The neovascular form of age-related macular degeneration (AMD) is the leading cause of registered blindness worldwide.\(^1\) Choroidal neovascularization (CNV) is the principal pathologic feature of this form of AMD and involves the growth of new blood vessels in the subretinal tissue, causing loss of central vision.\(^2\) AMD is a multifactorial and complex disease in which the molecular pathogenesis has not yet been fully elucidated. However, dysregulation of the complement system, associated with a number of well-defined single nucleotide polymorphisms (SNPs) in genes of the alternative pathway (AP) of complement activation, has a contributory role in many patients with AMD. Although haplotypes in complement factor H are the most common in AMD, genetic studies have revealed significant associations between SNPs in several complement genes, including complement factor B (CFB), and AMD risk.\(^3,4,5\)

CFB is mainly produced by hepatocytes but is also expressed in the choroid plexus in humans (EMBL-EBI database), in the mouse retina, and at low levels in retinal pigment epithelial (RPE) cells.\(^6\) CFB is a fluid-phase positive regulator of the AP, with its main activity being driven by the Bb subunit that binds C3b, the activated portion of C3. The C3bBb complex in turn drives the formation of the C5 convertase, the downstream C5 convertase, and generation of the membrane attack complex.

The H1 haplotype, common to both the CFB and complement C2 genes, has been shown to be associated with a significantly higher risk of developing AMD. However, in contrast to most genetic associations of complement genes with AMD, the L9H and the R32Q variants of CFB are protective against the neovascular form of AMD.\(^5\) Furthermore, it has been demonstrated that the R32W variant of CFB has an intermediate protective effect against neovascular AMD compared to R32Q.\(^9\) The greater protection afforded by CFB\(^{R32}\) correlates with its decreased hemolytic activity, which is explained by its reduced ability to bind C3b and to form the proenzyme C3bB; therefore, activation of the AP is less efficient.\(^5\) CFB variants distinct to those associated with AMD risk were also found to be associated with retinopathy of prematurity, which involves abnormal neovascularization in the retinas of preterm babies.\(^10\) Collectively, these reports lend weight to the idea that CFB may be particularly associated with the vascular complications of retinal disease.

Animal models of retinal vascular disease also support a role for CFB in the development of neovascularization in the eye. Thus, CFB is upregulated in CNV, and the AP was shown to be involved in the development of CNV in the...
mouse model of laser-induced CNV, as gene knockout of Cfb led to reduced pathologic ocular angiogenesis compared to wild type and to mice with a compromised classical or lectin pathway. In addition, AP activation was shown to be necessary, but not alone sufficient, for the development of laser-induced CNV because mice with a functional AP, but no classical and lectin pathway (C1q −/− MBL −/−), developed similar lesion sizes to CFB knockout mice. Studies using human serum, human CFB R32 (200 μg/mL), PBS, 10% heat-inactivated human serum, 10% of CFB having greater angiogenic activity. A role in vascular pathology in the eye with the R32 variant with AMD risk. Our data suggest that CFB may indeed play a role in vascular pathology in the eye with the R32 variant of CFB having greater angiogenic activity.

**Materials and Methods**

**Animals**

C57Bl/6j mice were purchased from Charles River Laboratories, Écully, France and bred in-house. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act and with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the Animal Welfare and the Ethical Review Bodies of the UCL Institute of Ophthalmology.

