Molecular Vision 2020; 26:370-377 <http://www.molvis.org/molvis/v26/370>
Received 26 February 2020 | Accepted 13 May 2020 | Published 15 May 2020

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The use of Matrigel combined with encapsulated cell technology to deliver a complement inhibitor in a mouse model of choroidal neovascularization

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Purpose: Risk for age-related macular degeneration (AMD), a slowly progressing, complex disease, is tied to an overactive complement system. Efforts are under way to develop an anticomplement-based treatment to be delivered locally or systemically. We developed an alternative pathway (AP) inhibitor fusion protein consisting of a complement receptor-2 fragment linked to the inhibitory domain of factor H (CR2-fH), which reduces the size of mouse choroidal neovascularization (CNV) when delivered locally or systemically. Specifically, we confirmed that ARPE-19 cells genetically engineered to produce CR2-fH reduce CNV lesion size when encapsulated and placed intravitreally. We extend this observation by delivering the encapsulated cells systemically in Matrigel.
Methods: ARPE-19 cells were generated to stably express CR2 or CR2-fH, microencapsulated using sodium alginate, and injected subcutaneously in Matrigel into 2-month-old C57BL/6J mice. Four weeks after implantation, CNV was induced using argon laser photocoagulation. Progression of CNV was analyzed using optical coherence tomography. Bioavailability of CR2-fH was evaluated in Matrigel plugs with immunohistochemistry, as well as in ocular tissue with dot blots. Efficacy as an AP inhibitor was confirmed with protein chemistry.
Results: An efficacious number of implanted capsules to reduce CNV was identified. Expression of the fusion protein systemically did not elicit an immune response. Bioavailability studies showed that CR2-fH was present in the RPE/choroid fractions of the treated mice, and reduced CNV-associated ocular complement activation.
Conclusions: These findings indicate that systemic production of the AP inhibitor CR2-fH can reduce CNV in the mouse model.

Age-related macular degeneration (AMD) is a slowly progressing, complex degenerative disease with typical onset at around 60 years of age. The disease involves pathology in the retina, the light-sensitive tissue at the posterior pole, the RPE, the blood–retina barrier, and the choroid, the ocular blood supply. AMD involves environmental and genetic risk factors [1], with an overactive complement pathway having been associated with all forms of AMD. Specifically, the Y402H single nucleotide polymorphism (SNP) in the complement inhibitor complement factor H (CFH) poses the greatest single genetic risk for AMD (reviewed in [2]). In addition, other variants that modify complement activation and are part of either complement inhibition [2-4] or activation [5-7] have been reported.

The complement system is an evolutionarily ancient part of the innate and adaptive immune system, and is involved in many different stress- and age-related diseases [8,9]. It is triggered in response to the generation of stress or injury-exposed antigens and produces three sets of biologic effector molecules: anaphylatoxins (C3a and C5a) that recruit phagocytes, opsonins (C3d and C3dg) that tag damaged cells or debris for removal, and the membrane attack complex (MAC), which lyses cells [10]. Based on the central role of the alternative pathway (AP) of complement in triggering complement dependent disease [8,9], we developed a designer complement inhibitor molecule (CR2-fH), which consists of the AP-inhibitory domain of CFH linked to the complement receptor 2 (CR2) targeting fragment that binds opsonins [11]. This protein was efficacious in a mouse model of wet AMD (laser-induced choroidal neovascularization [CNV]) when injected systemically [12] or delivered via gene therapy [13]. Recently, we confirmed that a biologic such as CR2-fH with potentially limited long-term stability in a 37 °C environment can successfully be delivered long-term using cell encapsulation technology (ECT). Specifically, we used immortalized RPE cells, stably transfected with an expression plasmid for CR2-fH and encapsulated in alginate for the treatment

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of mouse CNV [14]. Local administration using intravitreal injection has been the administration route of choice for AMD therapeutics [15-17]; however, based on the potential global effect of the complement system, systemic approaches are being considered [18].

