Genetic knockout and pharmacological inhibition of galectin-3 blocks microglia reactivity and protects from retinal degeneration

INAUGURAL-DISSERTATION

Zur

Erlangung des Doktorgrades

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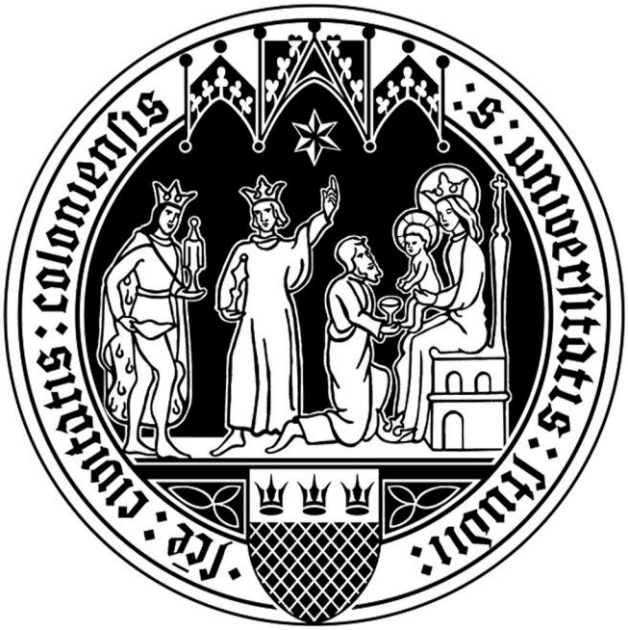

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For my Family

"Keep away from people who try to belittle your ambitions.

Small people always do that, but the really great make you feel

that you, too, can become great."

— *Mark Twain*

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Zusammenfassung

Die altersbedingte Makuladegeneration (AMD) ist eine der häufigsten degenerativen Erkrankungen in der Netzhaut in der westlichen Welt. Es handelt sich hierbei um eine komplexe genetische und multifaktorielle degenerative Erkrankung, die bei einem schweren Verlauf zur Erblindung führen kann. Eine fehlerhafte Funktion des angeborenen Immunsystems führt zu einer chronischen Aktivierung von speziellen Gewebsmakrophagen, den Mikrogliazellen, in der Netzhaut. Diese Zellen sind in ihrer ursprünglichen Form für die Aufrechterhaltung der Homöostase essentiell. Eine chronische Aktivierung dieser Zellen führt jedoch durch ihre proinflammatorischen und neurotoxischen Eigenschaften zu einem schnellen Fortschreiten der degenerativen Erkrankung. Ziel vieler Studien ist es daher eine Therapie zu entwickeln, die neurotoxische Eigenschaften der Mikroglia hemmt, jedoch die Fähigkeit die Homöostase aufrechtzuerhalten erhält. Solche immunmodulatorischen Zielstrukturen stellen nicht nur für die AMD eine therapeutische Möglichkeit dar. Eine mögliche Zielstruktur hierfür ist die Modulation des Proteins, Galektin-3, welches ein ß -Galaktose bindendes Protein ist, das in Mikrogliazellen nach Reaktivierung hochreguliert wird. Auch in anderen Krankheiten, wie Alzheimer, Huntington und Parkinson, könnte eine Modulation von Galectin-3 zielführend sein, da dort eine erhöhte Mikrogliaaktivität und zugleich eine verstärkte Galektin-3 Expression festgestellt werden konnte.

Die Ergebnisse dieser Arbeit zeigen, dass Mikroglia bei AMD Patienten ein hohes Maß an Galektin-3 exprimieren, welches in gesunden Menschen nur wenig detektiert werden kann. Mit Hilfe von molekular biologischen Expressionsanalysen konnte festgestellt werden, dass die Galektin-3 Expression mit der für Mikroglia spezifischen *Allograft inflammatory factor-1*, (*AIF-1)* Expression kolokalisiert ist. Im Licht-induzierten Degenerationsmodell der Netzhaut, welches ein etabliertes Mausmodell der trockenen AMD darstellt, zeigen die Ergebnisse, das sowohl der Verlust des funktionierenden Genes für Galektin-3 (*Lgals3*-KO Mäuse) sowie auch die Inhibition mittels eines kleinen chemischen Moleküls, TD139, eine hemmende Wirkung auf residente Mikrogliazellen haben. Analysen zum Migrationsverhalten der Mikrogliazellen deuten darauf hin, dass Galectin-3 die Migration von Mikrogliazellen begünstigt, welches durch eine Inhibition oder einem genetischen Verlust reduziert werden konnte. Zudem wurden die Level proinflammatorischer Zytokine in der Netzhaut und dem retinalen Pigmentepithel, sowie die Degeneration der Netzhaut, inklusive der Photorezeptorzellschicht reduziert.

Zusammenfassend zeigt diese Arbeit einen Zusammenhang der Mikrogliaaktivität und der erhöhten Galectin-3 Expression im Gewebe bei Mäusen im Licht-induzierten Degenerationsmodell, sowie auch in AMD Patienten. Eine Immunmodulation von Galektin-3 mit einem Inhibitor, TD139, stellt somit eine mögliche Zielstruktur für die Therapie neurodegenerativer Erkrankungen, wie AMD, dar.

Summary

Age-related macular degeneration (AMD) is one of the most common degenerative diseases of the retina in the western world. It is a complex genetic and multifactorial degenerative disease, which can lead to blindness. A dysfunction of the innate immune system leads to a chronic activation of tissue macrophages, microglia, in the retina. These cells are responsible for maintaining homeostasis in healthy tissue. However, chronic activation of these cells leads to rapid progression of degenerative diseases due to their pro- inflammatory and neurotoxic properties. The goal of many studies is to develop a therapy that inhibits the neurotoxic properties of microglia while maintaining their ability for homeostasis. Such immunomodulatory targets represent a therapeutic possibility not only for AMD. One possible target is the modulation of the protein, galectin-3, which is a ß-galactose binding protein that is upregulated in microglia after reactivation. In other diseases, such as Alzheimer's disease, Huntington's disease and Parkinson's disease, modulation of galectin-3 may also be beneficial, in these diseases increased microglia activity and galectin-3 expression have been observed.

The results of this work demonstrate that microglia in AMD patients express a high level of galectin-3, which is not the case in healthy individuals. Using molecular biological expression analysis, galectin-3 expression was found to be co- localized with Allograft inflammatory factor-1, *AIF-1*, expression specific for microglia. In the light induced degeneration model of the retina, which is an established mouse model of dry AMD, the results show that the deficiency of galectin-3 (*Lgals3*-KO) and the inhibition by a small chemical molecule, TD139, have an inhibitory effect on resident microglia cells. Analyses of the migration of microglia suggest that galectin-3 promotes microglia migration, which could be reduced by inhibition or genetic loss of galectin-3. In addition, the level of pro-inflammatory cytokines in the retina and retinal pigment epithelium was reduced, as well as the degeneration of the retina, including the photoreceptor cell layer.

In conclusion, this study shows a correlation of microglia activity and increased galectin-3 expression in tissues of mice in the light-induced degeneration model, as well as in AMD patients. Thus, immunomodulation of galectin-3 with an inhibitor, TD139, represents a potential target for the therapy of neurodegenerative diseases, such as AMD.

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List of abbreviations

α	alpha
β	beta
γ	gamma
µm	micrometer
Aß	amyloid ß
AIF-1	Allograft inflammatory factor-1
AMD	Age-related macular degeneration
AP	Activator protein
ARMS2	Age-related maculopathy susceptibility 2
ATP	Adenosintriphosphate
BBB	Blood brain barrier
BCB	blood-cerebrospinal fluid barrier
BDNF	brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
BM	Bruch's membrane
Bp	Base pair
BRB	Blood retina barrier
BSA	Bovine serum albumin
C1q	complement component 1q
C3	Complement factor 3

C3aR	Complement receptor 3
CCL2	C-C Motif Chemokine Ligand 2
CD200	cluster of differentiation-200
CD200R	cluster of differentiation receptor
cDNA	Complementary DNA
CFH	Complement factor H
CNS	Central nervous system
CNV	Choroidal neovascularization
cAMP	Cyclisches Adenosinmonophosphat
CRD	carbohydrate recognition domain
CREB	cAMP response element-binding protein
CRE-Motif	cAMP-dependent response element
CSFR1	Colony stimulating factor 1 receptor
Cx3cr1	C-X3-C Motif Chemokine Receptor 1
DAMP	Damage-associated molecular patterns
DAP12	DNAX-activating protein of 12 kDa
DBI	Diazepam binding inhibitor protein
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DOX	Doxorubicin
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid

Ets	Erythroblast Transformation Specific
GA	Geographic atrophy
GCL	Ganglion cell layer
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
H	Hour
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HTRA1	High temperature requirement A serine peptidase 1
i.p.	Intraperitoneal
IBA-1	Ionizing calcium-binding adaptor molecule-1
IFN	Interferon
IL	Interleukin
INL	Inner nuclear layer
iNOS	Inducible nitric oxide-synthase
IPL	Inner plexiform layer
IRF8	Interferon regulatory factor 8
IS	Inner segments
IVC	Individual ventilated cages
Kb	kilo base
kDa	Kilodalton
KO	Knockout

Lgals3	galectin 3 gene
LPS	Lipopolysaccharide
min	Minute
MMP	Matrix metalloproteinases
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NDS	Normal donkey serum
NF-κB	Nuclear factor ‘kappa light chain enhancer’ of activated B-cells
NO	nitrogen monoxide
NASH	non-alcoholic steatoHepatitis
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OS	Outer segments
P	Postnatal day
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-L1-	Programmed cell Death protein 1
pH	Potenia Hydrogenii, negative decadic logarithm of the H3O+ concentration
PRR	Pattern recognition receptor

PU.1	PU box binding 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RT	Room temperature
Sec	Seconds
SD-OCT	Spectral domain optical coherence tomography
SEM	Standard error of the mean
SIE	sis-inducible element
Siglecs	Sialic acid binding immunoglobulin type lectin
Sp1	specificity protein 1
SRS	Subretinal space
TBE	Tris-borate-EDTA buffer
TD139	thio-digalactoside inhibitor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
TRIS	Tris (hydroxymethyl) aminomethane
TSPO	Translocator protein
U	Units
UV	ultraviolet

V	Volt
VEGF	Vascular endothelial growth factor
WT	Wild type

1. Introduction

1.1 The retina: structure and function

One of the five sensory organs in the human body is the eye, which enables the perception of the environment. The eye consists of many different components that perform their own tasks (Giblin *et al.*, 2016). Besides the sclera, pupil and iris, which are familiar to most people because they can be seen immediately, there are other parts that are very important for the visual ability. Most of the eye is inconspicuous, but the inner parts of the eye, such as the cornea, lens, aqueous humor, ciliary muscle, rectus medialis muscle, rectus lateralis muscle, vitreous humor, choroid, optic nerve, retinal pigment epithelium, retina and medullary sheath, all have specific functions and are essential for vision (Malhotra *et al.*, 2011). The vitreous fills the center of the eye, and its clear structure allows light to pass unobstructed through the transparent cornea and pupil onto the retina (Fig. 1a). The lens focuses the light that has entered the eye through the pupil and controls the amount of light transmitting to the retina.

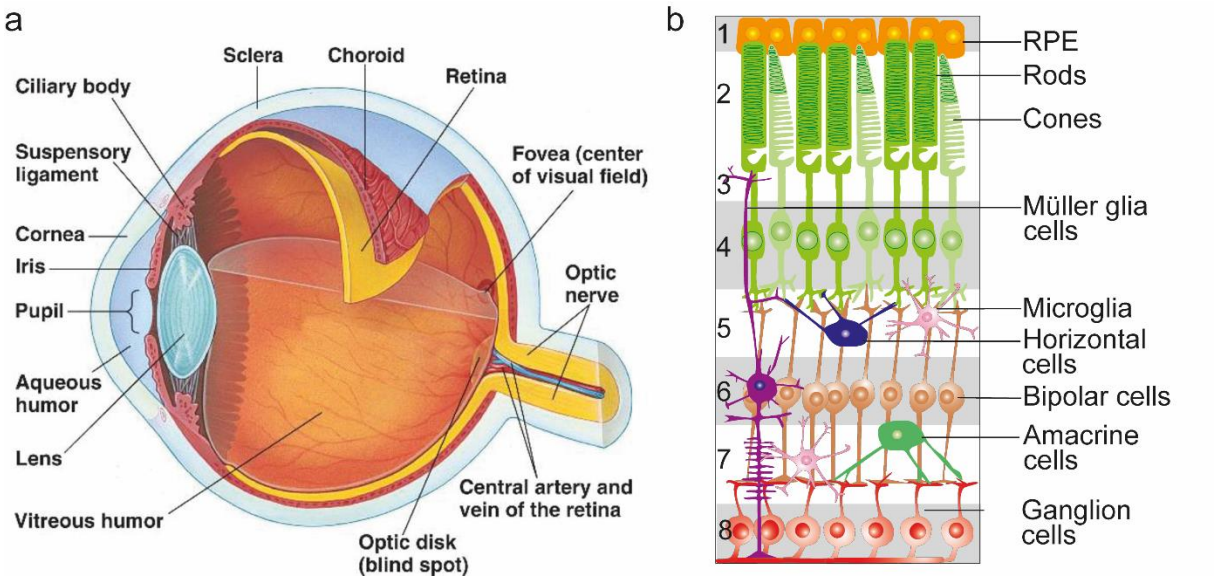

Figure 1: Anatomy of the human eye and the retina. **a** Schematic representation of a human healthy eye. Illustration from Chader *et al.*, 2013 (Chader *et al.*, 2013). **b** Schematic overview of all cells of the retina in homeostasis state. The mammalian retina is distributed in three cellular layers and two synaptic (plexiform) layers. 1: RPE; 2: outer segment; 3: inner segment; 4: outer nuclear layer; 5: outer plexiform layer; 6: inner nuclear layer; 7: inner plexiform layer; 8: ganglion cell layer. Modified from Karlstetter, Marcus *et al.*, 2010.

