A Chimeric Cfh Transgene Leads to Increased Retinal Oxidative Stress, Inflammation, and Accumulation of Activated Subretinal Microglia in Mice

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PURPOSE. Variants of complement factor H (Cfh) affecting short consensus repeats (SCRs) 6 to 8 increase the risk of age-related macular degeneration. Our aim was to explore the effect of expressing a Cfh variant on the in vivo susceptibility of the retina and RPE to oxidative stress and inflammation, using chimeric Cfh transgenic mice (chCfhTg).

METHODS. The chCfhTg and age-matched C57BL/6J (B6) mice were subjected to oxidative stress by either normal aging, or by exposure to a combination of oral hydroquinone (0.8% HQ) and increased light. Eyes were collected for immunohistochemistry of RPE-choroid flat mounts and of retinal sections, ELISA, electron microscopy, and RPE/microglia gene expression analysis.

RESULTS. Aging mice to 2 years led to an increased accumulation of basal laminar deposits, subretinal microglia/macrophages (MG/MΦ) staining for CD16 and for malondialdehyde (MDA), and MDA-modified proteins in the retina in chCfhTg compared to B6 mice. The chCfhTg mice maintained on HQ diet and increased light showed greater deposition of basal laminar deposits, more accumulation of fundus spots suggestive of MG/MΦ, and increased deposition of C3d in the sub-RPE space, compared to controls. In addition, chCfhTg mice demonstrated upregulation of NLRP3, IP-10, CD68, and TREM-2 in the RNA isolates from RPE/MG/MΦ.

CONCLUSIONS. Expression of a Cfh transgene introducing a variant in SCRs 6 to 8 was sufficient to lead to increased retinal/RPE susceptibility to oxidative stress, a proinflammatory MG/MΦ phenotype, and a proinflammatory RPE/MG/MΦ gene expression profile in a transgenic mouse model. Our data suggest that altered interactions of Cfh with MDA-modified proteins may be relevant in explaining the effects of the Cfh variant.

Keywords: CFH, AMD, MDA, malondialdehyde, CD16, hydroquinone, oxidative stress, 402H, inflammation, gene expression, microglia, macrophages, MG/MΦ, basal laminar deposits

Age-related macular degeneration (AMD) is a leading cause of blindness in the developed world.^{1,2} Age-related macular degeneration has a big impact on quality of life and is a significant risk factor for depression.³ The early stages of the disease are characterized by an accumulation of debris (altered proteins, lipids and inflammatory mediators) in the subretinal pigment epithelium (RPE) space and also external to the basal lamina of the RPE.^{4,5} This process is thought to be triggered by oxidative damage to photoreceptors and RPE.^{6–8} An immune response to this debris could promote more tissue damage, a neovascular response, or both.^{9,10} Recent genetic, epidemiologic, and histopathology data suggest an important role for complement and the immune system in the development of AMD. Complement factor H (CFH or Cfh) is an important regulator of the alternative complement pathway. A single nucleotide polymorphism, leading to a single amino acid substitution (Y402H) in CFH, significantly increases the risk for AMD.^{11–14} The mechanisms behind this increase in risk are not well understood.

Work in Cfh-deficient mice has provided very valuable information, including demonstrating the essential role of Cfh and complement components in retinal homeostasis.^{15–17} However, in contrast to AMD patients, Cfh-deficient mice have constant uncontrolled activation of complement, and depletion of complement components in the serum. Trying to mimic the process at play in AMD, we have developed Cfh transgenic mice.¹⁸ The Cfh transgenic mice express chimeric Cfh molecules: they consist of short consensus repeats (SCRs) 1 to 5 of mouse Cfh, followed by SCRs 6 to 8 of human Cfh, followed by SCRs 9 to 20 of mouse Cfh. The chimeric Cfh molecules should recognize mouse glycosaminoglycans (GAGs) and sialic acid through mouse SCR 9 to 20 and should recognize mouse C3b through its mouse SCR 1 to 5 and SCR 9 to 20. The SCR 6-to-8 segment is one of the main areas of interaction with C-reactive protein (Crp),¹⁹ malondialdehyde (MDA) adducts,²⁰ and may also be involved in interactions with GAGs.²¹ This segment also contains the sequence where the Y402H variant that has been associated to an increased risk of AMD occurs in

human patients. We have previously shown that the chimeric transgenic molecules are expressed at a level similar to that of mouse Cfh. Chimeric Cfh molecules are functional *in vivo*, rescuing serum C3 levels (C3 is depleted in mice deficient in Cfh owing to uncontrolled spontaneous activation).¹⁸ Yet, the transgenic Cfh molecules appear to be ineffective in the retina-RPE environment; the Cfh transgenic mice develop sub-RPE deposits, accumulations of wide-banded collagen, and increased lipofuscin granules.¹⁸ Basal laminar deposits (BLDs) and wide-banded collagen have been associated with early AMD.^{22–25} While some leading groups argue that lipofuscin has not been shown to be increased in the RPE cells of AMD patients,^{26–28} others have suggested that lipofuscin accumulation (or increased sensitivity to it due to complement dysregulation) plays a role in AMD pathogenesis.^{29–31}

