2-CD59, 149.0 ± 35.94 µm²; PBS 351.1 ± 73.01 µm²; Fig. 3L).

MAC deposition after CNV

As CD59 inhibits formation of the MAC, RPE/choroid flatmounts were stained with anti-C5b-9 to examine MAC levels in CNV lesions of CD59^{-/-} animals, as well as in wild type animals after CR2-CD59 treatment. Similar to previously published data by Cashman et al.,¹⁹ images taken by Zeiss fluorescent microscopy demonstrated staining of MAC beyond the region of CD102 staining (Fig. 4A).

Z-stack profile images were taken for each lesion using confocal microscopy to determine the mean fluorescence amplitude. MAC levels after laser-induced photocoagulation were significantly increased in the CD59-deficient mice when compared with those in wild type controls (mean amplitude, CD59^{-/-}, 139.0 ± 28.65; WT, 36.74 ± 29.29; $P < 0.001$; Fig. 4B, C, and F). In comparison, wild type mice treated with CR2-CD59 after CNV demonstrated a decreasing trend in MAC pixel intensity in the remaining lesions, although the difference did not reach significance (CR2-CD59: 26.39 ± 3.396, PBS: 33.54 ± 3.396; $P = 0.0928$) (Fig. 4D, E, and G).

FIG. 2. CR2-CD59 in CNV-lesioned ocular tissues after intraperitoneal injections. Dot blot analysis was used to confirm targeting of CR2-CD59 to the eye after laser-induced photocoagulation. CR2-CD59 was present in the RPE/choroid and retina extracts of mice treated with CR2-CD59 (100 µg, IP). Intravenous injections of CR2-fH (250 µg) was used as a positive control for CR2-mediated targeting to the RPE/choroid and retina. As expected, no CR2-positive material was present in mice injected with PBS (1×). In addition, only minimal CR2-positive material was found in the spleen of CR2-CD59-treated mice, whereas no reactivity was found in spleen of PBS-treated mice. IP, intraperitoneal injection; PBS, phosphate-buffered saline.

FIG. 3. OCT analysis of CNV in the presence and absence of CD59. Argon laser photocoagulation of the Bruch's membrane was used to generate 4 laser spots in each eye of wild type ($n=6$) and CD59-deficient mice (CD59^{-/-}; $n=16$). Mice were analyzed by OCT on day 5 postlaser-induced photocoagulation. Representative OCT images of C57BL/6 and CD59^{-/-} using fundus reconstruction (A, B) as well as quantification of lesion area, (C) demonstrate an increase in CNV size in CD59^{-/-} mice (**** $P \leq 0.0001$) compared with that in wild type control. Intraperitoneal injection of CR2-CD59 (100 μg in PBS; $n=8$) in equal volume on days 0, 2, and 4 compared with PBS-injected group (1×; $n=18$) resulted in a significant decrease in CNV size in the CR2-CD59 treatment group (E), than in the PBS-injected group (D, F) (*** $P \leq 0.001$). Assessment of fluid accumulation demonstrated a significant increase in fluid in CD59^{-/-} mice ($n=9$) (G, arrowhead) when compared with that in WT mice ($n=7$) (H, I, arrowhead; ** $P \leq 0.01$). Alternatively CR2-CD59 treatment ($n=7$) (J, arrowhead) resulted in a decrease in fluid accumulation when compared with that in PBS-injected mice ($n=11$) (K, L, arrowhead; * $P \leq 0.05$). Data shown are average values ($\pm$ SEM) per lesion. OCT, optical coherence tomography; SEM, standard error of the mean.

Cell-type-specific responses to MAC inhibition

CNV involves breakdown of the RPE (including loss of barrier function) followed by angiogenesis of the choroidal vasculature and fluid leakage from these newly formed vessels. For mechanistic studies, assessing the potential effects of CR2-CD59 on different aspects of cell behavior, we switched to assessing barrier function, wound healing, and cell adhesion in ARPE-19 and HMEC-1 cells, 2 human cell lines.