The retina plays an important role in the perception of our environment and enables us to see our counterpart. It has a multilayered structure with about 55 different cell types in the retina, and each cell type has a different function (Masland, 2001). The retina lines, the inside of the eye, is covered by the sclera and the choroid (Bhutto *et al.*, 2012; Borrelli *et al.*, 2018). In order for the environment to be perceived, the cornea and lens collect light and focus it to the macula, the center of the retina. In the macula lies the *fovea*, the point of the sharpest vision (Chader *et al.*, 2013). The retina, which is adjacent to the retinal pigment epithelium in the back of the eye, is a very light-sensitive tissue that creates an image, converts that image into electrical signals, and transmits those signals to the brain via the optic nerve (Bhutto *et al.*, 2012; Delgado-Bonal *et al.*, 2016). The retina is part of the central nervous system since it originates from the embryonic diencephalon and has an immune- privilege, because of the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCB) and blood-retinal barrier (BRB) (Galea *et al.*, 2007; Wraith *et al.*, 2012). The arrangement of the retina is structured in such a way that light must first pass through the various complex layers of the retina in order to transport the signal from the light-sensitive photoreceptors to the optic nerve (Lamb, 2016). The mammalian retina is composed of different highly specific cells distributed in three cellular layers and two synaptic (plexiform) layers (Godinho *et al.*, 2005; Hoon *et al.*, 2014). The outermost layer of the retina is the photoreceptor layer. There are 5 classes of neurons in the retina: Photoreceptors, horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells (Fig. 1b). There are two different types of photoreceptors in the eye, rods and cones, where the encoding of visual information begins (Ingram *et al.*, 2016). The perception of light energy in photoreceptors leads to changes in membrane potential, which alters the release of neurotransmitters. Both cell types are connected to the outer segment by the connecting cilium and differ in their perception of light (Fain *et al.*, 1973; Horst *et al.*, 1990). Rods are very light-sensitive cells that can detect single photons (Fain *et al.*, 1973). Therefore, these cells enable vision in dim light and at night. Cones handle the perception of bright and colored light. In the human retina, there are about 20 times more rods than cones. However, it is striking that in the center of the sharpest vision, the *fovea centralis*, an enrichment of cones is characteristic, whereas rods are little represented there. At the point of the sharpest vision, there is one cone per bipolar cell and one ganglion cell, which is crucial for the visual function of this central point of the retina (Provis *et al.*, 2013).

Important for the visual cycle are the so-called opsins, which are enriched in the photoreceptor outer segments (OS). Opsins belong to the family of heptahelical

transmembrane proteins (G-protein coupled receptors) and are considered light-sensitive proteins (Fredriksson *et al.*, 2005; Hur *et al.*, 2002). There are two different groups of opsins; in cones there are mainly opsins of the photopsin group, leading to the capability of color vision. In rods, there are scotopsins, which enable vision in darkness (Ebrey *et al.*, 2001). Absorption of a photon of an opsin pigment in rods leads to a conformational change of chromophore 11-cis-retinal (Chen, P. *et al.*, 2001). The conformational change of opsins in turn leads to subsequent binding of intracellular G protein. Opsins, together with chromophores, form light-sensitive pigment molecules such as rhodopsin, which trigger visual signal transduction (Nickle *et al.*, 2007; Radu *et al.*, 2008). This signal from the outer plexiform layer (OPL) is transduced through the dendrites of bipolar cells and horizontal cells in the inner nuclear layer (IPL) (Wässle, 2004). Horizontal cells are neurons that control lateral interactions within the retina (Schubert *et al.*, 2010). The bipolar cells then transmit the signal via their dendrites to another type of neuron, the amacrine cells; they belong to the interneurons, in the inner plexiform layer to the ganglion cell layer (Purves *et al.*, 2001). Whereby the ganglion cells transmit the signal via their axons to the optic nerve though the signal reaches the brain (Dhande *et al.*, 2014; Tian, N. *et al.*, 2003).

In addition to the five major groups of neurons that structure the different layers of the retina, the retina has other essential cells that maintain and function the retina (Masland, 2001). In the mammalian retina there are among others the glial cells, which include Müller glia cells, astroglia cells and microglia cells (Vecino *et al.*, 2016). Müller glia cells, along with neurons, are the most abundant cell types in the retina. They are elongated, slightly branched cells that extend from the inner to the outer retina and contribute to the boundary of the retina. In addition to providing stability to the retina, Müller cells are also responsible for supplying the ganglion cells and removing excess metabolites. Müller cells are thought to have light-conducting functions as well, allowing light to pass through unobstructed (Bringmann *et al.*, 2006; Newman, E. *et al.*, 1996; Vecino *et al.*, 2016). Microglia are probably the most abundant glial cells in the mammalian retina. Mainly, these cells are located in the nerve fiber layers and are involved in supplying neurons via blood vessels (De Hoz *et al.*, 2016; Vecino *et al.*, 2016). Mostly, microglia are directly connected to neurons, assume the fluid regulation of the brain and are significantly involved in the development of the blood-retinal barrier (Nickle *et al.*, 2007; Vecino *et al.*, 2016). Microglia are the smallest group of glial cells, accounting for approximately 20% of all glial cells. As cells of the central nervous system, microglia undertake phagocytosis, antigen presentation and have an impact on

embryonic development. They possess highly branches with which they scan their environment (Langmann, 2007; Silverman *et al.*, 2018).

1.2 Age-related macular degeneration

Age-related macular degeneration (AMD), the most common chronic eye disease in the western world, predominantly affects people over 55 years and impairs vision (Congdon *et al.*, 2004). Around 50% of age-related blindness is due to AMD. Worldwide, it affects about 170 million people (Pennington *et al.*, 2016). First signs of the disease can be detected by optical coherence tomography (OCT) even before there are limitations in vision (Cicinelli *et al.*, 2018). However, if both eyes are affected, there are often severe limitations in central vision (Coleman *et al.*, 2008). As the name implies, this eye disease affects the macula, which is the point of the sharpest vision in the retina (Fig. 2). The cells in the macula affects the central vision, that enables face recognition and the ability to read books (Mitchell *et al.*, 2018). In AMD, faces are perceived more and more distorted and the ability to see colors and contrasts are impaired, until finally the impairment is significant that only a black spot can be perceived in the central field of vision (Komatsu, 2006).

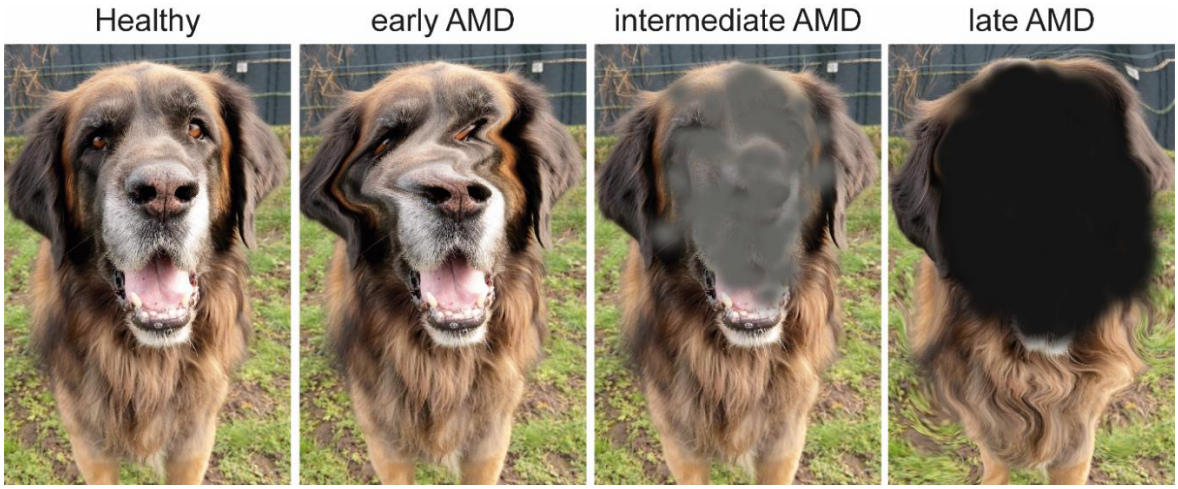

Figure 2: Perception of the different stages in AMD patients. At the beginning of AMD, early AMD, images/faces are often perceived distorted. In the further course, intermediate AMD, blind spots appear and colors are no longer recognized. The late AMD is characterized by loss of vision in the central area.

There are two different forms of AMD, wet and dry AMD, but even within the forms are distinguished among different levels. Characteristic for dry AMD, which occurs most

frequently, are deposits of metabolic products also called drusen, locating directly under the macula. Drusen contain proteins, lipids and other cellular components (Johnson *et al.*, 2001; Mullins *et al.*, 2001). The presence of drusen alone rarely affects vision initially, is asymptomatic, and is common in the population (Cohen *et al.*, 2007; Fritsche *et al.*, 2014; Klein *et al.*, 1992). In the case of AMD, these drusen become more frequent and increase in size during the course of the disease, leading to an undersupply of the retinal center by this the cells of the retina can no longer be adequately supplied (Green *et al.*, 1985). Progressively, dry AMD leads to a loss of function in the macula and slowly destroys vision in the central visual field. This is a result of degeneration of photoreceptor cells and choriocapillaris, as well as loss of RPE cells (Mcleod *et al.*, 2009). The advanced form of dry AMD is also known as geographic atrophy (GA), progresses very slowly, and affects approximately 90% of all AMD patients (Danis *et al.*, 2015; Mcleod *et al.*, 2009). Wet AMD is less common than dry AMD, but it progresses more rapidly. This form is characterized by choroidal neovascularization (CNV). There is pathological ingrowth of blood vessels from the choriocapillaris through Bruch's membrane into the subretinal space of the retina. Leaky blood vessels lead to accumulation of vascular fluids or hemorrhage, causing swelling and scarring in the retinal area, which can quickly lead to distorted vision and blind spots in the visual field (Colijn *et al.*, 2017; Mcleod *et al.*, 2009). Although both forms of AMD have a very different clinical presentation, it can not be ruled out that they occur bilaterally (Joachim *et al.*, 2017).

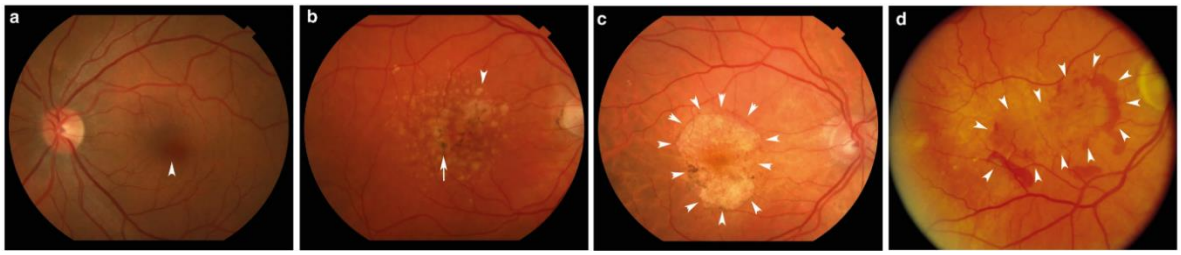

Figure 3: Fundus images of AMD patients in different stages. **a** Fundus image of a healthy macula. Fovea is marked with an arrowhead. **b** Fundus image of a patient in the intermediate stage of dry AMD. Arrowhead points to druse, arrow to RPE hyperpigmentation. **c** Fundus image of geographic atrophy (GA). Arrowheads point to GA. **d** Outline of neovascular AMD. Arrowheads indicate choroidal neovascularization. Images from Handa *et al.*, 2019.

A therapy exists so far only for the advanced stage of the wet AMD. With a timely diagnosis and the right therapy, the progression of the disease can be slowed or even stopped. Therapy options such as intravascular administration of anti-vascular endothelial growth factor A

(anti- VEGF-A) slow down the pathological ingrowth of blood vessels in many patients (Freund *et al.*, 2015; Kozhevnikova *et al.*, 2018). In contrast, there are no approved treatment options for dry AMD to date, which is why early detection to avoid risk factors is particularly important. Risk factors include smoking, sun exposure, UV light and diet (Armstrong *et al.*, 2015). These factors can be positively influenced and reduce the risk of developing AMD or living with a progressive form. Smoking is one of the biggest risk factors to be influenced. Depending on the frequency and intensity of smoking, smokers additionally increase their risk, as increased smoking increases oxidative stress and decreases the antioxidant response of immune cells. This results in smokers being up to two to three times more likely to be affected by AMD than non-smokers (Armstrong *et al.*, 2015; Espinosa-Heidmann *et al.*, 2006; Khan *et al.*, 2006). Excessive exposure of UV light should also be avoided, as this can damage not only the skin but also the retina, including the macula. A balanced healthy diet is also essential as well as a healthy weight, also taking supplements can affect the progression of AMD if micronutrients are deficient (Andriessen *et al.*, 2016; Parekh *et al.*, 2009; Schmidl *et al.*, 2015; Singh *et al.*, 2017). Diseases such as hypertension and diabetes mellitus are often associated with eye diseases, including AMD. For this reason, it is necessary to minimize the effects of the diseases. Well-controlled patients significantly reduce their risk of developing eye disease (Chakravarthy *et al.*, 2010; Chen, X. *et al.*, 2014; Joslin *et al.*, 2017). It is important to minimize these factors, as there are also some risk factors that cannot be controlled. These include genetic makeup, age, gender, skin and eye color. The genetic makeup plays a key role in the susceptibility to AMD. It is known that changes on the long (q) arm of chromosome 10, in the region 10q26, where the genes ARMS2 and HTRA1 are encoded, result in an increased risk of AMD. It is unclear whether both genes are related to the onset of the disease or only one of the two genes, as they are so close to each other that this has not yet been clearly established. These two genes are only a small part of the 52 known gene variations that have been linked to AMD. Most known genes that are related to retinal homeostasis, are involved in the formation of complement components, or are involved in inflammatory processes (Deangelis *et al.*, 2017; Francis *et al.*, 2009; Fritsche *et al.*, 2016; Fritsche *et al.*, 2008; Thakkinstian *et al.*, 2008). Crucially, the regulation of the innate immune response is also controlled by the complement system. The innate immune response, in addition to the adaptive immune response, is designed to respond to organisms and substances classified as foreign antigen. This can be achieved by a rapid and efficient engagement of a range of soluble factors and cell types against the foreign bodies. Dysregulation/overreaction of this immune response leads to an excessive

response of immune cells, such as microglial cells, and the complement system (Fritsche *et al.*, 2014; Gupta, N. *et al.*, 2003). With aging, the risk of developing AMD increases, approximately 20% of all 65- to 74- years- olds suffer from an early form of AMD. By the age of 75 to 84 years, 84% of these are also affected by more severe forms of AMD (Armstrong *et al.*, 2015; Klein *et al.*, 1992). This can be explained by increasing age, oxidative stress is increased and the function of photoreceptors and RPE cells disturbed, making the retina more susceptible to injury and often degeneration (Beatty *et al.*, 2000; Winkler *et al.*, 1999). Women are more likely to develop AMD than men; they have a 1.3-fold increased risk of developing AMD. Likewise, ethnic origin plays a decisive role, as light-skinned people and people with blue eyes are more susceptible than dark-skinned people with dark eyes (Beatty *et al.*, 2000; Winkler *et al.*, 1999).

