Drusen complement components C3a and C5a promote choroidal neovascularization

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Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in industrialized nations, affecting 30–50 million people worldwide. The earliest clinical hallmark of AMD is the presence of drusen, extracellular deposits that accumulate beneath the retinal pigmented epithelium. Although drusen nearly always precede and increase the risk of choroidal neovascularization (CNV), the late vision-threatening stage of AMD, it is unknown whether drusen contribute to the development of CNV. Both in patients with AMD and in a recently described mouse model of AMD, early subretinal pigmented epithelium deposition of complement components C3 and C5 occurs, suggesting a contributing role for these inflammatory proteins in the development of AMD. Here we provide evidence that bioactive fragments of these complement components (C3a and C5a) are present in drusen of patients with AMD, and that C3a and C5a induce VEGF expression *in vitro* and *in vivo*. Further, we demonstrate that C3a and C5a are generated early in the course of laser-induced CNV, an accelerated model of neovascular AMD driven by VEGF and recruitment of leukocytes into the choroid. We also show that genetic ablation of receptors for C3a or C5a reduces VEGF expression, leukocyte recruitment, and CNV formation after laser injury, and that antibody-mediated neutralization of C3a or C5a or pharmacological blockade of their receptors also reduces CNV. Collectively, these findings establish a mechanistic basis for the clinical observation that drusen predispose to CNV, revealing a role for immunological phenomena in angiogenesis and providing therapeutic targets for AMD.

angiogenesis | inflammation | injury

Age-related macular degeneration (AMD) is the leading cause of permanent vision loss among the elderly in many industrialized countries (1). The majority of vision loss due to AMD is a result of pathologic new blood vessels, termed choroidal neovascularization (CNV), invading the retina from the underlying choroid through fractures in Bruch membrane, the extracellular matrix between the choroid and the retinal pigmented epithelium (RPE). The earliest clinical hallmark of AMD is the appearance of drusen (2), localized lipoproteinaceous deposits between the RPE and Bruch membrane. Although their presence is an epidemiological risk factor for the development of CNV (3, 4), the mechanism of how, or whether, drusen provoke CNV remains undefined. Some investigators have suggested that drusen are epiphenomena, whereas others have claimed that drusen constituents act as a focal stimulus for inflammatory cells that secrete angiogenic molecules such as VEGF, and still others have suggested that drusen disturb RPE homeostasis by impairing transport across Bruch membrane (reviewed in ref. 5).

Recent work has demonstrated that complement components C3 and C5 are constituents of drusen in patients with AMD (6–9). Their presence, as well as that of the membrane-attack-complex (MAC) C5b-9 and other acute-phase reactant proteins in RPE cells overlying drusen, has fueled speculation that drusen biogenesis involves chronic inflammatory processes that either can trigger complement activation and formation of MAC acting to lyse RPE cells or disturb physiological homeostasis in RPE cells (9).

Recently, we described an animal model of AMD in aged *Ccl2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice, which develop many salient pathological features seen in the human condition (10). Interestingly, RPE and choroidal deposits of C3 and C5 also are found in these mice at a young age. The inability of these mice, which are impaired in induced macrophage trafficking, to clear these complement deposits, is thought to promote the later development of CNV via up-regulation of RPE cell secretion of VEGF (10). These findings suggest a mechanistic link between deposition of complement components in drusen and the development of CNV.

There is growing evidence that complement components are more than mere mediators of innate immunity (11). To date, their influence on the molecular regulation of angiogenesis *in vivo* has not been fully elucidated. C3a and C5a are the bioactive fragments of C3 and C5 that effect biological responses through their receptors C3aR and C5aR. Because drusen predispose to and predate CNV, we sought to obtain direct evidence that C3a and C5a are present in drusen and play a role in CNV development. We studied their impact on VEGF expression and on the development of laser-induced CNV, an accelerated model of neovascular AMD that reproduces much of the pathology and immunophenotype of human CNV (reviewed in ref. 5), by using neutralizing antibodies against C3a or C5a, small-molecule antagonists of C3aR or C5aR, and mice deficient in C3aR or C5aR.

Results

C3a and C5a were immunolocalized to hard and soft drusen, the proximity of RPE cells, and Bruch membrane in the eye of a patient with AMD, providing direct evidence of complement activation in AMD (Fig. 1 *A–F*). The specificity of immunolocalization was demonstrated by omitting the primary mouse monoclonal antibodies against complement fragments and staining only with the secondary antibody (Fig. 1*G*). Neither C3a nor C5a were detected in the posterior segment of an eye of a patient without AMD (Fig. 1 *H* and *I*). The demonstration of the complement anaphylatoxins in the pathological specimen indicates ongoing and continual deposition, because these soluble

Conflict of interest statement: J.A. is listed on a patent application filed by the University of Kentucky describing these findings.

