Local Production of the Alternative Pathway Component Factor B Is Sufficient to Promote Laser-Induced Choroidal Neovascularization

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PURPOSE. Complement factor B (CFB) is a required component of the alternative pathway (AP) of complement, and CFB polymorphisms are associated with age-related macular degeneration (AMD) risk. Complement factor B is made in the liver, but expression has also been detected in retina and retinal pigment epithelium (RPE)-choroid. We investigated whether production of CFB by the RPE can promote AP activation in mouse choroidal neovascularization (CNV).

METHODS. Transgenic mice expressing CFB under the RPE65 promoter were generated and crossed onto factor B-deficient (CFB-KO) mice. Biological activity was determined in vitro using RPE monolayers and in vivo using laser-induced CNV. Contribution of systemic CFB was investigated using CFB-KO reconstituted with CFB-sufficient serum.

RESULTS. Transgenic mice (CFB-tg) expressed CFB in RPE-choroid; no CFB was detected in serum. Cultured CFB-tg RPE monolayers secreted CFB apically and basally upon exposure to oxidative stress that was biologically active. Choroidal neovascularization sizes were comparable between wild-type and CFB-tg mice, but significantly increased when compared to lesions in CFB-KO mice. Injections of CFB-sufficient serum into CFB-KO mice resulted in partial reconstitution of systemic AP activity and significantly increased CNV size.

CONCLUSIONS. Mouse RPE cells express and secrete CFB sufficient to promote RPE damage and CNV. This further supports that local complement production may regulate disease processes; however, the reconstitution experiments suggest that additional components may be sequestered from the bloodstream. Understanding the process of ocular complement production and regulation will further our understanding of the AMD disease process and the requirements of a complement-based therapeutic.

Keywords: alternative complement pathway, complement factor B, RPE-specific transgenic mouse, choroidal neovascularization, age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness for Americans 60 years of age and older. Early AMD is characterized predominantly by the presence of extracellular deposits between Bruch's membrane (BrM) and the retinal pigment epithelium (RPE), with little consequence to vision. Late AMD, on the other hand, is divided into two forms: geographic atrophy (GA) associated with RPE cell loss, and choroidal neovascularization (CNV) associated with leakage of the new vessels and acute hemorrhage. Irrespective of the form of the disease, the affected tissues include the choriocapillaris, BrM, and the RPE, leading to dysfunction or death of the underlying photoreceptors. Despite the difference in pathology between the two forms of AMD, histologic and genetic studies have indicated that an overactive complement system might represent a fundamental component of the disease process driving the inflammatory response. In particular, complement deposition has been associated with the pathological structures of both GA and CNV¹; genetic variations

in the complement inhibitory protein factor H (CFH),^{2–6} the complement activation proteins complement factor B (CFB) and the complement components 2 (C2) and 3 (C3)^{7,8} are associated with AMD in general; and progression from early to late AMD has been associated with genetic variations in some of the same complement genes.⁹

The complement system is part of the innate and adaptive immune system, with differences in biological effects ranging from cell lysis (lytic activation), attraction of leukocytes (chemotaxis), and opsonization of membranes (cell removal) to activation of intracellular signaling cascades (sublytic activation) mediated by different effector molecules. The effector molecules are all generated by the different steps in the common terminal pathway. Irrespective of the downstream effects, the complement system can be triggered by three different pathways, the classical (CP), lectin (LP), and alternative (AP) pathways, all of which have their specific pattern-recognition receptors that trigger complement activa-

tion on membranes that present their respective ligands. The AP, however, is the exception. While the AP can be activated by ligands, it can also be activated spontaneously, and it serves an important function as an amplification loop. As part of the amplification loop, C3b deposited onto cell membranes by either the classical or the lectin pathway is recognized by the AP activator protein CFB, which together with complement factor D (CFD) and properdin (complement factor P; CFP) generates the AP C3 convertase to generate more C3b opsonins. This cycle can be interrupted by the AP inhibitor, CFH. Complement factor B is a glycosylated, single-chain polypeptide with a molecular weight of 93 kDa and approximate plasma concentration of 200 µg/mL. In the human, the gene for *CFB* is located on chromosome 6; in the mouse, on chromosome 17. In both species, the *CFB* gene lies in close proximity to *C2*. Specific effects for *CFB* when compared to *C2* have been identified in the mouse,^{10,11} but could not be dissociated in the human.¹²

Most of the soluble complement components are generated by the liver, although mRNA and protein expression of several complement components have been identified elsewhere, including the RPE.¹³ This dominant role of the liver in the production of complement has triggered the question whether global or local production of complement is relevant for progression of AMD. Evidence exists that the retina and RPE-choroid in AMD patients,¹ or RPE-choroid of animals with CNV, have increased mRNA and protein of complement components,¹⁴ providing evidence for a possible importance of local complement production in disease. Liver transplantation studies have supported this hypothesis. Lotery and colleagues^{15(p1612)} have shown that AMD is associated with liver recipient *CFH* genotype, leading them to conclude that “local intraocular complement activity is of greater importance in AMD pathogenesis.” Here we used mice expressing CFB uniquely in a RPE cell-specific manner to demonstrate that local CFB expression is sufficient to drive pathology in the eye. However, in the absence of locally produced CFB (CFB-KO), systemic CFB derived from wild-type serum injected via tail vein can drive pathology.

MATERIALS AND METHODS

Animals

CFB-KO mice on a C57BL/6J background that were originally generated by Matsumoto¹⁰ were generously donated to us by VM Holers (University of Colorado Health Science Center, Denver, CO, USA). C57BL/6J mice were generated from breeding pairs (stock number 000664; Jackson Laboratories, Bar Harbor, ME, USA). The CFB-KO strain has previously been confirmed as the 6J substrain (i.e., negative for the RD8 locus) by PCR using published primers.¹⁶ Animals were housed under a 12:12 hour light:dark cycle with access to food and water ad libitum. All 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.

Generation of RPE-Specific CFB-Transgenic (CFB-tg) Animals

Mouse factor B cDNA was isolated from the pBluescript-KS vector (generously provided by B Winston, University of Calgary) by *Xba*I and *Nof*I digestions and ligated into the *Xba*I and *Nof*I sites of the pGEM-mAlb-SVpA vector (generously provided by TJ Liang, now at the National Institute of Diabetes and Digestive and Kidney Diseases, Washington, DC, USA; referenced in Shiota et

al.¹⁷) containing the murine albumin promoter (mAlb) and enhancer with SV40-polyA cassette (SVpA) to generate pGEM-mAlb-SVpA-CFB. Positive transformants were verified by sequencing. The mAlb-SVpA-CFB fragment was then isolated from the pGEM-mAlb-SVpA-CFB vector by digesting with *Bsa*AI and *Nsi*I. The *Nsi*I site was filled in and ligated into the pWhere vector (InvivoGen, San Diego, CA, USA) to generate pWhere-mAlb-SVpA-CFB. Positive transformants were verified by sequencing. The mAlb promoter/enhancer sequences were then removed from the pWhere-mAlb-SVpA-CFB vector by digesting with *Xba*I. The *Xba*I ends were filled in to blunt, and the pWhere-SVpA-CFB fragment was isolated. The mouse RPE65 promoter was isolated from the pCH126-RPE65-TR4 plasmid (kindly provided by Ana Boulanger¹⁸) and ligated into the pWhere-SVpA-CFB vector to generate pWhere-RPE65-SVpA-CFB. Positive transformants in the forward orientation were verified by sequencing, and CFB expression was confirmed in transiently transfected D407 RPE cells. pWhere-RPE65-CFB was digested with *Pac*I, and the RPE65-CFB transgene construct flanked by H19 insulators was isolated by agarose gel electrophoresis using the Qiaex gel extraction kit (Qiagen, Valencia, CA, USA) per the manufacturer's instructions. The isolated transgene construct was provided to the transgenic facility at the Medical University of South Carolina (MUSC; in the public domain at <http://clinicaldepartments.musc.edu/transgenicmouse>) for the generation of transgenic animals using blastocysts generated from C57BL/6J mice (stock number 000664; Jackson Laboratories). Lines positive for the *CFB* transgene were identified by PCR genotyping. Select mice (see Results section for criteria for selection) carrying the *CFB* transgene were crossed onto the CFB-KO strain to generate mice that express CFB only in the RPE (the final cross is referred to as CFB-tg).

