IFN-β immunomodulation and complement receptor analysis in a mouse model of age-related macular degeneration (AMD)

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Zusammenfassung

Chronische Aktivierung von Mikroglia ist assoziiert mit retinaler Degeneration, wodurch diese als potentielle therapeutische Zielstrukturen für retinale Degenerationskrankheiten wie die altersabhängige Makuladegeneration (AMD) dienen können. Interferon-beta (IFN-β) ist ein potenter Immunregulator, der gewöhnlich zur Behandlung von Patienten mit Multipler Sklerose genutzt wird. In einer vorangegangenen Studie konnten wir zeigen, dass IFN-β Mikrogliose und choroidale Neovaskularisation in einem Laser-induzierten Mausmodell der feuchten AMD verhindert. Außerdem wird übermäßige Aktivierung des alternativen Komplementwegs als Hauptursache für das Fortschreiten der AMD angesehen. Mikroglia sind wichtige Produzenten von lokalem Komplement und besitzen selbst Komplementrezeptoren. In dieser Studie soll aufgeklärt werden, ob die Modulation von Mikroglia mittels IFN-β auch in einem Licht-induzierten Degenerationsmodell, welches einige Merkmale der trockenen AMD nachahmt, die Reaktivität von mononukleären Phagozyten dämpfen und dadurch vor Netzhautdegeneration schützen kann. Des Weiteren soll mithilfe von Anaphylatoxinrezeptor Knockout Mäusen die Beteiligung der Anaphylatoxin Signalübertragung an der Netzhautdegeneration untersucht werden. BALB/cJ Mäuse erhielten jeden zweiten Tag intraperitoneale Injektionen von 10.000 U IFN-β oder Vehikel, beginnend am Tag der Lichtexposition mit 15.000 Lux weißen Lichts für eine Stunde. Die systemische Behandlung mit IFN-β verstärkte teilweise die IFN-α/β-Rezeptor (IFNAR) Signalübertragung in der Netzhaut und verringerte die Anzahl an reaktivierten Mikroglia im subretinalen Raum. Vier Tage nach Lichtschaden wurde jedoch weder eine verringerte Expression an Komplementfaktoren noch eine Verbesserung der Netzhautdicke festgestellt. Bei den Mauslinien Komplementkomponente 3a Rezeptor 1 (*C3ar1*) oder Komplementkomponente 5a Rezeptor 1 (*C5ar1*) wurde 30 Minuten lang helles weißes Licht mit einer Intensität von 10.000 Lux angewendet. C3aR1 oder C5aR1 Ganzkörper Knockout Mäuse wurden getestet, aber keiner der Knockouts führte zu einer verminderten Migration von Mikroglia in den subretinalen Raum oder einer verringerten Expression von Komplementfaktoren nach Lichtschaden im Vergleich zum Wildtyp. Bei C3aR1 Knockout Mäusen konnte jedoch eine teilweise Rettung der Netzhautdicke gezeigt werden, was sich in einem signifikant geringeren Vorkommen des Membranangriffskomplexes (MAC) in der äußeren Netzhaut widerspiegelte. Zusammenfassend schlussfolgern wir, dass die IFNAR Signalübertragung retinale Mikroglia moduliert, aber eine starke Netzhautdegeneration durch akute Lichtschädigung nicht verhindern kann. Im Gegensatz dazu kann die Deletion des Anaphylatoxinrezeptors C3aR1 retinale Mikroglia nicht modulieren, aber die Degeneration der Netzhaut durch die Störung des Komplementwegs und die dadurch verringerte MAC-Assemblierung verlangsamen. Obwohl eine Verbindung zwischen der IFN-β Immunmodulation und dem Komplement Signalweg nicht

gezeigt werden konnte, können Therapien mit IFN-β und/oder gegen C3aR1 gerichtet für Patienten mit trockener AMD in Betracht gezogen werden. Weitere Studien werden erforderlich sein, um den genauen Wirkmechanismus von IFN-β und den optimalen Interventionspunkt in der Anaphylatoxin Signalachse zu bestimmen.

Summary

Chronic activation of microglia is associated with retinal degeneration, which makes them a potential therapeutic target for retinal degenerative diseases including age-related macular degeneration (AMD). Interferon-beta (IFN-β) is a potent immune regulator, commonly used for the treatment of multiple sclerosis patients. We have previously shown that IFN-β prevents microgliosis and choroidal neovascularization in a laser model of wet AMD. Furthermore, overactivation of the alternative complement pathway is generally accepted as the main driver for AMD disease progression and microglia are important producers of local complement and express complement receptors themselves. Here, we aim to elucidate whether modulation of microglia via IFN-β may also dampen mononuclear phagocyte reactivity and thereby protect from retinal degeneration in a light damage paradigm mimicking some features of dry AMD. We further investigate the involvement of anaphylatoxin signaling during retinal degeneration using anaphylatoxin receptor knockout mice. BALB/cJ mice received intraperitoneal injections of 10,000 U IFN-β or vehicle every other day; starting at the day of exposure to 15,000 lux white light for one hour. Systemic treatment with IFN-β partially enhanced IFN-α/β receptor (IFNAR) signaling in the retina and reduced the number of reactivated microglia in the subretinal space. However, four days after light damage neither decreased expression of complement factors nor rescue of retinal thickness was observed. Bright white light with an intensity of 10,000 lux for 30 minutes was applied for complement component 3a receptor 1 (*C3ar1*) or complement component 5a receptor 1 (*C5ar1*) mice. Full body knockout mice of either C3aR1 or C5aR1 were tested, but none led to mitigated microglia migration to the subretinal space or decreased expression of complement factors after light damage compared to wildtype. However, a partial rescue of retinal thickness was shown in C3aR1 knockout mice, which was mirrored by significant less membrane attack complex (MAC) occurrence in the outer retina. We conclude that IFNAR signaling modulate retinal microglia but cannot prevent strong retinal degeneration as elicited by acute white light damage. Contrarily, deletion of the anaphylatoxin receptor C3aR1 cannot modulate retinal microglia but decelerate retinal degeneration through interference in the complement pathway and thus decreased MAC assembling. Although a link between IFN-β immunomodulation and complement pathway signaling could not be underpinned, IFN-β- and/or C3aR1-targeted therapy may be considered for dry AMD patients. Further work will be necessary to elucidate the exact mechanism of action for IFN-β and the optimal intervention point in the anaphylatoxin signaling axis.

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A2E	*N*-retinylidene-*N*-retinylethanolamine
AAV2	Adeno-associated virus serotype 2
AF	Autofluorescence
Akt	Protein kinase B
AMD	Age-related macular degeneration
Atp5b	ATP synthase, H+-transporting, mitochondrial F1 complex, β polypeptide
BAF	Blue laser autofluorescence
BBB	Blood-brain barrier
bp	Base pair
BRB	Blood-retina barrier
BSA	Bovine serum albumin
C	Complement component
C1qa	Complement C1q A chain
C3aR	Complement component 3a receptor
C5aR	Complement component 5a receptor
C5L2	C5a receptor-like 2
CCL2	Chemokine (C-C motif) ligand 2
CCR2	CC receptor 2
CD	Cluster of differentiation molecule
CD200R	CD200 receptor
CD35	Complement receptor 1
cDNA	Complementary DNA
CF	Complement factor
CNS	Central nervous system
CNV	Choroidal neovascularization
COVID-19	Coronavirus disease 2019
$Crb1^{rd8}$	Crumbs family member 1, photoreceptor morphogenesis associated; retinal degeneration 8
CSF1R	Colony-stimulating factor 1 receptor
CX3CL1	C-X3-C motif chemokine ligand 1
CX3CR1	CX3C receptor 1
Cys	Cysteine
d	Day/days
DAMP	Damage-associated molecular patterns
ddH_20	Double-distilled water

DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveoretinitis/uveitis
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
FB	Factor B
FCS	Fetal calf serum
FD	Factor D
FI	Factor I
GA	Geographic atrophy
GCL	Ganglion cell layer
GSK3	Glycogen synthase kinase 3
GWAS	Genome wide association studies
h	Hour/hours
HCL	Hydrochloric acid
HET	Heterozygous
HOM	Homozygous
i.p.	Intraperitoneal
I3C	Indole-3-carbinol
Iba1	Ionized calcium binding adapter molecule 1
IFN	Interferon
IFNAR	Interferon-α/β receptor
IL	Interleukin
INL	Inner nuclear layer
iNos	Inducible nitric oxide synthase
IPL	Inner plexiform layer
IRF	Interferon regulatory factor
IS	Inner segment
ISG	IFN-stimulated genes
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
IVC	Individually ventilated cage
JAK1	Janus kinase 1
KO	Knockout
Leu	Leucine

LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAP	Mitogen-activated protein
Met	Methionine
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex proteins
min	Minute
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
Mx	Mx GTPase pathway
Mx1	Myxovirus influenza resistance 1
Mx2	Myxovirus influenza resistance 2
n.d.	Not detected
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDS	Normal donkey serum
NLRP3	NLR family pyrin domain-containing protein 3
nM	Nanomolar
nm	Nanometer
NO	Nitric oxygen
OAS	2’5’Oligoadenylate-synthetase-directed ribonuclease L
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segment
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
pH	Potentio Hydrogenii, negative decadic logarithm of the H_3O^+ concentration
PI3K	Phosphoinositide 3-kinase
PKR	Protein kinase R
PRRs	Pattern-recognition receptors
PU.1	Purine-rich box 1
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species

RPE	Retinal pigment epithelium
RPE65	RPE-specific protein (65 kDa)
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
SD-OCT	Spectral-domain optical coherence tomography
SDS	Sodium dodecyl sulfate
sec	Seconds
SEM	Standard error of the mean
Ser	Serine
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
SR	Subretinal space
STAT	Signal transducer and activator of transcription
TBE	Tris-borate-EDTA buffer
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRIS	Tris(hydroxymethyl)aminomethane
TSPO	Translocator protein (18 kDa)
TUNEL	Transferase dUTP nick end labeling
TYK2	Tyrosine kinase 2
U	Units
V	Volt
v	Version
VEGF	Vascular endothelial growth factor
w	week
WT	Wildtype

1. Introduction

1.1 The mammalian retina

The eye is the most important sensory organ, allowing orientation in the environment and non-vocal communication. Loss of sight is a life-changing event. Out of many compartments, the retina is the most important for vision, located posterior in the eye bulb (Figure 1). It inversely processes visual stimuli with wavelengths between 400 and 750 nm by initiating nerve impulses and forwarding them as action potentials through the optic nerve into the visual cortex of the brain (Sung & Chuang, 2010). Although the mammalian retina is a neural tissue with an average thickness of only 250 µm, it is composed of several highly organized layers containing more than 55 distinct cell types (Masland, 2001).

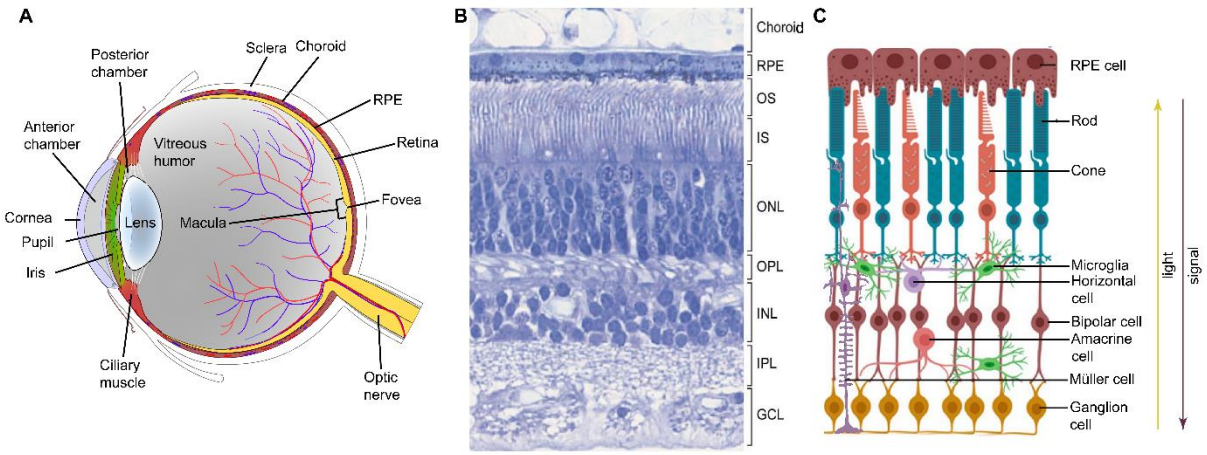

Figure 1: Overview of the human eye and retina. A Schematic anatomy of the eye. **B** H&E-stained cross-section of a human retina. RPE: retinal pigment epithelium; OS: outer segments (of photoreceptors); IS: inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer. GCL: ganglion cell layer. **C** Schematic cross-section of retinal cell types. Retinal pigment epithelial cells (RPE cells) form the RPE, photoreceptor nuclei reside in the ONL, horizontal, bipolar, amacrine and Müller cell nuclei build the INL, ganglion cells form the GCL. Microglia are found in the two plexiform layers (OPL, IPL). **A** Modified from Jmarchn - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=34828874. **B** Modified from Sung & Chuang, 2010. **C** Modified from Hadziahmetovic & Malek, 2021, Müller cell adapted from Wolf et al., 2020.

The light enters the eye through the cornea and passes through aqueous humor, pupil, lens and the whole retina in order to reach the light-sensitive photoreceptors. While the pupil is relevant to control the amount of incoming light, the cornea and lens focus it onto the macula, the central area of the retina. The eye contains two types of photoreceptors: rods and cones. While rods are responsible for vision under low light conditions (scotopic vision), cones cover day and color vision with high visual acuity (photopic vision) (Baylor et al., 1979; P. K. Brown & Wald, 1964). The human retina contains approximately 95 % rods that are mainly found in the periphery (Sung & Chuang, 2010). Cones are enriched in the fovea centralis, the center of

highest visual acuity within the macula (Chader & Taylor, 2013). The photoreceptor outer segments (OS) hold numerous opsins that absorb photons which triggers isomerization of its 11-*cis*-retinal chromophore to all-*trans* retinal that is further processed in the phototransduction cascade (Radu et al., 2008). OS and inner segments (IS) of the photoreceptors are joint by a connecting cilium with the underlying tightly packed photoreceptor nuclei (Horst et al., 1990), that constitute the outer nuclear layer (ONL). The outer plexiform layer (OPL) holds synapse connections from photoreceptors to bipolar cells, which are further connected to ganglion cells by synapses in the inner plexiform layer (IPL). The optic nerve is formed by the axons of the ganglion cells, their nuclei form the ganglion cell layer (GCL), that transports chemotactic signals to the brain, where the information are processed into a picture (Figure 1). Besides this vertical signal transduction, horizontal and amacrine cells provide lateral signal transduction. Their nuclei lie together with these of bipolar and Müller cells in the inner nuclear layer (INL). Synaptic transmission of photoreceptor and bipolar cells are hereby modulated by horizontal cells whereas amacrine cells regulate transmission to ganglion cells (Purves et al., 2001).

The retinal pigment epithelium (RPE) is a monolayer connecting with the apical side to the OS. Its function involves the absorption of scattered or unabsorbed light, regeneration of visual pigments, transport of nutrients, phagocytosis of photoreceptor debris and secretion of immunosuppressive factors (Bok, 1985; Bonilha, 2014; Strauss, 2005). RPE and Bruch´s membrane form the blood-retina barrier (BRB) separating the inner retinal tissue from the blood stream, ensuring the immune privilege of the eye (Cunha-Vaz et al., 2011).

Besides neuronal cell types, glia cells such as astrocytes, Müller cells and microglia, are also present in the retina. Müller cells span the whole retina and fill all space that is not occupied by neurons and blood vessels. They support neuronal cell survival by maintaining the retinal extracellular environment, including the BRB, and function as optic fibers (Bringmann et al., 2006; Cunha-Vaz et al., 2011; Franze et al., 2007). Astrocytes reside in vascular zones and influence the activity of the BRB (Cunha-Vaz et al., 2011). Microglia are the native immune cells within the immune-privileged retina. They are located in both plexiform layers and monitor the environment with their protrusions (Karlstetter et al., 2015) (for further information see section 1.3).

Due to the overall consistency of the basic organization of the retina across vertebrates, different mouse models are often used as research tools on eye diseases. Nevertheless, some differences between the human and mouse eye need to be noted. The murine retina neither has a macula nor a fovea and contains only 3 % cones (Carter-Dawson & Lavail, 1979). This minimum number of cones can further only detect short (S) and middle (M) wavelength light (G. H. Jacobs et al., 1991), while humans also express opsins that are sensitive for long (L) wavelength light (Nathans et al., 1986).

1.2 Age-related macular degeneration (AMD)

Age-related macular degeneration (AMD) is one of the leading causes of irreversible vision loss amongst the elderly of the western world. The two known terminal stages of AMD, namely dry and wet AMD, lead to complete vision loss that has a severe negative impact on the patient's quality of life (Jonas et al., 2014; Subhi & Sørensen, 2016). Additional therapy options are urgently needed, as the number of affected people is rising rapidly due to the demographic change, with an estimate of around 288 million patients worldwide in 2040 (Wong et al., 2014).

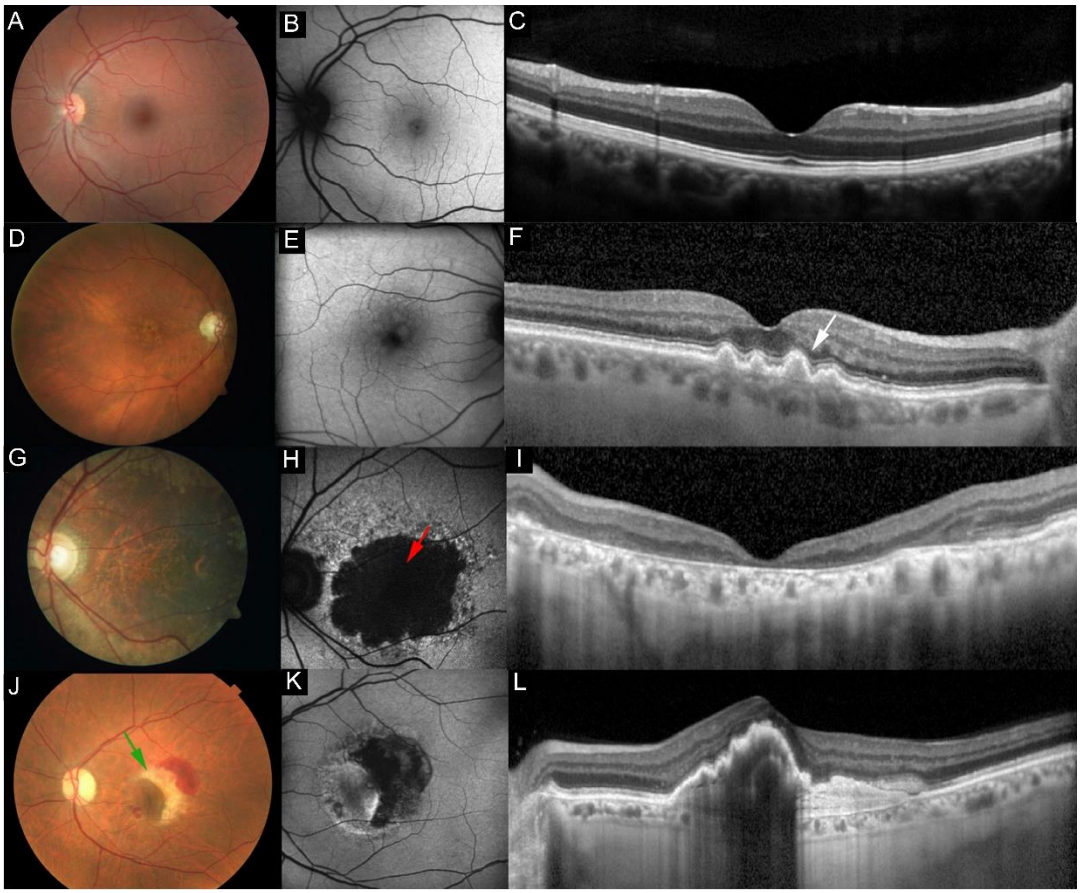

Figure 2: Fundus photographs and OCT scans of human healthy and AMD eyes. A-C Healthy eye (fundus photograph, fundus autofluorescence, and optical coherence tomography (OCT), respectively); **D-F** Intermediate, non-exudative AMD with drusen (F, white arrow); **G-I:** Dry AMD showing large, central geographic atrophy (H, red arrow); **J-L** Wet AMD showing choroidal neovascularization and retinal hemorrhage (J, green arrow). Modified from Hadziahmetovic & Malek, 2021.

Clinically, AMD is divided into three disease stages (Bird et al., 1995). In early AMD, asymptomatic lipid-rich deposits, called soft drusen (up to 63 µm diameter), occur within the Bruch´s membrane in the macula region (Figure 2, white arrow). Pigmentation changes as well as the increase in drusen size (64-125 µm diameter) and number mark the intermediate symptomatic stage of disease. Drusen in the subretinal space can worsen to central geographic atrophy (GA) (Figure 2H, red arrow). This late stage of disease is called dry AMD, accounts for about 90 % of AMD cases (Akyol & Lotery, 2020), and is characterized by

progressive degeneration of photoreceptors. In the rare late stage, the exudative or wet form of AMD, choroidal neovascularization (CNV) occurs. This case is accompanied by leaking blood vessels growing through the impaired Bruch´s membrane (Figure 2J, green arrow). Fortunately, wet AMD can be treated with anti-angiogenic substances, while no therapy option is known so far for dry AMD (Hubschman et al., 2009). Monthly intravitreal injections of anti-VEGF (vascular endothelial growth factor) drugs significantly reduced the incidence of blindness in wet AMD patients, however, it is only applicable to a limited number of patients and involves side effects (Finger et al., 2020; Miller, 2016). Therefore, there is an urgent need for novel therapy approaches and immunomodulation has been recognized as a new application field (Akhtar-Schäfer et al., 2018).

AMD pathogenesis and pathophysiology is extremely complex due to the complexity of the retina as well as the variety of risk factors and molecular mechanism involved in disease onset and progression. However, despite the existing relative immune privilege of the eye, several studies have implicated that the immune system may contribute to AMD development and progression. The current hypothesis implicates that an extensive dysregulation of different arms of the immune system is associated with the pathophysiology of AMD. Thus, a system initially devised to function in immune surveillance exceeds a critical threshold of activation and fosters the development of para-inflammation (Penfold et al., 1985; Xu et al., 2009). Moreover, the pathological changes observed in the retinal tissue are evoked by dysregulation of the innate immune system, mainly the complement system and reactive mononuclear phagocytes (Fritsche et al., 2014; Gupta et al., 2003). Although there is convincing evidence that different immune cell populations are related to the pathophysiology of AMD, their interactions and mechanisms of activation are still unclear.

1.2.1 AMD associated risk factors

AMD is a complex multifactorial disease, where a combination of genetic risk factors together with advanced age, smoking, hypertension and environmental factors define an individual's predisposition to AMD (Y. Chen et al., 2010; Wong et al., 2014).

Oxidative stress is high in the retina and especially in the macular region since photoreceptors have the highest oxygen consumption and metabolic rate of any tissue in the body (Blasiak et al., 2017). With age, this leads to a decline of mitochondria function in RPE cells and secondary photoreceptors which increases their vulnerability to injury (E. E. Brown et al., 2019; Feher et al., 2006). Moreover, the choroidal layer thins with increasing age, its elasticity weakens and the ability to process metabolites is diminished (Chirco et al., 2017; Wakatsuki et al., 2015). This ultimately leads to impaired Bruch´s membrane function and further, through constant debris deposition, to the formation of drusen (Curcio et al., 2009; Mullins et al., 2000). Smoking

additionally reduces the antioxidant defense of the retina and RPE (Espinosa-Heidmann et al., 2006). Furthermore, hyperglycemic diets, with high intake of fatty acids like linolenic acid and refined carbohydrates, are positively associated with the risk of AMD (Chiu et al., 2007; Cho et al., 2001). Contrary, Mediterranean diets, that are rich in vitamins and carotenoids, as well as fish intake may reduce the risk of AMD (Cho et al., 2001; B. M. J. Merle et al., 2013, 2019; Mozaffarieh et al., 2003). In addition, women are 1.4 times more likely to develop late AMD, especially with Caucasian heritage (Rudnicka et al., 2015).

However, genetic variations were found to account for 46-71 % of the variation in the overall severity of AMD (Seddon et al., 2005). Genome wide association studies (GWAS) revealed 52 genetic variants in genes of the immune system; especially those regulating the complement system are highly associated with AMD (Fritsche et al., 2016; Klein et al., 2005; Mullins et al., 2017). By this means, a common single nucleotide polymorphism (SNP) in complement factor H (CFH), a soluble regulator of the alternative complement pathway, has been strongly associated with AMD. Noteworthy, 42 % Europeans are heterozygous for the Y402H variant, with 2- to 3-fold greater odds for disease, while homozygous individuals hold even about 7-fold greater odds of developing AMD (Tzoumas et al., 2021) (for further information see section 1.5.2). However, little is known about how these genetic variants drive AMD progression (Van Lookeren Campagne et al., 2014).

1.3 Retinal microglia

Microglia are the resident active immune cells of the central nervous system (CNS) (Priller et al., 2001). Approximately 0.2 % of all retinal cells are microglia (F. Li et al., 2019). They build a regularly spaced network of ramified cells and are essential for homeostatic functions of the retina (Karlstetter et al., 2015; Karlstetter & Langmann, 2014). Microglia also play a key role in the initiation and perpetuation of chronic inflammation in the aging retina (Damani et al., 2011; Sierra et al., 2007). Like macrophages, microglia are mononuclear phagocytes and recognize small potentially pathogenic insults (Karlstetter et al., 2010).