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Abbreviations: AMD, age-related macular degeneration; CNV, choroidal neovascularization; C3/5, complement components 3/5; C3a/5a, bioactive fragments of C3/5; C3aR/5aR, Cea/5a receptors; RPE, retinal pigmented epithelium.

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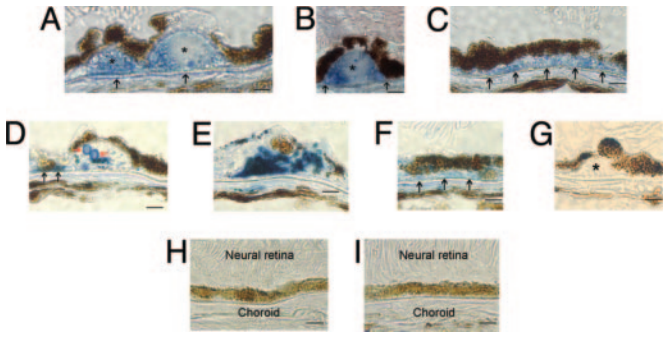

Fig. 1. C3a and C5a were present in human drusen. Representative images from a 91-year-old woman with AMD (*A–G*) and an 87-year-old man without AMD (*H* and *I*). Examples of hard drusen (asterisks) and Bruch membrane (black arrows) immunoreactivity (blue) to C3a (*A* and *B*) or C5a (*D* and *E*) in the eye of patients with AMD and lack of immunolabeling in neural retina, choroid, or intervening RPE in the eye of control (*H* and *I*). C3a (*C*) and C5a (*F*) staining in soft drusen deposits between RPE (brown pigmented cells) and Bruch membrane (black arrows). C5a is localized to vesicular structures (red arrows) in hard drusen (*D*). Lack of nonspecific labeling in the AMD eye demonstrated by omitting primary anticomplement anaphylatoxin antibody (*G*). (Scale bars, 10 μm.)

proteins generally diffuse away from the site of complement activation. Although C3a immunoreactivity was diffuse, C5a was present in vesicular structures within hard drusen (Fig. 1*D*) that have previously been shown to contain C3 fragments (9) and amyloid β (12).

Both C3a and C5a up-regulated the secretion of VEGF by primary human RPE cells, both in confluent and subconfluent conditions (Fig. 2*A*), but not in human choroidal endothelial cells (data not shown) *in vitro*, extending our previous observations with C5a (10). Similar results were obtained with the D407 human RPE cell line (13) (data not shown). This response was preserved *in vivo*, because intravitreous injection of C3a or C5a in wild-type mice induced VEGF expression in the RPE/choroid in a dose-dependent fashion within 4 h of administration (Fig. 2*B*). We found no increase in the number of macrophages or neutrophils in the choroid 4 h after intravitreous injection of C3a or C5a (Fig. 2 *C* and *D*), indicating that the increase in VEGF expression was due to resident cells. The absence of a similar effect in the neurosensory retina demonstrated that VEGF up-regulation was not a nonspecific response to intraocular injection.

In the laser-injury model, we detected enhanced C3a and C5a generation by ELISA in the RPE/choroid within 4 h after injury, with a peak at 12 h, and approached baseline levels within 1 day (Fig. 3*A*). Immunostaining revealed significant amounts of C3 and C5 deposition in and near RPE cells, as well as spillover into the adjacent subretinal space, in the area of injury (Fig. 3 *B* and *C*). Uninjured areas exhibited minimal, if any, staining for these complement components (Fig. 3 *D* and *E*). VEGF levels in the RPE/choroid 1 day after laser injury were significantly reduced in $C3aR^{-/-}$ (67.7 ± 6.8%; $P = 0.05$) and $C5aR^{-/-}$ mice (76.5 ± 7.1%; $P = 0.04$) compared with wild-type mice (Fig. 4*A*). Reduced VEGF levels persisted even 3 days after injury, the time point of the maximal VEGF response (14), in $C3aR^{-/-}$ (60.9 ± 4.9%; $P = 0.01$) and $C5aR^{-/-}$ mice (62.5 ± 3.1%; $P = 0.004$) compared with wild-type mice (Fig. 4*B*). Maximal neutrophil infiltration into the choroid occurred 1 day after injury (Fig. 5*A*) and was reduced in $C3aR^{-/-}$ (57.8 ± 9.0%; $P = 0.05$) and $C5aR^{-/-}$ mice (66.6 ± 1.7%; $P = 0.02$) compared with wild-type mice (Fig. 5*B*). Maximal macrophage infiltration into the choroid occurred 3 days after injury (Fig. 5*C*) and was reduced in $C3aR^{-/-}$ (49.1 ± 4.6%; $P = 0.04$) and $C5aR^{-/-}$ mice (42.1 ± 2.1%; $P = 0.05$) compared with wild-type mice (Fig. 5*D*).

