Moss-derived complement factor H variants attenuate light-induced retinal degeneration

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Zusammenfassung

Die altersabhängige Makuladegeneration (AMD) ist eine multifaktorielle Erkrankung der Netzhaut und einer der häufigsten Gründe für Sehkraftverlust. Neben vielen anderen Risikofaktoren wie Rauchen, Ernährung und Alter, trägt auch die Genetik zur komplexen Krankheitsätiologie bei. Ein Polymorphismus im Faktor H Gen der Komplementkaskade ist einer der am häufigsten vorkommenden genetischen Risikofaktoren. Faktor H ist ein wichtiger Regulator der alternativen Komplement Kaskade, die Teil der angeborenen Immunantwort ist. Eine gestörte Regulation des Komplementsystems steht auch in Verbindung mit anderen Erkrankungen wie dem atypischen Hämolytisch-Urämischen Syndrom, bei dem eine Behandlung mit Komplement Faktor H bereits Erfolge zeigen konnte.

In dieser Arbeit wurden zwei im Moos *Physcomitrella patens* hergestellte humane Komplementfaktor H varianten in einem licht-induzierten Netzhautdegenerations Maus Modell getestet. Die beiden Faktor H varianten CPV-101 und CPV-104 unterscheiden sich in ihrer Glykosylierung. Beide Varianten, beziehungsweise eine PBS Kontrolle wurden einen Tag vor der Lichtexposition intravitreal injiziert. Anschließend wurden die Mäuse für 30 Minuten hellem weißem Licht mit einer Intensität von 10.000 lux ausgesetzt.

Bei beiden mit rekombinantem Faktor H behandelten Gruppen konnte eine reduzierte Komplementsystem Aktivität gezeigt werden die in weniger Membranangriffskomplex resultierte. Zudem wurde die Mikroglia und Müller Zell Reaktivität reduziert. Damit einhergehend wurden weniger pro-inflammatorische Zytokine und Chemokine exprimiert und sekretiert. Auch die Anzahl der TUNEL positiven Zellen sowie die Netzhautdegeneration wurden in beiden behandelten Gruppen reduziert. Einen deutlichen Einfluss der Glykosylierung auf die Wirksamkeit der Faktor H Varianten konnten wir nicht zeigen. Ein möglicher Einfluss auf die pharmakokinetischen Eigenschaften sollte jedoch Thema zukünftiger Studien sein. Die Ergebnisse sind zusammengefasst vielversprechend für eine mögliche Nutzung von rekombinantem Komplement Faktor H als Behandlungsoption.

Summary

Age related macular degeneration (AMD) is one of the leading causes of vision loss. Various factors are part of the complex disease etiology including genetic risk factors. Polymorphisms in the Complement factor H (CFH) gene are the most prevalent genetic factors for increased AMD risk. CFH is an important regulator of the alternative complement pathway, which is part of the innate immune response. A miss regulation of the complement system is also related to other diseases, like atypical hemolytic syndrome, that were already successfully treated with CFH supplementation.

In this study, two moss-derived human CFH variants were tested in a light-induced mouse model of retinal degeneration that is reproducing important aspects of AMD. Two different variants, CPV-101 and CPV-104, which differ in their glycosylation were injected intravitreal one day prior light exposure. The mice were exposed to bright white light for 30 min with an intensity of 10.000 lux.

Both variants were capable of reducing the complement pathway activity, resulting in less formation of membrane attack complex. Additionally microgliosis and Müller cell reactivity was reduced, leading to less pro inflammatory cytokine and chemokine signaling. Finally, the number of TUNEL positive cells as well as the retinal thinning was attenuated in mice treated with CPV-101 or CPV-104. This is a promising outcome for a possible future use of moss derived CFH as a therapeutic strategy.

Abbreviations

AAVs	Adeno-associated viruses
aHUS	Atypical hemolytic uremic syndrome
AF	Autofluorescence
AMD	Age related macular degeneration
APOE	Apolipoprotein E
ASGPR	Asialoglycoprotein receptors
BAF	Blue autofluorescent laser
BM	Bruch's membrane
BRB	Blood retina barrier
BSA	Bovine serum albumin
C1-INH	C1 inhibitor
CCL2	C-C Motif Chemokine Ligand 2
CFH	Complement factor H
CNS	Central nervous system
CNV	Choroidal neovascularization
C2fh	Fusion molecule of iC3b binding region of complement receptor C2 (CR2) and the N-terminal region of CFH
CR3	Complement receptor 3
C3aR1	C3a receptor
C5aR1	C5a receptor
CRP	C-reactive protein
CX3CLR1	Chemokine (C-X3-C motif) ligand 1 receptor
DAF	Decay-accelerating factor
DR	Diabetic retinopathy
ELISA	Enzyme-linked immunosorbent assay
FB	Factor B
FcRn	Neonatal Fc-Receptor
FDA	Food and drug administration
FI	Factor I
GA	Geographic atrophy
GAG	Glycosaminoglycan
GCL	Ganglion cell layer
GEWAS	Genome-wide association study
Gfap	Glial fibrillary acidic protein
GlcNAc	N-Acetylglucosamine
Gs	Glutamate synthase

Iba1	Ionized calcium-binding adapter molecule 1
Il	Interleukin
INL	Inner nuclear layer
ip	Intraperitoneal
IPL	Inner plexiform layer
ivt	Intravitreal
kDa	Kilodalton
MAC	Membrane attack complex
Man	Mannose
MASP	MBL associated serine proteases
MBL	Mannose-binding lectin
mTOR	Mechanistic/mammalian Target of Rapamycin
N.D.	Not detectable
NDS	Normal donkey serum
Neu5Ac	N-Acetylneuraminic acid
NFL	Nerve fiber layer
O.C.T.	Optimal cutting temperature
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segments
PEG	Polyethylene glycol
PRR	Pattern recognition receptor
RGC	Retinal ganglion cell
RGC	Retinal ganglion cell
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RPE	Retinal pigment epithelium
RT	Room temperature
SD-OCT	Spectral domain optical coherence tomography
SEM	Standard error of the mean
SIRPα	Signal regulatory protein α
sMAP	Small MBL associated protein
SNP	Single nucleotide polymorphism
SRS	Subretinal space
Tnf	Tumor necrosis factor
Tspo	Translocator protein (18 kDa)
TSP-1	Thrombospondin 1

VEGF	Vascular endothelial growth factor
WT	Wild type

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1. Introduction

1.1. The eye

Throughout various animal phyla, diverse variants of light sensitive organs have evolved (Halder *et al*, 1995). Most mammals possess similar composed eyes (Figure 1 A). The white sclera serves as a protective layer, maintaining the eye´s shape. The transparent cornea covers iris and pupil and is refracting light together with the lens and the anterior chamber. In humans, adjacent to the lens, is the large vitreous cavity which is confined by the retina. The retina, retinal pigment epithelium (RPE) and the choroid form three layers covering the focal plane of the eye (Forrester *et al*, 2016).

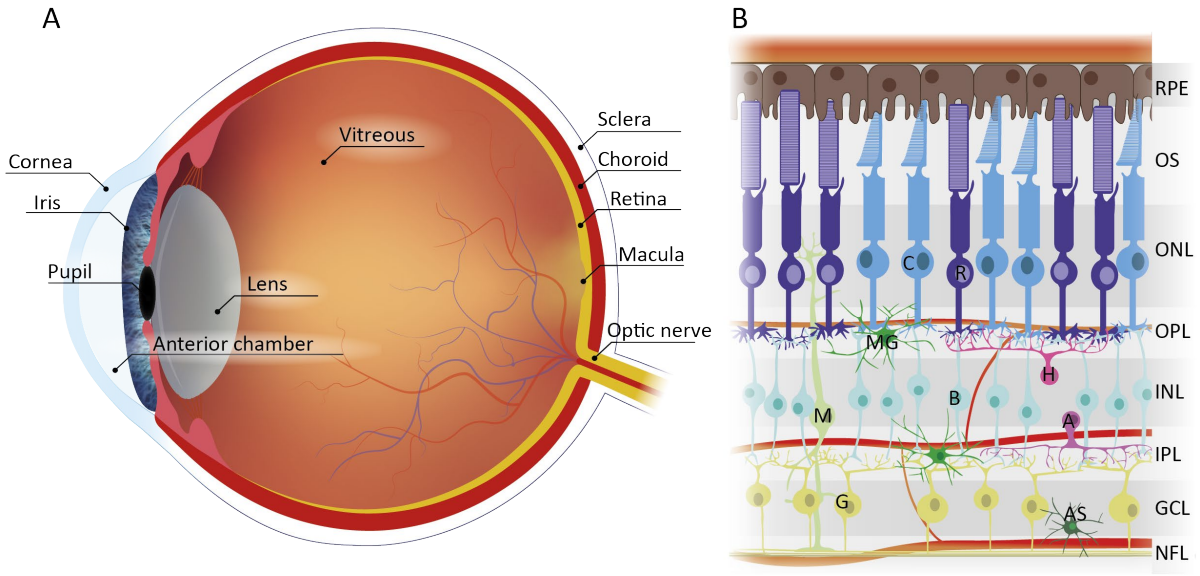

Figure 1: Anatomy of the human eye and retina. A Schematic anatomy of the human eye. **B** Schematic illustration of the retinal layers and cell types. The retinal pigment epithelium (RPE) is adjoined to the photoreceptor outer segments (OS). The photoreceptors can be distinguished into rods (R) and cones (C). The photoreceptor soma and nuclei form the outer nuclear layer (ONL). In the outer plexiform layer (OPL), the photoreceptors and bipolar cells (B) are connected. Horizontal cells (H) are also part of this synaptic network. The nuclei of H and B are in the inner nuclear layer (INL) together with the amacrine cells (A). Synaptic connections of A, B and ganglion cells (G) are made in the inner plexiform layer (IPL). The ganglion cell layer (GCL), consists of ganglion cell somata and the corresponding axons form the nerve fiber layer (NFL). Microglia (MG) are located in the plexiform layers, while astrocytes (AS) are mainly found in GCL and NFL. The Müller cells (M) span throughout the retina, with their somata located in the INL.

To process an image in the brain, light enters through the pupil and is refracted mainly to the *macula lutea* which is the central part of the retina, responsible for the most accurate vision. The retina is highly organized into distinct layers and capable of transducing light into neuronal activity (Figure 1 B). In brief, light reaches the photoreceptor outer segments (OS), where different photopigments undergo photon-triggered isomerization, initiating a phototransduction cascade. The photoreceptor nuclei are located in the outer nuclear layer (ONL). The next layer, in the direction towards the vitreous is the outer plexiform layer (OPL), where the emerging signal from phototransduction is forwarded by bipolar cells to the ganglion cells. Cell bodies of the bipolar cells, along with amacrine and horizontal cells,

form the inner nuclear layer (INL). The synaptic connections between ganglion cells and bipolar cells form in the inner plexiform layer (IPL), followed by the ganglion cell layer (GCL) (Forrester *et al.*, 2016). Finally, the axons of the ganglion cells constitute the innermost retinal layer, the nerve fiber layer (NFL). These axons reach the optic nerve head, which, due to its absence of photoreceptors, is a blind spot, and the signal is transmitted along the optic nerve towards the brain.

Our ability of color vision and adaption to dim light conditions is caused by the different kinds of photoreceptors. They can be divided into rods, which are sensitive to dim light (scotopic vision), and three types of cones, each sensitive to different wavelengths, enabling color perception under bright light conditions (photopic vision).

While the rods are predominantly distributed in the periphery of the eye, the cones exhibit their highest density in the central macula, the *fovea centralis.* A part of the fovea is an avascular area that is mostly supplied with oxygen by the RPE. The RPE is also crucial for maintaining the visual cycle by phagocytizing shed photoreceptor outer segments and reverting the isomerization that is essential for the photo transduction (Yang *et al*, 2021). An active visual pigment consists of an opsin and an 11-cis retinal. Upon light exposure, the 11-*cis* retinal is photoisomerized to an all-*trans* retinal. The RPE65 gene encodes for the retinoid isomerohydrolase that is crucial for reverting this reaction and therefore maintaining the visual cycle (Cai *et al*, 2009). Besides the RPE, the retinal neurons are also kept in a homeostatic surrounding with support of glia cells. However, various retinal diseases lead to disruption of this homeostasis, also inducing immune reactions that can deteriorate retinal damage.

1.2 Age-related macular degeneration

Age-related macular degeneration (AMD) is a progressive ocular disease that mainly affects the central part of the retina and is the leading cause of irreversible vision loss among the elderly population.

Early AMD is characterized by the formation of drusen, extracellular deposits that accumulate between the RPE and Bruch's membrane (BM). These drusen impair normal retinal function, resulting in visual disturbances (Figure 2 B). The late disease stages can be classified as either exudative (wet) or nonexudative (dry). Exudative AMD, although less prevalent, poses a severe threat to vision due to the growth of abnormal blood vessels from the choroid through BM towards the retina, a condition known as choroidal neovascularization (CNV). These abnormal blood vessels are prone to leakage, leading to detachment of the retina from the RPE (Figure 2 C). Exudative AMD can be treated by anti-angiogenic substances that need to be intravitreal (ivt.) injected regularly. Injections of anti-vascular endothelial growth factor (VEGF) therapeutics are regularly used gold standard in the clinics. Although they diminish the leaking blood vessels and lead to improved visual acuity, there are still patients that do not respond to this therapeutic approach (Sharma *et al*, 2013).

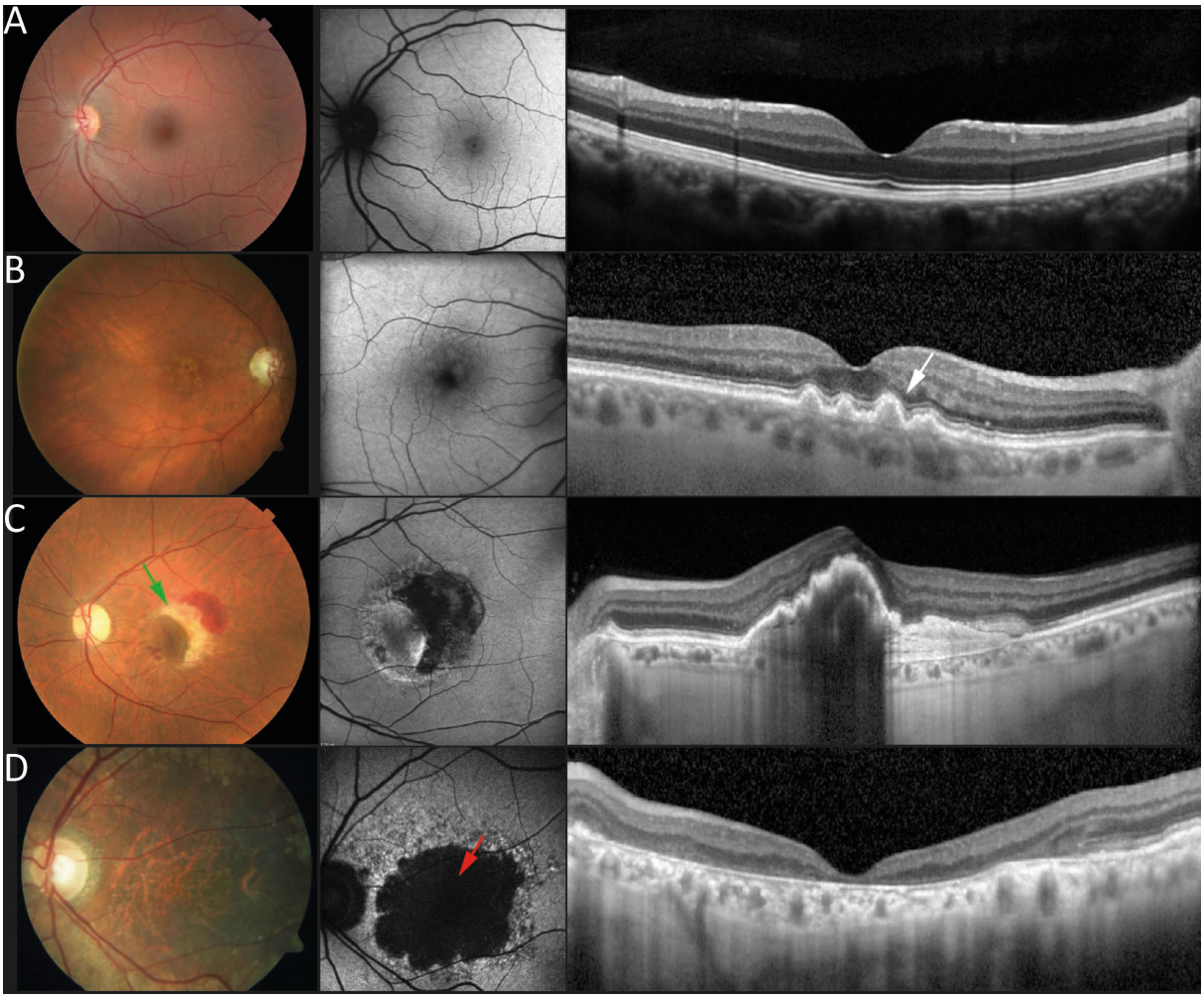

Figure 2: Stages of AMD progression. A Fundus photography, fundus autofluorescence and optical coherence tomography (OCT) of a healthy eye. **B** intermediate AMD patient, drusen are indicated with a white arrow. **C** patient with exudative (wet) AMD fluid accumulation indicated with a green arrow. **D** patient with late stage dry AMD, GA area indicated with a red arrow. Picture modified from (Hadziahmetovic & Malek, 2020)

Unlike the exudative form, dry AMD is more prevalent and typically has a slower disease progression. It is characterized by the accumulation of drusen that finally lead to geographic atrophy (GA) (Figure 2 D). The formation of these atrophic areas results in photoreceptor loss, which consequently reduces visual acuity. The treatment options for dry AMD are limited at the moment.