PCR Genotyping

Polymerase chain reaction primers to determine the presence of the transgene and the *CFB* genotype were as follows: CFB transgene: 5'-GCTGGTACCTCTTATGC-3' and 5'-GCTCCATT CATCAGTTCCA-3'; *CFB* genotype: 5'-CCGAAGCATTCC TATCCTCC-3', 5'-CAGATGGGCTGACCGCTTCC-3', and 5'-CTAGTCTTGTCTGCTTTCTCC-3'.¹⁹ Reactions for both genes were initially denatured at 95°C for 5 minutes followed by their respective amplification cycles: *CFB* transgene—40 cycles at 94°C for 60 seconds, 60°C for 10 seconds, 72°C for 10 seconds, and a final extension at 72°C for 10 minutes; *CFB* genotype—38 cycles at 95°C for 60 seconds, 54°C for 60 seconds, 72°C for 60 seconds, and a final extension at 72°C for 10 minutes. Amplified DNA samples were run with an aliquot of GulereneR 100 bp Plus DNA ladder (SM0323; Fermentas, Hanover, MD, USA). Presence of the amplicon for the *CFB* transgene was assessed; amplicon size for the *CFB* wild-type and knockout allele is 748 and 610 bp, respectively.

CNV Lesions

For CNV lesions, 3- to 4-month-old mice were anesthetized (xylazine and ketamine, 20 and 80 mg/kg, respectively) and pupils dilated (2.5% phenylephrine HCl and 1% atropine sulfate). Argon laser photocoagulation (532 nm, 100-µm spot size, 0.1-second duration, 100 mW) was used to generate four laser spots in each eye surrounding the optic nerve (positioned at 0°, 90°, 180°, and 270°) using a handheld coverslip as a contact lens. Bubble formation at the laser spot indicated the rupture of BrM.^{20,21} Any laser spots not creating a bubble, or those that accidentally ruptured a blood vessel, were indicated on each animal's data sheet and subsequently excluded from size determination by InterCellular Adhesion Molecule 2 (ICAM2) staining or optical coherence tomography (OCT)

analysis. Choroidal neovascularization lesions (and subsequent follow-up analyses) were performed in groups of three or four per genotype or treatment, and data were combined for the final analysis and presentation of the data.

OCT Analysis

Choroidal neovascularization size was determined in vivo using OCT. On day 5 post laser treatment, OCT was performed using a spectral-domain (SD)-OCT BiopTigen Spectral Domain Ophthalmic Imaging System (BiopTigen, Inc., Durham, NC, USA). Prior to imaging, mice were anesthetized and eyes were hydrated with normal saline. Using the BiopTigen InVivoVue software, rectangular volume scans set at 1.6×1.6 mm, consisting of 100 B-scans (1000 A-scans per B scan), were performed. Cross-sectional area of the lesions was used to measure CNV size. As described by Giani et al.,²² 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-BrM rupture with the axial interval positioned at the level of the RPE/choroid complex. 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, USA; available at <http://rsb.info.nih.gov/ij/index.html> [in the public domain]) was used to measure the area around the hyporeflective spot produced in the fundus image. Data were expressed as mean $\pm$ SEM.

Immunofluorescence

On day 6 or 7 post laser treatment (see individual experiments), eyes were enucleated and fixed in 4% paraformaldehyde. Flat-mount preparations of RPE-choroid-sclera (referred to as RPE-choroid) were stained for CD102 (also referred to as ICAM2; 0.5 mg/mL at 1:200; BD Pharmingen, San Diego, CA, USA) to determine CNV lesion sizes or C5b-9 (5 mg/mL at 1:200; Abcam, Cambridge, MA, USA) to analyze membrane attack complex (MAC) deposition, and visualized using Alexa 488-coupled secondary antibodies (2 mg/mL at 1:400; Invitrogen, Grand Island, NY, USA). Imaging was performed by confocal microscopy (Leica TCS SP2 AOBs; Leica, Bannockburn, IL, USA) followed by Alexa 488-coupled secondary antibodies (2 mg/mL at 1:400; Invitrogen, Grand Island, NY, USA). For CD102 staining, fluorescence measurements, taken from 2- μ m sections using confocal microscopy (40 $\times$ oil lens), were used for size determination. A Z-stack of images through the entire depth of the CNV lesion was obtained, using the same laser intensity setting for all experiments. For each slice the overall fluorescence was determined to obtain pixel intensity against depth, from which the area under the curve (indirect volume measurement) was calculated as described by us previously.²¹ A Z-stack outside the region of interest was collected for background subtraction. For C5b-9 analyses, the overall fluorescence was quantified using the stack profile setting of the Leica software to obtain the intensity mean amplitude of the lesion, and background was subtracted. Data were expressed as mean $\pm$ SEM.

Electroretinography

Retinal function of the mice was measured under scotopic conditions to assess rod function using electroretinography (ERG). Electroretinography recordings and data analyses were performed as previously described¹⁶ using the EPIC-2000 system (LKC Technologies, Inc., Gaithersburg, MD, USA). In short, mice were dark-adapted overnight prior to testing. Under scotopic conditions, responses to 10- μ s single flashes of white light (maximum intensity of 2.48 cd-s/m²) between -40

and 0 dB of attenuation were measured. Peak a-wave amplitude was measured from baseline to the initial negative-going voltage, whereas peak b-wave amplitude was measured from the trough of the a-wave to the peak of the positive b-wave.²³

Histology

Paraffin sections were prepared using a previously published protocol.²⁴ In short, enucleated eyes were fixed by immersion in 4% paraformaldehyde. Eyes were then dehydrated and transversely oriented in paraffin. Sections (7 mm) were cut, dried on poly-L-lysine-coated glass slides, dewaxed, rehydrated for toluidine blue staining, coverslipped using distyrene plasticizer xylene (DPX) medium, and photographed using a Zeiss (Dublin, CA, USA) microscope with a photographic attachment. The number of rows of photoreceptor nuclei was counted in the superior and inferior retina (each within 350 μ m of the optic nerve head and the ora serrata). Five measurements were made per field, which were averaged to provide a single value for each area, and the two area values were averaged to give a value for the entire retina. Each group consisted of four eyes.

Primary Mouse RPE Cell Cultures

Primary mouse RPE cells were prepared following the published protocol by Gibbs and colleagues.²⁵ Pups (10 days old; P10) were anesthetized on ice. Eyes were removed in lots of 20, washed in Dulbecco's modified Eagle's medium (DMEM) containing high glucose, and incubated with 5 mL 2% (wt/vol) dispase (DMEM, 45 minutes at 37°C). Dispase activity was quenched in growth medium (GM; DMEM [high glucose], 10% bovine fetal calf serum [FBS], 1% penicillin/streptomycin, 2.5 mM L-glutamine, and 1 $\times$ MEM nonessential amino acids). Posterior eyecups were dissected and incubated in GM for 20 minutes at 37°C, which facilitates the removal of the neural retina. Intact sheets of RPE were then peeled off and washed with GM (3 $\times$), followed by Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (2 $\times$) (5 mM KCl/0.5 mM KH₂PO₄/4 mM NaHCO₃/150 mM NaCl/3 mM Na₂HPO₄·7H₂O/5 mM glucose/0.05 mM phenol red, pH 7.4). Retinal pigment epithelium sheets were triturated using a Pasteur pipette, cells sedimented by centrifugation (200g, 5 minutes), resuspended in GM, and plated on transwell plates (12 well; 25,000 cells per well). Cells were cultured until confluent colonies were formed, at which point the cells were subcultured onto transwell plates for final experiments (see next section for transepithelial resistance measurement methods).

