Complement factor H inhibits CD47-mediated resolution of inflammation

Bertrand Calippe\textsuperscript{1*}, Sebastien Augustin\textsuperscript{1*}, Fanny Beguier\textsuperscript{1}, Hugo Charles Messance\textsuperscript{1}, Lucie Poupel\textsuperscript{2}, Jean-Baptiste Conart\textsuperscript{1}, Shulong Hu\textsuperscript{1}, Sophie Lavalette\textsuperscript{1}, Alexandre Fauvet\textsuperscript{1}, Julie Rayes\textsuperscript{3}, Olivier Levy\textsuperscript{1}, William Raoul\textsuperscript{1}, Catherine Fitting\textsuperscript{10}, Thomas Denèfle\textsuperscript{8}, Matthew C Pickering\textsuperscript{4}, Claire Harris\textsuperscript{5}, Sylvie Jorieux\textsuperscript{6}, Patrick M. Sullivan\textsuperscript{7}, José-Alain Sahel \textsuperscript{1}, Philippe Karoyan\textsuperscript{8}, Przemyslaw Sapieha\textsuperscript{9}, Xavier Guillonneau\textsuperscript{1}, Emmanuel L. Gautier\textsuperscript{2}, Florian Sennlaub\textsuperscript{1†}

\textsuperscript{1}Institut de la Vision, 17 rue Moreau, Sorbonne Universités, UPMC Univ Paris 06, INSERM, CNRS, 75012 Paris, France.
\textsuperscript{2}Sorbonne Universités, UPMC Univ Paris 06, INSERM UMR_S 1166, Faculté de médecine Pitié-Salpêtrière, 91 Boulevard de l’hôpital, 75013, Paris, France.
\textsuperscript{3}Université Paris Descartes, Unité Mixte de Recherche en Santé 872, Centre de Recherche des Cordeliers, Paris, France;
\textsuperscript{4}Centre for Complement and Inflammation Research, Department of Medicine, Imperial College, London W12 0NN, UK.
\textsuperscript{5}Institute of Infection and Immunity, Cardiff University, Cardiff, UK.
\textsuperscript{6}LFB Biotechnologies, Lille, France.
\textsuperscript{7}Department of Medicine, Centers for Aging and Geriatric Research Education and Clinical Center, Durham Veteran Affairs Medical Center, Duke University, Durham, North Carolina 27710.
\textsuperscript{8}Laboratoire des Biomolécules, UMR 7203 and FR 2769, Sorbonne Universités, Université Pierre et Marie Curie, Paris, France; Centre National de la Recherche Scientifique, UMR 7203, Paris, France; Département de Chimie, École Normale Supérieure, Paris, France.
\textsuperscript{9}Department of Ophthalmology, Maisonneuve-Rosemont Hospital Research Centre, University of Montreal, Quebec, Canada.
\textsuperscript{10}Unité Cytokines & Inflammation, Département Infection et Epidémiologie, Institut Pasteur, 75015 Paris, France.

*these authors have contributed equally

†Correspondence should be addressed to the lead contact: Dr Florian Sennlaub, Inserm, UMR_S 968, Institut de la Vision, Paris, F-75012, France. Tel: (33) 1 53 46 26 93, Email: florian.sennlaub@inserm.fr.
ABSTRACT

Variants of the *CFH* gene, encoding Complement factor H (CFH), show strong association with age-related macular degeneration (AMD), a major cause of blindness. Here we used murine models of AMD to examine the contribution of CFH to disease etiology. *Cfh*-deletion protected the mice from the pathogenic subretinal accumulation of mononuclear phagocytes (MP) that characterize AMD, and showed accelerated resolution of inflammation. MP-persistence arose secondary to binding of CFH to CD11b, which obstructed the homeostatic elimination of MPs from the subretinal space mediated by thrombospondin-1 (TSP-1) activation of CD47. The AMD-associated CFH(H402) variant markedly increased this inhibitory effect on microglial cells, supporting a causal link to disease etiology. This mechanism is not restricted to the eye, as similar results were observed in a model of acute sterile peritonitis. Pharmacological activation of CD47 accelerated resolution of both subretinal and peritoneal inflammation, with implications for the treatment of chronic inflammatory disease.
INTRODUCTION
Age-related Macular Degeneration (AMD) is a heritable neuroinflammatory disorder characterized by deposits of lipoproteinaceous debris called large drusen (early AMD), choroidal neovascularisation (wet AMD, late form), and by an extending lesion of the retinal pigment epithelium (RPE) and photoreceptors (geographic atrophy, GA, late form) (Sarks, 1976). Early AMD afflicts more than 150 million people worldwide and 10 million people suffer from late AMD (Wong et al., 2014). AMD is strongly associated with common and rare genetic variants of the \textit{CFH} gene, encoding Complement factor H (CFH), suggesting a causal role for alterations in CFH expression or function in the pathogenesis of this disease (Fritsche et al., 2014). However, the mechanisms through which these alterations may lead to or contribute to AMD are not well understood.

CFH is an abundant soluble plasma factor composed of 20 Short Consensus Repeat domains (SCR) with important roles in inflammation (Kopp et al., 2012), coagulation (Rayes et al., 2014) and as an antioxidant (Weismann et al., 2011). The SCR7 domain of CFH binds to glycosaminoglycans (GAG) on cell surfaces where it inhibits complement activation (Kopp et al., 2012). This domain also allows CFH to bind to myeloid cells via the integrin CD11b (that forms with CD18 the Complement 3 Receptor), supporting myeloid cell adhesion and migration as well as the phagocytosis of microbes and cell debris (DiScipio et al., 1998; Kang et al., 2012; Losse et al., 2010). At inflammatory sites, CFH is strongly secreted by mononuclear phagocytes (MP), such as microglial cells (MC) and macrophages (Mφ) (Gautier et al., 2012; Luo et al., 2011; Schlaf et al., 2001), adding to the exudated plasma CFH and CFH produced by certain stromal cells such as the retinal pigment epithelium (RPE) in the eye (Anderson et al., 2010).

A genetic variant that result in the substitution of histidine 402 for tyrosine (Y402H) in the SCR7 domain of CFH are associated with the highest risk of AMD (Fritsche et al., 2014),
as well as with other conditions such as smoking-associated lung cancer (Zhang et al., 2012) and increased mortality after cerebral hemorrhage (Appelboom et al., 2011). This genetic variant is associated with both early and advanced forms of AMD (Fritsche et al., 2014), suggesting that CFH(H402) may drive disease onset.

