

## **Supplemental Information**

### **Complement Factor H Inhibits**

### **CD47-Mediated Resolution**

### **of Inflammation**

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**Figure S1: CFH-deficiency does not alter *in vitro* pathogenic cytokine secretion of microglial cells and monocytes.**

We previously showed that MP derived IL-1 $\beta$ , IL-6, and TNF $\alpha$  induce photoreceptor degeneration and deregulate RPE-cell functions (Eandi et al., 2016; Hu et al., 2015; Levy et al., 2015a; Mathis et al., 2016). Cytokine multiplex analysis of supernatants from cultured primary bone marrow monocytes (BM-Mos, 100 000 cells/well) and brain microglial cells (MCs, 200 000 cells/well), incubated for 24 h in serum free DMEM medium or stimulated with APOE3 (5  $\mu$ g/ml as previously described (Levy et al., 2015b)) of *Cx3cr1<sup>GFP/GFP</sup>* and *Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup>* mice revealed no significant CFH-dependent differences in the secretion of these pathogenic cytokines in basal or stimulated conditions, suggesting that CFH did not significantly affect the secretion of these cytokines.

**Figure S2: Plasma C3 concentrations and C3, and C3 fragment, and CFH immunohistochemistry in the transgenic mice.**

A: Complement factor C3 (C3)-ELISA measurements of plasma C3 concentrations from the indicated mouse strains indicate that C3 plasma levels in *Cx3cr1<sup>GFP/GFP</sup>* mice do not differ from WT C57BL6/J mice and TRE2 mice from TRE3 mice and *Cfh*-deficiency induces low circulating levels of C3 in both strains, likely due to un-inhibited plasma complement activation and exhaustion (Pickering et al., 2002).

B-D: Immunohistochemistry (red) for C3 (B, clone 11H9 Hycult biotech), C3b/iC3b/C3c (C, clone 3/26 Hycult biotech), and CFH (A, ab8842 Abcam) in 4d light-challenged *Cx3cr1<sup>GFP/GFP</sup>* and *Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup>* mice (GFP in green, nuclear stain in blue). (B) C3 was strongly detected in the choriocapillaries (arrow) of *Cx3cr1<sup>GFP/GFP</sup>* mice, but not in and around subretinal MPs. In *Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup>* mice that are characterized by low plasma C3 levels the signal in the choriocapillaries (adjacent to the RPE) was reduced. (C) Immunohistochemistry using the anti-mouse-C3b/iC3b/C3c antibody (clone 26/3 (Mastellos et al., 2004)) that specifically recognizes C3b and its fragments revealed a faint staining in the choriocapillaries (arrows), but no staining in the subretinal space of both mouse strains. (D) CFH was detected surrounding subretinal MPs (arrows), RPE and in the choriocapillaries (arrow head) in *Cx3cr1<sup>GFP/GFP</sup>*, but not in *Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup>* mice.

ONL: outer nuclear layer; RPE retinal pigment epithelium. negative control: omitting the primary antibodies revealed no staining (not shown); the experiment was repeated three times with similar results. Scale bar = 50 $\mu$ m.

**Figure S3: *Cfh* induction in monocytes *in vitro* and monocyte elimination after subretinal adoptive transfer.**

A: Quantitative RT-PCR of *Cfh* mRNA normalized with *Rps26* mRNA in WT C57BL6/J or *Cx3cr1<sup>GFP/GFP</sup>* monocytes cultivated for 18 h with or without photoreceptor outer segments to simulate the subretinal environment (POSSs, one way Anova/Bonferroni test \*p=0,0082 versus C57BL6/J Monocytes without POS,  $\dagger$ p<0,0001 versus C57BL6/J Monocytes with and without POS). *Cfh* mRNA is robustly induced in BM-Mos from WT C57BL6/J-, and even more so from *Cx3cr1<sup>GFP/GFP</sup>*-mice in contact with POS.