METHODS

Cell encapsulation: Stably transfected ARPE-19 cells (ATCC® CRL-2302™; purchased from ATCC with required specifications examining short tandem repeat profiling to verify the human unique DNA profile and rule out intraspecies contamination) with plasmid constructs of CR2 and CR2-fH [12] have already been described and long-term CR2-fH secretion confirmed [14]. Likewise, cell encapsulation using the electrospray method was published by us in detail, including a video protocol [14,19]. In short, the encapsulation was performed by spraying cells at a final cell concentration of $1x10^6$ in 2% w/v alginate solution pumped through a 30G blunt tip needle connected to a high voltage generator producing a flowrate of 60 mm/h at 8.0 kV voltage. The capsules were dropped into a gelling bath containing a HEPES-buffered saline solution (100 mM $CaCl_2$ and 0.5% w/v poly-L-ornithine) to add a second coating. Microcapsules were stored in Dulbecco's Modified Eagle's Medium at 37 ºC and 5% CO_2 until further use.

Animals: Cell-containing microcapsules were suspended in 100 µl of serum-free media and then mixed with 100 µl of cold Matrigel (Corning Cat # 354234, Corning, NY) [20]. C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were bred in house and used at 2 months old in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the approval of the University Animal Care and Use Committee. Subcutaneous injections were performed on mice of both sexes with isoflurane anesthesia, using a 23-gauge needle.

Immunohistochemistry: One month after injection, the presence of CR2-fH was confirmed in cryosections (14 µm sections) of Matrigel plugs and surrounding tissues fixed overnight with 4% paraformaldehyde (PFA) and probed with anti-CR2 (7G6) antibody (1:200), followed by secondary antibody binding (Alexa Fluor 488 goat anti-mouse IgG; 1:500, Invitrogen, Carlsbad, CA) [12]. Fluorescence microscopy (Zeiss, Thornwood, NY) equipped with a digital black-and-white camera (Spot camera: Diagnostic Instruments, Sterling Heights, MI) was used to identify CR2-fH positive cells.

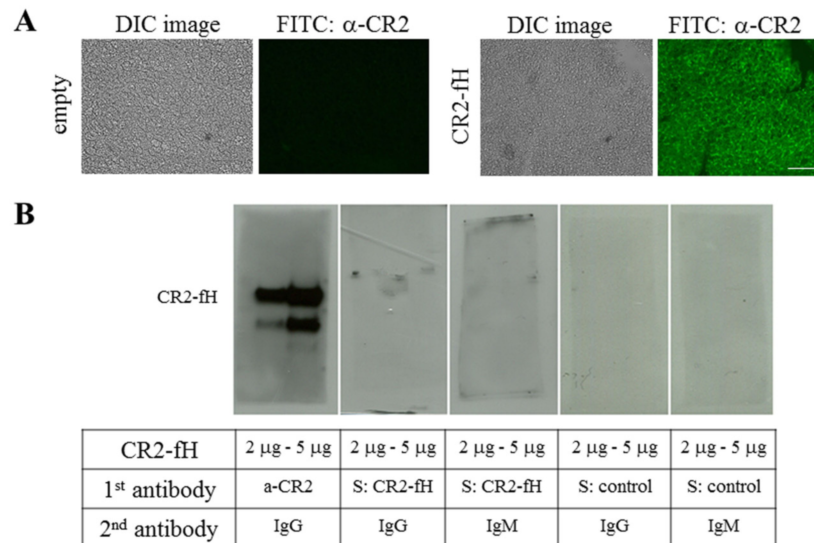
Choroidal neovascularization and optical coherence tomography: One month following the injection, argon laser photocoagulation (532 nm, 100 µm spot size, 0.1 s duration, 100 mW) was used to generate four laser spots around the optic nerve of each eye [12]. Five days after laser-induced CNV, the mouse eyes were imaged with optical coherence tomography (OCT) using an SD-OCT Bioptigen® Spectral Domain Ophthalmic Imaging System (Bioptigen Inc., Durham, NC) as previously described [21-23], before being sacrificed by isoflurane inhalation for tissue collection on day 6.

Protein analyses: Dot blot analyses (Bio-Dot® Microfiltration Apparatus; Bio-Rad Laboratories Inc., Hercules, CA) of the total RPE/choroid protein was used to detect CR2-fH and CR2 using the anti-CR2 antibody as published previously [14]. In short, total protein (25 µl per well of a 96 well plate) was transferred onto a nitrocellulose membrane, blocked for 2 h with 5% nonfat milk in TBST (Tris-buffered saline, 0.1% Tween 20), probed with an anti-CR2 primary antibody (10 µg/ml; rat anti-mouse CD21, clone 7G6; purified in house [24]) and visualized with Clarity™ Western ECL Blotting Substrate optimized for horseradish peroxidase substrates (Bio-Rad Laboratories Inc). C3a levels were measured using a sandwich enzyme immunoassay (LifeSpan Biosciences, Inc; Seattle, WA) in the RPE/choroid tissue homogenates as we have previously described [23,25]. In short, tissues were rinsed with ice cold PBS to remove excess blood, lysed by ultrasonication in the presence of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and homogenates cleared by centrifugation. Measurements were obtained according to the manufacturer's instructions. To determine whether mice generate antibodies against CR2-fH secreted from the ARPE-19 cells, CR2-fH was electrophoresed on a 4–20% Criterion™ TGX™ Precast Gels (Bio-Rad Laboratories, Inc.), proteins transferred to a PVDF membrane, and membranes incubated with primary antibody against CR2 (7G6), or serum (1:50) from mice treated with capsules, as previously described [14].