These findings translated into functional inhibition as well. The volume of laser-induced CNV was significantly reduced in $C3aR^{-/-}$ (41.5 ± 5.2%; $n = 13$; $P < 0.001$) and $C5aR^{-/-}$ mice (40.8 ± 4.3%; $n = 14$; $P < 0.001$) compared with wild-type mice ($n = 17$) (Fig. 6 *A–D*). To corroborate the genetic ablation studies, we used neu-

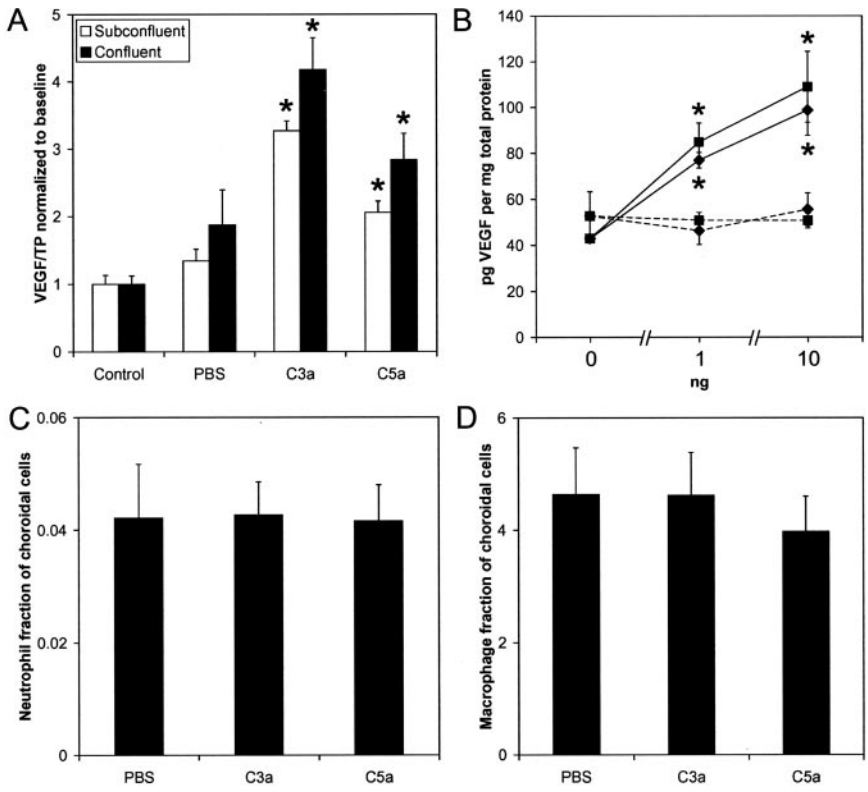

Fig. 2. C3a and C5a up-regulated VEGF *in vitro* and *in vivo*. C3a (50 ng/ml) and C5a (50 ng/ml) up-regulated human RPE cell secretion of VEGF 8 h after stimulation compared with unstimulated (control) or PBS-stimulated cells (*A*). Intravitreous injection of C3a (square) or C5a (circle) in wild-type mice increased VEGF levels in the RPE/choroid (solid lines) but not in the neurosensory retina (dotted lines) in a dose-dependent fashion 4 h after injection compared to PBS injection (0 ng) (*B*). *, $P < 0.05$ compared with PBS. No difference in the fraction (%) of neutrophils (*C*) or macrophages (*D*) in the choroid 4 h after intravitreous injection of C3a or C5a was observed compared with PBS.