B: Representative micrograph of CFSE<sup>+</sup> Monocytes on RPE flatmounts of the indicated strains 24h after subretinal adoptive transfer to *Cfh<sup>-/-</sup>* mice. Quantification of CFSE<sup>+</sup> Mos of the indicated strains 24h after adoptive transfer to *Cfh<sup>-/-</sup>* mice (one way Anova/Bonferroni test

\*p<0,001 versus C57BL6/J CFSE<sup>+</sup> Mos, \$p=0,0043 versus *Cx3cr1*<sup>GFP/GFP</sup> CFSE<sup>+</sup> Mos, †p=0,0178 versus *Cx3cr1*<sup>GFP/GFP</sup> *Cfh*<sup>-/-</sup> CFSE<sup>+</sup> Mos).

POS: photoreceptor outer segments, Mos: monocytes; n=number of replicates indicated in the graphs, for A n=replicates represent different culture wells of the indicated conditions, the experiment was repeated twice with similar results; for B n=replicates from individual mice from two experiments with two different cell preparations. Scale bar B = 50µm.

#### Figure S4: Complement component expression in retina, liver, and MPs.

Quantitative RT-PCR of *Cfh* (A-C), *C3* (D-E), *Cfb* (G-I), and *Cfi* (J-K) mRNA, normalized with *Rps26* mRNA, in retina (1), retina without MCs (2, after sorting of MCs), liver (3), bone marrow monocytes (BM-Mo), retinal microglia (5, MC Retina) and brain microglia (6, CNS MC) from WT C57BL6/J mice (left column), and of BM-Mo, and MCs from retina and CNS of the indicated mouse strains (middle and right column). (A) MCs robustly expressed *Cfh* mRNA (~1/4 of liver expression level) significantly (~20-40 fold) more than BM-Mo (B and C). In contrast, *C3* mRNA was found in BM-Mos (B, ~1/60 of liver expression level), significantly (~50 fold) more than in MCs (E and F). *Cfb* mRNA transcription in MPs was detectable (~1/1000 of liver expression level, G) in all MPs with a relatively stronger expression in MCs (H and I). Interestingly, *Cfi* mRNA, robustly expressed in the liver (J), but was not detectable in MPs (K and L). Retinal and CNS MCs expressed the transcripts similarly and except for *Cfh* mRNA in *Cfh*<sup>-/-</sup> strains, we did not detect strain-dependent differences in the MPs.

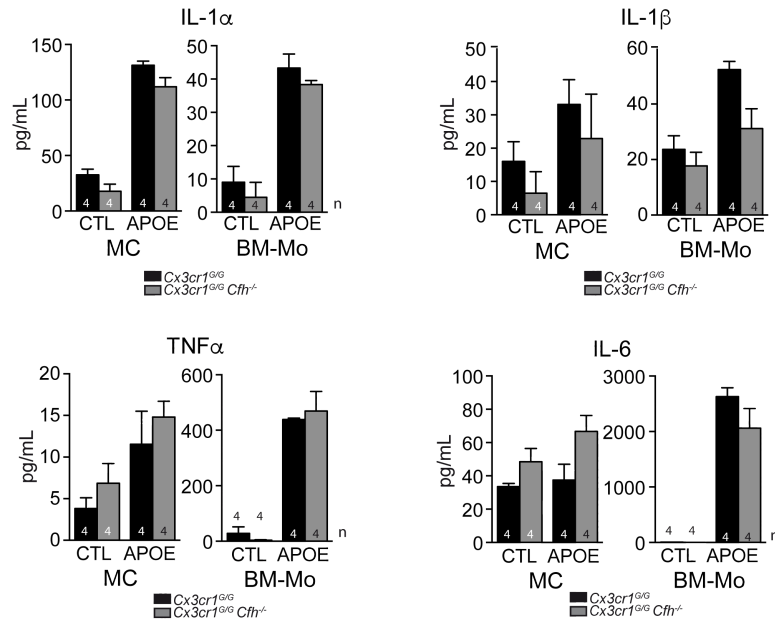
BM-Mo: bone marrow monocytes; MC: microglial cell. CNS: central nervous system. A, D, G, and J n= replicates from individual mice; B, C, E, F, H, I, K, and L n= replicates from mRNA pooled from two individual mice.