1.3.1 Origin and maintenance of microglia

Microglia were first described by del Río-Hortega 1919 as cells with small cell bodies and long cellular processes with phagocytic capacity within the brain parenchyma (Sierra et al., 2016). As Río-Hortega already predicted, microglia have a primitive erythro-myeloid origin, derive from hematopoietic progenitors and represent a distinct cellular entity (Gomez Perdiguero et al., 2015; Karlstetter et al., 2015). Their development is driven by colony-stimulating factor 1 receptor (CSF1R), purine-rich box 1 (PU.1) binding and interferon regulatory factor 8 (IRF8) signaling (Elmore et al., 2014; Erblich et al., 2011; Kierdorf et al., 2013). To circumvent the

blood-brain barrier (BBB) and BRB microglia invade from the yolk sac into the CNS during early embryonic development (Ginhoux et al., 2010; Kettenmann et al., 2011; Schulz et al., 2012). As both barriers are physiological borders (Engelhardt & Liebner, 2014), microglia represent a long lived, self-sustained cell population that persist for month or even the entire life span of the organism (Ajami et al., 2007; Lawson et al., 1992). Microglia repopulation and self-renewal in the retina was shown to be derived from residual microglia in the optic nerve and macrophages in the ciliary body/iris (Y. Huang et al., 2018).

1.3.2 Microglia functions in health and disease

Within the lifespan of an organism microglia fulfill different significant functions in the retina. During development, infiltrating microglia actively promote programmed cell death of developing neurons that are excessively generated (Marín-Teva et al., 2004). Hereto, microglia migrate to the respective site and phagocyte dead neurons without inducing inflammation and tissue necrosis (Ravichandran, 2003). Besides their clearance function microglia scalp neuronal circuits by eliminating excessive synaptic connections from retinal ganglion cells into the thalamus (Schafer et al., 2012). Furthermore, communication between microglia and endothelial cells via secreted soluble factors was shown to shape vascular growth as well as vascular branching and thus exerts an influence on the retinal vasculature development (Rymo et al., 2011). According to their functions, amoeboid microglia are predominantly found at the basal side of the retina at birth and become progressively ramified during the maturation process (F. Li et al., 2019).

Within the adult CNS microglia are primarily related to immune responses and tissue homeostasis. Under homeostatic conditions they reside in an equally distributed, non-overlapping network in the plexiform layers and monitor their environment through their protrusions (Karlstetter et al., 2010, 2015; Langmann, 2007; Nimmerjahn et al., 2005). Microglia are essential for neuronal homeostasis in the mature retina by maintaining synaptic function and plasticity (X. Wang et al., 2016). Intercellular signaling between microglia and neurons is essential to limit microglia activation and is tightly controlled through the release of soluble factors or direct physical interaction (Karlstetter et al., 2015). Following an insult or the detection of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Kettenmann et al., 2011), microglia activation occurs in a graded change of morphology from ramified to amoeboid (Kreutzberg, 1996) whilst migration to the site of damage (Figure 3) (Gupta et al., 2003). In this reactivated state microglia recruit immune cells from the periphery (Karlstetter et al., 2015; Langmann, 2007), increase their phagocytic activity (Kettenmann et al., 2011) and upregulate the expression of numerous cell surface receptors including major histocompatibility complex proteins (MHC) class II, cluster of

differentiation molecule 11b (CD11b, also integrin alpha-M beta-2) and ionized calcium binding adapter molecule 1 (Iba1, gene name *allograft inflammatory factor 1*) (Autieri, 1996; Dick et al., 1995; Ito et al., 1998; Xu et al., 2007). The latter is usually used to immunohistochemically distinguish microglia from other retinal cell types and to visualize their morphology (Kettenmann et al., 2011). Modes of action at the corresponding site therefore involve phagocytosis of cellular debris and apoptotic cell material, release of pro- and anti-inflammatory factors and antigen presenting (Gehrmann et al., 1995; Jurgens & Johnson, 2012; Karlstetter et al., 2015; Nimmerjahn et al., 2005; Van Rossum & Hanisch, 2004; Wynn & Vannella, 2016). Chemokine (C-C motif) ligand 2 (CCL2) thereby plays a crucial role in recruiting other mononuclear phagocytes (Hinojosa et al., 2011), which are previously activated by interleukin-6 (IL-6) and IL-1β in a paracrine manner (R. Ferreira et al., 2012; Krady et al., 2008).

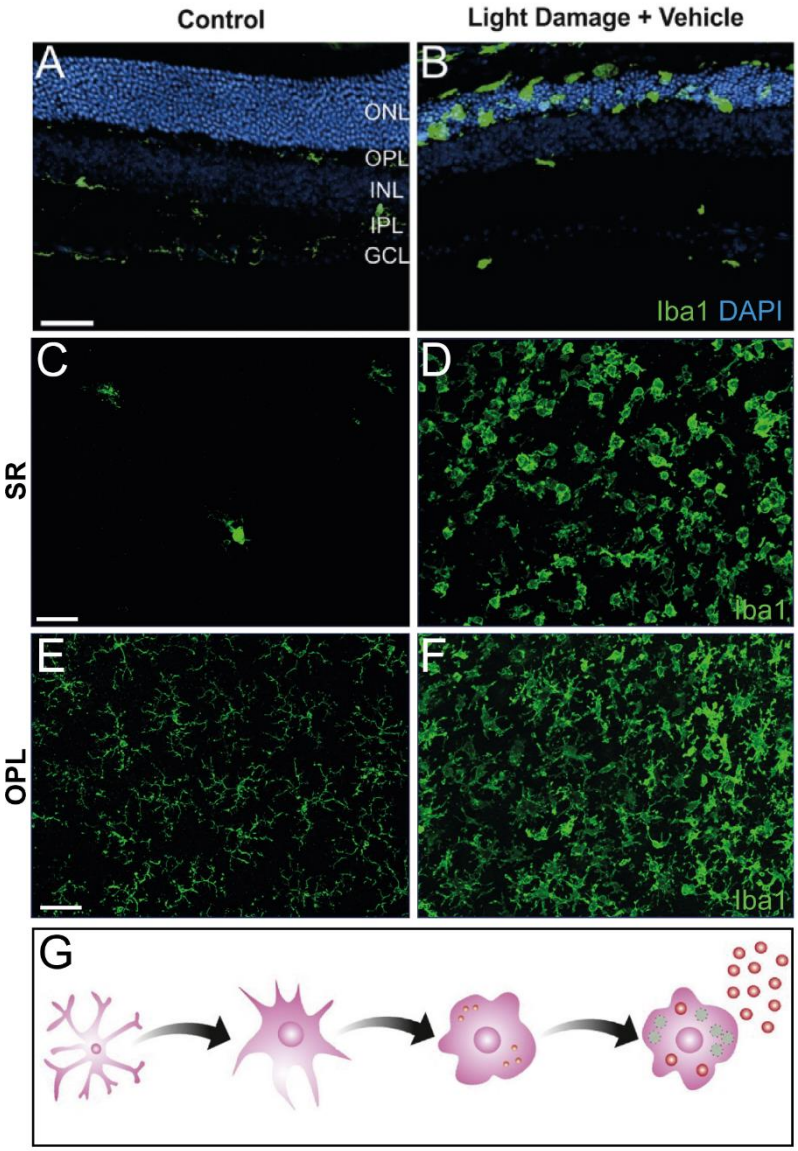

Figure 3: Microglia morphology and localization in health and disease. Ionized calcium-binding adapter molecule 1 (Iba1) staining of retinal sections **(A, B)** and flat mounts **(C-F)** in the healthy **(A, C, E)** and degenerated retina **(B, D, F)**. **G** Scheme of progressive morphological changes in microglia from ramified to amoeboid after activation. In the healthy retina, ramified microglia reside in an equally distributed, non-overlapping network in the plexiform layers and monitor their environment through their protrusion. During retinal degeneration, microglia change their morphology from ramified to amoeboid and migrate to the outer nuclear layer (ONL) and subretinal space (SR), where they increase their phagocytic activity and release pro- and anti-inflammatory factors, therefore contribute to the degenerative processes. Iba1: ionized calcium-binding adapter molecule 1; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar: 50 µm. Panels A, B, and G adapted from Karlstetter et al., 2015.

Following CNS injury microglia on the one hand promote synaptic stripping and neuronal regrowth (Gehrmann et al., 1995), but on the other hand release cytotoxic substances like nitric oxygen (NO) and reactive oxygen species (ROS) to fight infectious triggers (Banati et al., 1993; Langmann, 2007). To control microglial neurotoxicity, neurons express two regulatory ligands CD200 and C-X3-C motif chemokine ligand 1 (CX3CL1, also fractalkine) that provide calming signals when bound to their receptors (Cardona et al., 2006; Combadière et al., 2007). The corresponding receptors CD200R and CX3CR1 are highly expressed on microglia and modulate microglia activity as well as migration (Carter & Dick, 2004; Kettenmann et al., 2011; Liang et al., 2009). In conclusion, limited microglia activation is considered neuroprotective, while chronic activation can lead to retinal degeneration (Karlstetter et al., 2010; Shastri et al., 2013).

1.3.3 Microglia reactivity in AMD

To date, there is accumulating evidence that altered cell numbers and expression of surface receptors of phagocytes are related to AMD (Behnke et al., 2020). Examination of patient tissue uncovered the presence of several proteins associated with inflammation and immune-related molecules and cells as especially concentrated in drusen (Anderson et al., 2002). Concurrent, a comprehensive histopathological analysis of donor eyes revealed that the presence of drusen and basal laminar deposits is likely a trigger for the recruitment of macrophages to Bruch´s membrane and the choroid in early AMD (Cherepanoff et al., 2010). Therefore, drusen and subretinal immune cells can be regarded as crystallization points at the outer retina and RPE/Bruch´s membrane interface. One major trigger for subretinal microglia migration could be the age-dependent accumulation of intracellular *N*-retinylidene-*N*-retinylethanolamine (A2E), a constituent of lipofuscin (Ma et al., 2013). Indeed, aged microglia show an exaggerated response to homeostatic disturbance than naïve microglia (Sierra et al., 2007; Xu et al., 2009). Recruitment is a key event in retinal para-inflammation (Figure 4). As mentioned before, the signaling axes of CCR2-CCL2 and CX3CR1-CX3CL1 are crucial for phagocyte recruitment to retinal lesions. CCL2 expression in the retina is increased with age and higher transcript levels were observed in patients with neovascular and atrophic AMD compared to controls (Newman et al., 2012). Furthermore, CX3CR1-deficient mice develop exaggerated microglia accumulation, drusen-like deposits and more leaky neovessels in the laser-CNV model (Combadière et al., 2007). These animals also displayed increased microglia migration into the subretinal space after exposure to bright light (Raoul et al., 2008), suggesting that the CX3CR1/CX3CL1 signaling axis may be required for limiting microglia migration and reactivity. Recruited microglia/macrophages display a predominantly pro-inflammatory phenotype, characterized by enhanced phagocytosis as well as the release of ROS, complement factors and pro-inflammatory cytokines (Behnke et al., 2020).

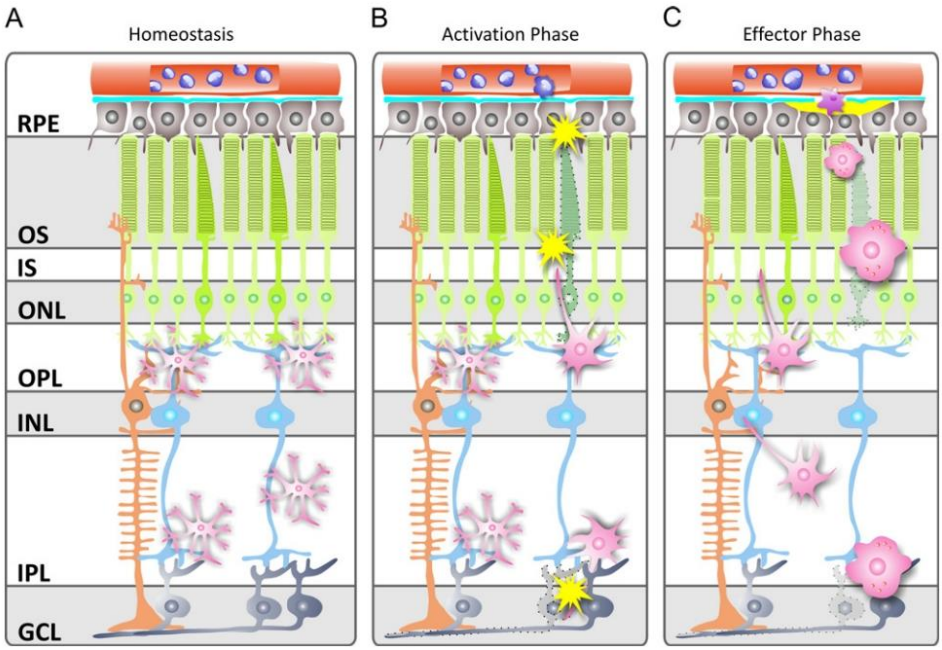

Figure 4: Microglia reactivity in AMD. A Under homeostatic conditions, microglia (pink) reside in an equally distributed, non-overlapping network in the plexiform layers and monitor their environment through their protrusion. **B** Following a sensed insult (yellow) microglia change their morphology in the activation phase from ramified to amoeboid and migrate to the designated tissue. **C** In this reactivated state microglia recruit immune cells from the periphery (dark purple cell), increase their phagocytic activity and release pro- and anti-inflammatory factors. In early AMD, drusen (yellow) are building between retinal pigment epithelium (RPE) and Bruch´s membrane (cyan). OS: outer segments (of photoreceptors); IS: inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Graphic adapted from Karlstetter et al., 2010.

In the subtle process of age-related retinal degeneration activated phagocytes successively migrate to these lesion hot spots and are therefore key inflammatory cells present at all stages of AMD (Krogh Nielsen et al., 2019). Furthermore, their reactivity is associated with human AMD and related mouse models (Combadière et al., 2007; Gupta et al., 2003; Levy et al., 2015). Large amounts of microglia are detected in the subretinal space and the outer nuclear layer of AMD patients and disease mouse models, harboring numerous engulfed photoreceptor particles (Gupta et al., 2003). Hence, degeneration of photoreceptors leads to activation of microglia, among others. Besides their phagocytic clearance functions, these cells can also act as antigen presenting cells, bridging the gap between innate and adaptive immunity. Transcriptome analysis of AMD patient donor retinas revealed an overexpression of complement and MHC class I genes, which indicate an activation and contribution of microglia in AMD pathogenesis (Newman et al., 2012). Overall, complex cell interactions fail during AMD and cause overactivation of microglia that drive retinal degeneration.

1.3.4 Microglia modulation as a therapeutic strategy in AMD

The previous section enlightened microglia/macrophage contribution in AMD, therefore, signaling pathways and molecular targets that modulate microglia and macrophage activity represent attractive therapeutic treatment options (Akhtar-Schäfer et al., 2018; Langmann,

2007). Minocycline, a semi-synthetic antibiotic agent with potent anti-inflammatory and neuroprotective capacity, counter-regulated pro-inflammatory gene transcription in the retina and prevented microgliosis as well as retinal degeneration in light-challenged mice (Scholz, Sobotka, et al., 2015). A synthetic ligand XBD173 of the microglia activation marker translocator protein (18 kDa) (TSPO) prevented microglia reactivity in a light-induced retinal degeneration mouse model as well as the laser-induced CNV mouse model (Scholz, Caramoy, et al., 2015; Wolf et al., 2020). The same results occurred using microglia-specific TSPO knockout animals (Wolf et al., 2020), highlighting microglia involvement in inflammatory processes during retinal diseases.

Natural compounds as the polyunsaturated fatty acid docosahexaenoic acid (DHA) as well as flavonoid luteolin and the turmeric component curcumin modulated the microglia transcriptome *in vitro* towards an anti-inflammatory and neuroprotective phenotype and thus alleviated microglia migration and morphological changes (Dirscherl et al., 2010; Ebert et al., 2009; Karlstetter et al., 2011; Khan & Langmann, 2020). Furthermore, indole-3-carbinol (I3C), a natural ligand of the aryl hydrocarbon receptor, inhibited microglia activation, migration, expression of anti-inflammatory transcripts and proteins and, as a consequence, protected from light-induced retinal degeneration *in vivo* (Khan & Langmann, 2020). Activation of interferon-beta signaling in retinal phagocytes diminished vascular leakage and inflammation (Lückoff et al., 2016), suppression of IL-27 reduced the extent of CNV and VEGF-production by migrating invading macrophages in the laser-CNV model (Hasegawa et al., 2012). Despite the fact that various cell culture and animal studies highlighted the beneficial effects of immunomodulation (Rashid et al., 2018), there are currently no clinical trials or approved therapies targeting mononuclear phagocyte reactivity.

1.4 Interferon-beta (IFN-β)

Interferon (IFN) was first described in 1957 by Isaacs and Lindemann as a substance for virus interference (Isaacs et al., 1957; Isaacs & Lindemann, 1957). Nowadays, IFNs are known to be large endogenous cytokines that exhibit potent immunomodulatory, anti-viral and anti-proliferative properties, which makes them applicable for different clinical treatments (Annibali et al., 2015; Borden et al., 2007; Jonasch, 2001; Pestka, 2007). They are produced and secreted by cells upon detection of PAMPs by pattern-recognition receptors (PRRs) (Wu & Chen, 2014).

IFN-β is one out of seven type I interferons (Pestka et al., 2004) and a key regulator of the innate immune system (Sadler & Williams, 2008). While IFN-α and -γ are promoting pro-inflammatory responses, IFN-β is mainly immuno-regulatory. Any nucleated cell can produce IFNs in response to an infection (Pestka et al., 2004), however, IFN-β is predominantly

produced by dendritic cells and fibroblasts (Stark et al., 1998; Swiecki & Colonna, 2011). The *IFN-β* gene contains no introns and is encoded by a single gene located on chromosome 9 in humans, encoding its pleiotropic protein consisting of 166 amino acids (Pestka et al., 1987).

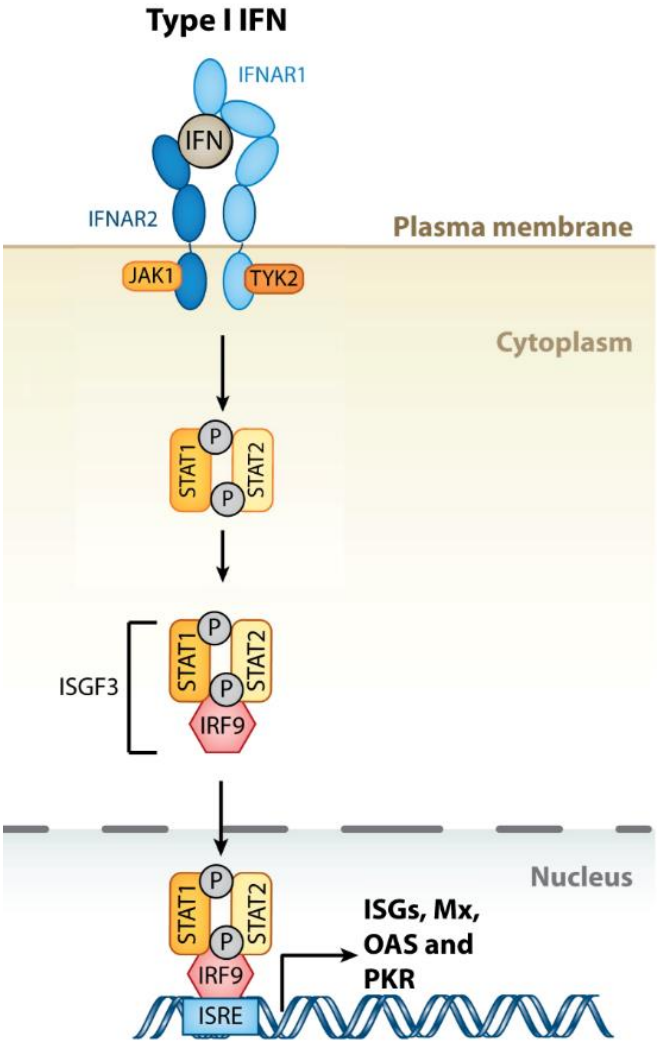

Figure 5: The Interferon signaling cascade. Type I interferons engage the interferon-α/β receptor 1 (IFNAR1) and IFNAR2 heterodimer and trigger activation of the associated tyrosine kinases janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) that subsequently induce signal transducer and activator of transcription (STAT) recruitment and phosphorylation. STAT1 and STAT2 associate to form a heterodimer, which in turn recruits interferon regulatory factor 9 (IRF9) to build the IFN-stimulated gene factor 3 (ISGF3). This complex translocates into the nucleus and binds upstream to the IFN-stimulated response element (ISRE) that regulates the downstream transcription of IFN-stimulated genes (ISGs). Effector pathways that further transduce IFN-β mode of action include Mx GTPase pathway (Mx), 2'5'oligoadenylate-synthetase-directed ribonuclease L (OAS) and protein kinase R (PKR). IFN: Interferon. Modified from Schneider et al., 2014.

All type I interferons connect to the ubiquitously expressed IFN-α dimer receptor composed of interferon-α/β receptor 1 (IFNAR1) and IFNAR2 (Figure 5). Receptor engaging activates the IFNAR associated tyrosine kinase 2 (TYK2) and janus kinase 1 (JAK1) respectively, that trigger recruitment and phosphorylation of signal transducer and activator of transcription (STAT) factors (Leonard, 2001). Subsequently, the STAT1 and STAT2 heterodimer recruits a DNA-binding protein like IRF9 to form the trimolecular complex IFN-stimulated gene factor 3 (ISGF3) (Platanias, 2005). This complex translocates into the nucleus and binds upstream to the IFN-stimulated response element (ISRE) that regulates the downstream transcription of numerous IFN-stimulated genes (ISGs). More than 300 different ISGs are induced by IFNAR signaling (de Veer et al., 2001). Information about type I, II, III interferon regulated genes from publicly available microarray datasets can be found in the Interferome database (http://interferome.its.monash.edu.au/interferome/) (Rusinova et al., 2013). Few ISGs have

direct anti-viral, anti-proliferative or immunomodulatory effects. Receptor activation can not only induce the canonical JAK-STAT, but alternative signaling pathways and their cooperative functions then result in a pleiotropic cellular, and thus, divergent biological response (De Weerd et al., 2007; Platanias, 2005). Other effector pathways transduce IFN-β mode of action namely Mx GTPase pathway (Mx), 2'5'oligoadenylate-synthetase-directed ribonuclease L (OAS) and protein kinase R (PKR) pathway (Sadler & Williams, 2008).

1.4.1 IFN-β as a therapeutic agent

Due to its immunomodulatory properties IFN-β has been used for clinical applications. As the first has Jacobs et al. treated in 1982 ten patients suffering from multiple sclerosis (MS), an inflammatory and demyelinating CNS disorder characterized by multifocal brain lesions, intrathecally with IFN-β (L. Jacobs et al., 1982) and 301 more patients later on intramuscularly (L. D. Jacobs et al., 1996). A beneficial effect on disease progression was evident after two years. In 1993, Betaseron, a recombinant IFN-β-1b produced by the Bayer AG, was released and further used as state-of-the-art treatment for relapsing-remitting MS patients (Bayas & Gold, 2003). Until today different IFN-β drugs are the first-line treatment option for MS patients (Annibali et al., 2015). However, some patients discontinue the treatment due to several adverse effects after IFN-β injection like for example flu-like symptoms, muscle pain, depression, leukopenia, lymphopenia, injection-site reactions and inflammation (Nikfar et al., 2010). To mention, IFN-β-1a is produced by mammalian Chinese hamster ovary (CHO) cells, while IFN-β-1b is purified from genetically modified *Escherichia coli (E. coli)* cultures and misses the native glycosylation pattern (Taniguchi et al., 1980).

Some studies focused on the biological activities of IFN-β and its mode of action. They highlighted the inhibitory effect of IFN-treatment on proliferation, migration and antigen presentation of T cells (Yong et al., 1998). Furthermore, IFN-β was shown to directly modulate cytokine production to an anti-inflammatory milieu and reduce trafficking of inflammatory cells across the BBB (Kieseier, 2011). Accordingly, chemokine gene expression and protein levels were elevated by IFN-β peripherally in blood of MS patients and suggested to reduce chemoattraction of immune cells to the CNS (Cepok et al., 2009). However, the exact mechanism of action is still unclear.

More recently, IFN-β was used to treat severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) on coronavirus disease 2019 (COVID-19) patients, based on an immune analysis study with 50 COVID-19 patients revealing highly impaired IFN type I response with no IFN-β present in blood samples (Hadjadj et al., 2020). However, the therapeutic efficiencies were rather diverse. Although IFN-β alone or in combination with other drugs prevalently reduced the time to clinical improvement in COVID-19 patients (Alavi Darazam et al., 2021;

Davoudi-Monfared et al., 2020; Khamis et al., 2021; Monk et al., 2021; Peiffer-Smadja & Yazdanpanah, 2021; Rahmani et al., 2020), a meta-analysis comparing 196 trials with all kind of treatments for COVID-19, including 7 IFN-β trials, could not substantiate a reduced risk of death or any other patient important outcome through IFN-β treatment (Siemieniuk et al., 2020).

1.4.2 Effect of IFN-β on microglia

Microglia are the major source of endogenous IFN-β during CNS autoimmunity (Kocur et al., 2015). In turn, there is strong evidence that IFN-β has an immunomodulatory effect on microglia and thereby counteracts autoimmunity in the CNS (Axtell & Steinman, 2008; Prinz et al., 2008). As mentioned before, IFN-β mediates neuroprotection against MS, including recruitment of microglia to the lesion sites and enhancing their phagocytic activity (Dendrou et al., 2015; Kocur et al., 2015; Stetson & Medzhitov, 2006). Mice suffering from experimental autoimmune encephalomyelitis (EAE), a disease model for MS, revealed a negative correlation of IFN-β/IFNAR signaling and disease severity with excessive microglia activation (Prinz et al., 2008; Teige et al., 2003).

