CFH exerts anti-oxidant effects on retinal pigment epithelial cells independently from protecting against membrane attack complex

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Age Related Macular Degeneration (AMD) is the first cause of social blindness in people aged over 65 leading to atrophy of retinal pigment epithelial cells (RPE), photoreceptors and choroids, eventually associated with choroidal neovascularization. Accumulation of undigested cellular debris within RPE cells or under the RPE (Drusen), oxidative stress and inflammatory mediators contribute to the RPE cell death. The major risk to develop AMD is the Y402H polymorphism of complement factor H (CFH). CFH interacting with oxidized phospholipids on the RPE membrane modulates the functions of these cells, but the exact role of CFH in RPE cell death and survival remain poorly understood. The aim of this study was to analyze the potential protective mechanism of CFH on RPE cells submitted to oxidative stress. Upon exposure to oxidized lipids 4-HNE (4-hydroxy-2-nonenal) derived from photoreceptors, both the human RPE cell line ARPE-19 and RPE cells derived from human induced pluripotent stem cells were protected from death only in the presence of the full length human recombinant CFH in the culture medium. This protective effect was independent from the MAC formation. CFH maintained RPE cells tight junctions’ structure and regulated the caspase dependent apoptosis process. These results demonstrated the CFH anti-oxidative stress functions independently of its capacity to inhibit MAC formation.

Age-related macular degeneration (AMD) is a complex multi-factorial degenerative disease that affects 50 million individuals worldwide and is the leading cause of vision loss in developed countries. Clinically, two advanced forms of AMD are recognized, the atrophic (aAMD) and the neovascular (nAMD) forms. Whilst anti-VEGF therapies are approved for nAMD, there is no validated treatment for aAMD1. Although photoreceptors die in the macula, the initial pathogenesis of AMD, involves the degeneration of retinal pigment epithelial cells (RPE)3, which is preceded by deposits between the RPE and the Bruch membrane (BrM), recognized as drusen, a whole mark of AMD. The RPE forms a monolayer of support cells essential for photoreceptor functions, ensuring retinoid cycle, phagocytosis of photoreceptor outer segments and for maintaining the blood-retina barrier, which is disrupted during nAMD4. Because of the close interaction between RPE and photoreceptors in both nutritional and metabolic aspects5, RPE dysfunction is associated with photoreceptor degeneration.

RPE cells are permanently submitted to oxidative stress as daily amount of oxidized phospholipids shed by photoreceptors are engulfed by RPE during phototransduction6,7. Exacerbated oxidative stress in RPE contributes to AMD8 eliciting decomposition of polyunsaturated fatty acid and the formation of 4-hydroxy-2-nonenal (4-HNE), a highly reactive but relatively stable end-product of lipid peroxidation which directly contributes to...

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oxidative cell damage\textsuperscript{9,10}. Substantial evidence indicates that 4-HNE production and their deleterious effects are associated with AMD\textsuperscript{12–14}. The polymorphism Y402H of the complement factor H (CFH) has been strongly associated with a risk of developing AMD\textsuperscript{12–16}. CFH is the major inhibitor of the complement alternative pathway. It interferes with the formation and activity of the C3 convertase (C3bBb), decreases the C5b9 membrane-attack complex (MAC) formation and the two anaphylotoxins C3a and C5a. Three major regions of the 20 existing complement control protein (CCP) modules of CFH are essential for its activity and its surface binding. The CCPs1–4 modules play a crucial role in the regulation of the anti-C3 convertase activity of CFH while CCPs6–8 interfere with the formation and activity of the C3 convertase (C3bBb), decreases the C5b9 membrane-attack complex (MAC) formation and the two anaphylotoxins C3a and C5a. Three major regions of the 20 existing complement control protein (CCP) modules of CFH are essential for its activity and its surface binding. The CCPs1–4 modules play a crucial role in the regulation of the anti-C3 convertase activity of CFH while CCPs6–8 interfere with the formation and activity of the C3 convertase (C3bBb), decreases the C5b9 membrane-attack complex (MAC) formation and the two anaphylotoxins C3a and C5a. Three major regions of the 20 existing complement control protein (CCP) modules of CFH are essential for its activity and its surface binding. The CCPs1–4 modules play a crucial role in the regulation of the anti-C3 convertase activity of CFH while CCPs6–8 interfere with the formation and activity of the C3 convertase (C3bBb), decreases the C5b9 membrane-attack complex (MAC) formation and the two anaphylotoxins C3a and C5a. 