We have shown previously that oxidative stress and complement activation can be mimicked in ARPE-19 cell monolayers treated with H₂O₂, leading to a reduction in

complement inhibition at the cell surface and resulting in sublytic complement activation requiring MAC. This level of complement activation is transient, as C5b-9 could be detected rapidly in cell supernatants. As MAC activation results in the secretion and mobilization of VEGF,^{13,37} which, in turn, reduces TER,³⁴ TER measurements have been found to be a convenient assay to probe the complement system in these cells. ARPE-19 cells grown as monolayers on Transwell filters develop TER levels of $\sim 40 \Omega \text{ cm}^2$ (Fig. 5A). In the presence of H₂O₂ + NHS, TER is reduced by $\sim 50\%$ ($P < 0.001$), and pretreatment with CR2-CD59 completely prevented this drop in TER. The effect of CR2-CD59 was comparable in magnitude to blocking the alternative pathway

FIG. 4. Membrane attack complex (MAC) deposition in CNV lesions in the absence and presence of CD59. MAC deposition was evaluated on day 6 postlaser-induced photocoagulation using C5b-9 immunohistochemistry. Representative images taken by fluorescence microscopy of wild type mice stained in the presence of anti-C5b-9 and anti-CD102. Scale bar 50 μ m (A). Representative images in wild type mice ($n=6$) mice (B), and CD59-deficient mice (CD59^{-/-}; $n=4$) (C), or wild type mice treated with either PBS (1 $\times$; $n=4$; D) or CR2-CD59 (100 μ g; $n=4$; E) on days 0, 2, and 4 postlaser-induced photocoagulation are provided. (B–E) were obtained at 40 $\times$ using confocal microscopy, with (C) demonstrating 4 images combined. Scale bar, 40 μ m. (F) A comparison of mean amplitude of each lesion suggested that the absence of CD59 resulted in more than 3 times the deposition of MAC when compared with that in wild type mice (*** $P \leq 0.001$). (G) Quantification of the mean pixel amplitude demonstrated no significant (n.s.) change in C5b-9 intensity in the CR2-CD59-treated mice when compared with that in PBS-injected mice. Data shown are average values ($\pm$ SEM) per lesion.

amplification loop with CR2-fH as we have previously reported,¹³ or to blocking at the level of C3 using CR2-Crry.

To assess wound healing and cell adhesion, epithelial ARPE-19 and endothelial HMEC-1 cell lines were used for the *in vitro* wound healing assays. A local wound was induced by making an equal sized scratch in the confluent monolayer culture, and then allowed to repair for up to 5 days in medium containing 5% NHS supplemented with the CR2-CD59 complement inhibitor or PBS (control) until the repair process reached a plateau level. The wound healing processes during repair were monitored by determining real-time cell impedance measured as CI.

Representative images for the 2 cell lines are depicted (Fig. 5B, C) and highlight important differences for the 2 cell types. First, after the scratch wound, in the presence of 5% NHS, ARPE-19 cells demonstrated a very slow increase in CI, reaching a plateau after multiple days, whereas HMEC-1 cells rapidly expand and proliferate, reaching stable plateau within less than 1 day. Second, the responses of the 2 cell types are opposite when exposed to NHS in the presence of CR2-CD59. In ARPE-19 cells, treatment with 150 or 500 nM CR2-CD59 accelerated wound repair, whereas in HMEC-1 cells, CR2-CD59 reduced the wound repair response.

Average kinetics and adhesion profiles (Fig. 5D, E) were obtained from multiple experiments, assessing slope (eg, ARPE-19 cells: 10–45 h and HMEC-1 cells: 1–3.5 h) and final CI value (eg, ARPE-19 cells: 60 h and HMEC-1 cells: 24 h). Taken together, we were able to identify cell-type-specific behavior of epithelial and endothelial cells in response to complement activation and injury from the wound healing studies. Epithelial ARPE-19 cells exhibit reduced barrier function (TER) in response to complement activation, which could be reversed by CR2-CD59. Increased barrier function was correlated with increased rate of wound repair shown by cell proliferation and adhesion in CR2-CD59-treated cells. On the flipside, in endothelial HMEC-1 cells, CR2-CD59 treatment resulted in a decrease in cell proliferation as well as decreased adhesion.