The complex disease etiology of AMD is composed of various risk factors. Modifiable factors such as smoking, obesity, and alcohol consumption have been implicated, alongside non-modifiable factors including age, gender, and genetic predisposition (Hyman & Neborsky, 2002). In addition, the innate immune system is influencing the pathophysiology of AMD. Especially chronic microglia and complement system activity contribute to disease progression (Ascunce *et al*, 2023).

1.2.1 Mouse models for AMD

The human eye as well as the complex AMD etiology, composed of environmental and genetic factors, is at the moment not fully mimicked with *in vitro* or *in vivo* models. Nevertheless, animal models can still contribute to development of novel therapeutic approaches. Although mouse eyes show many similarities to the human eye, some important differences in their ocular anatomy need to be considered. Mouse retinas do not have a macula in contrast to the human retina. Additionally, mice are lacking the cones that can detect long wavelengths from 564–580 nm, which makes them much less sensitive to red light (Bridges, 1959). Independent of these differences, it is still possible to replicate certain important aspects of AMD in mouse models. A variety of models is available – models using high fat diet or a high glycemic index, genetic models, cigarette smoke models, light- and laser-induced degeneration (Pennesi *et al*, 2012).

For neovascular AMD, an often used model is the laser-induced CNV. The disruption of the BM induces angiogenesis which leads to the ingrowth of leaking blood vessels (Shah *et al*, 2015). Alternative models increase the amount of pro-angiogenic factors like VEGF, by injection or with genetic models (Rastoin *et al*, 2020).

As slow progressing models for dry AMD, genetic modification of pathways that are also known to be influenced in patients are often used. One option is the use of complement system disruption using e.g. a complement factor H (CFH) knockout (KO) mouse, which develops drusen-like structures within the retina. However, these animals also develop kidney conditions and visual impairment were seen only in two-year-old mice (Coffey *et al*, 2007). Another possibility is influencing the cytokine/chemokine signaling. One example here is the CC-chemokine ligand 2 (CCL2) KO mouse that is also developing drusen-like accumulations and dysfunctional photoreceptors after nine months of age (Ambati *et al*, 2003). The slow progression in these models makes it also more difficult to see differences a potential treatment is achieving.

The model used in this work is a model of acute retinal degeneration using bright white light, leading to a synchronized photoreceptor cell death (Grimm & Reme, 2013). Mice that carry a variant of the RPE65 gene (L450M) are more susceptible to light damage, which enables a less strong intensity and shorter exposure time (Wenzel *et al*, 2001). The light-induced retinal degeneration replicates important key aspects of dry AMD, like the photoreceptor cell death, an inflammatory response consisting of activation of complement system (Schäfer *et al*, 2017), gliosis (Karlstetter *et al*, 2015) and cytokine secretion (Rutar *et al*, 2015).

1.3. Retinal glia cells

The retina as part of the central nervous system (CNS) contains three types of glia cells: Astrocytes, Müller cells and microglia (Reichenbach & Bringmann, 2020). Astrocytes contribute to integrity of blood retinal barrier (BRB) and are mainly located in NFL and GCL. Their processes are connected to blood vessels, thereby stabilizing tight junctions. Furthermore, astrocytes are crucial for the development of retinal vasculature (Provis *et al*, 2000; Stone & Dreher, 1987). Astrocytes and Müller cells share several homeostatic functions, such as buffering the potassium levels, removing CO_2 and regulating the pH (Bringmann *et al*, 2006; Ramírez *et al*, 1998). In contrast to the astrocytes, the soma of Müller cells is located in INL and they form two main processes that span throughout the retina. They are with about 90% of glia cells the most abundant glia type within the retina. Besides their role in maintaining homeostasis, their unique characteristics like a high reflective index, the funnel shape and the long processes work as an optical fiber for light (Franze *et al*, 2007). Activation of Müller cells and astrocytes can be triggered by pathogens, injury or retinal degeneration. This gliosis leads to cytokine and chemokine secretion and expression of complement components that can contribute to disease progression (Telegina *et al*, 2018). Similar to these macroglia, also microglia are capable of reacting to retinal damage.

1.3.1. Microglia

Microglia are the resident macrophages of CNS (Graeber & Streit, 1990). They have a variety of different functions in the retina that differ between developmental and mature retina. During development, microglia influence the synapse pruning, vascularization and neurogenesis. The synaptic pruning of microglia is regulated in complement system dependent manner. Complement receptor 3 (CR3, composed of CD11b and CD18), is expressed on microglia surface, capable of sensing complement component C3, which is binding to retinal ganglion cells (RGC). This signaling induces phagocytosis of the corresponding RGCs which is crucial for normal development of the retina (Schafer *et al*, 2012). Also the chemokine (C-X3-C motif) ligand 1 receptor (CX3CLR1) on the microglia surface was associated with cone maturation but also maintenance in adults (Jobling *et al*, 2018). In line with their function, microglia also vary in their morphology. While the cells are amoeboid during development, they become more ramified in the mature retina, where they are mainly located in the plexiform layers during homeostatic conditions (Figure 3 A) (Li *et al*, 2019).

For maintaining the microglia homeostasis several ligand-receptor signaling pairs are crucial. A functional impatient of the CX3CL1- CX3CLR1 signaling due to sequence variations can elevate the AMD risk, but can also be used as an AMD mouse model (Combadiere *et al*, 2007; Tuo *et al*, 2004). Similarly the CD200–CD200R signaling is important for limiting potentially excessive immune responses. A CD200R KO consequentially leads to increased inflammatory responses in an AMD model (Horie *et al*,

2013). Sialic acids, as well as CD47 are important for self-recognition, Siglecs and Signal regulatory protein α (SIRPα) as the corresponding receptors on microglia surface prevent phagocytic activity. Besides their function in homeostasis, microglia are capable of sensing retinal damage or pathogens by multiple pattern recognition receptors (PRR) that lead to reactivation. The cell morphology changes towards an amoeboid shape, additionally the cells migrate towards the damage site, express pro-inflammatory cytokines like interleukins (Il-6, Il-1β) and tumor necrosis factor-α (Tnf-α), reactive oxygen species (ROS) and increase their phagocytic activity (Figure 3 B) (Murenu *et al*, 2022).

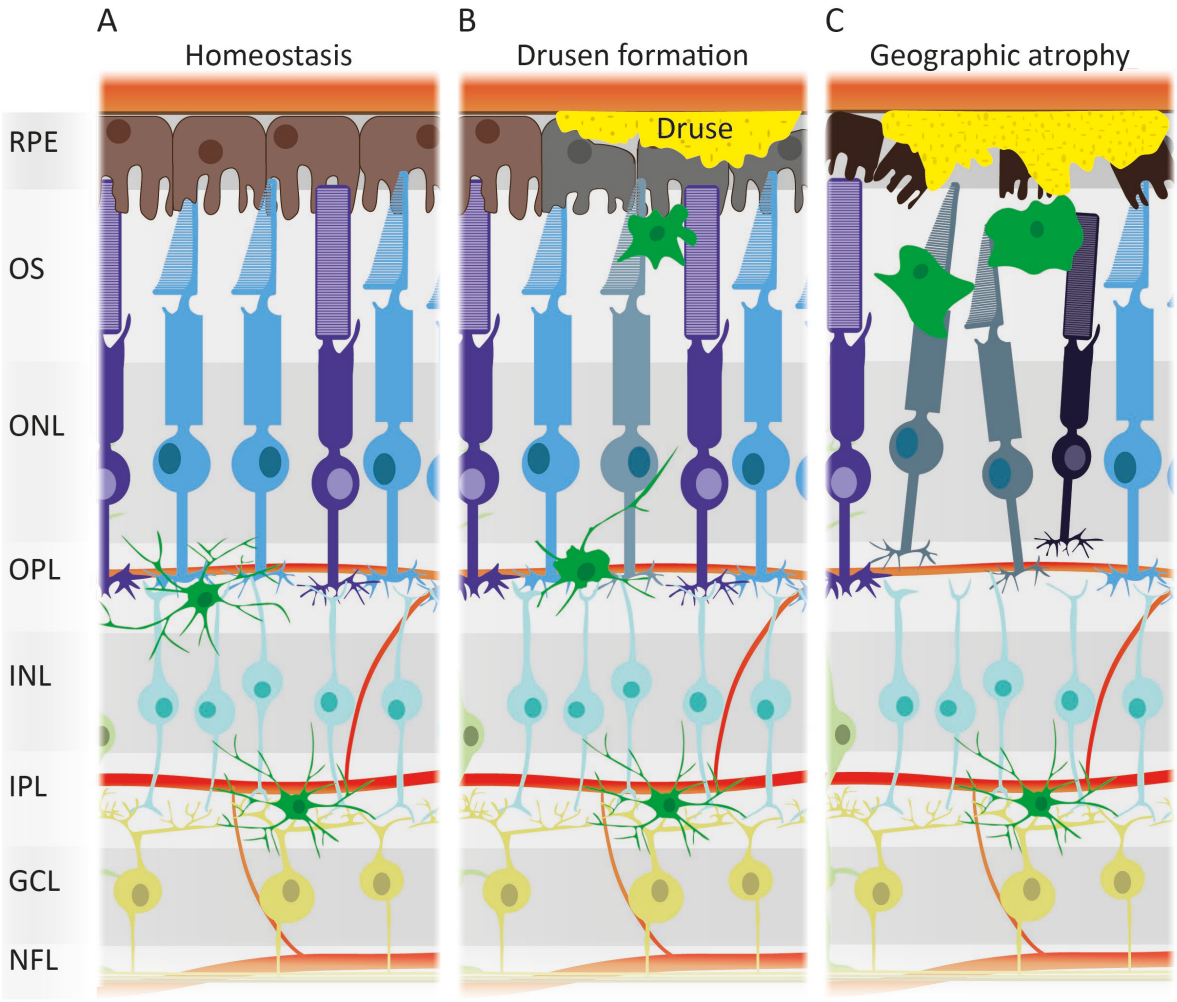

Figure 3: Schematic overview of microglia in GA. A In homeostatic conditions ramified microglia (green) are located within the plexiform layers with long protrusions. **B** Microglia are capable of detecting retinal damage resulting from drusen formation. They migrate towards the affected area, which is mainly the subretinal space in AMD, where they phagocytose dead cells and secrete pro-inflammatory factors to restore homeostasis. **C** In a late stage of AMD, chronic activity of microglia is exacerbating the retinal degeneration. RPE retinal pigment epithelium, OS outer segments, ONL outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer, GCL ganglion cell layer, NFL nerve fiber layer

This immune reaction plays a crucial role in tissue repair, facilitating a rapid return to homeostasis. However, chronic microglia reactivity is known to not only contribute to various neurodegenerative diseases such as Alzheimer´s, Amyotrophic lateral sclerosis and Parkinson's disease, but also to retinal

degenerative diseases like diabetic retinopathy (DR), glaucoma and AMD (Graeber & Streit, 2010; Rashid *et al*, 2019). Amoeboid microglia are found in the ONL and in the SRS of patients with GA (Gupta *et al*, 2003; Rashid *et al.*, 2019). The secretion of pro-inflammatory factors can induce photoreceptor cell death and therefore exacerbate the ongoing retinal degeneration. The same effects were described in corresponding animal models recreating important aspects of AMD (Karlstetter *et al*, 2010). The microglia migration is e.g. mediated by chemoattractants like Chemokine (C-C motif) ligand (Ccl2) and the corresponding receptor (CCR2). Low doses of Ccl2 are needed for survival of ganglion cells, whereas high doses that can also be expressed by microglia itself can induce a more rapid cell loss (Chiu *et al*, 2010). Due to this role of microglia in retinal degeneration, they became an interesting target for modulation.

Modulating the microglia reactivity with various substances was shown to influence retinal degeneration in different AMD mouse models. One way of modulating microglia is the use of sialic acids, which are natural occurring on the neuron surface proteins and lipids. The sialic acid binding immunoglobulin-like lectin 11 (Siglec11) receptor on microglia cells is capable of sensing sialic acids enabling self-recognition as part of the microglia regulatory mechanisms. Consequently, ivt. application of polysialic acid reduced microglia reactivity, CNV and retinal degeneration in a laser model of CVN and in a light-induced retinal degeneration, respectively (Karlstetter *et al*, 2017; Krishnan *et al*, 2023). Another example is the modulation via the translocator protein (Tspo). Tspo is an activation marker for microglia that was shown to be upregulated in various retinal disease models, but also in patients with AMD (Hector *et al*, 2024). The modulation of microglia using the Tspo ligands was shown to reduce microglia reactivity in models for dry and neovascular AMD. In both models, the modulation led to reduced retinal damage (Scholz *et al*, 2015a; Wolf *et al*, 2020). Reducing microglia reactivity is therefore beneficial for dampening AMD progression.

1.4. The complement system

The complement system is a complex cascade of about 50, mainly liver-derived, proteins that are part of the innate immune response. It has an important role in pathogen recognition, removal of dead cells and cell debris, but also synaptic pruning in development. Three different pathways lead to activation of the complement system. The classical pathway as well as the lectin pathway are mainly a reaction to pathogen contact, whereas the alternative pathway can be activated rather spontaneously (Figure 4). All three pathways can be subdivided into an initiation, C3 convertase activity, C5 convertase activity and finally formation of the membrane attack complex (MAC) (Ricklin *et al*, 2010).