#### Figure S5: Cd11b, Cd18, Cd47 and Thbs1 transcription in MPs and TSP-1 plasma levels of the transgenic mice.

Quantitative RT-PCR of *Cd11b* (A-C), *Cd18* (D-E), *Cd47* (G-I), and *Thbs1* (J-L) mRNA, normalized with *Rps26* mRNA, in retina (1), retina without MCs (2, after sorting of MCs), liver (3), bone marrow monocytes (BM-Mo), retinal microglia (5, MC Retina) and brain microglia (6, CNS MC) from WT C57BL6/J mice (left column), and of BM-Mo, and MCs from retina and CNS of the indicated mouse strains (middle and right column). Both, *Cd11b* and *Cd18* mRNA were expressed specifically in MPs and not detected in mRNA from retina without the MC population (A and D). While *Cd11b* mRNA was more strongly expressed in MCs than in BM-Mo, no significant difference was detected in *Cd18* transcription (B, C, E, and F). (G-I): *Cd47* mRNA was expressed ubiquitously with a tendentially higher expression in MCs compared to BM-Mos (C57BL6/J-, *Cx3cr1*<sup>GFP/GFP</sup>, *Cx3cr1*<sup>GFP/GFP</sup> *Cfh*<sup>-/-</sup> mice). (J-L) *Thbs1* mRNA expression was more strongly expressed in MCs than in BM-Mo of C57BL6/J-, *Cx3cr1*<sup>GFP/GFP</sup>, *Cx3cr1*<sup>GFP/GFP</sup> *Cfh*<sup>-/-</sup> mice, with differences less marked in TRE3- versus TRE2-mice. Retinal and CNS MCs expressed the transcripts similarly and we did not detect strain-dependent differences in the MPs.

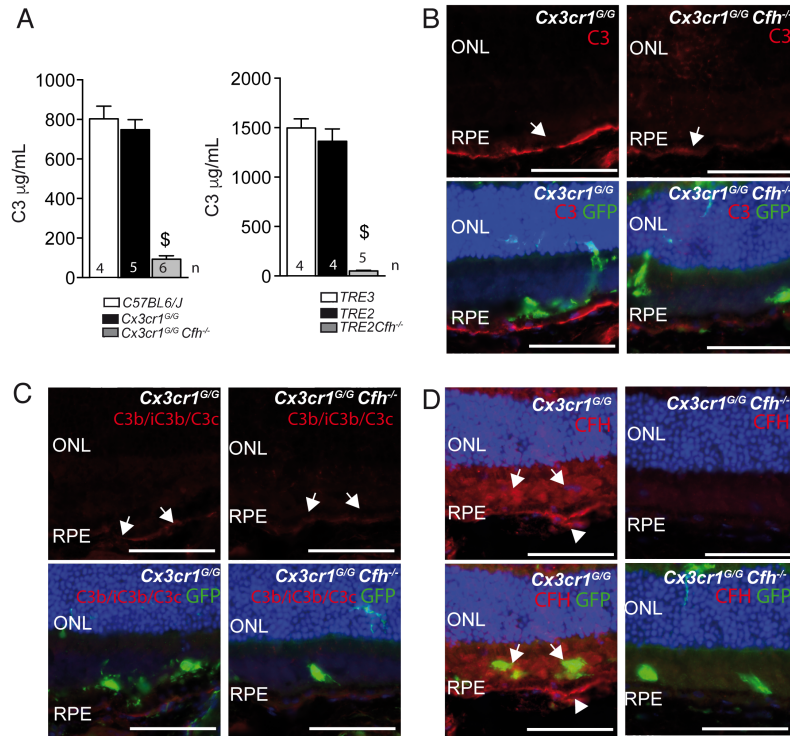
(M and L) TSP-1 plasma concentrations of the indicated mouse strains measured by Elisa.