Several immunomodulatory mechanisms have been proposed for IFN-β (Figure 6). After engaging its respective receptors IFANR1 and IFNAR2, IFN-β induces the expression of suppressor of cytokine signaling 1 (SOCS1) and SOCS3 as part of a negative feedback loop to dampen prolonged signaling by pro-inflammatory cytokines, either via the previously described JAK-STAT pathway (Ivashkiv & Donlin, 2014) or the non-canonical activation of the phosphoinositide 3-kinase (PI3K)–protein kinase B (Akt)–mammalian target of rapamycin (mTOR) pathway (Burke et al., 2014; Platanias, 2005). Here, STAT3 phosphorylation is also mediated by PI3K-Akt downstream activated mTOR (Zhu et al., 2015). ISGF3 and phosphorylated STAT3 translocate to the nucleus, where they induce various ISGs (Leonard, 2001). Once produced, SOCS1 and SOCS3 are recruited to cytokine receptors where they negatively regulate inflammatory responses by inhibiting the catalytic activity of JAK tyrosine kinases (Krebs & Hilton, 2001; Yoshimura et al., 2007). Consequently, in an experimental autoimmune uveoretinitis (EAU) mouse model myeloid-specific SOCS3-deficient mice displayed exaggerated retinal degeneration (M. Chen et al., 2018). Furthermore, overexpression of SOCS1 in EAU mice significantly diminished disease severity by suppressing inflammatory chemokine expression and inhibiting recruitment of inflammatory cells into the retina (C.-R. Yu et al., 2011).

Additionally, IFN-β mediated activation of Akt during the PI3-Akt pathway causes inhibition of glycogen synthase kinase 3 (GSK3) by serine phosphorylation (Jope et al., 2017). GSK3 is a defining mediator of inflammatory responses in the CNS and has been shown to alter the

production of anti- and pro-inflammatory cytokines (W.-C. Huang et al., 2009; Jope et al., 2017). The ISG ubiquitin-specific protease 18 was found as a negative regulator of IFNAR signaling in microglia, hindering STAT1 phosphorylation via interaction with IFNAR2 and therefore pruning microglia activation (Goldmann, 2015).

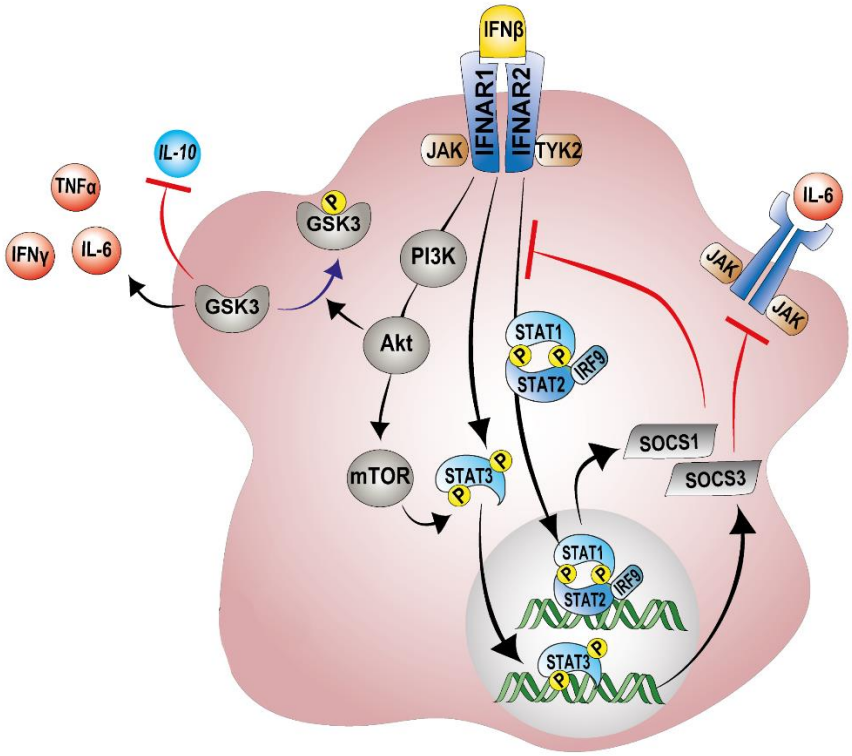

Figure 6: Signaling of IFN-β in microglia. IFN-β engages interferon-α/β receptor 1/2 (IFNAR1/2) and triggers activation of the associated tyrosine kinases janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) that subsequently induces signal transducer and activator of transcription 1 (STAT1), STAT2, and STAT3 transcription factor phosphorylation. The resulting dimer of STAT1 and STAT2 recruit interferon regulatory factor 9 (IRF9) and translocates to the nucleus, where it induces the transcription of a variety of IFN-stimulated genes like suppressor of cytokine signaling 1 (SOCS1) and SOCS3. Through the non-canonical PI3K-Akt-mTOR pathway STAT3 can be phosphorylated as well as glycogen synthase kinase 3 (GSK3), negatively regulating its inflammatory response and promoting IL-10 expression. These actions will further inhibit the negative feedback loop of inflammatory compounds in microglia through blockage of the JAK catalytic activity. PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; mTOR: mammalian target of rapamycin; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon. Modified from Rashid et al., 2019.

Previous results using a laser-induced CNV mouse model, that mimics features of exudative AMD (Lambert et al., 2013), demonstrated that IFN-β strongly inhibits microglia/macrophage activation and recruitment, and induces a transition towards a neuroprotective ramified microglia morphology (Lückoff et al., 2016). Conversely, global or microglia-specific conditional deletion of IFN-β/IFNAR1 signaling in mice resulted in an exacerbated disease (Lückoff et al., 2016). Remarkably, IFN-β treatment reversed subfoveal neovascularization and choroiditis in a MS patient (Cirino et al., 2006). These findings strongly suggest that IFN-β/IFNAR signaling, particularly in microglia, prevents chronic inflammation and pathological neovascularization in the retina and could be a therapeutic target for retinal inflammatory disorders. However, the precise mode of action of IFN-β on microglia remains unknown.

1.5 The complement system

The complement system is composed of more than 40 proteins and links adaptive and innate immune responses (N. S. Merle et al., 2015; Walport, 2001). A variety of complement proteins, receptors, and regulators are expressed in the retina (Anderson et al., 2010). Microglia are, alongside the RPE, important producers of local complement and are equipped with complement receptors themselves (Luo et al., 2011). In terms of disease, the complement system is often referred to as a double-edged sword, as under normal conditions it is the most significant mechanism to restore homeostasis of damaged tissue, while dysfunction or impaired regulation can lead to a variety of acute and chronic diseases (Kawa et al., 2014).

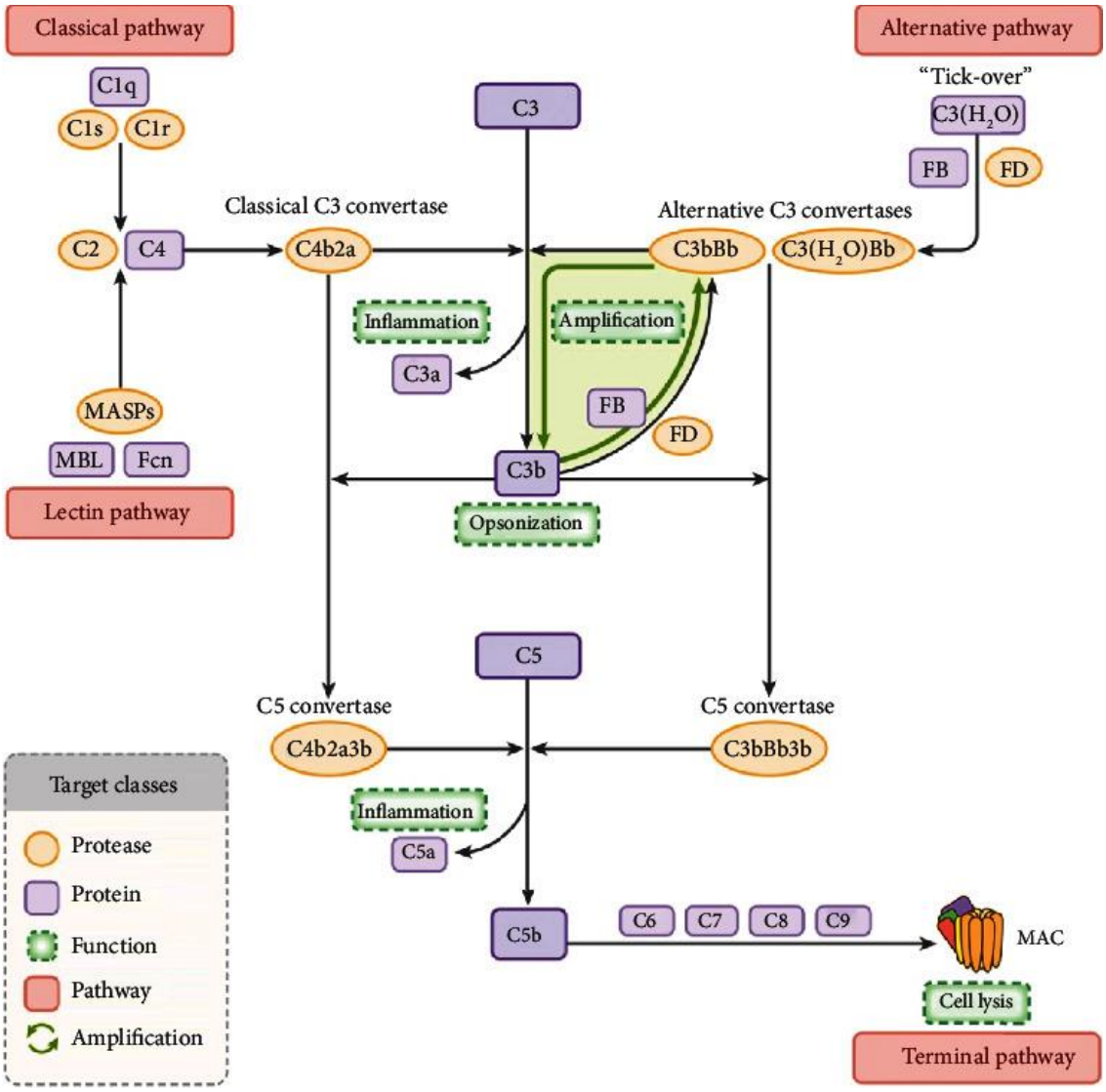

Figure 7: Overview of the complement signaling pathways. The complement cascade can be activated through three proteolytic pathways: classical, lectin and alternative. Spontaneous hydrolysis of C3 functions as a "tick-over", resulting in low grade continuous activation of the alternative pathway. All pathways result in formation of convertases that cleave C3 and C5, which ultimately leads to the terminal pathway, the formation of the membrane attack complex (MAC). Other cleavage products can further induce opsonization (C3b) or inflammation (C3a, C5a). Furthermore, ample amounts of C3b are used to assemble alternative C3 convertases, which is regarded as the amplification loop for all pathways. Adapted from Qin et al., 2021.

Three proteolytic pathways enable complement cascade activation: (1) the classical pathway, responding to antigen-antibody complexes, (2) the lectin pathway, engaged by mannose-binding lectin and damaged cell surface, (3) the alternative pathway, activated by cleavage of complement component 3 (C3) (N. S. Merle et al., 2015). The alternative pathway, in contrast, is spontaneously and in a low grade continuously activated, also named “tick-over”, due to spontaneous hydrolysis of C3 (C3(H_2O)) (Figure 7). Despite different types of activation through several patterns or pathogens, all three complement pathways function through the same main mechanism. The cleavage of C3 and C5 by their respective convertases are the central steps in all complement pathways. Therefore, C3 and C5 convertases are built upon activation. The membrane-bound classical C3 convertase (C4b2a) is produced by the classical and lectin pathways, while factor B (FB) is cleaved by factor D (FD) to build the alternative C3 convertases (C3bBb, C3(H_2O)Bb). C3b, resulting from C3 cleavage, is participating in opsonizing pathogens or dead cells and promotes their phagocytosis in a non-inflammatory manner. In addition, the alternative pathway is generating a feedback loop for all pathways, independent of the initial trigger, as sufficient amounts of the C3b produced are also used to assemble the alternative C3 convertase. Converging C3 convertases with an additional C3b results in the formation of membrane bound C5 convertases (C4b2a3b, C3bBb3b). C5b, resulting from C5 cleavage, initiates the formation of the membrane attack complex (MAC), which is built up by recruiting C6, C7, C8 and C9. MAC formation represents the terminal pathway, as it is a lipophilic transmembrane channel that, at high levels, leads to pore formation on the cell surface and cell lysis (Lueck et al., 2011). Sublytic MAC levels on the target cell can induce intracellular inflammatory signaling via NLR family pyrin domain-containing protein 3 (NLRP3) inflammasome activation (Laudisi et al., 2013; Triantafilou et al., 2013). Noteworthy, 80-90 % of MAC formation is induced by the alternative pathway, regardless of the initiating pathway (Harboe et al., 2004). Toxic parts of C3, C4, or C5 cleavage are complement proteins C3a, C4a, and C5a, respectively, so called anaphylatoxins.

Indeed, soluble and membrane-bound regulators tightly control complement cascade actions. For example, soluble CFH dissociates C3 convertases (Lambris, 1988), while CD59 is known to block MAC assembly (Kimberley et al., 2007). Furthermore, regulators such as CFH, CD46, CD55 and CD35 act as cofactors enabling factor I (FI) to cleave opsonins into their inactive forms, in which they can still promote phagocytosis, but can no longer form convertases, therefore slowing down complement activation (Schmidt et al., 2016).

1.5.1 Anaphylatoxins and their receptors

Anaphylatoxins C3a and C5a are the most potent pro-inflammatory fragments generated during the complement pathway and function as chemoattractants for mononuclear

phagocytes (Brandstetter et al., 2015; Nozaki et al., 2006). Inflammation is further stimulated through induction of oxidative burst, histamine production, degranulation, cell apoptosis of immune cells and modulation of the adaptive immune response (Klos et al., 2009; N. S. Merle et al., 2015). Of note, studies have also highlighted an anti-inflammatory role for C3a in different contexts (Coulthard & Woodruff, 2015), possibly based on surface- and intracellularly induced signaling (Liszewski et al., 2013).

C3a and C5a drive inflammation by engaging their corresponding chemotactic receptors C3aR1 and C5aR1 (also named CD88) (Engelke et al., 2014; M. Yu et al., 2012; L. Zhang et al., 2016). Both receptors belong to the rhodopsin family of G-protein-coupled 7 transmembrane-domain receptors and are primarily expressed on myeloid and lymphoid cells throughout the body (Ames et al., 1996; Gasque et al., 1998; C. Gerard et al., 1992; Laumonnier et al., 2017; Quell et al., 2017; Soruri et al., 2003). In human mast cells, C3aR1/C3a-induced chemokine synthesis has been shown to be mediated by distinct signaling pathways, including phosphorylation of extracellular-signal-regulated kinase (ERK) and Akt (Venkatesha et al., 2005). Using *in vivo* and *in vitro* approaches, C5aR1/C5a signaling was connected to activation of the PI3K/Akt signaling and the mitogen-activated protein (MAP) kinase pathway (Buhl et al., 1994; la Sala et al., 2005).

Another C5a anaphylatoxin receptor C5aR2 (also named C5a receptor-like 2, C5L2) was described and initially suggested to be non-signaling (T. Zhang et al., 2017). However, there is increasing evidence that the receptor has pro- and anti-inflammatory signaling roles (R. Li et al., 2013). Here, *in vivo* data indicate that C5aR2 acts as a positive modulator for anaphylatoxin-induced responses, proposing the formation of receptor heterodimers (N.-J. Chen et al., 2007). Furthermore, crosstalk between anaphylatoxin receptors and others, such as toll-like receptors (TLRs), have been described to alter anaphylatoxin effects on immune response (N. S. Merle et al., 2015).

1.5.2 Complement in AMD

The involvement of the complement cascade in AMD disease progression is generally accepted. Solid evidence from numerous large scale genetic studies, meta-analyses and molecular studies confirmed the significant role of the complement system including genetic variants, overactivation of the alternative pathway, chronic inflammation, oxidative stress, lipid accumulation, and energy metabolism as drivers of AMD pathogenesis (Armento et al., 2021; Kawa et al., 2014; Mullins et al., 2017; Qin et al., 2021). In the CFH gene a total number of 160 coding variants have been discovered in AMD patients (de Jong et al., 2021), including the extremely rare variant R1210C, which is even more strongly associated with AMD than the common Y402H mutant (Raychaudhuri et al., 2011). While the Y402H variant is assumed to

decrease the overall binding affinity of CFH (Clark et al., 2010; Laine et al., 2007; Sjöberg et al., 2007), the R1210C variant is known to have a decreased binding affinity to C3b, C3d and heparin, that in turn leads to increased C3 convertase activity (V. P. Ferreira et al., 2009; Józsi et al., 2006; Recalde et al., 2016). Furthermore, a total of 91 rare variants have been demonstrated in the C3 gene, the key component among all complement pathways (Geerlings et al., 2017; Rodriguez et al., 2014). The most common polymorphism R102G (de Jong et al., 2021) reduces CFH cofactor activity, which usually mediates C3b cleavage by complement factor I (CFI) to inactivate the C3 convertase (Heurich et al., 2011). Additionally, a number of 37 variants in the C9 gene, the most downstream component of the terminal pathway, were discovered in AMD (de Jong et al., 2021). Notably, it has been suggested that a combination of risk variants in one individual can lead to an additive risk of disease (Harris et al., 2012).
Nevertheless, there are even a few negatively linked variants. Complement factor B (CFB) mutations L9H and R32Q are considered to have a protective effect on AMD pathogenesis (Gold, Merriam, Zernant, Hancox, Taiber, Gehrs, Cramer, Neel, Bergeron, Barile, Smith, AMD Genetics Clinical Study Group, et al., 2006; Maller et al., 2006), through decreased formation of the alternative C3 convertase (Montes et al., 2009). Additionally, the R95X variant in the C9 gene is considered to be negatively linked to AMD risk as well (Nishiguchi et al., 2012).

Besides the mentioned SNPs in complement genes, that are associated with AMD, various complement components were found in drusen of AMD patients, including MAC and anaphylatoxins C3a and C5a (Crabb et al., 2002; Gold, Merriam, Zernant, Hancox, Taiber, Gehrs, Cramer, Neel, Bergeron, Barile, Smith, Hageman, et al., 2006; Mullins et al., 2001, 2011, 2014; Nozaki et al., 2006). In fact, an *in vitro* model with human fetal RPE cells demonstrated that C3a triggers the formation of this sub-RPE deposits (Fernandez-Godino & Pierce, 2018). The additional drusen components C-reactive protein, lipofuscin, and amyloid-β serve as complement activators (Sparrow et al., 2012; Zhou et al., 2006, 2009). The regulatory proteins, CD46 and CD59 were significantly lower expressed on $CD14^+$ monocytes from patients with neovascular AMD than controls (Singh et al., 2012). Additionally, the complement receptor 1 (CD35) was significantly higher expressed on monocytes in AMD patients (Haas et al., 2011). Among other functions, CD35 is involved in the clearance of complement opsonized immune complexes, degradation of C3b and co-stimulation of B cells. Overall decreased levels of regulatory complement proteins were found in eyes of AMD patients (Clark et al., 2010; Ebrahimi et al., 2013).

Mouse models are used to better understand the involvement of the complement system in the pathogenesis of AMD. An overall increase in proteins of the complement system was detected after light exposure in a mouse model mimicking dry AMD (Rutar et al., 2014; Schäfer et al., 2017). The most important effect of complement activation in AMD is the recruitment

and activation of immune cells mediated by C3a and C5a (Behnke et al., 2020; Ogura et al., 2020). In this regard, anaphylatoxin receptors C3aR1 and C5aR1 were highly expressed on microglia/macrophages after light exposure (Song et al., 2017).

CFH can also bind to mononuclear phagocytes, which then leads to phagocyte persistence at the inflammatory area. This cell trapping effect was markedly enhanced by an AMD-associated variant in CFH (Calippe et al., 2017). C3 is crucial for macrophage/monocyte recruitment and $C3^{-/-}$ mice had significantly smaller lesions and lower immune cell numbers in the laser-induced CNV model (Tan et al., 2015). After exposure to continuous bright light, retinal microglia/macrophages synthesize *C3* mRNA and deposit it in the ONL (Rutar et al., 2014). Overall, reactivation of microglia was shown to be fostered by a dysregulated complement system among others (Karlstetter et al., 2015). Furthermore, complement activation stimulates RPE cells to secrete a variety of inflammatory factors (Lueck et al., 2015). RPE cells and photoreceptors of CFH-deficient mice showed mitochondrial abnormalities and dysfunction (Ferrington et al., 2017; Sivapathasuntharam et al., 2019). Recently, a link between autophagy-lysosomes and the complement system has been reported (Cerniauskas et al., 2020).

1.5.3 Complement inhibitors in AMD therapy

Although numerous studies raise convincing evidence that the overactive complement system in retinas can lead to AMD, effective treatment options are lacking. Therefore, several studies using mouse models were carried out with genetically modified animals and/or complement system inhibitors. Antibody-mediated neutralization of anaphylatoxins, pharmacological blockade or knockout of both anaphylatoxin receptors resulted in reduced neovascular areas in the laser-CNV model of wet AMD (Nozaki et al., 2006). Likewise, reduced microgliosis could be detected in C5aR1-deficient mice after light exposure (Song et al., 2017). Furthermore, ablation of C3 in a photo-oxidative damage mouse model diminished microglia/macrophage numbers in the outer retina and mitigated their phagocytic capacity (Jiao et al., 2020). Aurintricarboxylic acid, a small molecule inhibitor of the MAC, protected from complement mediated lysis *in vitro* and attenuated CNV, MAC deposition and macrophage infiltration in a mouse model for wet AMD (Lipo et al., 2013). Intravitreal injection of a recombinant CD59 potently inhibited CNV growth in mice (Bora et al., 2010). While systemic administration of an anti-C5a antibody also inhibited the subretinal recruitment of phagocytes in aged $Cfh^{+/-}$ mice, retinal pathology remained unchanged (Toomey et al., 2018).

Based on these findings, several clinical studies focus on complement inhibition in AMD (Akhtar-Schäfer et al., 2018; Kassa et al., 2019; Xu & Chen, 2016). To date, more than 14 complement inhibitors have been generated and 26 clinical studies in dry AMD and 10 in wet

AMD are completed or ongoing (Qin et al., 2021). The following will feature the most promising candidates. Targeting the key complement component C3, APL-2 (pegcetacplan), a peptide compstatin analogue, was able to reduce GA growth rate by 20-29 % in phase II trial (NCT02503332) (Liao et al., 2020). Phase III clinical trial and a long-term safety assessment are currently running (NCT03525613, NCT03777332). Zimura (avacincaptad pegol), a pegylated RNA aptamer specifically targeting C5, significantly reduced the mean rate of GA growth, while unfortunately increasing the risk of CNV (Jaffe et al., 2020). A verifying phase III trial is under way (NCT04435366). Contrarily, Zimura achieved in combination with ranibizumab, a common anti-VEGF drug, improved visual acuity in wet AMD patients (NCT03362190).

To mention, delivery of therapeutics directly into the eye is the preferred administration route, but regular intravitreal injections increase the risk of bacterial infections. In addition, the compounds have to reach the appropriate anatomical site in an effective concentration; therefore, gene therapy introduces a promising new therapy direction. Adeno-associated virus serotype 2 (AAV2) gene therapy with HMR59 induces the generation of soluble complement terminal pathway regulator CD59 (Bordet & Behar-Cohen, 2019; Puliafito & Wykoff, 2019) and was able to slow GA growth rate in phase I clinical trial (NCT04358471). Furthermore, a combination of gene therapy and anti-VEGF treatment was predicted a promising approach for wet AMD patients (De Guimaraes et al., 2021). IBI302, the first bispecific recombinant human fusion protein, can bind and block VEGF with its N-terminus, while its C-terminus bins C3b and C4b to block initiation of the classical and alternative complement cascades (Ren et al., 2016). Safety and tolerability were proven by the phase I clinical trial (NCT03814291).

Despite the recent setback through disappointing results of the phase III anti-complement factor D (CFD)-inhibitor (lampalizumab) trial for geographic atrophy (Heier et al., 2020; Holz et al., 2018), the complement system is still a good candidate for pharmacological intervention. Further work will be necessary to identify the best targets in the complement cascade.

1.6 Aims of the thesis

Microglia are the resident, active immune cells of the CNS. Chronic activation of microglia is associated with retinal degeneration, which makes microglia-directed immunomodulation a promising pharmacological therapeutic strategy for retinal degenerative diseases including AMD. IFN-β is a potent immune regulator, commonly used for the treatment of MS patients. We have previously shown that IFN-β prevents microgliosis and CNV in a laser model of wet AMD. Thus, we hypothesized that microglia modulation via IFN-β may also dampen mononuclear phagocyte reactivity and thereby protect from retinal degeneration in a light damage paradigm mimicking some features of dry AMD. Furthermore, overactivation of the alternative complement pathway is generally accepted as the main driver for AMD disease progression and microglia are important producers of local complement and even express complement receptors themselves. Here, we aim to elucidate whether modulation of microglia via IFN-β may also dampen mononuclear phagocyte reactivity by altering the production of complement components in the retina and further investigate the involvement of anaphylatoxin signaling during retinal degeneration using anaphylatoxin receptor knockout mice.

2 Material and Methods

2.1 Cell Culture

2.1.1 Maintaining and subculturing of cells

Murine BV-2 cells (Blasi et al., 1990) from passages 8-20 were used and cultured at 37 °C and 5 % CO_2 humidity in T75 flasks. Culture media RPMI 1640 contained 5 % fetal calf serum (FCS), 1 % Penicillin/Streptomycin, 3mM L-Glutamine, and 50 µM β-Mercaptoethanol. Media was changed every three days and cells were split at a confluency of 95 %.

2.1.2 IFN-β and LPS stimulation

Human IFN-β-1a was purchased from PeproTech GmbH (#300-02BC) and diluted in phosphate-buffered saline (PBS) with 0.1 % bovine serum albumin (BSA). BV-2 cells were cultured in 6 well plates, with $3*10^5$ cells per well and treated after overnight attachment with 50 ng/ml Lipopolysaccharide (LPS) together with 1000 U/ml IFN-β or IFN-β alone for 6, 24, and 72 hours.