For the classical pathway, the C1 complex, consisting of C1q and two molecules C1s and C1r, bind to a cell surface. Specifically C1q can either directly bind certain antigens, bind to the constant region of IgG or IgM antibodies, or bind to the C-reactive protein (CRP), which is capable of marking apoptotic

cells (Kaplan & Volanakis, 1974). The binding of the C1 complex implements a conformational change that leads to a C1r dependent cleavage of C1s. C1s becomes an active serine protease, capable of cleaving C4. The emerging C4b is than bound to the cell surface and to C2, which is then also cleaved by C1s. Emerging C2b, which is also a serine protease, remains bound to C4b, both form a C3 convertase (C4b, C2b). The cleavage of C3 leads to C3b accumulating at the cell surface, where it binds to C4bC2b, finally forming a C5 convertase (C4b, C2b, C3b). The cleavage product of C5, C5b is finally part of MAC. C6, C7, C8 and C9 are all soluble complement components that undergo drastic conformational chances upon binding to C5b or each other, resulting in β-hairpin structures within the lipid bilayer (Xie *et al*, 2020). After forming an initiative complex (C5b, C6, C7, C8), 12-22 C9 molecules form an oligomer resulting in pore formation within the membrane (Dudkina *et al*, 2016; Podack *et al*, 1982).

Similar to the classical pathway, the lectin pathway requires pathogen surface binding for activation. Both pathways only differ in their initiation phase. Either the mannose-binding lectin (MBL) or ficolin bind to carbohydrates or glycoproteins on the pathogen surface. Three different MBL associated serine proteases (MASP1, MASP2 and MASP3) as well as the small MBL associated protein (sMAP) are forming a complex that reassembles C1 complex, likewise facilitating complement activation. MASP2 is in this case cleaving C4 and C2, which also results in formation of C3 convertase (C4bC2b). The following cascade is identical with the classical pathway.

The alternative pathway, unlike the other pathways, is initiated independently of both pathogens and C3 convertase activity. Instead, it is activated by a mechanism often referred to as the “C3 tick over”. The thioester bond of C3 is hydrolyzed spontaneously, leading to a conformational change (C3(H_20)). However, the contact to various surfaces such as gas bubbles and lipids was shown to make the generation of C3(H_20) more likely (Hamad *et al*, 2010; Nilsson Ekdahl *et al*, 1992). C3(H_20) is capable of binding Factor B (FB), which enables cleaving of FB by the serine protease Factor D (FD). The emerging Bb remains bond to C3(H_20), forming a C3 convertase (C3(H_20),Bb). Thus C3 can be cleaved to C3b and C3a. The emerging C3 can also bind to FB, resulting in the C3 convertase C3b,Bb. This self-amplification loop, that results in more cleavage of C3 and therefore more formation of the C3 convertases is not only occurring in the alternative pathway, but also applies for C3b from classical and lectin pathway. Additionally, C3b can bind to alternative pathway C3 convertases, resulting in the formation of a C5 convertase (C3b, Bb, C3b). Cleaving of C5 finally results in MAC formation and cell lysis, as for the other two pathways.

The cleavage products C3a and C5a are not directly part of the further cascade steps, but act as anaphylatoxins, that can influence apoptosis and immune response (Klos *et al*, 2009). Anaphylatoxins were also related to increased VEGF expression and therefore promotion of CNV (Nozaki *et al*, 2006). Additionally, high serum levels of anaphylatoxins were suggested to increase the risk of subretinal

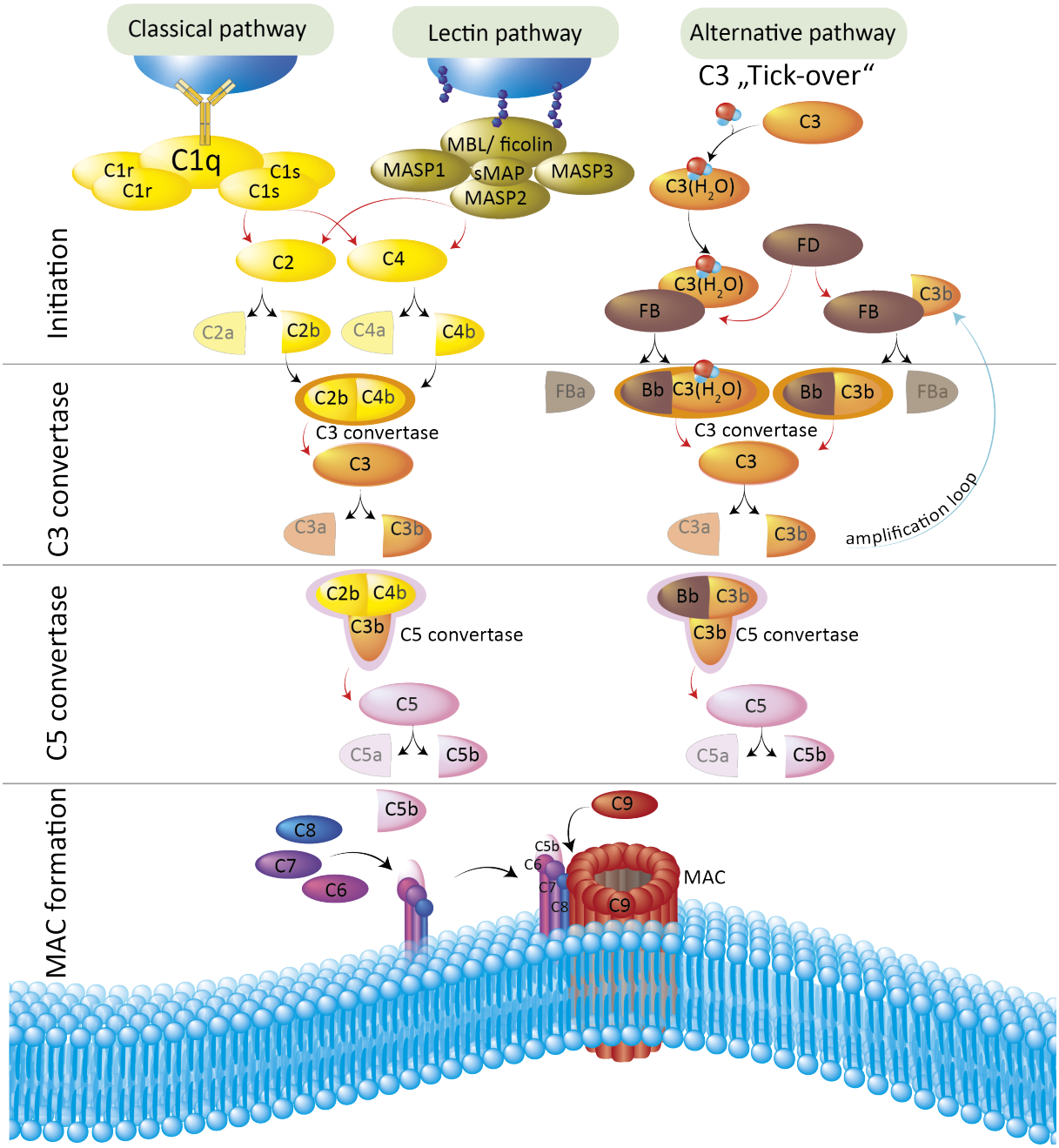

Figure 4 Schematic overview of the complement system pathways. The complement system can be activated by classical, lectin and alternative pathway. The classical and lectin pathway are both dependent on binding to pathogen surfaces for activation and only differ in their initiation. While the classical pathway is activated by binding of the C1 complex (C1q, 2xC1s, 2xC1r) to the Fc region of antibodies, the lectin pathway senses pathogens via binding to carbohydrates with a complex that reassembles C1 complex (MBL/ficolin, MASP1,MASP2,MASP3 and sMAP).both pathways lead to the formation of C3 convertase C4bC2b, followed by the formation of C5 convertase by adding C3b to form C4bC2bC3b. The cleavage of C5 results in C5b, which finally leads to formation of the membrane attack complex (MAC). The alternative pathway is independent of pathogen surface binding and can be activated by hydrolysis of C3, also called C3 “tick-over”, leading to activation of the complement system. All pathways have C3 and C5 convertase activity in common, although the convertases of the alternative pathway have a slightly different composition. C3 convertase is formed with hydrolyzed C3 ($C3H_2O$) or C3b and the cleaved factor B resulting in $C3H_2OBb$ or C3bBb. C5 convertase is achieved by binding of another C3b to C3bBb, forming C3bBbC3b. Finally the alternative pathway also results in formation of MAC, which does not differ between pathways. Red arrows indicate cleavage, black arrows depict the pathway flow and the cyan arrow shows the amplification loop of the alternative pathway. MBL - mannose-binding lectin, MASP - MBL associated serine proteases, sMAP - small MBL associated protein, MAC – membrane attack complex.

fibrosis in neovascular AMD patients (Lechner *et al*, 2016). C5a is the most potent anaphylatoxins and capable of recruiting several immune cells like, neutrophils, monocytes and T-cells as a chemoattractant. Additionally, C5a can enhance the inflammatory response of microglia as well as their migration (An *et al*, 2018; Song *et al*, 2017).

1.4.1. Complement system regulation

The complement pathways especially the alternative pathway, which is independent of pathogen contact, can also harm healthy cells. Hence complement activity needs to be tightly regulated. Several different plasmatic and membrane bound factors serve as regulators for the cascades. The C1 inhibitor (C1-INH) belongs to the group of plasmatic inhibitors and can bind irreversible to C1r, C1s, MASP1 and MASP2, which leads to inactivation of classical and lectin pathway respectively (Arlaud *et al*, 1979). The serine protease factor I (FI) can cleave C4b in a cofactor-dependent manner, which is influencing the classical pathway. For cleaving of C4b, the C4b binding protein servers as a cofactor (Ermert & Blom, 2016). However, FI can also cleave C3b, in this case complement factor H (CFH) can serve as a cofactor (Pangburn *et al*, 1977). CFH regulates especially the alternative pathway in different ways. Besides being a cofactor for FI dependent degradation, it can make the C3 convertase C3bBb more prone to dissociation (Weiler *et al*, 1976). Additionally, it can bind to C3b and therefore avoid binding of FB and formation of the C3 convertase C3bBb (Whaley & Ruddy, 1976).

Besides the soluble factors, also membrane bound proteins like CD46, serve as a cofactor for FI facilitated degradation of both C4b and C3b (Liszewski *et al*, 1991). Similar to CFH, CD55 also known as the decay-accelerating factor (DAF) can accelerate dissociation of C3 convertases C4bC2b and C3bBb (Fujita *et al*, 1987). Finally, also the MAC formation itself can also be inhibited by binding of the cell surface protein CD59 to C8 and C9 preventing the formation of a C9 oligomer (Couves *et al*, 2023; Davies *et al*, 1989).

This tight regulation of cascades is necessary to prevent various pathologies caused by an over reactive complement system. Nevertheless, a complement system not responding to stimuli can also contribute to disease pathologies.

1.4.2. The role of complement system in different disease etiologies

A dysregulation of the complement system contributes to a variety of diseases. Deficiencies in classical pathway components, such as C2 deficiency, which reduce complement activity, can increase susceptibility to infections, rheumatic diseases, and atherosclerosis (Jonsson *et al*, 2005; Sjoholm *et al*, 2006). Similar findings have been demonstrated for lectin pathway deficiencies. Low MBL levels in patients have for example been linked to higher risk for infections, but also influences cardiovascular diseases (Koch *et al*, 2001; Thiel *et al*, 2006).

Moreover, deficiencies of the regulatory complement components are also known to contribute to different disease etiologies resulting in an overactive complement system. Hereditary angioedema can be caused by a lack of C1-INH (Bork *et al*, 2019). Mutations in the regulatory alternative pathway genes, specifically, are associated with various diseases like atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathy and AMD (Zipfel *et al*, 2006). The most affected protein in context of these diseases, besides others like FI and CD46, is CFH.

In addition to these proteins being involved in AMD pathology, overall complement system activity has also been observed in other ocular diseases. In bacterial infections of cornea, it was observed, that certain bacteria are capable of enzymatically removing DAF and CD59 from the ocular surface which in leading to massive immune reaction and tissue damage (Cocuzzi *et al*, 2000). In other infectious diseases like uveitis, elevated serum concentration of C3 and C4 were observed. Also, for DR and glaucoma, a potential role of the complement system was discussed (Rathi *et al*, 2023).

In AMD specifically, this dysregulation of the complement system can be observed through the accumulation of complement components, like C3, C5, C6-9, FB, CRP and CFH in drusen and enhanced blood levels of these complement components (Crabb *et al*, 2002; Seddon *et al*, 2004).

1.4.3. Complement factor H

CFH plays a central role in regulating the alternative pathway, which partly also serves as an amplification loop for classical and lectin pathway. It is mainly expressed by the liver, but can also be expressed locally in the eye by RPE cells, choroidal endothelial cells and even photoreceptors (Chen *et al*, 2007; Schwaeble *et al*, 1987; Smit-McBride *et al*, 2015). The regulative effect of CFH is facilitated by the acceleration of C3 convertase decay, inhibition of C3 convertase formation and the ability of CFH to function as a cofactor for FI mediated C3b cleavage (Figure 5 A). These mechanisms reduce the formation of C3b deposits and thus a degradation of healthy host cells.

CFH is a 155 kDa glycoprotein composed of 20 complement control protein domains (CCP) each with about 60 amino acids that are arranged in a flexible chain (Figure 5 B) (Ripoche *et al*, 1988). It is active not only in serum, but also binds to the cell surfaces. The C3b binding is facilitated by domain 1-4 and domain 19 and 20 (Gordon *et al*, 1995; Kuhn *et al*, 1995). Whereas the binding to glycosaminoglycan (GAG) on the cell surface was shown to be dependent on domain 7 and 20 (Ormsby *et al*, 2006; Pangburn *et al*, 1991). Notably also domain 9-15 were discussed to bind C3b as well as GAGs (Ferreira *et al*, 2010). CFH has 9 potential N-glycosylation sites, 8 of them were shown to be glycosylated (Asn511, Asn700, Asn784, Asn804, Asn864, Asn893, Asn1011 and Asn1077) (Fenaille *et al*, 2007). The glycosylation itself is not influencing the CFH binding ability to C3b or GAGs, but can influence the

serum half-life (Jouvin *et al*, 1984; Tschongov *et al*, 2024). In contrast, mutations in CFH can have a tremendous impact on CFH function and patients' health.