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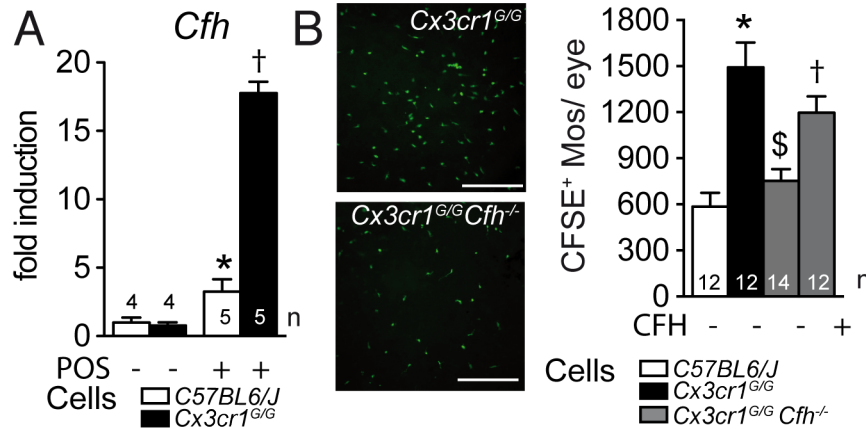


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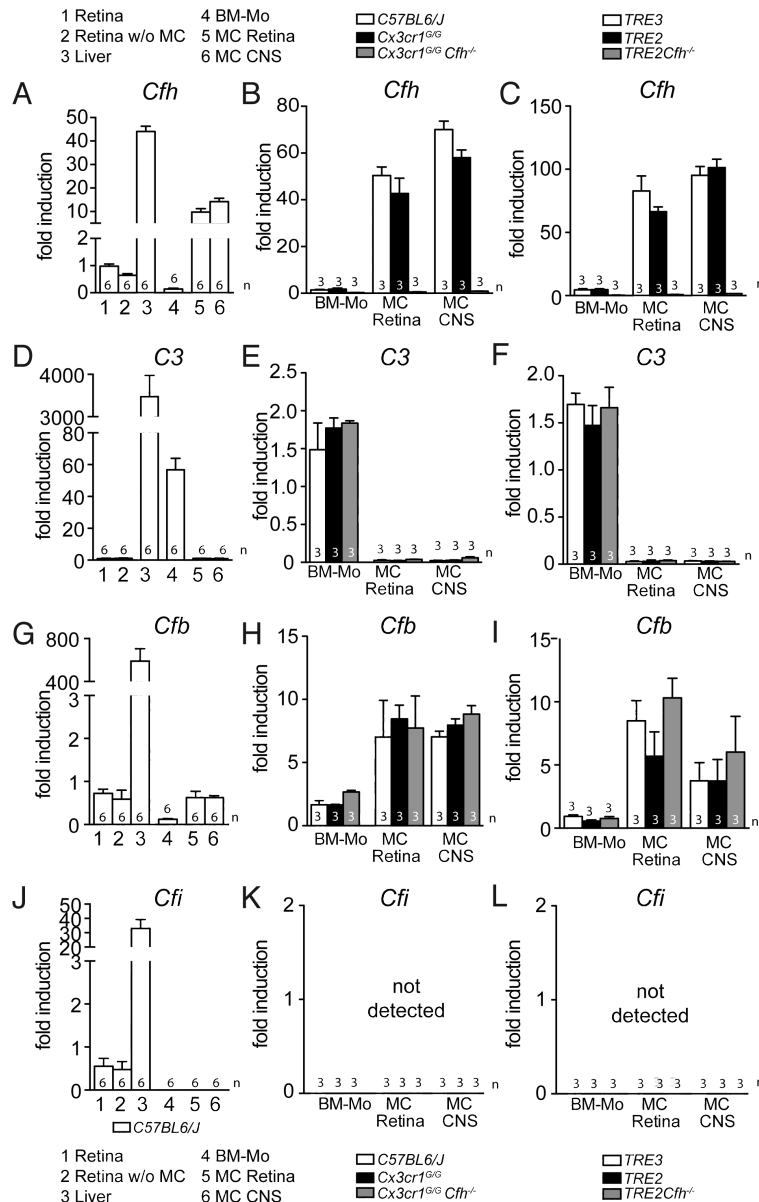