Discussion

MAC in RPE/choroid is found to be increased in eyes from aged donors, those with AMD, or those with the high-risk complement factor H genotype.^{38,39} Alterations in expression and localization of CD59 in ocular tissues⁴⁰ and leukocytes⁴¹ are associated with AMD. To further understand the role of CD59 and MAC in CNV, we first examined

FIG. 5. Oposing effects of CR2-CD59 in epithelial and endothelial cells. **(A)** TER measurements were obtained in stable ARPE-19 cells grown as monolayers on transwell filters at baseline and 4 h post-treatment. Cells treated with H₂O₂ in the presence of NHS demonstrated a 50% reduction in TER ($P < 0.001$). Pretreatment with CR2-CD59 (10 μg) prevented this decrease. Additional complement inhibitors were used to confirm the effect [alternative pathway inhibitor CR2-fH (25 μg) and terminal pathway inhibitor CR2-Crry (25 μg)] ($n = 3-6$). **(B)** ARPE-19 and **(C)** HMEC-1 cells were allowed to develop a plateau of CI before a scratch wound was applied. In the presence of CD59 (150 nM and 500 nM), ARPE-19 cells demonstrated a significant increase in wound repair, whereas wound repair was impaired in HMEC-1 cells. **(D)** Cell proliferation determined by slope analysis in the early phase of the response and **(E)** cell adhesion determined as CI at the plateau phase are provided for statistical analysis (* $P \leq 0.05$ for comparison with PBS). CI, cell index; NHS, normal human serum; TER, transepithelial resistance.

the membrane localization of mouse CD59 after the induction of injury (Fig. 1).

We report that by day 3 postlaser-induced photocoagulation, CD59 mislocalized from the basal side of the RPE to the apical side in areas just adjacent to the CNV lesion. By day 14 postinjury, CD59 partially returned to the basolateral

side, while some was still localized to the apical side. By day 28, however, localization of CD59 was again exclusively found on the basolateral side of the RPE. This time course correlates with some of the repair processes described by others. Chan-Ling and coworkers have shown that although hematopoietic stem cells participated in the

wounding response by forming astrocytes, macrophages, pericytes, and RPE, the closure of the wound is still present 3 weeks postlesion.⁴²

In addition, Giani et al. have shown that CNV lesion size as assessed by choroidal fibrovascular tissue involution into the subretinal space and SRFA as analyzed by OCT peaked by day 5, decreased rapidly between days 5 and 7, and decreased slowly such that by 28 days postlesion, choroidal fibrovascular tissue no longer penetrates into the subretinal space. In comparison, when examining the localization of Crry, an upstream inhibitor of the complement pathway, we found that Crry surface expression was completely lost on day 3 after laser-induced photocoagulation (Fig. 1), but returned to its correct prelesion location at the basolateral side of the RPE by day 7.

This novel finding on Crry in the RPE coincides with previously reported data that observed a loss of basolateral expression of Crry after ischemic injury in mouse proximal tubule cells, in which the loss of Crry polarity is believed to be necessary for the activation of the alternative pathway and concomitant pathology.³⁵ Interestingly, prior findings have reported an increase in CD59 in early AMD, in particular on the apical side of the RPE with patchy labeling of CD59 in areas of drusen.¹⁴ Similarly, the complement regulatory protein CD46 is also localized basolaterally in the RPE, with polarity and amount decreasing with progression of AMD.^{40,43} Our data taken together with the human data suggest that early in the progression of AMD, CD59 is recruited to the apical side of the RPE where it functions in the inhibition of MAC formation.

In the mouse, CNV lesions regress spontaneously after 14–21 days,⁴⁴ and CD59 gradually returns to its normal location. The mechanisms leading to the reduction in complement inhibitors in the prelesion area, most likely, involve the generation of oxidative stress. We have shown previously that CNV triggers the production of super oxide and reactive oxygen species in the lesion and perilesion area⁴⁵; in our *in vitro* work on RPE monolayers, we have shown that oxidative stress leads to the reduction of membrane-bound complement inhibitors from the apical and basolateral regions of the cells.¹³ However, future studies aimed at better understanding the relationship between CNV regression and the expression of complement inhibitors would provide important information.