In the current work we used the Cfh transgenic mice (chimeric CfhTg or chCfhTg) to explore the role of Cfh, and in particular SCRs 6 to 8 of the Cfh molecule, in regulating susceptibility to oxidative stress at the retina/RPE level. Our results suggest that a chimeric molecule in which SCRs 6 to 8 are mutated (human sequence instead of mouse sequence) can lead to increased susceptibility to oxidative damage (induced by aging or by oral intake of hydroquinone [HQ] and exposure to light). The expression of this chimeric Cfh molecule also led to increased accumulation of MDA-modified proteins in the retina, increased accumulation of activated subretinal microglia/macrophages (MG/MΦ), and upregulation of proinflammatory genes in the RPE/MG/MΦ of these mice.

METHODS

Animals and Genotyping of Mice

Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved by the UT Southwestern Medical Center (UTSW) Institutional Animal Care and Use Committee (Protocol No. 2009-0352). Before performing all procedures, mice were anesthetized with a ketamine-xylazine cocktail (100 mg/kg–5 mg/kg) one at a time.

The generation of the CfhTg and CfhTg/mCfhKO mice (CfhTg/mCfhKO mice express the Cfh chimeric transgene and are deficient in mouse Cfh molecules) has been described before.¹⁸ Transgenic mice expressing the 402H variant of Cfh were identified by PCR using tail DNA and used for all experiments (see Supplementary Table S1 for a list of primers). Given the C57BL/6J (B6) background, we did not expect to have the recently reported *rd8* mutation of the *Crb1* gene³²; however, genotyping and sequencing was done to confirm that they were indeed negative for this mutation by using the primers and protocols described before^{32,33} (Supplementary Figs. S1, S2). C57BL/6J mice used as controls were obtained from a UTSW breeding core facility or from the National Institutes of Aging mouse colony (both are originally derived from the Jackson Laboratories, and have been confirmed to be C57BL/6J). Mice were acclimated to our animal facility for at least 1 month before being used for experiments. Mice were bred and kept in a barrier animal facility at UTSW under normal lighting conditions with 12-hour-on/12-hour-off cycles.

Fundus Photography

Fundus photographs of mice were obtained by using a Micron III mouse fundus camera (Phoenix Research Laboratories, Pleasanton, CA, USA) as described before.¹⁸

Semiquantitative Analysis of Fundus Spots

To determine the amount of fundus spots, a semiquantitative scale was developed. Fundus images of chCfhTg and age-matched B6 mice (centered on the optic disc) were obtained with the Micron III camera. Based on the quantity and distribution of the white/yellow spots on the fundus photos, the following categories were assigned by a masked investigator: (1) 0 to few spots: if up to 10 spots were seen on the photo; (2) moderate spots: if the spots densely occupied only a limited area (within one quadrant) of the fundus, or the spots were less densely distributed but occupied less than half of the fundus; and (3) extensive spots: if the spots densely occupied more than one quadrant of the fundus or sparsely occupied more than half of the fundus. The number of eyes (observed counts) in each category and genotype group was arranged in a contingency table for χ^2 analysis.

Flat Mount Preparation and Immunostaining

Eyes from CfhTg/mCfhKO and age-matched B6 mice were enucleated, fixed in 4% paraformaldehyde, and RPE-choroid flat mounts were prepared for immunostaining as described before.¹⁸ The flat mounts were stained with (1) rabbit anti-Iba1, (2) rat anti-CD16/CD32, or (3) goat anti-MDA singly or in combination and probed by using appropriate secondary antibodies (Supplementary Table S2). The flat mounts were photographed with a Zeiss AxioObserver and/or a Zeiss Stereo Discovery fluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

Immunohistochemistry of Retinal Paraffin Sections

Eyes from 2-year-old CfhTg/mCfhKO and B6 mice were enucleated, immediately frozen in liquid nitrogen-cooled isopentane, and processed by freeze substitution for paraffin embedding as described before.³⁴ Hematoxylin and eosin stains were done for morphologic evaluation of the retina. Six-micrometer sections were deparaffinized and rehydrated in xylene and graded ethanol. Sections were then blocked, and double stained for IP-10 and Iba-1 or for NLRP3 and Iba-1, or triple stained for CD16, MDA, and Iba-1. Appropriate secondary antibodies were then applied (see Supplementary Table S2). Immunofluorescence was visualized by using a Zeiss AxioObserver epifluorescence microscope equipped with a Hamamatsu Orca 1024BT camera with high sensitivity at visible to near-infrared imaging (Hamamatsu Photonics, Middlesex, NJ, USA).