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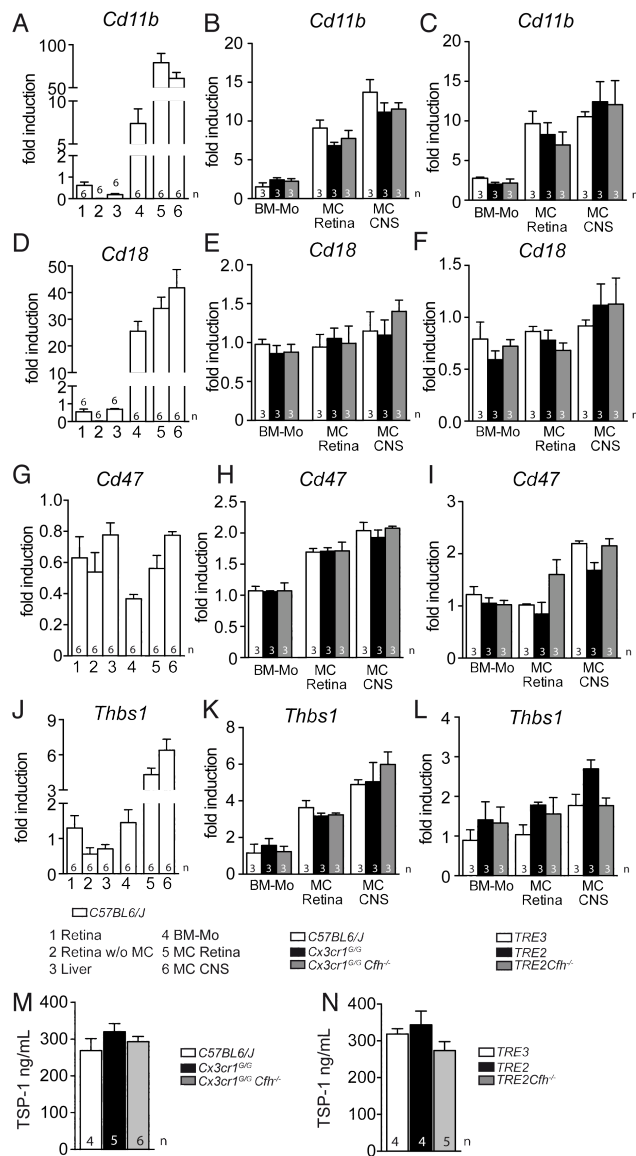
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## Supplemental Experimental Procedures:

### Animals

*Cfh*<sup>-/-</sup> and targeted replacement mice that express human APOE isoforms (TRE2, TRE3) were generous gifts from Mathew Pickering and Patrick Sullivan, respectively. *Cx3cr1*<sup>GFP/GFP</sup>, *Thbs1*<sup>-/-</sup>, *Cd47*<sup>-/-</sup>, and *Cd36*<sup>-/-</sup> mice were purchased (Charles River Laboratories, Jackson laboratories) and *Cx3cr1*<sup>GFP/GFP</sup> *Cfh*<sup>-/-</sup> and TRE2 *Cfh*<sup>-/-</sup> mouse strains were generated in-house. All mouse strains were either negative or backcrossed to become negative (*Thbs1*<sup>-/-</sup>) for the *Pde6b*<sup>rd1</sup>, *Gnat2*<sup>cpfl3</sup>, and *Crb1*<sup>rd8</sup> mutations that can lead to AMD-like features (Mattapallil et al., 2012). Mice were housed under specific pathogen-free condition, in a 12h/12h light/dark (100 lux) cycle with no additional cover in the cage, and with water and normal chow diet available *ad libitum*. All experimental protocols and procedures were approved by the local animal care ethics committee, “Comité d’éthique en expérimentation animale Charles Darwin” (N° Ce5/2010/011, Ce5/2010/044, Ce5/2011/033).

### Light challenge model

Two- to three-months old male and female mice were adapted to darkness for 6 hours, pupils dilated daily and exposed to green LED light (starting at 2AM, 4500 Lux, JP Vezon Equipements) for 4 days and subsequently kept in cyclic 12h/12h normal facility conditions as previously described (Sennlaub et al., 2013). MP count was assessed at the end of light exposure (day 4) or 10 days later (day 14).