Bora et al. were the first group to demonstrate the importance of the MAC in mouse CNV *in vivo*, as they showed an acceleration of CNV growth in CD59-deficient mice as measured by histology, although absolute sizes were not reported.¹⁸ Our data further support and extend this work by using OCT to show that mice deficient in CD59a have significantly larger CNV lesion sizes (40%) when compared with those of wild type mice (Fig. 3). In addition, measurements of fluid accumulation also demonstrated a significant increase of greater than 60% in the fluid present in CD59^{-/-} mice after CNV when compared with those in WT mice. In CNV lesions, the presence of MAC was confirmed using a C5b-9 antibody, in which a nearly 4-fold increase in MAC staining levels was demonstrated in CD59^{-/-} mice when compared with those in wild type mice (Fig. 4).

Based on the known involvement of CD59 in CNV, this protein has been examined in mouse CNV as a potential therapy, using soluble forms of CD59 injected as IgG2a-fusion proteins into the vitreous cavity or locally expressed in the

RPE using gene therapy.^{17,19,46} Both strategies were shown to reduce CNV lesion sizes as assessed by immunohistochemistry for newly formed blood vessels.

We explored a different proven strategy to effectively target complement inhibitors to sites of complement activation, and that is to link a given complement inhibitor to a portion of the CR2 receptor that binds to deposited long-lasting, covalently fixed C3 activation products (opsonins) on affected membranes. Such an approach to target complement inhibitors has been shown to significantly increase efficacy because inhibitors are not wasted on non-pathophysiologically important target molecules.³¹ This method also bypasses the need for more invasive treatments, as we show that CR2-CD59 is able to specifically target the site of CNV injury using intraperitoneal injection (Fig. 2). Importantly, as was shown with other CR2-fusion protein,^{27,28} unaffected tissues such as the spleen did not bind CR2-CD59.

The design of this complement inhibitor ensures that the only way CR2-CD59 can bind to tissues is by binding either to C3b (opsonin recognized by CR2) or to C8 α in the C5b-8 and to unfolded C9 in the assembling C5b-9 complex,⁴⁷ all of which only occur in areas of complement activation. In this study, we demonstrate a >30% decrease in CNV lesion size with intraperitoneal treatment of CR2-CD59 after OCT analysis (Fig. 3), which is comparable to our earlier observations in CR2-fH-treated mice.²⁶ In addition to a reduced CNV lesion size, fluid accumulation was decreased by nearly 60% in the presence of CR2-CD59. This effect of CD59 in reducing fluid accumulation is of great importance, because leakage from newly formed blood vessels and/or impaired capacity for fluid removal results in vision impairment and loss in wet AMD patients. The effects of CD59 were found to have correlates in our *in vitro* cell-based assays. As already indicated, CNV involves damage and breakdown of the RPE followed by angiogenesis and fluid leakage and accumulation. An effect on CD59 on reducing RPE cell damage was confirmed by TER and cell impedance assays (Fig. 5A, B), in which CD59 prevented loss of barrier function induced by complement activation and accelerated wound repair and cell adhesion, respectively. Likewise, a reduction in fibrosis is supported by our observation *in vitro* using HMEC-1 cells (Fig. 5C), in which we observed that CD59 reduced wound healing and cell adhesion. This effect on fluid accumulation may be direct, by improving barrier function as we have shown for ARPE-19 cells (Fig. 5A), or indirect, by reducing complement-mediated VEGF secretion, as we have shown previously for RPE cells.⁴⁸

CR2-CD59 targets only the terminal product of the complement cascade, which may provide potential therapeutic benefits compared with inhibitors acting earlier in the pathway. In this regard, if the MAC is one of the primary mediators of injury (as is suggested by available data), then inhibiting only the terminal product will still allow generation of anaphylatoxins and C3 opsonins that can promote inflammation and chemotaxis of immune cells, as well as marking injured and dying cells for clearance, processes essential to maintain tissue homeostasis. In addition, anaphylatoxins have been found to be essential in promoting tissue regeneration and repair in retina and CNS.^{49,50}

Staining with the C5b-9 antibody illustrated that when CR2-CD59 was administered, MAC was still able to form in

the existing, although significantly smaller, wounds (Fig. 4). This characteristic may be very important for those suffering from additional ailments, such as bacterial infection, as the formation of MAC would allow for removal of invading cells.