Protein Isolation From Mouse Retina for MDA ELISA

After enucleation, the posterior eye cups were immediately dissected out. The RPE and retina were separated and the retina was homogenized in 120 μ L T-PER tissue protein extraction reagent (catalog No. 78510; Thermo Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail. The homogenate was centrifuged at 10,000g for 20 minutes at 4°C and the supernatant was transferred to a new tube. Protein concentration was determined by NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The OxiSelect MDA-Adduct Competitive ELISA Kit (catalog No. STA-832; Cell Biolabs, Inc., San Diego, CA, USA) was used to determine the MDA-protein complex in the retinal samples according to the kit's protocol. This kit uses an affinity-purified rabbit polyclonal antibody that was generated against MDA-KLH (malondialdehyde-keyhole limpet hemocyanin) and

FIGURE 1. Increased accumulation of yellow spots with age in the posterior fundus of chCfhTg/mCfhKO (chCfhTg) mice. Eyes of 3-month-old and 2-year-old mice were photographed with a Micron III fundus camera, centered on the optic nerve. Fundus spots were only rarely seen in 3-month-old mice (A, B). At 2 years of age a few yellow spots can be seen in the posterior retina of the B6 mice (C, E), but many more such spots are seen in the chCfhTg mice (D, F). The mice were confirmed to be negative for the *rd8* mutation (see Supplementary Figs. 1, 2).

specifically binds to MDA-modified proteins.^{35–38} Absorbance of each well was read on a BioTek Synergy2 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm. Samples were run in duplicates.

Electron Microscopy

Mice were perfused through the heart with 1% glutaraldehyde and 2% paraformaldehyde in phosphate-buffered saline (pH 7.4). Fixed eyes were removed and sectioned behind the limbus, and posterior eye cups were processed as described before.¹⁸ For electron microscopy, 70-nm-thin sections were cut (UTSW Electron Microscopy Core), stained with 2% aqueous uranyl acetate and lead citrate, imaged with a JEOL 1200EX II transmission electron microscope (JEOL USA, Inc., Peabody, MA, USA), and analyzed by a masked investigator using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public

domain by the National Institutes of Health, Bethesda, MD, USA) for quantification of the BLDs (see Fig. 5 legend).

Retinal Pigment Epithelium Cell Isolation, RNA Extraction, and Real-Time Quantitative RT-PCR (qPCR)

Eyes were enucleated and the anterior segment and retina were removed. The remaining posterior eye cup was processed by using the SRIRS (simultaneous RPE isolation and RNA stabilization) method³⁹ in order to isolate RPE cells, together with the overlying subretinal MG/MΦ, and extract the RNA. The primers used are shown in Supplementary Table S3. The fold changes in expression of the genes in the RPE/microglia cell isolates were calculated by using the formula $RQ = 2^{-\Delta\Delta Ct}$. *GAPDH* served as an endogenous reference gene.

FIGURE 2. Increased fundus spots in old chCfhTg eyes. Eyes were photographed with the Micron III fundus camera. An actual spot count was only feasible for the young mice (**A**), which showed that there was no difference between B6 and chCfhTg mice at a young age. A semiquantitative scale was also developed: eyes were classified as having few fundus spots (fewer than 10 spots in the photographs of the posterior fundus), moderate fundus spots (if the spots occupied up to half of the fundus, usually sparsely distributed), or extensive spots if they densely occupied more than half of the fundus (see Fig. 7 for example photos for each grade). In young mice (1–8 months), all eyes fell under the first category in both chCfhTg and B6 mice (**B**). In old mice (13–16 months), there were an increased number of spots in chCfhTg eyes compared to B6 (**C**; $P < 0.01$ by χ^2). The following number of eyes was included: 11 young B6, 19 young chCfhTg, 86 old B6, and 71 old chCfhTg eyes. ON, optic nerve.

Hydroquinone Diet, Light Exposure, and C3d Staining

A grain-based rodent diet containing 0.8% HQ (AD3053; Custom Animal Diets, LLC, Bangor, PA, USA) was fed to 11.1 ± 0.5 -month-old chCfhTg/mCfhKO mice and age-matched C57BL/6J (11.0 ± 0.5 months) for 8 weeks. The diet had the following composition: 0.936 kcal/g protein, 0.405 kcal/g fat

and 2.076 kcal/g carbohydrates, and was provided as ½-inch pellets. Mice were kept in a room with special white fluorescent lights (1000 lux) directed at one side of the cages and on a 12-hour-on/12-hour-off light cycle. The cages did have normal bedding and the mice were free to roam around the cage. Fundus photographs were obtained 2 months after starting the diet. Control mice were maintained on regular diet and under normal light conditions. Eyes were collected for immunohistochemistry staining with anti-C3d antibody (goat anti-mouse C3d, 1:50; R&D Systems, Inc., Minneapolis, MN, USA) followed by rabbit anti-goat secondary antibody conjugated to AlexaFluor555 (Invitrogen, Inc., Grand Island, NY, USA) by using a previously described method.¹⁸ Sections were then mounted in gel-mount anti-fading medium, and immunostaining was visualized by using a Zeiss AxioObserver.D1 microscope equipped with an Axiocam MRm camera (Carl Zeiss Microscopy). ImageJ software was used to measure the area of the sub-RPE staining.

Statistical Analyses

SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA) was used for statistical analysis. Data are presented as the mean $\pm$ standard error of mean (SEM). The Shapiro-Wilk normality test was first applied. A two-tailed Student's *t*-test or the Mann-Whitney *U* test were performed when comparing two groups and a one-way analysis of variance (ANOVA) or Kruskal-Wallis test was done when comparing more than two groups, followed by pairwise multiple comparison procedures (Holm-Sidak, Tukey's, or Dunn's tests as recommended by the SigmaPlot software) when necessary. For the HQ diet and increased light experiment, a χ^2 test was performed for categorical data. A *P* value < 0.05 was considered significant for all statistical analyses.