### Choroidal and retinal flatmounts for mononuclear phagocyte and cone quantification

Eyes from male and female mice (as we never noticed a sex-dependent difference in subretinal MP accumulation in the past) (Combadiere et al., 2007; Hu et al., 2015; Levy et al., 2015a; Levy et al., 2015b; Sennlaub et al., 2013) were enucleated, fixed for 30 min in 4% PFA and sectioned at the limbus while the cornea and lens were discarded. The retinas were carefully peeled from the RPE/choroid/sclera. Retina and choroid were incubated with Peanut agglutinin Alexa 594 (ThermoFisher; 1:50), anti-IBA-1 antibody (Wako chemicals; 1:400), and anti-mouse cone-arrestin antibody (Millipore, #AB15282; 1:10 000) followed by secondary anti-rabbit antibody coupled to Alexa 488 and Alexa 647 (Thermo Fisher; 1:500) and nuclear staining using Hoechst. Choroids and retinas were flatmounted and viewed with a fluorescence microscope DM5500B (Leica). IBA-1<sup>+</sup> cells were counted on whole RPE/choroidal flatmounts and on the outer segment side of the retina. PA<sup>+</sup> cone arrestin<sup>+</sup> cells were counted on oriented retinal flatmounts in the central and peripheral retina.

### Laser-injury model

Laser-coagulations were performed on male mice with an 532nm ophthalmological laser mounted on an operating microscope (Vitra Laser, 532nm, 450 mW, 50ms, and 250µm). Intravitreal injections of 2µl of PBS, recombinant human TSP-1 (10µg/ml), the 4NGG control peptide (200µM) and the PKHB1 CD47-activating peptide (200µM), anti-CD11b antibody (50µg/ml, clone 5C6 Abd Serotec), isotype control rat IgG2 (50µg/ml, Invivogen), were performed at day 4 and 7 using glass capillaries (Eppendorf) and a microinjector, and mice were sacrificed at day 10. RPE and retinal flatmounts were stained and quantified as previously described (Sennlaub et al., 2013) using polyclonal rabbit anti-IBA-1 (Wako) and rat anti-mouse CD102 (clone 3C4, BD Biosciences) appropriate secondary antibodies and counterstained with Hoechst if indicated. Preparations were observed under a fluorescence microscope (DM5500, Leica) and IBA-1<sup>+</sup> MPs on the RPE were counted in a diameter of 500µm around the CD102<sup>+</sup> neovascularizations.

### Histology and Immunohistochemistry

Eyes were fixed in 0.5% glutaraldehyde, 4% PFA for 2h, cryoprotected (sucrose) and mounted in HistoResin (Leica). Oriented sections (5µm) crossing the inferior pole, optic nerve and superior pole were cut and stained with toluidin blue. Rows of nuclei in the outer nuclear layer (ONL) were counted at different distances from the optic nerve (Sennlaub et al., 2013). For immunohistochemistry, eyes from 4d light-challenged mice were fixed for 2h in 4% PFA, incubated in 30% sucrose overnight at 4°C, embedded in OCT and sectioned (10µm), and stained with anti-C3 antibody (clone 11H9 Hycult biotech; 1:50), anti-C3b/iC3b/C3c antibody (clone 3/26 Hycult biotech; 1:50), and anti-CFH antibody (ab8842 Abcam; 1:100) and appropriate secondary antibodies and Hoechst nuclear stain.

### Hydrodynamic injection

Murine Complement Factor H was cloned into pLIVE vector (Mirus) using NheI and SacII restriction sites, so that, its expression was under the control of a mouse minimal albumin promoter. The construct was sequenced and the plasmid amplified with endotoxin-free Megaprep kits (Qiagen). Control groups were injected with the empty pLIVE plasmid and test groups with pLIVE expressing murine CFH. 100 µg of plasmid diluted in NaCl 0.9% were

injected per mouse. The volume injected in the venous tail was 10% of the body weight (Rayes et al., 2010), which leads to robust transfection of the plasmid in the liver. Four days later, mice were exposed to the light challenge model and 50µl of blood were taken (mandibular vein) at day 0, 4 and 14 to quantify plasma C3 concentration by ELISA (Innovative Research).