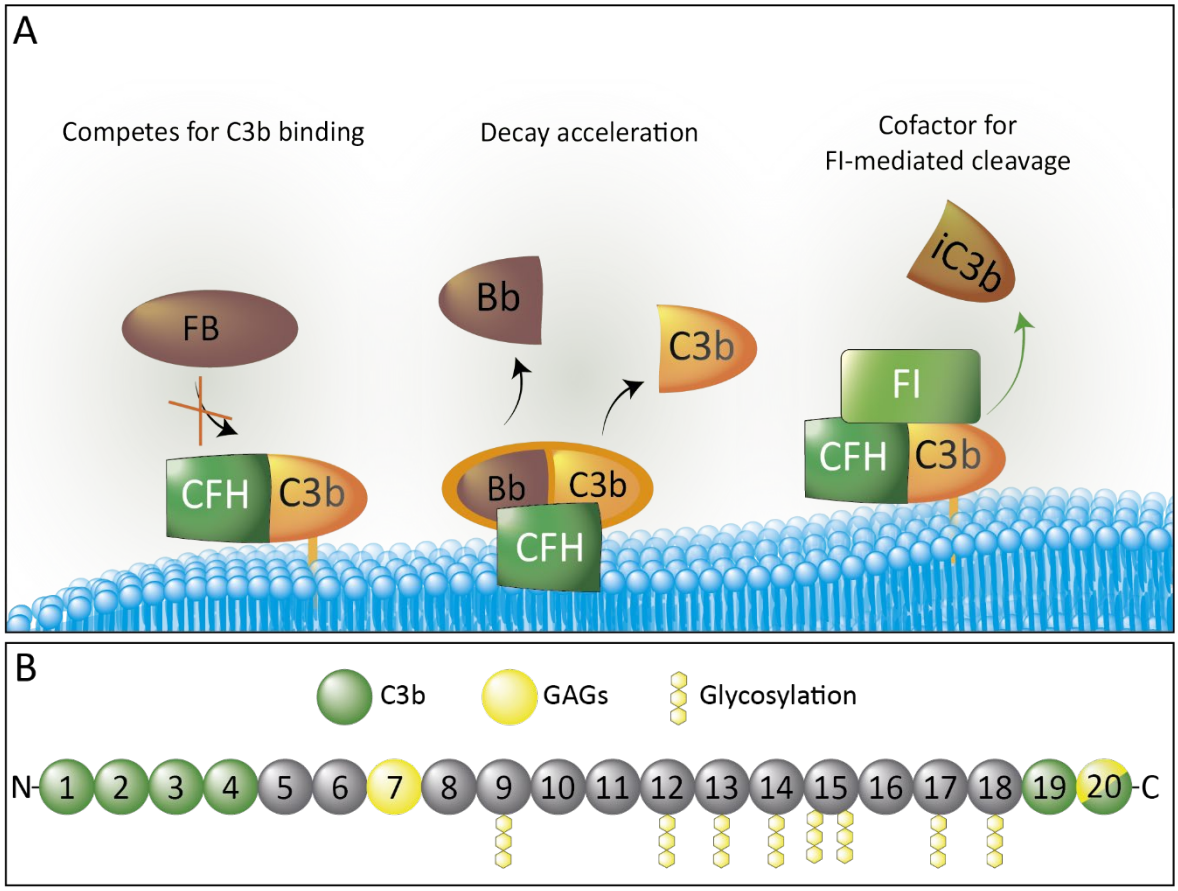

Figure 5 Schematic depiction of CFH functions and structure. A CFH is capable of regulating the alternative complement pathway in fluid phase and on cell surfaces. It either competes with factor B for C3b binding which is decreasing formation of C3 convertase. CFH can also accelerate the decay of C3 convertases and function as a cofactor for factor I mediated C3b inactivation. **B** CFH consists of 20 complement control protein domains (CCP). Domains that were shown to facilitate C3b binding are show in green, domains that bind glycosaminoglycans (GAGs) are shown I yellow. Glycosylation sites are indicated with three hexagons.

One of the first and also most studied single nucleotide polymorphisms (SNPs) that was found in genome wide association studies (GEWAS) is the Y402H polymorphism (rs1061170) affecting CFH (Edwards *et al*, 2005; Haines *et al*, 2005; Klein *et al*, 2005). Several groups found this polymorphism to be highly related with AMD risk, increasing the susceptibility in people heterozygous for this variant already about 2.5 times, whereas the AMD risk for homozygous carries is even about 6 times higher (Thakkinstian *et al*, 2006). Besides the increased risk, neovascular AMD patients homozygous for this SNP also show a poor long term response to anti-VEGF drugs compared to patients without this polymorphism (Hong *et al*, 2016; Veloso *et al*, 2014). The Y402H polymorphism is located in the 7th CCP, which is capable of GAG binding, specifically of heparin binding, which is also affecting the binding affinity to BM (Clark *et al*, 2017). CCP 7 is also associated with binding CRP, mutations can therefore reduce the regulatory function of CFH. Consequentially, increased level of CRP were find already in AMD patients with Y402H variant (Seddon *et al.*, 2004).

Besides the common Y402H variant, there are more rare mutation of the CFH gene known to be related to AMD. R1210C is affecting the GAG binding of CCG 20 which is leading to increased drusen numbers and a higher chance of developing GA (Ferrara & Seddon, 2015; Raychaudhuri *et al*, 2011). Other rare variants like D90G and R53C that inhibit the decay accelerating and the cofactor abilities are located in CCP 1 and 2 respectively, influencing the C3b binding. (Yu *et al*, 2014). The mutation P503A in CCP7 was found in an Amish population (Hoffman *et al*, 2014; Waksmunski *et al*, 2022). Although carriers of this mutation were younger in AMD onset than control patients, the exact impairment in CFH functions was not yet found. *In silico* modeling however proposed conformational changes (Waksmunski *et al.*, 2022). Another SNP that is influencing AMD risk, is the I62V variant in the 2nd CCP, influencing the C3b binding ability (Hageman *et al*, 2005). This gene variant is considered protective for AMD, underlining the impact of CFH and its regulatory function for AMD pathology.

Although the role of CFH in AMD makes it an interesting target for therapy, CFH was barely tested, as its expression remained challenging. However, targeting the complement system as a therapeutic approach was attempted in AMD patients already.

1.4.4. The complement system as a target for AMD therapy

Whilst there are several different anti-VEGF antibodies, Fabs and fusion molecules available for neovascular AMD therapy, there were no therapies available for GA until 2023. Most clinical trials were done with peptides or antibodies binding to C3 or C5. Notably, most of these studies were not successful due to a lack of a beneficial therapeutic effect in GA patients (Rathi *et al.*, 2023).

However, two complement system modulating substances were approved by Food and Drug Administration (FDA) recently. Avacincapad pegol (Izervay®), which is an aptamer specific for C5 and pegcetacoplan (Syfovre®), which is a polypeptide binding to C3 and C3b (Heier *et al*, 2023; Khanani *et al*, 2023). Both were capable of reducing progression of GA lesion size, slowing down the disease progression. Notably, both were not improving the visual acuity of patients so far. Additionally, both use polyethylene glycol (PEG) to improve pharmacokinetic characteristics of the molecules (Veronese, 2001). This PEGylation is known to induce production of anti-PEG antibodies and hypersensitivity in some patients (Zhang *et al*, 2014). Subretinal injections of PEG were also shown to induce CNV in mice (Lyzogubov *et al*, 2011). Accordingly, the clinical studies using avacincapad pegol and pegcetacoplan showed an increased risk of developing a nonvascular AMD compared to the sham treated controls (Jaffe *et al*, 2021; Liao *et al*, 2020). Notably, both substances also influence all complement cascades which could potentially increase the risk of infections. It is also discussed, at what point within the cascade the inhibition needs to act. Both therapeutic approaches hinder the emerging of the anaphylatoxin C5a and the formation of MAC. However, C5 inhibition of avacincaptad pegol still enables the formation of anaphylatoxin C3a, which still can influence immune cells.

The supplementation of CFH was also considered, especially for those patients carrying a CFH SNP that is influencing its functionality. A treatment with serum-derived CFH in patients with other CFH dysfunction related diseases like aHUS and C3 glomerulopathy already showed promising results (Haffner *et al*, 2015; Licht *et al*, 2006). GEM103, a recombinant produced CFH variant was tested in a Phase II clinical trial that was discontinued to a lacking effect in GA patients. Interestingly, there are also no data published showing an effect *in vivo*, only *in vitro* data showing the binding to C3b are available (Biggs *et al*, 2022; Khanani *et al*, 2022). CFH was expressed before in insect cell, mammalian cells, as for GEM103 and yeast, however none of these approaches achieved therapeutic success so far.

1.4.5. Moss-derived human CFH

Since 1962, the spreading earth-moss *Physcomitrella patens* is a regular used model organism due to its ability for homologous recombination enabling easy gene editing (Rensing *et al*, 2020). In contrast to other expression systems like bacteria or mammalian cells, *P. patens* can express complex proteins with correct folding whilst keeping the production costs low (Nosaki *et al*, 2021). These capabilities make it a compelling system for large-scale protein productions. *P. patens* also has the capacity of N-glycosylation as a post-translational modification, with the same N-Acetylglucosamine (GlcNAc) and mannose (Man) core as in human glycoproteins ($GlcNAc_2Man_3GlcNAc_2$)(Koprivova *et al*, 2003). However, the plant-specific sugar residues xylose and fucose (Figure 6 A) are known to cause immune reactions in mammals (Bardor *et al*, 2003).

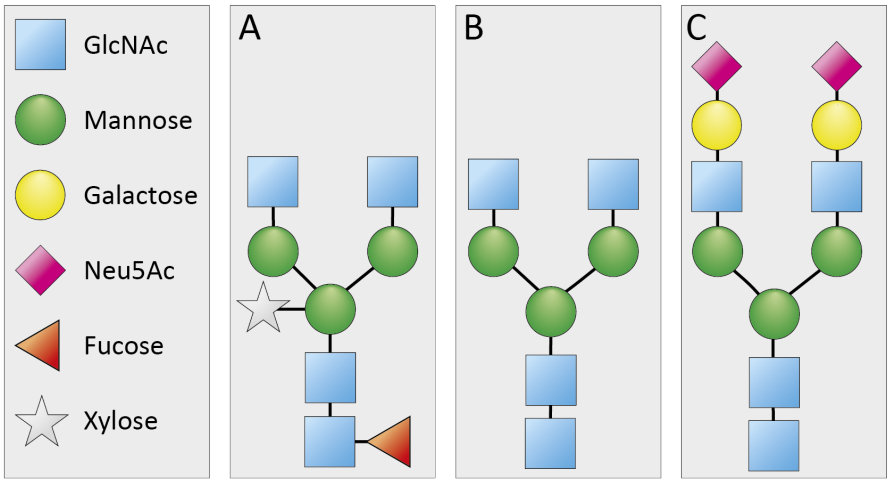

Figure 6: Differences in N-linked glycosylation A glycosylation of *Physcomitrella patens* including plant specific residues fucose and xylose, that cause immunoreaction in mammals. **B** glycosylation of CPV-101, that is achieved by using *P. patens* stain with a KO of β1,2-xylosyltransferase and α1,3-fucosyltransferase. **C** glycosylation of CPV-104. Reassembling human glycosylation using β-1,4-galactosyltransferase and α-2,6-sialyltransferase. GlcNAc - N-Acetylglucosamine, Neu5Ac - N-Acetylneuraminic acid.

Hence, although the first attempt of producing functional recombinant CFH in *P. patens* was successful, the plant specific residues limited the use to *in vitro* models (Büttner-Mainik *et al*, 2011). To avoid

potential immune reactions, a modified moss stain with a KO for the α1,3-fucosyltransferase and β1,2-xylosyltransferase needs to be used (Koprivova *et al*, 2004). The recombinant human CFH CPV-101, was the first expressed in this glycosylation optimized *P. patens* lacking the immunogenic xylose and fructose residues (Figure 6 B) (Michelfelder *et al*, 2017). Although CPV-101 was functional already in a CFH KO mouse, the circulatory half-live was reduced compared to the one of human plasma derived CFH, which can be caused by remaining differences in glycosylation.

Asialoglycoprotein receptors (ASGPR) on hepatocyte surfaces can bind to glycoproteins that are lacking terminal sialic acids in their N-linked glycosylation leading to a rapid plasma clearance (Jones *et al*, 2007). Additionally, one of the PRRs expressed by macrophages, is the mannose receptor CD206, that is capable of recognizing not only terminal mannose residues, but also fucose and GlcNAc (Cummings, 2022). Terminal sialic acids improve the pharmacokinetic capabilities of proteins by covering the binding sites for ASGPR and CD206 (Chia *et al*, 2023). The only sialic acid that is present in humans is the N-Acetylneuraminic acid (Neu5Ac) (Chou *et al*, 1998). Consequently, CPV-104 was additionally *in vitro* sialylated using β-1,4-galactosyltransferase and α-2,6-sialyltransferase which is recreating human glycosylation pattern by adding additional galactose and Neu5Ac (Figure 6 C). This glycosylation optimized CFH version was shown to have an about 5 times elongated serum half-life compared to CPV-101, whilst not influencing the binding abilities (Tschongov *et al.*, 2024). However, both were not yet evaluated for a potential application in ophthalmology.

1.5. Aim of this study

As one of the leading causes of irreversible vision loss among the elderly, AMD poses significant public health challenges. Developing effective therapeutic strategies is crucial, as especially for dry AMD the therapy options are limited so far. The excessive immune response of the innate immune system, especially of microglia and the complement system is known to contribute to disease progression. SNPs in the complement regulating protein CFH like the well described Y405H variant lead to a significantly elevated AMD risk. Furthermore, a supplementation of plasma-derived CFH in other complement associated diseases like aHUS already led to promising outcomes, as well as other complement inhibitors in GA patients. However, production of CFH, as a complex glycoprotein was challenging so far. The production of CFH in the moss *Physcomitrella patens*, gives the opportunity to produce high amounts of human like glycosylated recombinant CFH, which might be a therapeutic option in AMD.

Hence, we aimed to test two different moss-derived human CFH variants CPV-101 and CPV-104, differing in their glycosylation, in a light-induced model of retinal degeneration. Our model is mimicking important aspects of dry AMD, like photoreceptor cell death and gliosis, which enables us to evaluate the impact of the CFH variants on complement system activity, glia cell response and retinal degeneration, as well as a potential impact of N-glycosylation.

2. Material and methods

2.1. *In vivo* methods

2.1.1. Animal housing

All animal procedures were approved by the local government (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). Mouse were kept in individually ventilated cages (GM500 for Mice, Greenline from Tecniplast®) with a maximal density of five animals per cage. Light was in a 12h light /dark cycle with light on at 6 a.m. Temperature and humidity were regulated constantly at 22 ± 2°C and 45-65% humidity. Mice were fed with an irradiated phytoestrogen-free standard diet for rodents (Altromin 1314; 59% carbohydrates, 27%protein, 14 %fat) *ad libitum.*
All mice were 8-10-week-old wild type (WT) albino BALB/cJ of both sexes. BALB/cJ mice are all carrier of a light sensitive variant of RPE65 gene. The Leu450Met amino acid substitution in the RPE65 protein leads to higher susceptibility for a light damage.

2.1.2. Intravitreal injections

The used substances, CPV-101 and CPV-104, were both obtained from eleva GmbH dissolved in PBS. The animals were anesthetized with ketamine (Ketavet, 100 mg/kg) and xylazine (Bayer, 2% Rompun, 5 mg/kg) diluted in 0.9% sodium chloride by intraperitoneal (i.p.) injection one day before light exposure. The ocular surface was then additionally locally anesthetized with Conjucain® EDO® 0.4 mg/ml (Bausch & Lomb) eye drops. 1 µl of either CPV-101 (5 µg), CPV-104 (5 µg) or PBS as vehicle control was injected bilateral ivt. with a 34G needle using a NanoFil syringe (Word Precision Instruments) with a flow rate of 150 nl/sec. Afterwards the eyes were covered with Bepanthen® eye and nose ointment (Bayer HealthCare).

2.1.3. Light exposure

The mice were dark adapted after ivt. injection for 16 h before light exposure in red cages (Leddy - Red IVC from Tecniplast®). The pupils were then dilated using topical drop of phenylephrine 2.5%–tropicamide 0.5% under dim red light. Mice were exposed to bright white light with an intensity of 10.000 lux for 30 min. After light exposure, the mice were housed in normal light conditions for the remaining experimental time.

2.1.4. Optical coherence tomography and blue autofluorescence

The impact of the light damage on retinal thinning and the accumulation of autofluorescent material was analyzed one, three and four days after light exposure. Therefore, mice were anesthetized with a mixture of ketamine (100 mg/kg body weight, Ketavet; Pfizer Animal Health) and xylazine (5 mg/kg body weight, 2% Rompun; Bayer HealthCare) diluted in 0.9% sodium chloride by i.p. injection and their pupils dilated with a topical drop of phenylephrine 2.5%–tropicamide 0.5% before image acquisition. Spectral domain optical coherence tomography (SD-OCT) and BluePeak autofluorescence (BAF) was performed on both eyes with a Spectralis™ HRA+OCT device (Heidelberg Engineering). Retinal thickness (µm) was measured using the Heidelberg Eye Explorer Software on OCT images, using a ring scan (diameters 3 and 6 mm), centered on the optic nerve head. BAF images showing the accumulation of autofluorescent material within the fundus, were analyzed using FIJI.

2.2. Immunohistochemistry

After euthanizing the mice by cervical dislocation, the eyes were enucleated and fixed in 4% ROTI®Histofix (Carl Roth) for 3 h at room temperature (RT).

