Complement Factor H mutation *W1206R* causes retinal thrombosis and ischemic retinopathy in mice

Running title: *FH* mutation causes ischemic retinopathy

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Abstract

Single nucleotide polymorphisms (SNPs) and rare mutations in *Factor H* (*FH*) are associated with age-related macular degeneration (AMD) and atypical hemolytic uremic syndrome (aHUS), a form of thrombotic microangiopathy (TMA). Mice with the *FH W1206R* mutation ($FH^{R/R}$) share features with human aHUS. Here, we report that $FH^{R/R}$ mice exhibited retinal vascular occlusion and ischemia. Retinal fluorescein angiography (FA) demonstrated delayed perfusion and vascular leakage in $FH^{R/R}$ mice. Optical coherence tomography (OCT) imaging of $FH^{R/R}$ mice showed retinal degeneration, edema, and detachment. Histological analysis of $FH^{R/R}$ mice revealed retinal thinning, vessel occlusion, as well as degeneration of photoreceptors and retinal pigment epithelium (RPE). Immunofluorescence showed albumin leakage from blood vessels into the neural retina and electron microscopy demonstrated vascular endothelial cell irregularity with narrowing of retinal and choroidal vessels. Knockout of *C6*, a component of the membrane attack complex (MAC), prevented the aforementioned retinal phenotype in $FH^{R/R}$ mice, consistent with MAC-mediated pathogenesis. Pharmacologic blockade of C5 also rescued retinas of $FH^{R/R}$ mice. This $FH^{R/R}$ mouse line represents a model for retinal vascular occlusive disorders and ischemic retinopathy. The results suggest complement dysregulation can contribute to retinal vascular occlusion and that an anti-C5 antibody might be helpful for C5-mediated thrombotic retinal diseases.

Keywords: Factor H, retinal thrombosis, ischemic retinopathy

Introduction

Retinal vascular occlusive disorders collectively constitute the most common causes of visual disability in the middle-aged and elderly population [1]. Activation of the complement cascade, a component of the innate immune system, can promote retinal vascular occlusion, as seen in some patients with atypical hemolytic uremic syndrome (aHUS) [2–4].

Complement is activated *via* three pathways: classical, lectin, and alternative pathways (AP). Factor H (*FH*) regulates the AP by inhibiting the activity of C3 convertase, C3bBb, both on the cell surface and in the fluid phase [5–7]. *FH* contains 20 short consensus repeat (SCR) domains; rare mutations in SCRs 19 and 20 of human *FH* are associated with aHUS [8–10] and age-related macular degeneration (AMD) [11,12]. The C3 convertase activates the C5 convertase, which cleaves C5 into anaphylatoxin C5a and C5b. The latter promotes formation of the membrane attack complex (MAC), which contains C5b, *C6*, C7, C8, and C9. The MAC can cause lysis of microbes and human red blood cells as well as activation and injury of nucleated eukaryotic cells by creating holes in the cell membrane.

We previously generated a *W1206R* mutation in the mouse *FH* gene corresponding to the *W1183R* mutation in human *FH* found in familial aHUS [8–10]. These mice developed thrombosis in multiple organs including, as shown *in vivo* by fluorescein angiography, the retinal vasculature [13]. In the present study, we tested the hypothesis that MAC formation is necessary for the thrombosis by determining whether $FH^{R/R}C6$ double knockout (DKO) mice are protected from the thrombotic phenotype of the eye. We also performed detailed *in vivo* and histologic characterization of the retinal phenotype. Our results showed that $FH^{R/R}$ mice developed retinal vessel occlusion, delayed

and insufficient retinal perfusion, retinal detachment, inner retinal thinning, retinal pigment epithelium (RPE) degeneration, and basal RPE complement deposits. Knocking out *C6*, but not the receptor for anaphylatoxin C5a (C5a receptor 1, *C5aR1*) rescued the retinal phenotype of $FH^{R/R}$ mice, suggesting that the MAC is involved in the pathogenesis. Anti-C5 antibody treatment also protected $FH^{R/R}$ mice from developing the observed retinal phenotype. Our findings suggest a causal relationship between *FH* dysfunction-induced MAC activation and retinal vascular thrombosis.

Materials and Methods

Animals: The generation of $FH^{R/R}$ mice on a C57BL/6 background were described previously [13]. $C6^{-/-}$ mice on C57BL/6 background were generated in house from *C6* gene targeted embryonic stem (ES) cells obtained from VELOCIGENE (VG16017)[14]. $FH^{R/R}$ mice were crossed to $C5aR1^{-/-}$ [15] and $C6^{-/-}$ to generate $FH^{R/R}/C5aR1^{-/-}$ and $FH^{R/R}/C6^{-/-}$. All animals were screened for *Rd8* mutation and were negative. Age-matched littermates were used as controls, and both sexes were studied. Numbers of mice used in each experiment are shown in the corresponding figure legends. Experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmology and vision research. All protocols were approved by the animal care review board of the University of Pennsylvania.