1.3 Microglia-immune cells of the brain and retina

1.3.1 Microglia in the central nervous system

Microglia cells are resident immune cells of the central nervous system (CNS) and represent the endogenous distribution network and immune system of the brain and retina. In addition to their role in the immune defense of the brain, they are also essential for various homeostatic functions, such as the development and maintenance of functional neural networks. In prenatal development, microglia control development of neural progenitor cells in the cerebral cortex and their degradation during neurogenesis, as well as for the connectivity of the forebrain. Postnatal, they are responsible for the formation and elimination of synaptic contacts and dendritic projections (Wake *et al.*, 2013; Wu *et al.*, 2015).

The BBB normally prevents immune system cells from entering the central nervous system. It precisely regulates the exchange of ions, molecules and cells between the blood and brain, protecting the CNS from toxins and pathogens (Daneman *et al.*, 2015). However, in case of blood vessel injuries and severe diseases, immune cells reach the CNS (brain and spinal cord). The brain and spinal cord are nevertheless protected from pathogens all the time, by a certain type of glial cell, the microglia, which take over the role of immune cells in the central nervous system (Ginhoux *et al.*, 2010; Streit *et al.*, 2005). These cells perform the same function as macrophages, which belong to the leukocytes and are also called

phagocytes, in the immune system. Their task is to recognize and absorb pathogens and dead cells (Ajami *et al.*, 2007; Persidsky *et al.*, 1999).

Initially, they were associated with phagocytes in the blood, but it has been found that they differ in origin and development (Kierdorf *et al.*, 2013). Microglia develop very early in embryonic development from embryonic stem cells, whereas phagocytes (white blood cells) develop from bone marrow stem cells (Nayak *et al.*, 2014). These cells are therefore referred to as a separate cell class (Kierdorf *et al.*, 2013) and are derived from primitive yolk sac macrophages migrating into the brain (Ginhoux *et al.*, 2016; Hoeffel *et al.*, 2012). The precursor cells from the yolk sac pass the blood-brain barrier on embryonic day (E) 8.5- 9.5 before the BBB is fully developed, and from E 11.5 microglia are found in the retina. (Fig. 4) (Ginhoux *et al.*, 2010; Li *et al.*, 2019). After immigration, the microglia remain in the tissue for the rest of their lives, where they maintain the population by means of self-control (Hashimoto *et al.*, 2013). However, not all microglia have the ability of self-control in the brain, as new microglia are produced from non-microglia precursor cells. In the retina, there are also only a few microglia that have the ability of expansion and renewal. Although, microglia from the postnatal (P) 0 time point migrate from the optic nerve into the retina and take over the function of previously duplicated microglia (Fig. 4) (Elmore *et al.*, 2014; Huang, Y. *et al.*, 2018). Furthermore, the development of microglia is dependent on several factors such as the key transcription factor of the erythroblast transformation specific (Ets) family, PU-Box-Binding -1 (PU.1) (Rosenbauer *et al.*, 2007), the interferon-regulator 8 (Irf8) (Kierdorf *et al.*, 2013; Minten *et al.*, 2012) and transcription factor Runt-related transcription factor 1 (Runx1) (Zusso *et al.*, 2012), which influences the differentiation of microglia. In addition, CSF1R seems to be important for the architecture of the brain, as embryos from knockout (KO) mice show deficits in brain development. (Erblich *et al.*, 2011).

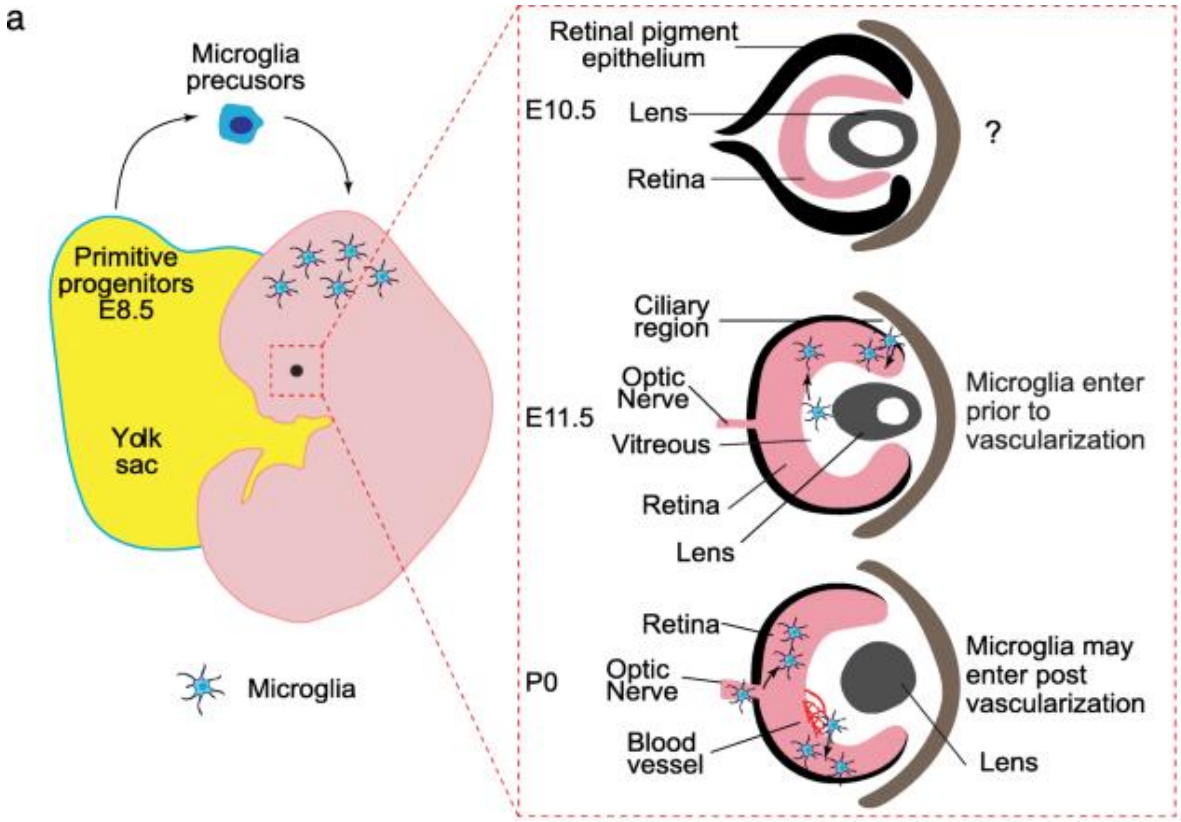

Figure 4: Microglia development in the mouse retina. Microglia develop in the yolk sac from primary yolk sac precursor cells and enter the CNS via the blood circulation and thus the brain and retina. First microglia are already found from E11.5 in the retina, where they organize themselves within the different layers of the retina. From P0 onwards, microglia migrate exclusively via the optic nerve. Image from Li *et al.*, 2019.

1.3.2 Resting microglia and homeostatic function

In the healthy brain, there are hundreds of thousands of microglia in the retina, 5-20% of all glial cells are microglia and stay in a ramified form under physiological conditions (Langmann, 2007; Pelvig *et al.*, 2008; Rathnasamy *et al.*, 2019). This form is characterized by a small cell body and numerous small flexible branches extending in different directions. The cell bodies remain largely in one place, with only the branching extensions of the microglia moving and continuously scanning the surrounding tissue (Huang, T. *et al.*, 2012; Langmann, 2007). These are flexible, and the branches move forward unless they hit a synapse where they linger for a while before moving on. A microglia cell controls a large area (Marinelli *et al.*, 2019). Therefore, this cell type forms an effective distribution network, which recognizes small changes within the shortest time and tries to eliminate them.

During development, in the still-maturing retina, microglia are responsible for phagocytosis of cell debris, control developmental apoptosis, and formation of neuronal connections (Marín-Teva *et al.*, 2004; Sierra *et al.*, 2010). Moreover, microglia are not only essential in neuronal development and population of the retina, but also in the formation of new neuronal circuits (Bialas *et al.*, 2013; Schafer *et al.*, 2012). This is already being studied by Paolicelli and colleagues, showing that the development of new synapses is underdeveloped in mice that do not express C-X3-C Motif Chemokine Receptor 1 (fractalkine receptor) (CX3CR1) (Paolicelli *et al.*, 2011). Microglia eliminates unnecessary synaptic connections. But this function is dependent on several factors. Furthermore, complement regulators such as complement component 1q (C1q) secreted by retinal ganglion cells activate the classical complement cascade, causing complement factor 3 (C3) to bind with complement receptor 3 (C3aR) and trigger phagocytosis (Bialas *et al.*, 2013; Schafer *et al.*, 2012). It is thought to be associated with the orientation of primary retinal vessel growth (Provis, 2001; Schafer *et al.*, 2012). Microglia colonize the retina before vessel formation begins and are associated with endothelial cells at the vessel front, resulting in interactions between these cells and promoting vessel growth (Checchin *et al.*, 2006; Rymo *et al.*, 2011). Moreover, studies have shown that the absence of microglia has a negative effect on CNS vascularity, but can be remedied by replenishing microglia through intravitreal injections (Arnold *et al.*, 2013; Checchin *et al.*, 2006).

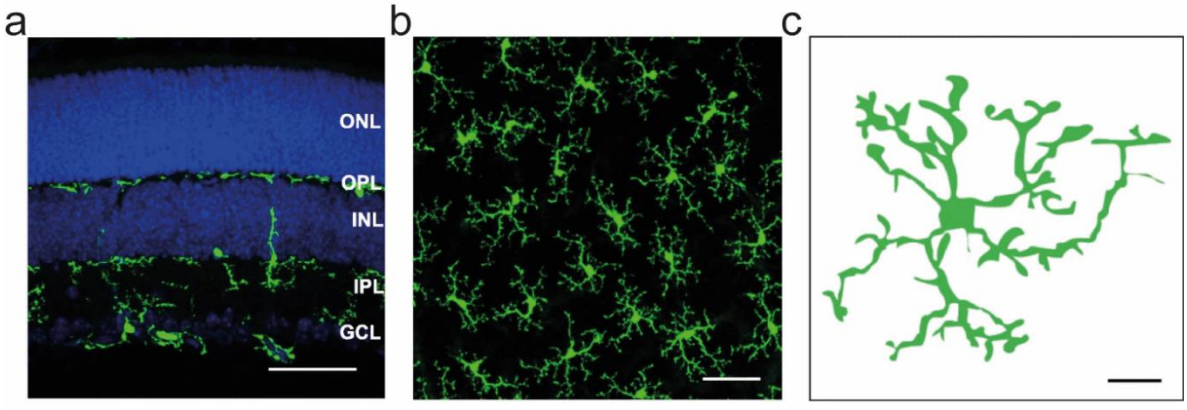

Figure 5: Spatial distribution of microglia in mouse retina. **a** Representative cross-sections of mouse retina in CX3CR1$^{GFP/+}$ mice. Microglia are labeled with GFP so that staining is no longer necessary. Microglia are located here in the IPL, OPL and in the GCL. Scale bar 50 µm. **b** Microglia morphology in retinal flat mount preparations of CX3CR1$^{GFP/+}$ mice. Scale bar 50 µm. **c** Representation of a single microglia reconstructions from whole mount. Scale bar: 10 µm. Image from Li *et al.*, 2019.

In the healthy adult retina, microglia are located in the plexiform layers (Fig. 5a) from where they monitor the retina. The cells are evenly distributed and do not overlap (Hume *et al.*, 1983; Karlstetter, M. *et al.*, 2015). Under homeostatic conditions, microglia remain in these layers, display a ramified morphology (Fig. 5b, c) and exclusively move their extensions to scan the environment (Damani *et al.*, 2011; Hume *et al.*, 1983; Langmann, 2007). This dynamic movement serves to maintain various processes, including homeostatic regulation of neuronal activity, removal of cellular debris and metabolic waste products (Nimmerjahn *et al.*, 2005; Wang *et al.*, 2016). Microglia regulate cytokines, chemokines, complement regulators, antibodies and detect cell changes by means of their surface proteins (Karlstetter, M. *et al.*, 2015; Kierdorf *et al.*, 2013). Since there are many factors that reactivate microglia, it is important that mechanisms exist that regulate microglia activation. One essential inhibitory receptor is the microglia-specific receptor cluster of differentiation-200 receptor (CD200R), which retains microglia in the resting state by binding to the transmembrane glycoprotein cluster of differentiation-200 (CD200) (Broderick *et al.*, 2002; Deckert *et al.*, 2006; Nathan *et al.*, 2001). In addition, binding causes an inhibitory intracellular signaling cascade to be triggered, preventing a conformational change in microglia and the associated pro- inflammatory immune response (Hoek *et al.*, 2000). Another inhibitory regulator is fractalkine (CX3CL1) which is released from neurons and endothelial cells and thereby inhibits neurotoxicity of microglia (Cardona *et al.*, 2006). As well as binding of translocator protein 18 kDa (TSPO) with its endogenous ligand diazepam binding inhibitor (DBI) also limits microglia activity (Karlstetter, M. *et al.*, 2014; Scholz *et al.*, 2015a).

1.3.3 Microglia in retinal damage

The immune system of the retina is strictly regulated and largely isolated from the rest of the body. The immune privilege of the eye, created by the existing blood-retinal barrier, makes it difficult for pathogens and other immune cells to invade (Carson *et al.*, 2006). If the surrounding tissue is injured or another type of damage is present, the innate immune system, microglia and complement system, of the retina responds (Chen, M. *et al.*, 2019; Xu *et al.*, 2009). Importantly, microglia activation is tightly regulated and highly complex with many receptors mediating the signals.

In general, these signals are divided into "Off" and "On" signals (Biber *et al.*, 2007). "Off" signals favor the activation of the cell by absent substances. Here, a substance that is actually

present in the tissue is either reduced in concentration or no longer present. If the substrate is present, this conveys a signal to the microglia that it can remain inactive (Deckert *et al.*, 2006; Hoek *et al.*, 2000; Karlstetter, M. *et al.*, 2015). "On" signals are signals that were not there before or are suddenly present in a greatly increased concentration. Using various pattern recognition receptors (PRR), which includes toll-like receptors (TLR), microglia recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). This leads to microglia activation and trigger an immune response (Hickman *et al.*, 2013) which include cell wall components of bacteria such as lipopolysaccharides (Arioz *et al.*, 2019; Orihuela *et al.*, 2016). As well as other molecules that trigger chemotactic movement including adenosine triphosphate (ATP), chemokines, cannabinoids, and bradykinin (Badimon *et al.*, 2020; Inoue, 2006). In addition, microglia possess purinergic receptors that respond to nucleotides released by neurons (Burnstock *et al.*, 2011; Calovi *et al.*, 2019; Illes *et al.*, 2020).