RPE cells are a major source of complement activator and inhibitor factors at the retina-choroid interface and in subretinal space\textsuperscript{22}. Photo-oxidative damaged RPE cells directly activate different complement components\textsuperscript{23,24}. The mechanisms that link the complement system and oxidative damage of RPE in early stages of AMD are insufficiently understood. A critical role of C3 has been demonstrated in the formation of sub-RPE deposits\textsuperscript{25,26} and C3a, the cleavage product of C3 induced by the activation of alternative pathway stimulates deposition of collagens IV and VI underneath the RPE and impairs the extracellular matrix turnover\textsuperscript{27}. The binding of C3a on the RPE membrane cells is also associated with oxidative stress and calcium mobilization, reticulum stress, and VEGF secretion\textsuperscript{28,29}. Sublytic levels of MAC alter the RPE barrier integrity, and induce secretion of VEGF and of pro-inflammatory cytokines\textsuperscript{30,31}. Additionally, Ramos and collaborators have demonstrated an association between C3a production and proteolytic activity of the proteasome in a mouse model of age-related RPE atrophy\textsuperscript{32}.

To better understand the role of CFH in degenerative processes underlying RPE death, we analyzed the effects of CFH and the contribution of its CCP domains on the RPE death induced by oxidative stress (4-HNE). We found that the three functional domains of CFH, CCPs1–4 anti-C3 convertase and its two binding domains CCPs6–8 and CCPs19–20 are mandatory to protect RPE cells from oxidative stress-induced cell death. CFH maintained the tight junction integrity. The protecting effect of CFH was independent from the inhibition of MAC formation and was associated with regulation of caspase-dependent apoptosis pathway.

**Results**

**Only full length CFH protects RPE from oxidative stress-induced cell death.** The effects of recCFH (300 nM), added at the time of 4-HNE (30 \( \mu \text{M} \)) exposure, was evaluated after 6 and 24 hours. The 4-HNE dose of 30 \( \mu \text{M} \) was chosen as it induced more than 50% of an ARPE-19 cells death (Supplemental Fig. 1a). A hundred times dose was chosen for recCFH (fragments or full length). Concentration of 4-HNE was chosen to reflect in vivo exposure as it was shown to accumulate in membranes at concentrations ranging from 10 \( \mu \text{M} \) to 5 \( \mu \text{M} \) in response to oxidative stimuli\textsuperscript{20,21}. We first showed that recCFH or recCFH fragments had no effect on ARPE-19 cell death. Contrariwise, the co-treatment of 4-HNE and recCFHY402H, carrying the Y402H polymorphism, did not protect ARPE-19 cells from death induced by 4-HNE (Fig. 1c). Thus, only the full length recCFH was effective to protect RPE cells from oxidative stress-induced cell death. CFH maintained the tight junction integrity. The protecting effect of CFH was independent from the inhibition of MAC formation and was associated with regulation of caspase-dependent apoptosis pathway.

**CFH protects RPE tight junctions from oxidative stress-induced disruption.** We first used an MTT (3 (4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide) colorimetric assay to investigate the effect of recCFH on the mitochondrial redox potential of ARPE-19 cultures. Co-treatment with 4-HNE and recCFH significantly protected ARPE-19 cells from death induced by 4-HNE (Fig. 1a), despite their presence in the culture medium (Fig. 1c). Only full length recCFH protects ARPE-19 cells from death induced by 4-HNE (Fig. 1a). This protection was abolished after 24 hours of culture and was associated with a decrease in the amount of recCFH in the culture medium (Fig. 1b,c). To identify the CCPs domains of recCFH, carrying the antioxidant activity, we tested several recCFH fragments. Because CCPs1–4 domains are essential for the anti-C3 convertase activity of CFH and both CCPs6–7 and CCPs19–20 are important for CFH membrane binding, we decided to test recCFH1–18 (without binding site CCPs19–20), recCFH 8–20 (with only the CCPs19–20 binding site), recCFH 1–7 (contains both anti-C3 convertase CCPs1–4 domains associated to binding site CCP7) and recCFH 7–20 (contains both binding sites without anti-C3 convertase domains). After 6 hours, none of the recCFH fragments significantly protected ARPE-19 cells from death induced by 4-HNE (Fig. 1a), despite their presence in the culture medium (Fig. 1c). Thus, only the full length recCFH was effective to protect RPE cells from 4-HNE-induced cell death. Contrariwise, the co-treatment of 4-HNE and recCFHY402H. Carrying the Y402H polymorphism, did not protect ARPE-19 cells from death, despite its presence in the culture medium (Fig. 1a–c). The protective effect of full length recCFH was investigated in hiPSC-derived RPE (iRPE) cells. iRPE cells grown in monolayers of polygonal pigmented cells (Udry et al., submitted), express most of RPE biomarkers (e.g. RPE65, RLBP1 and BEST) and show phagocytic ability. RecCFH also protected iRPE cells from 4-HNE toxicity (Fig. 1d).