**Metatarsal Angiogenesis Assay**

The metatarsal angiogenesis assay was performed as described with minor changes. Metatarsal bones were exposed to various treatments, including 10% fetal bovine serum, PBS, 10% heat-inactivated human serum, 10% human serum, human CFB (200 μg/mL), and CFB (200 μg/mL). The concentration of CFB used in these studies is in the physiologic range of CFB in human serum. At day 11 of culture, conditioned media were collected for analysis, the metatarsals were fixed in 4% paraformaldehyde (PFA), permeabilized in 10% BSA with 0.1% Triton, and stained overnight at 4°C for CD31 (553570; BD Pharmingen/BD Biosciences, CA, USA), complement C3 (555000; Cappel/MP Biomedicals, Cambridge, UK), F4/80 (MCA497R; AbD Serotec, Kidlington, UK), and Arginase-1 (SC-18351; Santa Cruz, CA, USA). The secondary antibodies were incubated for 2 hours at room temperature and samples were imaged. After imaging in ImageJ (National Institutes of Health, Bethesda, MD, USA) to mask the cartilage, the total length of CD31-positive tubular structures and the number of junctions were quantified by Angiosys (TCS Cellworks, Buckingham, UK) using manual thresholding. The area of staining was quantified using ImageJ, statistical analysis was performed using GraphPad Prism (6.01; GraphPad Software, La Jolla, CA, USA), and one-way ANOVA was used to determine statistical significance between test groups. For confocal imaging, metatarsals were cultured as described but on coverslips, then mounted on glass slides and imaged using maximum-intensity projections (ZEISS LSM 700 confocal microscope; ZEISS, Cambridge, UK).

**ELISA**

ELISA was used to measure C3 and VEGF in the conditioned media collected from the metatarsal assays. For the C3 ELISA, plates were precoated overnight at 4°C with polyclonal goat IgG to mouse complement C3 (554563; L8000; MP Biomedicals, Cambridge, UK). Plates were washed with 0.2% Tween in PBS and blocked with 2% BSA and 0.2% Tween for 1 hour at room temperature. After washing, conditioned media from metatarsals and standards (normal human serum with known concentration of C3 protein) were added for 1 hour at room temperature in blocking buffer in 12 serial 2× dilutions (1:1000 to 1:2,048,000). Standards ranged from 0.1 to 220 ng/mL and the experimental readings were between 0 and 27 ng/mL. All samples were analyzed in duplicate. After washing, horseradish peroxidase (HRP)–conjugated goat anti-mouse C3 (55557, 1/25,000; MP Biomedicals) was added for 1 hour at room temperature. After washing, HRP Substrate Reagent (DY993; R&D, Systems, Minneapolis, Minnesota, USA) and stopping solution (2N sulfuric acid, 895032; R&D) were added and the optical density was measured at 450 nm, with 540 nm set as the reference. Data were analyzed with GraphPad Prism (6.01) using nonlinear regression analysis, with a dose-response equation by interpolating unknown values from a standard curve of known values. For VEGF, the DuoSet ELISA kit for mouse VEGF (DY943-05; R&D) was used following the manufacturer's instructions.

**Quantitative PCR**

RNA from metatarsal cultures was extracted (74106; QIAGEN, Hilden, Germany) 24 hours after the final treatment and transcribed into cDNA (205310; QIAGEN) following the manufacturer's instructions. Quantitative PCR reactions were performed using PCR master mix (4367659; ThermoFisher Scientific, Loughborough, UK) and relative gene expression was determined using the ΔCt method. Results were normalized to the housekeeping gene and to PBS for each experiment. Primer sequences used were as follows: C3, forward primer 5′-AGACACAAAAAGGCCATCGGAACTGCT-3′, reverse primer 5′-AGGCCAGTCTCTCCTGGTGTTGAA-3′; Cfb, forward primer 5′-TACCCTGGTCACTGCGAGA-3′, reverse primer 5′-TGGGGACGACGTCTGGAT-3′; VEGF, forward primer 5′-GACTTGTGTTGGGAGAGGA-3′, reverse primer 5′-TCTGGAAGTGACCAATGCT-3′; β-tubulin, forward primer 5′-CAAGATGTCGATACCGTGTC-3′, reverse primer 5′-GCTTGAAGGTAGCGAACAGA-3′.