Once microglia perceive damage, they leave their site of origin and change their shape from ramified to amoeboid. The cells almost completely retract their extensions, become round cells, and begin to secrete a series of molecules (Fig. 6) (Jurgens *et al.*, 2012). Amoeboid cells are flexible and can easily move around in the tissue. Microglia migrate to an injured site, germs or pathogens. In the case of photoreceptor damage, microglia travel to the ONL and subretinal space (Karlstetter, M. *et al.*, 2015; Lew *et al.*, 2020). At the lesion side, these cells can either activate and attract other cells through signaling or phagocytose dead cells, cellular debris, pathological protein aggregates, and bacterial and viral pathogens (Jurgens *et al.*, 2012; Karlstetter, M. *et al.*, 2015). In addition, these cells can even destroy bacteria and other invaders through the release of immune mediations, such as oxygen and nitrogen radicals and cytokines (Jurgens *et al.*, 2012). Reactivated amoeboid microglia secrete pro-inflammatory cytokines in large quantities. These cytokines include tumor necrosis factor alpha (TNF-a), interleukin 1 beta (Il-1ß), and chemokine (C-C motif) ligand 2 (Ccl2) (Balzano *et al.*, 2020; Cherry *et al.*, 2020; Scholz *et al.*, 2015a; Swaroop *et al.*, 2018). Ccl2 is a chemokine that not only promotes the release of other molecules, but also activates microglia and attracts them to the site of damage (Ferreira *et al.*, 2012; Hinojosa *et al.*, 2011). The release of oxygen radicals from microglia leads to neuronal damage and death (Park *et al.*, 2015; Simpson *et al.*, 2020).

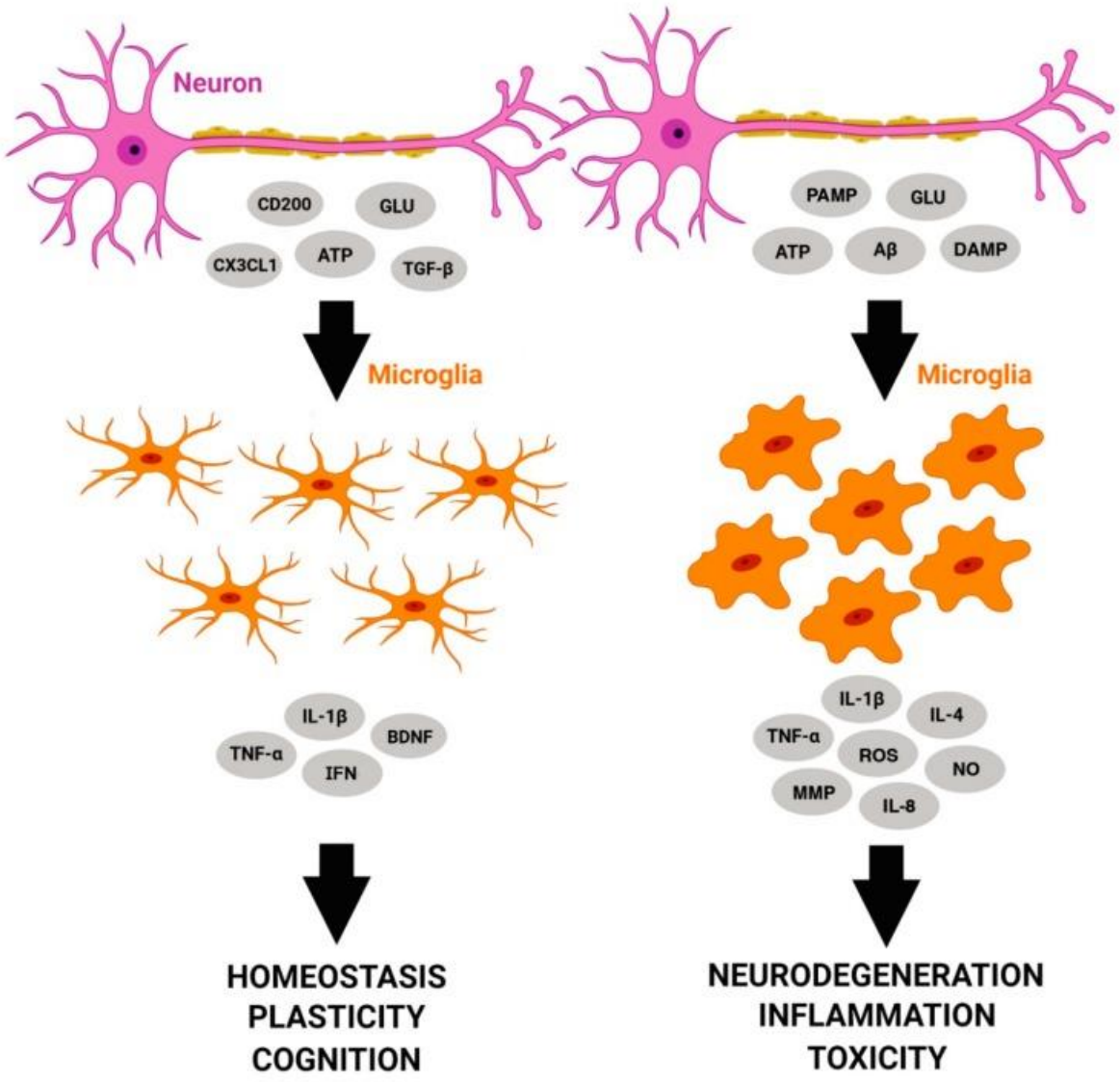

Figure 6: Microglia react to different molecules in their environment, e.g., molecules released by neurons. Anti-inflammatory molecules are CX3CL1, CD200, glutamate, ATP and TGF-β, which signal to the microglia that everything is fine and let them stay in homeostasis. In this state, microglia secrete TNF-α, IL-1β, IFN and BDNF, among others. In a damaged state, there is a release of ATP, glutamate, threat-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), and amyloid-beta proteins that cause microglia to reactivate and release pro-inflammatory cytokines, such as IL-1β, TNF-α, IFN-γ, IL-4, ROS, NO, IL-8, and MMP. If this state persists for a prolonged period, it promotes toxicity, neuroinflammation and neurodegeneration. (Augusto-Oliveira et al., 2019)

In addition to their activity in maintaining retinal homeostasis, microglia are particularly important in aging. During aging, changes in the immune system occurs, leading to increased tissue stress and damage. Aging or degenerative diseases may lead to a dysregulation and

chronical activation of microglia. Compared to naïve microglia, aged microglia eventually transition from a balanced activation state to a hyperreactive state, which may persist for an extended period of time and cause damage (Buchanan *et al.*, 2008; Chen, M. *et al.*, 2019; Sierra *et al.*, 2007; Xu *et al.*, 2009). If this state persists, greater amounts of pro-inflammatory mediators are released, exacerbating diseases (Karlstetter, M. *et al.*, 2015). In the case of AMD, activated microglia have been associated with the formation of drusen in the early AMD stage (Killingsworth *et al.*, 1990). Interestingly, the drusen components provide a chemotactic stimulus, causing microglia to become increasingly activated and migrate to the site of the drusen accumulation. Therefore, in patients with geographic atrophy, many amoeboid microglia are also found in the granular layers and subretinal space (Fig. 7) (Gupta, N. *et al.*, 2003; Penfold *et al.*, 2001). Microglia in the ONL usually phagocytose apoptotic photoreceptors, but dysfunction and chronical activation also kills surrounding healthy but stressed photoreceptors (Zhao *et al.*, 2015). However, transcriptional analysis has shown that AMD patients have elevated chemokine levels and an excess of complement regulators and inflammatory genes (Newman, A. M. *et al.*, 2012).

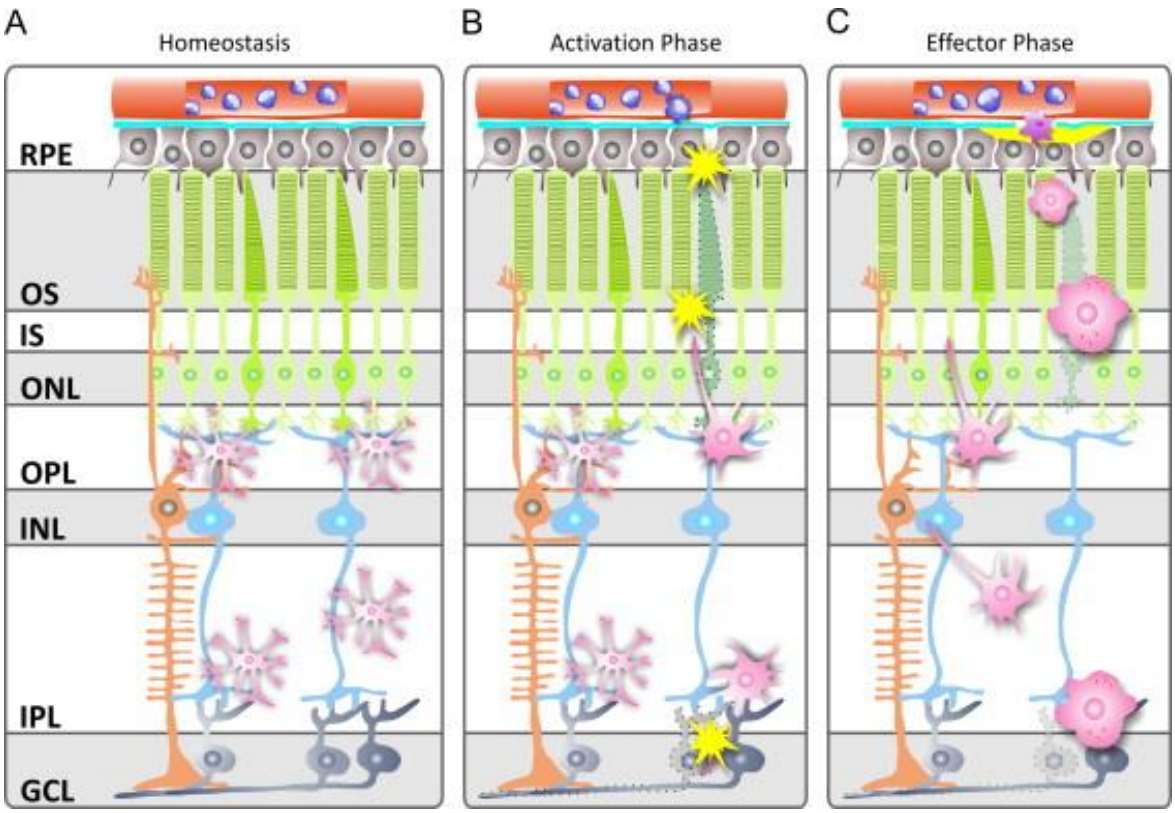

Figure 7: Schematic representation of microglial activity in the retina. **a** Under homeostatic conditions in retina, resident microglia mainly occupy the plexiform layers (IPL and OPL). With

their extensions, they scan the surrounding tissue, pick up cell debris, and secrete a variety of supporting mediators, including neurotrophins. **b** Stimuli in the other cell layers, here RPE, OS, and GCL, lead to activation of resident microglia. **c** Retinal microglia and recruited progenitor cells migrate to the damage site and develop into amoeboid phagocytes. RPE: retinal pigment epithelium; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Image from Karlstetter *et al.*, 2010 (Karlstetter, Marcus *et al.*, 2010).

1.3.4 Microglia as a therapeutic target

Excessive reactivity of microglia and associated immune activation is a common phenomenon in neurodegenerative diseases. Few retinal diseases have an exclusively genetic trigger, so modulation of microglia activity could be a targeted therapy option for retinal diseases (Amor *et al.*, 2014; Karlstetter, M. *et al.*, 2015; Langmann, 2007; Scholz *et al.*, 2015b). Early targeted therapy that inhibits microglia activity could decrease the progression of some degenerative diseases. Several studies are known to deplete microglia via CSF1R inhibition, thereby reducing neuroinflammation in numerous retinal diseases. When microglia are eliminated using CSF1R inhibitor, PLX5622, fewer plaques can be detected in Alzheimer's disease mouse models (Spangenberg *et al.*, 2019). Increased proliferation of microglia and the associated increase in pro-inflammatory cytokines were also detected in Parkinson's disease patients and mouse models. Inhibition of CSF1R, with GW2580, also shows a decrease in pro-inflammatory cytokines in mouse models (Neal *et al.*, 2020). However, CSF1R inhibition is mostly specific to microglia and also affects other macrophages. Depletion from these cells has a major impact on homeostasis (Lei *et al.*, 2020) and, when inhibited during embryonic development, affects the behavior of female adult mice (Rosin *et al.*, 2018). Furthermore, depletion of microglia can lead to transient immune deficiency and degeneration of synapses (Parkhurst *et al.*, 2013; Wang *et al.*, 2016). According to these reasons, immunomodulation of existing microglia is often targeted rather than microglia depletion. Microglia are attempted to be modulated in such a way that the immune response is reduced, but their homeostatic properties remain present. More and more therapeutic options are being explored, with injected molecules designed to inhibit activating signaling pathways or in case that signal cells return to their resting initial position (Akhtar-Schäfer *et al.*, 2018; Rashid *et al.*, 2018). Minocycline, which blocks the TLR-2 and TLR-4 signaling pathways, has been shown an immune inhibitory effect on microglia (Hu

et al., 2014; Teng *et al.*, 2004). This drug is already used as an antibiotic in acne and arthritis (Garrido-Mesa *et al.*, 2013), with the ability to cross both the BBB and the BRB (Domercq *et al.*, 2004). Additionally, it has been shown to inhibit the release of pro- inflammatory cytokines to microglia proliferation and migration (Teng *et al.*, 2004). While docosahexaenoic acid (DHA), a plasma membrane phospholipid, decreases microglia activity, it has been shown to significantly reduce retinal degeneration in a mouse model of X-linked retinoschisis (Bazan *et al.*, 2010; Ebert *et al.*, 2009; Karlstetter, M. *et al.*, 2015). Furthermore, the administration of TSPO-ligands serves as a potential therapeutic option. The binding of TSPO to its ligand signals the cell that activation is not necessary and prevents an immune response (Karlstetter, M. *et al.*, 2014; Wolf *et al.*, 2020). Moreover, the application of exogenous Il-1β results in a reduction of microglia activation, whereby blocking the signaling pathway reduces the release of pro-inflammatory cytokines (François *et al.*, 2013; Shi *et al.*, 2012).