2.2.1. Retinal whole mounts

For whole mount staining, the eyes were dissected into RPE/choroid and retina. Both tissues were permeabilized and blocked over-night at 4°C using PermBlock buffer. The whole mounts were then incubated with the corresponding 1st antibody for 24 h. After washing three times with PBST-X, the 2nd antibody was incubated for 1 h in the dark. Subsequently, the tissues were washed again three times in PBST-X and once in PBS. For microscopy the flat mounts were then embedded in Vectashield HardSet H-1400 fluorescence mounting medium for retina and Dako S3023 Fluorescence Mounting Medium for RPE. Images were taken with a Zeiss Imager.M2 equipped with an ApoTome.2. The used antibodies are listed in Table 1.

2.2.2. Cryosections

For cryosectioning, the eyes were embedded in Tissue-Tek® optimal cutting temperature (O.C.T) compound (Sakura Finetek) and 10 µm thick cross sections were cut using a cryostat. For immunohistochemical staining, the sections were rehydrated in 1x PBS for 10 minutes and blocked in BLOTTO for 30 min. Afterwards, 1st antibody was applied on the slides and incubated for 24 h at 4°C.

After washing 3 times with PBST-X, the 2nd antibody was incubated for 1 h in the dark. After another three times of washing with PBST-X, slides were embedded in Fluoromount-GTM with DAPI (00-4959-52, Thermo Scientific). Images were taken with a Zeiss Imager.M2 equipped with an ApoTome.2. The used antibodies are listed in Table 1.

Table 1: Antibodies for IHC

	Antibody	**Species**	**Dilution**	**Manufacturer, Cat#**
Primary antibody	Iba1	Rabbit, polyclonal	1:500	Wako, 019-19741
	Glutamine synthetase (GS) GS-6	Mouse, monoclonal	1:200	Merck, MAB302
	Glial fibrillary acidic protein (GFAP)	Rabbit, polyclonal	1:400	Sigma-Aldrich, G9269
	Membrane attack complex, C5b-9 (MAC)	Mouse, monoclonal	1:100	Biozol, FGI-10-1801
Secondary antibody	Alexa Fluor® 488	Donkey anti-rabbit	1:1000	Invitrogen, A21206
	Alexa Fluor® 647	Donkey anti-rabbit	1:1000	Invitrogen, A-31573
	Alexa Fluor® 488	Goat anti-mouse	1:1000	Thermo Fisher Scientific, A11001

2.2.3. TUNEL assay

Retinal cryosections were labeled with an in situ cell death detection kit (TMR red, Roche) according to the manufacturer's instructions. Fluoromount-G™ with DAPI was used to mount the sections and to counterstain the nuclei. Images were taken with a Zeiss Imager.M2 equipped with an ApoTome.2 and analyzed by counting all nuclei in the ONL and the TdT-mediated dUTP-biotin nick end labeling (TUNEL) + cells in the ONL. Images were taken with a Zeiss Imager.M2 equipped with an ApoTome.2.

2.3. ELISA

Cytokine, chemokine and complement pathway protein concentrations of retina and RPE lysates were measured by ELISA. Tissue samples were sonicated in 1x PBS supplemented with protease and phosphatase inhibitors (Complete protease inhibitor cocktail, Roche). Ccl2/JE/Mcp-1 (DY479), Il-1beta/IL-1F2 (DY401) and Il-6 (DY406) were purchased from R&D Systems. ELISA for C3 (ab263884), C5 (ab264609) and C5a (ab193718) were purchased from abcam. Each ELISA was used accordingly to manufacturer's instructions. Absorbance was measured with a TECAN infinite M1000.

2.4. Quantitative real-time PCR

Total RNA was isolated from retinas and RPE/choroidal tissue using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions and elution was done in 11 µl. First-strand complementary DNA (cDNA) was synthesized from the total mRNA using the RevertAid™ H Minus First strand cDNA Synthesis Kit (Thermo Scientific). Transcript levels of *Tspo*, *Il-6*, *Ccl2*, *Tnf*, *Casp3* and *C3* were analyzed by quantitative real-time PCR performed in LightCycler® 480 II (Roche) with SYBR® Green (Takyon No Rox SYBR Master Mix dTTP blue, Eurogentec). Amplification of *Atp5b* served as a control. Measurements were performed in technical duplicates and ΔΔCT threshold calculation was used for relative quantification of results. All primers were ordered from IDT (Table 2).

Table 2: Primers for qPCR

Gene	NM number	Forward primer (5' – 3')	Reverse primer (5' – 3')
Atp5b	NM_016774.3	ggcacaatgcaggaaag	tcagcaggcacatagatagcc
C3	NM_009778.3	accttacctcggcaagtttct	ttgtagagctgctggtcagg
Ccl2	NM_011333.3	catccacgtgttggctca	gatcatcttgctggtgaatgagt
Il-6	NM_001314054.1, NM_031168.2	gatggatgctaccaaactggat	ccaggtagctatggtactccaga
Tnf	NM_001278601.1, NM_013693.3	ctgtagcccacgtcgtagc	ttgagatccatgccgttg
Tspo	NM_009775.4	ggaacaaccagcgactgc	gtacaaagtaggctcccatgaa

2.5. Data analysis

2.5.1. Image analysis

The cell morphology of Iba1+ cells was analyzed using the MotiQ FIJI plugin (https://github.com/hansenjn/MotiQ). Therefore images were converted to 16 bit tif images using CellProfiler. Afterwards, single cells were marked manually using the FIJI MotiQ tool. The following analysis was automated. The total area and the spanned area, ramification index (RI) as well as the outline, number of junctions, branches and endpoints were used to analyze morphology. Analysis of Iba1+ area, C5b-9+area and BAF images was done using the FIJI tools for area analysis.

2.5.2. Statistical analysis

Data were analyzed using GraphPad Prism 8 software. Significance of difference between means was determined by one-way ANOVA followed by Sidak's multiple comparison test. Data are presented as mean ± SEM, *P < 0.05, **P < 0.01 and ***P ≤ 0.001.

2.6. Buffers

Table 3: Used buffers

Buffer	Ingredients
PBST-X	0.3% Triton X-100 in PBS
Perm/Block	5% NDS 0.2% BSA 0.3% Triton X-100 in 1x PBS
PBS	Gibco

2.7. Devices

Table 4: Used devices

Device	Manufacturer
Cryostat CM3050 S	Leica Biosystems
LightCycler® 480 Instrument II	Roche Applied Science
NanoDrop 2000 Spectrophotometer	Thermo Scientific
PeqSTAR 2x Cycler	Peqlab
SpectralisTM HRA+OCT	Heidelberg Engineering
Vibracell 75115 Sonicator	Fisher Bioblock Scientific
Zeiss Imager M.2 with ApoTome.2	Zeiss

2.8. Software

Table 5: Used software

Software	Manufacturer
Adobe Illustrator	Adobe Systems
EndNote X9	Clarivate Analytics
GraphPad Prism 8 (v8.4.3)	GraphPad Software; Inc.
Heidelberg Eye Explorer (HEYEX)	Heidelberg Engineering
FIJI (v2.90) Version	Wayne Rasband; NIH
FIJI plugin MotiQ	Jan Hansen, https://github.com/hansenjn/MotiQ/
Light Cycler® 480 Software (v1.5.1)	Roche Applied Science
Microsoft Office 2016	Microsoft Corporation
NanoDrop 2000 Software	Thermo Scientific
Zen Blue Edition (v3.1)	Zeiss

3. Results

A dysregulation of the complement system is known to increase the risk for various diseases like aHUS, C3 glomerulopathy and AMD. In other diseases involving complement dysregulation, the substitution of CFH has been shown already to have a positive effect (Fakhouri *et al*, 2010). Therefore, we assessed the effect of two different variants of moss-derived recombinant human CFH in the light-induced model of retinal degeneration that is mimicking key features of dry AMD like the emerging gliosis and the photoreceptor cell death (Grimm & Reme, 2013). The mice received one ivt. injection of either CPV-101 (5 µg) or CPV-104 (5 µg) or a PBS control. Afterwards they were dark adapted for 16 hours and exposed to 10.000 lux light for 30 min.

3.1. Influencing the complement system with application of moss-derived CFH

One indicator for immune reactivity is the cytokine secretion of cells. Hence, we assessed the expression level of selected pro-inflammatory cytokines/chemokines and markers in retinas after light damage (Figure7 A). The expression levels of the pro-inflammatory markers Ccl2, Il6 and Tnf were all increased upon light exposure. Both recombinant CFH variants led to a reduction in expression levels of CCl2 and Il6, whereas Tnf expression was not influenced by the treatments. The microglia activation marker *Tspo*, was also increased upon light damage. CPV-101 and CPV-104 treatment reduced *Tspo* expression, which indicates reduced microglia reactivity. *Caspase 3* expression was not influenced by light damage or treatment.

Beside the changes in cytokine signaling, we also assessed the complement system activation itself, since CFH should directly influence this cascade. Complement factor C3 expression in the retina was increased in all light-exposed mice compared to the healthy controls four days after light exposure. However, CVP-101- and CPV-104-treated mice showed decreased local C3 expression compared to the PBS controls. The expression of C5 was not detectable in retinal tissue using qPCR.

Additionally, we measured the concentration of cytokines and complement components in retinal tissue (Figure 7 B). The concentration of Ccl2, IL-6 and IL-1b were increased in light-exposed retinas compared to the control mice. Especially the CPV-104-treated mice showed a reduction in Ccl2 and IL-6 concentrations compared to the PBS control. The Il-1b expression was to a lesser extent influenced by recombinant CFH treatments, but still reduced compared to the PBS treated mice. Gene expression and concentration of the same cytokines was also observed in RPE (Supplement Figure 1). Only Il-6 and *Tspo* gene expression were here influenced by CFH treatment. Effects were in general less pronounced in RPE. Notably, the C3 levels were in general higher in RPE than in the retina.

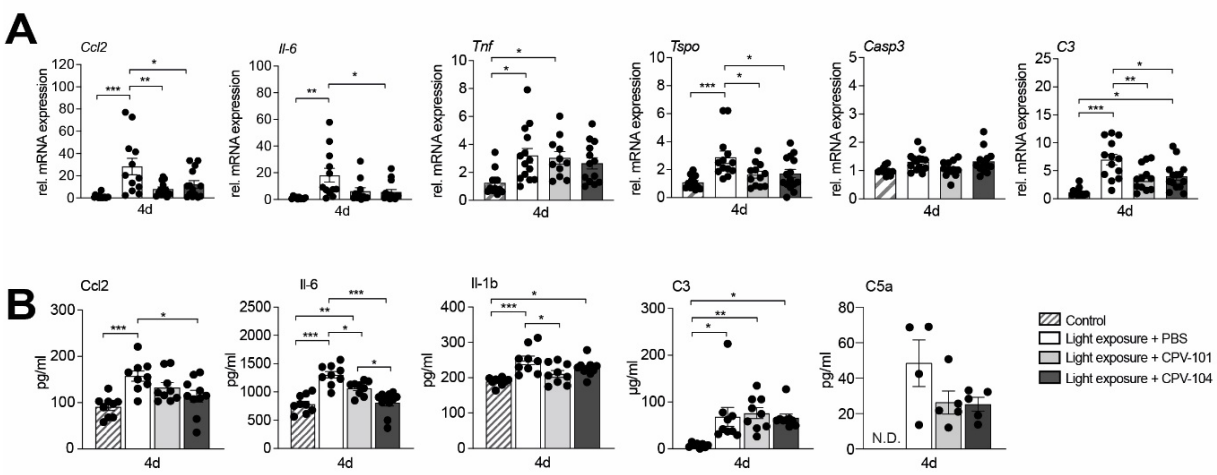

Figure 7: Effect of CPV-101 and CPV-104 on light-induced pro-inflammatory marker transcript levels and secretion. A qRT-PCR and **B** ELISA were performed from retinas of light-damaged and control mice four days after light exposure. Data are presented as mean ± SEM from n= 4-12 retinas. One-way ANOVA followed by Sidak's multiple comparison test was used for statistical analyses; *P< 0.05, **P < 0.01 and ***P ≤ 0.001, N.D. - not detectable.

The C3 concentration in the tissue was strongly increased upon light damage, the recombinant CFH treatments were not influencing this effect. However, the used ELISA is using C3c alpha chain as standard. Hence, it is not possible to differentiate between C3, C3b, iC3b and C3c, disguising potential effects of CFH treatment on C3 levels. While C5 was also not detectable using ELISA, the cleavage product C5a had increased concentrations in light-exposed retinas. The treatment with the moss-derived CFH variants had no significant effect on C5 cleavage, although a clear trend towards lower C5a lever could be observed indicating reduced complement activity.

Only in Il-6 ELISA, a significant difference between the two glycosylation variants could be observed, showing a stronger effect in the CPV-104-treated mice.

The MAC is the final step of all three complement cascades, leading to cell lysis. Since, both human CFH variants influenced the complement cascade, we evaluated the impact of CPV-101 and CPV-104 on MAC formation with an immunohistochemical staining for C5b-9 in cryosections of the whole mouse eye. The formation of MAC can be observed already one day after light exposure, mainly in the ONL and SRS (Figure 8 A and D). Both of the recombinant CFH treated groups showed significantly decreased MAC formation compared to the vehicle control.

As not only the complement components revealed an impact of the CFH treatment, but also the pro-inflammatory signaling, we were also interested in the effect on gliosis. Therefore, we used a staining for the microglia marker Iba1. As expected, the microglia were mainly located in the plexiform layers in healthy conditions (Figure 8 A). Upon light exposure the microglia migrate towards the ONL and the SRS (Figure 8 B and C). The migration to the ONL was reduced in CPV-101- and CPV-104-treated mice,

compared to the PBS control, at all time points. For the SRS microglia, this effect was only significant four days after light exposure.