2.2 Mouse experiments

All experimental protocols complied with the ARRIVE guidelines and were carried out in accordance to the German animal welfare act, which is in line with the European Directive 2010/63/EU, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal experiments used in this study were reviewed and approved by the governmental body responsible for animal welfare in the state of North Rhine-Westphalia, Germany (Landesamt für Natur, Umwelt und Verbraucherschutz).

2.2.1 Mouse husbandry

The animals were housed in an individually ventilated cage (IVC) system (GM 500, Tecniplast® Greenline) under SPF-conditions. Air-conditioned environment with a temperature of 22±2 °C and relative humidity of 45-65 % was obtained. Mice were kept in a 12 hour light/dark cycle, with light on at 6 am. Irradiated phytoestrogen-free standard diet for rodents (Altromin 1314) and acidified water were provided *ad libitum*.

2.2.2 Experimental mouse lines

8-10-week-old male and female mice were used in the experiments (Lückoff et al., 2016; Scholz, Caramoy, et al., 2015; Scholz, Sobotka, et al., 2015). BALB/cJ and C57BL/6J were

from house. C.129S4-*C3ar1*^{tm1Cge}/J and C.129S4(B6)-*C5ar1*^{tm1Cge}/J, further referred to as *C3ar1* and *C5ar1*, were purchased from Jackson Laboratory (stock number 005712 and 006845, respectively), backcrossed two times with BALB/cJ and bred in house. In both mice a neomycin-resistant gene driven by the phosphoglycerate kinase (PGK) promoter was used to replace the endogenous gene; for *C3ar1* the N-terminal region including the start codon and for *C5ar1* the entire coding region was deleted (Höpken et al., 1996; Humbles et al., 2000). Genetic modified mouse lines were bred heterozygous, wildtype littermates were used as controls. All animals used in this study (excluding C57BL/6J) had a genetic BALB/cJ background with the light-sensitive RPE-specific protein (65 kDa) (RPE65) Leu450 variant, Ser103 substitution in tyrosinase, and wildtype *Crb1*^{rd8} (crumbs family member 1, photoreceptor morphogenesis associated; retinal degeneration 8) alleles.

2.2.3 Light exposure regime

Littermates were dark adapted for 16 hours prior to light exposure. Pupils were dilated with 2.5 % phenylephrine and 0.5 % tropicamide under dim red light and mice were placed separately in reflective, aluminum-foil-coated cages to prevent covering. Bright white light, that contains a blue peak in its spectrum, with an intensity of 15,000 lux for one hour (BALB/cJ) or 10,000 lux for 30 minutes (*C3ar1/C5ar1*) was applied. After light exposure animals were transferred to normal light cycle until further analysis.

2.2.4 IFN-β administration

Human IFN-β-1a was purchased from PeproTech GmbH (#300-02BC) and diluted to 10,000 U/10 µL in PBS and 0.1 % BSA, if not stated otherwise. Intraperitoneal (i.p.) application of 10,000 U per mouse started one hour prior to light damage and continued every other day until the end of the experiment.

2.2.5 *In vivo* imaging – Anesthesia, SD-OCT, and BAF

For imaging mice were anesthetized with ketamine (Ketavet, 100 mg/kg) and xylazine (2 % Rompun, 5 mg/kg) diluted in 0.9 % sodium chloride and pupils were dilated with 2.5 % phenylephrine and 0.5 % tropicamide. Retinal thickness and autofluorescence in fundus (only *C3ar1/C5ar1*) were analyzed four days after light damage via spectral-domain optical coherence tomography (SD-OCT) and BluePeak Blue laser autofluorescence (BAF), respectively, using the Spectralis™ HRA/OCT device. Retinal thickness heatmaps were calculated through Heidelberg Eye Explorer (HEYEX) software. Analysis was carried out using 3 mm and 6 mm diameter values; the average of four sections around the optic nerve circle of 1 mm was accounted for one n.

2.3 Molecular methods

2.3.1 Isolation of genomic DNA

Ear punches were obtained between P21-24 and genomic DNA was isolated using a modified HotSHOT method (Truett et al., 2000). Samples were incubated 20 minutes in alkaline lysis buffer (Table 6) at 95 °C, then chilled on ice for 10 minutes. An equal amount of neutralizing solution (Table 6) was added and DNA diluted to a working concentration of 25 ng/µl.

2.3.2 Quantification of nucleic acids

Purity and integrity of RNA/DNA was assessed with NANODrop 2000 Spectrophotometer. 260/280 nm absorbance ratio between 1.8 and 2.0 was accepted as pure.

2.3.3 Genotyping

Genotype of *C3ar1* and *C5ar1* mice was determined by polymerase chain reaction (PCR), based on genotyping protocols from Jackson Laboratories, with customized primer (purchased from IDT) using the Taq-S PCR kit (Genaxxon). All amplifications were performed in a total reaction volume of 25 µl containing a minimum of 50 ng DNA, 25 pmol of each primer (50 pmol: *C3ar1* forward common), 25 µM dNTP mix, 1x reaction buffer S and one unit of Taq polymerase. Primer and PCR programs are listed in Table 1 and Table 2, respectively. After amplification RPE65 samples were digested with 1 unit HpyF10VI, Tyrosinase samples with 1 unit HpyF3I enzymes in 1x Buffer Tango at 37 °C for two hours followed by thermal inactivation at 80 °C or 65 °C for 20 minutes, respectively. Amplified DNA from genotyping was analyzed by gel electrophoresis. DNA samples were mixed with loading dye (6x) and electrophorized at 120 V in an 1-2 % (w/v) agarose gel regarding their size. Amplicons were visualized with UV-light using the Intas Gel iX20 Imager.

Table 1: List of all genotyping primer used in this study.

Gene	Primer	Primer Sequence	Amplicon size [bp]
C3ar1	Forward common	5'-agccattctaggggcgtatt-3'	WT: 250
	Reverse wildtype	5'-tggggttatttcgtcttctgc-3'	KO: 400
	Reverse mutant	5'-tggatgtggaatgtgtgcgag-3'	
C5ar1	Forward wildtype	5'-ggtctctccccagcatcata-3'	WT: 386
	Forward mutant	5'-gccagaggccacttgtgtag-3'	KO: 244
	Reverse common	5'-ggcaacgtagccaagaaaaa-3'	
RPE65	Forward	5'-cactgtggtctctgctatcttc-3'	Met: 674
	Reverse	5'-ggtgcagttccattcagtt-3'	Leu: 437, 236 (after digest)

Gene	Primer	Primer Sequence	Amplicon size [bp]
Tyrosinase	Forward	5'-aagaatgctgcccaccatg-3'	Cys: 165, 110, 73, 12 (after digest)
	Reverse	5'-gacatagactgagctgatagtatgtt-3'	Ser: 130, 110, 73, 35, 12 (after digest)
Crb1^{rd8}	Forward wildtype	5'-gtgaagacagctacagttctgatc-3'	WT: 220
	Forward mutant	5'-gcccctgtttgcatggaggaaacttgga agacagctacagttcttctg-3'	HOM: 244
	Reverse common	5'-gccccatttgcacactgatgac-3'	

Table 2: List of all genotyping PCR programs used in this study.

Gene of interest	Step	°C	Time (Cycles)
C3ar1	Initial denaturation	95	2 min
	Denaturation	95	30 sec
	Annealing	57	20 sec (x35)
	Elongation	72	30 sec
	Final elongation	72	3 min
C5ar1	Initial denaturation	94	2 min
	Denaturation	94	20 sec
	Annealing	65	15 sec (-0.5 °C/cycle) (x10)
	Elongation	68	10 sec
	Denaturation	94	15 sec
	Annealing	60	15 sec (x28)
	Elongation	72	10 sec
	Final elongation	72	2 min
RPE65	Initial denaturation	95	2 min
	Denaturation	95	20 sec
	Annealing	56	15 sec (x35)
	Elongation	72	20 sec
	Final elongation	72	2 min
Tyrosinase	Initial denaturation	95	2 min
	Denaturation	95	20 sec
	Annealing	58	15 sec (x40)
	Elongation	72	20 sec
	Final elongation	72	2 min

Gene of interest	Step	°C	Time (Cycles)
Crb1^{rd8} (2 PCRs)	Initial denaturation	95	2 min
	Denaturation	95	20 sec
	Annealing	58 (WT)/65 (HOM)	15 sec (x35)
	Elongation	72	20 sec
	Final elongation	72	2 min

2.3.4 RNA isolation, cDNA synthesis, and qRT-PCR

RNA from cultured microglial cells was isolated with the NucleoSpin RNA isolation kit following the manufacturer's instructions (Machery & Nagel). RNA from retinas was isolated with the Qiagen RNeasy® Micro kit according to the manufacturer´s protocol. cDNA was synthesized with the Thermo Fischer Reverse Transcriptase kit according to the company's protocol. Subsequent transcripts measurements were performed in technical duplicates with the probe-based (Takyon™ No ROX Probe MasterMix blue dTTP) detection according to the manufacturer´s instructions using the LightCycler® 480 II. Primer sequences and Roche Universal Probe Library probe numbers are listed in Table 3. *ATP synthase, H+-transporting, mitochondrial F1 complex, β polypeptide (Atp5b)* was used as reference gene and the $^{\Delta\Delta}$Ct method was applied using the LightCycler® 480 software 1.2.1 for relative quantification of results. For this, technical duplicates of one retina were averaged for one n.

Table 3: List of all qRT-PCR primer and probes used in this study.

Gene	Forward primer	Reverse primer	Probe
Atp5b	5'-ggcacaatgcaggaaagg-3'	5'-tcagcaggcacatagatagcc-3'	#77
C1qa	5'-ggagcatccagtttgatcg-3'	5'-catccctgagaggtctccat-3'	#16
C3	5'-accttacctcggcaagtttct-3'	5'-ttgtagagctgctggtcagg-3'	#76
C3ar1	5'-gtggctcgcagatcatca-3'	5'-aagactccatggctcagtcaa-3'	#1
C5ar1	5'-gcatccgtcgctggttac-3'	5'-tgctgttatctatggggtcca-3'	#63
Cfb	5'-ctcgaacctgcagatccac-3'	5'-tcaaagtcctgcggtcgt-3'	#1
Cfh	5'-gaaaaaccaaagtgccgaga-3'	5'-ggaggtgatgtctccattgtc-3'	#25
iNos	5'-ctttgccacggacgagac-3'	5'-tcattgtactctgagggctga-3'	#13
Mx1	5'-ttcaaggatcactcatacttcagc-3'	5'-gggaggtgagctcctcagt-3'	#53
Mx2	5'-cagttcctctcagtcccaagat-3'	5'-tgcggttgtgagcctctt-3'	#11
Tspo	5'-cccttgggtctctacactgg-3'	5'-aagcagaagatcggccaag-3'	#21

2.4 Biochemistry

2.4.1 Immunohistochemistry

Eyes were enucleated and fixed in 4 % Roti Histofix for 2.5 h at room temperature (RT). For retinal flat mounts, eyes were dissected and permeabilized and unspecific antigens then blocked with Perm/Block Buffer (Table 6) overnight at 4 °C. For cryosections, whole eyes were transferred in 30 % sucrose for one hour before embedding in optimal cutting temperature (O.C.T.) compound. 10 µm sections were prepared with a Leica CM3050 S Cryostat. Frozen slides were thawed at RT and dehydrated in PBS for 10 minutes before unspecific antigens were blocked with Blotto (Table 6) for 30 minutes at RT. Retinal flat mounts and sections were incubated with primary antibody overnight at 4 °C. Antibodies and dilutions listed in Table 4. Afterwards, retinal flat mounts (1:1500) and sections (1:1000) were incubated with secondary antibody for one hour at RT. Retinal flat mounts were mounted on microscopic slides and embedded with Vectashield® HardSet™ H-1400. Sections were mounted in Fluoromount-G with DAPI. Z-stack images of the central retina with 20x magnification were taken with a Zeiss Imager M.2 equipped with ApoTome.2.

Table 4: List of antibodies used for immunohistochemistry.

Antibodies	Species	Dilution	Manufacturer, Cat. No.
anti-Iba1	Rabbit, polyclonal	1:500	Wako, 019-19741
anti-C5b-9	Rabbit, polyclonal	1:500	Abcam, ab55811
Alexa Fluor® 488	Donkey anti-rabbit IgG	1:1000/1:1500	Invitrogen, A21206
Alexa Fluor® 647	Donkey anti-rabbit IgG	1:1000	Invitrogen, A-31573

2.4.2 TUNEL assay

Retinal cell death was detected and quantified on cryosections via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the *in situ* cell death detection kit RED (Roche) following the manufacturer's instructions.

2.5 Computational Analysis

2.5.1 Image Analysis

For one retinal flat mount four pictures of the SR and two of the OPL, for cryosections 5-10 pictures of one section were analyzed and averaged for one retinal n. The total number of $Iba1^+$ or $TUNEL^+$ cells were counted in all images of each retinal flat mount or in the ONL of

cryosections. Counting was performed with the particle analyzer plugin or the multi-point tool of ImageJ in flat mounts and cryosections, respectively. Percentage of autofluorescent or C5b-9^{+} area was measured within the region of interest (ROI) using ImageJ.

2.5.2 Statistical analysis

Data were obtained from 8-10 individual experiments for each mouse line, except for supplementary material where it is stated in the figure legend. All data were plotted and analyzed with GraphPad Prism. After normality tests, *in vitro* data were analyzed using Mann-Whitney U test. For *in vivo* data Mann-Whitney U test, Kruskal-Wallis test with Dunn´s multiple comparison, one-way or two-way ANOVA followed by Tukey´s or Sidak´s multiple comparison post-tests was used as indicated (*$p < 0.05$, **$p < 0.01$, ***$p \leq 0.001$, and ****$p < 0.0001$). Error bars show mean ± SEM (standard error of the mean).

2.6 Buffers, chemicals, and kits

All chemicals used in this study are listed in Table 5, buffers/solutions and kits used in this study are listed in Table 6 and Table 7, respectively.

Table 5: List of chemicals and reagents.

Name	Manufacturer, Cat. No.
Agarose	Biozym, 84004
Artelac® Splash EDO®	Bausch + Lomb, PZN 07706996
Boric acid	Sigma-Aldrich, B6768
Bovine serum albumin (BSA)	Sigma-Aldrich, A9418
Bromophenol blue	Sigma-Aldrich, B-6131
DNase I recombinant	Roche, 04536282001
Ethanol, absolute	AppliChem, A3678
Ethidium bromide	Sigma-Aldrich, 46067
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, E9884
Fetal calf serum (FCS)	Gibco, 10270
Fluoromount-G with DAPI	Invitrogen, 00-4959-52
GeneRuler 100 bp plus	Thermo Scientific, SM0332
Glycerol	Sigma-Aldrich, 49781
HpyF10VI (Mvol)	Thermo Scientific, ER1731
HpyF3I (Ddel)	Thermo Scientific, ER1881
Hydrochloric acid (HCl), 37 %	Roth, X942.1
IFN-β-1a	PeproTech GmbH, 300-02BC
Ketaset 100 mg/ml	Zoetis, PZN 12467832
L-Glutamine, 200mM	Gibco, 25030

Name	Manufacturer, Cat. No.
Lipopolysaccharide (LPS), ultrapure from *E. coli* O111:B4	InvivoGen, tlrl-eblps
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, 68475
Normal donkey serum (NDS)	Linaris, ADI-NDKS-10
Penicillin/Streptomycin	Gibco, 15140
Phenylephrine 2.5 % / Tropicamide 0.5 %	University Hospital Cologne, Pharmacy
Powdered milk	Roth, T145.3
Rompun 2 % (Xylazine)	Bayer, PZN 1320422
Roti Histofix 4 %	Roth, P087.4
Sodium Chloride (NaCl) 0.9 %, injection	Fresenius Kabi, PZN 06605514
Sodium hydroxide (NaOH)	Merck, 1.06462
Sucrose	Roth, 4621.1
Tissue-Tek® optimal cutting temperature (O.C.T.™) compound	Sakura Finetek, 4583
TRIS	Roth, 4855.3
Tri-Sodium citrate dihydrate	Roth, 3580
Triton X-100	Sigma-Aldrich, X100
Trypan Blue 0.4 %	Thermo Scientific, 15250061
Vectashield® HardSet™ Mounting Medium	Vectashield®, H1400
β-Mercaptoethanol	Sigma-Aldrich, M-7154

Table 6: List of buffers and solutions.

Buffer / Solution	Chemical composition / Manufacturer, Cat. No.
10x Buffer Tango	Thermo Scientific, BY5
Alkaline lysis buffer	25 mM NaOH
	200 mM EDTA pH 12 in ddH_2O
Antibody solution	2 % BSA
	0.02 % NaN_3
	0.3 % Triton X-100 in 1x PBS
Blotto	1 % Milk powder
	0.3 % Triton X-100 in 1x PBS
DNA loading dye (6x)	30 % w/v Glycerol
	0.25 % w/v bromophenol blue in ddH_2O
DNase I dilution buffer	50 mM TRIS-HCl
	10 nM $MgCl_2$
	1 mg/ml BSA
DPBS (1x)	Gibco, 14190
Neutralization buffer	40 mM TRIS-HCl pH 5 in ddH_2O
PBST-X	0.3 % Triton X-100 in PBS

Buffer / Solution	Chemical composition / Manufacturer, Cat. No.
Perm/Block buffer	5 % NDS 0.2 % BSA 0.3 % Triton X-100 in 1x PBS
Permeabilization Solution	0.1 % Sodium Citrate 0.1 % Triton X-100
RPMI medium 1640	Gibco, 31870
TBE buffer (10x)	1 M Boric acid 1 M Tris pH 7.5 20 mM EDTA pH 8.0 in ddH_2O

Table 7: List of all kits used in this study.

Kit	Manufacturer, Cat. No.
In Situ Cell Death Detection Kit	Roche, 12156792910
NucleoSpin RNA Kit	Machery & Nagel, 740955.250
RevertAid RT Kit	Thermo Scientific, K1691
RNeasy® Micro Kit	Qiagen, 74004
Takyon™ No ROX Probe MasterMix blue dTTP	Eurogentec, UF-NPMT-B0701
Taq-S PCR Kit	Genaxxon bioscience, M3313

2.7 Devices and Software

All devices used in this study are listed in Table 8 and software are listed in Table 9.

Table 8: List of all devices used in this study.

Device	Manufacturer
Adventurer Pro balance	Ohaus®
Centrifuge 5415 R	Eppendorf
Centrifuge Mini Star	VWR International
Cryostat CM3050 S	Leica Biosystems
Explorer R Ex 124 balance	Ohaus®
Galaxy 170S CO_2 incubator	Eppendorf
Heraeus Megafuge 40R Centrifuge	Thermo Scientific
Intas Gel iX20 Imager	Intas
LightCycler® 480 Instrument II	Roche Applied Science
Matrix™ Multichannel Pipette	Thermo Scientific
MSC-Advantage hood	Thermo Scientific
NanoDrop 2000 Spectrophotometer	Thermo Scientific

Device	Manufacturer
Neubauer counting chamber	OptikLabor
PeqSTAR 2x cycler	Peqlab
See-saw rocker SSL4	Stuart®
Spectralis™ HRA+OCT	Heidelberg Engineering
Thermomixer compact	Eppendorf
Vibracell 75115 Sonicator	Fisher Bioblock Scientific
Vortex-Genie™	Scientific Industries
Zeiss Imager M.2 with ApoTome.2	Zeiss
Zeiss Stemi 508 Stereo microscope	Zeiss

Table 9: List of software used in this study.

Software	Manufacturer
Adobe creative suite	Adobe Systems
GraphPad Prism 7 (v7.05)	GraphPad Software, Inc.
Heidelberg Eye Explorer (HEYEX)	Heidelberg Engineering
Image J (v1.53e)	Wayne Rasband, NIH
Intas Gel Documentation Software (v3.39)	Intas Science Imaging
LightCycler® 480 Software (v1.5.1)	Roche Applied Science
Mendeley (v1.19.6)	Elsevier
Microsoft Office 365 pro plus	Microsoft Corporation
Nanodrop 2000/2000c Software	Thermo Scientific
ZEN blue edition (v3.1)	Zeiss

3 Results

3.1 Effect of IFN-β application on microglia activity and retinal degeneration

Previous *in vivo* studies in mouse models of EAE or laser-induced CNV showed exacerbated disease symptoms including extensive microglia activation and inflammatory response through *Ifn-β* or *Ifnar1* gene knockout (Lückoff et al., 2016; Prinz et al., 2008; Teige et al., 2003). Similar findings were observed in the laser-CNV mouse model for wet AMD using microglia specific *Ifnar1* knockout mice, while IFN-β therapy clearly attenuated microgliosis and reduced CNV size (Lückoff et al., 2016). However, little is known about the exact mechanism of IFN-β signaling on microglia activity in dry AMD. We hypothesized, that modulation of microglia via IFN-β might also dampen mononuclear phagocyte reactivity by altering the production of complement components. The current study sought to fill this gap using a light-induced retinal degeneration mouse model mimicking some features of dry AMD. Analysis at day four after light exposure allows to investigate microglia activity and concurrently retinal degeneration.

3.1.1 Biological activity of human IFN-β in murine BV-2 cells

It was previously shown, that human recombinant IFN-β is active in the murine BV-2 microglia cell line (Lückoff, 2016). First, the biological activity of IFN-β in BV-2 cells that were previously untreated or simultaneously activated with Lipopolysaccharide (LPS) was evaluated. The BV-2 cells were treated for 6, 24, and 72 hours, respectively, to analyze time-dependent mRNA expression levels for the candidate genes (Figure 8).

The mRNA expression of *inducible nitric oxide synthase (iNos)* severed as a pro-inflammatory control and two well-known IFN-stimulated transcripts, *Myxovirus influenza resistance 1* (*Mx1*) and *Mx2* (Lückoff et al., 2016; Verhelst et al., 2013) were analyzed using qRT-PCR. In the six hour short term stimulation experiments, all three analyzed transcripts were significantly increased by stimulation with either LPS or IFN-β or both together (Figure 8A-C). This indicates that the BV-2 cells were sensitive to LPS and IFN-β in this early phase and that both signaling pathways were active. At 24 hours after stimulation, only LPS was able to trigger expression of all three genes (Figure 8H-J), whereas the effect of IFN-β was only seen for the two interferon-responsive genes (Figure 8I, J). In late phase experiments, LPS could only stimulate *iNos*, whereas *Mx1* and *Mx2* were exclusively responsive to IFN-β single or co-treatment (Figure 8O-Q). These data indicate that human IFN-β is biologically active in murine microglia cells and that interferon responsive genes *Mx1* and *Mx2* are activated over a longer time span.

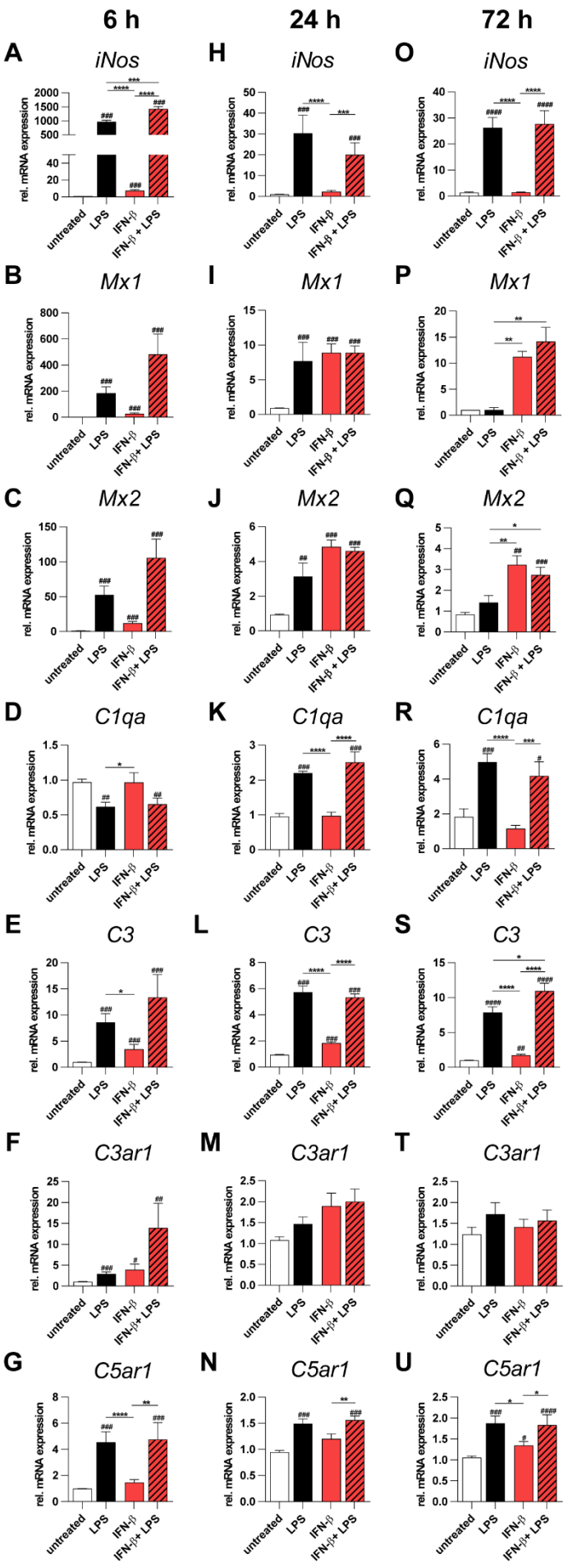

Figure 8: Biological activity of human IFN-β in murine microglia cells. BV-2 cells were treated with LPS (50 ng/ml) and IFN-β (1000 U/ml) for 6 h **(A-G)**, 24 h (**H-N)**, and 72 h **(O-U)**. Gene expression analysis using probe-based qRT-PCR was performed and $^{\Delta\Delta}$Ct analysis was used for quantification. For this, technical duplicates of one retina were averaged for one n. *Atp5b* was used as reference gene. Bars represent mean ± SEM (*p < 0.05, **p < 0.01, ***p ≤ 0.001, and ****p < 0.0001; # depicts significance versus control). Data were analyzed using Mann-Whitney U test (n = 6-12).