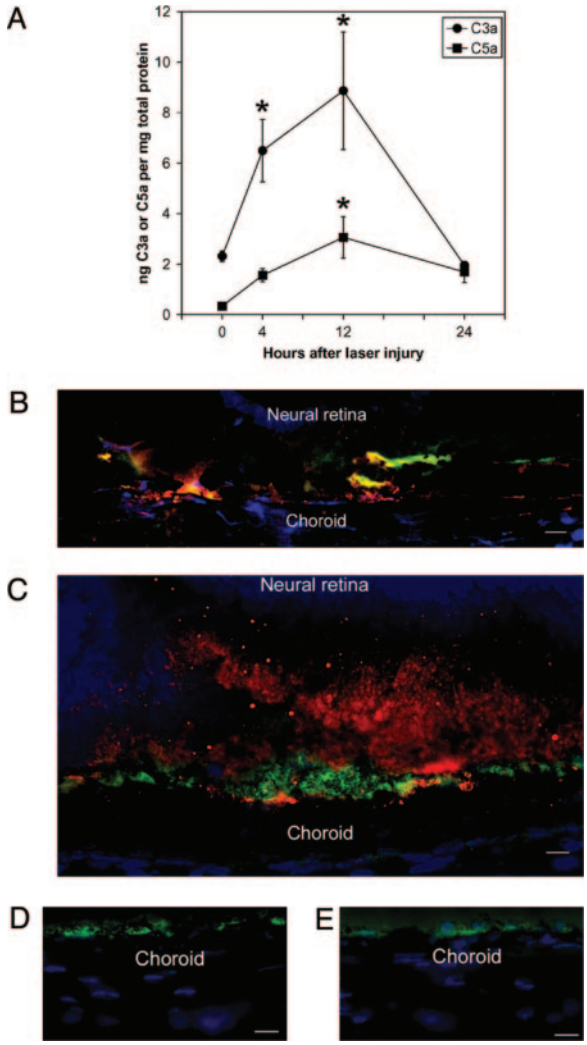

Fig. 3. Laser injury-induced complement in wild-type mice. ELISA demonstrated increased levels of C3a and C5a in the RPE/choroid within 4 h of injury and a maximum at 12 h after injury. (*A*) *, $P < 0.05$ compared with PBS. C3 (*B*) and C5 (*C*) were deposited (red) in the proximity of RPE cells (green) in the area of injury 4 h after injury. Areas of complement colocalization with RPE cells appear yellow. No deposition of C3 (*D*) or C5 (*E*) was identified in unlasered areas. (Scale bars, 5 μm.) DAPI staining appears blue.

tralizing antibodies against C3a or C5a and small-molecule antagonists of their receptors in wild-type mice. CNV was significantly reduced by treatment with both peptide antagonists (60–73%; $n =$ 14–16) compared with control peptide ($n = 10$; $P < 0.001$) and by both neutralizing antibodies (58–80%; $n = 10$–13) compared with isotype control antibodies ($n = 10$; $P < 0.01$) (Fig. 6 *E* and *F*).

Discussion

Prior studies convincingly demonstrated the presence of numerous complement components in drusen that strongly, although indirectly, suggested complement activation in AMD (6–9). However, these data provide direct demonstration of complement anaphylatoxins in AMD. Given the diffusible nature of these proteins, identification of C3a and C5a in drusen indicates that the overlying RPE cells would be continually exposed to these bioactive complement fragments. Ongoing studies of C3a and C5a generation in a larger group of eyes will provide more insight into the extent of complement activation in AMD.

In AMD, the integrity of the RPE cell monolayer is compromised in some, but not all, regions, and thus RPE cells would exist

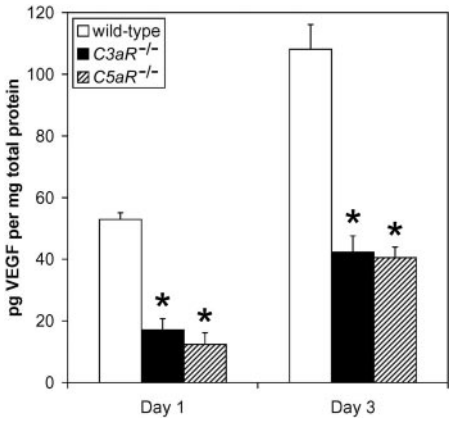

Fig. 4. VEGF induction was diminished in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice. VEGF levels in the RPE/choroid were significantly reduced in both knockout strains at 1 and 3 days after laser injury. *, $P < 0.05$ compared with wild-type mice.

both in confluent (quiescent) and subconfluent (proliferating) conditions. In both states, C3a and C5a induced VEGF in RPE cells *in vitro*. Significant quantities of C3a and C5a were present within the RPE/choroid soon after laser injury, and CNV was significantly reduced in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice via concerted suppression of leukocyte recruitment and VEGF expression. C3a and C5a up-regulated RPE/choroid production of VEGF in the uninjured eye in a dose-dependent manner, consistent with the induction of VEGF from RPE cells *in vitro*, and VEGF up-regulation induced by laser injury was abrogated in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice. Although C3a and C5a individually increased VEGF (Fig. 2), VEGF levels were reduced in both *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice after laser injury (Fig. 4), suggesting the possibility of cooperation between C3a and C5a or crosstalk between their receptors in inducing VEGF.