Statistical analysis: Data are presented as mean ± standard error of the mean (SEM). Single comparisons were analyzed using unpaired *t* tests, data consisting of multiple groups were analyzed with analysis of variance (ANOVA); Fisher's protected least significant difference). Mean value differences were considered statistically significant at p values less than or equal to 0.05 (StatView).

RESULTS

Subcutaneous delivery of encapsulated ARPE-19 cells, dose-finding study: Stably transfected ARPE-19 cells expressing CR2 or CR2-fH were encapsulated and injected subcutaneously into the back of the mice within a Matrigel suspension [20]. Then, the Matrigel plugs were collected for analysis. The presence of cells containing CR2-fH was documented with



CR2-fH	2 μg - 5 μg	2 μg - 5 μg	2 μg - 5 μg	2 μg - 5 μg	2 μg - 5 μg
1st antibody	a-CR2	S: CR2-fH	S: CR2-fH	S: control	S: control
2nd antibody	IgG	IgG	IgM	IgG	IgM

Figure 1. Encapsulated cell technology to deliver CR2-fH: Tool development. **A**: Detection of CR2-fH in the tissue surrounding the ARPE-19 cell-containing matrigel plug with immunohistochemistry using an antibody against CR2. The corresponding differential interference contrast (DIC) and fluorescence image is presented. CR2 antibody staining was negative in control tissues containing empty alginate capsules. Scale bar: 100 µm. **B**: Systemically produced CR2-fH could trigger an immune response. Lack of immunoglobulin G (IgG) or IgM antibody production was confirmed 1 month after capsule injection. Purified CR2-fH was run at two different concentrations and probed for the presence of CR2-fH using the anti-CR2 antibody (positive control). Identical lanes were probed with serum from experimental animals (S: CR2-fH) or control animals (S: control) at 1:50, followed by the appropriate secondary antibodies.

fluorescence microscopy using an antibody against the CR2 portion of the molecule [12] (Figure 1A).

After the presence of the therapeutic fusion protein in Matrigel plugs was confirmed, 1 month following the injection, argon laser photocoagulation was used to generate four CNV lesions per eye [12]. After 5 days, the CNV lesions sizes were assessed with OCT. To identify an effective concentration to reduce CNV progression, depots of CR2- versus CR2-fH expressing cells were compared and analyzed based on the ability of the proteins to target binding sites in the RPE/choroid of eyes with CNV lesions, reduce the CNV-induced generation of C3a in the eye, and reduce the CNV lesion size in a dose-finding study with a small set of animals. In dot blot analyses of the total RPE/choroid protein, CR2-fH and CR2 were detected using the anti-CR2 antibody also used for immunohistochemistry, and confirmed to increase in levels with increasing numbers of capsules (Figure 2A). CNV-induced generation of C3a in the RPE/choroid fraction was reduced by the presence of CR2-fH when compared to CR2 alone (Figure 2B). A preliminary CNV analysis testing Matrigel depots containing 500–25,000 capsules suggested that CR2-fH secreted from 1,000 capsules was most effective in reducing CNV lesion sizes (Figure 2C). Importantly, as we reported for gene therapy [13] or intravitreal ECT delivery [14], complement inhibition by CR2-fH has an optimal level; too much inhibition is ineffective or potentially has cytotoxic effects. Finally, to confirm that long-term production of the mouse protein-based CR2-fH in Matrigel plugs does not induce anti-CR2-fH antibody production, the mouse serum was examined for the presence of anti-CR2-fH antibodies. One month after the implantation of the capsules, serum from experimental animals with CR2-fH capsules was used as the source of primary antibodies and compared to that from control animals (empty capsules). Neither immunoglobulin G (IgG) nor IgM antibodies recognizing CR2-fH could be identified (Figure 1B). These results confirmed previous results we found in mice treated with CR2-fH with ECT [14] or gene therapy [13].