RecCFH added in the culture medium was found, not only on the ARPE-19 cell membrane upon 4-HNE treatment, but also, in the cytosolic compartment (Fig. 2a). Once in contact with ARPE-19 cells exposed to 4-HNE treatment, recCFH protected C3 from cleavage to its C3 fragments (C3 Frag.), resulting in a higher C3/C3 Frag. ratio as compared to 4-HNE treated cells (Fig. 2b). Exposure to 4-HNE increased by 19% the deposit of MAC on ARPE-19 cell membranes, identified by C5b9 immunodetection (Fig. 3a,b). Treatment with full length recCFH significantly prevented MAC deposition, but treatment with its polymorphism form recCFHY402H did not (Fig. 3a,b). Although recCFH 1–7 and 1–18 did not protect from death cells exposed to 4-HNE, they significantly decreased MAC deposit on ARPE19 cells by respectively 86% and 56% (Fig. 3c), suggesting that CFH protection resulted from mechanisms unrelated to MAC deposit (Figs 1a and 3c).
while a significant increase of inos oxidative gene expression in ARPE-19 cells could be observed as compared to untreated cells (Fig. 4a). One of RPE functions is to maintain the outer blood-retina barrier by expressing tight and adherence junction proteins, such as ZO-1. 4-HNE treatment altered ZO-1 immunostaining at ARPE-19 (Fig. 4b) and iRPE (Fig. 4c) cell membranes. RecCFH protected RPE cells junction integrity (Fig. 4b,c), as quantified by count the number of ZO-1-immunolabeled fragments according to their length (Fig. 4b,c). The protective effect of recCFH from oxidative stress on the ARPE-19 or iRPE cells structure was confirmed by immunofluorescent experiments using Phalloidin with or without ZO-1 co-labeling (Supplemental Fig. 2).

CFH preserves mitochondria and nucleus structure of RPE submitted to oxidative stress. Using electron microscopy, exposure of ARP-19 to 4-HNE induced morphological features of apoptotic cells, including chromatic margination, nuclear condensation and cell fragmentation in apoptotic membrane-bound bodies (Fig. 5a,b,d). Treatment with recCFH preserved the normal cell nucleus morphology (Fig. 5g,h). Dynamic remodeling of mitochondrial morphology is also an important indicator of healthy cells. In 4-HNE-treated ARPE-19 cells, mitochondria showed fractured tubular cristae with a round form compared to untreated ARPE-19 cells, which had many of continuous tubular cristae and an egg-shaped form (Fig. 5c,f). Co-treatment with 4-HNE/recCFH preserved the framework of mitochondria (Fig. 5c,i). All together, these data demonstrated a protective effect of CFH on oxidative stress-induced cellular organelles. Because the cellular volume of ARPE-19 cells co-treated with 4-HNE and recCFH was reduced compared to 4-HNE treatment cells (Fig. 5e,h), the expression profile of genes implicated in osmotic flow was investigated. 4-HNE treatment up-regulated Kir7.1 and Kir4.1 potassium channel and aquaporin 1 (Aqp1) gene expression, while recCFH reduced significantly these gene expression (Fig. 5j).
CFH protects RPE from caspase-dependent apoptosis. Mechanisms of CFH on 4-HNE-induced cell death were studied. RecCFH reduced the number of TUNEL positive cells by 60% (vs 4-HNE treatment, \( p < 0.001 \)) (Fig. 6a). To explore whether apoptosis was caspase dependent, we measured the levels of caspase3 activation using immunohistochemistry. In untreated ARPE-19 cells, pro-caspase3 immunolabelling was observed in contrast to active caspase3 and caspase9 (Fig. 6b). Exposure to 4-HNE increased the immunolabelling signal of caspase9 and active-caspase3 (Fig. 6b). Co-treatment with recCFH reduced caspase9 and active caspase3, as shown by semi-quantified immunostaining (Fig. 6b). Pro-caspase3 is activated in the apoptotic cell death both by extrinsic (death ligand cascade involving caspase 8) and intrinsic (mitochondrial cascade implicating caspase9) pathways. In this study, expression of caspase 8 mRNA was also reduced 25 times (\( p < 0.05 \)) on RT-qPCR compared to 4-HNE ARPE-19 cells treatment (Fig. 6c). These data show that CFH regulated both extrinsic and intrinsic apoptosis pathways by modulating caspases expression.

CFH protects RPE from necrosis. Necrosis is a type of cell death morphologically characterized by swelling, rupture of intracellular organelles, and cell membrane permeabilization, measured by the release of lactate dehydrogenase (LDH). Compared to untreated ARPE-19 cells, exposure to 4-HNE showed a 430% increase of LDH levels in culture medium (Fig. 7a). Treatment with recCFH reduced the LDH increased by 56% (\( P < 0.01 \)) (Fig. 7a). On western-blot, the levels of receptor-interacting protein kinase 3 (RIP3), identified as a crucial regulator of death receptor-induced necrosis, was decreased by 36% (\( P < 0.01 \)) in ARPE-19 cells in presence of recCFH compared to 4-HNE only (Fig. 7b). In addition, necrotic cells induce pro-inflammatory cytokines. Quantitative mRNA expression measurements revealed a major increase of several interleukins (Il1β, Il6 and Il8) in 4-HNE-exposed cells compared to untreated cells (Fig. 7c). RecCFH treatment significantly reduced the expression of these inflammatory mediators (Fig. 7c).