**Cloning of CFB Variants**

The PCR fragment CFB-Hisx6 (I.M.A.G.E clone, ID: 2959706/AT29 G01 M13F; Source Bioscience, Nottingham, UK) containing the DNA sequence of the W32 variant of human CFB was ligated into the mammalian expression vector pcDNA3.1 (Invitrogen, Loughborough, UK) at the AflII/ApaI site (forward primer: GTCTCAGCTGCT-TAACCTGGTCATGGGAGCAATCTCGA; reverse primer: TACTATATCCGGCCGCTTTACTGATGATGGTGTAGTGATGTTAGAAAAACCATCCTCTCACT). Site-directed mutagenesis (210518; Agilent Technologies, CA, USA) was used to obtain the R32 and Q32 variants: hCFB and hCFB (Q32 forward primer: CAGGATCCCCTGGGCTGGGACCCAAA-GACCATG, reverse primer: CCATGGCTTTTGGCCAGTTT...
TAGGGATCTCG; R32 forward primer: GATCCCTGGGCGGCGCAAGACCAT, reverse primer: ATGGCTTTTGGCGCGGGCCCCAGGGATC). All DNA sequences were verified by Sanger sequencing.

CBF Expression in Mammalian Cells

The pcDNA3.1-CBF expression vectors were stably expressed in human embryonic kidney (HEK) 293T cells (R790-07; Invitrogen) maintained in freestyle expression medium supplemented with Glutamax. Cultures were grown to confluency at 37°C in a humidified atmosphere of 8% CO₂ on an orbital shaker. Transfections were carried out using 40 μg of each plasmid, Opti-MEM (31985-070; Life Technologies, Loughborough, UK) and Lipofectamine 2000 (11668019; Life Technologies). Forty-eight hours following transfection, cells were resuspended in medium containing 0.2 mg/mL G418 (Sigma, Dorset, UK) to select for transfected cells only. Three weeks after transfection, supernatants containing the secreted recombinant Human Complement Factor B (hCBF) proteins were collected.

Recombinant CBF Purification

The His-tagged hCBF protein variants were purified using the ÄKTA pure 150 Protein Purification System (GE Healthcare Life Sciences, Buckinghamshire, UK) via affinity chromatography. The HisPrep FF16/10 column (total column volume [VT]: 20 mL; GE Healthcare Life Sciences) was used in conjunction with His Buffer Kit (GE Healthcare Life Sciences) according to the manufacturer’s instructions with minor changes. The purified protein was buffer exchanged into HEPES-buffered saline using the HiPrep 26/10 Desalting column (void volume [V0]: 15 mL and VT is 53 mL; GE Healthcare). All the proteins used in this study had endotoxin levels below 2 EU/mL (PSD250-30; Cape Cod, Massachusetts, USA) at the working concentration.

Protein Detection and Analysis Techniques

Purified proteins were resolved by SDS-PAGE and gels stained with Coomassie blue. NuPAGE Novex precast 4% to 12% Bis-Tris gels (NP0321BOX; ThermoFisher Scientific) were run in NuPAGE MOPS (3-N-Morpholino) propanesulfonic acid) SDS running buffer (NP0001-02; ThermoFisher Scientific). NuPAGE lithium dodecyl sulfate sample buffer (Life Technologies, NP0008, with 100 mM DL-dithiothreitol; ThermoFisher Scientific, R0861) was added to the samples before heat denaturation for 5 minutes at 95°C. Gels were run at 150 V and 400 mA for 90 minutes, then fixed in 25% isopropanol and 10% acetic acid in water for 30 minutes, as well as stained in 10% acetic acid in double-distilled water containing Coomassie Brilliant Blue R 250 (27816; Sigma) at 60 mg/L.

RESULTS

Generation of Recombinant Human CBF Variants

Following the discovery of a significant association between human CBF variants (CBF R32 and CBF W32) and a reduced risk of developing neovascular AMD,4,9 we wanted to generate the recombinant proteins to test whether they have any influence on angiogenesis when compared to the most common variant (CBF Q32). Figure 1A shows the codon of interest of the three proteins, the single amino acid change (R, W, or Q) and their allelic frequencies in the population, with CBF Q32 being the most common.

Following expression in HEK cells, the secreted recombinant hCBF proteins were visualized on SDS-PAGE gels to confirm their identity, purity, and concentration (Fig. 1B). A single polypeptide band of the expected size was observed in each case. Figure 1C shows in schematic form the relative biological activities of the three variants: CBF R32 has the highest binding affinity to C3b and therefore activates complement with the greatest efficiency, and CBF W32 binds C3b with lower affinity and thus complement activity is reduced. CBF Q32 exhibits an intermediate level of complement activation.