Transepithelial Resistance (TER) Measurements

Mouse RPE cells were grown as mature monolayers on six-well transwell inserts (0.4- μ m PET; Corning, Corning, NY, USA) in the presence of 1% FBS for 2 to 3 weeks as described previously for a human RPE cell line, seeding 50,000 cells per well.²⁶ For the final 2 to 3 days prior to the experiments, cells were changed to serum-free media. Complement activation was induced as reported previously,²⁶ exposing cells to 0.5 mM H₂O₂ in the presence of 10% normal human serum (NHS). Since we have shown previously that sublytic complement activation results in VEGF release, which in turn reduces barrier function,²⁶ TER measurements are a convenient readout for the level of activity in the complement cascade.²⁷ Transepithelial resistance was determined by measuring the resistance across the monolayer with an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL, USA). The value for cell monolayers was determined by subtracting the TER for filters without cells, and percentage was calculated using the starting value as the reference. To determine whether cells secrete sufficient CFB to drive complement activation, TER measurements were performed in

FIGURE 1. Generation of RPE-specific factor B transgenic mice. **(A)** Cartoon of the transgene assembled in the pWhere vector: Complement factor B (*CFB*) expression is driven from the promoter of the *RPE65* gene. **(B)** Supernatants of transiently transfected D407 RPE cells (pWhere-RPE65-SVpA-CFB [RPE65-CFB] versus pWhere- β -actin-GFP [RPE65-GFP]) were probed for full-length CFB (93,000 Da) using two different amounts of supernatant (5 and 10 μ L out of 2 mL). Complement factor B levels were quantified using densitometry. Complement factor B levels in the supernatants were elevated by ~40% in pWhere-RPE65-SVpA-CFB-transformed cells when compared to those transformed by the control GFP vector.

the presence of 0.5 mM H_2O_2 and 10% CFB-depleted human serum (Comptech, Tyler, TX, USA). For reconstitution experiments, we used CFB-depleted human serum rather than serum from CFB-KO mice to increase the dynamic range of the response, since mouse cells tend to be less susceptible to mouse complement components. Each condition was examined in duplicate three independent times.

Quantitative RT-PCR (QRT-PCR)

Tail DNA from genotyping samples was used for QRT-PCR to determine the relative amount of transgene present in the different transgenic mouse lines. The same primers as for genotyping were utilized and compared, relative to a known plasmid concentration (see Fig. 1B). To assess mRNA levels for genes of interest, RPE-choroid-sclera (referred to as RPE-choroid) fractions were isolated from control and CNV eyes and stored at -80°C until used. For RNA extraction, three to five samples were pooled to obtain good-quality RNA, to reduce sample variability, and to reduce the number of experiments needed to generate reliable data.²⁸ Primers used are listed in Table 1. For either approach, quantitative RT-PCR analyses were performed as described in detail previously.²⁹ Real-time PCR analyses were performed in triplicate in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using standard cycling conditions. Quantitative values were obtained by the cycle number. Significance required both a ± 2 -fold difference and $P < 0.05$ between the relevant comparisons.

Western Blotting

Retinal pigment epithelium-choroid-sclera fractions (referred to as RPE-choroid) or RPE cell supernatants were collected. Some of the samples were concentrated using concentrators with a 10-kDa cutoff (Amicon, EMD Millipore Corporation, Billerica, MA, USA). Samples were separated by electrophoresis on a 10% Bis-Tris polyacrylamide gel (Invitrogen), and proteins were transferred to a nitrocellulose membrane. The membrane was probed with polyclonal antibody to CFB (ICN Pharmaceuticals, Costa Mesa, CA, USA) and antibody binding was visualized using a chemiluminescence detection kit (Amersham Life Science,

Pittsburgh, PA, USA). Some of the membranes were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Life Technologies, Grand Island, NY, USA). The intensity of the bands was quantified using the Alpha Innotech Fluorchem 9900 imaging system running Alpha Ease FC software version 3.3 (Alpha Innotech, San Leandro, CA, USA).

Reconstitution of Systemic Alternative Pathway

Wild-type mouse serum was collected from adult mice and stored at -80°C until used. CFB $^{-/-}$ mice were injected intravenously (heat-dilated tail vein) four times with 200 μ L serum each on days -2 , 0 , 2 , and 4 , with day 0 being the day of laser CNV induction.

C3a ELISA

Microtiter (Immulon2; Dynatech Laboratories, Chantilly, VA, USA) plates were first coated with 5 μ g/mL purified rat anti-mouse C3a capture antibody (BD Biosciences, San Jose, CA, USA) overnight at 4°C . The plates were then washed three times with PBS and blocked with 2% BSA in PBS for 1 hour at room temperature, followed by exposure to the antigen (mouse serum) for 1 hour at 37°C . Zymosan-activated wild-type mouse serum (60 μ L/mL serum, incubated at 37°C for 30 minutes) dilutions were used as standards. The plates were again washed and incubated with biotin-conjugated anti-mouse C3a (BD Biosciences) followed by alkaline phosphatase-conjugated streptavidin and color development using pNPP phosphatase substrate (KPL, Inc., Gaithersburg, MD, USA).

Zymosan Assay

Activity in the AP was assessed using the zymosan bead assay. Zymosan beads (50-mg beads in 10 mL 0.15 NaCl; Sigma-Aldrich Corp., St. Louis, MO, USA) were prepared by boiling (60 minutes), centrifuged at 10,000g for 1 minute, and resuspended in 1 mL water, aliquoted, and stored at -80°C . Ninety-six-well plates were coated with 2×10^8 particles/mL zymosan (1:10 dilution of the stock) in coating buffer overnight at 4°C . The plates were washed three times with PBS and blocked with 2% BSA in PBS for 1 hour at room temperature. A dilution series of wild-type serum and samples was prepared in triplicate in PBS/EGTA/Mg $^{2+}$ buffer and added to the zymosan-coated plates, then incubated for 45 minutes at 37°C . The plate was again washed with PBS and incubated with horseradish-peroxidase-conjugated anti-mouse C3 (1:2000 in PBS; ICL, Inc., Portland, OR, USA), followed by color development using Turbo-TMB ELISA (Pierce, Thermo Scientific, Rockford, IL, USA).

Statistics

For OCT, image analysis, and histologic counts, samples were randomized. For data consisting of multiple groups, repeated measures ANOVA or one-way ANOVA followed by Fisher post hoc test ($P < 0.05$) was used (Statview; Scientific Computing, Cary, NC, USA); single comparisons were examined by *t*-test analysis ($P < 0.05$); QRT-PCR was examined by Z-test analysis (Excel; Microsoft, Redmond, WA, USA) ($P < 0.05$).

RESULTS

Generation of RPE-Specific Factor B Transgenic (CFB-tg) Mice

Transgenic mice were generated, expressing mouse factor B cDNA under the RPE-specific promoter from the mouse

TABLE 1. Quantitative RT-PCR Primer Sequences

Gene Name	Symbol	Forward Primer	Reverse Primer
Retinal pigment epithelium 65	<i>Rpe65</i>	5'-TTCTGAGTGTGGTGGTGAGC-3'	5'-AGTCCATGGAAGGTACACAGG-3'
Complement component 3	<i>C3</i>	5'-TGAACAAGGGGAGCTCCT-3'	5'-CAGGCGAAATGAAGGAATA-3'
Hemolytic complement*	<i>Hc</i>	5'-TAAACAAGGGGTGGTGAGC-3'	5'-CACCTGTCCAAGCACTCTCA-3'
Complement component 9	<i>C9</i>	5'-AGCATTTTAGCCCTTCGGACA-3'	5'-CCTGCCCTGTCTCACACTGAA-3'
Complement factor D (adipsin)	<i>Cfd</i>	5'-CAAGCGATGGTATGATGTC-3'	5'-CTCGTATTGCAAGGGTAGGG-3'
Complement component factor H	<i>Cfb</i>	5'-TGGACTTCTTGTGGACCTC-3'	5'-CCATCAATTCCAAGCCTGT-3'
CD55 antigen	<i>CD55</i>	5'-TAATGCCGAGGGGAAAGTGAC-3'	5'-GTGGACTGCTCCATTTGTCCT-3'
CD59a antigen	<i>CD59a</i>	5'-CTGCTTCTGGCTGTGTTCTG-3'	5'-TCTGGTCAGGAGAGCAAGT-3'
Clusterin	<i>Clu</i>	5'-GCCTTGGCAAGAGATAAGCATT-3'	5'-GGACTGTTCCGTGTCAACTGAA-3'
Ceruloplasmin	<i>Cp</i>	5'-ATAATCAACCTGTTCCCTGCCA-3'	5'-GCAACCCAGCTTTCAGATGGT-3'
Lysozyme 1	<i>lyz 1</i>	5'-CCAGTGTCACGAGGCATTTA-3'	5'-TGATAACAGGCTCATCTGTCTCA-3'
Microtubule-associated protein 1 light chain 3 α	<i>Map1lc3a</i>	5'-CGGCTTCTGTACATGGTTT-3'	5'-ATGTGGTGCCTACGTTCTC-3'
Serine (or cysteine) peptidase inhibitor, clade F, member 1†	<i>Serpinf1</i>	5'-CCTCAGCATCCTTCTCCTTG-3'	5'-TGACATCATGGGACTCTCA-3'
Vascular endothelial growth factor A	<i>Vegfa</i>	5'-CTGGACCCCTGGCTTACTGC-3'	5'-TGAACTTGATCACTTCATGGGACT-3'
Hypoxia inducible factor 1, alpha subunit	<i>Hif1a</i>	5'-GAAATGGCCCACTGAGAAAA-3'	5'-CTTCCACGTTGCTGACTTGA-3'
Mitochondrially encoded cytochrome c oxidase I	<i>mt-Co1</i>	5'-TGACTTGC AACCCCTACACGGAGGTA-3'	5'-TCGTGAAGCACGATGTCAAGGGATG-3'
Dynamin 1-like	<i>Dnm1l</i>	5'-GGCGCTGATCCCGGTCATCAA-3'	5'-GAGCTCTTCCCACTGCTCTGCG-3'
Heme oxygenase (decycling) 1	<i>Hmox1</i>	5'-AGGCTAAGACCCGCTTCTCTGCT-3'	5'-GGCAGTATCTTGCAACCAGGCTAGC-3'
Mitofusin 1	<i>Mfn1</i>	5'-GGAGGATGTGCGCAGACAGCA-3'	5'-CCGATTCGAGCTTCCGACGG-3'
NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	<i>Ndufb8</i>	5'-AGGAATCGTGTGGACACGTACCT-3'	5'-CCACAGGCTGGTAGGAAGGGAA-3'
6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	<i>Pfkfb2</i>	5'-GGAACAGCTTTACGCCCTCTG-3'	5'-TTCTTGATGTCCAGAGCAC-3'
Actin, β	<i>Actb</i>	5'-AGCTGAGAGGGGAAATCGTGC-3'	5'-ACCAGACAGCACTGTGTTG-3'