#### **Plasma, monocyte and microglial cell preparations, analysis, and culture**

Blood samples from 2-3m old male mice were collected for determination of plasma C3 (Innovative Research) and TSP-1 (Elabsience) levels by ELISA. Bone marrow monocytes, circulating monocytes, central nervous MC and retinal MC were purified. Mos were isolated by negative selection (EasySep Mouse Enrichment Kit, Stemcell Technologies). MCs were prepared from dissociated PBS-perfused brains or retinas (Neural Dissociation Kit, Miltenyi Biotec). After dissociation, 70µm filtered cell suspensions were washed and myelin was eliminated Percoll density gradient centrifugation. Then cells labeled with anti-CD11b microbeads (clone M1/70.15.11.5, Miltenyi Biotec) were purified by MS Columns (Miltenyi Biotec) and washed. No serum was used in any step of the purification to avoid cell contamination with serum derived CFH. The cells were used for adoptive transfer experiments, analyzed by RT-qPCR or cultured for 24h in serum free DMEM, in presence of recombinant human APOE3 (Interchim; 10µg/mL), and finally their supernatants were analyzed by cytokine multiplex array (MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel, Merck Millipore).

#### **Subretinal adoptive mononuclear phagocyte transfer and clearance**

Brain MCs from male mice of the indicated strains were sorted as described above, labeled in 10µM CFSE (Thermo Fisher Scientific), washed and resuspended in PBS. Brain MCs express the molecules of interest similarly to retinal MCs (Fig. S4, S6, and S7). 12000 cells (in 4µL) were injected in the subretinal space of anesthetized WT or *Cfh*<sup>-/-</sup> male mice (10-14 weeks old) using glass microcapillaries (Eppendorf) and a microinjector as previously described (Levy et al., 2015a). A decompression hole was pierced with the glass capillary prior to the injection in order to allow retinal detachment with 4µl of solution. The subretinal injection was verified by funduscopy. In specific experiments, cells were co-injected with purified commercial human CFH (500µg/ml; Tecomedical), purified native CFH(Y402) and CFH(H402) (500µg/ml, obtained from Claire Harris), recombinant CFH (500µg/ml, LFB Biotechnologies, Lille), recombinant human TSP-1 (10µg/ml, R&D Systems), anti-CD11b antibody (10µg/ml, clone 5C6 Abd Serotec), anti-C3b/iC3b/C3c antibody (10µg/ml, clone 3/26 Hycult biotech), isotype control rat IgG2 (10µg/ml, Invivogen), anti-TSP-1 antibody (10µg/ml, clone A4.1, Thermo Fisher), and isotype control mouse IgM (10µg/ml, Invivogen). Eyes were enucleated after 24 hours, fixed 30 minutes in PFA 4% and counterstained with Hoechst nuclear stain. Eyes with hemorrhages were discarded. CFSE<sup>+</sup> cells in the subretinal space were quantified on flatmounts on the RPE side of the retina and on the apical side of the RPE.

#### **Thioglycollate induced peritonitis and flow cytometry**

Intraperitoneal injections of 0.5 ml of 3% (wt/vol) thioglycollate (T 9032 Sigma Aldrich) broth were used to elicit peritonitis in male *Cfh*<sup>-/-</sup> mice and wildtype C57BL6/J controls. Where indicated, mice undergoing peritonitis were injected at day 1 with 100µl of PBS containing native or heat inactivated (1h at 96°C) purified commercial human CFH (500µg/ml; Tecomedical), recombinant human TSP-1 (50µg/ml, R&D Systems), the CD47-activating peptide PKHB1 (500µM) or the 4NGG control peptide (500µM), and exudate cells were analyzed at the indicated days. At the indicated times, peritoneal lavage was performed after injecting 4 ml PBS containing 1% BSA and 0.5mM EDTA. Cells were labeled with antibodies directed against CD115 (clone AFS98), F4/80 (clone BM8), Gr-1 (anti-Ly6C and Ly-6G, clone RB6-8C5), ICAM-2 (clone 3C4), CD11b (M1/70) and MHC-II (clone M5/114.15.2). Antibodies were purchased from eBioscience. Cells suspensions were run on a BD Fortessa flow cytometer (BD biosciences) and analysis was performed using Flowjo software (TreeStar).