Discussion
Oxidative stress is a recognized pathogenic factor in the complex and multi-factorial occurrence of AMD. CFH was previously shown to protect RPE from hydrogen peroxide, but the exact mechanisms of CFH on oxidative stress-induced damages in RPE has remained imperfectly understood. In this study, we showed that only the full length CFH protected RPE cells from death, contrariwise, this effect was abolished by the polymorphism CFHY402H, demonstrating the importance of the CFH-CCP7 domain binding site. The binding CCP7 seems
mandatory to mediate protection against 4-HNE-induced cell death. Factor H, the main alternative complement pathway (AP) regulatory protein that circulates in the plasma, controls AP activation and MAC formation on the surface of host cells through its interaction with GAGs, anionic molecules and complement C3 fragment displayed on the cell membrane. In this study, we showed that full length recCFH reduced C3 cleavage and C5b9 deposit on ARP19 cell surface upon 4-HNE treatment, demonstrating a CFH functional activity on AP activation and MAC formation. The CFH domains (CCPs1-4), that has regulatory effect on the AP activation but do not bind on cell membrane, did not protect RPE from oxidative stress death. On the other hand, reduction of C5b9 deposit did not seem to be the major mechanism of cell death inhibition as there was no correlation between MAC and cell death inhibition using the different CFH fragments. Indeed, to be active on cell death, CFH needed to have the AP-regulatory domains responsible for the MAC formation and its two binding sites (with an intact CFH-CCP7 domain). Consistent with our data, it has been shown that protection against cell death is not achieved with CFH anti-C3 convertase domains but that it requires a cooperative bivalent binding of CFH at the cell membrane surface. The CCP7 domain seems to play a major role as the recCFHY402H, carrying the Y402H polymorphism, did not show any protecting effect, which could be one of the mechanisms of susceptibility to AMD in the population carrying this polymorphic variant.

The identification of CFH ligands at the surface of apoptotic cells remains unclear. Lipids are unlikely to be ligands for CFH on apoptotic cells but the calcium-dependent phospholipids-binding protein Annexin-II, involved in communication between cell membranes and the cytoplasm and in membrane trafficking and remodeling, could be a CFH binding partner on apoptotic cells. Interestingly, using different constructs of CFH, Leffler and collaborators show that fragments comprising CCPs6-8 and CCPs19-20 bind on apoptotic cells surface with higher affinity than one binding site, consistent with a stronger survival effect of full length CFH.

Despite complement activation by down regulation of membrane bound complement regulatory proteins expression, apoptotic cells do not undergo lysis process but the calcium-dependent phospholipids-binding protein Annexin-II, involved in communication between cell membranes and the cytoplasm and in membrane trafficking and remodeling, could be a CFH binding partner on apoptotic cells. Interestingly, using different constructs of CFH, Leffler and collaborators show that fragments comprising CCPs6-8 and CCPs19-20 bind on apoptotic cells surface with higher affinity than one binding site, consistent with a stronger survival effect of full length CFH.

**Figure 3.** CFH protects ARPE-19 cells from oxidative stress independently of reduced MAC deposit. (a) C5b9 immunostaining on ARPE-19 cells 6 h after 4-HNE (30 μM) with or without recCFH or recCFHY402H (300 nM) co-treatment. (b) As compared to 4-HNE treatment, semi-quantification of C5b9 immunostaining showed less MAC formation in ARPE-19 cells co-treated with recCFH full length, in contrast to its mutated form recCFHY402H. (c) Semi-quantification of C5b9 immunostaining revealed less MAC deposit with recCFH 1–7 or 1–18 but not with recCFH 7–20 or 8–20 fragments as compared to 4-HNE treatment only. All data were presented as mean ± s.e.m. Statistical significance was assessed using Mann-Whitney test. *P < 0.05; **P < 0.01; NS = no significant. Scale bars: 50μm.
ARPE-19 cells were protected from oxidative stress death independently of MAC formation inhibition but by specific functions of CFH. Indeed, CFH maintained RPE tight junctions and decreased caspase activation pathway just after exposure to oxidative stress. It has been demonstrated that activation of Caspase3 and C-Jun N-terminal kinase (JNK) are observed in many 4-HNE induced apoptosis cell lines. In this study, we showed that CFH down regulated, two pro-activators of Caspase3, Caspase8 and 9 expression previously inducing by exposure to 4-HNE. CFH regulated apoptotic cells death by modulating both extrinsic (caspase8) and intrinsic (caspase 9) apoptotic process. All together these data indicate that exposed to oxidative stress, CFH protected RPE tight junctions and reduced caspase activation pathway in these dying cells, but when damages were so high CFH was internalized and facilitated the removal of apoptotic cells by producing iC3b. CFH binding domains were shown to have stronger attachment to necrotic cells as compared to apoptotic cells, suggesting also a protective effect of CFH against necrosis. In our experiments, full length CFH also protected cells from necrosis as shown by the down regulation of RIP3 expression and LDH measurement.