Furthermore, four complement genes were chosen to determine expression changes by IFN-β treatment; *complement C1q A chain (C1qa)* as engaging protein of the classical pathway, central component *C3* and chemotactic anaphylatoxin receptors *C3ar1* and *C5ar1*. In case of the latter ones, anaphylatoxins C3a and C5a drive inflammation by engaging their corresponding receptors (Engelke et al., 2014; M. Yu et al., 2012; L. Zhang et al., 2016) and are chemoattractants for mononuclear phagocytes (Brandstetter et al., 2015; Nozaki et al., 2006). In single or co-treatment, LPS significantly decreases *C1qa* expression after six hours (Figure 8D), while it significantly enhanced it at 24 and 72 hour time points (Figure 8K, R). IFN-β was not able to induce expression at any time point (Figure 8D, K, R), suggesting no engaging of the classical pathway by IFN-β. *C3* transcripts were significantly enriched at all time points and all conditions compared to the untreated control (Figure 8E, L, S), showing the only significant difference between LPS only and co-stimulation with IFN-β treatment at 72 hours (Figure 8S). Anaphylatoxin receptor *C3ar1* was only increased at the early six hour time point with either LPS or IFN-β or their combination without significant differences amongst the groups (Figure 8F, M, T), while LPS or co-treatment triggered *C5ar1* expression at all time points (Figure 8G, N, U). IFN-β single treatment enhanced *C5ar1* transcription solely at the late time point of 72 hours (Figure 8U). The results indicate an enrichment of complement signaling in microglia through IFN-β, which is further enhanced in LPS-activated microglia.

3.1.2 IFN-β administration in a murine light damage paradigm

We were next interested to study the biological effects of human IFN-β in a murine model of light-induced retinal degeneration. The mouse model of light-induced retinal degeneration mimics some features of dry AMD. For this, 8-10-week-old BALB/cJ mice were dark adapted for 16 hours and treated with 10,000 U human IFN-β every other day starting 1 hour prior to light exposure with 15,000 lux for 1 hour (Figure 10A).

Transcriptome analysis via qRT-PCR of retinal tissue was performed four days post light exposure to examine changes in expression of IFN-stimulated genes, complement components and inflammation modulators (Figure 9). For this, technical duplicates of one retina were averaged for one n. In accordance with the cell culture experiments, *Mx1* and *Mx2* expression was significantly upregulated by light damage in both vehicle-treated and IFN-β-treated conditions (Figure 9A, B). Additionally, *iNos* transcripts and the early microglia activation marker *Tspo* were unchanged four days after light exposure (Figure 9C, D). As was seen for *Mx1* and *Mx2* genes, *C3* and *C1qa* transcripts were significantly upregulated by light damage in both vehicle-treated and IFN-β-treated conditions (Figure 9E, F). These findings implicate that light damage alone already induced IFNAR signaling in the retina and that

systemic application of human IFN-β showed only a trend towards higher expression levels of IFNAR responsive and complement genes.

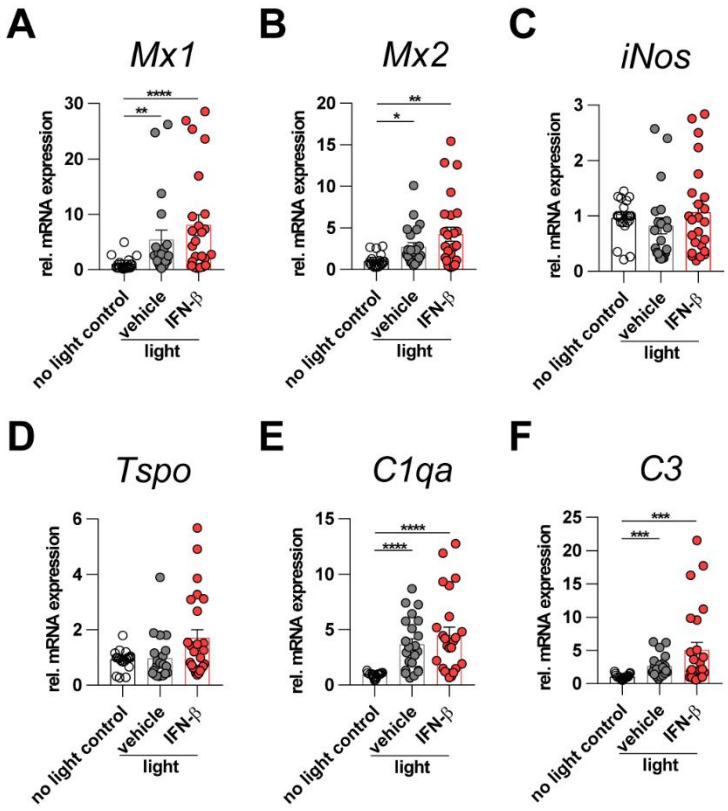

Figure 9: Expression analysis of retinal tissue. A-F BALB/cJ mice were treated with IFN-β (10,000 U) or PBS as vehicle every other day starting 1 h prior to light damage with 15,000 lux for 1 h until 4 d afterwards. Gene expression analysis using probe-based qRT-PCR was performed and $^{\Delta\Delta}$Ct analysis was used for quantification. For this, technical duplicates of one retina were averaged for one n. *Atp5b* was used as reference gene. Bars represent Mean ± SEM (*p < 0.05, **p < 0.01, ***p ≤ 0.001, and ****p < 0.0001). Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparison (n = 20-25).

For a systematic morphometric analysis of retinal microglia behavior, microglia numbers, location, and morphology were determined using Iba1-stained retinal flat mounts (Figure 10) as well as cryosections (Figure 11). Only few cells were present in the SR (Figure 10C) and mainly ramified microglia were detected in the OPL of control animals (Figure 10D). After light exposure, microglia were activated and recruited to the SR (Figure 10C). Four images of the central retina were taken around the optic nerve of each eye and averaged for one n (Figure 10B). Interestingly, IFN-β treatment resulted in significantly less amoeboid microglia numbers in the SR (Figure 10E) and in the OPL compared to vehicle-treated animals (Figure 10F). Engulfed cell debris is visible as autofluorescent material in microglia cells in the SR (Figure 10C), which is significantly elevated after light exposure with only a tendency of reduction by IFN-β compared to vehicle treatment (Figure 10E).

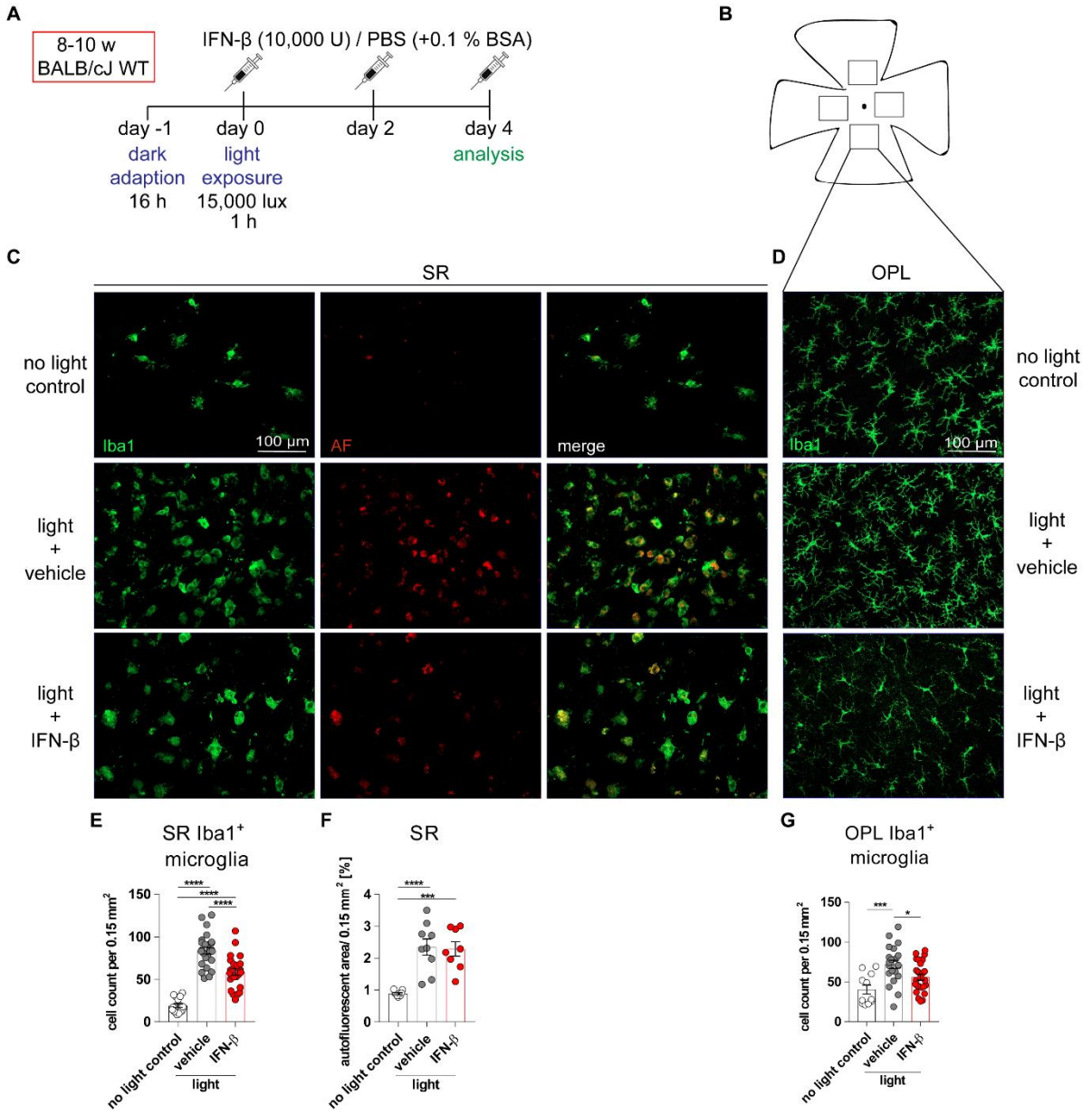

Figure 10: Iba1 staining of microglia in retinal flat mounts. A 8-10-week-old BALB/cJ mice were dark adapted for 16 h and treated with IFN-β (10,000 U) or PBS as vehicle every other day starting 1 h prior to light damage with 15,000 lux for 1 h until 4 d afterwards. **B** Four images of the central retina were taken around the optic nerve of each eye and results averaged for one n. **C, D** Microglia were stained in retinal flat mounts using ionized calcium-binding adapter molecule 1 (Iba1). **E, G** Cell numbers were counted using the particle analyzer plugin of ImageJ (n = 17-25). **F** Percentage of autofluorescent area was measured with ImageJ (n = 8-9). **E-G** Data were analyzed using one-way ANOVA and Tukey's multiple comparison. Bars represent mean ± SEM (*p < 0.05, ***p ≤ 0.001, and ****p < 0.0001). SR: subretinal space; OPL: outer plexiform layer; AF: autofluorescence.

These findings were also confirmed by Iba1-stained cryosections, where less amoeboid microglia were present in the OPL and in the SR after IFN-β therapy compared to the vehicle group (Figure 11A). Results out of 5-10 images of the central retina of each eye were averaged for one n (Figure 11B, black-rimmed area). However, a significant reduction of infiltrating microglia numbers in the ONL could not be observed (Figure 11C). Photoreceptor outer

segments and by-products of the visual cycle are recycled in the RPE, which was visible as autofluorescent material (Figure 11A).

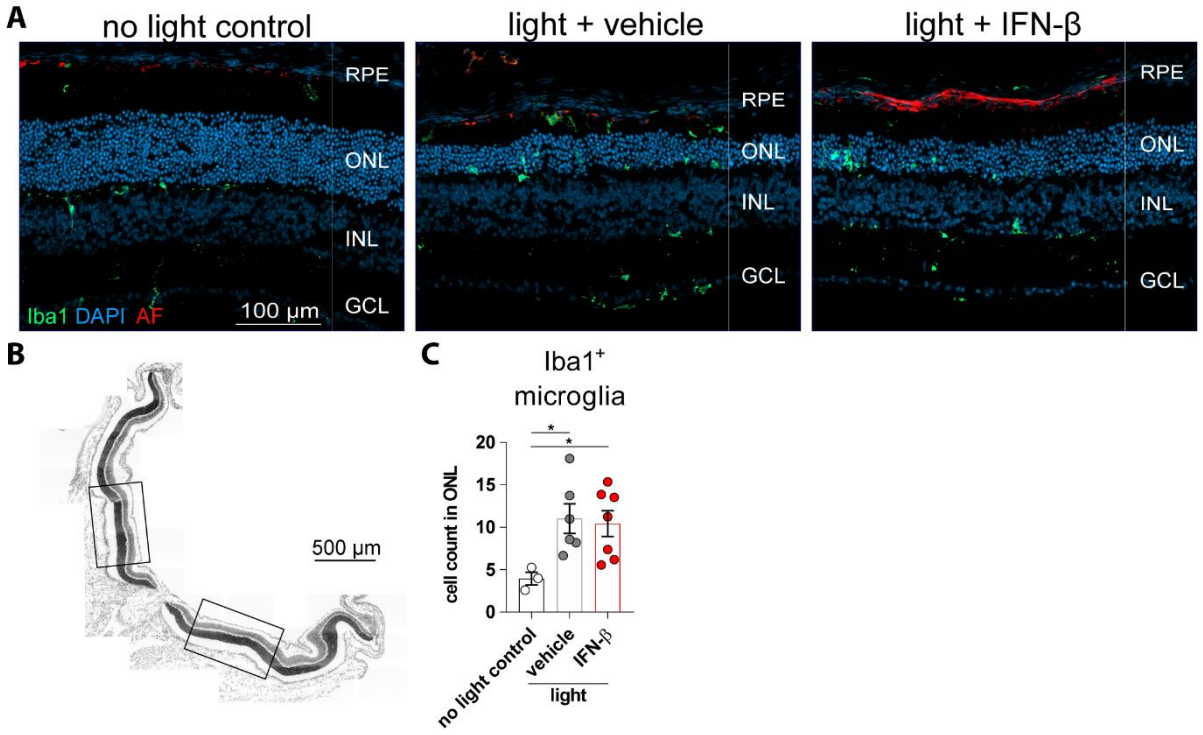

Figure 11: Iba1 staining of microglia in cryosections. BALB/cJ mice were treated with IFN-β (10,000 U) or PBS as vehicle every other day starting 1 h prior to light damage with 15,000 lux for 1 h until 4 d afterwards. **A** Cryosections were stained with DAPI and ionized calcium-binding adapter molecule 1 (Iba1). **B** Results out of 5-10 images of the central retina of each eye were averaged for one n. **C** Cell numbers were counted using the multi-point tool of ImageJ. Data were analyzed using Mann-Whitney U test (n = 3-7). Bars represent mean ± SEM (*p < 0.05). AF: autofluorescence; RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

The DAPI-staining in Figure 11A already indicated that the light damage induced thinning of the ONL was not affected by IFN-β treatment. We therefore quantified the overall retinal thickness in these experimental groups using SD-OCT (Figure 12). These OCT-scans indicated that light exposure changed the overall reflectance pattern of the retina (Figure 12A). As clearly seen from the retinal heatmaps of individual animals (Figure 12B) and combined analyses of larger animal numbers (Figure 12D) a significant reduction of retinal thickness was observed after light damage, which was not influenced by IFN-β therapy. Analysis was carried out using 3 mm and 6 mm diameter values; the average of four sections around the optic nerve circle of 1 mm was accounted for one n (Figure 12C).

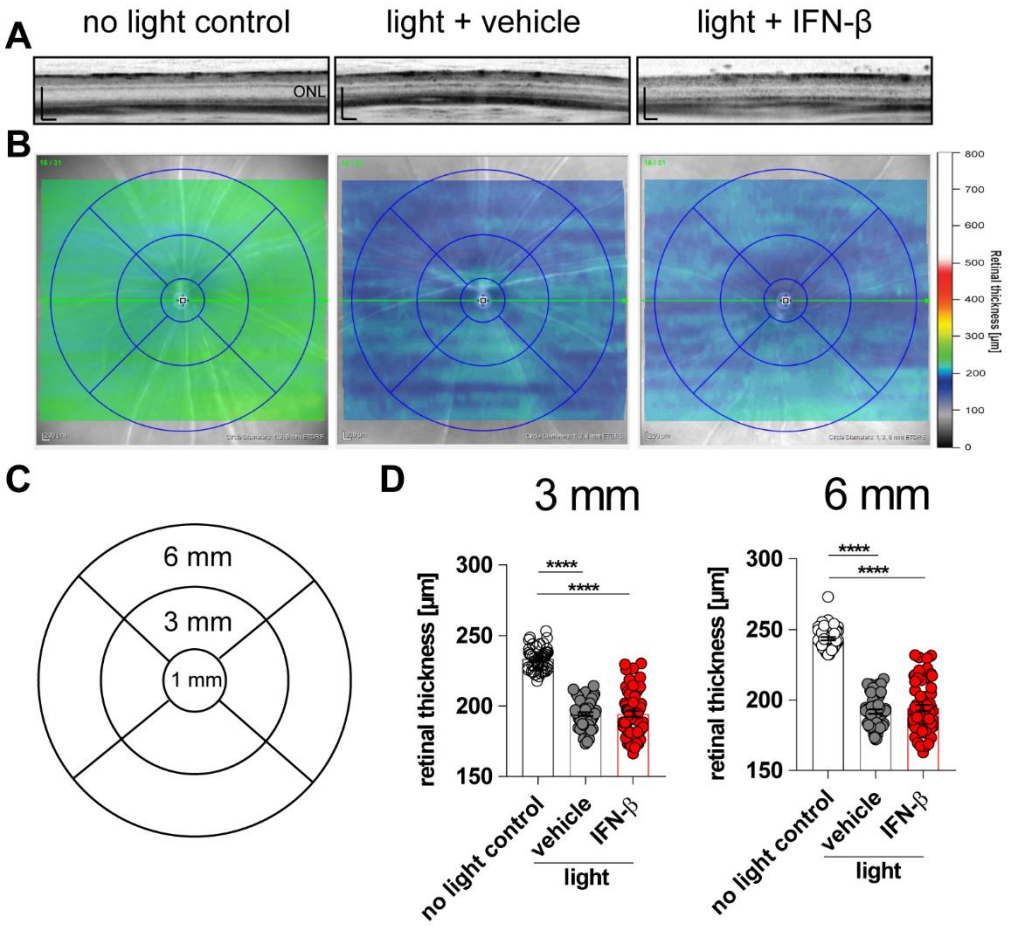

Figure 12: Retinal thickness analysis using SD-OCT. BALB/cJ mice were treated with IFN-β (10,000 U) or PBS as vehicle every other day starting 1 h prior to light damage with 15,000 lux for 1 h until 4 d afterwards. **A** Representative scans and **B** corresponding heatmaps compiled by spectral-domain optical coherence tomography (SD-OCT) displaying a retinal overview. **C** Four 3 mm and 6 mm section values of each eye were averaged and accounted for one n, respectively. **D** Retinal thickness was plotted, bars represent Mean ± SEM (****p < 0.0001). Data were analyzed using one-way ANOVA and Tukey's multiple comparison (n = 55-63). Scale bar: 200 µm.

3.2 Anaphylatoxin receptor knockout in a murine light damage paradigm

As demonstrated, IFN-β-treated animals showed reduced microglia numbers in the subretinal space and outer plexiform layer after light exposure compared to controls and complement signaling was upregulated by light exposure. Anaphylatoxins C3a and C5a function as chemoattractants for mononuclear phagocytes (Brandstetter et al., 2015; Nozaki et al., 2006) and drive inflammation by engaging their corresponding chemotactic receptors C3aR1 and C5aR1 (Engelke et al., 2014; M. Yu et al., 2012; L. Zhang et al., 2016). Therefore, we used anaphylatoxin receptor knockout mice to elucidate whether modulation of mononuclear phagocyte reactivity may be due to altered production of complement components in the retina and further investigate the involvement of anaphylatoxin signaling during retinal degeneration.

Genotypes of genetically modified mice were determined using PCR (Figure 13A, B). Initial experiments with the beforehand introduced light exposure settings (Supplementary Figure 1) revealed a tendency of low-grade inflammation in knockout (KO) animals (Supplementary Figure 2) and no differences of microglia recruitment to the outer nuclear layer upon light exposure (Supplementary Figure 3A-D & Supplementary Figure 4A-D). For C5aR1 KO animals even a significant thinning of the retina compared to WT light-challenged animals was shown (Supplementary Figure 4E, F), indicating a higher light susceptibility for genetically modified animals, as previously reported for C3-deficient and *Cln3*$^{\Delta ex7/8}$ mice (Dannhausen et al., 2018; Rogińska et al., 2017). Therefore, the light exposure settings were changed to low light conditions, previously used by different light-induced retinal degeneration studies (Dannhausen et al., 2018; Song et al., 2017). For this, 8-10-week-old wildtype (WT) or KO animals of mouse lines *C3ar1* and *C5ar1* with BALB/cJ background were dark adapted for 16 hours before light exposure with 10,000 lux for 30 minutes (Figure 13C). Analysis assessed below were performed four days after light exposure.

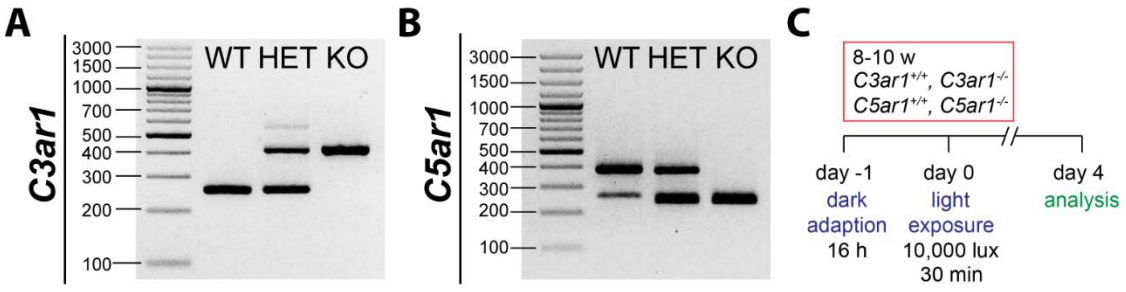

Figure 13: Genotyping and light damage paradigm. A PCR samples were separated on a 2 % agarose gel. A 250 bp amplicon was considered for WT and 400 bp for KO. **B** PCR samples were separated on a 1 % agarose gel. A 386 bp amplicon was considered for WT and 244 bp for KO. **C** 8-10-week-old *C3ar1* or *C5ar1* WT and KO were dark adapted for 16 h before light exposure with 10,000 lux for 30 min until analysis 4 d afterwards.

Gene expression analysis via qRT-PCR of retinal tissue was performed four days post light exposure to examine changes in expression of complement components and inflammation modulators (Figure 14 & Figure 15). Transcripts for *iNos* and *Tspo* remained unchanged four days after light exposure (Figure 14B & Figure 15A, B), except for a significant induction of *iNos* after light damage in *C3ar1* WT mice (Figure 14A). *C3* and *C1qa* transcripts were significantly upregulated by light damage in both WT and KO animals (Figure 14C, D & Figure 15C, D). Additionally, CFB, which is cleaved by CFD to build the alternative C3 convertases, and regulatory protein CFH, which dissociates C3 convertases and acts as a cofactor enabling CFI to cleave opsonins into their inactive forms, were monitored. *Cfb* transcripts could not be detected in untreated samples; thus data were normalized to WT light exposed samples. However, *Cfb* and *Cfh* transcripts were upregulated in light-challenged mice, but unaltered between WT and KO (Figure 14E & Figure 15E). To mention, light-exposed *C5ar1* WT mice displayed a high, but not significant tendency of elevated *Cfh* transcripts compared to untreated controls, with p-values of 0.0539 and 0.0725 to WT and KO, respectively (Figure 15F). These data indicate that light damage induces complement signaling in the retina, with a tendency of lower induction of central factor *C3* and higher levels of regulatory *Cfh* in C3aR1 KO mice compared to WT (Figure 14).

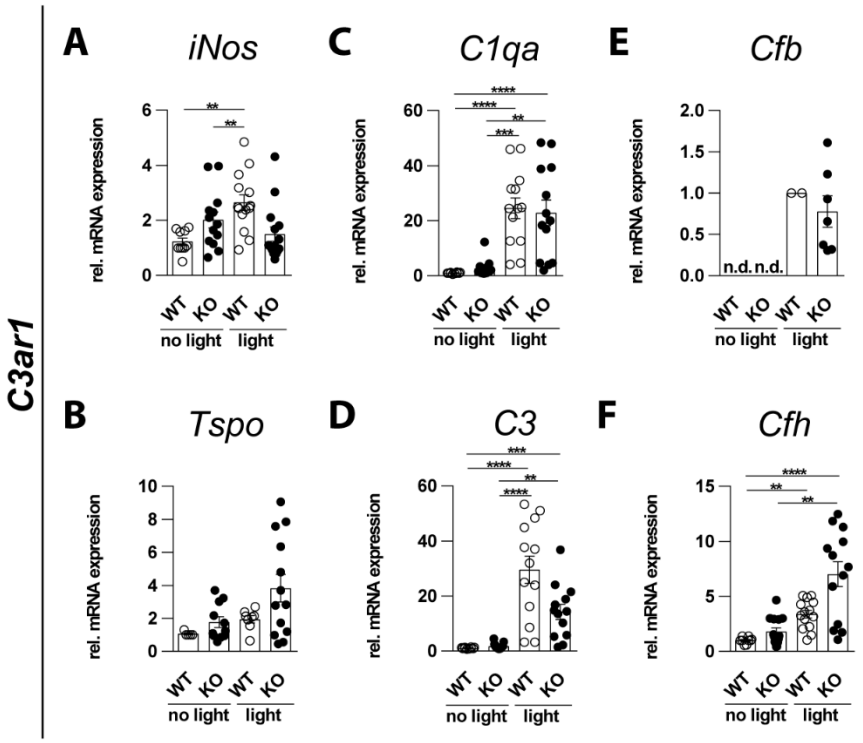

Figure 14: Expression analysis of *C3ar1* WT and KO retinal tissue. A-F Gene expression analysis using probe-based qRT-PCR was performed 4 d after light exposure with 10,000 lux for 30 min. *Atp5b* was used as reference gene and $^{\Delta\Delta}$Ct analysis was used for quantification. For this, technical duplicates of one retina were averaged for one n. Bars represent Mean ± SEM (**p < 0.01, ***p ≤ 0.001, and ****p < 0.0001). Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparison (n = 10-15); and for *Cfb* using Mann-Whitney U test (n = 2-7), n.d. = not detected.