Fundus imaging and fluorescence angiography: After anesthesia with a mixture of (in mg/kg body weight) 100 ketamine, 2 xylazine, and 2 acepromazine, bright-field imaging of the fundus was performed using a Micron III intraocular imager (Phoenix Research Labs; Pleasanton, CA). After fundus images were acquired, 100 µl fluorescein sodium (0.5mg/L, AK-Fluor, Akorn Inc.,

Lake Forest, IL) was injected intraperitoneally and fluorescence image was captured immediately at 30 second intervals.

Morphologic Analysis: Enucleated eyes were immersion-fixed in 2% paraformaldehyde/2% glutaraldehyde overnight. Then eye cups were made by removing the cornea and lens before dehydration in increasing concentrations of ethanol, infiltrated overnight and embedded the next day in plastic (JB4; Polysciences, Inc., Warrington, PA). For standard histology, 3-μm-thick plastic sections were cut in the sagittal plane and stained with 1% toluidine blude O and 1% sodium tetraborate decahydrate (Sigma-Aldrich, St. Louis, MO) for 5 seconds. Stained sections were then dried and imaged using bright-field microscopy (TE300, Nikon, Tokyo, Japan).

Immunofluorescence: After the globes were fixed in 4% paraformaldehyde, eyecups were generated by removing the anterior segment. The eyecups were infiltrated in 30% sucrose overnight and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA). Immunofluorescence was performed on 10-μm-thick frozen sections with FITC-conjugated C3 antibody (#0855500 MP Biomedicals, OH, USA), albumin (A90-134A, Bethyl, Montgomery, TX) and CD31 (ab76533, Abcam, Cambridge, MA) Control sections were treated identically with isotype matched antibodies (ab37374, Abcam, Cambridge, MA).

For immunolabeling of flat mounts, after enucleation, eyes were immersion fixed in 4% paraformaldehyde on ice for 20 minutes. Eye cups were then generated by circumferential incision at the pars plana, removing the cornea, iris, ciliary body and lens. After blocking in 10% normal donkey serum in TBS plus 0.1% Triton X-100 (TBST), eye cups were incubated in 100ul of primary antibody in 2% normal donkey serum in TBST at 4°C overnight. Eye cups were then washed 3 times by transferring the eye cup to a well with 200ul of 1xTBST, at room temperature for 10min. The secondary antibody was then applied by transferring the eye cup to a well with

100ul diluted the secondary antibody in 2% Normal Donkey serum in 1xTBST (1:200 dilution) and incubating at room temperature for 2-3hr. The eye cup was then washed three times by transferring to a well with 200ul of 1xTBST, at room temperature for 10min, then washed once by transfer of the eye cup to a well with 200ul of 1xTBS, at room temperature for 10min to remove the Triton. The retina was then dissected from the RPE/choroid/sclera, flattened with 4-6 radial incisions, placed on a slide and dried by wicking surrounding fluid with a Kimwipe. Mounting medium was applied to coverslips, which were then inverted onto the slides. Antibodies used were as follows: Rabbit anti mouse CD31 (1:100 dilution, Abcam, ab28364), Rat anti mouse CD31 (1:100 dilution, Abcam, ab7388), BV421 Rat anti mouse CD41 (1:100 dilution, BD HorizonTM, 562957), Rabbit Anti-Human Fibrinogen/FITC, (1:100 dilution, Dako, F0111, Cy3-Donkey anti Rabbit IgG (H+L) (1:200 dilution, Jackson Immuno Research Laboratories, Inc, 711-165-152, Cy3-Donkey anti Rat IgG (H+L) (1:200 dilution, Jackson Immuno Research Laboratories, Inc, 712-165-153), Alexa FluorTM 488 Donkey anti Rat IgG(H+L) (1:200 dilution, Invitrogen, A21208).

The sections were analyzed by fluorescence microscopy with identical exposure parameters (model TE300 microscope, Nikon, Tokyo, Japan) with ImagePro software (Media Cybernetics, Silver Spring, MD, USA).

Spectral domain OCT imaging: Mice were anesthetized and their pupils dilated with 1% tropicamide (Bausch & Lomb, Inc., Bridgewater, NJ). Mice were placed in the Bioptigen AIM-RAS holder and artificial tears were used throughout the procedure to maintain corneal clarity. OCT images of the retina were acquired with the Envisu R2200-HR SD-OCT device (Bioptigen, Durham, NC) with the reference arm path length set at 950 mm. Image acquisition software was provided by Bioptigen.

Electron Microscopy: Retinas (including RPE) fixed in 2% paraformaldeyhyde, 2% glutaraldehyde were postfixed in 1% osmium tetroxide, 0.1 M sodium cacodylate buffer. Specimens were dehydrated and embedded in Epon (Ted Pella, Inc., Redding, CA). Ultrathin sections were cut, stained with uranyl acetate, and examined with a JEOL 1010 transmission electron microscope.