It is recently shown that CFH, actively internalized by apoptotic RPE cells, forms complexes with nucleosomes which facilitates their phagocytosis by monocytes, ensuring an efficient removal of dying cells. The binding between CFH with nucleosomes also modulates phagocytes cytokines towards an anti-inflammatory profile. In our experiments, CFH also reduced the expression of pro-inflammatory cytokines by RPE cells, in agreement with a demonstrated reduction of IL-8 by CFH on malondialdehyde-acetaldehyde-induced RPE cell death. However, CFH has no effect on IL-8 expression induced by phorbol myristate acetate oxidative stress, suggesting that the CFH inflammatory regulation is dependent on the nature of oxidative stress.

In conclusion, this study showed that only full length CFH protected both human ARPE-19 cell line and iRPE from oxidative stress-induced cell death created by exposure to 4-HNE. Both necrosis and caspase-dependent apoptosis were reduced by CFH. Exposure to 4-HNE increased MAC deposit on RPE cells while full length CFH as well as CCPs1-7 and CCPs1-18 decreased MAC, but only full length CFH protected from oxidative stress-induced cell death, suggesting an effect independent from MAC formation. The Y402H polymorphism.
form of CFH, that is associated with the risk of AMD, lost the protective effect. Taken together, these results suggest that CFH per se exerts antioxidant protective effects on RPE cells and that blocking the alternative complement pathway activation, without restoring the activity of CFH might not be sufficient to exert full preventive and therapeutic effects in AMD.

Materials and Methods

Culture and treatment of ARPE-19 cells. The human retinal pigment epithelial cells ARPE-19, a no transformed human RPE line that displays many differentiated properties typical of RPE in vivo, were established and characterized previously. ARPE-19 were grown in 6 flat bottom cell culture dishes to a confluency in a standard incubator (37°C, 5% CO2) in DMEM: F12 (Invitrogen, France), supplemented with 10% calf serum, 2 mM glutamine, and 15 mM Hepes (complete culture medium). Confluent cells were cultured with medium containing 1% fetal calf serum for 2 weeks and then exposed to 30 μM 4-HNE (Merck, France) for 1, 6 or 24 h. Time zero of the kinetics corresponds to the moment of the stimulation with 4-HNE. To study the influence of CFH, cells were co-exposed to 300 nM of recCFH or one of its fragments (recCFH CCP1-7; CCP1-18; CCP8-20; CCP7-20 and rec CFH Y402H) produced by the Laboratoire Français du Fractionnement (LFB). Control cell cultures consist of ARPE-19 cultured in complete culture medium without treatment. Cells were washed twice with PBS 1X, detached from the flask by treatment with trypsin (Invitrogen, France), washed with complete cell culture medium and then harvested as pellet for transcriptomic or proteomic analysis.

Culture and treatment of hiPSC-derived RPE cells. General protocol modified from Singh, R. et al. was used to expand and differentiate in 60 days human induced pluripotent stem cells (hiPSC) into hiPSC-derived RPE cells (iRPE) and was recently submitted (Udry et al.) In brief, starting form hiPSC obtained from a healthy
donor, 250 to 500 embryonic body-like aggregates were plated and cultured in P60 (60 mm) cell culture dishes coated with a matrigel matrix (Corning). Following 30 days of differentiation, pigmented foci were micro dissected, collected, seeded in matrigel-coated P60 cell culture dishes and grown for an additional 30 days. Mature pigmented RPE patches were micro dissected, purified by removal of non-RPE like cellular structures, dissociated with Trypsin-EDTA and reseeded in 24-well matrigel-coated plates for further expansion and maturation until passage 3 (P3). From cells at passage 1 (P1) to cells at P3, additional 2 to 3 months of cell culture were required.

iRPE cells were incubated in a serum- and antibiotic-free retinal differentiation medium containing DMEM (high glucose, GlutaMAX Supplement, HEPES, ThermoFisher, France), Ham’s F-12 Nutrient Mix (ThermoFisher) (3:1 ratio) and 2% B-27 supplement minus vitamin A (ThermoFisher, France). Characterization of iRPE cells and experimentations took place at P3 on day 42. iRPE cells cultured on transwell plates were characterized and compared to human fetal RPE and postmortem human RPE controls (Udry et al., submitted). The expression of specific RPE markers was assessed by RT-PCR, RT-qPCR and immunofluorescence. iRPE cells grew in monolayers of polygonal pigmented cells, demonstrated specific RPE markers expression and generated high TER levels (300 Ω • cm²). For experimentations, iRPE cells were seeded and grown at P3 in 6-well (cell viability assay, semi quantitative Western blot analysis and RT-qPCR) or 24-well (immunocytochemistry) cell culture plates for 42 days. One week prior to experimentations, 1% fetal bovine serum was added to cell culture medium. At day 42, iRPE cells were treated for 6 h with 30 μM 4-hydroxy-2-nonenal (4-HNE) (Merck, France) ± 300 nM recCFH (Laboratoire français du fractionnement et des biotechnologies LFB, France). Untreated cells served as control.