Encapsulated ARPE-19 cells expressing CR2-fH reduce complement activation and attenuates CNV development: Having established proof of principle, we confirmed the effectiveness of encapsulated ARPE-19 cells (1,000 capsules per Matrigel plug) expressing CR2-fH when compared to CR2 on CNV lesion sizes. Mice were injected with Matrigel containing 1,000 capsules containing either CR2- (control) or CR2-fH-expressing ARPE-19 cells. After 1 month, CNV lesions were induced in all four quadrants of the eye, using argon laser-induced rupture of Bruch's membrane [12], and OCT was used to evaluate lesion sizes based on two parameters: diameter [23] and maximum height or depth

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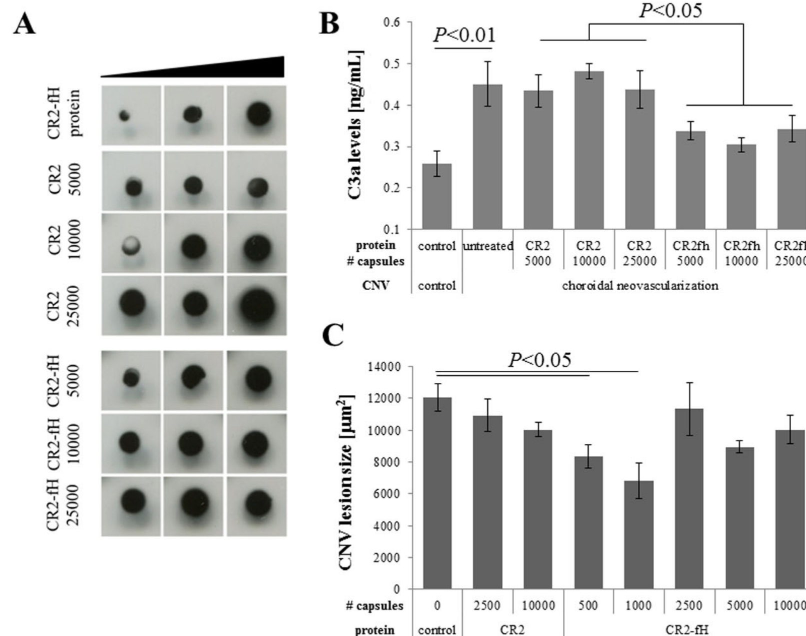

Figure 2. Encapsulated cell technology to deliver CR2-fH: Identification of a therapeutic dose. **A**: After systemic capsule delivery, CR2-fH was detectable in the RPE/choroid fraction of eyes with choroidal neovascularization (CNV) lesions. A dot blot of RPE/choroid samples with twofold dilution steps is presented documenting a dose-dependent increase in CR2 and CR2-fH delivery and binding to the tissues. **B**: CNV-induced complement activation in untreated animals was demonstrated by elevated levels of C3a when compared to animals with no lesions (control). C3a levels remained elevated in animals exposed to CR2, and statistically significantly reduced by CR2-fH. **C**: CNV sizes were reduced in animals injected systemically with ARPE-19 cells expressing CR2-fH as opposed to those expressing CR2, with an apparent efficacious dose of 1,000 capsules. Data shown are average values (± standard error of the mean [SEM]; n = 2–3 animals per condition).

[26]. Both factors were statistically significantly decreased in the presence of CR2-fH containing capsules compared to those receiving the cells expressing CR2 (size: $p = 0.03$ and lesion height $p<0.0001$; Figure 3A,B), and a repeated-measure ANOVA revealed a treatment by measure interaction ($p<0.01$). Complement activation in the RPE/choroid fractions was assessed with enzyme-linked immunosorbent assay (ELISA) for C3a. As expected in the presence of the AP inhibitor CR2-fH, a reduction in C3a was identified (Figure 3C). The ELISA measurements demonstrated that CNV (four lesions per eye) resulted in an approximate threefold difference in C3a in the eyes of animals exposed to capsules containing CR2-expressing cells when compared to the control mice without CNV. This increase was blunted statistically significantly by CR2-fH ($p<0.01$). Importantly, the therapeutic dose (as well as the higher doses above 1,000 capsules) of CR2-fH delivered via systemic ECTs reduced complement activation, but never below levels present in animals without CNV. We have argued that these baseline levels would allow for normal homeostatic functions of the complement system in the eye and low-level surveillance of the immune system [27].