RESULTS

Aging Leads to Increased Accumulation of Subretinal MG/MΦ-Expressing Markers of Activation in chCfhTg Mice

We followed up chCfhTg/mCfhKO (chCfhTg) and age-matched B6 mice for up to 2 years. In young mice (up to 8 months of age; B6, $n = 11$, chCfhTg, $n = 19$) there were very few yellow spots in the posterior fundus of both chCfhTg and B6 mice (Figs. 1A, 1B, 2A, 2B). Fundus examination in aging mice (13 months and older; B6, $n = 86$, chCfhTg, $n = 71$) revealed a clear increase in the number of yellow spots suggestive of subretinal MG/MΦ in chCfhTg mice compared to B6 mice (Figs. 1C–F, 2C).

Immunohistochemistry of RPE-choroid-scleral flat mounts revealed the presence of Iba-1+ subretinal MG/MΦ (Fig. 3). We found a strong 1:1 correlation between the number of yellow spots in fundus photos and the number of subretinal Iba-1+ cells in flat mounts ($n = 70$, $r = 0.922$, and $P < 0.001$; Supplementary Fig. S3), which is in line with observations by others.^{40–42} Quantification of the Iba-1+ cells on the flat mounts demonstrated a significant increase in aging chCfhTg ($n = 9$) mice when compared to age-matched B6 ($n = 8$) mice ($P = 0.02$), and also when compared to young chCfhTg ($n = 10$) mice ($P = 0.003$; Fig. 3C). Double staining showed that aging chCfhTg mice had an increased number of Iba-1+ CD16+ cells in the posterior fundus (Fig. 3D), compared to age-matched B6 mice ($P = 0.01$) and to young chCfhTg mice ($P = 0.04$). CD16 is considered to be a marker for proinflammatory MG/MΦ both in humans^{43–45} and mice.^{46–49}

FIGURE 3. Increased accumulation of Iba-1+ and Iba-1+/CD16+ cells in the RPE-choroid-scleral flat mounts of chCfhTg mice. (A) Representative flat mount from an old chCfhTg mouse showing a posterior flat mount (black rectangle), and a photographic field (white rectangle). A posterior flat mount is composed of four photographic fields. (B) Iba-1 staining in a photographic field, similar to that used for quantitation of cells. (C) Iba-1+ cells and (D) Iba-1+/CD16+ cells in posterior flat mounts of age-matched chCfhTg versus B6 mice. Young mice were 2 to 6 months old, while old mice were 14 to 21 months old. The following eyes were included: young B6 ($n = 10$ eyes), old B6 ($n = 8$ eyes), young chCfhTg ($n = 10$ eyes), and old chCfhTg ($n = 9$ eyes). Statistical significance: * $P < 0.05$, *** $P < 0.005$.

Increased MDA Staining in the Retina and in Subretinal MG/MΦ of Aging chCfhTg Mice

Malondialdehyde is a marker of oxidative stress and has been proposed to be important in AMD pathogenesis, perhaps partially explaining the role of Cfh variants in increasing AMD risk.²⁰ Some groups have reported an elevation of MDA in the serum^{50,51} and in the retina of AMD patients.^{20,52,53}

To test the hypothesis that chCfhTg mice have increased oxidative stress, we again prepared flat mounts from chCfhTg and age-matched B6 mice, and double stained for Iba-1 and MDA. We imaged the posterior flat mounts by taking four photographs around the optic disc (Figs. 3A, 3B) and called each photograph a “photographic field.” The four photographic fields combined (“posterior flat mount”) accounted for approximately the posterior 10 disc diameters of fundus (centered on the optic disc). Since young mice had minimal numbers of central subretinal MG/MΦ, we only tested aging mice (21-months-old). First, we corroborated an increased number of Iba1+ cells in the posterior flat mounts of chCfhTg mice ($P = 0.002$, $n = 32$ –40 photographic fields per group; Fig. 4A). Staining for MDA in the Iba-1+ cells was mainly seen in the cell body of the MG/MΦ and not on the extensions (Figs. 4B, 4C). The MDA+ cells tended to have a more activated morphology, with fewer extensions (see lower rectangular area in Figs. 4B, 4C). We found that there was an increased proportion of Iba-1+ cells that was also positive for MDA in the posterior flat mounts of chCfhTg mice compared to B6 mice ($P = 0.031$; Fig. 4D). Immunohistochemistry of retinal sections in

2-year-old chCfhTg mice confirmed that the Iba-1+/CD16+/MDA+ MG/MΦ localized to the subretinal space (B6, $n = 4$ eyes and chCfhTg, $n = 5$ eyes; Figs. 4E–G).