**Cell viability assays.** Cell viability was assessed by counting trypan blue-excluding cells after adding 0.5% trypan blue and by monitoring LDH (lactate dehydrogenase) release into the culture, with a cytotoxicity detection kit (Roche Diagnostics, Meylan, France) according to manufacturer’s recommendations. A micro plate reader calibrated with 600 and 490 nm directly measured the absorbance.

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**Figure 6.** CFH regulates caspase dependent apoptosis. (a) TUNEL staining was performed and quantified in ARPE-19 cells 1 h after 4-HNE (30 μM) or after 4-HNE (30 μM) and recCFH (300 nM) treatment. RecCFH protected ARPE-19 cells from apoptosis (b) Immunostaining of pro-caspase3, active caspase 3 and caspase 9 was performed 1 h after ARPE-19 4-HNE or 4-HNE/recCFH treatment (30 μM). Semi-quantification of caspase-immuno staining showed an increase of pro-caspase 3 cleavage by caspase 9 in ARPE-19 upon 4-HNE treatment as compared to a co-treatment with recCFH. (c) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis showed a decrease of caspase 8 mRNA expression in ARPE-19 cells 1 h after treatment in presence of recCFH (300 nM) as compared to 4-HNE (30 μM) treatment alone. All data are presented as mean ± s.e.m. Statistical significance was assessed using Mann-Whitney test. *P < 0.05; **P < 0.01; ***P < 0.005, NS = no significant. Scale bars: 50 μm.
Measurement of mitochondrial redox potential. Mitochondrial redox potential was assessed spectrophotometrically with an MTT assay (Sigma-Aldrich, France). Cells were seeded at 20,000 cells per well in a 12-well plate. At 30 days after the final culture medium change, cells were stimulated with 4-HNE or both 4-HNE and recCFH for 6 hours. After cell stimulation, cells were washed once with PBS pre-warmed to 37 °C and incubated at 37 °C in 5% CO₂ in a solution of MTT (1 mg mL<sup>−1</sup> in PBS) pre-warmed to 37 °C. After 1 h, isopropanol (final concentration 50%) was directly added to the MTT solution, and the 12-well plates were slowly rotated for 10 min at room temperature. The absorbance was directly measured at 570 nm in a microplate reader.

Transmission electron microscopy. ARPE-19 cells were fixed in 2.5% glutaraldehyde cacodylate buffer (0.1 M, pH 7.4) and then fixed in 1% osmium tetroxide in cacodylate buffer (0.2 M, pH 7.4) and progressively dehydrated in graduated ethanol solution and finally in propylene oxide. Cells were contrasted by uranyl acetate and analyzed with a transmission electron microscope (Philips CM10).

Tunnel experiments. Apoptotic cells were visualized by the terminal deoxynucleotidyl transferase-mediated dUTP end-labeling (TUNEL) technique using the Dead End Colorimetric TUNEL system (Promega, France) after 6 hours of 4HNE and/or recCFH co-treatments.

Immunohistochemistry. For immunohistochemical studies, ARPE-19 or iRPE cells were fixed for 15 min at 4 °C with paraformaldehyde 4% diluted in PBS solution, incubated in PBS/BSA 0.1%, permeabilized in 0.1% Triton X-100 for 20 min, saturated with normal goat serum (Cliniscience, France) 10%/PBS for 30 min at 4 °C and then stained one night at room temperature in selective primary antibodies diluted in 0.2% Triton X-100 in PBS: anti-C5b9 (1:500, rabbit, Abcam, France), anti-C3 (1:300, rabbit, Invitrogen, France), anti-C3 fragments (1:100, mouse, Hycult, France), anti-ZO-1 (1:300, rabbit, Invitrogen, France), Phalloidin (1:700, rabbit, Invitrogen, France), anti-Caspase3 (1:700, rabbit, Santa-Cruz, France), anti-Caspase3 active (1 :700, rabbit, BD Biosciences, France) and anti-Caspase9 (1:700, rabbit, Cell signaling, France) and anti-CFH (1 :700, mouse, R&D system, France). After three washes in PBS/triton 0.1%, ARPE-19 cells were incubated in a solution of 1:200 of secondary antibody conjugated to Alexa (red 594 nm or green 488 nm; Molecular Probes, Interchim, France) and corresponding
Table 1. List of forward and reverse primers for qPCR experiments.