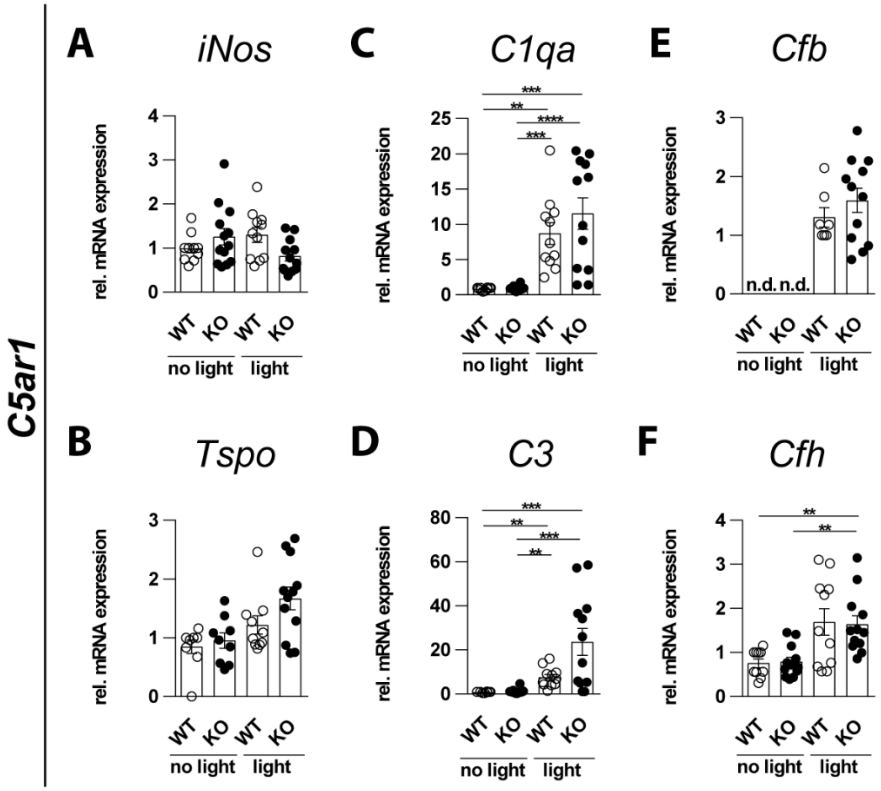

Figure 15: Expression analysis of *C5ar1* WT and KO retinal tissue. A-F Gene expression analysis using probe-based qRT-PCR was performed 4 d after light exposure with 10,000 lux for 30 min. *Atp5b* was used as reference gene and $^{\Delta\Delta}$Ct analysis was used for quantification. For this, technical duplicates of one retina were averaged for one n. Bars represent Mean ± SEM (**p < 0.01, ***p ≤ 0.001, and ****p < 0.0001). Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparison (n = 9-13); and for *Cfb* using Mann-Whitney U test (n = 7-12), n.d. = not detected.

Systematic morphometric analysis of retinal microglia in unchallenged and light-challenged *C3ar1* and *C5ar1* mice were performed using Iba1-stained retinal flat mounts (Figure 16 & Figure 17). The untreated controls had very few activated microglia in the SR, while light damage significantly increased the number of migrated microglia (Figure 16A & Figure 17A). Concurrent with activation and migration to the SR microglia became amoeboid and enhanced their phagocytic reactivity, shown by increased autofluorescence co-localized with subretinal microglia (Figure 16A & Figure 17A). Nevertheless, there were no differences in microglia numbers and autofluorescent area found in the SR of light-challenged retinas comparing KO to WT (Figure 16B, C & Figure 17B, C). An evenly spaced network of few microglia was evident in the OPL of both, WT and KO mice (Figure 16D & Figure 17D). After light damage the number of microglia significantly increased and cells showed a more amoeboid shape with some rudiment protrusions, which remained unchanged among genotypes (Figure 16E & Figure 17E).

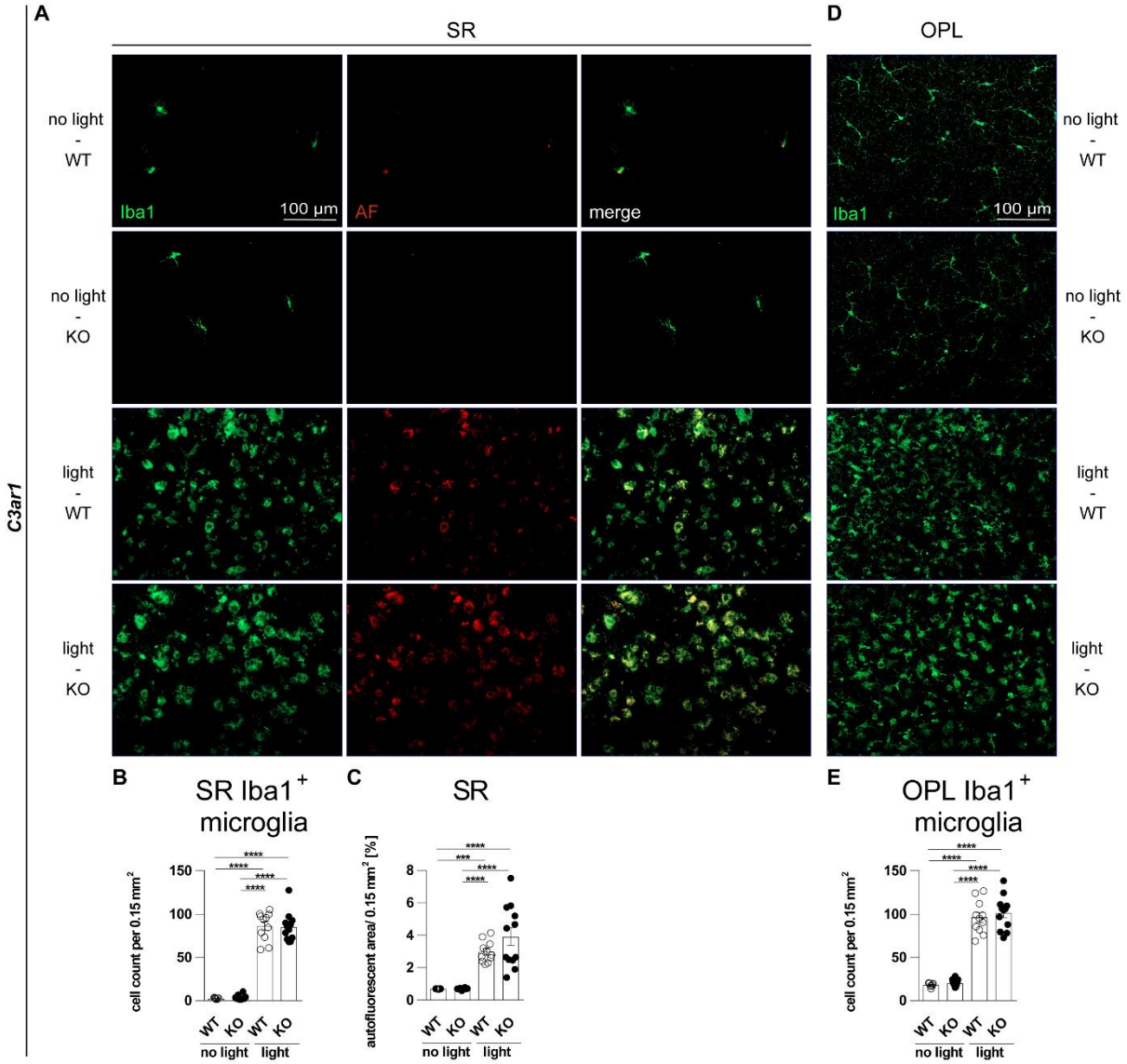

Figure 16: Iba1 staining of microglia in retinal flat mounts of *C3ar1* WT and KO mice. A, D Microglia were stained in retinal flat mounts 4 d after light exposure with 10,000 lux for 30 min using ionized calcium-binding adapter molecule 1 (Iba1). **B, C, E** Four 4 images of the central retina were taken around the optic nerve of each eye and results averaged for one n. Cell numbers were counted using the particle analyzer plugin of ImageJ. Percentage of autofluorescent area was measured with ImageJ. Data were analyzed using one-way ANOVA and Tukey's multiple comparison (n = 9-12). Bars represent mean ± SEM (***p ≤ 0.001 and ****p < 0.0001). SR: subretinal space; OPL: outer plexiform layer; AF: autofluorescence.

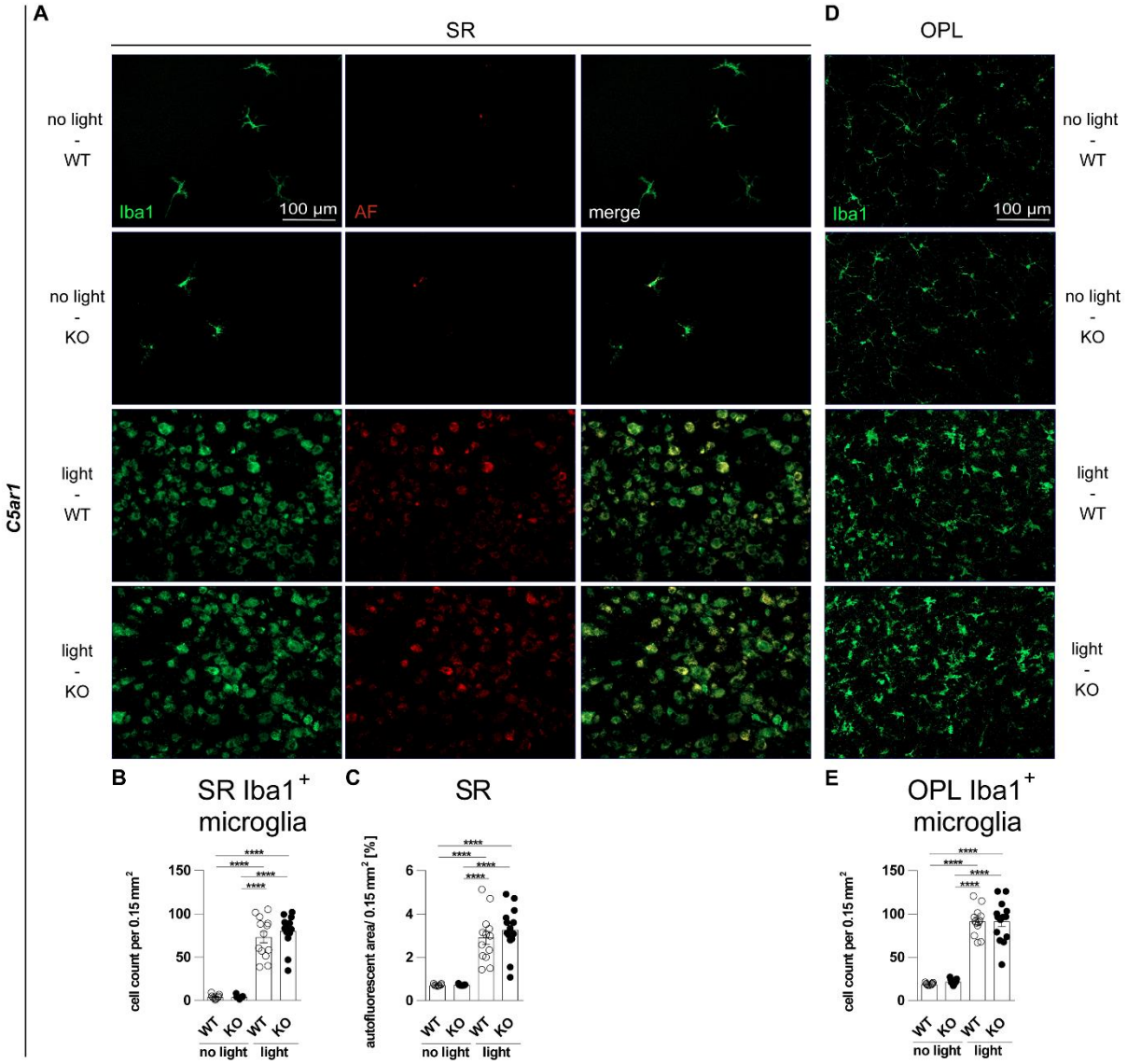

Figure 17: Iba1 staining of microglia in retinal flat mounts of *C5ar1* WT and KO mice. A, D Microglia were stained in retinal flat mounts 4 d after light exposure with 10,000 lux for 30 min using ionized calcium-binding adapter molecule 1 (Iba1). **B, C, E** Four images of the central retina were taken around the optic nerve of each eye and results averaged for one n. Cell numbers were counted using the particle analyzer plugin of ImageJ. Percentage of autofluorescent area was measured with ImageJ. Data were analyzed using one-way ANOVA and Tukey's multiple comparison (n = 13-15). Bars represent mean ± SEM (****p < 0.0001). SR: subretinal space; OPL: outer plexiform layer; AF: autofluorescence.

Cryosections were used to obtain an overview of retinal changes after light exposure (Figure 18 & Figure 19). Iba1-stained cryosections confirmed the results from retinal flat mounts, where amoeboid microglia are more abundant after light exposure in the SR (Figure 18A & Figure 19A). Furthermore, phagocytosed autofluorescent material co-localized with subretinal microglia (white arrows). Photoreceptor outer segments and by-products of the visual cycle are recycled in the RPE, which was also visible as autofluorescent material (Figure 18A & Figure 19A). Counting of microglia in the ONL revealed migration after light damage. However, a significant reduction of infiltrating microglia numbers in the ONL of light challenged KO mice compared to WT could not be observed (Figure 18C & Figure 19C).

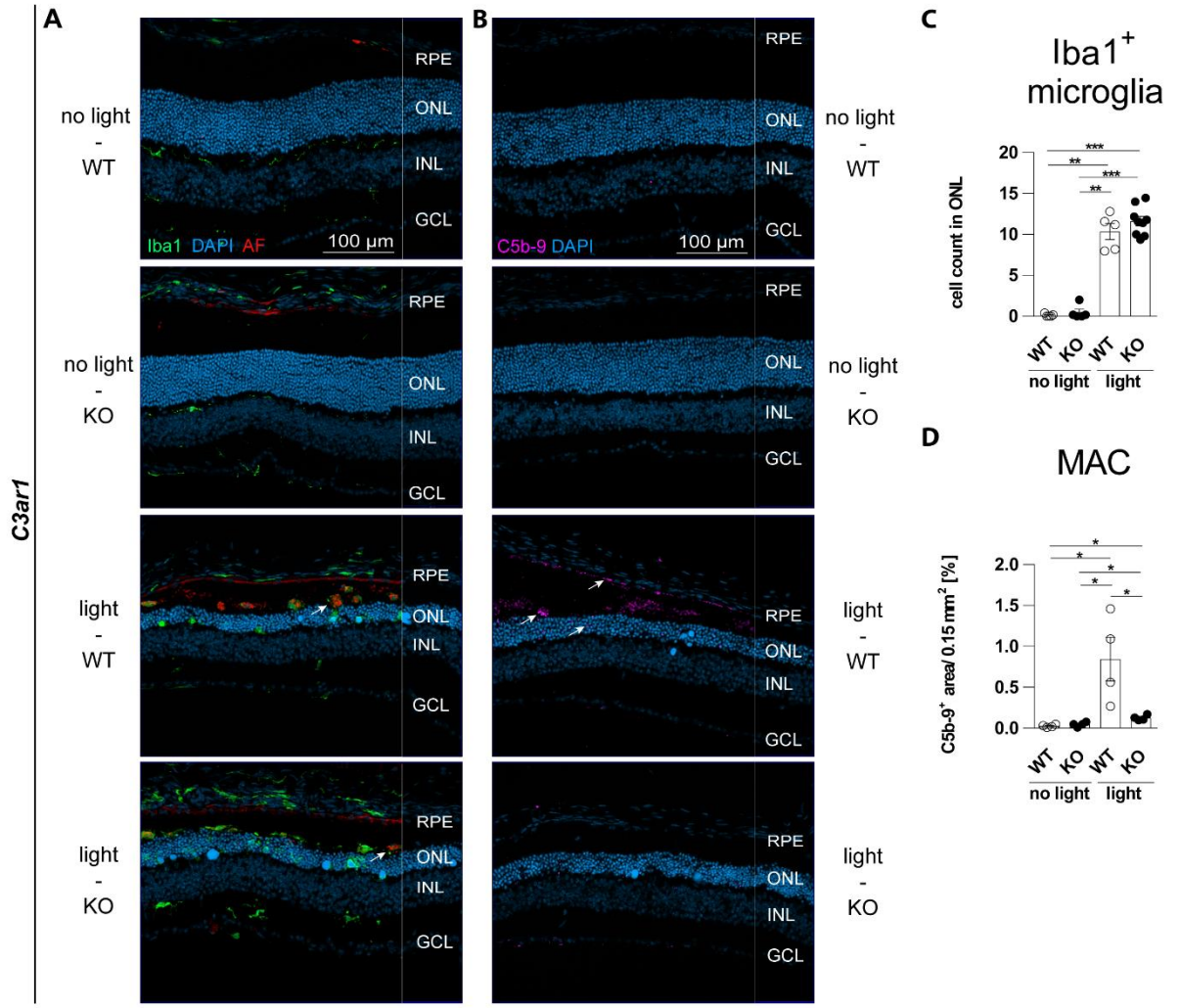

Figure 18: Iba1 and C5b-9 staining in cryosections of *C3ar1* WT and KO mice. Cryosections were stained 4 d after light exposure with 10,000 lux for 30 min with DAPI and either ionized calcium-binding adapter molecule 1 (Iba1) to visualize microglia (**A**) or C5b-9 to visualize the membrane attack complex (MAC, **B**). Results out of 5 images of the central retina of each eye were averaged for one n. **C, D** Cell numbers were counted using the multi-point tool of ImageJ. Percentage of C5b-9$^+$ area was measured with ImageJ. Data were analyzed using Mann-Whitney U test (n = 4-9). Bars represent mean ± SEM (*p < 0.05, **p < 0.01, and ***p ≤ 0.001). AF: autofluorescence; RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

Formation of the membrane attack complex (MAC), which is built up by C5b recruiting C6, C7, C8 and C9, denotes the terminal pathway that leads to pore formation on the cell surface and cell lysis, subsequently. Cryosections stained for C5b-9 revealed increased MAC formation after light damage in the outer retina of *C3ar1* and *C5ar1* mice (Figure 18B, D & Figure 19B, D; white arrows). Interestingly, significantly less MAC was detected in C3aR1 KO mice than WT (Figure 18B, D), indicating a less pronounced activation of complement signaling as proposed from transcript levels (Figure 14).

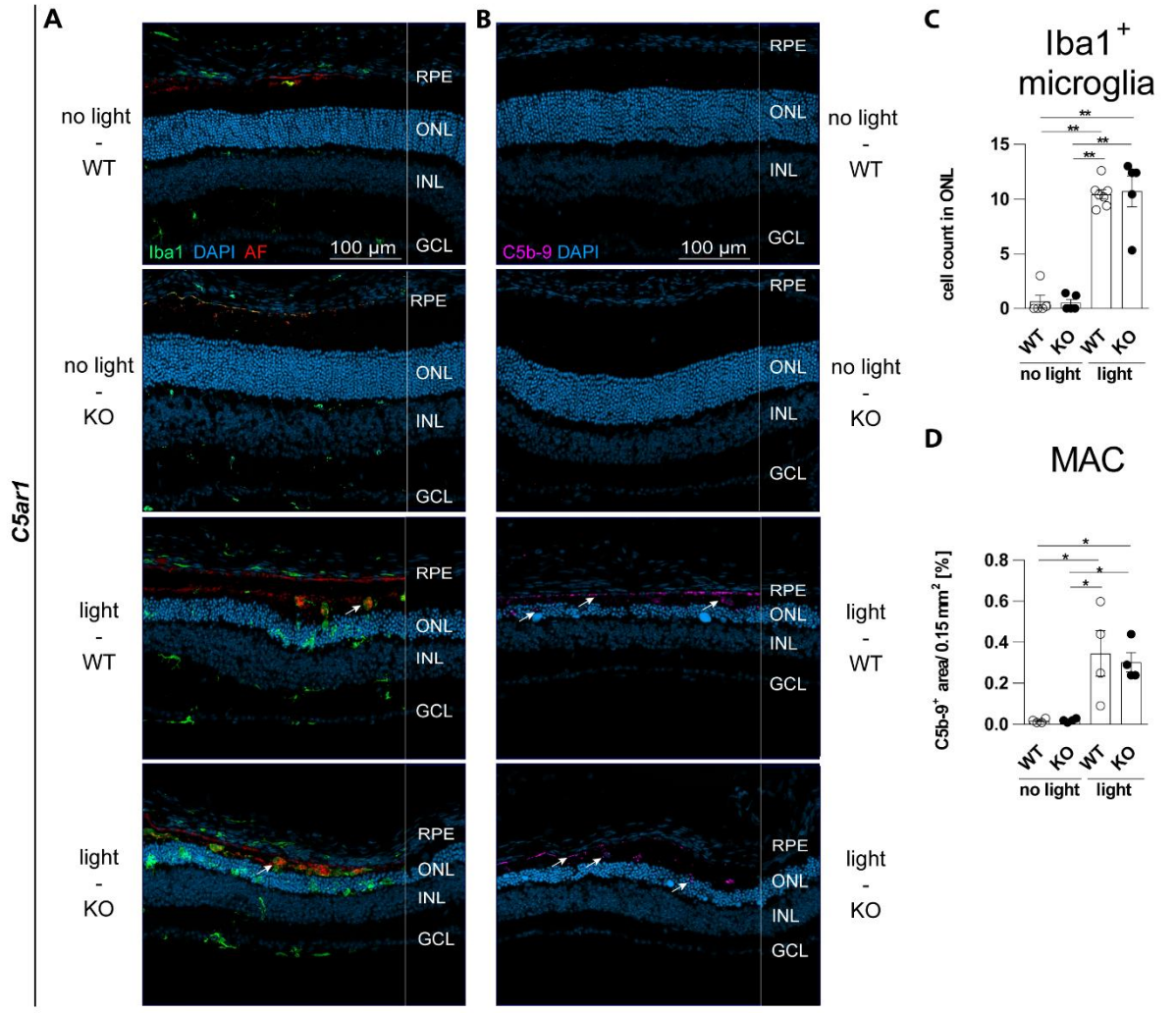

Figure 19: Iba1 and C5b-9 staining in cryosections of *C5ar1* WT and KO mice. Cryosections were stained 4 d after light exposure with 10,000 lux for 30 min with DAPI and either ionized calcium-binding adapter molecule 1 (Iba1) to visualize microglia (**A**) or C5b-9 to visualize the membrane attack complex (MAC, **B**). Results out of 5 images of the central retina of each eye were averaged for one n. **C, D** Cell numbers were counted using the multi-point tool of ImageJ. Percentage of C5b-9$^+$ area was measured with ImageJ. Data were analyzed using Mann-Whitney U test (n = 4-7). Bars represent mean ± SEM (*p < 0.05 and **p < 0.01). AF: autofluorescence; RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

DAPI-staining in cryosections already indicated light damage induced thinning of the outer nuclear layer. We therefore quantified the overall retinal thickness using SD-OCT scans (Figure 20A, B, D & Figure 21A, B, D). Here, OCT-scans confirmed that light damage changed the overall reflectance patterns with thinning of the ONL (Figure 20A & Figure 21A). As clearly indicated by color changes in retinal heatmaps of individual animals (Figure 20B & Figure 21B), combined analysis of larger animal numbers revealed a significant reduction of retinal thickness after light damage (Figure 20D & Figure 21D). However, in the 3 mm diameter the retinal thickness of light damaged C3aR1 KO animals significantly exceeded that of WT mice (Figure 20D).