Anti-C5 antibody treatment: $FH^{R/R}$ mice were treated with an anti-C5 monoclonal antibody [16] intraperitoneally, 1mg twice weekly for 8 weeks starting at 4 weeks of age. The same concentration of MOPC, an isotype IgG, was used as a control.

Statistical analysis: The means ± SD were calculated for each comparison pair using a two-group student's t-test. Statistical analyses for quantification of C3 immunostaining were performed in GraphPad Prism 6.0 (San Diego, CA) by the one-way ANOVA with a Tukey posttest comparing means. $P < 0.05$ was considered to be statistically significant for all analyses.

Results

$FH^{R/R}$ but not $FH^{R/R}/C6^{-/-}$ mice developed vascular occlusion and white spots visible with in vivo retinal photography

We performed retinal photography on 8 week old mice (Fig. 1) to profile ophthalmic changes in $FH^{R/R}$, $FH^{R/R}/C5aR1^{-/-}$, $FH^{R/R}/C6^{-/-}$, and in $FH^{W/W}$ (wild-type) controls. $FH^{W/W}$ mice had a normal retinal appearance. However, in $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice, we observed occluded vessels appearing as white lines (black arrows in Fig. 1 *E-H, J,* and *K*). In addition, white ischemic patches were found in both $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice (black arrowheads in Fig. 1 *E, I*), with variable numbers of white spots (Fig. 1 *E-L*). In contrast, $FH^{R/R}/C6^{-/-}$ mice were rescued, exhibiting normal retinal appearance.

$FH^{R/R}$ mice showed delayed and incomplete retinal perfusion

Fluorescein angiography (FA) was performed at 8 weeks of age. The whole retina was perfused within 30 seconds in $FH^{W/W}$ and $FH^{R/R}/C6^{-/-}$ mice, and no fluorescein leakage was observed (Fig. 2A). In contrast, both $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice exhibited delayed and/or incomplete retinal perfusion and fluorescein leakage around the optic nerve head. There was almost no perfusion at even 90 seconds after fluorescein injection in $FH^{R/R}$ mice.

Quantification of abnormal phenotypes was performed using retinal and FA images (Fig. 2B). None of the $FH^{W/W}$ (n=8) and $FH^{R/R}/C6^{-/-}$ mice (n=15) showed retinal abnormalities. In contrast, 82% of $FH^{R/R}$ (black bars in Fig. 2B, n=17) and 50% of $FH^{R/R}/C5aR1^{-/-}$ mice (white bars, n=14) had retinal vessel occlusion. 88% of $FH^{R/R}$ and 71% of $FH^{R/R}/C5aR1^{-/-}$ mice had white spots. 53% of $FH^{R/R}$ and 43% of $FH^{R/R}/C5aR1^{-/-}$ mice had ischemic patches. 100% of $FH^{R/R}$ (n=8) and 89% of $FH^{R/R}/C5aR1^{-/-}$ mice (n=9) showed perfusion delay. 100% of $FH^{R/R}$ and 67% of $FH^{R/R}/C5aR1^{-/-}$ mice showed vascular leakage. There were significant associations between genotype and likelihood of exhibiting the pathological changes.

Pharmacological blockade of C5 ameliorated retinal pathology in $FH^{R/R}$ mice

Because $FH^{R/R}/C6^{-/-}$ mice were protected from retinal vascular occlusion, we tested the therapeutic efficacy of a systemically administered anti-C5 antibody (Fig. 3 *D-F, J-L, P-R*) compared to a mouse IgG1-kappa monoclonal isotype control (Fig. 3 *A-C, G-I, M-O*). This anti-C5 antibody has been shown previously to block activation of the terminal complement cascade [16]. Retinal photos from a control antibody treated $FH^{R/R}$ mouse show hypopigmented spots (red arrowheads in Fig.

3 *A*, *G*) with leakage on FA (Fig. 3*M*) at 4 weeks of age. After 4 weeks of treatment with the control antibody, this representative mouse showed pathological progression that was indicated by ischemic patches (black arrowheads in Fig. 3*B*) and by hypopigmented spots (red arrowheads in Fig. 3*H*). There was also retinal vascular occlusion (black arrows in Fig. 3 *C*, *I*) leading to decreased retinal perfusion (Fig. 3*O*) after 8 weeks of control antibody treatment. In comparison, hypopigmented spots (red arrowheads in Fig. 3*D*), ischemic patches (black arrowhead in Fig. 3*J*), and occluded vessels (black arrows in Fig. 3*J*) that were observed before anti-C5 treatment improved after 4 and 8 weeks of treatment, as demonstrated by imaging the same mice over time (Fig. 3 *E*, *F*, *K*, *L*). Note specifically that the vessel at 5:30 o'clock in Fig. 3*P* re-opened after anti-C5 treatment (Fig. 3 *Q* and *R*); and leakage around the optic nerve head (Fig. 3*P*) stopped after anti-C5 treatment (Fig. 3 *Q* and *R*). Only one anti-C5 treated $FH^{R/R}$ mouse (Fig. 2B, gray bars, n=5) showed vessel occlusion and white spots; the others exhibited no pathological changes.