We then used a competitive ELISA technique to measure the level of MDA-adducts in the retina of chCfhTg versus B6 mice. Although there was no difference in the level of MDA-modified proteins in the retinas of young chCfhTg versus B6 mice (data not shown), there was a significant increase ($P < 0.01$, $n = 15$ eyes/group; Fig. 4H) in MDA-adducts in the retina of 21- to 22-month-old chCfhTg mice compared to age-matched B6 controls.

Aging Leads to Increased Accumulation of BLDs in the Sub-RPE Space of chCfhTg Mice

The accumulation of BLDs has been found to be a marker of retinal oxidative stress in various animal models.^{54,55} No BLDs were seen in electron microscopy of young B6 or chCfhTg mice (Fig. 5A). Despite no clear differences in histologic sections between aging chCfhTg and B6 mice (Supplementary Fig. S4), 2-year-old chCfhTg mice showed an increase in BLDs, compared to age-matched B6 mice (Fig. 5B). Standardized analysis of the electron microscopy images was performed by a masked investigator using ImageJ software (Fig. 5C, details in the legend). Aging chCfhTg mice had close to a doubling of the amount of electron-dense BLDs accumulating in the sub-RPE space, when compared to age-matched B6 mice ($P = 0.034$, $n = 10$ –11 eyes/group; Fig. 5D).

FIGURE 4. An increased fraction of subretinal microglia in chCfhTg mice stain intracellularly for MDA. (A) Increased Iba1+ cells in posterior flat mount photographic fields in chCfhTg mice ($n = 40$ photographic fields) compared to age-matched B6 mice ($n = 32$ photographic fields). (B, C) Digitally magnified views from the photographic fields showing cells stained with Iba-1 (B) and MDA (C). Note that while Iba-1 stains the whole microglia including the extensions, MDA mostly stains the cell bodies (compare *upper highlighted rectangles*). Also, note in the *lower highlighted rectangle* that the cell with no extensions has strong MDA staining, while the two cells with strong Iba-1 staining and many extensions do not stain positively for MDA. (D) The chCfhTg mice have an increased fraction of Iba-1+ cells that are also MDA+, compared to B6 mice (same number of fields analyzed in [A]). (E–G) Retinal section of a 2-year-old chCfhTg mouse demonstrating a subretinal cell staining positively for Iba-1 (E), CD16 (F), and MDA (G). (H) There is increased MDA level in the retinas of aging chCfhTg mice. Protein was isolated from the retinas of 21- to 22-month-old chCfhTg and age-matched B6 mice. An ELISA kit was used to determine the MDA concentration in these samples (nanogram of MDA-adduct per microgram of retinal protein). The *graph* represents the combined results of two separate experiments (15 chCfhTg eyes and 15 age-matched B6 eyes). Scale bar in (E): 20 μ m. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Gene Expression Changes in chCfhTg Mice Suggest an Increased Proinflammatory State in the RPE and Subretinal MG/M Φ

With the goal of studying the gene expression profile of RPE cells and subretinal MG/M Φ in chCfhTg mice, we used a recently published method³⁹ to isolate RPE cells. This method allows us to perform simultaneous RPE cell isolation and RNA stabilization (SRIRS), and generates high-quality RNA that has very little contamination from conjunctiva, sclera, choroid, or retina. It does contain, in addition to the RNA from RPE cells, the RNA from the subretinal MG/M Φ that is normally adhered to the RPE layer. A preliminary Illumina microarray (San Diego, CA, USA) analysis on SRIRS-generated RNA isolates (individual eyes from 2-year-old chCfhTg [$n = 3$] and B6 [$n = 3$] mice) yielded low signals. Still, interestingly, analysis of the results with Ingenuity Pathway Analysis software (Qiagen, Redwood City, CA, USA) revealed that the top three biological functions affected in chCfhTg mice compared to B6 mice were inflammatory response, hypersensitivity response, and immunologic disease (Supplementary Table S4). Gene expression analysis by qPCR (Figs. 6A, 6B) was then performed on a larger number of SRIRS-generated RNA samples from 2-year-old mice (no pooling of samples; $n = 15$ –17 eyes/group, except *NLRP3*, which included 10 eyes/group). The data demonstrated that chCfhTg mice had an increased expression of *CD68* (marker of

activated MG/M Φ ,^{56–59} $P = 0.02$), and *TREM2* (a microglial gene that is increased in activated microglia,^{60–63} $P = 0.004$). We also found a significant increase in *IP-10* (CXCL10, a chemoattractant⁶⁴ for monocytes and lymphocytes that has been found to be increased in the aqueous humor, serum, and RPE of AMD patients^{65,66} ($P = 0.008$); and *NLRP3* (the NLRP3 inflammasome plays a key role in innate immunity⁶⁷ by initiating inflammatory signals⁶⁸ and priming/activating microglia^{69,70}; it has been recently proposed to be an important, although still ill-defined, player in AMD pathogenesis,^{71–73} $P = 0.032$). These findings were corroborated on a subset of genes in a separate experiment using 1-year-old chCfhTg versus age-matched B6 mice (six eyes/group; Fig. 6C). Furthermore, immunohistochemistry of retinal sections demonstrated that subretinal microglia from chCfhTg mice (Figs. 6H–K), but not B6 mice (Figs. 6D–G), were positive for IP-10 and NLRP3.