Primers were designed to probe six categories of genes: RPE cell health, complement activation, control of angiogenesis, oxidative stress, autophagy, and mitochondrial function/metabolism.
* Complement component 5, C5.
† Pigment epithelium-derived factor, Pedf.

FIGURE 2. Genotyping and quantification of transgene expression. (A) Nine pups that were found to be positive for the *CFB* transgene were analyzed for their relative level of transgene expression using quantitative RT-PCR analysis. Their respective delta CT levels were plotted against delta CT levels of known pWhere-RPE65-SVpA-*CFB* plasmid concentrations. Mouse #J (high expresser) and mouse #L (low expresser) are indicated. These two founders were used to generate *CFB* transgenic mice on a *CFB*-KO background. (B) Gels to identify offspring that expressed the *CFB* transgene (bottom gel, CFB-tg) and lacked systemic *CFB* (top gel; wild type [WT] or knockout [KO]) are presented. Tail DNA from four mice (lanes 1–4) are presented, documenting a WT mouse lacking the CFB-tg (lane 1), a mouse heterozygous for *CFB* carrying the CFB-tg (lane 2), a KO mouse for *CFB* carrying the CFB-tg (lane 3), and a WT mouse for *CFB* carrying the CFB-tg (lane 4). (C) RPE-choroid extracts from the #J line were found to contain CFB detectable by Western blotting. No CFB-positive band was present in the samples from the *CFB*-KO mice. Note that *CFB*-KO and *CFB*-tg samples were concentrated ~10-fold as shown by GAPDH.

RPE65 gene. The RPE65-promoter-*CFB* construct was assembled in the pWhere vector to generate pWhere-RPE65-SVpA-*CFB* (see Fig. 1A), and positive transformants were verified by sequencing. This vector, as well as the pWhere- β -actin-GFP control vector, was used to transiently transfect D407 RPE cells, and *CFB* protein expression and secretion were confirmed by Western blotting. Both cell extracts (data not shown) and supernatants (Fig. 1B) contained full-length *CFB* of 93,000 Da as determined by Western blotting, which was elevated by ~40% in pWhere-RPE65-SVpA-*CFB*-transformed cells when compared to those transformed by the control GFP vector.

After linearization of the expression vector with *PacI*, the RPE65-SVpA-*CFB* construct flanked by H19 insulators was isolated and used for C57BL/6J blastocyst injections at the MUSC Transgenic Mouse Core Facility. Nine pups were identified by PCR genotyping that were positive for the *CFB* transgene, and backcrossed to C57BL/6J mice to establish lines. The relative levels of the transgene in the nine resulting lines, when compared to a known plasmid concentration of

the transgene, were identified based on cycle number using QRT-PCR (Fig. 2A). Two lines were selected, one with high levels of transgene expression (#J) and one with low levels (#L), and crossed onto the *CFB*-KO strain to generate mice that expressed *CFB* only in the RPE. The resulting mice were negative for *CFB*, but positive for the *CFB*-tg (see lane 3 in Fig. 2B as an example).

RPE-Derived CFB Is Functionally Active

Retinal pigment epithelium–choroid extracts from the #J-line (3 months of age; unstressed RPE-choroid) were found to contain measurable amounts of *CFB* (Fig. 2C) as identified by Western blotting, whereas *CFB* was not detectable in the #L-line (data not shown). Likewise, *CFB* was absent in tissues isolated from the *CFB*-KO mice. Since the *CFB* measurements were obtained from RPE-choroid samples combined, and choroid appears to be the predominant local source of *CFB* when compared to the RPE,³⁰ the percentages of wild-type levels of *CFB* in the *CFB*-tg tissues were not quantified. Finally, *CFB* could not be detected in the serum of the #J-line animals (data not shown), suggesting that the levels of *CFB* secreted from the RPE were too low to be detected in the bloodstream. Any further experiments were performed in the #J-line animals only, which are henceforth referred to as *CFB* transgenic mice or *CFB*-tg.

To test whether *CFB* present in the RPE of the *CFB*-tg mice was biologically active, we performed an *in vitro* assay of complement activation. We have shown previously that in monolayers of primary human RPE,³¹ as well as in immortalized human RPE cells (ARPE-19 cells),²⁶ combined treatment with H_2O_2 (to induce oxidative stress) and NHS (as a source of complement) disrupts barrier function as determined by TER measurements, whereas H_2O_2 or NHS alone had no effect. The effect on TER is due to the transient formation of the MAC²⁶ and triggered by the lectin pathway,²⁷ as shown by depletion and reconstitution experiments. Importantly for this experiment, loss of TER by the combined treatment with 0.5 mM H_2O_2 and 10% NHS requires the AP amplification loop, since it can be blocked completely with the AP inhibitor CR2-fH.²⁶ Primary mouse RPE cells were isolated from wild-type mice as reported²⁵ and grown as monolayers on transwell plates until stable TER values of 80 to 100 Ωcm^2 were achieved (typically within 3–4 weeks after reaching confluency). Exposure of monolayers to 0.5 mM H_2O_2 and 10% NHS resulted in the reduction of TER by $48\% \pm 4\%$ in mouse RPE, which is similar to that seen in ARPE-19 cells in response to H_2O_2 and NHS.²⁶

After having confirmed that mouse RPE monolayers are susceptible to complement attack similarly to human RPE, we used this culture system to determine whether wild-type RPE monolayers secrete sufficient amounts of *CFB* to drive complement activation under oxidative stress conditions. First, to determine the polarity of complement *CFB* secretion as well as the polarity of susceptibility to complement attack, stable monolayers grown on transwell plates were stimulated either apically or basally once a day for 2 days using 0.5 mM H_2O_2 to generate oxidative stress, without changing the media between treatments. This allowed for the accumulation of secreted proteins in the supernatants and confirmed the presence of *CFB* in both the apical and basal supernatant (Fig. 3A). In comparison, supernatants from untreated wild-type cells (labeled as controls in Figs. 3A, 3B) contained no detectable levels of *CFB*. The addition of H_2O_2 + *CFB*-depleted human serum to the accumulated supernatant resulted in a significant reduction in TER of both apically and basally treated cells when compared to control cells (in percent reduction from baseline: 15.4 ± 1.03 and 10.6 ± 1.03 , respectively; $P = 0.02$). Since apical effects of complement

FIGURE 3. Complement factor B expressed in transgenic mice is functionally active in vitro. (A) Mouse RPE monolayers derived from 10-day-old pups were grown on transwell plates until they reached stable transepithelial resistance (TER). Treatment of monolayers with 0.5 mM H_2O_2 two times (every 24 hours) resulted in the secretion of CFB protein both apically (Ap) and basally (Ba) from wild-type and CFB-tg, but not from CFB-KO mice. Note that all samples were concentrated equally. Controls represent supernatants from untreated wild-type cells and demonstrate that unchallenged cells secrete undetectable levels of CFB. (B) Retinal pigment epithelium monolayers grown from wild-type, CFB-tg, and CFB-KO pups were treated with H_2O_2 as in (A) to accumulate CFB in the supernatant, followed by apical exposure to 0.5 mM H_2O_2 + 10% CFB-depleted serum. In wild-type cultures, CFB accumulation was sufficient to partially reconstitute the CFB-depleted serum, resulting in a significant reduction in TER, as did supernatant from the CFB-tg animals. Supernatants from CFB-KO mice and unchallenged wild-type mice were used as negative controls. Data are expressed as mean $\pm$ SEM ($n = 3$ per condition).