Early and advanced forms of AMD are associated with chronic accumulation of MPs in the subretinal space located between the RPE and photoreceptor outer segments (Combadiere et al., 2007; Gupta et al., 2003; Lad et al., 2015; Levy et al., 2015a; Sennlaub et al., 2013). Functional studies in animal models suggest that subretinal MP accumulation play a critical role in neovascularization and photoreceptor degeneration that characterize late AMD (Cruz-Guilloty et al., 2013; Sennlaub et al., 2013; Tsutsumi et al., 2003). Similarly, non-resolving, low-grade inflammation and MP persistence contributes significantly to the pathogenesis of many chronic, age-related diseases such as metabolic diseases (obesity, atherosclerosis), neurodegenerative diseases and cancers (Nathan and Ding, 2010). In these settings, MP persistence is not considered causal, but rather is associated with considerable collateral damage to host cells, which fuels further inflammation (Nathan and Ding, 2010).

Here we examined the contribution of CFH to the etiology of AMD. We found a noncanonical role for CFH in the regulation of MP accumulation in chronic inflammation and in the resolution of acute inflammation. Our findings shed light on the mechanisms by which CFH(H402) drives sub-retinal inflammation, and further suggest that the role for CFH in resolution of inflammation may be relevant in various settings.
RESULTS

**CFH deficiency prevents chronic pathogenic subretinal inflammation**

Physiologically, the subretinal space is devoid of immune cells, including resident MCs (Combadiere et al., 2007; Levy et al., 2015a), due to the potent immunosuppressive pro-apoptotic factors produced by the RPE that eliminate infiltrating leukocytes (Griffith et al., 1995; Levy et al., 2015a). We have previously shown that high levels of Apolipoprotein E, as observed in subretinal MPs of AMD patients, Cx3cr1-deficient mice and humanized transgenic mice expressing the AMD-risk APOE2 isoform (TRE2-mice), induce chronic, age-related and pathogenic subretinal MP accumulation (Levy et al., 2015a; Levy et al., 2015b; Sennlaub et al., 2013). Indeed, Cx3cr1GFP/GFP and TRE2 mice model the age-dependent subretinal MP accumulation and associated photoreceptor degeneration observed in human AMD (Combadiere et al., 2007; Levy et al., 2015b; Sennlaub et al., 2013). We therefore sought to determine whether Cfh deficiency would alter disease onset and progression in Cx3cr1GFP/GFP and TRE2 mice. Quantification of subretinal IBA-1+ MPs on retinal and RPE/choroidal flatmounts of 2-3 month and 12 month old animals showed that the age-related increase in subretinal MPs observed in both models was nearly completely blunted in the absence of CFH (Fig. 1A and 1B). Micrographs revealed that Cx3cr1GFP/GFP Cfh−/− mice were protected against the thinning of the outer nuclear layer, which hosts photoreceptor nuclei, usually observed in Cx3cr1GFP/GFP mice (Sennlaub et al., 2013) (Fig. 1C). Photoreceptor nuclei row counts (Fig. 1D) and calculation of the area under the curve showed Cfh deficiency protected against the photoreceptor cell loss observed in Cx3cr1GFP/GFP mice (Fig. 1E) and TRE2-mice (Levy et al., 2015b) (Fig. 1F), while no difference was observed in Cfh−/− compared to control-mice. Similarly, Cfh deficiency completely protected against cone loss observed on peanut agglutinin / cone arrestin stained retinal flatmounts from 12m-old Cx3cr1GFP/GFP mice and TRE2-mice (Fig. 1G-J). CFH did not alter the secretion of key pathogenic cytokines by MPs in vitro (Fig. S1), confirming that the numerical decrease of
MPs rather than an altered ability to produce inflammatory mediators protected $Cfh$ deficient animals against the degeneration. Thus, we showed that CFH was required for the chronic, age-related subretinal MP accumulation and associated photoreceptor degeneration observed in both mouse models of AMD. A similar age- and CFH-dependent increase in MPs was also described in the choroid of $Cfh^{+/}$ compared to $Cfh^{-/-}$ mice (Toomey et al., 2015). In humans, ocular CFH immunoreactivity is invariably stronger in AMD donor tissues (Hageman et al., 2005; Johnson et al., 2006; Weismann et al., 2011) and CFH autoantibodies are protective in AMD (Dhillon et al., 2010). Together with these observations, our results strongly suggest CFH critically controls subretinal MP accumulation in AMD.

**CFH inhibits the resolution of acute subretinal inflammation**

Next, to better understand how CFH controls subretinal inflammation, we evaluated its impact on acute light-induced stress. The intensity of the light-challenge model used herein was calibrated to induce substantial subretinal MP infiltration in AMD-prone $Cx3cr1^{GFP/GFP}$ and $TRE2$ mice but not in controls (Levy et al., 2015b; Sennlaub et al., 2013). Quantification of subretinal IBA-1$^+$ MPs revealed similar early subretinal MP accumulation in $TRE2$ $Cfh^{-/-}$ (Fig. 2A) and $Cx3cr1^{GFP/GFP}$ $Cfh^{-/-}$ mice (Fig. 2B) and their respective controls at the end of the four-day light-challenge. However, after an additional 10 days under normal light conditions to allow for MP clearance and inflammation resolution (Hu et al., 2015), subretinal MPs were eliminated significantly faster in $TRE2$ $Cfh^{-/-}$ and $Cx3cr1^{GFP/GFP}$ $Cfh^{-/-}$ mice compared to controls (Fig. 2A and B). Thus, our data suggest that CFH controlled MP persistence at the inflammatory site rather than their initial accumulation.

Similar to $Cfh^{-/-}$ mice (Pickering et al., 2002), $Cx3cr1^{GFP/GFP}$ $Cfh^{-/-}$ and $TRE2$ $Cfh^{-/-}$ mice have low circulating levels of complement factor C3 (Fig. S2), likely due to un-inhibited plasma complement activation and exhaustion. To test whether the systemic lack of C3 would accelerate the elimination of subretinal MPs as observed in $Cx3cr1^{GFP/GFP}$ $Cfh^{-/-}$ mice, we
replaced hepatic CFH using hydrodynamic injection of a plasmid encoding Cfhl under the albumin promoter. Liver Cfhl complementation restored circulating C3 concentrations in Cx3cr1GFP/GFP Cfhl-/- mice to 40-60% of the Cx3cr1GFP/GFP levels over the 14 days of the experimental protocol (Fig. 2C). However, the significant increase in circulating C3 levels did not affect the number of subretinal MPs in Cx3cr1GFP/GFP Cfhl-/- mice at day 14 (Fig. 2D).