C3 and C5 were localized to RPE cells and their immediate vicinity within 4 h after injury, when there was minimal neutrophil infiltration (Fig. 5*A*), and macrophage infiltration had not yet occurred (Fig. 5*B*). At the time of the peak neutrophil response, 1 day after injury (Fig. 5*A*), the levels of C3a and C5a approached baseline (Fig. 3*A*), suggesting that the principal source of complement anaphylatoxin generation was resident cells. However, this does not formally exclude the contribution of infiltrating cells to the generation of complement proteins.

In the laser-injury model, the initial rise in VEGF levels occurs before the infiltration of leukocytes (14), suggesting that resident cells such as RPE cells are responsible. The peak of the VEGF response 3 days after injury parallels the infiltration of macrophages and can be blunted by macrophage depletion (14), suggesting these recruited cells that are responsible for the second surge in VEGF. VEGF levels were blunted both at 1 and 3 days after injury in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice. The lack of an initial rise in VEGF immediately after injury in these animals is attributed to the disruption of C3a- and C5a-induced VEGF. The attenuated secondary surge in VEGF in these mice might be due both to the interference with complement component receptor-mediated signaling and the reduction in leukocyte recruitment.

Leukocyte recruitment, which plays a pivotal role in laser-induced CNV (14, 15), was markedly reduced in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice after injury. This might be due to direct interception of the chemotactic function of C3aR and C5aR on leukocytes or reduction in VEGF, which itself is a chemoattractant (16, 17). It also might result from indirect reduction of chemokines such as Ccl-2 and Cxcl-2, which we and others have shown can be induced by complement components (10, 18–20). Suppression of CNV in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice and in wild-type mice treated with antagonists of the activated complement components or their

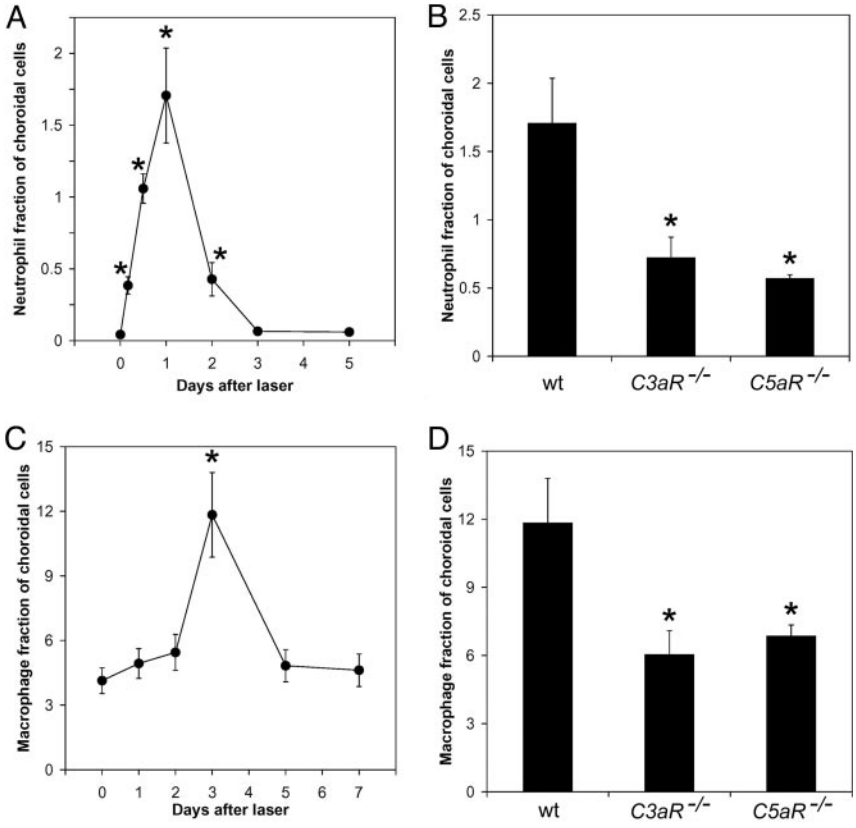

Fig. 5. Leukocyte recruitment to the choroid was diminished in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice. The fraction (%) of neutrophils in the choroid, maximal at 1 day after injury (*A*), and of macrophages in the choroid, maximal at 3 days after injury (*C*), was significantly reduced in both knockout strains (*B* and *D*). *, $P < 0.05$ compared with wild-type (wt) mice.

receptors also may be due to down-regulation of leukocyte–endothelial adhesion molecules, which can be promoted by C3a and C5a (21–23) and are critical for CNV formation (15).