activation on TER appeared to be more pronounced, all subsequent experiments were performed using apical applications only. Increased susceptibility of RPE cells to apical when compared to basal complement attack has already been shown by us using human RPE cells.²⁶

Second, RPE monolayers derived from wild-type, CFB-tg, and CFB-KO RPE cultures were treated with 0.5 mM H_2O_2 as described above to trigger the release and accumulation of CFB in the supernatant (Fig. 3A). Complement factor B released from the cultured CFB-tg mouse RPE after H_2O_2 stimulation could also be found in the apical and basal compartment, and the accumulated levels of CFB were sufficient to drive

complement activation. Specifically, we tested whether the supernatant containing secreted CFB could reconstitute CFB-depleted human serum using TER as the readout. Transepithelial resistance measurements in monolayers after apical addition of H_2O_2 + CFB-depleted human serum revealed significant levels of TER reduction in wild-type and CFB-tg cells (17.4 ± 0.80 vs. 14.3 ± 0.30 , $P = 0.02$). In comparison, supernatants from CFB-KO cultures resulted in minimal TER reduction (5.7 ± 0.11), levels that were indistinguishable from those in untreated control cells (2.9 ± 0.97 ; $P = 0.2$) (Fig. 3B). With all this taken together, the RPE from CFB-tg mice generates biologically active CFB.

Characterization of CFB Transgenic (CFB-tg) Mouse Retina Structure and Function

Retinal structure was assessed in horizontal sections of 3- to 4-month-old eyes stained with toluidine blue (Figs. 4A–C). Representative images were taken from the ventral retina within 100 μ m of the optic nerve. Comparable thickness of retinal layers was observed between the three genotypes (Fig. 4D), and the RPE had a normal appearance based on pigmentation and spacing of the nuclei.

Retinal function was assessed by single-flash, dark-adapted ERG to assess rod function. The CFB-tg mice were compared against wild-type and CFB-KO mice at 4 months of age (Fig. 5). Using repeated measures ANOVA over the different light intensities, we determined a significant difference between the groups and established a genotype-by-light intensity interaction for ERG a-waves (genotype: $P = 0.01$; genotype $\times$ amplitude: $P < 0.0001$) and b-waves (genotype: $P = 0.007$; genotype $\times$ amplitude: $P = 0.04$). A Fisher post hoc protected least significant difference (PLSD) test demonstrated that both CFB-tg mice and CFB-KO mice were different from the wild-type mice for both a- ($P < 0.001$) and b-waves ($P < 0.005$), but that CFB-tg and CFB-KO mice were not different from each other ($P > 0.5$). Likewise, the b- to a-wave ratios in the ERG plateau phase (brightest three light intensities; -10 , -6 , and 0 dB) were not different between the three different groups (genotype: $P = 0.3$; genotype $\times$ ratio: $P = 0.5$).

RPE-Derived CFB Promotes Choroidal Neovascularization

Activation of the AP and an associated inflammatory response have been shown to be involved in the development of CNV in mice and humans. In the mouse model of CNV, eliminating CFB by gene knockout²¹ or siRNA³² or inhibiting the AP using CR2-fH, a novel targeted inhibitor that is specific for the AP, all reduced the size of the lesions. And in patients, genetic

FIGURE 4. Retina structure of CFB transgenic (CFB-tg) mice. (A–C) No apparent structural difference in toluidine blue-stained paraffin sections between genotypes (wild-type, CFB-tg, and CFB-KO mice) at 4 months of age. RPE, retinal pigment epithelium; OS, (photoreceptor) outer segments; IS, (photoreceptor) inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; RGC, retinal ganglion cell layer. (D) Rows of photoreceptor nuclei were counted in the superior and inferior retina, and the two area values were averaged to give a value for the entire retina. There was no difference in the number of rows of nuclei in the ONL between the three genotypes ($n = 4$ per condition).

FIGURE 5. Retina function of CFB transgenic (CFB-tg) mice. Retina function was compared between age-matched wild-type, CFB-tg, and CFB-KO mice using dark-adapted ERG recordings (single-flash ERG recordings; max light intensity of 2.48 photopic $cd \cdot s/m^2$). A- and b-wave amplitudes revealed a significant reduction in amplitude in CFB-tg mice when compared to wild-type mice. CFB-tg responses were indistinguishable from those recorded from age-matched CFB-KO mice. Data are expressed as mean $\pm$ SEM ($n = 11$ –14 animals per genotype). Statistical significance as determined by repeated measures ANOVA is indicated for the range of light intensities included in the analysis.

polymorphisms in CFB are related to prevalence of advanced AMD⁹ or the risk of CNV.³³

The mouse model of CNV is triggered by the generation of reproducible lesions by laser photocoagulation of RPE/BrM, which results in VEGF-dependent vessel growth through the RPE/BrM and into the subretinal space (see, e.g., Nozaki et al.,²⁰ Espinosa-Heidmann,³⁴ and Bora et al.³⁵). The development of CNV following laser photocoagulation was assessed in

three cohorts of mice (C57BL/6J, CFB-KO, and CFB-tg) at 3 months of age. On day 5 after CNV induction, CNV size was measured using OCT (Figs. 6E–G); 7 days after CNV induction, mice were euthanized and CNV lesions measured histologically by CD102 staining of RPE-choroid flat mounts (Figs. 6A–C). These two methods were chosen for their close correlation between the obtained histologic measurements and fluorescence angiography.^{22,36} As reported previously,²¹ CNV development was significantly reduced in CFB-KO mice when compared to wild-type mice ($P \leq 0.01$; Figs. 6D, 6H). In contrast, levels of CFB in the RPE-choroid of CFB-tg mice supported CNV development and progression, resulting in a size $\sim 85\%$ of wild-type (Figs. 6D, 6H).

Complement activation in the lesions was assessed using immunohistochemistry for the MAC (C5b-9), the final step in the complement cascade. C5b-9 staining could be demonstrated in the CNV lesions of all three groups of mice (C57BL/6J, CFB-KO, and CFB-tg; Figs. 7A–C) on day 6 post laser, with levels scaling with lesion size but not differing when quantified as intensity per mm^2 (Fig. 7F).

To gain further insight into how CFB may be modulating RPE cell function and angiogenesis, we examined the local expression of genes that fall into one of six categories: RPE health, complement activation, control of angiogenesis, oxidative stress, autophagy, and mitochondrial function¹⁶ in the absence and presence of CNV. Overall, RPE-choroid gene expression was more similar between CFB-KO and CFB-tg transgenic mice at 3 months of age, and differed from the profile obtained from wild-type mice (Table 2). Importantly, no differences were observed in mRNA levels of *Rpe65*, a gene whose mRNA levels are susceptible to RPE cell health (see, e.g., Alizadeh et al.³⁷) and is hence used as an indicator of RPE cell integrity. Minimal changes in expression levels were observed for genes controlling angiogenesis, autophagy, and energy metabolism and mitochondrial function. However, significant elevations in gene expression levels were observed in genes controlling complement activation of the terminal pathway (C5 and C9) and complement inhibition (CD55 and CD59) between CFB-KO and CFB-tg when compared to wild-type mice.

FIGURE 6. Choroidal neovascularization development in CFB transgenic (CFB-tg) mice. Choroidal neovascularization lesions were induced in cohorts of age-matched wild-type, CFB-tg, and CFB-KO mice using laser photocoagulation. (A–C) The size of the lesions was assessed using CD102 staining of RPE-choroid flat mounts or (E–G) by OCT in separate cohorts of mice. Representative images are presented for each assessment mode. (D) Choroidal neovascularization size as obtained by CD102 analysis; (H) CNV size as obtained by OCT analysis. Mice with CFB expression in the RPE supported a lesion size of $\sim 85\%$ that in wild-type mice; this is in contrast to the CFB-KO mice, in which the size is reduced by $\sim 40\%$. Data are expressed as mean $\pm$ SEM (numbers in bar graphs indicate number of animals and spots analyzed).