CBF and Angiogenesis

To investigate whether the CBF Q32 and/or CBF W32 variants have a modulatory role in angiogenesis, the ex vivo mouse metatarsal model was used. First, we examined 11-day metatarsals treated with 10% heat-inactivated fetal bovine serum (control), as well as 10% heat-inactivated (HI) and non-heat-inactivated (NIH) human serum and stained for CD31 (Fig. 2A). Vessel outgrowth and branching were both increased in NIH-serum-treated metatarsals compared to HI-serum-treated metatarsals (Fig. 2B). The positive impact of human serum on murine angiogenic sprouting and branching provides evidence of the functional activity of the human proteins in a mouse system, and the reduced activity of the...
HI-serum shows that heat-labile serum components, such as complement proteins, contribute to the observed angiogenic growth.

Because CFB is a key complement activator, we next examined the impact of CFBQ32, CFBW32, and the commoner CFBR32 variant on angiogenesis in the metatarsal assay. Mouse metatarsals (E17.5) were prepared using tissues from wild-type C57/Bl6; treated with 200 μg/mL CFBQ32, CFBW32, or CFBR32; and stained for CD31 and imaged at 11 days postplating (Fig. 2C). Vessel outgrowth and branching from explanted metatarsals were analyzed following image analysis (Fig. 2E), which revealed a significant increase in the presence of exogenous CFBR32. In contrast, no significant impact on angiogenesis was observed when metatarsals were cultured with the two protective CFB variants (Fig. 2D). Note that because the CFBW32 variant showed equivalent activity to CFBQ32 in these assays, we did not pursue it further.

**CFB and Complement Activation**

CFB activation results in the catalytic conversion of C3 to C3b and the generation of the proenzyme C3bB, and previous studies have reported that CFBQ32 is inferior to CFBR32 in driving this conversion. We therefore sought to identify C3 in the metatarsal model system and to determine its abundance in relation to the presence of the CFB variants. To do this, we maintained metatarsals in the presence of CFBQ32 and CFBR32 and determined the abundance of C3/C3b signal by immunocytochemical staining using a C3 antibody that is specific for both inactive and active (C3b, iC3b, C3c) forms of C3 (Fig. 3A). For the two CFB variants, the area of C3/C3b staining was quantified in low-magnification images, revealing significantly less C3/C3b staining after CFBR32 treatment compared to PBS or CFBQ32 (Fig. 3B). In line with this and previous observations,6 we showed by ELISA that secreted C3/C3b was increased in the conditioned media of CFBQ32-treated metatarsals compared to PBS control and CFBR32 (Fig. 3C), consistent with the activity profiles of the two CFB variants. To test whether CFBR32 was stimulating blood vessel growth in a VEGF-dependent manner, we quantified VEGF levels by ELISA in the conditioned media in metatarsals treated with the two CFB variants. This analysis indeed revealed a significant difference in the level of VEGF between the control and the two CFB variants and between the variants themselves (Fig. 3D), suggesting that
angiogenesis driven by CFBR32 may occur, at least in part, via a VEGF-dependent mechanism and that CFBR32 is less active in this model. To assess if there was correlation between protein and mRNA levels, we analyzed gene expression of Cfb, C3, and Vegf in the metatarsal cultures treated with PBS, 200 μg/mL CFBQ32 and CFBR32. Cfb transcripts appeared similar across treatments (Fig. 3E). Interestingly, C3 mRNA levels (Fig. 3F) did not closely correlate with C3 protein levels (Fig. 3C), suggesting that the CFB variants have little impact on C3 expression themselves and that C3 protein levels are instead a reflection of the different activities of the two variants. Additionally, Vegf transcripts were found to be markedly upregulated following CFBQ32 compared to CFBR32 treatment (Fig. 3G), in line with the increased VEGFA concentration in conditioned media and increased angiogenesis with CFBQ32.