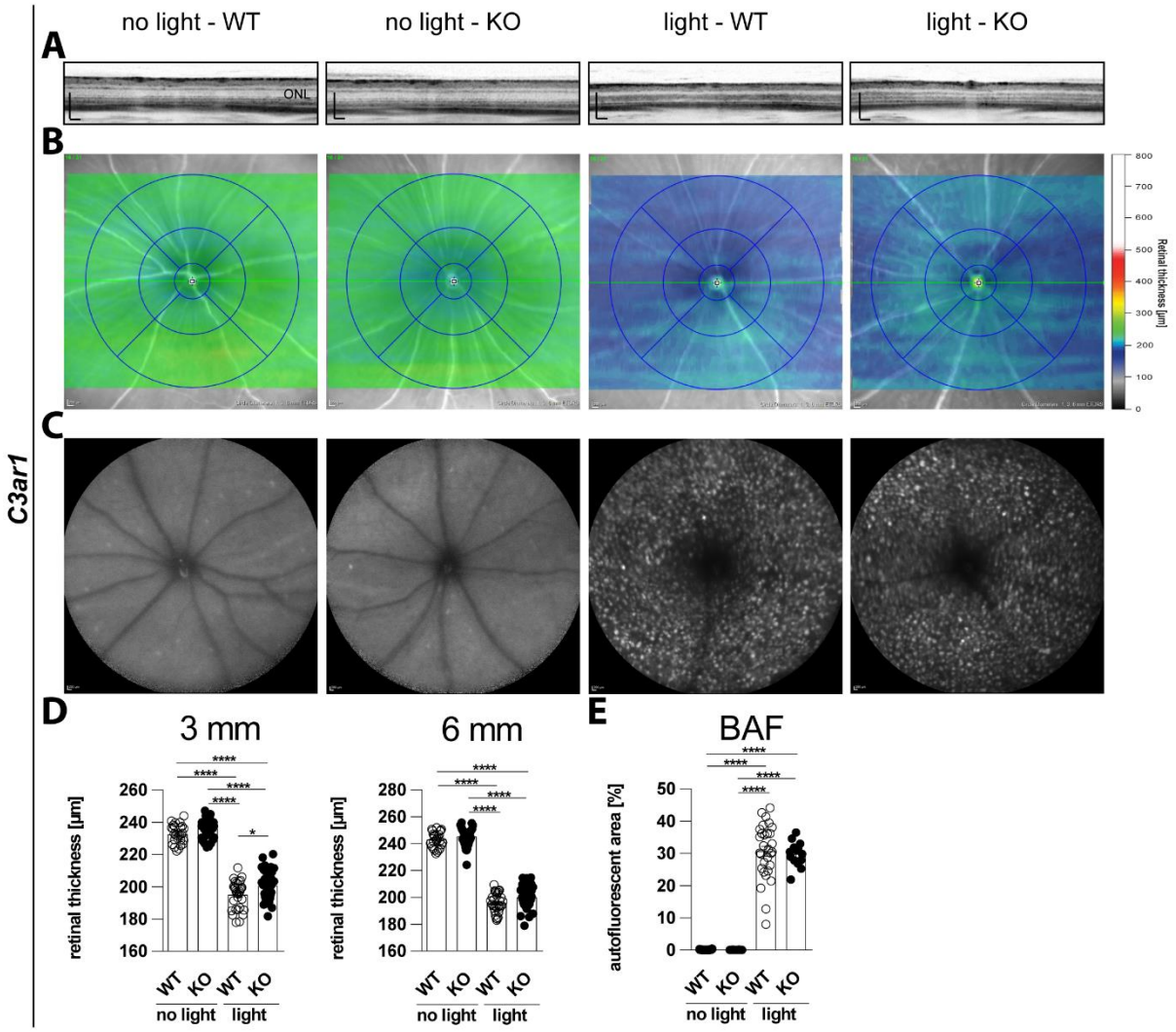

Figure 20: Analysis of retinal thickness and fundus of *C3ar1* WT and KO mice. Analysis was performed 4 d after light exposure with 10,000 lux for 30 min. **A** Representative scans and **B** corresponding heatmaps compiled by spectral-domain optical coherence tomography (SD-OCT) displayed a retinal overview. **C** Fundus images were obtained using BluePeak blue laser autofluorescence (BAF). **D** Retinal thickness was averaged from quartiles in 3 mm and 6 mm diameters (n = 28-36). **E** Percentage of autofluorescent area was measured with ImageJ (n = 12-30). **D, E** Bars represent mean ± SEM (****p < 0.0001). Data were analyzed using one-way ANOVA and Tukey's multiple comparison. Scale bar: 200 µm.

During disease progression, accumulation of autofluorescent material is a hallmark, that appear as hyperreflective foci in retinal fundus images, which are already detectable in intermediate AMD patients. Anaphylatoxins and other complement components are, besides lipids, main components of drusen; therefore, BAF images were analyzed (Figure 20C, E & Figure 21C, E). The autofluorescent area was significantly increased after light damage, which was even more pronounced in C5aR1 KO than WT (Figure 21E).

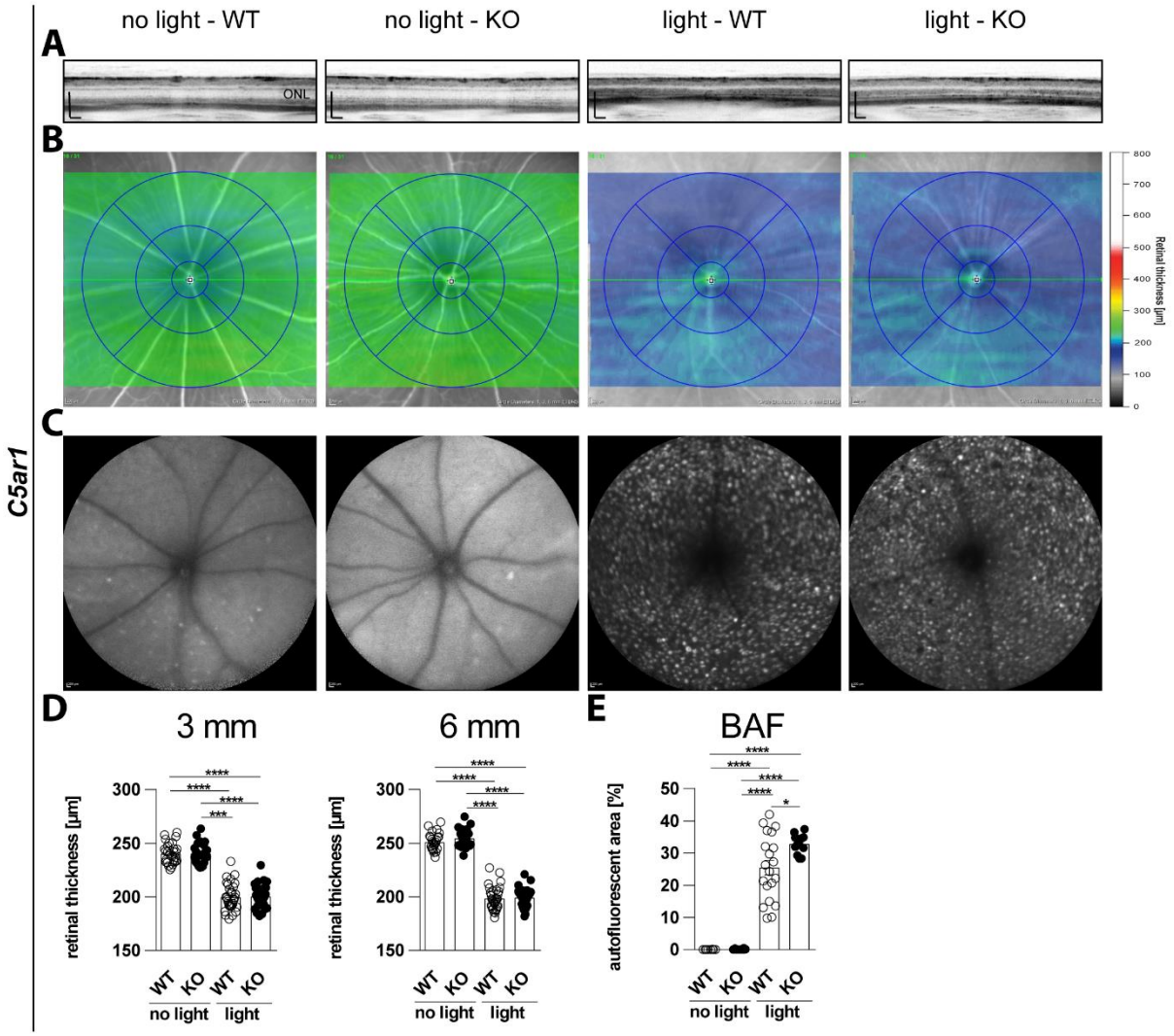

Figure 21: Analysis of retinal thickness and fundus of *C5ar1* WT and KO mice. Analysis was performed 4 d after light exposure with 10,000 lux for 30 min. **A** Representative scans and **B** corresponding heatmaps compiled by spectral-domain optical coherence tomography (SD-OCT) displayed a retinal overview. **C** Fundus images were obtained using BluePeak blue laser autofluorescence (BAF). **D** Retinal thickness was averaged from quartiles in 3 mm and 6 mm diameters (n = 28-34). **E** Percentage of autofluorescent area was measured with ImageJ (n = 820). **D, E** Bars represent mean ± SEM (*p < 0.05, ***p ≤ 0.001, and ****p < 0.0001). Data were analyzed using one-way ANOVA and Tukey's multiple comparison. Scale bar: 200 µm.

In addition, *in situ* retinal cell death was detected and quantified in cryosections of *C3ar1* mice via TUNEL (Figure 22). Following light damage, TUNEL positive cells were enriched predominantly in the outer nuclear layer (Figure 22A), without significant changes between WT and KO (Figure 22B).

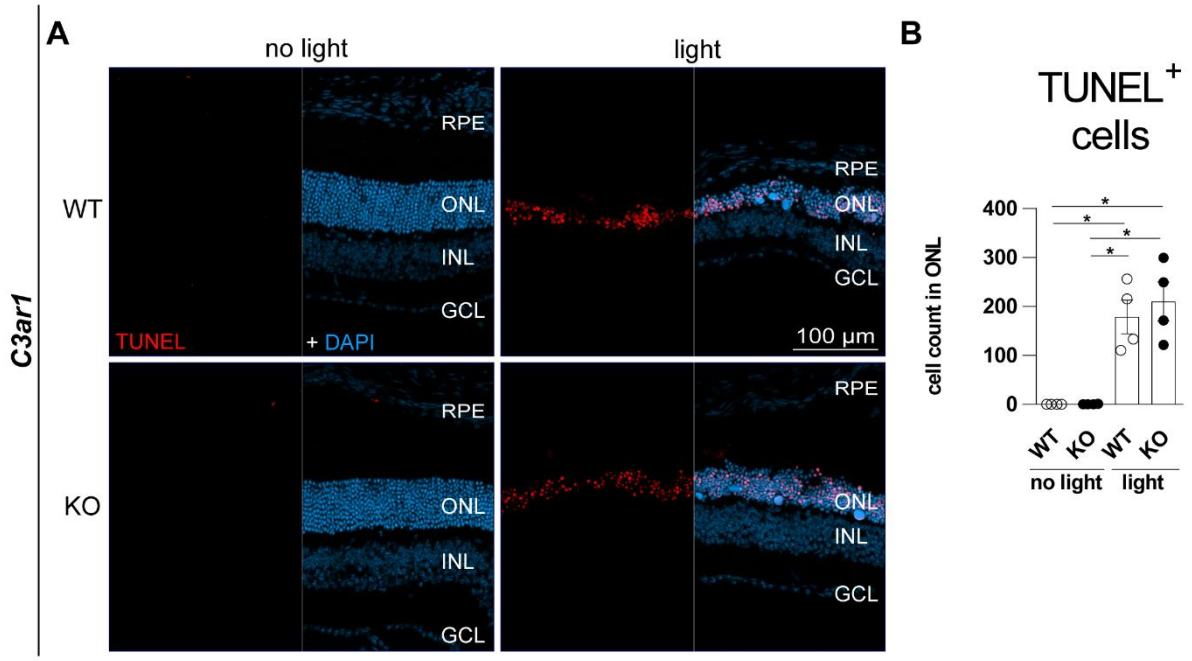

Figure 22: Photoreceptor cell death in cryosections of *C3ar1* WT and KO mice. A Cell death was detected 4 d after light exposure with 10,000 lux for 30 min using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in cryosections. Results out of 5 images of the central retina of each eye were averaged for one n. **B** Cell numbers were counted using the particle analyzer plugin of ImageJ. Data were analyzed using Mann-Whitney U test (n = 4). Bars represent mean ± SEM (*p < 0.05). RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

4 Discussion

4.1 IFN-β administration in a mouse model of dry AMD

Type I IFNs are known to play an important role in activating the innate and adaptive immune response, but excessive IFN signaling has been implicated in the pathogenesis of several diseases (Crow & Ronnblom, 2019). Here we showed that IFN-β is a potent immunomodulator of retinal microglia activity but cannot prevent the photoreceptor cell loss in an experimental mouse model of dry AMD.

IFN-β signaling has several complex effects (Frohman et al., 2006), dictated by STAT activation and differential complex formation (Wesoly et al., 2007). In a mouse model of EAE, genetic ablation of *IFNAR* or *IFN-β* resulted in exacerbated chronicity of the clinical disease, accompanied by extensive microglia activation and inflammatory response (Prinz et al., 2008; Teige et al., 2003). Engagement of myeloid-specific IFNAR by locally produced IFN-β reduced inflammation in effector phases (Prinz et al., 2008). Nonetheless, the number of infiltrating mononuclear cells was not altered in this experimental setting (Prinz et al., 2008). These results in brain tissue are somewhat contradictory to our findings in the retina, since we detected less mononuclear phagocytes in the subretinal space. In line with this, IFN-β can dose-dependently reduce the expression of MHC class II molecules on murine macrophages (Ling et al., 1985) and microglia (Kawanokuchi et al., 2004), as well as human monocytic cells (Q. Li et al., 1998). A possible mechanism of immune modulation by IFN-β via antigen presentation could be a potential interference with T cell activation. Indeed, there is evidence that both innate and adaptive immune mechanisms are linked to AMD pathogenesis (Behnke et al., 2020).

The effects of IFN-β on microglia depend on mode and conditions of activation (Jin et al., 2007). Likewise, IFN-β did not prevent from neuronal cell death despite its suppression of glutamate and superoxide production (Jin et al., 2007). IFN-β blocked the expression of cytotoxins (tumor necrosis factor α and lymphotoxin) by stimulating human blood mononuclear cells, albeit with large interindividual variations in response to the suppressive effects of IFN-β (Abu-khabar et al., 1992). Hence, variability in IFN-β effects could be due to different species, source of recombinant IFN-β and experimental setup and thus needs further research. Nonetheless, local immunomodulation in the retina has been shown to be effective in another light damage paradigm. While intravitreal injection of cerium oxide nanoparticles showed a full rescue of the retinal phenotype and function via reduction of microglia activation and reactive oxygen species (Tisi et al., 2019), intravenous injection was not affective (Fiorani et al., 2015).

IFN-β is clinically used for MS therapy since 1993 (Annibali et al., 2015), modulating antigen presentation and cytokine expression patterns. Dose and frequency of IFN-β administration affect its efficacy in patients (Sharief, 2003). Higher doses at a more frequent level decreased the number of relapses in patients. However, raising dose did not yield different results in our study (Supplementary Figure 5 & Supplementary Figure 6). Interestingly, earlier studies in our lab showed that IFN-β treatment attenuated microglia reactivity and significantly reduced CNV size in a laser-induced mouse model for wet AMD (Lückoff et al., 2016). Synergistic action of IFN-β and tumor necrosis factor α (TNFα), engaging a non-canonical STAT2/IRF9-dependent pathway, was suggested (Fink et al., 2013). Indeed, TNFα is known to be induced and secreted at great extend in the laser-induced CNV mouse model (Shi et al., 2006) A recent study proposed that genetic polymorphisms predict the response to IFN-β therapy in MS patients (Carrasco-Campos et al., 2021). Despite the use of C57BL/6J mice in the previous study, minimal differences in the *Mx* and none in the inflammatory response of retinal tissue were evident (Supplementary Figure 7), eliminating potential influences of the genetic background. However, differences in alternative complement component expression between C57BL/6J and BALB/cJ mouse strains were found (Pauly et al., 2019).

4.2 Anaphylatoxin receptor knockout analysis in a mouse model of dry AMD

Anaphylatoxins are potent inflammatory mediators that induce cellular degranulation, pro-inflammatory cytokine release and cellular chemotaxis, therefore are implicated in inflammatory disease (Klos et al., 2009). The proteins C3a and C5a drive inflammation by engaging their corresponding chemotactic receptors C3aR1 and C5aR1 (Engelke et al., 2014; M. Yu et al., 2012; L. Zhang et al., 2016). Thus, full body knockout mice of either *C3ar1* or *C5ar1* were tested in an experimental mouse model of dry AMD. Here we showed that none of the knockouts led to mitigated microglia migration to the subretinal space, albeit C3aR1 KO partially preserved retinal thickness.

The involvement of complement in AMD disease progression is generally accepted, with dysregulation of the alternative complement pathway as a main driver for AMD disease progression. Thus, several mouse models and clinical studies focused on complement inhibition in AMD. However, effective treatment options for intermediate and dry AMD are still lacking. Previous results identified anaphylatoxin receptors C3aR1 and C5aR1, among other complement proteins, to be highly expressed on immune cells after light exposure (Song et al., 2017). In the same study, reduced microgliosis could be detected in C5aR1-deficient mice, but not C3aR1-deficient mice, after light exposure. However, neither of the KO were able to preserve retinal thickness (Song et al., 2017). In line with this, C3aR1 KO and C3aR1/C5aR1-double knockout mice, but not C5aR1 KO mice, had progressive retinal cell loss and

dysfunction after light damage (M. Yu et al., 2012). However, Song et al. proposed a pro-inflammatory role for C5aR1 and a protective one for C3aR1, while Yu et al. concluded that in either case receptor-mediated signaling is mandatory for retinal function and structure. Our results observed no change in mononuclear phagocytes in the subretinal space and only a partial rescue of the retinal thickness in C3aR1-deficient mice. All studies used BALB/cJ mice, but furthermore Yu et al. compared the results with C57BL/6J mice and could not report differences, eliminating potential influences of the genetic background. However, experimental settings as age, sex, light regime and analysis time points differed between all studies. Noteworthy, the unchanged findings in C5aR1-deficient animals may possibly be due to pro-inflammatory functions of the second C5a ligand C5aR2 in this model, that have been suggested previously (T. Zhang et al., 2017). Antibody blockade or targeted deletion of C5R2 resulted in augmented C5a-mediated chemotaxis (Bamberg et al., 2010; N. P. Gerard et al., 2005), suggesting the absence of C5aR1 prohibits heterodimerization and therefore alter C5R2 function. However, most pro-inflammatory effects of C5a binding occur through C5aR1 (Klos et al., 2009).

Aside from that, studies targeting complement in inflammatory mouse models yield diverse results. Antibody-mediated neutralization of anaphylatoxins, pharmacological blockade or knockout of both anaphylatoxin receptors resulted in reduced neovascular areas in the laser model of wet AMD (Nozaki et al., 2006). In another CNV study, alternative pathway antibody blocking with the fusion protein CR2-fH, combining the iC3b/C3d-binding region of CR2 and the N-terminus of the regulatory CFH, normalized anaphylatoxin levels and reduced lesion size (Parsons et al., 2019). In contrast, pharmacological or genetic blockade of anaphylatoxin receptors was not sufficient to alter the course of lesion repair significantly in the same study (Parsons et al., 2019). Following spinal cord injury, a dual time-dependent effect of C5aR1/C5a signaling was demonstrated (Brennan et al., 2015). Interestingly, pharmacological blockade or genetic ablation of C5aR1 during the acute phase of injury reduced inflammation and improved functional recovery, while prolonged signaling impairment exacerbated immune cell infiltration and reduced functional recovery (Brennan et al., 2015). Counterfactual, C3aR signaling during ischemic injury of the adult brain was found to be detrimental during the acute phase, but contributed to the recovery in the chronic phase (Pekna et al., 2021). In a mouse model of experimental autoimmune uveitis (EAU) less severe uveitis in C3aR1/C5aR1-double knockout than control mice was evident, involving reduced T cell response (L. Zhang et al., 2016). Indeed, in the laser-induced CNV mouse model C5a was shown to recruit T cells to the lesion site and concomitant pharmacological blocking reduced CNV size and T cell infiltration (Coughlin et al., 2016).

In our study, a partial rescue in the 3 mm radius of SD-OCT heatmaps of C3aR1 KO mice was shown. This was mirrored by significant less MAC staining in the outer retina of C3aR1-deficient mice compared to control, which was also indicated through less pronounced transcripts of complement proteins. However, *in situ* detection of retinal cell death on cryosections of *C3ar1* mice via TUNEL did not indicate less apoptotic cell in KO compared to WT mice. In fact, unpublished data of our group showed highest TUNEL positive cell levels one day after light damage and intermediate levels on day four. In addition, the significant rescue was only partial and the picture range of cryosections may have exceeded this area or C3aR1 KO may only decelerate retinal degeneration. In line with our data, *C3ar1* deficiency changed the microglial neuroinflammatory expression pattern and lowered the risk of degeneration in ocular hypertensive DBA/2J mice (Harder et al., 2020). Recently, the C3/C3aR/STAT3 pathway was revealed to mediate immune response and subsequent photoreceptor cell degeneration and ablation of C3 or pharmacological inhibition of C3aR/STAT3 reversed this effect under oxidative stress (S. Wang et al., 2020). Furthermore, C3a stimulation of monocytes was shown to induce IL-1β secretion, subsequently to NLRP3 inflammasome activation (Asgari et al., 2013), which resulted in recruitment of choroidal macrophages in Pentraxin3-deficient mice (L. Wang et al., 2016). Drusen are a hallmark in the development of AMD and C3a triggered the formation of sub-RPE deposits *in vitro* (Fernandez-Godino & Pierce, 2018).

Indeed, anaphylatoxin receptor signaling subsequently recruits immune cells and promotes the pro-inflammatory surrounding, which is considered the most important effect of complement dysregulation in AMD (Behnke et al., 2020; Ogura et al., 2020). Nevertheless, their signaling is also needed for homeostasis and regeneration. For example, C3aR regulates proper chick eye morphogenesis (Grajales-Esquivel et al., 2017) and postnatal cerebellum developmental in mice (Young et al., 2019), while engaging of C3aR and C5aR are essential for liver regeneration (Strey et al., 2003). Therefore, genetic compensatory mechanisms due to full body knockout, as previously reported (El-Brolosy & Stainier, 2017), should be taken into account, when interpreting study data. *In vitro* analysis of RPE cells suggest a combination of signaling between C3aR and C5aR1 in order to implement their precise immune regulatory functions (Busch et al., 2017).

4.3 Link among IFN-β and complement signaling

During AMD, dysregulation of the complement system and migration of mononuclear phagocytes are hallmarks of AMD disease progression. Complex cell interactions fail and cause overactivation of microglia that drive retinal degeneration. Microglia are important producers of local complement and express complement receptors themselves. Thus, in this

study we aimed to elucidate, whether modulation of microglia via IFN-β may also dampen mononuclear phagocyte reactivity by altering the production of complement components in the retina. Although IFN-β application was able to decrease microgliosis and C3aR1-deficiency in mice partly decelerated retinal degeneration, no direct link was found between both treatment options in this study. However, IFN-β treatment increased complement transcripts in retinal tissue. Therefore, concomitant or double treatment targeting both, immunomodulation of microglia and the complement system, could be a possible strategy for interfering in AMD disease progression. Recently, evidence was raised linking the inflammatory cytokine storm in SARS-CoV-2 to elevated C5a and downregulation of IFN-α/β secretion (Shibabaw et al., 2020). Chemokine CCL2 expression, which is mediated by Type I IFN receptor (Lehmann et al., 2016) and known to play a crucial role in recruiting mononuclear phagocytes (Hinojosa et al., 2011), was increased in MS patients treated with IFN-β (Comini-Frota et al., 2011) and just shown to modulate classical and lectin complement pathways in microglial cell cultures (Popiolek-Barczyk et al., 2020). To our knowledge, however, no study could identify a mechanistic involvement of IFN-β and complement signaling. Overall, our study highlights the complex interactions of AMD pathogenesis and disease progression, also keeping in mind, that AMD is not entirely a genetic disease.

5 Conclusion

In summary, our study showed that IFNAR signaling in the retina significantly reduced the number of reactivate microglia in the subretinal space of light damaged mice. However, neither a decreased expression of complement factors nor a rescue of retinal thickness was detectable after IFN-β therapy. Furthermore, full body knockout mice of either *C3ar1* or *C5ar1* were tested, but none led to mitigated microglia migration to the subretinal space. However, a partial rescue of retinal thickness was shown in C3aR1 KO mice, which was mirrored by significant less MAC occurrence in the outer retina. Although a link between IFN-β immunomodulation and complement pathway signaling could not be underpinned, IFN-β- and C3aR-targeted therapy may be considered for dry AMD patients. Further work will be necessary to elucidate the exact mechanism of action for IFN-β and the optimal intervention point in the anaphylatoxin signaling axis. As previously suggested, a combined genetic and immunomodulatory treatment should also be considered.