Finally, MAC localization has been demonstrated in Bruch's membrane and the choriocapillaris in human eyes as early as 2 years of age with a significant increase by age 21.⁵¹ As this age range is well less than the age of normal AMD development, Mullins et al. hypothesize that this accumulation might provide a beneficial role in decreasing the risk of bystander injury, and may possibly aid in the phagocytosis of photoreceptor cells by the RPE. Understanding the time course and conditions of both the loss and recovery of cell surface complement inhibitors will clearly provide important insights into our understanding of the AMD disease process and the requirements of any complement-based therapeutic.

In summary, altered localization of the complement inhibitors CD59 and Crry in the RPE, which are an early consequence of the laser injury, is thought to increase the susceptibility of the RPE to complement-mediated injury, contributing to CNV. This hypothesis is supported by results using CD59^{-/-} mice, which exhibit augmented CNV and increased complement deposition in the lesion sites, and the corresponding protective effect of CR2-CD59. Finally, the differential effects of CD59 on epithelial and endothelial cells suggest that inhibition of MAC may be an effective means of reducing CNV and promoting wound repair.

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Author Disclosure Statement

The authors declare the following financial or proprietary interests: B.R. and S.T. hold licensed patents for CR2-targeted complement fH inhibitor. G.S. and M.K.B. have no competing financial interests.

References

- Friedman, D.S., O'Colmain, B.J., Munoz, B., Tomany, S.C., McCarty, C., de Jong, P.T., Nemesure, B., Mitchell, P., Kempen, J., and Eye Diseases Prevalence Research Group. Prevalence of age-related macular degeneration in the United States. *Arch. Ophthalmol.* 122:564–572, 2004.
- Macular Degeneration Partnership. What is AMD? www.amd.org/what-is-macular-degeneration.html (accessed July 9, 2013).
- Haines, J.L., Hauser, M.A., Schmidt, S., Scott, W.K., Olson, L.M., Gallins, P., Spencer, K.L., Kwan, S.Y., Noureddine, M., Gilbert, J.R., Schnetz-Boutaud, N., Agarwal, A., Postel, E.A., and Pericak-Vance, M.A. Complement factor H variant increases the risk of age-related macular degeneration. *Science*. 308:419–421, 2005.
- Klein, R.J., Zeiss, C., Chew, E.Y., Tsai, J.Y., Sackler, R.S., Haynes, C., Henning, A.K., SanGiovanni, J.P., Mane, S.M., Mayne, S.T., Bracken, M.B., Ferris, F.L., Ott, J., Barnstable, C., and Hoh, J. Complement factor H polymorphism in age-related macular degeneration. *Science*. 308:385–389, 2005.
- Nicholson-Weller, A., and Halperin, J.A. Membrane signaling by complement C5b-9, the membrane attack complex. *Immunol. Res.* 12:244–257, 1993.
- Muller-Eberhard, H.J. Molecular organization and function of the complement system. *Annu. Rev. Biochem.* 57:321–347, 1988.
- Tegla, C.A., Cudrici, C., Patel, S., Trippe, R., 3rd, Rus, V., Niculescu, F., and Rus, H. Membrane attack by complement: the assembly and biology of terminal complement complexes. *Immunol. Res.* 51:45–60, 2011.
- Rollins, S.A., and Sims, P.J. The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. *J. Immunol.* 144:3478–3483, 1990.
- Meri, S., Waldmann, H., and Lachmann, P.J. Distribution of protectin (CD59), a complement membrane attack inhibitor, in normal human tissues. *Lab. Invest.* 65:532–537, 1991.
- Okada, N., Harada, R., Fujita, T., and Okada, H. A novel membrane glycoprotein capable of inhibiting membrane attack by homologous complement. *Int. Immunol.* 1:205–208, 1989.
- Holguin, M.H., Fredrick, L.R., Bernshaw, N.J., Wilcox, L.A., and Parker, C.J. Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. *J. Clin. Invest.* 84:7–17, 1989.
- Davies, A., Simmons, D.L., Hale, G., Harrison, R.A., Tighe, H., Lachmann, P.J., and Waldmann, H. CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J. Exp. Med.* 170:637–654, 1989.
- Thurman, J.M., Renner, B., Kunchithapautham, K., Ferreira, V.P., Pangburn, M.K., Ablonczy, Z., Tomlinson, S., Holers, V.M., and Rohrer, B. Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. *J. Biol. Chem.* 284:16939–16947, 2009.
- Ebrahimi, K.B., Fijalkowski, N., Cano, M., and Handa, J.T. Decreased membrane complement regulators in the retinal pigmented epithelium contributes to age-related macular degeneration. *J. Pathol.* 2012.
- Hageman, G.S., Luthert, P.J., Victor Chong, N.H., Johnson, L.V., Anderson, D.H., and Mullins, R.F. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog. Retin. Eye Res.* 20:705–732, 2001.
- Hakulinen, J., and Meri, S. Shedding and enrichment of the glycolipid-anchored complement lysis inhibitor protectin (CD59) into milk fat globules. *Immunology*. 85:495–501, 1995.
- Bora, N.S., Jha, P., Lyzogubov, V.V., Kaliappan, S., Liu, J., Tytarenko, R.G., Fraser, D.A., Morgan, B.P., and Bora, P.S. Recombinant membrane-targeted form of CD59 inhibits the growth of choroidal neovascular complex in mice. *J. Biol. Chem.* 285:33826–33833, 2010.