Although VEGF levels after laser injury in *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice were markedly reduced, CNV was not completely abolished, suggesting there are both VEGF-dependent and- redundant pathways of angiogenesis in this model. Although RPE cells constitutively produce both Ccl-2 (24, 25) and VEGF (26), the stimuli inducing their overexpression in human CNV (27–29) are unknown. The demonstration that bioactive complement fragments can trigger Ccl-2 (10) and VEGF production makes them attractive CNV-triggering candidates given their presence in drusen deposits in patients with AMD, as well as after laser injury, particularly because they can be locally synthesized in the RPE (6, 7). As such, there is a rationale for testing complement inhibitors in the prophylaxis or treatment of CNV. These data also reinforce the potential significance of *Ccl2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice as models for studying the progress of human AMD.

After submission of this report, a paper describing the importance of C3 and membrane attack complex in laser-induced CNV appeared (30). The recent definition of complement factor H, which inhibits C3 deposition and C5a release after complement activation (31), and toll-like receptor-4, which influences C3 deposition and is negatively regulated by C5a (32, 33), as major susceptibility loci in AMD (34–39) adds to the mounting evidence of complement dysfunction in this disease. These findings and ours enrich the growing recognition that the complement system is not dedicated solely to immunological surveillance but also can play a versatile role in angiogenesis.

Intercepting specific receptor-mediated pathways, we have identified a mechanistic association between the complement and cytokine networks in promoting laser-induced CNV. Our findings introduce bioactive complement fragments as modulators of angiogenesis in the eye and highlight an intersection of immunology and vascular biology. If these complement components act similarly in the human eye via leukocyte recruitment and VEGF production, the hypothesis of a causative link between drusen and CNV in neovascular AMD is strengthened, given the many parallels and conserved pathways of pathophysiology in both human disease and animal models of CNV (5, 10).

Materials and Methods

Animals. All animal experiments were in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology. Male *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice backcrossed six and nine times, respectively, to C57BL/6J generated as described (40, 41) and wild-type C57BL/6J mice (The Jackson Laboratories) between 6 and 8 weeks of age were used to minimize variability. For all procedures, anesthesia was achieved by i.p. injection of 50 mg/kg ketamine hydrochloride (Fort Dodge Animal Health, Wyeth) and 10 mg/kg xylazine (Phoenix Scientific, San Marcos, CA), and pupils were dilated with topical 1% tropicamide (Alcon Laboratories, Fort Worth, TX).

Patients. Donor eyes were obtained within 3–4 h after death from a 91-year-old woman with an ophthalmic diagnosis of confluent soft drusen and an 87-year-old man with a normal fundus examination and no signs of drusen. Eye globes were dissected to remove the anterior segment, and the posterior segment was fixed with 4% paraformaldehyde in PBS for 2 h on ice. Globes were dehydrated by transferring to 15% sucrose for 30 min and maintained in 30% sucrose at 4°C overnight. TBS-embedded blocks were frozen on dry ice and cut into 12-μm sections on polylysine-coated slides. All human tissue experiments were approved by the University of Utah and University of Kentucky Institutional Review Boards and conformed to the Declaration of Helsinki.

CNV. Laser photocoagulation (532 nm, 200 mW, 100 ms, 75 μm) (OcuLight GL, IRIDEX, Mountain View, CA) was performed