The comparable subretinal MP counts at the beginning of acute inflammation (day 4) (Fig. 2A and B) and the lack of influence of circulating C3 levels during the resolution phase (Fig. 2D) suggested that systemic C3 was not involved in MP recruitment or their elimination. We next assessed relative Cfhl mRNA levels in retinal and RPE/choroid tissue homogenates, bone marrow (BM-Mos) and circulating (Mos) monocytes as well as MCs isolated from the brain and retina. Our data showed that RPE/choroid and MCs expressed the highest levels of Cfhl mRNA in WT and Cx3cr1GFP/GFP mice (Fig. 2E), in accordance with CFH protein localization around subretinal MPs in vivo (Fig. S2). To evaluate whether MCs- or RPE-derived CFH would impact subretinal MP elimination, we adoptively transferred CFSE-labeled MCs from different mouse strains into the subretinal space of either WT or Cfhl-/- mice. The surviving CFSE+ MCs were then enumerated 24h after injection. We previously showed that subretinally injected WT Mos, MCs or Mφs quickly undergo apoptosis and are eliminated (Levy et al., 2015a), and that such clearance is significantly delayed when MPs lack CX3CR1 (Levy et al., 2015a). We found Cx3cr1GFP/GFP MCs lacking Cfhl were eliminated faster than controls (Fig. 2F). This difference could be reversed by human CFH (Fig. 2F). Experiments using BM-Mos revealed comparable results (Fig. S3). Interestingly, recipient derived-CFH only had a very minor impact on MCs elimination (Fig. 2F), suggesting MP-derived CFH is more important. Similarly, TRE2 Cfhl-/- MCs injected in WT recipients were eliminated faster than controls, and adding CFH protein again reversed the effect (Fig. 2G).
In summary, our data showed that CFH did not influence initial MP recruitment but inhibited MP elimination during inflammation resolution. They also demonstrated that RPE- and liver-derived CFH had little influence on this phenotype. Indeed, MP-derived CFH was sufficient to inhibit MP clearance. Similar to our experiments, the observation that recipient, but not liver Cfh genotype, confers the AMD-risk in liver transplant patients (Khandhadia et al., 2013) suggests that plasma CFH is not involved in AMD pathogenesis and points to the importance of MP-derived CFH in the disease.

**CFH binding to CD11b inhibits MP elimination mediated by TSP-1 activation of CD47**

CFH can act as a cofactor of complement factor I (CFI) to cleave C3b into iC3b, which reduces C3 convertase formation, formed by C3b and activated complement factor B (CFB) and opsonizes (iC3b) apoptotic bodies (Martin and Blom, 2016). However, we did not detect C3 or activated C3 fragments in subretinal MPs of Cx3cr1<sup>GFP/GFP</sup> or Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup> mice (Fig. S3) and BM-Mos and MCs only expressed low and very low levels of C3 mRNA and no detectable levels of Cfi mRNA in both cell types (Fig. S4). Together, these observation made a significant implication of locally produced C3, C3b, or iC3b in CFH-mediated inhibition of MP elimination unlikely.

CFH also binds directly to the integrin CD11b (DiScipio et al., 1998; Kang et al., 2012; Losse et al., 2010) that was strongly expressed by MPs with no detectable differences in our mouse strains (Fig. S5). Flow cytometry analysis showed fluorescently labeled CFH bound dose-dependently to MCs (Fig. 3A) and BM-Mos isolated from Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup> mice (Fig. 3B). Pre-incubation with an anti-CD11b antibody inhibited CFH binding (Fig. 3B), as previously demonstrated for neutrophils (DiScipio et al., 1998). As shown above (Fig. 2F), adding CFH to Cx3cr1<sup>GFP/GFP</sup> Cfh<sup>-/-</sup> MCs transferred subretinally in WT recipients delayed their elimination. This effect was no longer observed when transferred MPs were treated with the anti-CD11b antibody (Fig. 3C). The antibody also significantly accelerated the
elimination of CFH-competent Cx3cr1\textsuperscript{GFP/GFP} MCs. By contrast, an anti-C3b/iC3b/C3c antibody that inhibits complement-induced hemolysis (clone 3/26 (Mastellos et al., 2004), had no effect (Fig. 3C). These results revealed that CFH binding to the CD11b integrin was necessary to inhibit MC elimination and suggested a C3-independent, non-canonical role of CFH in this mechanism.

CD11b co-localizes with the integrin-associated protein (IAP, CD47) in lipid-rafts (Pfeiffer et al., 2001), a thrombospondin 1 (THBS1, TSP-1) receptor known to potentiate FasL-induced endothelial cell and T cell death (Manna et al., 2005; Quesada et al., 2005). We therefore investigated whether CFH binding to CD11b could limit CD47 activation and impair MP elimination in Cx3cr1\textsuperscript{GFP/GFP} and TRE2 mice. First, we found the expression level of TSP-1 and CD47 as well as the plasma TSP-1 levels were similar in our different mouse strains (Fig. S5). Next, proximity ligation assay revealed numerous complexes formed by CD11b and CD47 on Cx3cr1\textsuperscript{GFP/GFP} Cfh\textsuperscript{−/−} MCs (Fig. 3D). We next analyzed the role of CD47 in subretinal MC clearance in adoptive transfer experiments. Subretinally injected CFSE-labeled MCs from WT, Thbs1\textsuperscript{−/−} or Cd47\textsuperscript{−/−} donors into WT recipients revealed MCs lacking Thbs1 or Cd47 had slower elimination rates than WT MCs (Fig. 3E). Co-injected recombinant TSP-1 accelerated the elimination of WT MCs, reversed the phenotype of Thbs1-deficient MCs but had no effect on MCs lacking Cd47 (Fig. 3E), suggesting the interaction of TSP1 with CD47 mediated MC elimination. Moreover, analysis of Thbs1\textsuperscript{−/−} and Cd47\textsuperscript{−/−} mice revealed a significant age-related (Fig. 3F) and light-induced (Fig. 3G) increase in subretinal MPs in these strains as compared to controls, but not in mice lacking Cd36, an alternative TSP-1 receptor. Overall, our results pointed to TSP-1 activation of CD47 as central in the maintenance of subretinal immunosuppression, and likely explained the previously reported increased and prolonged subretinal inflammation observed in Thbs1\textsuperscript{−/−} mice (Chen et al., 2012; Ng et al., 2009; Wang et al., 2012). Interestingly, binding of TSP-1 to CD36 that mediates
endothelial cell apoptosis and is necessary for latent TGF-β activation (Febbraio et al., 2001) had no significant influence on subretinal MP accumulation, as observed here in Cd36−/− mice.