FIGURE 7. Membrane attack complex (C5b-9) deposition in CNV. Flat mounts of RPE-choroid were assessed for MAC deposition using an antibody against C5b-9. (A–C) Immunolabeling could be detected in the CNV lesions of C57BL/6J, CFB-tg, and CFB-KO mice as well as (D, E) CFB-KO mice treated with PBS or wild-type serum. (F) Levels quantified as intensity per mm² did not differ significantly across genotypes or treatment. Data are expressed as mean $\pm$ SEM ($n = 4$ –8 animals per genotype).

Gene expression was subsequently compared between all three genotypes at 7 days post CNV induction (Table 2). In wild-type animals, genes involved in complement activation (C3, C5, fD) and inhibition (CD55 and CD59), control of angiogenesis (Vegf), oxidative stress (Clu), mitochondrial function (Cox1, Hmox1, Ndufb8), and autophagy (Lys, Map1lc3a) were all found to be significantly elevated when compared to the values in untreated age-matched controls. In contrast, in CFB-KO mice, the majority of these changes was reversed or attenuated (Table 2). In particular, the increase in complement activation (C3, C5, fD) and inhibition (CD55 and CD59), control of angiogenesis (Vegf), oxidative stress (Clu), mitochondrial function (Cox1, Drp1, Mf1, Ndufb8), and autophagy (Lys, Map1lc3a) was normalized. Gene expression in the CFB-tg mice after CNV was more similar to the CFB-KO, despite the fact that the pathology (CNV development) was more similar to that observed in the wild-type mice. Two possibly notable exceptions were the effects on complement factor H (Cfh) and pigment epithelium-derived factor (Pef) expression. Significantly reduced CFH and PEDF levels observed in the CFB-tg when compared to the CFB-KO mice might allow for increased complement and VEGF activation in the CFB-tg mice, potentially driving angiogenesis.

Systemically Derived CFB Promotes Choroidal Neovascularization

The animal data thus far suggest that locally derived CFB is sufficient to drive complement activation leading to pathology in the murine eye, correlating with human data, which suggest that local complement is the driver in AMD pathology.¹⁵ However, the mouse data do not rule out the possibility that systemically derived CFB contributes to complement activation in the eye. To determine whether CFB derived locally from the RPE is both required and sufficient to drive pathology in the eye, reconstitution experiments were performed, using wild-type serum in CFB-KO mice.

Three-month-old CFB-KO mice were injected intravenously every 48 hours with either 200 μ L wild-type serum or PBS, starting 2 days prior to the laser lesion. As for the analysis of the CFB-tg mice, development of CNV following laser photocoagulation was assessed on day 5 using OCT; on day

6, 48 hours after the final bolus of serum, mice were euthanized for collection of tissues and blood. Optical coherence tomography analysis (Fig. 8A) revealed that CNV development was significantly augmented in CFB^{-/-} mice injected with wild-type serum when compared to those treated with PBS ($P = 0.02$; Fig. 8B).

The anatomic data correlated with $\sim$ 6-fold increased levels of C3a (in μ g/mL; $P < 0.01$; Fig. 8C) and a $\sim$ 1.5-fold increase in AP activity (measured as μ g/mL C3b generated in zymosan assay; $P < 0.05$; Fig. 8D) in serum of serum-treated when compared to PBS-treated animals (ratio of available/consumed C3: $\sim$ 0.4) at the 6-day time point. A separate set of animals was treated using a single bolus of 200 μ L wild-type serum for 4 hours prior to being euthanized. At this earlier time point, less C3 had been consumed by complement activation, resulting in lower levels of C3a (67.33 ± 15.81) and higher levels of AP activity (104.67 ± 4.10), leading to a higher ratio of available to consumed C3 of $\sim$ 1.7. To compare these values as percent wild-type values, the amount of C3a or C3b that can be generated by zymosan-induced C3 cleavage in wild-type serum was determined. The obtained values of 482 ± 18.8 μ g/mL C3a and 545.6 ± 46.8 μ g/mL C3b were consistent with the estimated total C3 concentration in adult mouse serum of 500 μ g/mL.³⁸ Finally, normal mouse serum contains 51.1 ± 1.7 μ g/mL C3a, which corresponds to $\sim$ 10% of the total C3. Hence the available AP activity present in serum after a single bolus at 4 hours in reconstituted CFB-KO mice corresponded to $\sim$ 20% of wild-type levels, which at 48 hours after four serum treatments was reduced to $\sim$ 14%; the levels of C3a correspond to $\sim$ 10% at 4 hours and $\sim$ 30% at 48 hours after injection.

Finally, complement activation in the lesions was assessed as described above, using immunohistochemistry for C5b-9 (Figs. 7D, 7E) and analyzing MAC deposition as intensity per mm² (Fig. 7F) 6 days post laser and 48 hours after the final serum injection. Similar to the results for the C57BL/6J, CFB-KO, CFB-tg comparison, similar amounts of C5b-9 were present in the CFB-KO whether they were injected with PBS or wild-type serum when analyzed as signal levels per mm²; however, less complement activation was present in the PBS-injected CFB-KO when compared to those mice receiving serum when C5b-9 levels were to be expressed per lesion.

DISCUSSION

The main results of the current study are as follows. (1) Transgenic mice, expressing CFB under the RPE65 promoter, were generated using C57BL/6J blastocysts and crossed with CFB-KO mice to generate mice that produce CFB solely in the RPE. (2) These transgenic mice expressed CFB, which is secreted apically and basally from the RPE. (3) Secreted CFB is biologically active, as determined in AP-dependent TER assays using primary mouse RPE cells. (4) Laser-induced CNV was comparable in size between wild-type and CFB-tg mice, whereas lesions were significantly blunted in CFB-KO mice. (5) Despite the CNV response similarity between wild-type and CFB-tg mice, the gene expression profile of CFB-tg mice was more similar to that of the CFB-KO mice. (6) Complement factor B-knockout reconstituted with wild-type serum developed larger CNV lesions when compared to those treated with PBS, concomitant with exhibiting elevated levels of systemic AP activation. (7) Finally, MAC deposition correlated with lesion size rather than with the genetic or treatment status of the mouse. Thus, in summary, both locally produced and systemically recruited CFB can promote CNV in mice. Our data on CFB, together with the human data on genotype-specific differences in CFH, suggest that under normal conditions when complement components are generated in the RPE and the