Deposition of fibrin and platelets was found in $FH^{R/R}$ mice

To detect clots, immunolabeling of retinal flat mounts was performed with anti-fibrin and anti-platelet antibodies. Retinal vessels were labeled with VEC marker anti-CD31 (red) plus either anti-fibrin (green) or anti- platelets (green). Retinas of $FH^{W/W}$ mice appeared normal (Fig. 4 *A* and *C*). However, strong staining for fibrin and platelets was observed within clots within the retinal vasculature in $FH^{R/R}$ retinas (Fig. 4 *B* and *D*).

***In vivo* OCT imaging showed retinal protection in $FH^{R/R}/C6^{-/-}$ mice**

OCT imaging was performed to assess retinal morphology *in vivo*. No abnormalities were found in $FH^{W/W}$ (Fig. 5 *A*-*E*) and $FH^{R/R}/C6^{-/-}$ mice (Fig. 5 *P*-*T*). In $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice, we

observed retinal detachment (white asterisks in Fig. 5 *F, K, M*), ONL palisading in regions of disorganized photoreceptor inner/outer segments (PR IS/OS) (red arrows in Fig. 5 *F, G, L*), thinning of inner retinas (white arrow in Fig. 5 *G, K*) and of total retinas (double white arrows in Fig. 5 *H, M*), occluded arteries (yellow arrowhead in Fig. 5 *H*), as well as retinal edema (red arrowheads in Fig. 5 *J, L, N, O*). No abnormalities were observed in anti-C5-treated $FH^{R/R}$ mice (Fig. 5 *U-X*).

Quantification of abnormalities in OCT images was performed (Fig. 5Y). 100% of both $FH^{R/R}$ (black bars in Fig. 5Y, n=5) and $FH^{R/R}/C5aR1^{-/-}$ mice (white bars, n=6) showed retinal detachment, vessel occlusion, and ONL palisading. 80% of $FH^{R/R}$ mice and 100% of $FH^{R/R}/C5aR1^{-/-}$ mice showed inner retinal thinning. 60% of $FH^{R/R}$ and 50% of $FH^{R/R}/C5aR1^{-/-}$ mice showed retinal edema. None of the $FH^{W/W}$ (n=4), $FH^{R/R}/C6^{-/-}$ (n=4), and anti-C5 treated (n=5) mice had these abnormalities. There were significant associations between genotype and likelihood of exhibiting pathological changes.

Retinal ischemia and degeneration in $FH^{R/R}$ mice

Morphologic analysis was performed at 8 weeks of age. Plastic sections of $FH^{W/W}$ (Fig. 6 *A-D*) and $FH^{R/R}/C6^{-/-}$ mice (Fig. 6 *M-P*) revealed normal retinal histology. In contrast, sections from $FH^{R/R}$ (Fig. 6 *E-H*) and $FH^{R/R}/C5aR1^{-/-}$ mice (Fig. 6 *I-L*) revealed occluded arteries and veins (yellow arrowheads in Fig. 6*I*), dilated veins (black arrowheads in Fig. 6 *G, K, L*), retinal thinning (black double arrows in Fig. 6 *F, H, L*), ONL palisading (Fig. 6 *E, F, J*), vacuolar degeneration of PR IS/OS (green arrowheads in Fig. 6 *E, G, J, L*), migration of PR nuclei towards the RPE (red arrowheads in Fig. 6 *E, G, I*), migration of RPE cells towards the ONL (green arrows in Fig. 6 *F*

and *G*), and vacuolar degeneration of the RPE (red arrows in Fig. 6 *E,J*). Among the five anti-C5 treated $FH^{R/R}$ mice, one mouse showed retinal thinning (Fig. 6Y) and another one showed focal RPE vacuolization in the peripheral retina close to the ora serrata (red arrows in Fig. 6*T*). The other three exhibited normal retinas (Fig. 6 *Q* and *R*). In comparison, control antibody treated $FH^{R/R}$ mice (n=3) showed vein dilation (black arrowheads in Fig. 6 *U, X*) and retinal thinning (black double arrowheads in Fig. 6*V*). One mouse showed minimal degeneration (Fig. 6 *U*), whereas another exhibited extensive total retinal thinning (Fig. 6 *W* and *X*). One possibility for this observation is that the $FH^{R/R}$ mutation may be associated with variable severity of retinal degeneration. This variability does not track with gender. It should be noted, however, that the three control antibody treated $FH^{R/R}$ mice were the ones that survived the full 8 weeks of antibody treatment; another five only survived between one and five weeks after treatment initiation. Therefore, there may be selection bias in that the three healthiest control antibody treated mice were analyzed, including a minimally affected one.