Taking into account the opposing effects of TSP1 and CFH, we next evaluated their interaction in MP elimination. Using our adoptive transfer assay, we found a TSP-1 blocking antibody reversed the accelerated elimination of Cx3cr1GFP/GFP Cfh−/− MCs compared to controls (Fig. 3H). Furthermore, the faster elimination rate observed after supplementation of recombinant TSP-1 to Cx3cr1GFP/GFP Cfh−/− MCs was completely lost when purified CFH was concomitantly added (Fig. 3I).

Next, we used the laser-injury model to test whether CFH binding to CD11b or CD47 activation can accelerate inflammation resolution in CFH-competent Cx3cr1GFP/GFP mice. Using this model, we take advantage of the fact that laser burn induces a thinning of the retina above the impact, facilitating diffusion of intravitreally-injected molecules to the subretinal space. Our results showed that TSP-1 (Fig. 3J), CD47-activating peptide PKHB1 (a derivate of the 4N1K CD47-agonist peptide with improved pharmacological properties (Denefle et al., 2016), Fig. 3K), or the anti-CD11b antibody (that blocks CFH binding to CD11b, Fig. 3L), injected at the height of laser-induced subretinal inflammation (d4 and d7), all significantly accelerated subretinal MP elimination as compared to their controls.

Taken together, our data showed that CFH binding to the integrin CD11b inhibited subretinal MC elimination. We demonstrated that CD47 co-localized with CD11b on MPs and mediated the physiological role of TSP-1 in subretinal MP elimination. The observation that TSP-1 blockade reversed the effect of CFH deficiency and that recombinant CFH blocked the effect of TSP-1 on subretinal MP elimination strongly suggested that CFH binding to CD11b interfered with TSP-1 activation of CD47. We showed that TSP-1 and more specifically CD47 activation efficiently accelerated MP elimination similar to Cfh-deficiency.
**CFH inhibits the resolution of acute sterile peritonitis**

Non-resolving inflammation contributes significantly to the pathogenesis of many chronic, age-related diseases (Nathan and Ding, 2010). To test whether CFH influences inflammation resolution in other pathological contexts, we used a model of acute thioglycollate-induced peritonitis, characterized by an early accumulation of neutrophils, followed by recruited monocyte-derived inflammatory macrophages (recMφ), both experiencing an apoptosis-driven elimination at different kinetics (Gautier et al., 2013). Analysis of the ImmGen dataset (Gautier et al., 2012) (GenBank no. GSE37448) showed Cfhl mRNA levels in thioglycollate-elicited peritoneal recMφ was approximately doubled compared to circulating blood Ly-6C+ Mo, from which they derive (Fig. 4A). Thus, recMφs likely participate to local CFH in peritonitis, in addition to extravasated plasma CFH. Quantification of recruited CD115+ F4/80+ ICAM2lo recMφs in Cfhl−/− mice and controls (Fig. 4B), revealed a robust and similar early accumulation 1 day after peritonitis induction (Fig. 4C). At day 3 however, while the numbers of recMφs continued to rise in WT mice, they had receded by 50% in Cfhl−/− mice (Fig. 4C). This observation was similar to the accelerated elimination of subretinal MPs observed in light-challenged TRE2 Cfhl−/− and Cx3cr1GFP/GFP Cfhl−/− mice (Fig. 2A and B). Human recombinant CFH injected into the peritoneal cavity of Cfhl−/− mice at day 1 significantly inhibited the enhanced clearance of recMφs observed in this strain at day 2 as compared to heat-denatured CFH (Fig. 4D), akin to the effect we observed in subretinally injected Cx3cr1GFP/GFP Cfhl−/− MCs (Fig. 2F). In addition, similar to our results in MCs (Fig. 3D), a proximity ligation assay revealed numerous and specific complexes CD11b and CD47 in WT recMφ retrieved at day 1 (Fig. 4E). Finally, a single intra-peritoneal injection of recombinant TSP-1 or the CD47-specific activating peptide PKHB1 at day 1 significantly accelerated the elimination of recMφs as observed at day 2 (Fig. 4F).

In summary, our results showed that CFH inhibited recMφ elimination in sterile peritonitis confirming findings for infiltrating MPs in the subretinal space. The observation
that complexes of CD11b and CD47 were present on peritoneal recMφ and that CD47 activation accelerated recMφ elimination during peritonitis strongly suggested that CFH inhibited CD47-dependent inflammation resolution similarly in both the eye and the peritoneum.

The CFH(H402) variant inhibits subretinal MC elimination more potently than the common CFH(Y402)

The substitution of histidine 402 for tyrosine (Y402H) in CFH sequence accounts for a major part of the genetic risk of AMD. To test whether the Y402H polymorphism influenced the elimination of distinct MPs differently, we transferred CFSE-labeled Cx3cr1GFP/GFP Cfh−/− MCs or BM-Mos to the subretinal space together with purified CFH(Y402) or the disease associated CFH(H402) to WT mice. CFSE+ cell counts after 24h revealed that both isoforms inhibited clearance of BM-Mos (Fig. 5A) and MCs (Fig. 5B) compared to cells injected without CFH. However, while CFH(Y402) and CFH(H402) had similar effects on BM-Mo elimination (Fig. 5A), CFH(H402) limited MC clearance by 37% compared to CFH(Y402) (Fig. 5B). In addition, recombinant CFH(Y402) reversed the accelerated elimination rate observed when subretinally injected Cx3cr1GFP/GFP Cfh−/− MCs were treated with recombinant TSP-1, and recombinant CFH(H402) was 50% more potent at inhibiting this phenomenon (Fig. 5C).

Taken together our results showed that CFH(H402) was significantly more potent to inhibit the subretinal elimination of certain MPs, such as MCs. The CFH(H402) variant might thereby spur non-resolving inflammation under the retina, and thus explain its association with early and late AMD (Fritsche et al., 2014) where subretinal MPs accumulate (Lad et al., 2015; Levy et al., 2015a; Sennlaub et al., 2013).