1.4 Galectin-3

1.4.1 Structure and localization of galectin-3

Galectin-3 belongs to the group of galectins. This group is part of the protein family animal lectins, which are equipped by means of a highly conserved carbohydrate recognition domain (CRD). This CRD is specific for ß-galactose containing oligosaccharides (Barondes *et al.*, 1994; Cooper, Douglas N.W. *et al.*, 1999; Rabinovich, Gabriel A. *et al.*, 2002). The CRDs usually contain 130 amino acids, which are responsible for carbohydrate bonds (Cooper, Douglas N. W., 2002). So far, 15 galectins are known in mammals, possessing a CRD. Some galectins, galectin-4, -6, -8, -9 and -12, have a second homologous CRD. There, both domains are separated in a single polypeptide chain only by a linker consisting of 70 amino acids. Among the galectins that have only one CRD (galectin-1, -2, -3, -5, -7, -10, -11, -13, -14 and -15), galectin-3 is unique in having a 120 amino acid sequence long N-terminal region (Brinchmann *et al.*, 2018). The N-terminal domain of galectin-3 is associated with the CRD, thus the only chimera is considered to be galectin in vertebrates (Fig. 8) (Barondes *et al.*, 1994; Di Lella *et al.*, 2011). Although it is usually present as a monomer, it can form a pentamer (oligomer) with other galectin-3 proteins in association with the N-

terminal domain (Kasai *et al.*, 1996). But even as a monomer, it has multivalent binding properties (Knibbs *et al.*, 1993).

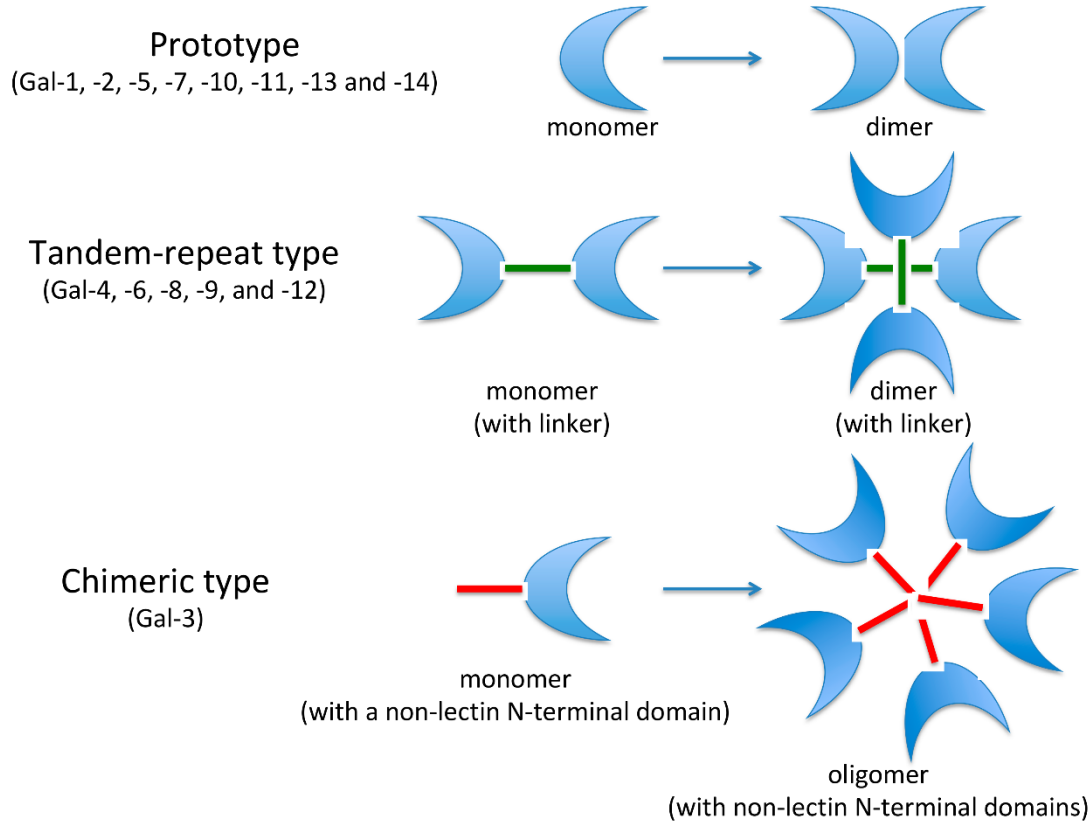

Figure 8: Schematic representation of the structure of galectins. Galectins are classified into three structural types: Members of the galectin family are divided into three types: 1. The prototype, which includes galectin-1, -2, -7, -10, -11, -11, -13 and -14. This type has a single carbohydrate recognition domain (CRD). 2. The tandem repeat type, to which galectin-4, -6, -8, -9 and -12 belong, is characterized by 2 CRDs connected by a linker. 3. The chimeric type, exhibited by only one galectin, galectin-3, with one CRD and one N-terminal domain. Some galectins have the ability to assemble into dimers or oligomers. Illustration from Sciacchitano *et al*. 2018 (Sciacchitano *et al.*, 2018)

Galectin-3 is a 29-35 kDa protein that is also known as Mac-2, εBP, RL-29, HL-29, L-34, LBP, or CBP-35 (De Boer *et al.*, 2009). Human galectin-3, (*LGALS3*) is localized on chromosome 14 locus 21-22 (Raimond *et al.*, 1997). The mouse gene (Lgals3) has the same structure and is 6.4 kb long (Fig. 9). The gene has six exons and five introns, of which exon 1 and 2 encode for the 5'untranslated region (UTR) of the mRNA. Exon 3 is responsible for encoding the N-terminal domain and exon 4 for the CRD. (Kadrofske *et al.*, 1998). *LGALS3* gene in humans contains several regulatory elements: five putative Sp1 binding sites (GC

boxes), five cAMP-dependent response element (CRE-motifs), four AP-1 and one AP-4-like site, two NF-κB-like sites, one sis-inducible (SIE) element, and one consensus basic helix-loop-helix (bHLH) core sequence (Dumic *et al.*, 2006; Kadrofske *et al.*, 1998). It has two antiparallel ß-sheets consisting of five or six ß-strands. With a sequence similarity of 20-25%, galectin-3 resembles the homodimer galectin-1 and -2 (Seetharaman *et al.*, 1998).

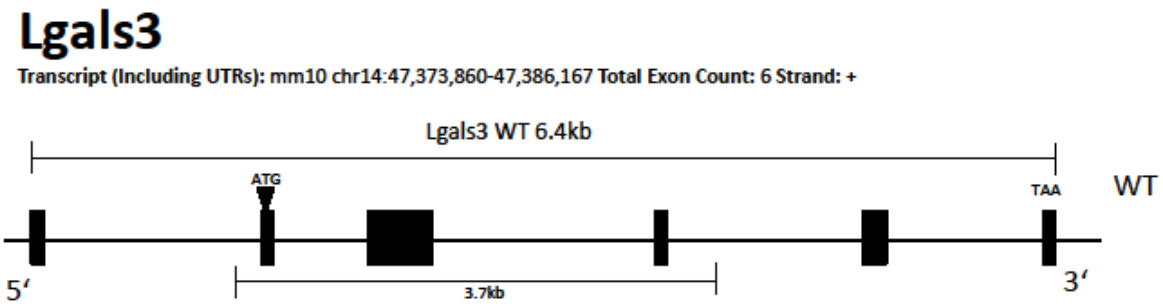

Figure 9: Gene structure of galectin-3. The mouse gene, *Lgals3*, is about 6.4 kb long, is characterized by five introns and six exons (black boxes). UTR: 5'untranslated region; WT: wild type.

The ability to bind carbohydrate is also present in galectin-3. The preferred ligand of galectin-3 is N-acetyllactosamine (LacNAc, Galβ1,4(3)GlcNAc) (Dumic *et al.*, 2006). Furthermore, galectin-3 has the ability to incorporate oligosaccharides as well as polylactosaminoglycans through an extended binding site (Seetharaman *et al.*, 1998; Vlassara *et al.*, 1995). In addition, galectin-3 binds to TLR4, triggering receptor expressed on myeloid cells 2 (TREM2), desialylated neurons, which it opsonizes via Mer tyrosine kinase for phagocytosis (Puigdellívol *et al.*, 2020). It is formed by reactivated microglia and it also activates microglia (feedback loop) (Puigdellívol *et al.*, 2020; Stapleton *et al.*, 2012).

In adult tissues, galectin-3 expression was detected predominantly in epithelial cells and myeloid cells. In numerous epithelial cells, such as in numerous epithelial cells, such as: epithelial cells of the small and large intestine, cornea and conjunctiva, kidney, lung, thymus, breast and prostate (Castronovo *et al.*, 1996; Dumic *et al.*, 2006; Flotte *et al.*, 1983; Gupta, S. K. *et al.*, 1997; Kasper *et al.*, 1996; Lotz *et al.*, 1993). Moreover, galectin-3 has been associated with immune cells. Neutrophils, eosinophils, basophils, mast cells, Langerhans cells, dendritic cells, monocytes and macrophages in various tissues express galectin-3 (Dietz *et al.*, 2000; Flotte *et al.*, 1983; Frigeri *et al.*, 1993; Kasper *et al.*, 1996; Liu *et al.*, 1995; Maeda *et al.*, 2003; Saada *et al.*, 1996; Smetana *et al.*, 1999; Truong *et al.*, 1993a;

Truong *et al.*, 1993b; Wollenberg *et al.*, 1993). Disease-related expression can also be initiated in lymphocytes, as well as in tumor cells (Danguy *et al.*, 2002; Van Den Brûle, F. *et al.*, 2002). During mouse embryogenesis, tissue and time-dependent expression was detected. The expression already starts at the E 4 (Poirier *et al.*, 1993). Notochord, galectin-3 is expressed at day E 8.5 to E 11.5 (Fowlis *et al.*, 1995) and later in the vertebrae, ribs and facial bones, epidermis, endodermis, and esophagus. In addition, punctate expressions were detected in liver and lung, which could be associated with macrophages (Dumic *et al.*, 2006; Van Den Brûle, F. A. *et al.*, 1997).

LGALS3 is synthesized at free ribosomes in the cytoplasm and is found in many different areas of the cell. It has been detected in the nucleus as well as on cell surfaces and in the extracellular space (Dumic *et al.*, 2006; Krześlak *et al.*, 2004). The exact transport away from the intracellular to the extracellular space has not yet been clarified, but it is known that this does not occur via the endoplasmic reticulum and the golgi apparatus. Since the protein lacks signal sequences for transfer via the golgi apparatus and the endoplasmic reticulum as well as for entry into the classical signaling pathway, it is not possible to determine the exact pathway (Menon *et al.*, 1999). Lactose-dependent endocytosis is thought to facilitate transport from the extracellular space to the intracellular space (Furtak *et al.*, 2001). However, the transport away from the cell nucleus has also not yet been clarified.

1.4.2 Galectin-3 in neurodegenerative diseases

When neurons lose function or structure, this is called neurodegeneration, which is a pathological condition of the nervous system. This condition favors the development of diseases such as Alzheimer's disease, AMD, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (Chen, W. W. *et al.*, 2016; Dorothée, 2018). The exact causes of neurodegeneration are not understood. Among the factors that promote neurodegenerative diseases are prolonged inflammatory reactions, genetic factors, mitochondrial stress and oxidative stress (Amor *et al.*, 2010). Inflammatory responses are caused by noxious stimuli that cause activation of glial cells (astrocytes and microglia). These cells try to eliminate and reduce structural damage and loss of function of neurons (Amor *et al.*, 2010). Regulation functions until acute inflammation becomes chronic and dysregulation and overreaction of glial cells leads to the development of neurodegenerative diseases (Glass *et al.*, 2010; Ramírez Hernández *et al.*, 2020). It was found that during neurodegeneration, glycan

expression in neurons is altered, which is why protein-glycan interactions are thought to be involved in the pathological condition (Espinosa *et al.*, 2001; Ramos-Martinez *et al.*, 2018). Therefore, proteins that interact with glycans are thought to have effects on neurodegenerative diseases. Proteins such as lectins, to which galectins also belong, interact with glycans (Cerliani *et al.*, 2017; Liu *et al.*, 2012; Rabinovich, G. A. *et al.*, 2009). The 15 galectins of the galectin family show different immunomodulatory functions in various neurodegenerative disorders, in which they act as regulators and enable the remodeling of damaged tissues (Burguillos *et al.*, 2015; Starossom *et al.*, 2012). The proteins differ in their affinity to bind carbohydrate and also the structure influences the ability to bind ligands (Di Lella *et al.*, 2011; Rabinovich, G. A. *et al.*, 2012). Due to the unique chimeric structure of galectin-3, it is known to modulate microglia and astrocytes through its lectin-glycan interaction, thus contributing to the maintenance of homeostasis under normal conditions (Dhirapong *et al.*, 2009; Liu *et al.*, 2010).

The regulation of galectin-3 expression, in immune cells such as astrocytes, macrophages, microglia, dendritic cells, eosinophils, mast cells, Natural killer cell (NK cells), and activated T and B cells is not yet known. However, because the gene has five CRE motifs and two NF-kB-like sites, regulation is thought to be related to cAMP response element-binding protein (CREB) or the transcription factor NF-κB (Dumic *et al.*, 2006; Kadrofske *et al.*, 1998). In chronic neurodegeneration, the release of interferon (IFN) γ promotes microglia activation and thus secretion of pro- inflammatory cytokines and chemokines. The strength of the response depends on the JAK/STAT signaling pathway and the expression of MHC II, CD86, and iNOS (Jeon *et al.*, 2010; Shin, 2013). The increase in galectin-3 expression mediated by IFN-γ, suggests that galectin-3 is an important component of the inflammatory response cascade. In brain injury models in mice, ionized calcium-binding adapter molecule 1 (Iba-1) positive cells, microglia, and glial fibrillary acidic protein (GFAP)-positive cells, astrocytes, could be co-stained with galectin-3. This co-expression indicates that galectin-3 influences the development of the inflammatory response, proliferation and apoptosis (Burguillos *et al.*, 2015; Sirko *et al.*, 2015; Yan *et al.*, 2009). Expression levels of galectin-3 increase in glial cells with aggravated inflammatory response (Dhirapong *et al.*, 2009). In Alzheimer's disease, depleting galectin-3 in the mouse model for Alzheimer's disease (5xFAD mice) was shown to result in a weaker microglia response. This reduced the amyloid-beta (Aß) load and improved the cognitive behavior of the mice. Depletion of galectin-3 was associated with TLR and TREM2/DAP12 signaling pathway in these models (Boza-Serrano *et al.*, 2019). Detection of galectin-3 in blood serum could be associated with

Parkinson's disease (Cengiz *et al.*, 2019). In cell cultures, the absence of galectin-3 was shown to attenuate peripheral inflammation in Parkinson's disease (Espinosa-Oliva *et al.*, 2021). A proteomic study in AMD patients also shows upregulation of galectin-3 in most forms of AMD. Galectin-3 is most elevated in patients with advanced dry AMD (Yuan *et al.*, 2010). Also, in Huntington patients and in mouse models of this disease, the level of galectin-3 in plasma correlates with the severity of the disease. Mice with elevated galectin-3 shown motor impairments (Siew *et al.*, 2019).