Quantification showed that 100% of both $FH^{R/R}$ (black bars in Fig. 6Y, n=5) and $FH^{R/R}/C5aR1^{-/-}$ mice (white bars in Fig. 6Y, n=5) had vessel occlusion. 100% of $FH^{R/R}$ and 80% of $FH^{R/R}/C5aR1^{-/-}$ mice had retinal thinning. 80% of both $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice had ONL thinning/palisading and RPE degeneration. None of the $FH^{W/W}$ (n=4) and $FH^{R/R}/C6^{-/-}$ mice (n=4) showed these pathologies. Only one anti-C5 treated $FH^{R/R}$ mouse exhibited inner retinal thinning and RPE degeneration (gray bars in Fig. 6Y, n=5). There were significant associations between genotype and likelihood of exhibiting pathological changes.

Extravascular Albumin and C3 leakage into the neural retinas of $FH^{R/R}$ mice

Albumin labeling was observed exclusively within the retinal vasculature in $FH^{W/W}$ (Fig. 7*B*), $FH^{R/R}/C6^{-/-}$ (Fig. 7*P*), and anti-C5 treated $FH^{R/R}$ mice (Fig. 7*T*). In contrast, albumin labeling was seen outside of the vasculature in $FH^{R/R}$ (Fig. 7*F*), $FH^{R/R}/C5aR1^{-/-}$ (Fig. 7*K*), and control antibody treated $FH^{R/R}$ mice (Fig. 7*X*), indicating vascular leakage.

We performed immunolabeling with anti-C3 to test for complement deposition. There was no C3 labeling in $FH^{W/W}$ mice (Fig. 7*A*, n=4). However, there was prominent sub-RPE C3 labeling in $FH^{R/R}$ (Fig. 7*E*, n=4) and $FH^{R/R}/C5aR1^{-/-}$ mice (Fig. 7*J*, n=6). Higher magnification images of sections co-labeled with a CD31 antibody against VECs showed that C3 co-localized with retinal VECs (white arrow in Fig. 7*N*) and was also present outside vessels within the NSR (red arrow in Fig. 7*N*). Patchy sub-RPE C3 labeling was still observed in $FH^{R/R}/C6^{-/-}$ (Fig. 7*O*, n=4) and anti-C5 treated $FH^{R/R}$ mice (Fig. 7*S*, n=5). Surprisingly, control antibody treated $FH^{R/R}$ mice (Fig. 7*W*, n=3) had minimal sub-RPE labeling. C3 labeling in the neural retina was similar to the albumin leakage patterns, consistent with C3 leaking into the neural retinas from the vasculature.

Pixel density analysis revealed a significantly higher sub-RPE C3 signal in $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice relative to the other groups (Fig. 7*AA*). In comparison, $FH^{W/W}$ mice had lower signal than all other groups. Notably, there was a reduction in sub-RPE C3 signal intensity, relative to $FH^{R/R}$ mice, in both anti-C5 treated $FH^{R/R}$ and control antibody treated $FH^{R/R}$ mice, suggesting a possible inhibition of sub-RPE C3 deposition conferred by antibody treatment in a non-epitope-specific manner. Regarding extravascular albumin in the NSR, the $FH^{R/R}$ group (with the exception of one mouse) along with the $FH^{R/R}/C5aR1^{-/-}$ and control antibody treated

$FH^{R/R}$ groups exhibited higher albumin signal in the NSR than the other groups (Fig 7*BB*). Only one of five anti-C5 treated $FH^{R/R}$ mice had a relatively high albumin signal.

Electron microscopy revealed extravascular pericyte swelling associated with constriction of superficial vessels in retinal and choroidal vasculature of $FH^{R/R}$ mice.

To better determine the nature of the observed vascular occlusion, ultrastructural analysis was performed using electron microscopy. Relative to $FH^{W/W}$ mice (Fig. 8 *A*, *B* and *C*, n=4), $FH^{R/R}$ mice (Fig. 8 *D-G*, n=5) exhibited decreased lumen diameters of both choroidal capillary (CC in *D*) and retinal vessels (*E-G*), and irregularly contoured VECs (black arrows in *D-G*).

Discussion

In this study, we report a mouse model of retinal vascular occlusion and ischemic retinopathy. Key pathological changes in eyes of $FH^{R/R}$ mice include retinal vascular occlusion leading to atrophic thinning of the retina. These features are also found in human retinal vascular occlusive disorders. Moreover, while previous mouse models of retinal vascular disease have utilized photothermal damage and photodynamic activation of rose bengal to induce occlusion [17], our model uniquely features occlusion secondary to a mutation in a complement regulator gene.

Previously, we showed that the renal phenotypes of $FH^{R/R}$ mice shared features of human aHUS [13]. The *W1206R* mutation in mouse *FH* is equivalent to *W1183R* mutation in human *FH* found in some aHUS patients [8–10]. This mutation impairs FH interaction with host cell surface but does not affect the complement-regulating function of FH. Thus, one major consequence of this and other similar mutations in *FH* C-terminus is susceptibility of host cells, particularly kidney and retinal

endothelial cells, to complement-mediated injury with increased risk of thrombotic vasculopathy. Glomerular thrombotic microangiopathy (TMA) is the pathological hallmark of aHUS [18]. Interestingly, ocular involvement in both adult [19–21] and pediatric patients [20,22–26] with aHUS has been reported. It is likely that most of the ocular findings in $FH^{R/R}$ mice occur secondary to TMA following MAC-mediated VEC damage, since we found extensive retinal non-perfusion and vascular leakage on FA, and normal retinas in $FH^{R/R}$ mice that also had deficiency in *C6*, a component of the MAC. Terminal complement activation most likely damaged VEC and caused stenosis of vessels, as observed by electron microscopy, predisposing the vessel wall to thrombosis, which was observed in retinal flat mounts immunolabeled with anti-fibrin and anti-platelet antibodies.