DISCUSSION
We report the previously unknown ability of CFH to favor subretinal MP accumulation in mice developing chronic, non-resolving, age-related inflammation in the eye. We extended
this finding to inflammation resolution in general by using models of acute inflammation in the peritoneum and the eye. Our work supports the long-standing association between CFH variant and AMD, and we uncovered the mechanisms by which CFH impacts MP infiltration, independently from its action in the complement cascade.

The subretinal space is prone to MP infiltration due to light-induced oxidative stress and high metabolic activity (Combadiere et al., 2007; Ng and Streilein, 2001), and this is physiologically counterbalanced by the expression of immune-suppressive factors by the RPE (Griffith et al., 1995; Levy et al., 2015a). We showed that CFH favored MP accumulation by inhibiting their elimination mediated by TSP-1 activation of CD47. Remarkably, Thbs1−/− and Cd47−/− mice developed age-related subretinal inflammation under normal living conditions. This can possibly be explained by the sensitizing role of TSP-1 on FasL-induced cell death (Manna et al., 2005; Quesada et al., 2005) as FasL expression by the RPE is necessary to prevent subretinal MP accumulation (Griffith et al., 1995; Levy et al., 2015a).

CFH was expressed at high levels in MCs and Mo-derived Mφs in peritonitis and we confirmed a step-wise binding interaction between CFH and CD11b (Gautier et al., 2012; Luo et al., 2011; Schlaf et al., 2001) and CD11b and CD47 (Pfeiffer et al., 2001) on MPs. The ligation of the sizeable CFH (1231 amino acids) to CD11b might thereby sterically hinder the ligation of the trimeric TSP-1 (3x1106 amino acids) to CD47, and increase MP lifespan. Although the exact mechanism remains to be elucidated, our data shows that complement activation or CFH-dependent production of iC3b, an alternative ligand of CD11b, did not play a detectable role in the process: C3b/iC3b/C3c fragments were not found in the subretinal space and CFI (necessary to produce iC3b) was not significantly transcribed in MPs. Lastly, a specific antibody that inhibits the alternative cascade (Mastellos et al., 2004) did not accelerate MP elimination.
In addition, we found that the AMD-associated CFH(H402) variant had an increased capacity to inhibit the elimination of certain MP populations, such as MCs, strengthening the causal link to AMD etiology. Although CFH(H402) affinity is decreased to certain GAG species, it is higher in particular to GAG sulfates (Clark et al., 2006). GAG sulfate profiles differ greatly between MPs and different microenvironments. For example, keratan sulfate proteoglycans (KPSG) are strongly present on ramified brain MCs but not on blood Mos (Wilms et al., 1999). The differential expression of GAG sulfates, such as KPSG, on MCs might explain why CFH(H402) differentially influenced MC but not BM-Mos elimination. Subretinal MPs originating from infiltrating Mo and MCs (Sennlaub et al., 2013) invariably express KPSG strongly (Combadiere et al., 2007; Ng and Streilein, 2001; Ng et al., 2009). This is also the case in spinal cord injury, but not in autoimmune neuritis (Jones and Tuszynski, 2002; Matsui et al., 2013). CFH(H402) might therefore have a particularly strong influence on subretinal inflammation observed in AMD but not necessarily in other chronic inflammatory diseases. In human evolution, the limited elimination of MPs and the increased inflammatory reaction associated with CFH(H402) might have increased survival to certain infectious diseases, leading to its high frequency as observed today in certain populations. In AMD, CFH(H402) might be pathogenic as it fuels subretinal inflammation and leads to chronic inflammation, which add to its decreased capacity to inhibit oxidative stress (Weismann et al., 2011) and to bind to Bruchs membrane (Clark et al., 2010) that protects the RPE against uncontrolled complement activation (Coffey et al., 2007; Toomey et al., 2015).

Current anti-inflammatory therapies, such as steroids, non-steroidal anti-inflammatory drugs (NSAID), or immunosuppressive drugs (Ciclosporin) can have paradoxical effects on macrophage function. They increase proinflammatory mediators (high-dose steroids) (Lim et al., 2007), upregulate toll-like receptors on macrophages (Ciclosporin) (Tedesco and Haragsim, 2012) and prolong macrophage infiltration (NSAID) (Gilroy et al., 1999), which
might explain their lack of therapeutic effect in AMD. Our findings introduce a new strategy to directly induce the elimination of pathogenic macrophage accumulation in AMD, and possibly other conditions, by pharmacological activation of CD47.
Experimental Procedures:

Mice

*Cfh*−/−, TRE2 and TRE3 mice were generous gifts from Mathew Pickering and Patrick Sullivan. *Cx3cr1*GFP/GFP, *Thbs1*+/−, *Cd47*−/−, and *Cd36*−/− mice were obtained for The Jackson laboratories, *Cx3cr1*GFP/GFP *Cfh*−/− and TRE2 *Cfh*−/− mouse strains were generated, all mice were either negative or backcrossed to eliminate the *Pde6b*rd1, *Gnat2*cpfl3, and *Crb1*rd8 mutations. The mice were kept to the indicated ages 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.

Aging, light challenge- and laser-injury model

Mice were exposed to green LED light (4500 Lux) for 4 days and subsequently kept in cyclic conditions (Sennlaub et al., 2013). Laser-coagulations were performed on male mice (Vitra Laser, 532nm, 450 mW, 50ms, and 250μm), intravitreally injected (day 4 and 7) with 2μl of PBS, recombinant human TSP-1 (10μg/ml), the 4NGG control peptide or the PKHB1 CD47-activating peptide (200μM), anti-CD11b antibody (50μg/ml), isotype control rat IgG2 (50μg/ml), and sacrificed at day 10. All experimental procedures were approved by the local animal care ethics committee C2EA-05 - Charles Darwin.

Histology and Immunohistochemistry

After fixation the eyes were cryo-sectioned (10μm), and stained with anti-C3-, anti-C3b/iC3b/C3c-, and anti-CFH-antibodies and appropriate secondary antibodies, or dissected for flatmount preparations. The retinas and choroids were incubated with Peanut agglutinin, anti-IBA-1, anti-cone arrestin, or anti-CD102 antibodies as indicated, followed by appropriate conjugated secondary antibodies. IBA-1+ cells were counted on whole RPE/choroidal flatmounts and on the outer segment side of the retina. PA+ cone arrestin+ cells were counted on oriented retinal flatmounts. For histology the eyes were fixed, embedded in Historesin, cut
(5µm sagittal sections) and stained with toluidin blue. Rows of nuclei in the outer nuclear layer (ONL) were counted at different distances from the optic nerve.