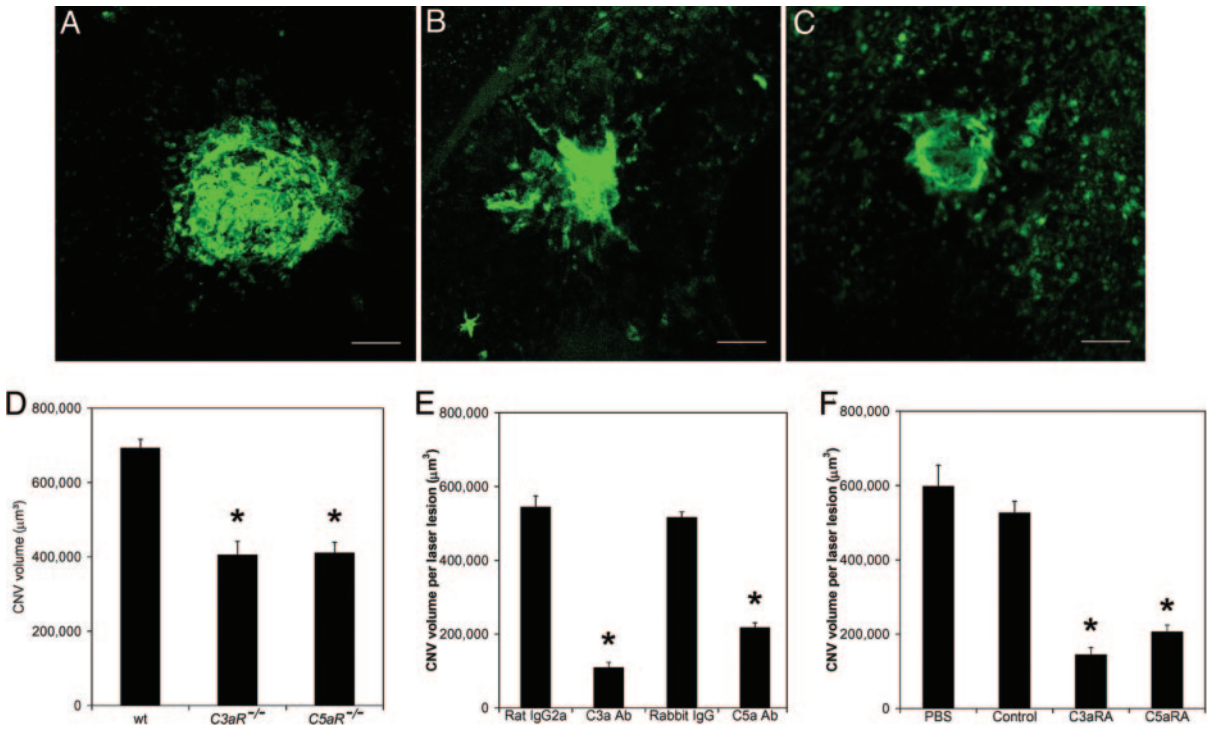

Fig. 6. Disruption of complement function inhibited CNV. Stacked confocal images (1-μm sections) of FITC–isolectin B4-labeled tissue within laser scars in wild-type (*A*), *C3aR*$^{-/-}$ (*B*), and *C5aR*$^{-/-}$ (*C*) mice demonstrate reduction in CNV volume in knockout animals (*D*). *, $P < 0.05$ compared with wild-type (wt) mice. (Scale bar, 100 μm.) CNV was reduced in wild-type mice treated with neutralizing anti-C3a or -C5a antibodies (2 μg) compared with isotype control antibodies (*E*) or with C3a receptor antagonist or C5a receptor antagonist (1 μg) compared with control peptide or PBS (*F*). *, $P < 0.05$ compared with control IgGs (*E*) or control peptide (*F*).

(volume studies, three per eye; protein analyses/flow cytometry, 12 per eye) on both eyes of each animal to induce CNV, as described (14, 15, 42). CNV volumes were measured by scanning laser confocal microscope (TCS SP, Leica), as reported (14, 15, 42), with 0.5% FITC–*Griffonia simplicifolia* Isolectin B4 (Vector Laboratories) or 0.5% FITC–rat antibody against mouse CD31 (BD Biosciences). Volumes obtained by lectin and CD31 staining were highly correlated ($r^2 = 0.95$).

Complement Inhibition. The C3a receptor antagonist N^2-[(2,2-diphenylethoxy)acetyl]-L-arginine (43) and the C5a receptor antagonist AcF[OPdChaWR] (44) were synthesized as described (45, 46). A control peptide (IAVVQDWGHHRAT), synthesized as described (47), was used as control. Rat IgG2a antibody against mouse C3a (clone 3/11; HyCult biotechnology, Uden, The Netherlands) and rabbit polyclonal IgG antibody against a synthetic peptide constructed from the C-terminal region of rat C5a (48) were used to block C3a and C5a. Rabbit IgG (Jackson Immunoresearch) and rat IgG2a (Serotec) were used as controls. These reagents were injected into the vitreous humor of wild-type mice by using a 33-gauge double-caliber needle (Ito, Tokyo) immediately after laser injury, as described (42).