Environmental Oxidative Stressors Lead to a Dramatic Increase in Fundus Spots and Markers of Retinal Oxidative Injury in chCfhTg Mice Compared to B6 Mice

To test the effect of the Cfh SCR 6-to-8 variant in the response of the retina to oxidative stress, we decided to combine two environmental oxidative stressors that may be relevant in AMD: light and a cigarette smoke-related toxin (HQ).^{54,55,74,75} The

FIGURE 5. Increased BLDs in 2-year-old chCfhTg mice. (A) Young (3-month-old) B6 and chCfhTg mice do not have BLDs under the RPE. (B) Old (2-year-old) chCfhTg mice have increased BLDs, compared to B6 age-matched controls. (C) Electron microscopy images were analyzed by using ImageJ software. The whole specimen (consisting of the posterior two-thirds of the retina/RPE) was imaged at $\times 4000$ magnification in a continuous nonoverlapping fashion. Then, alternating images (a total of approximately 15 images per eye) were analyzed by using ImageJ. The total area of BLDs was measured (shown in bottom half of Fig. 4C, between two arrows) and divided by the total length of RPE measured (top half of Fig. 4C, above arrowhead), and this ratio was reported as BLD units. (D) The chCfhTg mice had close to twice the amount of BLDs when compared to age-matched B6 mice ($n = 10$ B6 eyes and 11 chCfhTg eyes). Statistical significance: $*P < 0.05$.

mice were fed a diet containing 0.8% HQ and were exposed to a moderate increase in the level of light (1000 lux directed at one side of the cages and on a 12-hour-on/12-hour-off light cycle; see Methods) for 8 weeks.

A semiquantitative scale for the amount of fundus spots was developed. Representative images are shown in Figures 7A through 7C, and the scale is discussed in the legend. In old chCfhTg mice kept on a normal diet/normal light environment, a larger number of fundus spots were observed ($P < 0.01$ compared to B6; B6, $n = 86$, chCfhTg, $n = 71$; Fig. 7D). This difference became a lot more pronounced when mice were exposed to the oxidative stressor combination ($P < 0.001$; B6, $n = 18$, chCfhTg, $n = 22$; Figs. 7E, 7F). This was confirmed in a separate repeated experiment ($P < 0.001$, data not shown). Looking specifically at chCfhTg mice, there was a marked increase in fundus spots after 2 months of HQ diet and increased light ($P < 0.001$ by χ^2). However, in B6 mice no difference was observed when they were exposed to the oxidative stressors. The fundus spots were identical in appearance to those confirmed by our group and others to be Iba-1+ subretinal MG/MΦ.^{18,76–79}

Eyes from age-matched chCfhTg and B6 mice exposed to HQ and increased light were collected and processed for immunostaining to measure deposition of C3d in the sub-RPE space. We used ImageJ software to measure the area of C3d staining and found that chCfhTg mice had roughly a doubling of the area of C3d staining compared to B6 mice ($P = 0.026$; Figs. 7G–I). Finally, electron microscopy also showed a 4-fold increase in BLD accumulation in the sub-RPE space of chCfhTg

mice compared to B6 mice, after exposure to HQ in the diet and increased light ($P < 0.05$; Figs. 7J–L).

DISCUSSION

It has been proposed that 402H increases the risk of AMD owing to a reduced affinity of this Cfh variant for either Crp,^{19,80–82} GAGs,^{21,83,84} or MDA,²⁰ leading to a decreased ability of Cfh to control complement activation in the subretinal/sub-RPE region. Malondialdehyde is generated from oxidative damage (lipid peroxidation) of membrane phospholipids in the retina. It can activate microglia and can also be recognized by Cfh as an oxidation-specific epitope, or a “danger signal.”²⁰ In a patient homozygous for the 402H variant of Cfh, a decreased affinity of 402H-Cfh to MDA could prevent Cfh from being brought into play.

To improve our understanding of these early events, and while recognizing the limitations of the mouse as a model for AMD, we have generated chCfhTg mice.¹⁸ The human SCRs 6-to-8 fragment in our chimeric Cfh molecules should allow us to recapitulate the interactions (or lack thereof) with Crp, GAGs, and/or MDA.^{19,21} We have previously shown that the chimeric Cfh molecules in these transgenic mice are functional in serum (preventing the spontaneous C3 depletion that occurs in CfhKO mice).¹⁸ In the subretinal/subRPE space, however, we hypothesize that this Cfh variant has a decreased ability to function, leading to poor regulation of oxidative stress, innate immune dysregulation, and tissue injury.