**Hydrodynamic injection**
Empty pLIVE plasmid and coding for murine CFH was intravenously injected to mice as previously described (Rayes et al., 2010). Four days later, mice were exposed to the light challenge model and their blood was sampled to quantify plasma C3 concentration by ELISA.

**Plasma and mononuclear phagocyte preparations, analysis, and culture**
Plasma C3 and TSP-1 levels were measured by ELISA. Mos from the blood and bone marrow were isolated by negative selection. MCs from brain and retina were isolated with anti-CD11b microbeads. No (CFH-containing) serum was used in any step of the purification. 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, and finally their supernatants were analyzed by cytokine multiplex array.

**Subretinal adoptive microglial cell transfer and clearance**
Brain MCs from male mice were sorted, labeled with CFSE, and injected in the subretinal space (verified by fundoscopy) of WT or Cfh−/− male mice in 4µl of PBS, purified human or recombinant CFH, CFH(Y402), and CFH(H402) (500µg/ml), recombinant human TSP-1 (10µg/ml), and following antibodies (10µg/ml); anti-CD11b, anti-C3b/iC3b/C3c, isotype control rat IgG2, anti-TSP-1, and isotype control mouse IgM. After 24 hours, CFSE+ cells in the subretinal space were quantified on whole RPE/choroidal and retinal flatmounts.

**Thioglycollate induced peritonitis and flow cytometry**
Peritonitis was induced by intraperitoneal injections of 0.5 ml of 3% (wt/vol) thioglycollate broth in male mice. At 24 hours mice were injected with 100µl of PBS containing native or heat inactivated purified commercial human CFH (500µg/ml), recombinant human TSP-1
(50µg/m), the CD47-activating peptide PKHB1 or the 4NGG control peptide (500µM), and exudate cells were analyzed using a BD Fortessa flow cytometer.

**CFH binding assay by flow cytometry**
MCs from Cx3cr1GFP/GFP Cfh−/− were stained 30 minutes with Cy5.5-conjugated human CFH at 37°C and washed before acquisition on a BD Fortessa flow cytometer. Bone marrow monocytes were preincubated with control IgG or anti-CD11b antibody (clone 5C6) at 10µg/ml, and incubated with hCFH-Cy3.

**CD11b-CD47 proximity ligation assay**
Duolink® PLA assay was performed following the manufacturer’s instructions with rabbit anti-CD11b and goat anti-CD47 on day 1 thioglycollate elicited peritoneal Mφs, and brain MCs.

**Statistical analysis**
Graph Pad 7 (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.

Please see supplemental information for more details.

**Author contributions:**
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Figure Legends

Figure 1. CFH deficiency prevents chronic pathogenic subretinal inflammation

A and B: (A) Representative images of 12 month-old IBA-1 (green) stained RPE flatmounts of Cx3cr1GFP/GFP and Cx3cr1GFP/GFP Cfh−/− mice and (A and B) quantification of subretinal IBA-1+ MPs in 2-3 months and 12 months old mice of the indicated strains (A: one-way Anova/Bonferroni test *p<0.0001 versus all other groups, Mann Whitney $p=0.0003$ versus Cx3cr1GFP/GFP at 12 months of age; B: one-way Anova/Bonferroni test *p<0.0001 versus all other groups, Mann Whitney $p=0.0003$ versus TRE2 at 12 months of age).

C-F: (C) Micrographs, taken 1000 μm from the optic nerve, of 12 month-old Cx3cr1GFP/GFP and Cx3cr1GFP/GFP Cfh−/− mice. (D) Photoreceptor nuclei rows at increasing distances (-3000μm: inferior pole, +3000μm: superior pole) from the optic nerve (0μm) in 12 month-old mice. (E and F) Quantification of the area under the curve of photoreceptor nuclei row counts of 12 month-old mice of the indicated transgenic mouse strains (E: one-way Anova/Bonferroni test: *p<0.0001 versus all other groups; Mann Whitney $p=0.0024$ versus Cx3cr1GFP/GFP mice; F: one-way Anova/Bonferroni test: *p<0.001 versus all other groups; Mann Whitney $p=0.0158$ versus TRE2 mice).

G-J: (G) Micrographs, taken in the superior periphery of peanut agglutinin (staining cone segments, red), cone arrestin (white), IBA-1 (green) triple stained 12 month-old Cx3cr1GFP/GFP and Cx3cr1GFP/GFP Cfh−/− mice. (H) Cone density quantifications on retinal flatmounts in peripheral and central, inferior and superior retina (-3000μm: inferior pole, +3000μm: superior pole, optic nerve: 0μm) and their average (I and J) in 12 month-old mice of the indicated transgenic mouse strains (I: one-way Anova/Bonferroni test: *p<0.0001 versus all other groups; Mann Whitney $p=0.0024$ versus Cx3cr1GFP/GFP mice; J: one-way Anova/Bonferroni test: *p<0.0001 versus all other groups; Mann Whitney $p=0.0158$ versus TRE2 mice).

TRE2 and TRE3: Targeted replacement mice expressing human APOE 2 and 3 isoforms

n= number of replicates indicated in the graphs; replicates represent quantifications of eyes from different mice of at least three different cages.
Figure 2. CFH inhibits the resolution of acute subretinal inflammation

A and B: (A) Representative images of IBA-1 (green) stained RPE flatmounts of light-challenged TRE2 and TRE2 CFh−/− mice and (A and B) quantification of subretinal IBA-1+ MPs in non-illuminated (NI), 4 days-light challenged (d4), and 4 days-light challenged followed by 10 days of recovery (d4+10) of 2-3 months old mice of the indicated strains. (A: one way Anova/Bonferroni test \( p<0.0001 \) versus the NI groups and TRE3 mice at d4, \( ^5p<0.0001 \) versus TRE2 d4+10; MannWhitney \( ^6p=0.0004 \) versus TRE2 at d4+10; B: one way Anova/Bonferroni test \( ^6p<0.0001 \) versus the NI groups and C57BL6/J mice at d4, \( ^5p<0.0001 \) versus Cx3cr1GFP/GFP at d4+10; MannWhitney \( ^6p=0.0002 \) versus Cx3cr1GFP/GFP at d4+10).