1.4.3 Galectin-3 as a target for immunomodulation

Increased expression of galectin-3 has been frequently associated with the acute course of neurodegenerative diseases. Therefore, galectin-3 may be important as a target for immunomodulation as a therapeutic target. Several studies have demonstrated that loss of galectin-3 reduces disease progression in neurodegenerative diseases. Furthermore, the study of Boza-Serrano, who investigated galectin-3 in a mouse model of Alzheimer's disease, has shown that the loss of galectin-3 slows down the development of the disease (Boza-Serrano *et al.*, 2019). Also, other studies showed a lower deposition of Aβ- plaques in this disease, the increased galectin-3 expression was associated with microglia. Galectin-3 and Iba-1 protein staining co- localization could be detected, these signals partially overlapped with those of amyloid-β- plaques (Tao *et al.*, 2020). In galectin-3 KO mice, in addition to the reduction of amyloid-β- plaques, an increased neprilysin concentration was detected (Tao *et al.*, 2020). Neprilysin is a zinc-dependent endopeptidase that is involved in the degradation of amyloid-β-peptide together with other peptidases (Bayes-Genis *et al.*, 2016; Malfroy *et al.*, 1978).

Unfortunately, knockout is not a viable therapeutic option, so many studies are already using inhibitors that prevent the binding of free galectin-3 to its ligands. This would be a choice for example in diseases with desialylation of neurons. Indirectly, galectin-3 has an effect on the desialylation of neurons because activated microglia have sialidases activity. Galectin-3 is not only produced by microglia but also activates them by binding to Toll-like receptor 4 (TLR4), TREM2, desialylated neurons and opsonizing them via Mer tyrosine kinase for phagocytosis (Boza-Serrano *et al.*, 2019; Burguillos *et al.*, 2015; Caberoy *et al.*, 2012; Nomura *et al.*, 2017; Tao *et al.*, 2020). Therefore, this means that if more galectin-3 is secreted, more microglia will be reactivated and initiate desialylation (Chen, H. L. *et al.*,

2014; Puigdellívol *et al.*, 2020; Shin, 2013). Desialylation can be inhibited in microglia with binding of activating sialic acid receptors (Siglecs) to their ligands. In wet AMD models, a polysialic acid ligand has been shown to inhibit microglial reactivity in the retina (Karlstetter, M. *et al.*, 2017). In section 1.3.5 it was described how microglia react in AMD and that dysfunction of microglia can be expected to play an important role in the progression of AMD. Since it is possible that galectin-3 is an antagonist of the Siglec ligands, it is possible that a therapy with galectin-3 inhibitors can stop the desialylation and positively influence the course of AMD.

Known inhibitors include belapectin (GR-MD-02), GB1107, TD139 (33DFTG), and G3-C12 (TFA). The effect of galectin-3 inhibition has been demonstrated in several disease models. Moreover, the inhibitor belapectin (GR-MD-02) reduces liver fibrosis and hypertension in rats and has already been tested in clinical studies in non-alcoholic steatohepatitis (NASH) patients and patients with hypertension. (Chalasani *et al.*, 2020). Unfortunately, an improvement could not be observed in NASH patients (Harrison *et al.*, 2016). Other studies with belapectin in combination with pembrolizumab, a PD-L1 inhibitor, however, yielded promising results in tumor cell apoptosis (Curti *et al.*, 2021). Another promising inhibitor is TD139, a thio- digalactoside galectin-3 inhibitor that has also been tested in clinical trials for idiopathic pulmonary fibrosis (St-Gelais *et al.*, 2020). This inhibitor also shows in mouse models that activation by galectin-3 in liver progenitor cells is reduced after administration of the inhibitor (Yang, F. *et al.*, 2020). Therefore, TD139 is not only known for its high affinity to human galectin-3, but also for its affinity to rat and mouse galectin-3 (Kumar *et al.*, 2021). In T cells, administration of TD139 reduces NK cells dependent-hepatitis as well as the expression of pro-inflammatory cytokines (Volarevic *et al.*, 2015). Comparing the two inhibitors, GB-1107 and TD139, both show an inhibitory effect on cell coherence and decrease both migration and phosphorylation. Evidence is accumulating that galectin-3 is a histological marker for thyroid cancer. However, the lectin-based pharmacological approach has not been well studied. In the present study, we aimed to investigate the therapeutic potential of novel galectin-3 inhibitors by treating thyroid cancer cells with different concentrations of GB1107 or TD139. However, TD139 also initiates apoptosis of thyroid cancer cells, which GB1107 did not show (Lee *et al.*, 2021). G3-C12 is also binds specifically to the CRD of galectin-3 and showed anti-tumor-promoting effect in nude mice (Yang, Y. *et al.*, 2012).

Taken together, the results show that knockout of galectin-3, as well as modulation of the protein, can reduce or slow neurodegenerative diseases and thus represent a good immunomodulatory target.

1.5 Aim of the study

Microglia are immune cells of the CNS, and key factors in tissues such as the retina and regulate homeostasis. When microglia are reactivated, these cells promote apoptosis of damaged cells, such as neurons, and phagocytose cell debris so that inflammation cannot occur. In neurodegenerative diseases, there is often a dysregulation/overreaction of microglia, which accelerates disease progression and worsens disease symptoms. Furthermore, chronic activation of microglia in the retina favors the development of age-related macular degeneration. Therefore, immunomodulatory targets are being investigated and the impact of the loss of such a target on neurodegenerative diseases is being explored.

One promising target structure is the protein, galectin-3, which plays an important role in the activation of microglia. Loss or inhibition of galectin-3 has been shown to inhibit the microglial response and reduce the release of pro-inflammatory cytokines. Modulation or loss of this protein has not been adequately studied in the retina, and the mechanism behind it remains largely unexplained. In the present study, we investigated the effect of galectin-3 expression on retinal degeneration in a light-induced degeneration model. This mouse model is an established model for the study of dry AMD, for which there is currently no potential treatment option. The study allows to investigate the immunomodulatory properties and the protective effect of galectin-3 inhibition.

2. Material and methods

2.1 Material

2.1.1 Mouse lines and husbandry

Table 1: List of mouse lines used in this study

Line	Original name	Genotype	Background
Lgals3-KO	B6.Cg-*Lgals3*^{tm1Poi}/J Jackson laboratory	Homozygous knockout of galectin-3	BALB/cJ x C57BL/6J
Lgals3 WT	WT littermates of *Lgals3*-KO	WT gene for galectin-3	BALB/cJ x C57BL/6J
BALB/cJ	BALB/cJ Charles River	WT gene for galectin-3	BALB/cJ

Table 2: Equipment for mouse husbandry

Equipment	Manufacturer
Individually ventilated cages	Tecniplast Greenline®
Litter, spruce granules	Rettenmaier und Söhne® FS14
wooden sticks for gnawing	Labodia
cardboard house	Plexx®
Chow (11 % fat, 24 % protein and 65 % carbohydrates)	Lage, GER #1324

2.1.2 Devices

Table 3: List of devices

Device	Manufacturer
Adventurer Pro balance	Ohaus®
BlueMarineTM 200 Electrophoresis unit	SERVA Electrophoresis GmbH

Centrifuge 5415 R	Eppendorf
Centrifuge Mini Star	VWR International
Cryostat CM3050	Leica
Explorer R Ex 124 balance	Ohaus®
HB-1000 Hybridizer	UVP
Heraeus Megafuge 40R Centrifuge	Thermo Scientific
HybEZ™ II Oven	ACD bio-techne®
Intas Gel iX20 Imager	Intas
LightCycler® 480 Instrument II	Roche Applied Science
MatrixTM Multichannel Pipette	Thermo Scientific
NanoDrop 2000 Spectrophotometer	Thermo Scientific
PeqSTAR 2x cycler	Peqlab
See-saw rocker SSL4	Stuart®
SpectralisTM HRA+OCT	Heidelberg Engineering
Thermomixer compact	Eppendorf
Vibracell 75115 Sonicator	Fisher Bioblock Scientific
Vortex-GenieTM	Scientific IndustriesTM
Zeiss Stemi 508 Stereo microscope	Zeiss

2.1.3 Chemicals and reagents

2.1.3.1 General Buffers and reagents

Table 4: List of general buffers and reagents

Buffer / reagents	Manufacturer, Cat. No.
DPBS (1x)	Gibco, 14190
Ethanol	Applichem, #A3678
PBS (10x)	Lonza, #51226
RNase away	Molecular Biopro., #70003

2.1.3.2 Chemicals and reagents for molecular biological analysis

Table 5: List of chemicals and reagents for genotyping

Chemical/ reagent	Manufacturer, Cat. No.
Agarose	Biozym, #84004
Boric acid	Sigma-Albrich, #B6768
Ethidium bromide	
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, E9884
GeneRuler 100 bp plus	Thermo Scienttific, #SM0332
Sodium hydroxide (NaOH)	Merck, #1.06462
Tris	Roth, #4855.3

Table 6: List of chemicals and reagents for *in situ* hybridization

Chemical/ reagent	Manufacturer, Cat. No.
ImmEdge® Hydrophobic Barrier Pen	ACD bio-techne®, # 310018
Opal 520	Akoya Biosciences, PN FP1487001KT
Opal 570	Akoya Biosciences, PN FP1488001KT
Opal 690	Akoya Biosciences, PN FP1497001KT
RNAscope 3-plex negative control probes	ACD bio-techne®, #320871
RNAscope 3-plex positive control probes	ACD bio-techne®, #320871
RNAscope H202 and Protease Reagents	ACD bio-techne®, #PN 322381
RNAscope Multiplex Fluorescent Detection	ACD bio-techne®, #PN 323110
RNAscope Target Retrieval reagents	ACD bio-techne®, #322000
TSA buffer	ACD bio-techne®, #PN 322809
RNAscope Wash Buffer	ACD bio-techne®, #PN 310091

Table 7: List of chemicals and reagents for RNA isolation

Chemical/ reagent	Manufacturer, Cat. No.
ß-Mercaptoethanol	Sigma-Aldrich, #M-7154
Ethanol 70%	Applichem, #A2192

Ethidium bromide	
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, E9884
Sodium hydroxide (NaOH)	Merck, #1.06462
Tris	Roth, #4855.3

2.1.3.3 Chemicals and reagents for immunhistochemical analysis

Table 8 : List of chemicals and reagents for immunhistological stainings

Chemical/ reagent	Manufacturer, Cat. No.
Bovine Serum Albumin (BSA)	Sigma-Aldrich, #A9418
Normal donkey serum (NDS)	Linaris, #ADI-NDKS-10
Fluorescence Mounting Medium	Dako, #S3023
Fluoromount- G™, with Dapi	Invitrogen, Thermo Fisher Scientific, #00-4959-52
Milk powder	Roth, #T145.3
Roti Histofix 4 %	Roth, #P087.4
Sucrose	Roth, #4621.1
Tissue- Tek	Sakura, #1913602823
Triton X-100	Sigma-Aldrich, #X100
Tween 20	Sigma-Aldrich, #P1379
Vectashield® HardSet™	Mounting Medium Vectashield®, H1400

2.1.3.4 Chemicals and reagents for mouse experiments

Table 9: List of chemicals and reagents for anesthesia and optical coherence tomography

Chemical/ reagent	Manufacturer, Cat. No.
Ketaset 100 mg/ml	Zoetis, PZN #12467832
Phenylephrine 2.5 % / Tropicamide 0.5 %	University Hospital Cologne
Rompun 2 % (Xylazine)	Bayer, PZN #1320422
Sodium Chloride 0.9 %, injection	Fresenius Kabi, PZN #06605514

Table 10: List of chemicals and reagents for pharmacological inhibition

Chemical/ reagent	Manufacturer, Cat. No.
33DFTG	AOBIOUS, #AOB 37408
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, #D5879
TD139	MedChem Express, #HY- 19940

2.1.4 Buffers and solutions

2.1.4.1 Buffers and solutions for molecular biological analysis

Table 11: Recipes for buffers and solutions used for genotyping

Buffer/solution	Chemical composition
Agarose gel 1%	1% Agarose
	0.5 µl/ml Ethidium bromide in TBE
Alkaline lysis buffer	25 mM NaOH 200 mM
	EDTA pH 12 in ddH2O
Neutralization buffer	40 mM TRIS-HCl pH 5 in ddH2O

Table 12: Recipes for buffers and solutions used for in situ hybridization

Buffer/solution	Chemical composition
RNAscope Target retrieval reagents (1x)	10x Target retrieval reagents in ddH_2O
Saline- sodium citrate (SSC) buffer	20x SSD: NaCl (M:58.4 g/mol) 175.3g-3M
	Sodium Citrate (M: 258g/mol) 77.4g-0.3M
	In 1 liter dH2O – pH=7
Wash buffer (1)	20x Wash buffer in ddH_2O

2.1.4.2 Buffers and solutions for immunohistochemically analysis

Table 13: Recipes for buffers and solutions used in whole mount staining

Buffer /solution	Chemical composition
PBS (1x)	10x PBS in ddH_2O
PBST-X	0.3 % Triton X-100 in PBS
Perm/ Block buffer	5 % NDS
	0.2 % BSA
	0.3 % Triton X-100 in 1x PBS

Table 14: Recipes for buffers and solutions used for cryostaining

Buffer /solution	Chemical composition
Antibody solution	2 % BSA
	0.1 % Triton X-100
Blotto	1 % Milk powder
	0.03 % Triton X-100 in PBS
PBS (1x)	10x PBS in ddH_2O

2.1.5 Enzymes

Table 15: List of enzymes for genotyping

Enzyme	Manufacturer, Cat. No.
Taq Polymerase	Genoxxon, #M3001
10x Reaction buffer	Genaxxon, #M3454

Table 16: List of enzymes for cDNA synthesis

Enzyme	Manufacturer, Cat. No.
RevertAit H minus Reverse Transcriptase	ThermoFischer Scientific Kit # K1622
10x Reaction buffer	ThermoFischer Scientific Kit # K1622

2.1.6 Primer und probes

Genotyping and quantitative real-time Primer were purchased from IDT (table 16 and 17). The probes for all the experiments were designed and produced by ACD Bio-techne®.