FIGURE 6. Gene expression analysis of candidate genes by qPCR and immunohistochemistry confirmation. (A) RNA was extracted (SRIRS technique) from the RPE/subretinal microglia of individual eyes of 2-year-old chCfhTg and B6 mice. The data represent the combined results from three separate experiments (two separate experiments for NLRP3) normalized for B6 gene expression level. (B) Summary of the results, including the number of eyes analyzed. (C) The experiment was reproduced in 1-year-old mice by using six chCfhTg eyes and six B6 eyes. (D–K) Immunolabeling of retinal cross-sections of 2-year-old mice for IP-10 and NLRP3 confirmed the qPCR results. B6 eyes (D–G) and chCfhTg eyes (H–K) were double-stained for either the combination of Iba-1 (D, H) and IP-10 (E, I) or the combination of Iba-1 (F, J) and NLRP3 (G, K). Paired images (e.g., [D] versus [E]) are the same section stained with either Iba-1 as positive control for microglia/macrophages, or the test antibody (IP-10 or NLRP3). Arrows show positively staining cells, while arrowheads indicate the location of nonstaining cells. Scale bar: 20 μm. Statistical significance for (A) and (C): * $P < 0.05$, ** $P < 0.01$.

Our findings in aging chCfhTg mice of increased numbers of yellow fundus spots and the corresponding subretinal Iba-1+ microglia, accompanied by the increased frequency of CD16 expression, may be relevant in a model of AMD. CD16 is a marker for activated and proinflammatory MG/MΦ (perhaps with an M1 phenotype).^{43–49} Multiple lines of evidence suggest that MG/MΦ may play a role in AMD. Drusen often contain amyloid β,⁸⁵ which has been shown in various settings to attract/activate MG/MΦ.^{86–88} Also, MG/MΦ have been described in AMD specimens.^{24,89–93} Light and electron microscopy specimens have often documented macrophages/microglia closely associated to Bruch's membrane both in early stages²⁴ and late stages of AMD.⁸⁹ Also, most surgically excised choroidal neovascularization (CNV) specimens in AMD patients contain macrophages.^{90,91} Of particular interest, given

the location of the MG/MΦ in our study, Combadiere et al.⁹² and Gupta et al.⁹³ have found that patients with AMD have an accumulation of microglial cells in the subretinal space at sites of retinal degeneration and CNV.

Most of the MG/MΦ reported in AMD histopathologic specimens have been found in advanced stages of the disease. Yet, the clinical appearance (20–50-μm yellow deep spots) and optical coherence tomography findings⁹⁴ of subretinal microglia in mice could be reminiscent of subretinal drusenoid deposits (SDDs) in early AMD (hyperreflective conical lesions in the subretinal space that protrude through the ellipsoid zone).^{95,96} Also, the variable appearances in multimodal imaging⁹⁷ suggest that there are multiple types of SDDs. Microglia have not been described in SDDs in AMD specimens,^{98,99} but we propose that this question should be specifically addressed.

FIGURE 7. Oxidative stress in the retina/RPE after feeding HQ in the diet and increasing light levels. The chCfhTg (11.1 ± 0.5 months) and age-matched B6 mice (11.0 ± 0.5 months) were fed a diet containing 0.8% HQ and were exposed to increased light (see Methods). Eyes were photographed with the Micron III fundus camera and classified as having (A) few fundus spots (fewer than 10 spots in the photographs of the posterior fundus), (B) moderate fundus spots (if the spots occupied up to half of the fundus, usually sparsely distributed), or (C) extensive spots if they densely occupied more than half of the fundus. Fundus photographs were obtained and evaluated in a masked fashion. They demonstrated an increase in fundus spots in chCfhTg compared to B6 mice in the absence of HQ/light ([D], $\chi^2 P < 0.01$), which became more pronounced after 2 months of HQ and light ([E], $\chi^2 P < 0.001$). A total of 18 B6 eyes and 22 chCfhTg eyes were evaluated for the HQ/light groups. A total of 86 B6 and 71 chCfhTg eyes were evaluated for the control groups (normal diet and light). (F) Graph showing only the eyes with extensive spots. (G–I) Retinal sections were obtained at the end of the experiment and stained for C3d. The area of C3d staining under the RPE was measured by using ImageJ software ([G]; $n = 4$ eyes and a total of 18 immunohistochemistry slides from B6 mice, and $n = 3$ eyes and a total of 12 immunohistochemistry slides from chCfhTg mice; $P = 0.026$). Examples of the C3d staining are shown for B6 (H) and chCfhTg (I) mice. (J–L) With the protocol described in

Figure 4, electron microscopy images from six B6 and five chCfhTg eyes were obtained, and the amount of BLDs was quantified in a masked fashion (J), $P < 0.05$). Representative electron micrographs from B6 (K) and chCfhTg eyes (L) are shown. Scale bars for (H) and (I): 20 μm . Scale bars for (K) and (L): 10 μm . Statistical significance: * $P < 0.05$.