| Gene  | Forward 5'→3' | Reverse 5'→3' |
|-------|---------------|---------------|
| Kir1.1 | CAGGACCCTGGGACACACTCT | GGGATCAAGGAGAGAAGG |
| Kir7.2 | CCCCACCTGAAAACACACTATCTG | GCCATGAGGCCTAGACAC |
| Asp1  | TGACCACCTCCTGGCTAATTG | GGGCCAGGAGTAAAGTCTAG |
| IL6   | GATGGATGCTCCAATCTGGGT | AGTCTGCATTAGAGAACACAATA |
| IL8   | CGATGTCATCGATAAAAGCA | TGAATTCTCAGGCCCTCTACAAA |
| IL1beta | CATCGCAACCTCTCAAGCAG | GAGTGCACATTGCAGGAGG |
| Caspase8 | CTGCTGGGATGGCCACTTGGT | TCCCTGAGGACATGGCTCTC |
| Catalase | TAAAGCCTAGCAGGACA | CAAACCCTTGGAGATCGAAA |
| Gpx   | CCTCAAGTGACCTCGGACCTG | CAATGTCCTGCGGCACACC |
| Inos  | GTCTCCAAGGCACAGTCT | GCAGGTCACTTTAGCTACTTAC |
| Actin | AGGGAGAGCCTTGCTACGTG | AGGGGCGCGACTCGTCATAC |

results are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 5 software. For data related to qPCR and western blot, comparison between two groups was performed using Mann–Whitney test.
References

1. Petrokhin, K. New therapeutic targets in atrophic age-related macular degeneration. Expert Opin. Ther. Targets 11, 625–639 (2007).

2. Bhutto, I. & Lutty, G. Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch’s membrane/choriocaillaris complex. Mol. Aspects Med. 33, 295–317 (2012).

3. Zarbin, M. A. Age-related macular degeneration: review of pathogenesis. Eur J Ophthalmol 8, 199–206 (1998).

4. Letelier, J., Bovolenta, P. & Martinez-Morales, J. R. The pigmented epithelium, a bright partner against photoreceptor degeneration. J. Neurogenet. 31, 203–215 (2017).

5. Marmorstein, A. D. The Polarity of the Retinal Pigment Epithelium. Traffic 2, 867–872 (2001).

6. Salomon, R. G., Hong, L. & Hollyfield, J. G. Discovery of carboxyethylpyrroles (CEPs): critical insights into AMD, autism, cancer, and wound healing from basic research on the chemistry of oxidized phospholipids. Chem. Res. Toxicol. 24, 1803–1816 (2011).

7. Beatty, S., Koh, H., Phil, M., Henson, D. & Boulton, M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 45, 115–134 (2000).

8. Datta, S., Cano, M., Ebrahimi, K., Wang, L. & Handa, J. T. The impact of oxidative stress and inflammation on RPE degeneration in non-neovascular AMD. Prog Retin Eye Res 60, 201–218 (2017).

9. Schutt, F., Bergmann, M., Holz, F. G. & Kopitz, J. Proteins modified by malondialdehyde, 4-hydroxynonenal, or advanced glycation end products in lipofuscin of human retinal pigment epithelium. Invest. Ophthalmol. Vis. Sci. 44, 3663–3668 (2003).

10. Choudhary, S. S. et al. Toxicity and detoxification of lipid-derived aldehydes in cultured retinal pigmented epithelial cells. Toxicol. Appl. Pharmacol. 204, 122–134 (2005).

11. Ethen, C. M., Reilly, C., Feng, X., Olsen, T. W. & Ferrington, D. A. Age-related macular degeneration and retinal protein modification by 4-hydroxy-2-nonenal. Invest. Ophthalmol. Vis. Sci. 48, 3469–3479 (2007).

12. Kaarniranta, K. et al. Geldanamycin increases 4-hydroxynonenal (HNE)-induced cell death in human retinal pigment epithelial cells. Neurosci. Lett. 382, 185–190 (2005).

13. Klein, R. J. et al. Complement factor H polymorphism in age-related macular degeneration. Science 308, 385–389 (2005).

14. Hageman, G. S. et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc. Natl. Acad. Sci. USA 102, 7227–7232 (2005).

15. Edwards, A. O. et al. Complement factor H polymorphism and age-related macular degeneration. Science 308, 421–424 (2005).

16. Haines, J. L. et al. Complement factor H variant increases the risk of age-related macular degeneration. Science 308, 419–421 (2005).

17. Oppermann, M. et al. The C-terminus of complement regulator Factor H mediates target recognition: evidence for a compact conformation of the native protein. Clin. Exp. Immunol. 144, 342–352 (2006).

18. Pangburn, M. K. Cutting edge: localization of the host recognition functions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. J. Immunol. 169, 4702–4706 (2002).

19. Hellwage, J. et al. Complement C2b/C3d and cell surface polyanions are recognized by overlapping binding sites on the most carboxyl-terminal domain of complement factor H, J. Immunol. 169, 6935–6944 (2002).

20. Shaw, P. X. et al. Complement factor H genotypes risk type of age-related macular degeneration by interaction with oxidized phospholipids. Proc. Natl. Acad. Sci. USA 109, 13757–13762 (2012).

21. Weismann, D. et al. Complement factor H binds malondialdehyde epitopes and protects from oxidative stress. Nature 478, 76–81 (2011).

22. Anderson, D. H. et al. The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis revisited. Prog Retin Eye Res 29, 95–112 (2010).