**CFB and Macrophages**

Since macrophages are responsive to C3/C3b and can either promote or inhibit angiogenesis depending on the microenvironment, we next examined their density and phenotype in metatarsal cultures treated with the wild-type CFBR32 and the less active variant CFBQ32. Macrophages and monocytes were identified by immunostaining in metatarsal cultures treated with PBS, 200 μg/mL CFBQ32, or 200 μg/mL CFBR32 (Fig. 4A, upper panel). Confocal images of metatarsals treated as described above revealed some C3/C3b-positive cells that were also F4/80 positive (white arrows in Figure 4A, lower panel). The double-stained cells acquired different phenotypes depending upon the treatment: control cells were generally more elongated with irregular perimeter consistent with these being in a resting state and typically appeared in groups, whereas CFB-treated cells were smaller with a regular round shape, indicative of activation, and more evenly distributed. The area of F4/80 staining was quantified revealing that upon CFBQ32 treatment, there was a significantly higher density of F4/80-positive cells compared to control and CFBR32-treated metatarsals (Fig. 4B). Additionally, the colocalization of C3/C3b and F4/80 and vice versa was evident in approximately 50% of cells across treatments, demonstrating that only half of the two cell populations were positive for both markers. However, the amount of F4/80 staining overlapping C3/C3b was significantly greater with hCFBQ32 compared to hCFBR32 (data not shown).

There are two main subtypes of macrophages: classically activated M1 macrophages, which are proinflammatory and antiangiogenic, and alternatively activated M2 macrophages, which are anti-inflammatory and proangiogenic. However, the distinction between the two types is neither absolute nor binary, and therefore the need to more accurately identify macrophage subtypes is important because of their distinct effects on angiogenesis and inflammation. Nevertheless, the distribution of the M2 macrophage marker, arginase 1, as assessed by immunocytochemistry, revealed an increase in total staining area, indicating that there were significantly more arginase 1–positive cells after CFBQ32 treatment compared to metatarsals treated with PBS or CFBR32 (Fig. 4D).

**DISCUSSION**

CFB is a key positive mediator in the alternative pathway of complement activation where it drives the formation of the C3 convertase. Its absence or loss of function would therefore be predicted to lead to complement dysregulation. An imbalance in the proteins of the alternative pathway of the complement system is not only involved in immune and inflammatory diseases such as AMD,17,18 but also other conditions such as atherosclerosis and cancer, as well as physiologic processes such as angiogenesis and lipid metabolism.19–22 Investigating the role of CFB in angiogenesis and inflammation is of interest since SNPs in this gene have been identified as being genetically associated with...
FIGURE 4. Impact of human CFB variants on macrophages. (A) Upper panel: Representative images of metatarsals treated with PBS, 200 μg/mL CFBQ32, or 200 μg/mL CFBR32 and stained for F4/80. Scale bar: 200 μm. Lower panel: Representative 40× maximum-intensity projection confocal images of metatarsals treated as described above and stained for F4/80 (red) and C3 (green). Cells that are double stained for both markers appear yellow (white arrows). In the PBS treatment, the double-positive cells are elongated and ramified, while in the CFB-treated metatarsals, they are round. Scale bar: 20 μm. (B) The area of F4/80 staining (μm²) is quantified from 4× images and normalized to PBS. (C) Upper panel: Representative images of metatarsals treated with PBS, 200 μg/mL CFBQ32, or 200 μg/mL CFBR32 and stained for arginase 1. Scale bar: 200 μm. Lower panel: Representative 40× maximum-intensity projection confocal images of metatarsals treated as described above and stained for F4/80 (red) and arginase 1 (arg-1) (green). Cells that are double stained for both markers appear yellow (white arrows). Scale bar: 20 μm. (D) The area of arginase 1 staining (μm²) is quantified from 4× images and normalized to PBS. Error bars: SEM. **P ≤ 0.01, ****P ≤ 0.001.

Reduced risk (CFBW32 and CFBQ32) in developing AMD.4–7 Since it is only the human variants of CFB that are known to be associated with ocular pathology and to enhance the relevance of our studies to human disease, we generated variants of human CFB as recombinant proteins rather than the orthologous mouse proteins.