Table 17: Genotyping primer

Target	Primer	Sequence (5´-3´)	Orientation
Lgals3	Lgals3_WT/Mut_f	GAC TGG AAT TGC CCA TGA AC	forward
	Lgals3_WT_r	GAG GAG GGT CAA AGG GAA AG	reverse
	Lgals3_Mut_r	TCG CCT TCT TGA CGA GTT CT	reverse

Table 18: Primer quantitative real-time PCR

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	UPL Probe
Atp5b	GGCACAATGCAGGAAAGG	TCAGCAGGCACATAGATAGCC	77
Ccl2	CATCCACGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT	62
Casp-3	GAGGCTGACTTCCTGTATGCTT	AACCACGACCCGTCCTT	80
Lgals3	GCCTACCCCAGTGCTCCT	GGTCATAGGGCACCGTCA	18
Il-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA	6
iNos	CTTTGCCACGGACGAGAC	TCATTGTACTCGAGGGCTGA	13

Table 19: List of probes used for in situ hybridization

Gene	Species	Accession No.	Target region	Cat. No.
HS-LGALS3-C1	Human	NM_002306.3	53 - 995	#477839
HS-AIF1-C3	Human	NM_032955.1	8 - 468	#433128-C3
mm-Lgals3-C1	Mouse	NM_001145953.1	55 - 1045	#461471
mm-Aif1-C3	Mouse	NM_019467.2	31 - 866	# 422741-C3

2.1.7 Antibodies

Table 20: List of primary and secondary antibodies used in this study

Antibodies /Stains	Species	Dilution	Manufacturer, Cat. No.
Anti-Iba-1	Rabbit, Polyclonal	1:500	Wako, #019-19741
Alexa Fluor® 488	Donkey anti-rabbit IgG	1:1000	Invitrogen, #A21206
Alexa Fluor® 647	Donkey anti-rabbit IgG	1:1000	Invitrogen, #A-31573

2.1.8 Kits and software

Table 21: List of kits

Kit	Manufacturer, Cat. No.
LightCycler ® 480 Probes Master	Roche Applied Science, #04707494001
RevertAid RT Kit	Thermo Scientific, #K1691
RNeasy® Micro Kit	Qiagen, #74004
RNAscope Multiplex Fluorescent Reagent Kit v2	ACD bio-techne®, #323100
Takyon™ No ROX Probe MasterMix blue dTTP	Eurogentec, #UF-NPMT-B0701
Taq-S PCR Kit Genaxxon	Bioscience, #M3313

Table 22: List of software used in this study

Software	Manufacturer
Adobe creative suite	Adobe system
EndNote 20	EndNote
FIJI/ Image J (V2.10/1.53c)	Wayne Rasband, NIH

GraphPad Prism 7 (V7.05)	Graphpad Software, Inc.
Heidelberg Eye Explorer (HEYEX)	Heidelberg Engineering
Intas Gel Documentation Software (V3.39)	Intas Science Imaging
LightCycler® 480 Software (V1.5.1)	Roche Applied Science
Microsoft Office 365 pro plus	Microsoft Corporation
Nanodrop 2000/2000c Software	Thermo Scientific
Zen blue 2012 (V3.1.0.00002)	Zeiss

2.2 Methods

2.2.1 Mouse experiments

2.2.1.1 Mouse husbandry

Mice were housed in individually ventilated cages under specific pathogen-free (SPF) conditions. Maximum five adult mice were kept in one cage, equipped with litter, cotton wool, wooden sticks for gnawing and cardboard house. The mice are kept in a 12 h/ 12 h light/ dark rhythm; the temperature is 22 ± 2 °C and 45- 65 % air humidity (air exchange rate 8/ h). Mice were fed with animal flour and low in nitrosamine-free standard diet (1324: 11 % fat, 24 % protein and 65 % carbohydrates) and acidified water ad libitum.

2.2.1.2 Experimental mouse line

B6.Cg-*Lgals3*^{tm1Poi}/J mice and BALB/cJ mice, acquired at the Jackson Laboratory (Bar Harbor, ME, US) were crossed to B6.Cg-*Lgals3*^{tm1Poi}/ BALB/cJ mice (*Lgals3*-KO). For the experiments 8-10 weeks old *Lgals3*^{tm1Poi}/ BALB/cJ mice with light sensitive RPE, inactive tyrosine and homozygote galectin- 3 knockout and corresponding wild type mice with intact galectin- 3 were used. BALB/cJ WT mice were also used for inhibition studies. All tests and protocols were carried out according to the German animal protection agency (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein- Westfalen: approval no. §81-02.04.2018.A303).

2.2.1.3 Galectin-3 inhibition

The pharmacological inhibitor TD139 (HY- 19940), was obtained by MedChem Express (NJ, US) and later von AOBIOUS (33DFTG; AOB 37408). It was dissolved in DMSO and stored -20°C (solved and powder). 8-10 weeks old BALB/cJ mice received daily intraperitoneal injections of 15 mg/kg body weight dissolved TD139. The vehicle control group received injections of DMSO. Injections started one day before light exposure. The inhibitor was incubated 10 min under shaking at 37 °C before use.

2.2.1.4 Experimental design

The light- induced degeneration model is a well-established mouse model (Scholz *et al.*, 2015b). To induce retinal degeneration in 8-10 weeks old BALB/cJ and *Lgals3*-KO mice, mice were dark adapted for 16 h before light exposure. Afterwards their pupils were dilated with eye drops (0.5 % tropicamide and 2.5 % phenylephrine) under dim red light and mice were exposed to bright white light with an intensity of 15,000 lux for 1 h respectively. The remaining experimental period the animals were housed under normal light conditions.

2.2.1.5 Optical coherence tomography (OCT)

For image acquisition mice were anesthetized by intraperitoneal injection of Rompun (5 mg/ kg body weight, 2 % Rompun) and Ketamine HCl (100 mg/ kg body weight) diluted in 0.9 % NaCl. The pupils were dilated by 0.5 % tropicamide and 2.5 % phenylephrine 5- 10 minutes before imaging with Spectralis^{TM} HRA/OCT (Heidelberg Engineering). At day one, three and four post light exposure spectral-domain OCT (SD-OCT) was performed to analyze post light-induced the retinal thickness. To measure the retinal thickness, two circular rings cantered 3 and 6mm around the optic nerve were scanned. The thickness (µm) was calculated by the mean of four fields in each circular ring using the Heidelberg Eye Explorer Software.

2.2.2 Human donor samples

Retinal donor samples were collected from volunteer registered donors and obtained from the Eye Bank of the center of Ophthalmology at the University of Cologne. The study was performed in accordance with the tenets of the Declaration of Helsinki and Medical Research Involving Human Subjects Act (WHO) and was approved by the local ethics committee of the University.

2.2.3 Molecular biological analysis

2.2.3.1 Isolation of genomic DNA for genotyping

Ear biopsies, 1 to 2 mm sample, from 21-day-old mice were used for the isolation of genomic DNA. The isolation was performed using the HotSHOT method. Samples were incubated with 75 µl alkaline lysis buffer (table 11.) in thermocycler at 95°C for 15-20 min, until the fragments are completely submerged. Followed by 5 min on ice before 75 µl of neutralization buffer (table 1.) was added to stop the reaction. The container vortexed and centrifuged.

2.2.3.2 Genotyping of *Lgals3*-KO

The genotype of the mice was examined by PCR, primers were generated covering the coding region of the protein (table 17). The reverse primer for the WT variant of the gene binds directly upstream of the exon 2 and the translated region then corresponds to a size of 224 bp (Fig.10). The primer designed for the knockout binds to the neomycin caste of the mutant. The neomycin caste replaces the region important for the gene and shortens the gene. Thus, the band of the mutant is only 150 bp in size and can be easily distinguished from the WT band on an agarose gel. The PCR is performed using the Genaxxon kit and a thermocycler PCR instrument. The PCR kit has a reaction volume of 25 µl and contains 50 ng DNA. The mix is composed of 1x reaction buffer, 0.2 µM dNTPs, 1.25 U/ µl Taq S, 0.2 µM primer each and the remaining volume ddH2O (table 22.). The PCR program starts with initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of 30 sec

denaturation again at 94°C, 30 sec annealing at 50 °C and an elongation step of 30 sec at 72°C follow. Finally, a final elongation step follows for 2 min at 72°C (table 23.). After cooling of the PCR product, it is applied to a 1 % agarose gel and separated by gel electrophoresis at 120 mV and analyzed after approximately 20- 30 min.

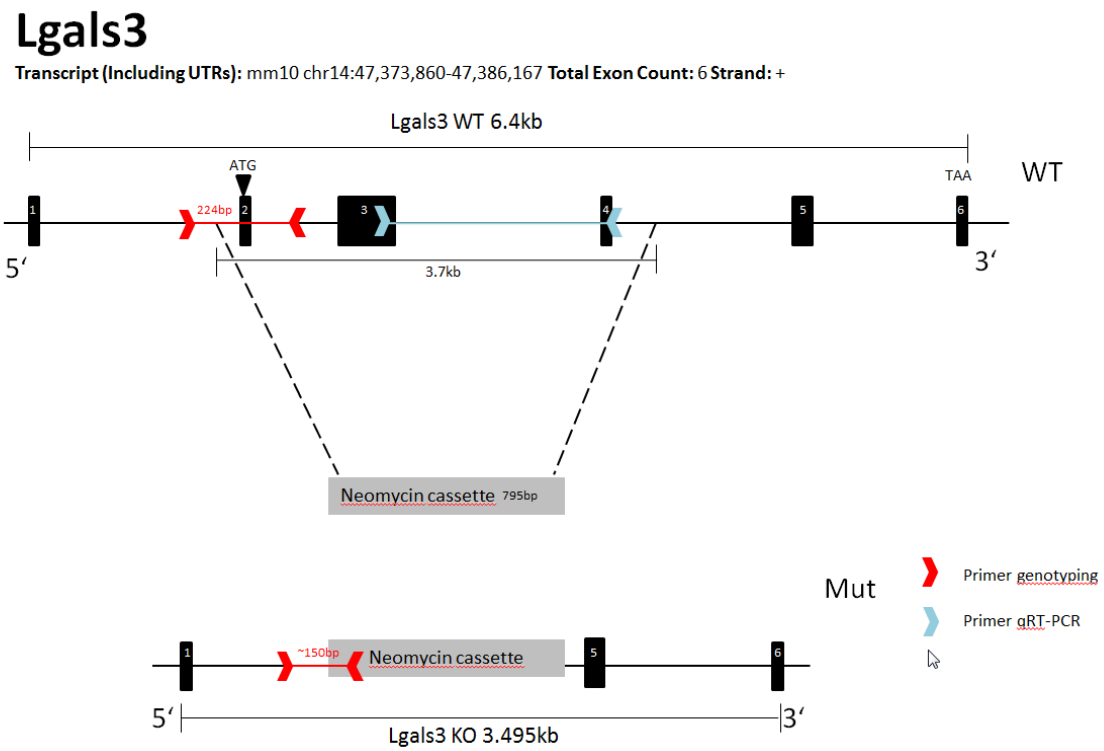

Figure 10: **Genetic background of the *Lgals3*-knockout mouse.** Mice with C57BL/6J background have a neomycin cassette inserted in the gene coding for galectin-3 in the region of chromosome 14, whereby exons 2, 3 and 4 can no longer be transcribed and the expression of galectin-3 is no longer possible.

Table 23: Recipe of galectin-3 genotyping PCR

Reaction component	Amount	Concentration
10x Reaction buffer S (15mM $MgCl_2$)	2.5 µl	1x
Lgals3_WT/Mut_f	0.5 µl	0.2 µM
Lgals3_WT_r	0.5 µl	0.2 µM
Lgals3_Mut_r	0.5 µl	0.2 µM
dNTPs	0.5 µl	0.2 µM
Taq S (5 U/µl)	0.25 µl	1.25 U/µl
Genomic DNA	2 µl	50 ng
ddH_2O	18.25 µl	

Table 24: Galectin-3 PCR temperature profile

Step		Temperature	Time
Initial denaturation		94°C	3 min
35 cycles	Denaturation	94°C	30 sec
	Annealing	50°C	30 sec
	Elongation	72°C	30 sec
Final extension		72°C	2 min
Hold		8°C	∞

2.2.3.3 RNA isolation and reverse transcription.

Total RNA was isolated from murine retina and RPE tissue using the RNeasy Micro Kit (Qiagen) according to the manufacturer´s instruction. RNA was quantified spectrophotometrically with a NanoDrop 2000. First-strand cDNA was synthesized from total mRNA using RevertAid^{TM} H Minus First-strand cDNA Synthesis kit (Thermo Scientific).

2.2.3.4 Quantitative real-time PCR

Transcript levels from different genes were analyzed by quantitative real- time PCR performed in LightCycler® 480 II from Roche with probe based (LightCycler 480 Probe Master Roche) detection, according to the manufacturer's instructions. To measure the gene transcription levels Atp5b, were used as housekeeping gene. Measurements were performed in technical duplicates, Atp5b expression was used as reference gene, and results were analyzed with the LightCycler 480 Software using delta delta CT threshold calculation for quantification.

2.2.3.5 In situ hybridization: RNA Scope®

In situ hypridization by ACD Biotechne is a method that allows to visualize mRNA transcripts under the light of a microscope. This imaging technique can be used for molecular biology purposes. RNA Scope® ISH was performed with the RNAScope® Multiplex Fluorescent Reagent Kit v2, according to the information's of the manufactures, with some modifications. In brief, fresh frozen ten micrometer sections were baked for 1 h at 60°C. After hybridization and washing according to the manufacture instructions, the mouse slides stays 5 min and human slides 10 min in 1x Target retrieval reagents heated on 95°C. All washing steps following the probe application are 5 min at room temperature (RT). The following probes were used for these studies: HS-LGALS3 C1, ACD 477839; HS-AIF1 C3, ACD 433128; Mm-Aif C3, ACD 319141; Mm-Lgals3 C1, ACD 461471. The probes were labeled with TSA® Plus Fluorophore cyanine 3 and Fluorescein. The sections were mounted in mounting medium (Dako, Fluorescence Mounting Medium). For localization of the galectin- 3 expression, we performed a co-immunohistochemical staining with Iba-1 polyclonal antibody 1: 500, secondary antibody AF 647 (A-31573 invitrogen). The same immunohistochemistry protocol was used for this, except that all steps are carried out protected from light. For analysis images were taken with a Zeiss Imager.M2 equipped with an ApoTome.2 and quantification were performed with ImageJ.