DISCUSSION

A successful therapeutic for a chronic disease such as AMD requires long-term drug delivery to the posterior pole. Although repeated ocular administration using intravitreal injections has been the administration route of choice for AMD therapeutics [15-17], systemic approaches are being considered based on the potential global effect of the complement system in AMD [18]. The use of Matrigel was identified to facilitate the systemic delivery of genetically engineered encapsulated ARPE-19 cells that produce the designer complement inhibitor CR2-fH for the treatment of mouse CNV. The main results of the present study are as follows: (1) ARPE-19 cells encapsulated in alginate can be implanted subcutaneously using Matrigel, and expressed or secreted CR2-fH can be visualized in tissues with immunohistochemistry. (2) CR2-fH secreted systemically does not lead to anti-CR2-fH antibody production. (3) CR2-fH when expressed and secreted by ARPE-19 cells systemically targets the lesions in the RPE/choroid where it reduces complement activation and leads to the reduction in CNV lesion sizes.

As discussed in the parent paper to this publication [14], and citing Wong and colleagues [28], “the pinnacle goal of

ECT is to provide sustained delivery of fresh therapeutics secreted by the encapsulated cells at the target sites without causing any immune reactions." As in the previous publication using intravitreal delivery, we achieved both goals. First, we confirmed using dot blot analysis of RPE/choroid tissues that CR2-fH (and CR2) can be detected 1–2 months after capsule injection in the injured tissue; this observation also indirectly confirmed the continued viability of the implanted ARPE-19 cells. Second, no anti-CR2-fH antibodies (IgG and IgM) were generated during that time frame. In accordance with our previous publication [14], we did not examine whether ARPE-19 cell epitopes elicit antibody production. First, human patients treated with encapsulated ARPE cells did not require immunosuppressants [29-31]; second, the potential immune response in the mouse to systemically implanted ARPE-19 is irrelevant for future preclinical work. We did not expect to be able to identify intact alginate capsules at the termination of the experiment (Figure 1A) as there are many enzymes able to degrade them; however, cells expressing CR2-positive material could be identified in the regions of the Matrigel plugs using immunohistochemistry. For future human studies, stable depots containing cells rather than alginate capsules will be explored.

ECT devices have been studied in the eye for scientific purposes, and Neurotech has tested them for therapeutic purposes using ARPE-19 cells. Specifically, two programs with intravitreal delivery are still being pursued: NT-501 was designed to deliver ciliary neurotrophic factor (CNTF) and NT-503 a soluble anti-vascular endothelial growth factor receptor (VEGF-R) protein. In short, although both had good safety profiles, neither reached their primary clinical endpoints in geographic and wet AMD respectively [29,31]. A comprehensive discussion was provided in our previous publication [14]. However, as we discussed previously, ARPE-19 cells secrete not only therapeutic proteins (e.g., CNTF, anti-VEGF, or CR2-fH) but also a large range of proteins, including some known to play a role in angiogenesis or the immune response [32]. These additional proteins when secreted into the vitreous could potentially interfere with the efficacy of the therapeutic, or worse, augment disease. Thus, cell encapsulation and delivery systemically appears advantageous, as in the subcutaneous space the small