TABLE 2. Quantitative RT-PCR Results

Gene Name	Symbol	Fold Change Compared to C57BL/6j			Fold Change Compared to No CNV						
		CFB-tg	Sign.	CFB-KO	Sign.	C57BL/6j	Sign.	CFB-tg	Sign.	CFB-KO	Sign.
Retinal pigment epithelium 65	<i>Rpe65</i>	1.85 ± 0.52		1.12 ± 0.10		-1.99 ± 0.33		-2.65 ± 0.02	‡	-1.45 ± 0.21	
Complement component 3	<i>C3</i>	4.26 ± 1.07	‡	2.16 ± 0.44	‡	2.90 ± 0.23	‡	1.14 ± 0.02		1.58 ± 0.03	
Hemolytic complement*	<i>Hc</i>	8.96 ± 1.12	‡	4.56 ± 0.04	‡	4.11 ± 1.09	‡	-5.00 ± 0.53	‡	-4.48 ± 0.19	‡
Complement component 9	<i>C9</i>	38.45 ± 9.61	‡	30.28 ± 3.00	‡	1.10 ± 0.37		1.23 ± 0.26		1.08 ± 0.27	
Complement factor D (adipsin)	<i>Cfd</i>	7.50 ± 1.93	‡	1.88 ± 0.62		8.95 ± 1.83	‡	-1.96 ± 0.14		5.13 ± 0.04	‡
Complement component factor H	<i>Cfb</i>	2.55 ± 1.08		1.35 ± 0.27		2.21 ± 1.06		-1.10 ± 0.06		5.70 ± 0.64	‡
CD55 antigen	<i>Cd55</i>	5.65 ± 0.73	‡	3.06 ± 0.09	‡	4.56 ± 0.09	‡	-3.92 ± 0.15	‡	-2.47 ± 0.19	‡
CD59a antigen	<i>Cd59a</i>	8.55 ± 2.03	‡	6.58 ± 0.79	‡	4.64 ± 0.77	‡	-1.79 ± 0.65		-1.23 ± 0.42	
Clusterin	<i>Clu</i>	2.82 ± 0.09	‡	1.49 ± 0.25		15.17 ± 2.59	‡	-3.06 ± 0.05	‡	-1.98 ± 0.10	
Ceruloplasmin	<i>Cp</i>	3.46 ± 0.06	‡	3.21 ± 0.20	‡	1.62 ± 0.13		-3.96 ± 0.63	‡	-3.56 ± 0.53	‡
Lysozyme 1	<i>Lyz1</i>	1.73 ± 0.09		-1.12 ± 0.14		2.51 ± 0.16	‡	-9.05 ± 0.50	‡	-5.54 ± 1.40	‡
Microtubule-associated protein 1 light chain 3 α	<i>Map1lc3a</i>	4.13 ± 0.24	‡	3.13 ± 0.03	‡	6.49 ± 1.56	‡	-1.70 ± 0.08		1.19 ± 0.06	
Serine (or cysteine) peptidase inhibitor, clade F, member 1†	<i>Serpinf1</i>	5.70 ± 0.03	‡	1.11 ± 0.19		1.77 ± 0.06		1.01 ± 0.06		2.07 ± 0.10	‡
Vascular endothelial growth factor A	<i>Vegfa</i>	1.42 ± 0.05		-3.61 ± 0.68	‡	4.04 ± 0.35	‡	1.03 ± 0.16		1.25 ± 0.01	
Hypoxia inducible factor 1, alpha subunit	<i>Hif1a</i>	1.59 ± 0.52		2.07 ± 0.28	‡	-3.43 ± 1.08	‡	-4.17 ± 1.92	‡	-2.87 ± 0.19	‡
Mitochondrially encoded cytochrome c oxidase I	<i>mt-Co1</i>	3.78 ± 0.01	‡	2.49 ± 0.07	‡	5.72 ± 0.11	‡	-1.77 ± 0.08		1.05 ± 0.08	
Dynamin 1-like	<i>Dnm1l</i>	-1.34 ± 0.13		-2.26 ± 0.47	‡	-2.84 ± 0.29	‡	-2.78 ± 0.73	‡	-1.11 ± 0.13	
Heme oxygenase (decycling) 1	<i>Hmox1</i>	2.71 ± 0.59	‡	1.39 ± 0.06	‡	4.69 ± 0.37	‡	2.23 ± 0.64	‡	3.22 ± 0.76	‡
Mitofusin 1	<i>Mfn1</i>	1.20 ± 0.01		-1.16 ± 0.01		-3.97 ± 0.87	‡	-3.19 ± 0.22	‡	-1.98 ± 0.06	
NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	<i>Ndufb8</i>	5.82 ± 0.08	‡	5.00 ± 0.02	‡	10.93 ± 0.08	‡	-1.84 ± 0.00		1.07 ± 0.02	
6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	<i>Pfkfb2</i>	4.75 ± 0.04	‡	2.35 ± 0.18	‡	1.42 ± 0.02		-1.76 ± 0.15		-0.85 ± 0.01	

Gene expression levels in cells derived from RPE-choroid in mice at baseline (fold change compared to C57BL/6j) and after CNV induction (fold change compared to no CNV) were examined by QRT-PCR. Six categories were analyzed: RPE cell health, complement activation, control of angiogenesis, oxidative stress, autophagy, and mitochondrial function/metabolism. Gene expression levels of CFB-tg mice were more similar to those in CFB-KO than in wild-type mice. The most significant differences were observed in genes controlling complement activation of the terminal pathway (C5 and C9) and complement inhibition (CD55 and CD59). After CNV induction, wild-type mice exhibited an increase in expression for transcripts involved in complement activation (C3, C5, fD) and inhibition (CD55 and CD59), control of angiogenesis (Vegf), oxidative stress (Cox1, Hmox1, Ndufb8), and autophagy (Lys, Map1lc3a). In CFB-KO mice, the majority of these changes were reversed or attenuated. Similar to expression levels at baseline, overall gene expression in CFB-tg mice was more similar to that observed in CFB-KO mice. However, the two exceptions were a significant reduction in the alternative pathway inhibitor, fH, and the antiangiogenic factor, Pedf, in the CFB-tg when compared to the CFB-KO mice. Sign., significance.

* Complement component 5, C5.

† Pigment epithelium-derived factor, Pedf.

‡ $P < 0.05$.

FIGURE 8. Reconstitution of CFB-KO mice with wild-type serum. Complement factor B-knockout mice were injected every 48 hours with either 200 μ L wild-type serum or PBS, starting 2 days prior to CNV induction. (A) Optical coherence tomography analysis was performed on day 5. (B) Choroidal neovascularization sizes were significantly increased in CFB-KO mice injected with CFB-sufficient mouse serum when compared to those receiving PBS. Numbers in bar graphs indicate number of animals and spots analyzed. (C) Treatment with wild-type serum increased the amount of C3a that could be detected in serum using a C3 ELISA, (D) as well as the amount of alternative pathway activity using a zymosan assay. Data are expressed as mean $\pm$ SEM.

liver, locally derived complement might dominate over that sequestered from the bloodstream.

The original studies that suggested an association of AMD with complement were based on an observation that various complement proteins were deposited in drusen and along BrM of patients with AMD.¹ More recently, this observation has gained support with the discovery that polymorphisms of the human factor H gene are a risk factor for all forms of AMD.^{2–5} Additional support for the close association between AMD and the AP of complement has been provided by the discovery that polymorphisms of genes encoding the AP proteins *CFB* and *C3*,^{7,8} as well as the CFH-like genes (e.g., complement factor H-related 4, *CFHR4*; complement factor H-related 5, *CFHR5*), are also associated with AMD, and those single nucleotide polymorphisms (SNPs) are related to prevalence of advanced AMD⁹ or the risk of CNV.³³

Complement factor B is a glycosylated protein composed of a single 93-kDa polypeptide chain. It is secreted mainly by the liver and is present in plasma at $\sim$ 200 μ g/mL. However, other sources of CFB have been reported, including the RPE.^{13,39} Complement factor B is a required component of the AP of complement. In the presence of Mg^{2+} , CFB binds to C3b deposited onto a cell membrane by either the classical or the lectin pathway. The resulting C3b,B complex can then be activated by CFD, which results in the cleavage of CFB, releasing the Ba fragment (33 kDa) and leaving the 60-kDa Bb fragment bound to C3b. C3b,Bb, a serine protease, which is also called a C3/C5 convertase as it cleaves both C3 and C5, releases the anaphylatoxins C3a and C5a and thereby generates the membrane-bound C3b and C5b components. Hence CFB contributes to the AP amplification loop. Another role for CFB is in the initiation of the AP in fluid phase in a process called tickover. Spontaneous hydrolysis of the thioester in C3 occurs continuously, resulting in the formation of C3(H₂O). C3(H₂O) then binds CFB, and CFB in the C3(H₂O),B complex can then be activated by CFD, triggering cleavage of CFB. This fluid phase C3 convertase, C3(H₂O),Bb, can cleave C3, resulting in C3b, which, if close to a membrane, can rapidly attach to the

carbohydrates present on cell surfaces, triggering the generation of the C3 convertase C3b,Bb as described above. Importantly, due to the presence of the AP amplification loop, over 80% of the C3 convertase deposited onto cells is derived from AP activation rather than the initiating classical or lectin pathways.^{40,41}

We have investigated the CFB-KO mice at different ages as part of other studies to investigate mechanisms of wet²¹ and dry¹⁶ AMD. At 3 months of age, rod ERG amplitudes were found to be equal between wild-type and CFB-KO mice,²¹ whereas both rod and cone ERG amplitudes were reduced by 20% to 30% across all light intensities by 9 months of age.¹⁶ Here we showed that by $\sim$ 4 months of age, rod ERGs were reduced by 15% to 20% over the same range of light intensities used in the other two studies, demonstrating an age-dependent decline in photoreceptor cell function due to the lack of CFB. The reduction in ERG amplitudes at 9 months of age was not due to a thinning of the outer nuclear layer (ONL) or inner nuclear layer (INL), but gene expression analysis suggests that it might be due to a reduction in opsin and RPE65 expression.¹⁶ Likewise, the reduction in ERG amplitudes at 4 months of age reported here in the CFB-KO mice was independent of changes in ONL thickness. Partially restoring CFB in the RPE (CFB-tg) did not reverse the changes in retinal function seen in the CFB-KO mice. Interestingly, and in support of the hypothesis that low levels of complement activation are required for proper tissue function, eliminating both activators and inhibitors in the complement cascade influenced retinal structure and function. Age-dependent losses in ERG and c-wave amplitudes, as well as photoreceptor cell numbers, have been reported for C3a receptor and complement C3, but not C5a receptor knockout mice⁴²; a trend for a reduction in ERG amplitudes at 3 months of age was shown in CFD knockout mice⁴³; and mice lacking CD59 have significantly reduced ERG amplitudes (Coughlin B, Rohrer B, unpublished results, 2012). Since these studies were conducted using age-matched wild-type controls bred from heterozygote matings⁴² as well as using nonlittermate controls (C57BL/6J¹⁶ and Balb/c⁴³), the age-dependent decline in ERG amplitudes is expected to be due to the elimination of the respective complement gene(s); however, a contribution due to inherent differences between the background strains cannot be excluded.