Our primary objective was to find out whether CFB has a role in the formation of new blood vessels, in light of previous studies linking CFB to pathologic neovascularization in mouse models, and its association with neovascular AMD. We first addressed the hypothesis that a mixture of complement proteins, rather than a single complement factor, is proangiogenic in the mouse metatarsal model. In these experiments, we used normal human serum, which contains thermo-labile components such as CFB, C5b-9, CFI, C2, C8, C7, and the thermo-stable complement C3,23,24 In the presence of 10% heat-inactivated human serum, we observed a significant reduction in angiogenic sprouting and branching in the metatarsal assay, showing that complement factors may contribute to the angiogenic process.

To specifically focus on the possible role(s) of CFB as a factor that contributes to angiogenesis in the metatarsal assay, we generated CFB variants as recombinant proteins and observed that the addition of exogenous CFBR32 at physiologic levels increased angiogenesis compared to the protective variants CFBQ32 and CFBW32. This suggests that increased vessel outgrowth and branching are indeed caused by complement activation since CFBR32 is the most active of the three variants. This is consistent with the observation of decreased membrane-bound C3/C3b in CFBR32-treated metatarsals and the accumulation of C3/C3b in the conditioned media from metatarsals after CFBQ32 treatment. C3 and C3b proteins are both present in the metatarsal assay media and potentially synthesized and expressed by several cell types, including endothelial cells, macrophages, and fibroblasts.25 Reduced C3/C3b staining is most likely the result of C3 being broken down by the C3 convertase into C3a and C3b fragments, of which the latter drives a positive feedback loop, leading to amplification of complement activation. In support of a role for endothelial cells in the production of C3, Human Umbilical Vein Endothelial Cells
(HUVECs) in culture have been reported to secrete C3 and also CFB, albeit in low amounts during basal conditions, and in previous work, we observed induction of CFB expression in the pathologic retinal blood vessels of several mouse models. Our observations here are in line with previous biophysical studies that revealed that CFB binds with highest affinity to C3b, and as such, it catalyzes the most effective C3 convertase formation and the most efficient C3 breakdown.

In this context, it was interesting to note that VEGFA protein levels in the conditioned media and Vegf transcripts of CFB-treated metatarsals were significantly increased, suggesting that this variant, apparently acting at the transcriptional level, stimulates angiogenesis via a VEGFA-dependent mechanism. Whether the elevated VEGFA levels were a consequence of increased C3 convertase formation or reflect another undescribed activity of CFB is not clear. Nevertheless, our observations fit with the known role of VEGF in neovascular AMD and also its involvement in tumor angiogenesis and in the interplay between the immune system, inflammatory cells, and cancer cells. As macrophages may be an important source of VEGF, we also investigated their phenotype in the metatarsal assay. Multiple macrophage subpopulations exist, and their identification can shed light on their role in pathology. In our study, addition of CFB to mouse metatarsals led to macrophage activation, at least on the basis of a phenotypic switch in which the shape typically became round compared to the ramified shape of macrophages in the control samples, although similar changes in macrophage cell morphology may also be due to de-differentiation. Whether the macrophage phenotype shift is a direct or indirect effect of CFB is unknown. There is evidence that CFB and C3 can be expressed by macrophages, and thus Cfb is upregulated in mouse macrophages via Toll-like receptor type 4 activation. Interestingly, we also found an increased number of F4/80-positive macrophages in mouse metatarsals in response to CFB, which may have been the result of increased cell proliferation.

An important distinction in macrophage phenotype and function is the separation into M1 and M2 subtypes. It is well established that a spectrum of phenotypes means that this is not always a simple binary issue. Here we observed that most F4/80-positive macrophages in CFB-treated metatarsals were at the M2 end of the phenotype spectrum due to the increased arginase I staining. These macrophages are typically alternatively activated, anti-inflammatory, and proangiogenic. However, despite promoting an increase in the proangiogenic M2 population, CFB stimulated significantly less angiogenesis than CFB. These observations, together with the correlation between CFB and higher levels of VEGFA, indicate that the effects of CFB on angiogenesis are not solely mediated by macrophages.

In summary, in this study, we have provided evidence that CFB, CFB, and CFB have distinct biological activities with regard to angiogenesis, with the more common CFB driving increased vessel growth accompanied by elevated levels of VEGFA.

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