18. Bora, N.S., Kaliappan, S., Jha, P., Xu, Q., Sivasankar, B., Harris, C.L., Morgan, B.P., and Bora, P.S. CD59, a complement regulatory protein, controls choroidal neovascularization in a mouse model of wet-type age-related macular degeneration. *J. Immunol.* 178:1783–1790, 2007.
19. Cashman, S.M., Ramo, K., and Kumar-Singh, R. A non membrane-targeted human soluble CD59 attenuates choroidal neovascularization in a model of age related macular degeneration. *PLoS One.* 6:e19078, 2011.
20. Czajkowsky, D.M., Hu, J., Shao, Z., and Pleass, R.J. Fc-fusion proteins: new developments and future perspectives. *EMBO Mol. Med.* 4:1015–1028, 2012.
21. Fraser, D.A., Harris, C.L., Williams, A.S., Mizuno, M., Gallagher, S., Smith, R.A., and Morgan, B.P. Generation of a recombinant, membrane-targeted form of the complement regulator CD59: activity in vitro and in vivo. *J. Biol. Chem.* 278:48921–48927, 2003.
22. Song, H., He, C., Knaak, C., Guthridge, J.M., Holers, V.M., and Tomlinson, S. Complement receptor 2-mediated targeting of complement inhibitors to sites of complement activation. *J. Clin. Invest.* 111:1875–1885, 2003.
23. Atkinson, C., Song, H., Lu, B., Qiao, F., Burns, T.A., Holers, V.M., Tsokos, G.C., and Tomlinson, S. Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. *J. Clin. Invest.* 115:2444–2453, 2005.
24. Qiao, F., Atkinson, C., Kindy, M.S., Shunmugavel, A., Morgan, B.P., Song, H., and Tomlinson, S. The alternative and terminal pathways of complement mediate post-traumatic spinal cord inflammation and injury. *Am. J. Pathol.* 177:3061–3070, 2010.
25. Alawieh, A., Elvington, A., Zhu, H., Yu, J., Kindy, M.S., Atkinson, C., and Tomlinson, S. Modulation of post-stroke degenerative and regenerative processes and subacute protection by site-targeted inhibition of the alternative pathway of complement. *J. Neuroinflammation.* 12:247, 2015.
26. Rohrer, B., Long, Q., Coughlin, B., Wilson, R.B., Huang, Y., Qiao, F., Tang, P.H., Kunchithapautham, K., Gilkeson, G.S., and Tomlinson, S. A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* 50:3056–3064, 2009.
27. Elvington, M., Blichmann, P., Qiao, F., Scheiber, M., Wadsworth, C., Luzinov, I., Lucero, J., Vertegel, A., and Tomlinson, S. A novel protocol allowing oral delivery of a protein complement inhibitor that subsequently targets to inflamed colon mucosa and ameliorates murine colitis. *Clin. Exp. Immunol.* 177:500–508, 2014.
28. Marshall, K.M., He, S., Zhong, Z., Atkinson, C., and Tomlinson, S. Dissecting the complement pathway in hepatic injury and regeneration with a novel protective strategy. *J. Exp. Med.* 211:1793–1805, 2014.
29. Holt, D.S., Botto, M., Bygrave, A.E., Hanna, S.M., Walport, M.J., and Morgan, B.P. Targeted deletion of the CD59 gene causes spontaneous intravascular hemolysis and hemoglobinuria. *Blood.* 98:442–449, 2001.
30. Sakurai, E., Anand, A., Ambati, B.K., van Rooijen, N., and Ambati, J. Macrophage depletion inhibits experimental choroidal neovascularization. *Invest. Ophthalmol. Vis. Sci.* 44:3578–3585, 2003.
31. Holers, V.M., Rohrer, B., and Tomlinson, S. CR2-mediated targeting of complement inhibitors: bench-to-bedside using a novel strategy for site-specific complement modulation. *Adv. Exp. Med. Biol.* 735:137–154, 2013.