Neutrophils or monocytes are activated by C5a via the C5aR1 which is highly expressed on these cells. Activation of the C5a/C5aR pathway causes release of tissue factor to participate in clot formation in both capillaries and large blood vessels [27,28]. Deletion of *C5aR1* from $FH^{R/R}$ mice prevented macro-vessel thrombosis in several organs such as the liver and spleen but did not prevent TMA in the kidney (Ueda et al, unpublished data). Results presented here suggested that the C5a/C5aR pathway is similarly not involved in the retinal pathologies of $FH^{R/R}$ mice; instead, MAC component *C6* was. Thus, it is likely that both the retinal phenotypes and kidney TMA are MAC-dependent. It is possible that direct injury by MAC leads to endothelial swelling and narrowing of capillary lumens as we have observed in the retinas of $FH^{R/R}$ mice (Fig 8). Released hemoglobin from sheared and fragmented red blood cells passing through the narrowed lumen can scavenge nitric oxide, a major vaso-protective and platelet activation inhibitory molecule, further promoting the occurrence of retinal thrombosis [29].

Interestingly, C3 label in the retina co-localizes with extravascular albumin in *FH*$^{R/R}$ and *FH*$^{R/R}$/*C5aR1*$^{-/-}$ neural retinas, indicating leakage across a damaged blood retinal barrier, consistent with the leakage shown by fluorescein angiography. In *FH*$^{R/R}$/*C6*$^{-/-}$ and anti-C5 mAb-treated *FH*$^{R/R}$ mice, probably due to the downstream blockade of MAC formation, there was less damage to VEC and no leakage of albumin or C3 into the NSR. However, C3 label on the basal RPE most likely occurs due to the loss of RPE membrane protection by FH. Treatment with anti-C5 mAb diminished this labeling. However, this effect of anti-C5 mAb is unlikely to be related to its MAC-inhibiting activity as the control antibody had a similar effect. Whether this represented a non-specific anti-inflammatory effect similar to IVIG treatment remains to be investigated.

In patients, Purtscher and Purtscher-like retinopathy are rare occlusive thromboembolic retinopathies. Purtscher retinopathy refers to a traumatic etiology, while Purtscher-like retinopathy has non-traumatic causes [30]. Although the pathogeneses of Purtscher and Purtscher-like retinopathy are multifactorial, embolization of the retinal circulation has been proposed as the common cause of the ocular findings [31–35]. The majority of patients with Purtscher-like retinopathy show cotton wool spots and intraretinal hemorrhages on funduscopic exams, and evidence of occlusive thromboembolic retinopathy with non-perfused areas and fluorescein dye leakage from retinal arterioles, capillaries, venules, and the optic disk [30,32,36]. Previous reports showed that select patients with Purtscher-like retinopathy were successfully treated with IV Eculizumab [2,31], a humanized monoclonal anti-C5 mAb that prevents activation of the terminal complement cascade, analogous to the protective effect of anti-C5 mAb in our *FH*$^{R/R}$ mice.

Our anti-C5 mAb therapy results indicate that C5 activation participates in the pathophysiology of vascular occlusion and ischemic retinopathy in $FH^{R/R}$ mice. Our finding that an anti-C5 mAb protects against retinal thrombosis and ischemia in this aHUS model highlights differences between this model and AMD. For AMD patients, intravenous anti-C5 therapy with Eculizumab was not clinically effective. These findings also highlight the disparate retinal effects of *FH W1206R* mutation and the AMD-associated Y402H mutation. The study supports the concept that anti-C5 antibodies may represent a valuable therapeutic approach, particularly in patients with Purtscher-like retinopathy in whom complement is the primary driving factor. It will be of interest to determine whether complement may play a role in additional patients with retinal vascular thrombosis.

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Figure Legends

Figure 1. Fundus images taken at 8 weeks of age. Representative normal fundus images of *FH*$^{W/W}$ mice (A-D) are shown. *FH*$^{R/R}$ mice showed occluded vessels (black arrows in E-H), white ischemic patches (black arrowhead in E) and white spots (E-H). Similarly, *FH*$^{R/R}$/*C5aR1*$^{-/-}$ mice showed occluded vessels (black arrows in J, K), ischemic patches (black arrowheads in I) and white spots (I-L). However, *FH*$^{R/R}$/*C6*$^{-/-}$ mice had normal fundus images (M-P). See Fig. 2B for quantification.