We speculated that the activated MG/M Φ in the chCfhTg mice were responding to an increased oxidative stimulus, which was supported by the findings of increased MDA-adducts in the retina of aging chCfhTg mice, an increased uptake of MDA by subretinal MG/M Φ , and the accumulation of BLDs under the RPE. Basal laminar deposits are associated with early AMD²⁵ and appear to correlate with RPE injury after oxidative stress in several animal models.^{54,55,100–102} Malondialdehyde-modified proteins can be recognized directly, and removed, by MG/M Φ via pattern recognition receptors (PRRs),^{103,104} but this process can promote monocyte activation and the induction of inflammatory pathways.¹⁰⁵ In the meantime, MDA can also be recognized by Cfh, which can play an anti-inflammatory role. This Cfh-MDA interaction seems to occur in a location on the MDA molecule that competes with its recognition by macrophages.²⁰ In other words, there may normally be a balance between MDA-microglial/macrophage and MDA-Cfh interactions. We propose that the SCR 6-to-8 variant in the chCfhTg mice may be disrupting the delicate balance between MDA-Cfh versus MDA-PRR interactions.²⁰ This would explain our observations in aging chCfhTg mice of (1) decreased regulation of complement activation and (2) increased microglial/macrophage activation. Of note, there is no consensus regarding the role of MDA in AMD pathogenesis. Several groups have proposed that the relevance of Cfh variants in AMD is due to altered Cfh-GAG interactions,^{21,83,84} or altered Cfh-Crp interactions,^{19,80–82} and not related to MDA. Also, while some groups have reported increased MDA in the retina of AMD patients,^{20,52,53} work from others^{106,107} raises questions about this conclusion.

A second model of chronic oxidative stress (HQ in the diet plus increased light) corroborated our findings of increased susceptibility to oxidative injury in the chCfhTg mice. Cigarette smoke is the most important environmental risk factor for AMD, and HQ is an abundant pro-oxidant in cigarette tar. Chronic cigarette smoke and HQ have been shown to cause oxidative stress to RPE, and lead to BLDs in mice.^{54,55,76,77} Although there is still debate regarding the relevance of light exposure in AMD pathogenesis,^{108–110} it has been demonstrated in vitro^{111,112} and in mouse models^{55,100,113} that light can lead to oxidative stress on RPE cells, retina, and choroidal vasculature.

Our goal was to use a mild-moderate oxidative stressor. Thus, it was not surprising that the findings in our control B6 mice were milder than those observed by Espinosa-Heidmann et al.⁵⁵ when they exposed B6 mice to HQ and blue light. Specifically, they have used B6 mice that were 4 months older than ours, and also exposed their retinas to direct pulses of blue light, using an argon laser, and have found a significant increase in BLDs. In our case the increase in light was only mild and was not directly applied to the retinas. Since mice are mostly nocturnal, and the cages still had bedding, and the light cycles were not altered, the mice were able to protect themselves from the light. Still, when exposed to HQ in combination with this mild increase in light, the chCfhTg mice accumulated a large number of subretinal MG/M Φ , had a more prominent deposition of C3d in the sub-RPE space, and had increased accumulation of BLDs, compared to age-matched B6 mice.

Gene expression analysis of the RPE/subretinal microglia from chCfhTg mice revealed several MG/M Φ -associated upregulated genes, from which we found *IP-10* and *NLRP3* to be of

particular interest. IP-10 is considered to be a marker for M1 MG/M Φ .¹¹⁴ It can be produced by MG/M Φ in response to many activating stimuli.¹¹⁵ Furthermore, it can act as a chemo-attractant for monocytes,¹¹⁶ and perhaps preferentially for proinflammatory macrophages.⁶⁴ On the other hand, upregulation of *NLRP3* by MG/M Φ in response to a variety of stressors (infections,⁶⁹ amyloid β ,^{67,70} and others⁶⁸) has recently been shown to be an important step in the priming/activation of proinflammatory MG/M Φ in a variety of diseases. We confirmed by immunohistochemistry that subretinal microglia of chCfhTg mice, but not B6 mice, were positive for IP-10 and *NLRP3*, suggesting again that Cfh variants may predispose to a proinflammatory microglial phenotype in the subretinal space.

The role of MG/M Φ in retinal homeostasis and disease is complex: they may play protective versus disease-promoting roles.^{117–119} While here we found a proinflammatory response heralded by gene expression changes (upregulation of *NLRP3*, *IP-10*, and *CD68*), increased expression of *CD16*, and increased uptake of MDA by MG/M Φ , we also detected the upregulation of *TREM-2*. *TREM-2* may be important in controlling microglial activation.^{60–63} These findings raise the question of whether subretinal MG/M Φ (or a subpopulation of these) are partly responsible for the relative difficulty in generating more advanced AMD-like changes in mice. Experiments aimed at further defining the role of different subpopulations of MG/M Φ in regulating the response of the retina/RPE to oxidative stress and inflammation will be of great interest. The results could spark interest in therapies for retinal conditions (including AMD), based on the manipulation of these important cells.

Although mice have many limitations in modeling AMD,¹²⁰ many mouse models^{54,55,77,78,101,121–135} have given us valuable information regarding retinal physiology and pathology that may be applicable to AMD and other retinal diseases. We think that our work adds to this body of knowledge. Our findings of increased MDA-modified proteins in the retina, increased ultrastructural evidence of oxidative injury (BLDs), increased microglial/macrophage activation, increased microglial/macrophage uptake of MDA, and increased proinflammatory gene expression in the chCfhTg mice are all consistent with the hypothesis that altered Cfh-MDA interactions may play an important role in generating the pathology seen in chCfhTg mice. Moreover, they confirm our hypothesis that a variant of Cfh affecting SCRs 6 to 8 is sufficient to increase the susceptibility of the retina/RPE to oxidative stress and inflammation.

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