32. Faktorovich, E.G., Steinberg, R.H., Yasumura, D., Matthes, M.T., and LaVail, M.M. Basic fibroblast growth factor and local injury protect photoreceptors from light damage in the rat. *J. Neurosci.* 12:3554–3567, 1992.
33. Giani, A., Thanos, A., Roh, M.I., Connolly, E., Trichonas, G., Kim, I., Gragoudas, E., Vavvas, D., and Miller, J.W. In vivo evaluation of laser-induced choroidal neovascularization using spectral-domain optical coherence tomography. *Invest. Ophthalmol. Vis. Sci.* 52:3880–3887, 2011.
34. Ablonczy, Z., and Crosson, C.E. VEGF modulation of retinal pigment epithelium resistance. *Exp. Eye Res.* 85:762–771, 2007.
35. Thurman, J.M., Ljubanovic, D., Royer, P.A., Kraus, D.M., Molina, H., Barry, N.P., Proctor, G., Levi, M., and Holers, V.M. Altered renal tubular expression of the complement inhibitor Crry permits complement activation after ischemia/reperfusion. *J. Clin. Invest.* 116:357–368, 2006.
36. Rohrer, B., Bandyopadhyay, M., Kunchithapautham, K., and Thurman, J. Complement pathways and oxidative stress in models of age-related macular degeneration. In: Stratton, R.D., Hauswirth, W.W., and Gardner, T.W., eds. *Studies on Retinal and Choroidal Disorders*. New York, NY: Humana Press; 2012, 47–63.
37. Kunchithapautham, K., and Rohrer, B. Sublytic membrane-attack-complex (MAC) activation alters regulated rather than constitutive vascular endothelial growth factor (VEGF) secretion in retinal pigment epithelium monolayers. *J. Biol. Chem.* 286:23717–23724, 2011.
38. Chirco, K.R., Tucker, B.A., Stone, E.M., and Mullins, R.F. Selective accumulation of the complement membrane attack complex in aging choriocapillaris. *Exp. Eye Res.* 146:393–397, 2016.
39. Mullins, R.F., Dewald, A.D., Streb, L.M., Wang, K., Kuehn, M.H., and Stone, E.M. Elevated membrane attack complex in human choroid with high risk complement factor H genotypes. *Exp. Eye Res.* 93:565–567, 2011.
40. Ebrahimi, K.B., Fijalkowski, N., Cano, M., and Handa, J.T. Decreased membrane complement regulators in the retinal pigmented epithelium contributes to age-related macular degeneration. *J. Pathol.* 229:729–742, 2013.
41. Singh, A., Faber, C., Falk, M., Nissen, M.H., Hviid, T.V., and Sorensen, T.L. Altered expression of CD46 and CD59 on leukocytes in neovascular age-related macular degeneration. *Am. J. Ophthalmol.* 154:193–199.e192, 2012.
42. Chan-Ling, T., Baxter, L., Afzal, A., Sengupta, N., Caballero, S., Rosinova, E., and Grant, M.B. Hematopoietic stem cells provide repair functions after laser-induced Bruch's membrane rupture model of choroidal neovascularization. *Am. J. Pathol.* 168:1031–1044, 2006.
43. Vogt, S.D., Curcio, C.A., Wang, L., Li, C.M., McGwin, G., Jr., Medeiros, N.E., Philp, N.J., Kimble, J.A., and Read, R.W. Retinal pigment epithelial expression of complement regulator CD46 is altered early in the course of geographic atrophy. *Exp. Eye Res.* 93:413–423, 2011.
44. Lambert, V., Lecomte, J., Hansen, S., Blacher, S., Gonzalez, M.L., Struman, I., Sounni, N.E., Rozet, E., de Tullio, P., Foidart, J.M., Rakic, J.-M., and Noel, A. Laser-induced choroidal neovascularization model to study age-related macular degeneration in mice. *Nat. Protoc.* 8:2197–2211, 2013.
45. Rohrer, B., Long, Q., Coughlin, B., Renner, B., Huang, Y., Kunchithapautham, K., Ferreira, V.P., Pangburn, M.K., Gilkeson, G.S., Thurman, J.M., Tomlinson, S., and Holers, V.M.