Figure 2. A. Representative fundus images with FA at the indicated time points. Both *FH*$^{W/W}$ (top row) and *FH*$^{R/R}$/*C6*$^{-/-}$ (bottom row) showed normal fundus and FA images. An *FH*$^{R/R}$ mouse (second row) had almost no perfusion up until 90 seconds, while a *FH*$^{R/R}$/*C5aR1*$^{-/-}$ mouse (third row) had delayed and incomplete retinal perfusion and fluorescence leakage around optic nerve head. B. Quantification of abnormalities on fundus images and FA images. Abnormalities including vessel occlusion, white spots, ischemic patches on fundus images, and perfusion delay and vascular leakage on FA images were observed in *FH*$^{R/R}$ (black bars, n=17) and *FH*$^{R/R}$/*C5aR1*$^{-/-}$ mice (white bars, n=14), but not in *FH*$^{W/W}$ (horizontally striped bars, n=8) and *FH*$^{R/R}$/*C6*$^{-/-}$ (dotted bars, n=15) mice. Only one anti-C5 mAb treated *FH*$^{R/R}$ mouse (gray bars, n=5) showed vessel occlusion and white spots; the others exhibited no pathological changes. Fractions represent ratios of abnormal mice to all experimental mice for each genotype. Fisher's exact test revealed a significant association between genotype and likelihood of exhibiting all of the observed pathological changes. ***p<0.001, ****p<0.0001. The dotted lines are used to separate different graphs.

Figure 3. Serial fundus images and FAs with increasing age from representative mice before and after anti-C5 mAb treatment. In a control antibody-treated mouse, white spots were found in both eyes (red arrowheads in A,G, H)with vascular leakage in left eye (M,N). After 4 weeks of treatment with control antibody, ischemic patches (black arrowheads in B) had developed in right eye and hypopigmented spots (red arrowheads in H) and vessel occlusion (black arrow in H) were found in left eye. After 8 weeks of treatment with control antibody, additional white spots and vessel occlusion (black arrows in C, I) were seen in both eyes with corresponding hypoperfusion on FA (O). Before anti-C5 mAb treatment, white spots (red arrowheads in D), ischemic patches (black arrowhead in J), and vessel occlusion (black arrows in J) were observed, which then disappeared after 4 weeks (E, K) and 8 weeks (F, L) of anti-C5 treatment. Insufficient retinal perfusion and leakage around the optic nerve head before treatment (P) improved after treatment with anti-C5 (Q, R).

Figure 4. Fluorescence photomicrographs of retinal flat mounts showing fibrin (green in B) and platelets (green in D) staining in the retinal vasculature of *FH*$^{R/R}$ mice. Minimal labeling was seen in *FH*$^{W/W}$ mice (A, C). Vascular endothelial cells were labeled with anti-CD31 (red). Scale bar=50 μm.

Figure 5. A. OCT images of retinas. Normal retinas of *FH*$^{W/W}$ mice (A-E). In *FH*$^{R/R}$ (F-J, n=4) and *FH*$^{R/R}$/*C5aR1*$^{-/-}$ (K-O, n=4) mice, retinal detachment (white asterisks in F, K, M), ONL palisading in regions of disrupted PR IS/OS (red arrows in F, G, L), occluded vessels (yellow arrowhead in H), inner retinal thinning (single white arrows in G, K), total retinal thinning

(double white arrows in H, M), and retinal edema (red arrowheads in J, L, N, O) were found. $FH^{R/R}/C6^{-/-}$ mice (P-T, n=4) showed normal retinas (P-T). $FH^{R/R}$ mice with anti-C5 mAb treatment (U-X, n=5) had normal retinas. Scale bar=50 μm. Y. Quantification of abnormalities on OCT images. Abnormalities including vessel occlusion, retinal detachment, ONL palisading, inner retinal thinning, and retinal edema were observed in $FH^{R/R}$ (black bars, n=5) and $FH^{R/R}/C5aR1^{-/-}$ (white bars, n=6) mice, but not in $FH^{W/W}$ (horizontally striped bars, n=4), $FH^{R/R}/C6^{-/-}$ (dotted bars, n=4), and anti-C5 mAb-treated $FH^{R/R}$ (gray bars, n=5) mice. Fractions represent ratios of abnormal mice to all experimental mice for each genotype. Fisher's exact test revealed a significant association between genotype and likelihood of exhibiting all of the observed pathological changes. *p<0.05, ****p<0.0001. The dotted lines are used to separate different graphs. INL: inner nuclear layer, ONL: outer nuclear layer, IS/OS: inner segment/outer segment, RPE: retinal pigment epithelium.