Immunohistochemistry. Mouse eyes were enucleated, snap-frozen in OCT compound, and cryosectioned. Frozen sections (10 μm), fixed in Histochoice MB (Amresco, Euclid, OH), and blocked with DakoCytomation Protein Block (DAKO) were incubated with rabbit antibody against mouse RPE65 (1:100; generous gift of T. M. Redmond, National Eye Institute, Bethesda) diluted in DakoCytomation Antibody Diluent (DAKO) and then with Alexa 488-conjugated goat antibody against rabbit IgG (Molecular Probes). We generated chicken antibodies against mouse C3 or C5 by immunizing hens with keyhole limpet hemocyanin-conjugated mouse C3 or C5 peptide (Aves Labs, Tigard, OR) and subsequently affinity-purified these polyclonal antibodies from the eggs by using C3 or C5 peptide coupled to agarose beads. Slides were incubated in chicken antibodies against mouse C3 (1:250) or C5 (1:5,000) and then with Cy3-conjugated donkey antibody against chicken IgG (Jackson Immunoresearch). Cell nuclei were stained with DAPI (1:25,000; Molecular Probes). Human eye sections, fixed in Histochoice MB and blocked in DakoCytomation Protein Block, were incubated with mouse monoclonal antibodies against human C3a (1:50) or C5a (1:25; HyCult biotechnology), which are specific to the neoepitopes generated upon complement activation, and then with biotinylated goat antibody against mouse IgG (1:1,000; Vector Laboratories). Immunostaining was developed by using Alkaline Phosphatase Vectastain ABC and Vector Blue Alkaline Phosphatase Substrate Kits (Vector Laboratories) according to the manufacturer's instructions. Endogenous alkaline phosphatase was blocked by using levamisole solution (Vector Laboratories). Specificity of staining was confirmed by omitting the primary antibodies.

Cell Culture. Human RPE cells isolated from donor eyes of patients with a normal fundus examination were cultured in DMEM (Invitrogen) containing 10% FBS, penicillin G (100 units/ml), streptomycin sulfate (0.1 mg/ml) (all from Sigma Aldrich) at 37°C under 10% CO_2 and 90% room air. Passage 2–4 cells were used for experiments upon attaining 80% or 100% confluence and stimulated with either C3a or C5a (50 ng/ml; Sigma Aldrich) in serum-free media. Supernatant fractions from the media were harvested at 8 h and analyzed for protein content.

ELISA. Cell culture supernatants were analyzed for total protein content by a modified Bradford assay (Bio-Rad) and for VEGF by ELISA kit (R & D Systems), at 450/570 nm (Emax, Molecular Devices). Normalized VEGF levels were calculated for each sample by dividing the VEGF ELISA outcome by the total protein determination for the same sample. For *in vivo* determination, the

RPE/choroid complex was sonicated in lysis buffer [20 mM imidazole HCl/10 mM KCl/1 mM $MgCl_2$/10 mM EGTA/1% Triton X-100/10 mM NaF/1 mM Na molybdate/1 mM EDTA with protease inhibitor (Sigma-Aldrich)] on ice for 15 min. VEGF levels in the supernatant were determined by ELISA and normalized to total protein as above. To detect C3a and C5a protein levels in the RPE/choroid lysates, we developed a sandwich ELISA (threshold of detection, 15.6 ng) by coating high-binding 96-well plates (Costar) with 1 μg/ml capture rat antibodies against mouse C3a (clone I87–1162) or C5a (clone I52–1486; BD Biosciences), which are specific to the neoepitopes generated upon complement activation, overnight at 4°C. Standards and unknowns were incubated in a sample buffer of 3% BSA in PBS at room temperature for 2 h. Detection was performed by using biotinylated rat antibodies (1 μg/ml) against mouse C3a (clone I87–419) and C5a (clone I52–278; BD Biosciences) followed by addition of streptavidin–horseradish peroxidase conjugate (BD Biosciences) and color development with TMB substrate (Pierce). C3a and C5a levels were normalized to total protein as above. Measurements were performed in masked fashion.

Flow Cytometry. Cell suspensions isolated from mouse RPE/choroids via collagenase D (20 units/ml; Roche Diagnostics) treatment were incubated in Fc block (0.5 mg/ml; BD Biosciences) for 15 min on ice; stained with Cy5-rat antibody against mouse F4/80 (1:30; Serotec), FITC–hamster antibody against mouse CD11c (1:100; Serotec), or PE-rat antibody against mouse Gr-1 (1:200; eBioscience, San Diego, CA); and subjected to FACS analysis (FACScalibur, BD Biosciences). Macrophages and neutrophils were defined as $F4/80^+CD11c^-$ and $Gr\text{-}1^+F4/80^-$ cells, respectively.

Statistics. ***Volume of CNV.*** Because the probability of each laser lesion developing CNV is influenced by the group to which it belongs (mouse, eye, and laser spot), the mean lesion volumes were compared by using a linear mixed model with a split plot repeated-measures design, as described (14, 15). The whole plot factor was the genetic group to which the animal belonged, whereas the split plot factor was the eye. Statistical significance was determined at the 0.05 level. Post hoc comparison of means was constructed with a Bonferroni adjustment for multiple comparisons.

Protein levels and flow cytometry. VEGF, C3a, and C5a levels and leukocyte numbers are represented as the mean ± SEM of at least three independent experiments and compared by using the Mann–Whitney U test. The null hypothesis was rejected at $P < 0.05$.

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