- V.M. A targeted inhibitor of the complement alternative pathway reduces RPE injury and angiogenesis in models of age-related macular degeneration. *Adv. Exp. Med. Biol.* 703:137–149, 2010.
46. Adhi, M., Cashman, S.M., and Kumar-Singh, R. Adeno-associated virus mediated delivery of a non-membrane targeted human soluble CD59 attenuates some aspects of diabetic retinopathy in mice. *PLoS One.* 8:e79661, 2013.
 47. Huang, Y., Qiao, F., Abagyan, R., Hazard, S., and Tomlinson, S. Defining the CD59-C9 binding interaction. *J. Biol. Chem.* 281:27398–27404, 2006.
 48. Kunchithapautham, K., Bandyopadhyay, M., Dahrouj, M., Thurman, J.M., and Rohrer, B. Sublytic membrane-attack-complex activation and VEGF secretion in retinal pigment epithelial cells. *Adv. Exp. Med. Biol.* 723:23–30, 2012.
 49. Guo, Q., Cheng, J., Zhang, J., Su, B., Bian, C., Lin, S., and Zhong, C. Delayed post-injury administration of C5a improves regeneration and functional recovery after spinal cord injury in mice. *Clin. Exp. Immunol.* 174:318–325, 2013.
 50. Haynes, T., Luz-Madrigal, A., Reis, E.S., Echeverri Ruiz, N.P., Grajales-Esquivel, E., Tzekou, A., Tsonis, P.A., Lambris, J.D., and Del Rio-Tsonis, K. Complement anaphylatoxin C3a is a potent inducer of embryonic chick retina regeneration. *Nat. Commun.* 4:2312, 2013.
 51. Mullins, R.F., Schoo, D.P., Sohn, E.H., Flamme-Wiese, M.J., Workamelahu, G., Johnston, R.M., Wang, K., Tucker, B.A., and Stone, E.M. The membrane attack complex in aging human choriocapillaris: relationship to macular degeneration and choroidal thinning. *Am. J. Pathol.* 184:3142–3153, 2014.

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