C: Quantification by ELISA of circulating plasma C3 in control animals and after hypervolemic liver transfection with a control plasmid (ctl PI) or a plasmid encoding murine CFH (mCFH) in 2-3 months old Cx3cr1GFP/GFP and Cx3cr1GFP/GFP Cfh−/− mice. Measurements were performed before (d0), at the end (d4) and ten days after (d14) the light-challenge. (one way Anova/Bonferroni test at d0 \( ^6p=0.0003 \), d4 \( ^6p=0.0018 \), d4+10 \( ^6p=0.0009 \) compared to control plasmid injected mice at each time point).

D: Quantification of subretinal IBA-1+ MPs/mm² in light-challenge model at day 14 in 2-3 month-old Cx3cr1GFP/GFP and Cx3cr1GFP/GFP Cfh−/− mice after hypervolemic liver transfection of the empty plasmid or the plasmid encoding murine CFH. (Mann Whitney = 0.1).

E: Quantitative RT-PCR of Cfh mRNA normalized to Rps26 mRNA expression in retina, choroid/RPE, circulating monocytes (cMo), bone marrow monocytes (BM-Mo), retinal microglia (MC Retina) and brain microglia from the indicated strains (n=replicates from individual mice except for retinal MCs which represent 3 preparations from 5 pooled mice each).

F: Representative micrograph of CFSE+ MCs on RPE flatmounts of the indicated strains 24h after subretinal adoptive transfer to Cfh−/− mice. Quantification of CFSE+ MCs of the indicated strains 24h after adoptive transfer to WT C57BL6/J or Cfh−/− mice, with or without purified human CFH (one way Anova/Bonferroni test \( p<0.001 \) versus C57BL6/J CFSE+ MCs in C57BL6/J recipients, \( ^5p=0.0043 \) versus Cx3cr1GFP/GFP CFSE+ MCs in C57BL6/J recipients, \( ^6p=0.0139 \) versus Cx3cr1GFP/GFP Cfh−/− CFSE+ MCs in C57BL6/J recipients; there were no significant differences between groups injected in WT C57BL6/J or Cfh−/− recipients for each cell genotype).

G: Quantification of subretinal CFSE+ MCs of the indicated strains 24h after adoptive transfer to C57BL6/J WT mice, with or without purified human CFH (one way Anova/Bonferroni test \( ^6p=0.0009 \) versus C57BL6/J CFSE+ MCs, \( ^5p=0.0012 \) versus TRE2 CFSE+ MCs, \( ^6p=0.0085 \) versus TRE2 Cfh−/− CFSE+ MCs).

n=number of replicates presented in the graphs; for A, B and D n=replicates represent quantifications of eyes from different mice of at least three different cages; for F and G n=replicates from individual mice from three experiments with three different cell preparations. TRE2 and TRE3: Targeted replacement mice expressing human APOE 2 and 3 isoforms; post-transf.: post-transfection; light-chall: light-challenge; ctlPL: control plasmid; mCFH: murine CFH plasmid; Scale bar A and F = 50μm.
Figure 3. CFH binding to CD11b inhibits MP elimination mediated by TSP-1 activation of CD47

A and B: Representative cytometry plots of (A) sorted brain Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> Microglial Cells ( gated on GFP<sup>high</sup>) incubated with increasing dose of hCFH::Cy5.5 (15,625μg/ml to 500μg/ml), and (B) sorted Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> bone marrow monocytes pre-incubated with an isotype IgG or anti-CD11b IgG (5C6 clone) before hCFH::Cy3 or PBS incubation. Bone marrow monocytes were gated as GFP<sup>+</sup> CD115<sup>+</sup> Ly-6G<sup>−</sup> cells (the experiments were repeated three times with similar results).

C: Representative micrograph of subretinal Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> CFSE<sup>+</sup> MCs on RPE flatmounts 24h after subretinal adoptive transfer injection together with CFH and control IgG or anti-CD11b IgG (clone 5C6) and Cx3cr1<sub>GFP/GFP</sub> CFSE<sup>+</sup> MCs with control IgG, anti-CD11b IgG (clone 5C6) or anti-C3b/iC3b/C3<sup>c</sup> IgG (clone 3/26) to WT C57BL6/J mice and quantification of the indicated groups (Anova/Bonferroni test: *p=0.0034 versus Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> CFSE<sup>+</sup> MCs without CFH, †p=0.0036 versus Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> + CFH + cIgG, Mann Whitney *p=0.0083 versus cIgG).

D: Representative confocal micrographs of CD11b-CD47 complexes (red dots) detected by proximity ligation assay on freshly isolated brain Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> MCs counterstained with Hoechst nuclear stain (blue; negative control: omitting the primary antibodies; the experiment was repeated three times with similar results).

E: Quantification of subretinal CFSE<sup>+</sup> MCs of the indicated strains 24h after adoptive transfer to WT C57BL6/J mice with and without recombinant TSP-1 (n=replicates from individual mice from experiments with three different cell preparations; one-way Anova/Bonferroni test: *p=0.0359 versus C57BL6/J CFSE<sup>+</sup> MCs without TSP-1; †p<0.0001 versus C57BL6/J CFSE<sup>+</sup> MCs without TSP-1; ‡p<0.0001 versus Tsp1<sup>−/−</sup> CFSE<sup>+</sup> MCs without TSP-1; †p=0.0002 versus C57BL6/J CFSE<sup>+</sup> MCs without TSP-1).

F and G: Representative images of 12 month-old IBA-1 (green) stained RPE flatmounts of C57BL6/J and Cd47<sup>−/−</sup> mice and quantification of subretinal IBA-1<sup>+</sup> MPs of WT C57BL6/J, Thbs1<sup>−/−</sup>, Cd47<sup>−/−</sup> mice and Cd36<sup>−/−</sup> mice at (F) 2-3 months and 12 months and (G) after a 4 day-light challenge, followed by 10 days of recovery (n=replicates represent quantifications of eyes from different mice of at least three different experiments and cages; one-way Anova/Bonferroni test: *p<0.0001 versus 2-3 months old Thbs1<sup>−/−</sup> mice and 12 months old C57BL6/J mice; †p<0.0001 versus 2-3 months old Cd47<sup>−/−</sup> mice and 12 months C57BL6/J mice; G: *p<0.0001 versus C57BL6/J; †p<0.0001 versus C57BL6/J).

H and I: Quantification of subretinal Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> CFSE<sup>+</sup> MCs with (G) control IgM or anti-TSP-1 IgM (clone A4.1) 24h after adoptive transfer (Mann Whitney *p=0.0002 IgM versus anti-TSP-1) or (H) recombinant TSP-1 (rTSP1) and rTSP1 + purified CFH (one-way Anova/Bonferroni test: *p=0.0024 versus Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> CFSE<sup>+</sup> MCs without rTSP1; *p=0.0003 versus Cx3cr1<sub>GFP/GFP</sub> Cfh<sup>−/−</sup> CFSE<sup>+</sup> MCs + rTSP).