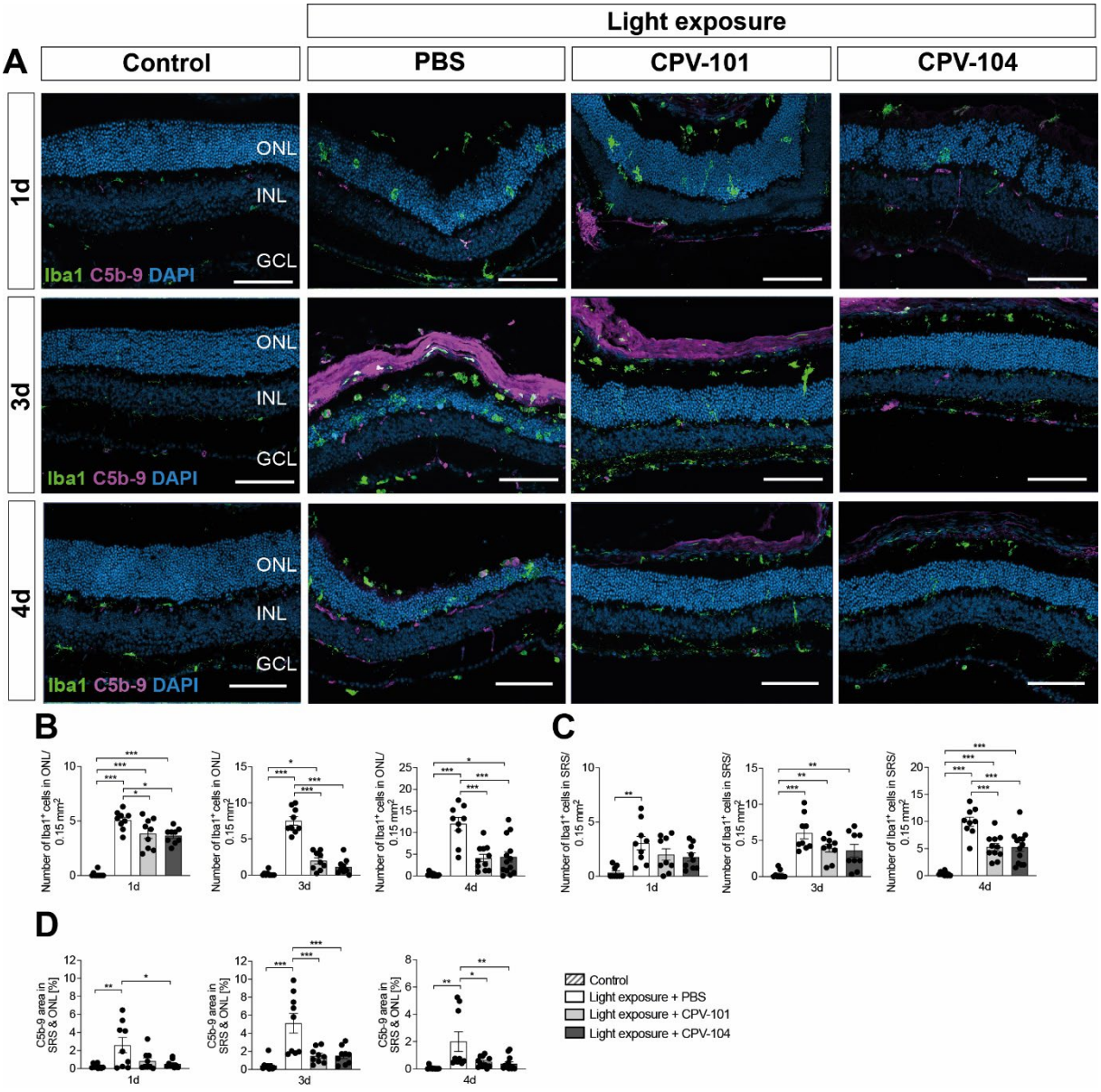

Figure 8: Effect of recombinant CFH on microglia localization in light-exposed retinas and membrane attack complex formation. A Representative images from Iba1, C5b-9 Membrane attack complex (MAC) and DAPI stained cryosections from light-exposed and control mice one, three and four days after light exposure. Scale: 100 µm. **B** Quantification of Iba1+ cells in ONL and **C** in SRS at indicated time points. **D** Quantification of C5b-9 area in SRS and ONL. Data show mean ± SEM; n= 9-13 retinas. One-way ANOVA following Sidak's multiple comparison test were used for statistical analysis; *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001.

3.2. Effects of moss-derived CFH variants on gliosis

Microgliosis is known to contribute to AMD disease progression, whereas a reduced reactivity was shown to attenuate retinal damage. Since the cryosections indicated less migration of microglia in CFH treated mice and the expression of microglia activation marker like Tspo was reduced in treated groups, we further analyzed microglia reactivity. Therefore, the migration of Iba1 positive microglia to SRS, was assessed in whole mount perpetrates of the eyes (Figure 9 A). The Iba1 positive area and the

number of Iba1 positive cells is increasing after light exposure compared to the healthy controls (Figure 9 B and C). The mice treated with CPV-101 or CPV-104 show significant less microglia migration, in line with the observations from the cryosections.
Upon light damage, autofluorescent (AF) material is accumulating in SRS, especially of vehicle-treated mice. Whereas both CFH-treated groups show reduced accumulation of AF material (Figure 9 D).

Besides the microglia migration we also assessed the morphological changes that occur upon microglial reactivation (Figure 10). The Iba1 positive cells within the OPL were analyzed individually for their total area and the spanned area, ramification index (RI) as well as their outline, number of junctions, branches and endpoints using the software tool MotiQ (Figure 10 B-D). In homeostatic conditions, the microglia build a network of non-overlapping ramified cells, which translated into high values for the measured parameters. The retinal degeneration that occurs after light exposure leads to a reactivation of the microglia and consequently to a morphological change towards a more amoeboid shape. This was also recapitulated by the analyzed parameters, showing a decrease in all factors after light exposure, already one day after light exposure (Figure 10 B). In retinas of CVP-101- and CPV-104-treated mice these parameters were less decreased compared to the vehicle control. The most distinct differences were observed on day 4 (Figure 10 D). This underlined the reduced reactivity of microglia of mice that received one of the moss derived CFH variants. Notably, CPV-104 had a more pronounced effect on microglia morphology than CPV-101 four days after light exposure.

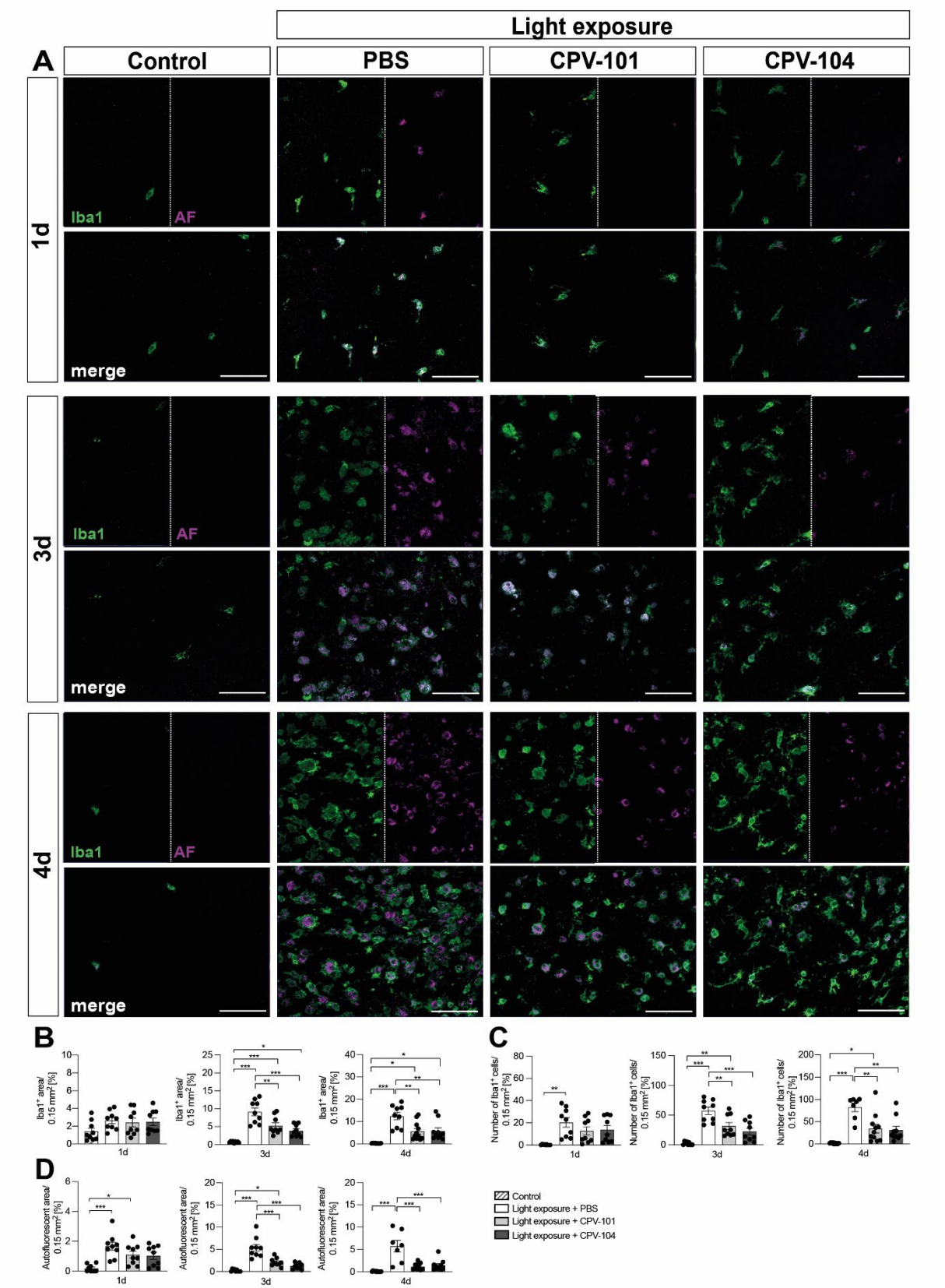

Figure 9: Recombinant human CFH variants reduce microglia accumulation in the subretinal space. A Representative images of Iba1-stained retinal flat mounts and autofluorescence (AF) in the subretinal space (SRS) one, three and four days after light exposure of mice treated with 5 µg CPV-101, CPV-104 or vehicle control. Scale bar: 100 µm. **B** Quantification of Iba1⁺ area in the SRS. **C** Quantification of Iba1⁺ cells in SRS. **D** Quantification of autofluorescent area. Data are presented as mean ± SEM from n=9-11 retinas. One-way ANOVA followed by Sidak's multiple comparison test was used for statistical analyses; *P< 0.05, **P < 0.01 and ***P ≤ 0.001

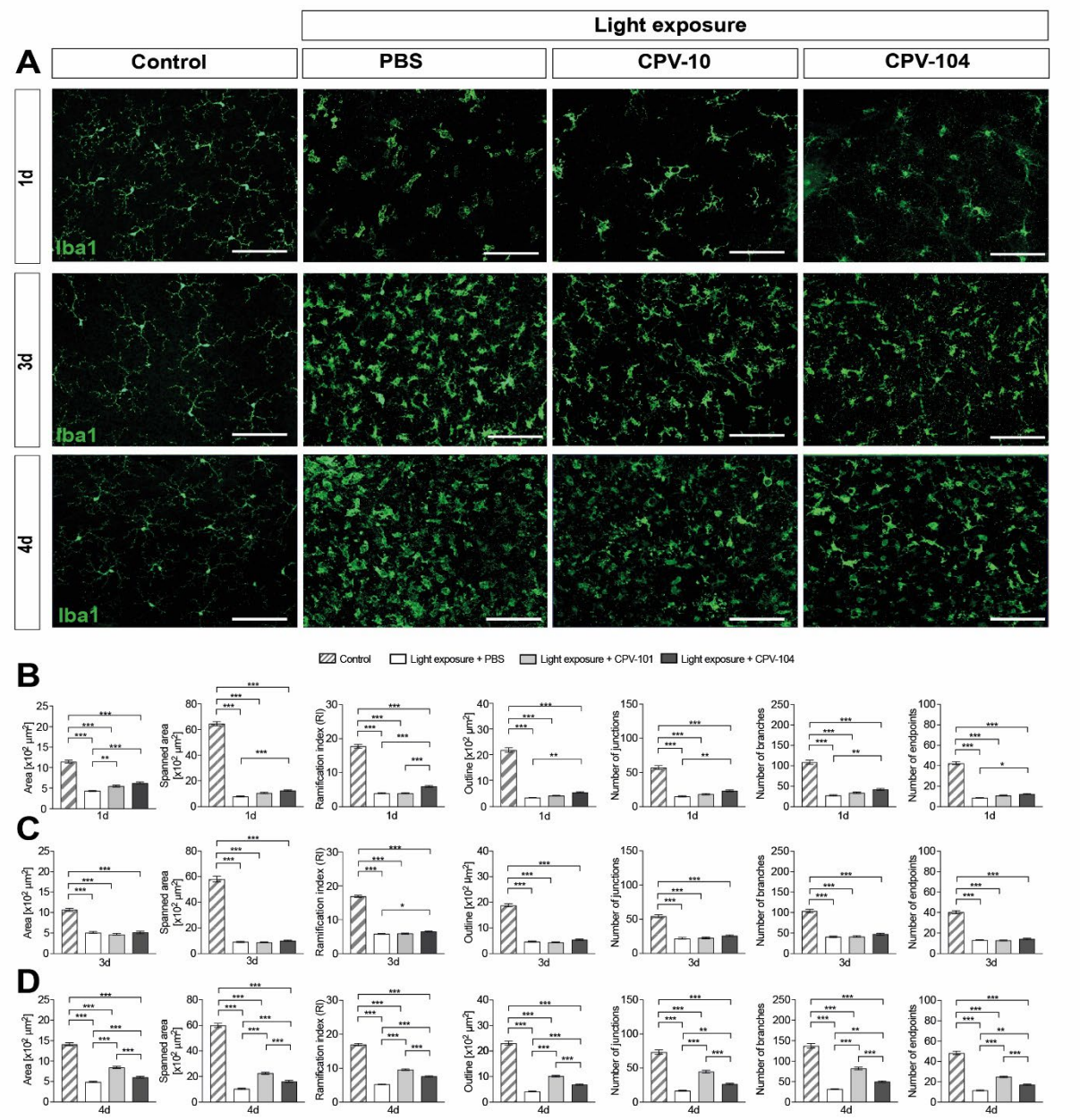

Figure 10: Recombinant human CFH variants reduce amoeboid microglia morphology in the OPL. A Representative images of Iba1-stained retinal flat mounts. Outer plexiform layer (OPL) one, three and four days after light exposure of mice treated with 5 µg CPV-101, CPV-104 or vehicle control. Scale bar: 100 µm. **B-D** Quantification of microglia morphology using MotiQ ImageJ tool, analyzing total area, spanned area, ramification index (RI), outline, number of junctions, branches and endpoints. One (**B**), three (**C**) and four (**D**) days after light exposure. Data are presented as mean ± SEM from n= 86-237 cells from 8-9 retinas. One-way ANOVA followed by Sidak's multiple comparison test was used for statistical analyses; *P< 0.05, **P < 0.01 and ***P ≤ 0.001.

Müller cells, as part of the retinal glia population also respond to retinal damage (Grosche *et al*, 1995). For an assessment of the Müller cell activity, we did a co-staining for glutamate synthetase (Gs) and glial fibrillary acidic protein (Gfap) (Figure 11). Gs is a constitutively expressed Müller cell marker, while Gfap is an astrocyte marker, that is also upregulated in activated Müller cells (Bignami & Dahl, 1979).