Figure 6. Photomicrographs of plastic sections of mouse retinas. Each image is representative of its source eye, as pathological severity did not noticeably change with retinal location. $FH^{W/W}$ mice had normal retinas (A-D, n=4). In contrast, $FH^{R/R}$ (E-H, n=4) and $FH^{R/R}/C5aR1^{-/-}$ (I-L, n=4) mice exhibited palisading of the ONL (E, F, J), vacuolar degeneration of PR IS/OS (green arrowheads in E, G, J, L), migration of photoreceptor nuclei towards the RPE (red arrowheads in E, G, I), migration of RPE cells towards the ONL (green arrows in F, G), vacuolar RPE degeneration (red arrows in E, J), dilated veins (black arrowheads in G, K, L), occluded arteries and veins (yellow arrowheads in I), and retinal thinning (double black arrows in F, H, L). $FH^{R/R}/C6^{-/-}$ mice (M-P, n=4) showed normal retinas. $FH^{R/R}$ mice with anti-C5 mAb treatment (Q-T, n=5) had normal retinas, except for one mouse that showed focal thinning of the retina (black double arrows in S) and another mouse with focal RPE vacuolization in the peripheral retina (red arrows in T). Control antibody-treated $FH^{R/R}$ mice (U-X, n=3, W and X from same mouse) showed vein dilation (black arrowheads in U, X) and retinal thinning (black double arrows in V), One mouse showed extensive total retinal thinning (W, X). Scale bar=50 μm. Y. Quantification of abnormalities in images of plastic sections. Abnormalities including vessel occlusion, inner retinal thinning, ONL thinning/palisading and RPE degeneration were observed in $FH^{R/R}$ (black bars, n=5) and $FH^{R/R}/C5aR1^{-/-}$ mice (white bars, n=5), but not in $FH^{W/W}$ (horizontally striped bars, n=4) and $FH^{R/R}/C6^{-/-}$ (dotted bars, n=4) mice. Only one anti-C5 mAb-treated $FH^{R/R}$ mouse (gray bars, n=5) showed inner retinal thinning and RPE degeneration; the others exhibited no pathological changes. Fractions represent ratios of abnormal mice to all experimental mice for each genotype. Fisher's exact test revealed a significant association between genotype and likelihood of exhibiting all of the observed pathological changes. **p<0.01, ***p<0.001, ****p<0.0001. The dotted lines are used to separate different graphs. GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer, RPE: retinal pigment epithelium.

Figure 7. Fluorescence photomicrographs showing C3, albumin, and CD31 localization. In $FH^{W/W}$ mice (A-D, n=4), no C3 labeling was seen in the retina and RPE. However, there was prominent retinal and sub-RPE C3 labeling in $FH^{R/R}$ (E-I, n=4) and $FH^{R/R}/C5aR1^{-/-}$ (J-N, n=6) mice. No retinal labeling and patchy sub-RPE C3 labeling were seen in $FH^{R/R}/C6^{-/-}$ (O-R, n=4) and anti-C5 mAb-treated $FH^{R/R}$ mice (S-V, n=5). Control antibody treated $FH^{R/R}$ mice (W-Z, n=3) exhibited retinal C3 labeling similar to $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice, but patchy sub-RPE labeling. Albumin labeling was exclusively within the retinal vasculature in $FH^{W/W}$ (B),

$FH^{R/R}/C6^{-/-}$ (P), and anti-C5 mAb-treated $FH^{R/R}$ mice (T). Extravascular albumin labeling was seen in $FH^{R/R}$ (F), $FH^{R/R}/C5aR1^{-/-}$ (K), and control antibody treated $FH^{R/R}$ mice (X), indicating vascular leakage. Scale bar=50 μm. Higher magnification showed co-labeling of C3 with endothelial cells (white arrow in N) and extravascular C3 labeling (red arrow in N). C3 also localized to the basolateral side of the RPE (white arrowheads in I, N) and albumin was localized to both apical and basolateral sides of RPE cells (red arrowheads in F, K). Quantification of sub-RPE C3 signal intensity (AA) revealed that $FH^{R/R}$ and $FH^{R/R}/C5aR1^{-/-}$ mice had significantly higher signals than the other groups, whereas $FH^{W/W}$ mice had a lower signal than all other groups. Quantification of extravascular retinal albumin signal intensity (BB) showed that $FH^{R/R}$, $FH^{R/R}/C5aR1^{-/-}$, and control antibody treated $FH^{R/R}$ mice had significantly higher signals than $FH^{W/W}$ and $FH^{R/R}/C6^{-/-}$ mice. All anti-C5 mAb-treated $FH^{R/R}$ mice except one, and one $FH^{R/R}$ mouse had intensities similar to $FH^{W/W}$ and $FH^{R/R}/C6^{-/-}$ mice. *p<0.05, **p<0.01, ****p<0.0001, ns= not significant. INL: inner nuclear layer, ONL: outer nuclear layer, RPE: retinal pigment epithelium.

Figure 8. Electron micrographs showing choroidal and superficial retinal vessels. All sections were taken from near the optic nerve. Relative to $FH^{W/W}$ mice (A-C, n=4), $FH^{R/R}$ mice (D-G, n=5) exhibited enlarged VECs (D), narrowed vessel lumens, irregularly contoured VECs, and enlarged pericytes in small arterioles (E-G). Black arrows indicate VECs. RPE: retinal pigment epithelium, CC: choroidal capillary. Scale bar=4 μm.