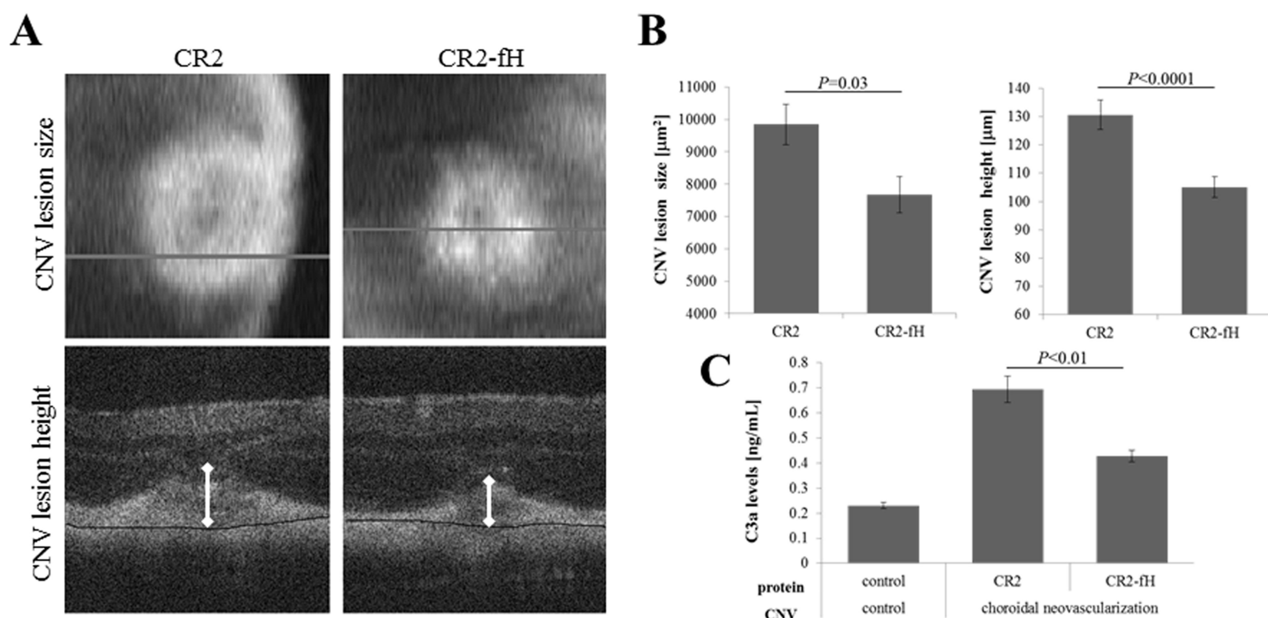

Figure 3. Systemic ECT-mediated delivery of CR2-fH reduces CNV and complement activation. One month following subcutaneous injection of alginate capsules, laser choroidal neovascularization (CNV) was induced. On day 5 after injury, the lesion sizes were analyzed with optical coherence tomography (OCT; lesion size and height), and complement activation was determined using enzyme-linked immunosorbent assay (ELISA) for the C3 breakdown product C3a. **A, B**: CNV sizes and lesion heights were statistically significantly reduced in animals treated with ARPE-19 cells expressing CR2-fH as opposed to those expressing CR2. The gray line in the en face image can be used to pick the locations of B-scans, but are not used here; CNV lesion height is measured between BrM (black line) and its peak. **C**: CNV induced complement activation as demonstrated by elevated levels of C3 breakdown product C3a when compared to animals with no lesions (control), an effect that was mitigated by CR2-fH but not CR2. Data shown are average values (± standard error of the mean [SEM]; n = 5–8 animals per condition).

amount of additional human protein secreted by these cells is not expected to alter systemic cell behavior. Although systemic delivery of therapeutic proteins aimed at the eye, such as CNTF and anti-VEGF, would require too many cells to be practical, a therapeutic with targeting potential, such as CR2-fH [11], can be used successfully. We and our collaborators on this program have shown that CR2-targeting systemic delivery can be used successfully to treat many different organ systems. Importantly, it has been shown that CR2-fH via its opsonin binding domain (CR2) will bind specifically to organs with complement activation where it is retained for multiple days [33], whereas unbound protein has a half-life of approximately 8–9 h [34] and will be rapidly eliminated by the kidneys. Thus, the inhibitor will be available at sites of complement activation and not wasted on non-pathophysiologically important complement target molecules. Although a residual inhibitory effect of CR2-fH in in vitro hemolysis assays has been observed, likely attributable to the fH moiety of CR2-fH acting in the fluid phase [35], the inhibitor had no measurable effect on serum complement activity in vivo in recipient mice, and therefore, is not expected to alter the homeostatic complement pathway [36]. Finally, CR2-fH has recently been humanized (TT30), and efficacy of TT30 in CNV was confirmed [37,38], allowing potentially for rapid development of this therapeutic approach.

ACKNOWLEDGMENTS

The study was supported in part by the National Institutes of Health (R01EY024581), the Department of Veterans Affairs (I01RX000444, I01BX003050 and IK6BX004858) and the South Carolina SmartState Endowment. The authors thank Marwa Belhaj and Jay Potts for helping with the cell encapsulation protocol and Stephen Tomlinson for providing reagents (plasmids for CR2 and CR2-fH). **Conflict of interest**: BR holds patents that describe the CR2-fH technology. The remaining authors declare that they have no conflict of interest.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 15 May 2020. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.