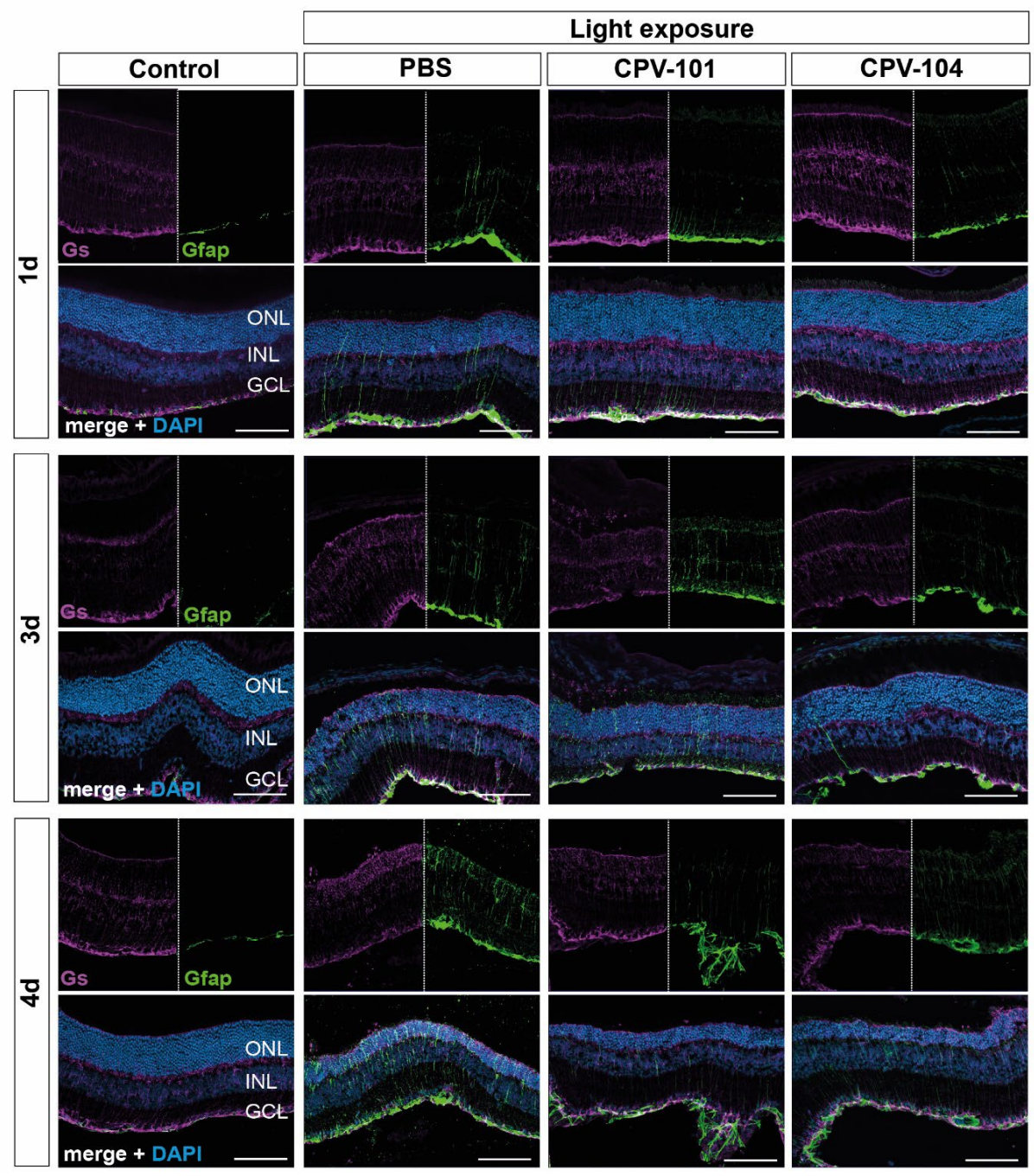

Figure 11: Effect of recombinant CFH on Müller glia. Representative images from the DAPI, glutamine synthetase (Gs) and glial fibrillary acidic protein (Gfap) stained cryosections from light-damaged and control mice one, three and four days after light exposure. Scale: 100 µm.

In the control retinas, the Gs positive areas were mainly located in the GCL and in the OPL. Upon light damage, there are more Gs positive cells visible in the ONL, especially 4 days after light exposure. This effect seemed stronger in the vehicle group compared to the CFH treated groups.

GFAP positive astrocytes were only visible in the GCL in healthy conditions. In the vehicle light exposure group, the Gfap positive area was spanning throughout the whole retina, indicating Müller cell activity. In both CPV-101 and CPV-104 treated groups, the Gfap positive fiber formation is less pronounced compared to the vehicle control.

3.3. Effect of moss-derived CFH variants on retinal degeneration

Since complement activation, as well as the gliosis was reduced in CFH treated groups, we were interested, if the treatments were also affecting photoreceptor survival after light damage. Therefore, we used a TUNEL assay, labelling double-strand breaks of the DNA (Figure 12 A). Upon light exposure, TUNEL positive cells appear in the ONL indicating photoreceptor cell death. This effect can be observed

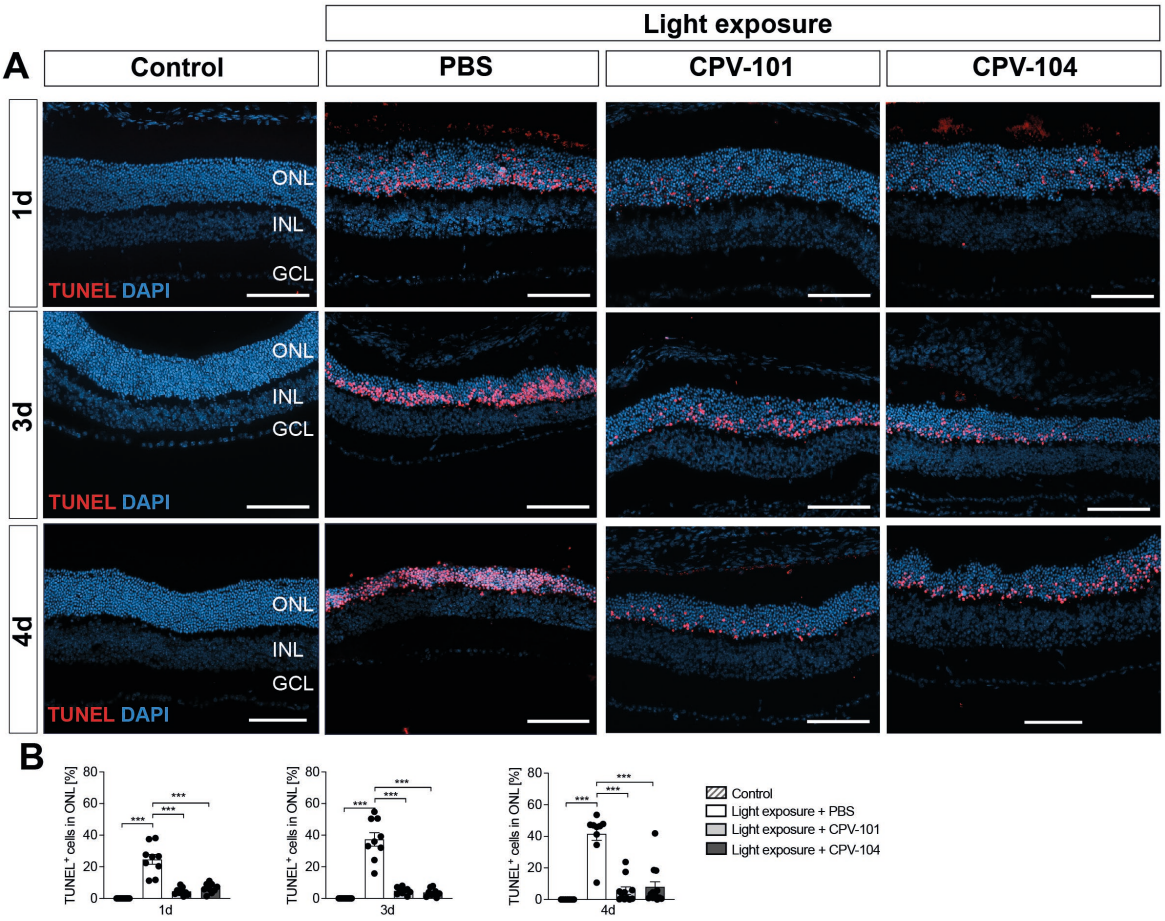

Figure 12: Effect of recombinant CFH on light-induced photoreceptor cell death. A Representative images from the DAPI and TUNEL-stained cryosections from light exposed and control mice, one, three and four days after light exposure. Scale: 100 µm**. B** Quantification of TUNEL+ cells in the ONL**.** Data are presented as mean ± SEM from n= 86-237 cells from 8-9 retinas. One-way ANOVA followed by Sidak's multiple comparison test was used for statistical analyses; *P< 0.05, **P < 0.01 and ***P ≤ 0.001.

already one day after light exposure. Up to day four, the number of TUNEL positive cells is increasing. In CPV-101 and CPV-104 treated mice, the amount of TUNEL positive cells was at all time points significant decreased compared to the vehicle control (Figure 12 B).

The DAPI staining, especially in the PBS group as well as the number of TUNEL positive cells, was indicating a reduction of nuclei in the ONL which is a sign of retinal degeneration. To further quantify this degeneration, we used SD-OCT to measure retinal thickness (Figure 13 A). The OCT scans show a light-induced retinal thinning in the PBS control group, three and for days after light exposure. The severity of this retinal degeneration was reduced in both recombinant CFH treated groups. This effect could be observed in the central field (3mm), but also in the more peripheral surrounding (6mm) around the optic nerve head (Figure 13, B & C).

The accumulation of autofluorescent material was also assessed using BAF images (Figure 14 A). The autofluorescent area was increased after light damage (Figure 14 B). This effect was less pronounced in CPV-101- andCPV-104-treated mice compared to the PBS control. In all light-exposed groups, the autofluorescent particles accumulate from day 1 to day 4.

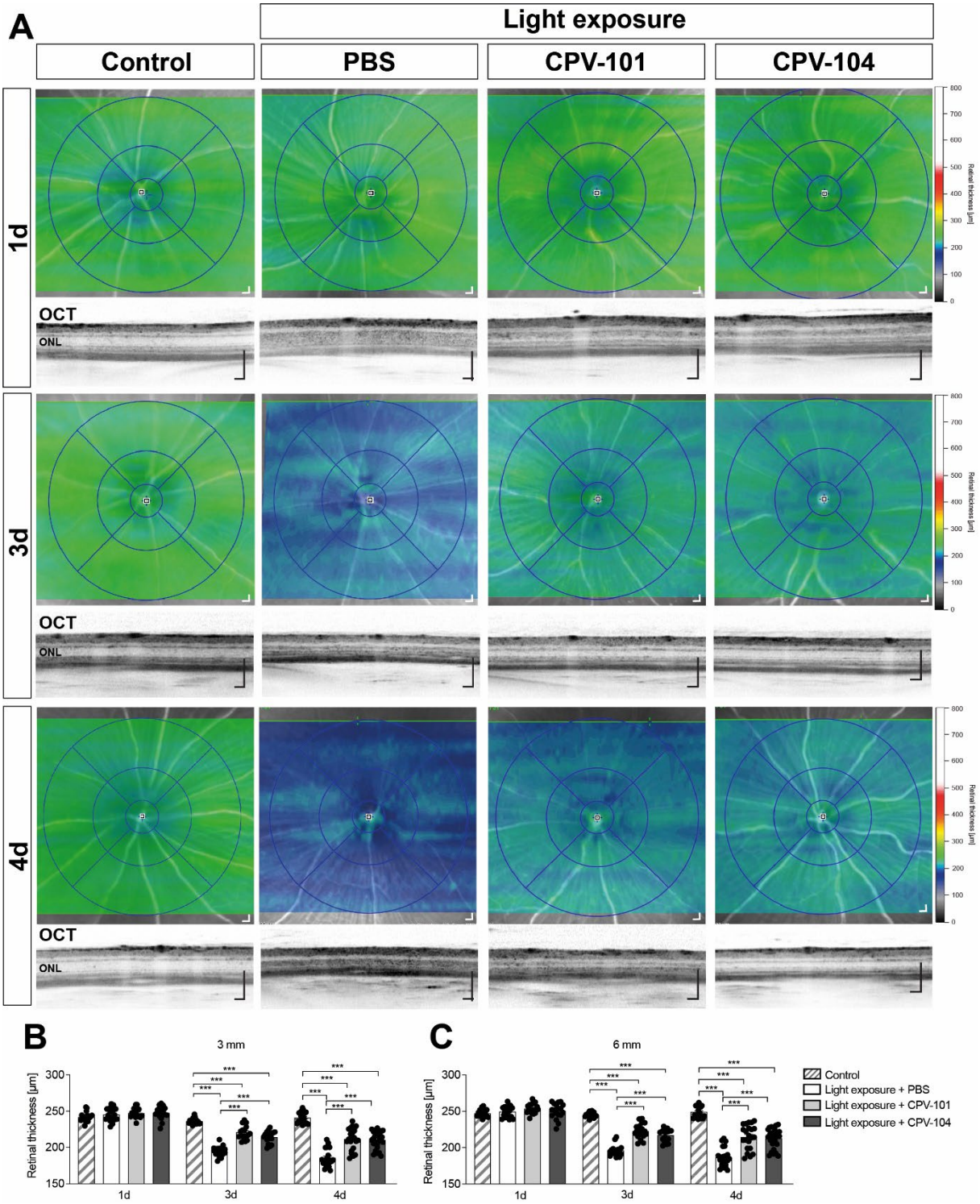

Figure 13: Effect of recombinant CFH on light-induced retinal degeneration. A Representative heat maps and OCT scan from control mice and treaded groups, one, three and four days after light exposure. Scale bar: 200 µm. **B** Quantification of retinal thickness in the central (3 mm) and **C** more peripheral area (6 mm). One data point represents the average thickness of the retina of one eye, calculated from four different areas around the optic nerve head in circle diameters of 3 mm and 6 mm, respectively. Data show mean ± SEM; n= 18-27 eyes. One-way ANOVA following Sidak's multiple comparison test were used for statistical analysis; *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001.

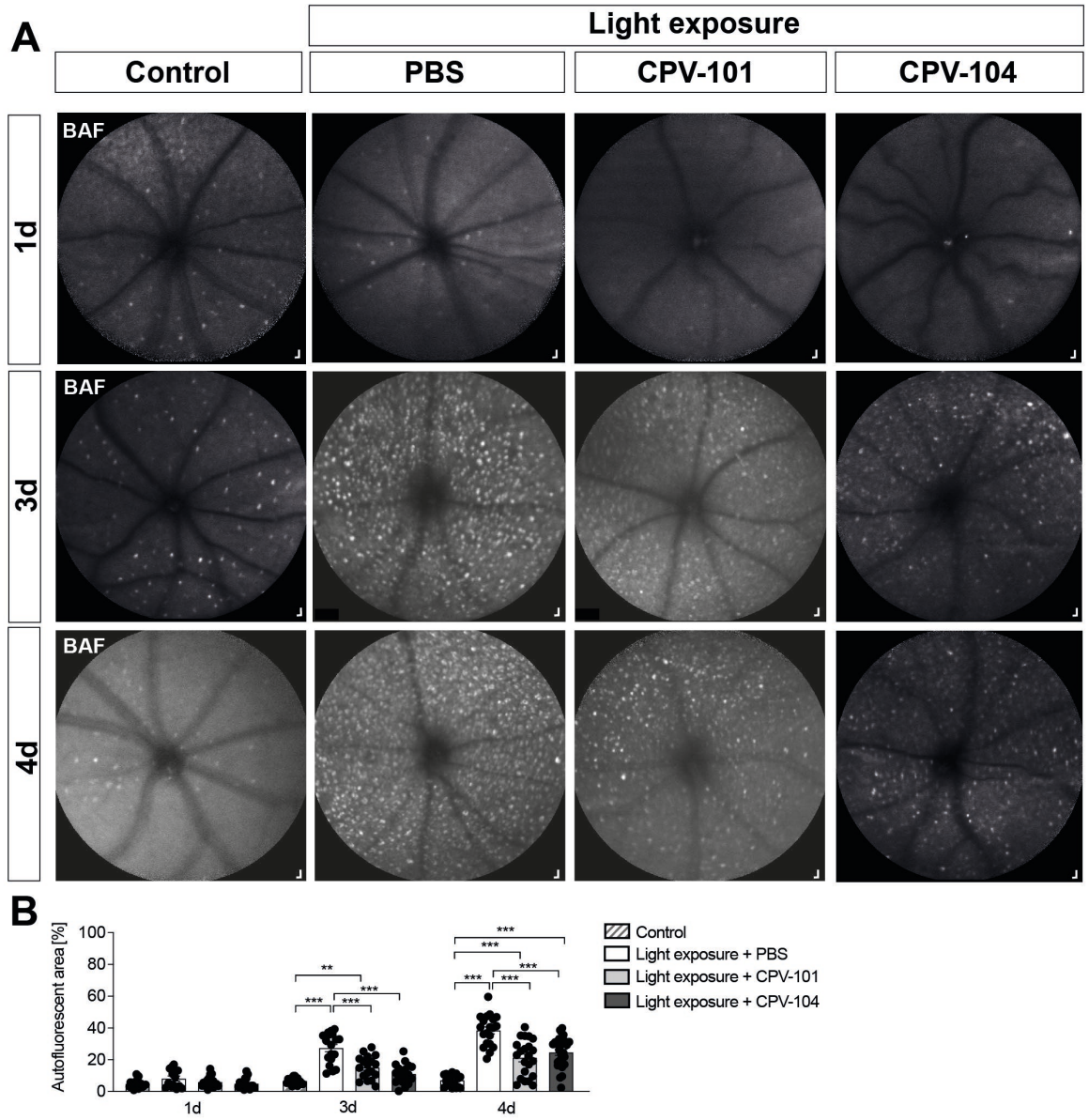

Figure 14: Effect of recombinant CFH on light-induced accumulation of autofluorescent material. A Representative fundus images from BluePeak autofluorescence (BAF) imaging, from control mice and treaded groups, one, three and four days after light exposure. Scale bar: 200 µm. **B** Quantification of autofluorescent area. One data point represents one eye. Data show mean ± SEM; n= 18-27 eyes. One-way ANOVA following Sidak's multiple comparison test were used for statistical analysis; *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.001.