23. Sokolov, V. S. et al. Interaction of pyridinium bis-retinoid (A2E) with bilayer lipid membranes. J. Photochem. Photobiol. B, Biol. 86, 177–185 (2007).

24. Brandstetter, C., Holz, E. G. & Krohne, T. U. Complement Component C5a Primes Retinal Pigment Epithelial Cells for Inflammassome Activation by Lipofuscin-mediated Phototoxic Damage. J. Biol Chem. 290, 31189–31198 (2015).

25. Fernandez-Godino, R., Garland, D. L. & Pierce, E. A. A local complement response by RPE causes early-stage macular degeneration. Hum. Mol. Genet. 24, 5555–5569 (2015).

26. Liu, L. et al. The R345W mutation in EFEMP1 is pathogenic and causes AMD-like deposits in mice. Hum. Mol. Genet. 16, 2411–2422 (2007).

27. Fernandez-Godino, R. & Pierce, E. A. C3a triggers formation of sub-retinal pigment epithelium deposits via the ubiquitin proteasome pathway. Sci Rep 8, 9679 (2018).

28. Kunchithapautham, K., Atkinson, C. & Rohrer, B. Smoke exposure causes endoplasmic reticulum stress and lipid accumulation in retinal pigment epithelium through oxidative stress and complement activation. J. Biol. Chem. 289, 14534–14546 (2014).

29. Busch, C. et al. Anaphylatoxins Activate Ca2+-, Akt/PI3-Kinase, and FOXO1/FOX3 in the Retinal Pigment Epithelium. Front Immunol. 8, 703 (2017).

30. Georgiannakis, A. et al. Retinal Pigment Epithelial Cells Mitigate the Effects of Complement Attack by Endocytosis of C5b-9. J. Immunol. 195, 3382–3389 (2015).

31. Kunchithapautham, K. & Rohrer, B. Sublytic membrane-attack-complex (MAC) activation alters regulated rather than constitutive vascular endothelial growth factor (VEGF) secretion in retinal pigment epithelium monolayers. J. Biol. Chem. 286, 23717–23724 (2011).

32. Ramos de Carvalho, J. E. et al. Complement factor C3a alters proteasome function in human RPE cells and in an animal model of age-related RPE degeneration. Invest. Ophthalmol. Vis. Sci. 54, 6489–6501 (2013).

33. Uchida, K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog. Lipid Res. 42, 318–343 (2003).

34. Krills, M. et al. Dual roles of different redox forms of complement factor H in protecting against age related macular degeneration. Free Radic. Biol. Med. 129, 237–246 (2018).

35. Meri, S. & Pangburn, M. K. Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. J. Biol. Chem. 287, 3982–3986 (1999).

36. Leffler, J. et al. Annexin-II, DNA, and histones serve as factor H ligands on the surface of apoptotic cells. J. Biol. Chem. 285, 3766–3776 (2010).

37. Perkins, S. J., Fung, K. W. & Khan, S. Molecular Interactions between Complement Factor H and Its Heparin and Heparan Sulfate Ligands. Front Immunol. 5, 126 (2014).

38. Waisman, D. M. Annexin II tetramer: structure and function. Mol. Cell. Biochem. 149–150, 301–322 (1995).

39. Renner, B. et al. Annexin A2 Enhances Complement Activation by Inhibiting Factor H. J. Immunol. 196, 1355–1365 (2016).

40. Trouw, L. A. et al. C5b-binding protein and factor H compensate for the loss of membrane-bound complement inhibitors to protect apoptotic cells against excessive complement attack. J. Biol. Chem. 282, 28540–28548 (2007).

41. Botto, M. & Walport, M. J. Clq, Autoimmunity and Apoptosis. Immunobiology 205, 395–406 (2002).

42. Martin, M. et al. Factor H uptake regulates intracellular C3 activation during apoptosis and decreases the inflammatory potential of neutrophils. Cell Death Diff. 23, 903–911 (2016).

43. Singh, S. S. et al. Antioxidant role of glutathione S-transferases: 4-Hydroxynonenal, a key molecule in stress-mediated signaling. Toxicol. Appl. Pharmacol. 289, 361–370 (2015).

44. Dunn, K. C., Aotaki-keen, A. E., Putkey, F. R. & Hjelmeland, L. M. ARPE-19, A Human Retinal Pigment Epithelial Cell Line with Differentiated Properties. Experimental Eye Research 62, 155–170 (1996).
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C.B., J.C., K.D., L.S. and M.C.N. performed the experiments; L.J. and M.S. highly contributed to obtain MET pictures; T.A. and S.J. synthesized all forms of recCFH and recCFH fragments; C.K., M.E.S., Y.A. and A.S. participate to the data analysis; S.J., V.D., F.B.C., T.A. and C.B. conceptualized experiments; V.D., F.B.C., C.B., T.A. and S.J. interpreted data; V.D., S.J., A.S., Y.A. and F.B.C. wrote the manuscript; V.D. was responsible for research supervision.

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