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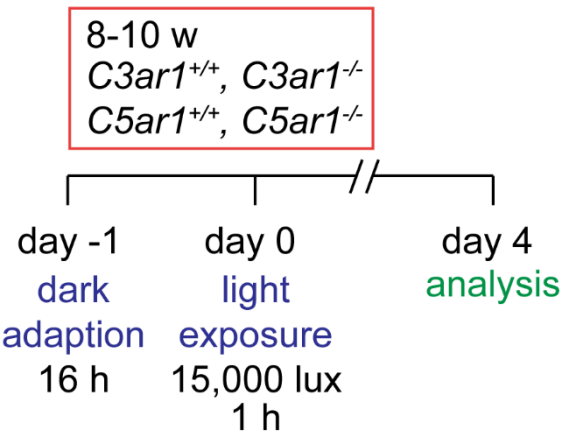
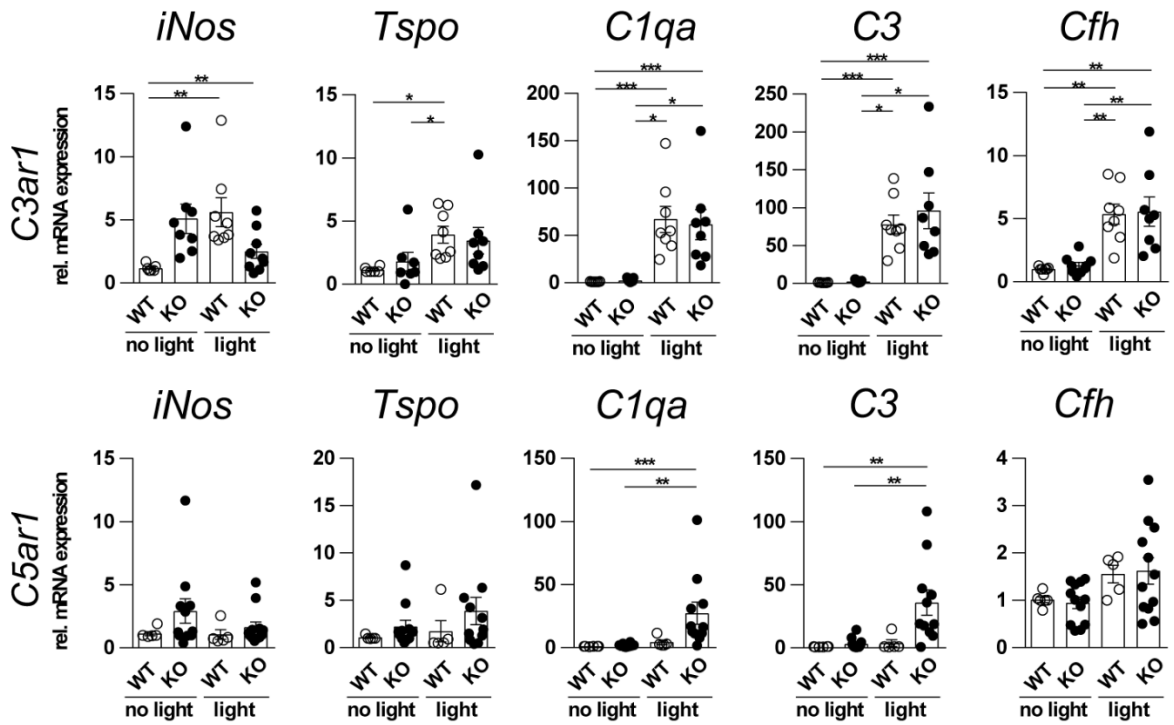
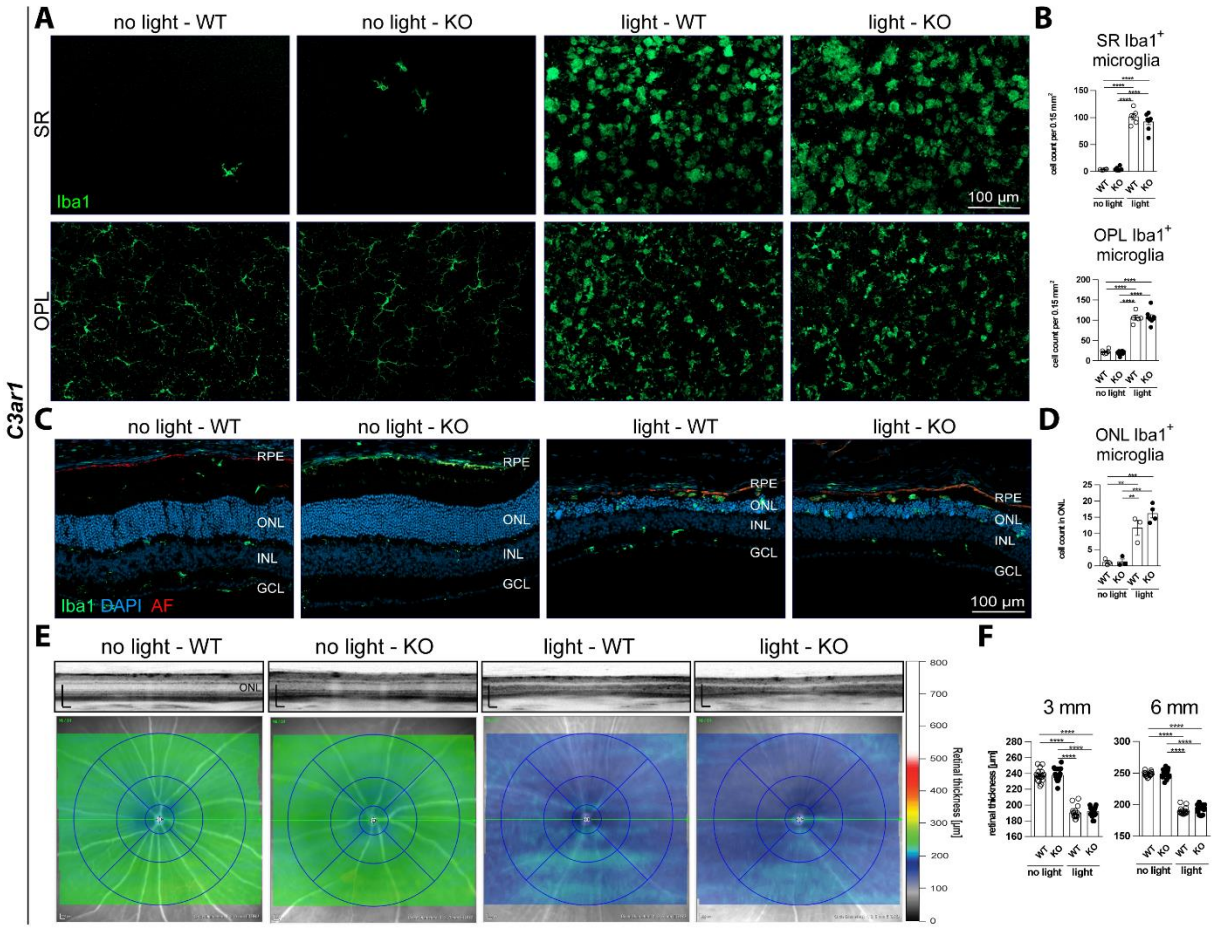
7 Attachments



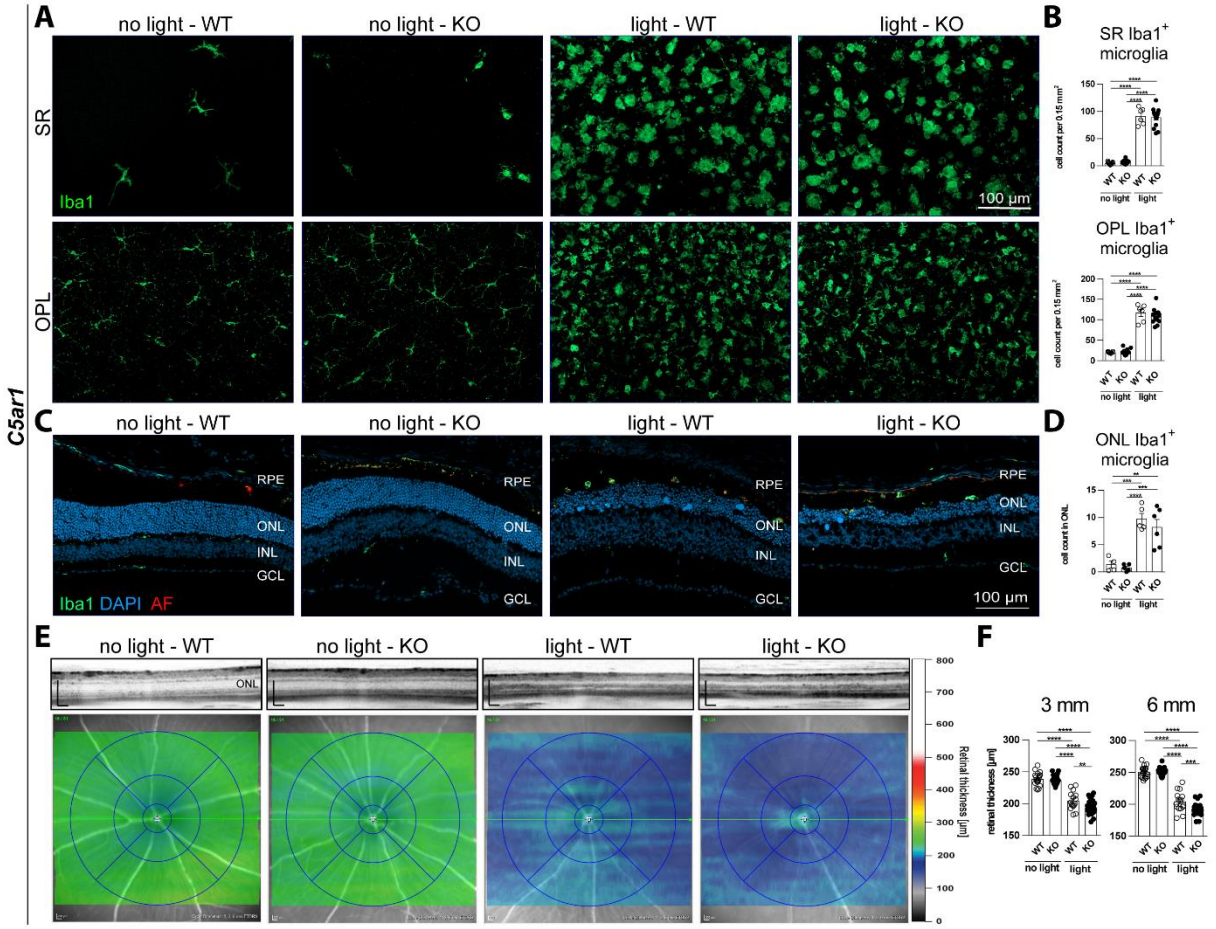
Supplementary Figure 1: Initial light damage paradigm for *C3ar1* and *C5ar1* mice. WT and KO mice were dark adapted for 16 h before light exposure with 15,000 lux for 1 h until analysis 4 d afterwards.



Supplementary Figure 2: Expression analysis of *C3ar1* or *C5ar1* WT and KO retinal tissue 4 d after light damage with 15,000 lux for 1 h. Gene expression analysis using probe-based qRT-PCR was performed 4 d after light exposure with 15,000 lux for 1 h. *Atp5b* was used as reference gene and $^{\Delta\Delta}$Ct analysis was used for quantification. For this, technical duplicates of one retina were averaged for one n. Data were obtained from 6/9 individual experiments. Bars represent Mean ± SEM (*p < 0.05, **p < 0.01, and ***p ≤ 0.001). Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparison (n = 5-12).

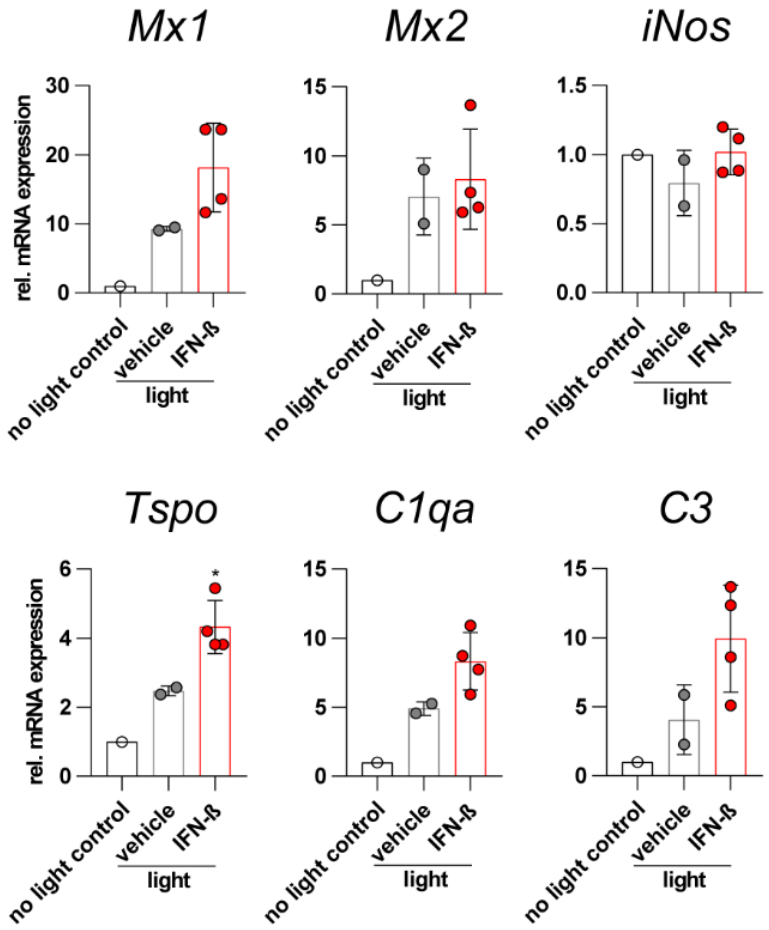


Supplementary Figure 3: Analysis overview of *C3ar1* WT and KO mice 4 d after light damage with 15,000 lux for 1 h. Microglia were stained in retinal flat mounts (**A**) or cryosections (**C**) using ionized calcium-binding adapter molecule 1 (Iba1). SR: subretinal space; OPL: outer plexiform layer; AF: autofluorescence; RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. **B** Four images of the central retina were taken around the optic nerve of each eye and results averaged for one n. Cell numbers were counted using the particle analyzer plugin of ImageJ (n = 5-8). **D** Results out of 5 images of the central retina of each eye were averaged for one n. Cell numbers were counted using the multi-point tool of ImageJ (n = 3-4).Representative scans and corresponding heatmaps compiled by spectral-domain optical coherence tomography (SD-OCT) displayed a retinal overview. Scale bar: 200 µm. **F** Retinal thickness was averaged from quartiles in 3 mm and 6 mm diameters (n = 20-26). **B, D, F** Data were obtained from 6 individual experiments. Bars represent Mean ± SEM (**p < 0.01, ***p ≤ 0.001, and ****p < 0.0001). Data were analyzed using one-way ANOVA and Tukey's multiple comparison.

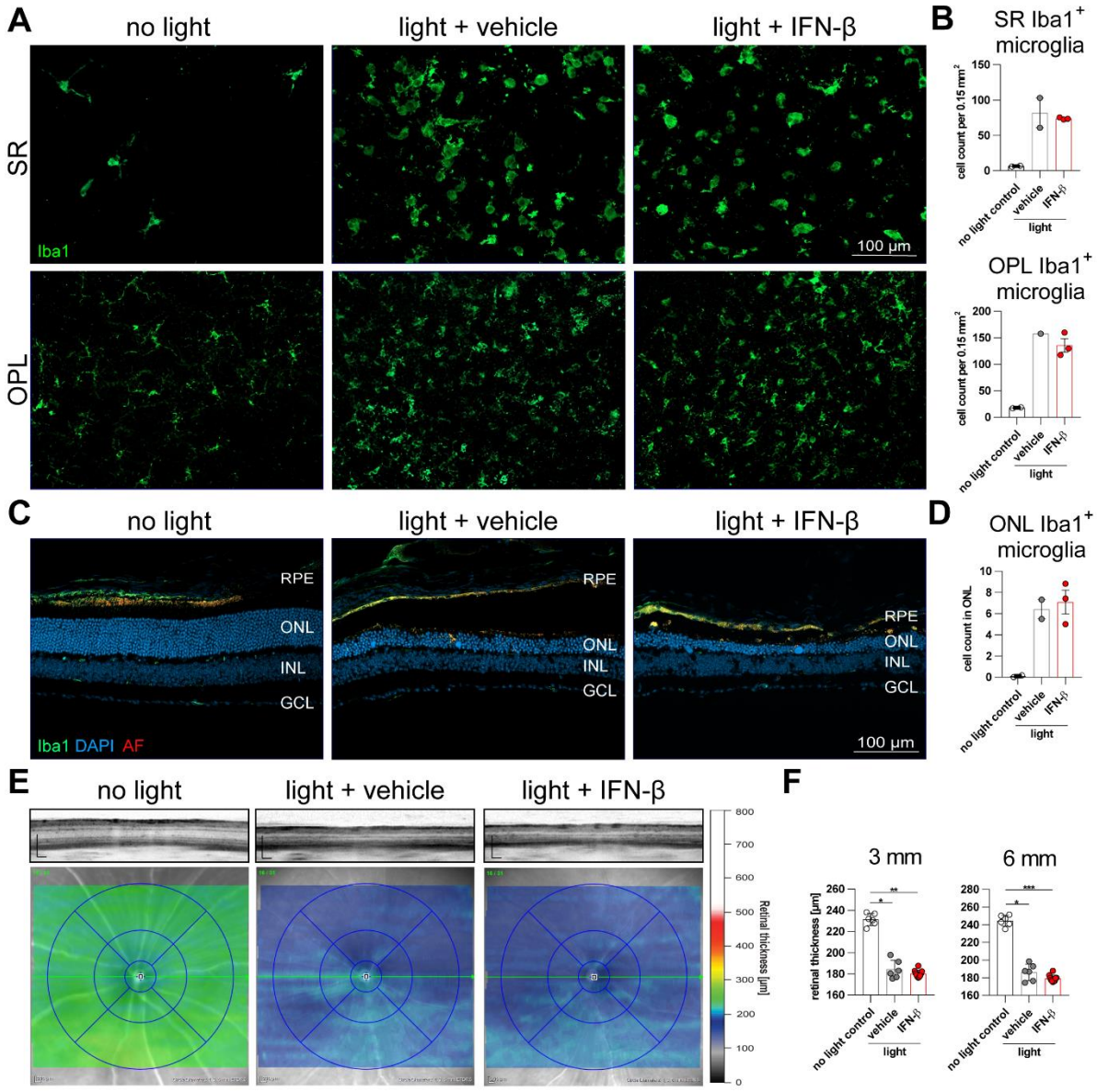


Supplementary Figure 4: Analysis overview of *C5ar1* WT and KO mice 4 d after light damage with 15,000 lux for 1 h. Microglia were stained in retinal flat mounts (**A**) or cryosections (**C**) using ionized calcium-binding adapter molecule 1 (Iba1). SR: subretinal space; OPL: outer plexiform layer; AF: autofluorescence; RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. **B** Four images of the central retina were taken around the optic nerve of each eye and results averaged for one n. Cell numbers were counted using the particle analyzer plugin of ImageJ (n = 6-15). **D** Results out of 5 images of the central retina of each eye were averaged for one n. Cell numbers were counted using the multi-point tool of ImageJ (n = 4-6). Representative scans and corresponding heatmaps compiled by spectral-domain optical coherence tomography (SD-OCT) displayed a retinal overview. Scale bar: 200 µm. **F** Retinal thickness was averaged from quartiles in 3 mm and 6 mm diameters (n = 16-30). **B, D, F** Data were obtained from 9 individual experiments. Bars represent Mean ± SEM (**p < 0.01, ***p ≤ 0.001, and ****p < 0.0001). Data were analyzed using one-way ANOVA and Tukey's multiple comparison.

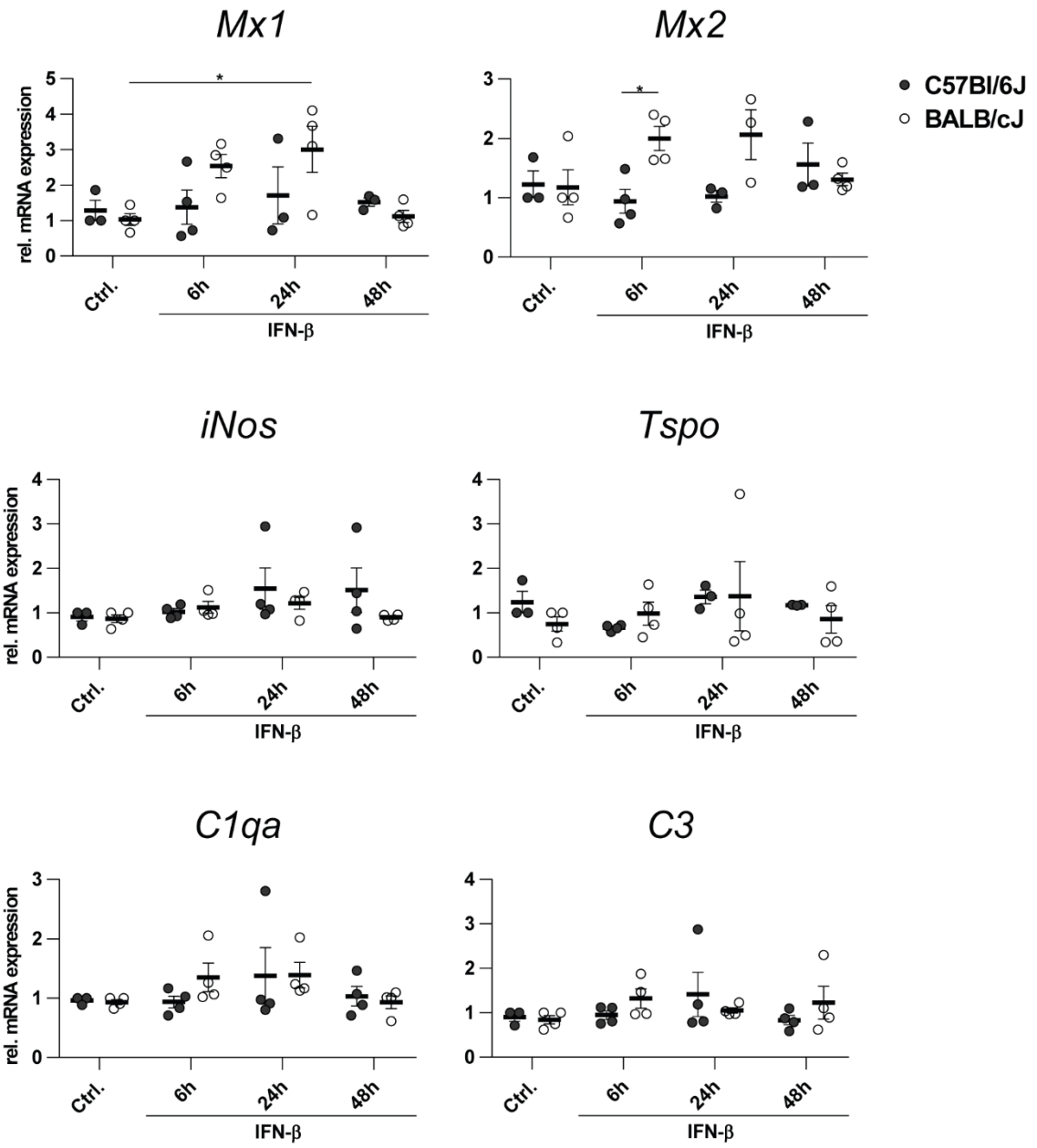
The dilution of IFN-β was adjusted to the manufacturer´s protocol, since the biological activity was changed. BSA was no longer needed in the dilution as it is only required for extended storage. Overall, no significant changes in inflammation and retinal thickness were discovered. Therefore, the following experiments were carried out with the old dilution in consultation with the manufacturer. Changes in the biological activity of IFN-β resulted from an experimental change of the same.



Supplementary Figure 5: Expression analysis of retinal tissue 4 d after light exposure with an adjusted dilution of IFN-β without BSA. BALB/cJ mice were treated with IFN-β (10,000 U) or PBS as vehicle every other day starting 1 h prior to light damage with 15,000 lux for 1 h until 4 d afterwards. Gene expression analysis using probe-based qRT-PCR was performed. *Atp5b* was used as reference gene and $^{\Delta\Delta}$Ct analysis was used for quantification. For this, technical duplicates of one retina were averaged for one n. Data were obtained from one experiment. Bars represent Mean ± SEM (*p < 0.05). Data were analyzed using one-way ANOVA followed by Sidak´s multiple comparison (n = 1-4).



Supplementary Figure 6: Analysis overview 4 d after light exposure with an adjusted dilution of IFN-β without BSA. BALB/cJ mice were treated with IFN-β (10,000 U) or PBS as vehicle every other day starting 1 h prior to light damage with 15,000 lux for 1 h until 4 d afterwards. Microglia were stained in retinal flat mounts (**A**) or cryosections (**C**) using ionized calcium-binding adapter molecule 1 (Iba1). SR: subretinal space; OPL: outer plexiform layer; AF: autofluorescence; RPE: retinal pigment epithelium; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. **B** Four images of the central retina were taken around the optic nerve of each eye and results averaged for one n. Cell numbers were counted using the particle analyzer plugin of ImageJ (n = 2-3). **D** Results out of 5 images of the central retina of each eye were averaged for one n. Cell numbers were counted using the multi-point tool of ImageJ (n = 2-3). **E** Representative scans and corresponding heatmaps compiled by spectral-domain optical coherence tomography (SD-OCT) displayed a retinal overview. Scale bar: 200 µm. **F** Retinal thickness was averaged from quartiles in 3 mm and 6 mm diameters (n = 6-10). **B, D, F** Data were obtained from two individual experiments. Bars represent Mean ± SEM (*p < 0.05 and **p < 0.01). Data were analyzed using Kruskal-Wallis test followed by Dunn´s multiple comparison.



Supplementary Figure 7: Comparison of gene expression in retinas after IFN-β stimulation. C57Bl/6J and BALB/cJ mice were injected with 10,000 U IFN-β and retinas were harvested after 6, 24 or 48 h. Gene expression analysis using probe-based qRT-PCR was performed. *Atp5b* was used as reference gene and $^{\Delta\Delta}$Ct analysis was used for quantification. For this, technical duplicates of one retina were averaged for one n. Data were obtained from one experiment. Bars represent Mean ± SEM (*p < 0.05, **p < 0.01, and ***p ≤ 0.001). Data were analyzed using 2way ANOVA followed by Tukey´s multiple comparison (n = 3-4).

8 Danksagung

Zunächst möchte mich bei Herrn Prof. Dr. Thomas Langmann für die Übernahme der Betreuung und Erstkorrektur sowie die Vergabe dieses interessanten Themas bedanken. Außerdem bedanke ich mich bei Frau Prof. Dr. Elena Rugarli und Herrn Prof. Dr. Marcel Bucher für die Übernahme des Amtes der Zweitprüferin und des Prüfungsvorsitzes.

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9 Erklärung

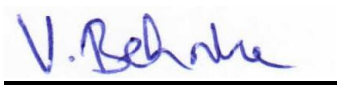
Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Thomas Langmann betreut worden.

Teilpublikationen:

Behnke, V., & Langmann, T. (2020). IFN-β signaling dampens microglia reactivity but does not prevent from light-induced retinal degeneration. *Biochemistry and Biophysics Reports*, *24*, 100866. https://doi.org/10.1016/j.bbrep.2020.100866

Behnke, V., Wolf, A., & Langmann, T. (2020). The role of lymphocytes and phagocytes in age-related macular degeneration (AMD). *Cellular and Molecular Life Sciences*, *77*(5), 781–788. https://doi.org/10.1007/s00018-019-03419-4

Köln, den 10.11.2021



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Publikationen

V. Behnke, A. Wolf, T. Langmann, The role of lymphocytes and phagocytes in age-related macular degeneration (AMD), **Cell. Mol. Life Sci**. 77 (2020) 781–788. https://doi.org/10.1007/s00018-019-03419-4

V. Behnke, T. Langmann, IFN-β signaling dampens microglia reactivity but does not prevent from light-induced retinal degeneration, **Biochem. Biophys. Reports** 24 (2020) 100866. https://doi.org/10.1016/j.bbrep.2020.100866

V. Behnke, T. Langmann, Neuroinflammation bei neuronalen Ceroid-Lipofuszinosen, **Der Ophthalmol.** (2021) 1–7. https://doi.org/10.1007/s00347-020-01301-4