4. Discussion

4.1. Moss-derived CFH variants reduce the complement system activity

A dysregulation of the complement system can contribute to the disease progression of AMD. Especially SNPs in the CFH gene are known to increase the AMD risk tremendously (Edwards *et al.*, 2005; Haines *et al.*, 2005; Klein *et al.*, 2005). In the present work, we demonstrated that the ivt. injections of moss-derived recombinant CFH variants reduced the complement activation in a light-induced mouse model of dry AMD. The C3 expression, C5a levels as well as the MAC level were decreased in CFH treated animals.

C3 and C5 are key proteins in the complement cascade and beside others they are part of the proteins aggregated in AMD patients' drusen (Mullins *et al*, 2000). Also in the light-induced retinal degeneration, the increased expression of complement components was described before in our group. C3, as well as the C3a receptor (C3aR1) were shown to be upregulated in retinas 4 days after light exposure using RNA sequencing (Scholz *et al*, 2015b; Tabel *et al*, 2022). In line with that, we showed an increased C3 expression and concentration in the light-exposed retinas. In the RPE, these effect were less pronounced, which was described before for light-induced retinal degeneration. Also the higher concentration of complement components in the RPE compared to the retinal tissue was previously described (Schäfer *et al.*, 2017). Interestingly, only the local C3 expression was influenced by CFH application, whereas the C3 level remained unaltered. A clear differentiation between C3 and its cleavage products is however not possible with the used ELISA, which could disguise potential effects of CFH treatment. A reduced C3 expression after inhibiting the alternative pathway was also observed in a model of cigarette smoke induced retinal damage. Treating the mice with a fusion molecule (C2fh) of the iC3b binding region of complement receptor C2 (CR2) and the N-terminal region of CFH was reducing the C3 expression after smoke application compared to PBS control (Woodell *et al*, 2016). A different study, making use of the same fusion molecule also revealed reduced C3 expression in a laser-induced CNV model (Rohrer *et al*, 2009).

C5 concentrations as well as the expression levels were not detectable in mouse retinas, which was observed before for mice (Luo *et al*, 2011). This might just be a species dependent difference, as for humans, the expression of C5 in the retina is known (Anderson *et al*, 2010). Similar differences were observed in brain, where C5 is expressed in healthy human tissue, but not in mice (Lackner *et al*, 2008; Yasojima *et al*, 1999).

The cleavage of C3 and C5 leads to emerging of anaphylatoxins that can further influence the immune response. Similar to C3 and C5, both cleavage products were also shown to be part of the drusen deposits of patients. C5a is the most stable anaphylatoxin, a potent chemoattractant for macrophages and enhances phagocytic activity (Aksamit *et al*, 1981). We could observe a strong increase in C5a

concentration after light exposure, which is in line with earlier observations after light damage (Song *et al.*, 2017). In CPV-101- and CPV-104-treated mice the C5a concentrations was decreased, yet missing statistical significance. Similar effects were observed using a C2fh fusions molecule that was reducing C5a and C3a level in a laser CNV model (Parsons *et al*, 2019).

Finally the complement cascade results in formation of MAC which is leading to cell lysis. The number of MAC deposits on the choriocapillaries and RPE is increasing with aging and elevated in AMD patients compared to healthy controls (Mullins *et al*, 2014). Also the plasma level of fluid phase C5-9 were five times higher in the plasma of AMD patients than in age-matched controls (Busch *et al*, 2023). Accordingly, we observed an increased MAC formation in the SRS and ONL after light exposure, which is in line with earlier, yet unpublished data from our group using the same model. In contrast to the light-induced retinal degeneration, other AMD mouse models like a Ccl2 KO are not recreating this part of AMD pathology (Chen *et al*, 2011). In our study, the increased MAC formation was reduced in both CFH treated groups compared to PBS controls. A correlation between CFH function and MAC formation was also shown before in a patient study, revealing 68% more MAC deposits in AMD patients being homozygous for the high risk CFH Y402H SNP that in the low risk variant patients (Mullins *et al*, 2011). An impact of CFH on MAC formation was also shown in a laser CNV model of wet AMD. CFH KD animals tend to have more MAC formation than WT mice after laser coagulation (Lyzogubov *et al*, 2010). Intravitreal application of plasma-derived human CFH was able to decrease MAC formation (Kim *et al*, 2013). A correlation between MAC formation and CNV was also found in a Japanese population with a high abundancy of a C9 nonsense mutation that was abolishing the ability of MAC formation. These cohort had a 4.7 fold decreased risk of developing wet AMD (Nishiguchi *et al*, 2012). Therefore, moss-derived CFH treatment and the associated reduction in MAC formation might also be a therapeutic option for avoiding the maturation of neovascular AMD in patients.

Overall, our results clearly reveal a reduced complement cascade activity in CPV-101- and CPV104-treated mice.

4.2. CFH variants decreases gliosis and pro-inflammatory signaling

It is known that the microglia reactivity is contributing to the AMD disease progression (Karlstetter *et al.*, 2015). Whereas reducing this chronic activity using microglia modulation was repeatedly shown to attenuate retinal degeneration in different ocular disease models. We could show less expression of the microglia activation marker Tspo, less shift towards a reactive amoeboid morphology and less migration of microglia cells. In addition, we could show a decreased Gfap positive Müller cell stress fiber formation in the light exposed mice with CFH treatments. Accordingly we also observed reduction of pro inflammatory cytokine and chemokine signaling.

A different AMD mouse model, making use of Cx3cr1$^{GFP/GFP}$ mouse line that is accumulating microglia in the SRS upon aging, broad up contrary results. A CFH KO was decreasing microglia accumulation in the SRS compared to the mice capable of expressing CFH (Calippe *et al*, 2017). The effect was hypothesized to be facilitated by a binding of CFH to the CD11b (one chain of CR3) on the microglia surface thus hindering the binding of thrombospondin 1 (TSP-1) to CD47, which is usually leading to microglia clearance. Other complement components like C3, iC3b or CFI were not found to influence the inflammation in this model. However, in our study microglia were reduced in SRS of CFH treated mice compared to the control group indicating that this pathway might at least not be the most significant one in the light induced retinal degeneration. Nevertheless, this non-canonical role of CFH needs to be further evaluated.

In line with our results, other researchers reported a decreased gliosis after reducing complement cascade activity. A C3 KO mouse in a photo-oxidative damage model was shown to decrease Müller cell stress fiber formation, but also microglia reactivity (Jiao *et al*, 2020). Gfap-dependent expression of truncated CFH variants in Müller cells using adeno-associated viruses (AAVs) was also shown to reduce Müller cell activity, but also microglia reactivity in an ischemia mouse model (Biber *et al*, 2024). The mechanisms linking the complement system with the glia activity are well described. Anaphylatoxins C5a and C3a are potent chemoattractants initiating microglia migration. A complement C5a receptor KO was able to diminish microglia response after light exposure (Song *et al.*, 2017). Additionally, an impact on cytokine secretion of Il-6 and Il-1β was also shown to be induced by C5a (Fukuoka *et al*, 2003). This signaling via anaphylatoxins is also shown for Müller cells that increase pro inflammatory cytokine expression depending on C5aR signaling (Cheng *et al*, 2013). Notably, also the soluble MAC was shown to induce microglia response and cytokine secretion (Yang *et al*, 2014).

The accumulation of autofluorescent materiel that can be observed in the SRS but also in BAF images, emerges from cell debris containing lipofuscin that is phagocytized (Luhmann *et al*, 2009). This was observed after light exposure already, as well as the reduction of autofluorescence after microglia modulation (Dannhausen *et al*, 2018). In line with that, our study revealed less accumulation of autofluorescent material in both CPV treated groups.

Close interactions between Müller cells and microglia are also known to influence each other's immune responses via neurotrophic factors (Harada *et al*, 2002), cytokines, but also via Tspo and its natural ligand diazepam binding inhibitor (DBI) (Wang & Wong, 2014). Accordingly, suppressing e.g. the Ccl2 expression of Müller cells can decrease microglia migration and reduced photoreceptor cell death in light-induced retinal degeneration (Rutar *et al*, 2012).

4.3. Influencing the complement activity is reducing retinal degeneration

The number of TUNEL positive cells was reduced in both CFH treated groups compared to the PBS control. Additionally, the light-induced retinal thinning, as well as the accumulation of autofluorescent material was reduced in CFH treated mice.

Besides the cells that are affected by MAC formation and the subsequent cell lyses itself, MAC on cell surface was also shown to induce caspase activation and apoptosis (Nauta *et al*, 2002). We could not see an impact on caspase expression in our study, nevertheless a reduction of TUNEL positive cells indicating less cell death, was overserved.

The reduced gliosis that was accomplished in CFH treated mice can also reduce the retinal degeneration. This impact of especially microglia reactivity on retinal degeneration was described before in various studies (Tabel *et al.*, 2022; Wolf *et al.*, 2020).

Other models were also able to confirm a positive effect of CFH treatment on cell survival. In a CS induced retinal degeneration model, the application of the C2fh fusion molecule was able to reduce retinal thinning (Woodell *et al.*, 2016). Whereas, the application of truncated CFH variants was not significantly influencing the number of TUNEL positive cells in an ischemia model. However, structural integrity of the cells in IPL was still better preserved in treated mice (Biber *et al.*, 2024).

Interestingly, in contrast to the use of CFH that is influencing the whole complement cascade, a reduction of anaphylatoxin signaling only, is not leading to a successful decrease of retinal degeneration. A KO of C5aR1 was shown to influence microglia reactivity in light induced retinal degeneration, whereas, retinal thinning was not attenuated (Song *et al.*, 2017). Similar results were described in a laser CNV model, were influencing the anaphylatoxin signaling with C3a and C5a antibodies or C3aR1/C5aR1 KOs was sufficient for influencing anaphylatoxin levels in the retina, but did not improve CNV size reduction. Whereas using the C2fh fusion molecule influencing the whole alternative pathway, had an impact not only on anaphylatoxins, but also reduced the CNV size (Parsons *et al.*, 2019).

Besides this well-described interactions of CFH influencing the complement pathway, gliosis and finally cell death, CFH was also discussed to influence mechanistic/mammalian target of Rapamycin (mTOR) signaling in the RPE (Merle *et al*, 2021). The mTOR pathway itself was also discussed to be a target for a potential therapy for retinal degenerative diseases including AMD (Wang *et al*, 2022)

Also a binding of CFH to apolipoprotein E (APOE) was observed already (Chernyaeva *et al*, 2023). Depending on the prevalent APOE variants the risk for Alzheimer's disease, but also for AMD are increased (Hu *et al*, 2021). The CFH binding was shown to influence the accumulation of amyloid-β in Alzheimer's, inducing neuroinflammation, however an interaction of CFH and APOE is not jet known to influence AMD.

4.4. The impact of N-Glycosylation on pharmacokinetics

The sialylated CFH variant CPV-104 and the non-sialylated variant CPV-101 showed overall no clearly notable differences, confirming earlier results revealing no direct impact of glycosylation on the CFH functions (Tschongov *et al.*, 2024). Nevertheless, future studies should determine the impact of glycosylation on vitreous half-life for the CFH variants for future applications. For other molecules, such as commonly used anti VEGF drugs, these data are already available. For example, the anti-VEGF antibody bevacizumab has a vitreous half-life in rabbit eyes of 4.32 days following a single 1.25 mg ivt. injection (García-Quintanilla *et al*, 2019). Although bevacizumab has a similar molecular weight (145 kDa) like CFH, the half-life cannot be considered similar, as differences is charge, protein structure, post-translational modifications and the binding ability of bevacizumab to the neonatal Fc-receptor (FcRn) influence pharmacokinetics (Datta-Mannan, 2019).

In the phase I clinical trial of the mammalian cell derived CFH GEM103, the concentration in aqueous humor of patients did not return to basal level 8 weeks after 100 µg ivt. injection (Khanani *et al.*, 2022). However, the post-translational modifications and therefore potential impact on the clearance of GEM103 is not known. Considering a half-life of CPV-101 and CPV-104 in a range of a few days, a slower-progressing AMD model might reveal differences that are not apparent in our acute retinal degeneration within an observation time of 4 days. Still some slight differences in microglia morphology four days after light exposure, or in Il-6 secretion levels can be observed in our study that might be explained by differences in pharmacokinetic features.

5. Conclusion

Our findings demonstrate that both moss-derived CFH variants, independent of their glycosylation, can reduce complement activity in a mouse model of dry AMD. Additionally, the gliosis of microglia and Müller cells was decreased, leading to less migration and release of pro-inflammatory factors. Finally both variants led to decreased light induced retinal degeneration. This underlines the potential of moss-derived CFH as a new therapeutic option. However, more research needs to be done to adjust the application route, dosing and the impact of glycosylation on half-life in vitreous humor.

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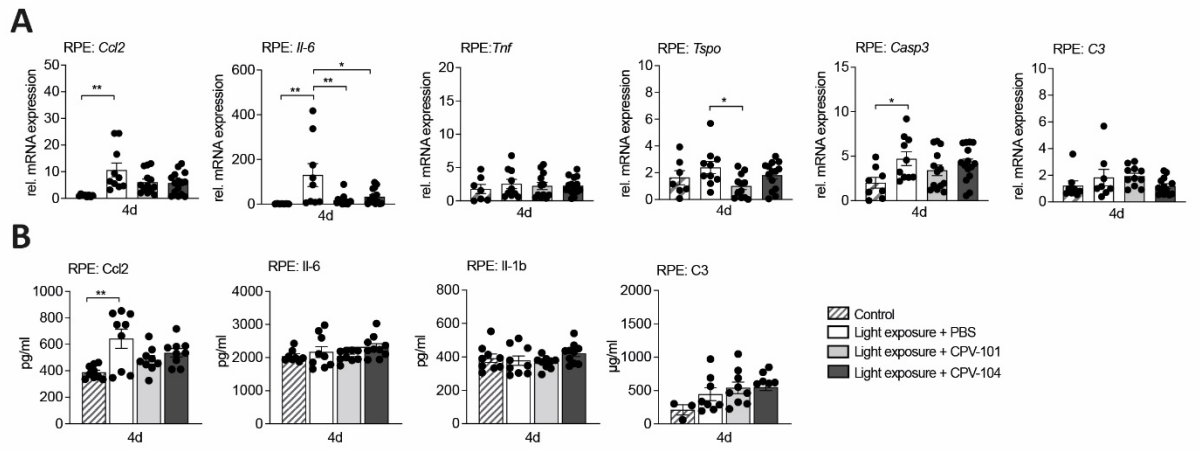
7. Attachments



Supplement Figure 1: Effect of CPV-101 and CPV-104 on light-induced pro-inflammatory marker transcript levels and secretion in RPE. A qRT-PCR and **B** ELISA were performed from RPEs of light-damaged and control mice four days after light exposure. Data are presented as mean ± SEM from n= 3-12 RPEs. One-way ANOVA followed by Sidak's multiple comparison test was used for statistical analyses; *P< 0.05, **P < 0.01 and ***P ≤ 0.001, N.D. - not detectable.

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9. Eidesstattliche Erklärung

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