#### **CFH binding assay by flow cytometry**

Human CFH was conjugated to Cyanin 5.5 (Abcam conjugation kits). MCs from *Cx3cr1*<sup>GFP/GFP</sup> *Cfh*<sup>-/-</sup> were stained 30 minutes with Cy5.5-conjugated human CFH in PBS at indicated concentrations at 37°C and washed two times before acquisition on a BD Fortessa flow cytometer (BD biosciences). Analysis was performed using Flowjo software (TreeStar). Briefly, doublets were eliminated with FSC-H, FSC-W, SSC-H and SSC-W and microglial cells were identified as GFP<sup>high</sup> cells. Bone marrow monocytes were blocked with anti CD16/CD32 antibodies (Seroblock, AbD Serotec) for 15 minutes, preincubated with control IgG or anti-CD11b antibody (clone 5C6, AbD Serotec) at 10µg/ml, and incubated with hCFH::Cy3, were labeled with rat anti-CD115-PE (AbD Serotec), rat anti-Ly-6G AlexaFluor 700 (BD Biosciences) to be defined as GFP<sup>+</sup>, CD115<sup>+</sup>, Ly-6G<sup>-</sup> cells. Note that the anti-CD11b clone M1/70.15.11.5 used for MC purification does not interfere with CFH binding as shown in Fig. 3A and the fact that

recombinant CFH is capable to reverse the accelerated elimination of *Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup>* MCs and *TRE2Cfh<sup>-/-</sup>* MCs sorted using clone M1/70 (Fig. 2). This difference is likely due to the fact clone M1/70 recognizes a distinct epitope of CD11b compared to the 5C6 clone (Rosen and Gordon, 1987).

#### CD11b-CD47 proximity ligation assay

Mouse peritoneal exudate cells (PECs) were elicited by i.p. injection of 0,5 ml 3 % thioglycollate (T9032, Sigma) into 10 weeks old male C57BL/6J and *Cd47<sup>-/-</sup>* mice. After 1 day, PECs were isolated by flushing of the peritoneum with ice-cold PBS. Mφs were negatively selected by magnetic sorting following the manufacturers protocol (EasySep Mouse Monocyte Enrichment Kit, Stemcell Technologies), resuspended in X-VIVO 15 medium (Lonza), and plated in Lab-Tek® Chamber Slide™ (Nunc®). Brain *Cx3Cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup>* MCs were prepared as described above, resuspended in X-VIVO 15 medium (Lonza), and plated in Lab-Tek® Chamber Slide™ similarly. After 2 h at 37 °C in a 5 % CO<sub>2</sub> atmosphere, cells were rinsed with PBS, fixed 10 minutes in 4% paraformaldehyde solution, rinsed and permeabilized by incubating cells 10 minutes in 0.1% Triton solution in PBS. Duolink® PLA assay was performed following the manufacturer's instructions (Sigma-Aldrich). In brief, rabbit anti-CD11b (ab75476, Abcam; 1:1000) and goat anti-CD47 (AF1866, R&D Systems; 1:1000) were incubated overnight at 4°C. Afterwards, anti-rabbit and anti-mouse oligonucleotides-labeled secondary antibodies (PLA probes) were incubated, followed by a ligase and polymerase reaction to amplify the signal. Images were taken on an Olympus FLUOVIEW FV1000 confocal laser-scanning microscope at imaging facility of the Institut de la Vision.

#### Statistical analysis

Sample sizes for our experiments were determined according to our previous studies (Combadiere et al., 2007; Hu et al., 2015; Levy et al., 2015a; Levy et al., 2015b; Sennlaub et al., 2013). Severe hemorrhage secondary to subretinal injection interferes with MP clearance and was used as exclusion criteria. Graph Pad 6 (GraphPad Software) was used for data analysis and graphic representation. All values are reported as mean ± SEM. Statistical analysis and variance analysis was performed by one-way ANOVA followed by Bonferroni post-test (for multiple comparison) or Mann-Whitney U-test (2-group comparison) among means depending on the experimental design. The n and P-values are indicated in the figure legends.

#### Supplemental references

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