We are currently exploring the beneficial effects of complement activation in ocular tissues, together with the effects of different genetic backgrounds on complement-dependent changes to retinal structure, function, and pathology (see, e.g., Schnabolk et al.⁴⁴), to answer these critical questions. Finally, gene expression changes related to RPE health, complement activation, control of angiogenesis, oxidative stress, autophagy, and mitochondrial function have been analyzed in RPE-choroid samples in CFB-KO and wild-type animals at 9 months of age¹⁶ as well as in CFB-KO, CFB-tg, and wild-type animals at 3 to 4 months of age (current study). In both studies, differences between genotypes were observed in the absence of stressors. Here we asked whether the expression of CFB in the CFB-tg mouse (generated in C57BL/6J-derived blastocysts and crossed onto the original CFB-KO mice) could reverse the genetic phenotype of the RPE. However, the genetic phenotype of the CFB-tg mice closely resembled that of the CFB-KO rather than the C57BL/6J strain. This suggests that the amount of CFB expressed in the RPE does not reverse the genetic phenotype of the RPE on a CFB-KO background, or that additional systemic changes due to the lack of global CFB contribute to the genetic phenotype of the RPE. Finally, the observation that the genetic as well as the functional phenotype of the CFB-tg (sharing the C57BL/6J background from the blastocysts used to generate the transgenics as well as that of the original C57BL/6J background used

to generate the CFB-KO in 1997) correlated with that of the CFB-KO—rather than falling between the CFB-KO and the C57BL/6J mice—suggests that RPE gene expression and retinal function are affected by the differences in genotype, rather than being indirectly affected by a potential genetic drift between the different C57BL/6J populations (the CFB-KO mice are separated from new C57BL/6J by ~45 generations).

In mouse CNV, a role for the alternative complement pathway has been demonstrated by inhibition of the AP by CFB siRNA,³² CFB gene knockout, and treatment with the inhibitor CR2-FH.²¹ In these studies, CFB deficiency or AP inhibition was associated with a significant reduction in the size of CNV following laser injury, preserved retinal function, and decreased VEGF mRNA expression. After siRNA injection, CFB mRNA and protein was reduced to <95% within the eye, liver, and spleen, eliminating the possibility to examine tissue-specific contributions. In patients, the question whether global, liver-derived, or local, RPE-derived, production of complement is relevant for progression of AMD has been addressed in liver transplant patients. Lotery and colleagues¹⁵ published a retrospective study on >200 liver transplant patients, documenting that AMD, 5 years after the transplant, was associated with recipient *CFH* genotype. Rather than performing liver transplants, we generated mice expressing CFB uniquely in the RPE (i.e., CFB driven by RPE-specific promoter and crossed onto CFB-KO mice), the results of which demonstrated that local CFB expression and secretion in the eye appears to be sufficient to drive pathology. However, additional contributions by CFB recruited from the bloodstream cannot be excluded under normal conditions, since CFB-KO mice partially reconstituted with CFB-sufficient wild-type mouse serum developed larger CNV lesions than littermates injected with PBS only. After 200- μ L serum application, assumed to result in maximum reconstitution of CFB to ~35% to 43% of wild-type levels (based on a blood volume of 1.2–1.5 mL for an adult mouse and a blood-to-serum ratio of 3:1), mice exhibited ~20% to 14% of wild-type levels of AP activity and generated 10% to 30% of maximal C3a levels when measured after 4 and 48 hours, respectively. These data suggest that mouse CFB is a relatively stable protein in blood, presumably supplying CFB for consumption in tissues, including the eye. Similar stability has been reported for human CFB, with a half-life of ~72 hours.⁴⁵ However, it is not clear how much of the systemically injected CFB is recruited to the CNV lesions. Likewise, the experiments utilizing the CFB-tg also did not provide a clear answer as to how much CFB is required to drive pathology in the eye, since we could not cleanly separate RPE and choroid in the mouse eye and hence determined the presence of CFB in the combined RPE-choroid samples from nonperfused animals. In addition, the amount generated, or importantly, the amount locally secreted might be significantly higher under CNV conditions; since we showed here that RPE cells from CFB-tg in culture secrete higher levels of CFB under oxidative stress conditions when compared to baseline.

A high concentration of MAC in the area of BrM and RPE has been documented in aged and AMD eyes,⁴⁶ with all basal laminar and linear deposits being positive for C5b-9 staining.⁴⁷ The amount of extractable MAC per tissue punch was found to correlate with the CFH genotype; however, there was no statistical difference between individuals with AMD and controls.⁴⁶ Here we asked whether the amount of C5b-9, as detected by immunohistochemistry in the mouse CNV lesions, correlated with the size of the lesion or varied in density based on genotype or treatment. Interestingly, we found that the amount of C5b-9 signal measured as intensity of immunofluorescence per mm² was identical between genotypes (C57BL/6J, CFB-KO, CFB-tg) and treatments (CFB-

KO treated with PBS or wild-type serum) and that rather the overall amount of C5b-9 signal correlated with the size of the lesions. These data suggest that in the presence of endogenous soluble and membrane-bound inhibitors, MAC deposition drives CNV in a dose-dependent manner; however, further data are required to corroborate this hypothesis. Likewise, the precise localization of MAC in CNV lesions awaits clarification; however, this uniform distribution of MAC throughout the regions of the laser burn appears to be a common observation.⁴⁸

Our data on blood levels of mouse CFB (no detection of CFB in serum of CFB-tg mice), as well as in human when blood levels of CFH are examined, suggest that the circulating complement components appear to be produced entirely by the liver.¹⁵ It is plausible that polarity of secretion might contribute to this phenomenon. It appears that CFH is localized predominantly to the apical cytoplasm of cultured human RPE cells and is secreted from these cells, but unfortunately, polarity of secretion was not investigated.⁴⁹ In mouse retina, there appears to be evidence of apical CFH secretion due to the presence of CFH in the interphotoreceptor cell space; yet additional staining is present in BrM, although the source for that staining might be due to choroidal secretion.⁵⁰ As in human RPE cells, CFB in the mouse is localized predominately in the apical area of RPE cells; however, that distribution changes with age. In addition to the apical distribution in 3-month-old mice, in 2-year-old mice RPE cells show additional staining in the central and basal RPE, and CFB immunoreactivity is detected in the ONL and the photoreceptor outer segment area.¹³ Here we showed that in primary RPE cell cultures derived from early postnatal animals (P10), CFB secretion was triggered upon exposure to oxidative stress, resulting in release toward both the apical and the basal side. While the TER experiments might suggest that higher levels are secreted toward the apical rather than the basal side, the greater sensitivity of the apical side of the RPE monolayer to complement attack might be due to the polarized localization of membrane-bound complement inhibitors. Taken together, these experiments suggested that the RPE is capable of producing and secreting an essential component of the AP of complement to mount a complement attack. It would be of great interest to determine which other components can be generated by the RPE and under what conditions. Anderson and coworkers³⁰ have analyzed RPE and choroid collected from control and AMD donors (40–84 years old) separately for the ability of these tissues to express mRNA of complement components. Both tissues express almost all complement components and regulatory molecules required for both alternative and classical pathway activation, while many genes involved in the lectin pathway or those required for assembly of the MAC are missing. Overall, mRNA levels for complement genes were found to be higher in samples derived from the choriocapillaris. No apparent differences in gene expression levels were, however, found between control and AMD samples when using real-time PCR analyses.

In summary, we showed that the mouse RPE can generate and secrete CFB under stress conditions (oxidative stress or CNV), sufficient to support complement activation requiring the AP. These data provide further evidence of a local complement system present in the eye. The ability of the eye to locally contribute complement components will be important to consider in designing complement therapeutics for ocular use.

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