J-L: (I) Representative micrographs of RPE flatmounts of CD102 (red) and IBA-1 (green) immunohistochemistry and quantification of subretinal IBA-1<sup>+</sup> MPs on the RPE counted at a distance of 0-500μm from CD102<sup>+</sup> CNV 10 days after the laser-injury in 2 month-old Cx3cr1<sub>GFP/GFP</sub> mice injected at day 4 and day 7 with (I) PBS or rTSP1, (J) control peptide 4NGG or CD47-activating peptide PKHB1, and (K) control IgG or anti-CD11b IgG (Mann Whitney I *p=0.0097; *p<0.0001; K *p=0.0298)

This: thrombospondin 1 gene; TSP-1: thrombospondin. n-number of replicates indicated in the graphs, for C, E, G and H n=replicates from individual mice from experiments with three different cell preparations, for F, I and J n=replicates represent quantifications of eyes from different mice of at least three different experiments and cages. Scale bar: C, F and I = 50μm; D left panels =20μm, right panel =10μm.
Figure 4. CFH inhibits the resolution of acute sterile peritonitis

A: Normalized signal intensity for Cfh mRNA levels extracted from Affymetrix transcription analysis of bone marrow monocytes (1), Ly-6C+ circulating blood monocytes (2), MHC-II and MHC-II+ monocyte-derived peritoneal macrophages at day 5 of thioglycollate-induced peritonitis in 3 months old WT C57BL6/J mice (3 and 4; one-way Anova/Bonferroni test \textsuperscript{\textdagger}p=0.0036 versus blood monocytes; \textsuperscript{*}p=0.0005 versus blood monocytes).

B: Representative cytometry plots of WT C57BL6/J (upper panels) or Cfh\textsuperscript{-/-} mice (lower panels) three days after thioglycollate-induced peritonitis. CD115\textsuperscript{+} F4/80\textsuperscript{+} cells represents macrophages, with CD115\textsuperscript{+} F4/80\textsuperscript{+} ICAM-2\textsuperscript{lo} representing Mo-derived Mφ and CD115\textsuperscript{+} F4/80\textsuperscript{+} ICAM-2\textsuperscript{hi} representing resident macrophages.

C: Quantification of CD115\textsuperscript{+} F4/80\textsuperscript{+} ICAM-2\textsuperscript{lo} Mo-derived Mφ in exudates of WT C57BL6/J and Cfh\textsuperscript{-/-} mice at indicated time points after thioglycollate-induced peritonitis (one-way Anova/Bonferroni test \textsuperscript{\textdagger}p=0.012 versus d0 WT C57BL6/J; \textsuperscript{*}p=0.005 versus d0 Cfh\textsuperscript{-/-}; \textsuperscript{\textdagger\textdagger}p=0.9697 between d1 groups; \textsuperscript{\textdagger\textdagger\textdagger}p<0.0001 versus d3 WT C57BL6/J).

D: Quantification of CD115\textsuperscript{+} F4/80\textsuperscript{+} ICAM-2\textsuperscript{lo} Mo-derived Mφ in exudates of Cfh\textsuperscript{-/-} mice at day 2 (d2) after mice were injected with native (CFH) or heat-denatured (dCFH) purified CFH at day 1 (d1) (MannWhitney \textsuperscript{*}p=0.0087 versus dCFH).

E: Representative confocal micrographs of CD11b-CD47 complexes (red dots) detected by proximity ligation assay on freshly harvested Mo-derived Mφ 1 day after thioglycollate injection in WT C57BL6/J and Cd47\textsuperscript{-/-} mice. Hoechst was used for nuclear stain (blue; negative control: omitting the primary antibodies; the experiment was repeated three times with similar results).

F: Quantification of CD115\textsuperscript{+} F4/80\textsuperscript{+} ICAM-2\textsuperscript{lo} Mo-derived Mφ in exudates of WT C57BL6/J mice at day 2 after mice were injected with PBS or rTSP-1 (Mann Whitney \textsuperscript{\$}p=0.0048); or control peptide 4NGG or CD47-activating peptide PKHB1 (Mann Whitney \textsuperscript{\$}p=0.0087) at day 1.

Mφ: macrophage; Mo: monocyte; Thio: thioglycollate; MHC: major histocompatibility complex; dCFH: heat-denatured CFH; negCTL: negative control. n= number of replicates indicated in the graphs; replicates represent quantifications of exudate cells from different mice of three (C) or two (E and F) different peritonitis inductions. Scale bar: E=10\textmu m.
**Figure 5. The CFH(H402) variant inhibits subretinal MC elimination more potently than the CFH(Y402) form.**

A: Quantification of subretinal $Cx3cr1^{GFP/GFP}$ $Cfh^{-/-}$ CFSE$^+$ monocytes on RPE and retinal flatmounts 24h after adoptive transfer to WT C57BL6/J mice with and without purified CFH(Y402) or CFH(H402) (Anova/Bonferroni test $^*p<0.0001$).

B: Quantification of subretinal $Cx3cr1^{GFP/GFP}$ $Cfh^{-/-}$ CFSE$^+$ microglial cells on RPE and retinal flatmounts 24h after adoptive transfer to WT C57BL6/J mice with and without purified CFH(Y402) or CFH(H402) (Anova/Bonferroni test $^*p=0.0025$ versus without CFH; $^†p=0.0052$ versus CFH(H402)).

C: Quantification of subretinal $Cx3cr1^{GFP/GFP}$ $Cfh^{-/-}$ CFSE$^+$ microglial cells on RPE and retinal flatmounts 24h after adoptive transfer to WT C57BL6/J mice without or with recombinant TSP-1 (rTSP1), rTSP1 + recombinant CFH(Y402) or rTSP1 + recombinant CFH(H402) (Anova/Bonferroni test $^*p=0.00236$ versus without TSP-1; $^†p=0.0002$ versus with TSP-1; $^‡p=0.0005$ versus with TSP-1 + CFH$_{Y402}$).

Mos: monocytes; MCs: microglial cells; rTSP1: recombinant TSP-1; n=number of replicates indicated in the graphs, replicates represent quantifications from individual mice from two (A) to three (B and C) experiments with three different cell preparations.