2.2.4 Immunohistological analysis

2.2.4.1 Immunohistochemistry analysis of retinal and RPE flat mounts

The analysis was done at day one, three and four days post light exposure. The mice were euthanized by cervical dislocation and the eyes enucleated. After fixation with 4 % Roti Histo fix (paraformaldehyde) for 3h at RT, retinal and RPE flat mounts were dissected from each other and incubated in Perm/Block buffer (table 13.) for permeabilization and blocking, overnight at 4°C. The flat mounts were incubated with primary antibodies diluted in Perm/Block buffer (table 20) for 48 h at 4°C. Subsequently, three washing steps with PBST-X are followed by the incubation with the secondary antibody diluted in PBST-X for one hour (table 20). After three washing steps with PBST-X for 15 minutes follows a washing step overnight before the retina and RPE were mounted on a microscope slide and embedded

with fluorescence mounting medium (Vectorshied HardSet). Images were taken with a Zeiss Imager.M2 equipped with an ApoTome.2.

2.2.4.2 Immunohistochemistry analysis of cryotome sections

The staining of the cryostat sections was also performed on eyes that have been fixed for 3h. For this purpose, the fixed eyes were dehydrated in 30 % sucrose until the eyes sunk to the ground and afterwards embedded in Tissue-Tek. After freezing on dry ice, the eyes were cut in ten micrometer sections. The sections were rehydrated with 1x PBS and blocked with dried milk solution for 30 min (table 13). Subsequently, followed by the incubation of the primary antibody, diluted in antibody solution, overnight at 4°C. After three washing steps with 1x PBS, the secondary antibody diluted in 1x PBS was added for 1h, which is followed by four more washing steps for 10 min. Further, the sections were mounted in mounting medium plus Dapi (Fluoromount- G^{TM}). Images were also taken with a Zeiss Imager.M2 equipped with an ApoTome.2.

3 Results

3.1 Microglia regulates *LGALS3* expression in AMD patients

It is known that the protein, galectin-3, is being secreted by microglia. Further, studies showed an upregulation of galectin-3 in stimulated microglia cell line (Nomura *et al.*, 2017). A recent review indicated the involvement of galectin-3 in the development and pathogenesis of different eye diseases (Caridi *et al.*, 2021). Under demanding conditions, the protein production is increased by microglia and other cells. In the research paper of Yuan and colleagues using proteomic data, it was shown that patients with AMD had an upregulated expression of galectin-3 (Yuan *et al.*, 2010). Since it is known that other cells also express galectin-3, we sought to prove that this expression originated from microglial cells in the retina. Using in situ hybridization, the expression of mRNA transcripts in healthy retinas and in retinas of AMD patients can be detected and visualized. Probes labeling human LGALS3 and human *Allograft inflammatory factor* (AIF-1) were used. The *LGALS3* probe encoded the expression of galectin-3, whereas AIF-1 is encoded the expression of the protein IBA-1 produced by microglia. We were able to confirm the results of the proteomic analysis. In healthy human retinas, only a very small amount of *LGALS3* is expressed (Fig. 11a). In contrast, this expression was strongly upregulated in AMD patients (Fig. 11b). The co-expression of *AIF-1* and *LGALS3* confirmed that in AMD microglia also take over the expression of *LGALS3* in the retinal tissue.

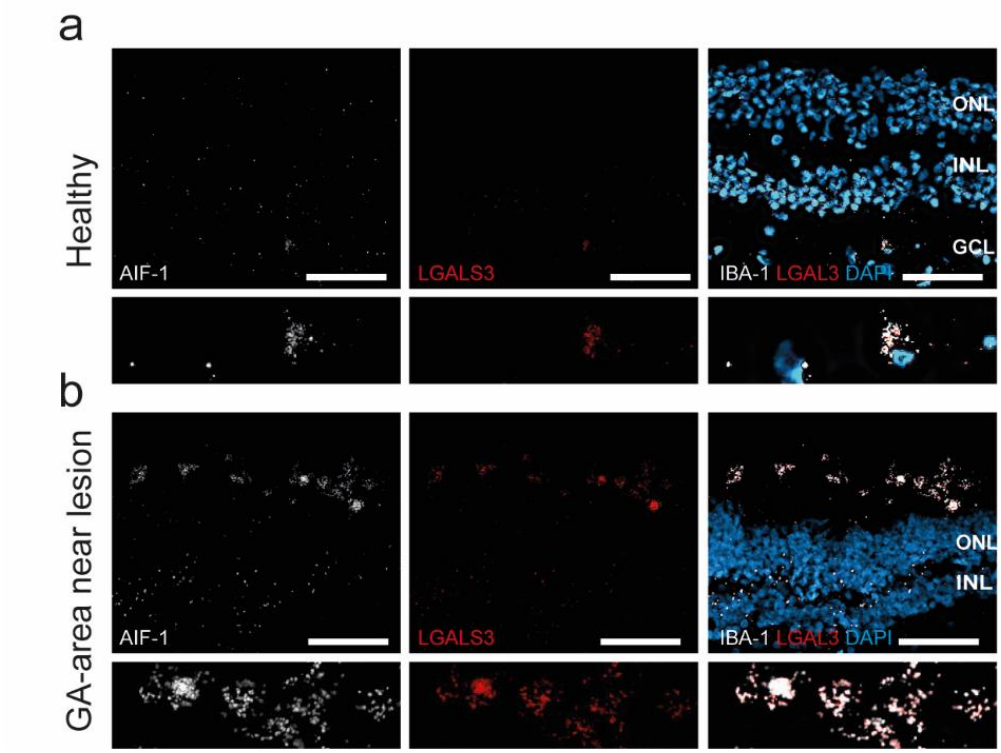

Figure 11: LGALS3 expression in human cryotome sections from healthy donor and AMD patient. **a** An in situ hybridization shows a very low expression of *LGALS3* (red) and rather a defused localization of *AIF-1* (white) in the healthy human control. **b** the donor of the AMD patient, on the other hand, shows an upregulated expression of *AIF* in the nuclei and a distinct upregulation of *LGALS3*. ONL: Outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 µm.

3.2 Immunological effect of genetic knockout of galectin-3 in light-induced degeneration model

3.2.1 The deficiency of galectin-3 in *Lgals3*-KO mice reduced microglia migration in the ONL

The mechanism of upregulation of galectin-3 and the associated reactivity in microglial cells is largely unexplained. To explore whether the inhibition of galectin-3 represents a promising treatment option for dry AMD, a light-induced model for retinal degeneration was performed. This light damage model mimics several features of dry AMD, including immune activation and apoptosis of photoreceptor cells (Grimm *et al.*, 2000). However, light damage occurs when dark-adapted enriched rhodopsin is bleached by bright white light. As a result, neurotransmitters are secreted from damaged cells and surrounding cells are alerted.

Within a few days, damaged photoreceptors are phagocytosed by microglial cells and the retina begins to degenerate (Scholz *et al.*, 2015b). First, the immune-related effect of the loss of galectin-3 in light induced degeneration model was investigated. In these mice, a 3.7 kb sequence was replaced by a neomycin cassette. The replaced region contains important coding features, including exon two, three and four (https://www.jax.org/strain/006338). The mice used in the first experiments were crossed with BALB/cJ mice to render this mouse strain sensitive to light damage. Mice on C57BL/6J background typically have methionine at position 450 in the RPE 65 protein, which makes these animals relatively insensitive to acute light damage. BALB/cJ mice have leucine-variants at this position of the RPE 65 protein. Furthermore, BALB/cJ mice have an inactive tyrosine gene and are therefore not pigmented. These two genes were decisive for our backcrossing. The experiments were also performed with wild type (WT) littermates for comparability. Therefore, the experiments were performed with 8- 10 weeks old *Lgals3*-KO mice, which were dark adapted for 16h and exposed to bright white light with an intensity of 15,000 lux for 1 hour (Fig. 12a). To test the effect of galectin-3 deficiency on microglia migration, the microglia population was examined in sections of healthy naïve *Lgals3*-KO and WT mice and compared with the microglia population one, three, and four days after light exposure. Under healthy conditions, without light exposure, microglia were found in the plexiform layers (OPL - outer plexiform layer and IPL - inner plexiform layer) and ganglion cell layer (GCL) (Fig 12b).

When microglia perceive a stimulus, such as a light induction, they migrate to the site of the lesion and change their morphology. This migration can easily be identified in cryostat sections labeled with the microglia and macrophages marker Iba-1 after light exposure (Fig. 12b). Microglia in WT mice formed more branches in the nuclear layers and moved toward photoreceptors as early as one day after light exposure. The movement was more strongly induced in a time-dependent manner from one to four days after light exposure. Further, nuclear layers (INL- inner nuclear layer and ONL- outer nuclear layer) have been colonized by many Iba-1^+ cells three days after light exposure and these cells appear to have changed their morphology. They exhibit a more rounded (amoeboid) morphology and hardly any filopodia were visible. In contrast, migration of Iba-1^+ cells in the ONL appears to be reduced in *Lgals3*-KO mice. Quantification of the number of Iba-1^+ cells in the ONL showed a significantly increased number of microglia in the ONL already on the first day post light exposure in WT mice and somewhat delayed from the third day post light exposure in *Lgals3*-KO mice. However, the number of Iba-1^+ cells was significantly reduced in *Lgals3*-

KO mice compared with WT mice (Fig. 12c). Next, the expression of *Lgals3* in the retina and RPE was measured by qRT-PCR. The expression of *Lgals3* increases in a time-dependent manner from one to four days post light exposure (Fig. 12d, e). Since the expression of *Lgals3* was increased in the light induced degeneration model, *Lgals3* could be a potential marker for the reactivity of microglia.

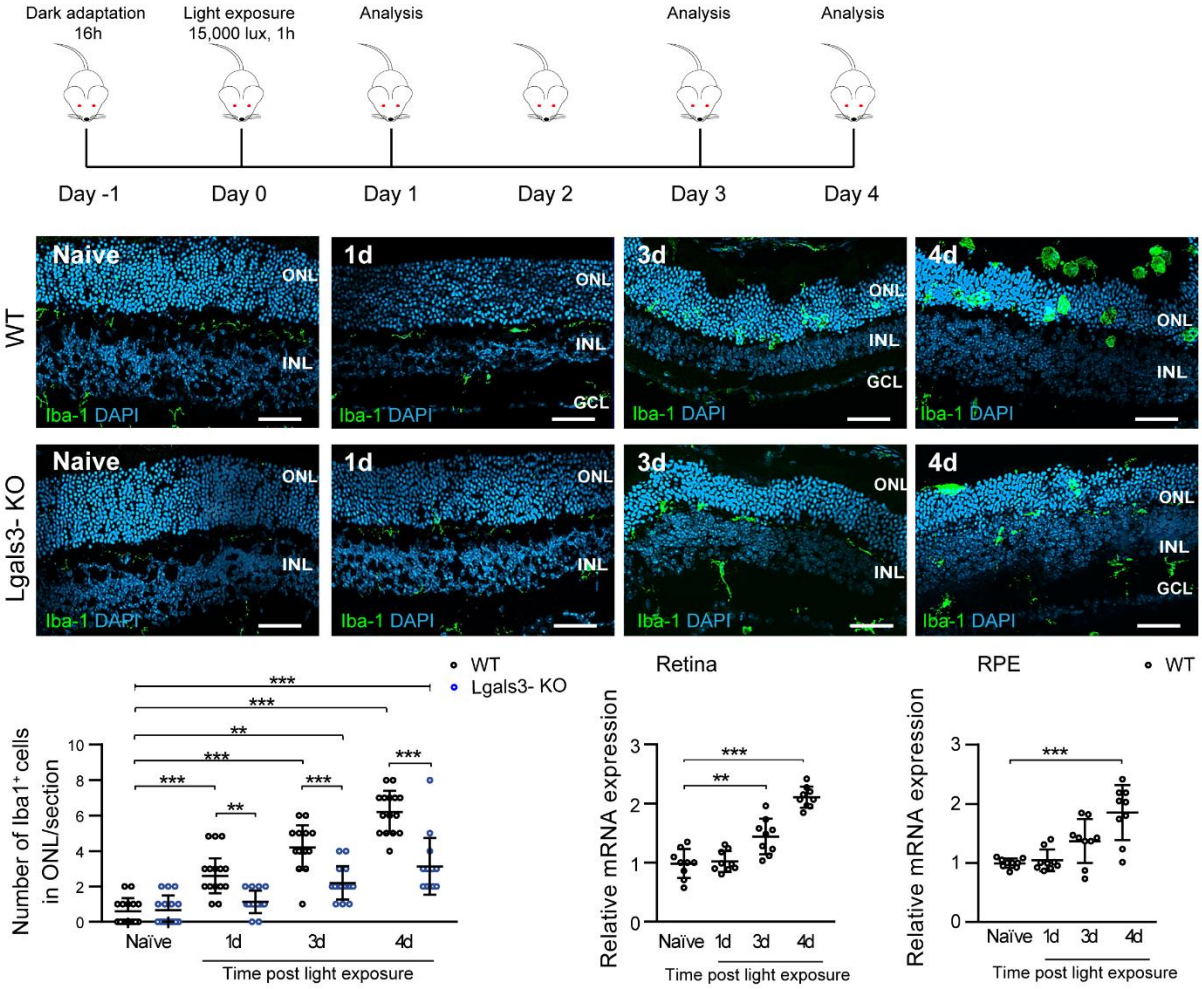

Figure 12: The deficiency of galectin-3 in *Lgals3*-KO mice reduced migration of Iba-1$^+$ microglia. a Experimental strategy of light induced degeneration model in Lgals3-KO mice and complementary WT littermates. **b** Representative images of Iba-1$^+$ phagocyte migration into the ONL in light exposed Lgals3 and WT mice one, three and four days post light damage. Scale bar: 50 µm. **c** Quantification of Iba-1$^+$ cells in the ONL in time dependent fashion from one to four days post light exposure (WT/ Lgals3-KO n= 15 eyes) **d** Relative mRNA expression of Lgals3 in retina and **e** RPE analyzed by qRT-PCR. ONL: Outer nuclear layer; INL: inner nuclear layer; GCL: Ganglion cell layer. Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.2.2 The deficiency of galectin-3 in *Lgals*3-KO mice reduces retinal microglia reactivity

The number of microglia in subretinal space and RPE correlates with the reduction of photoreceptor cells. Microglia in these layers are mostly amoeboid and reactivated. To analyze the morphology and migration of microglia in subretinal space, flat mounts were stained with anti- Iba-1 antibody. Here, images of retinal flat mounts from WT mice revealed a strong accumulation of reactive amoeboid Iba-1^+ cells in the subretinal space three days post light exposure. In light-exposed *Lgals3*-KO mice, the number of Iba-1^+ cells also increased in the subretinal space, but the number was less than the number of cells in WT retinas and these cells mainly exhibited a more branched morphology (Fig. 13a). Quantification described a significantly higher amount of Iba-1^+ area in WT mice three and four days after light exposure, whereas the area was only very slightly increased in *Lgals3-KO* mice (Fig. 13b). The area at one day post light exposure was not significantly increased in any mouse model. In addition, the number of Iba-1^+ cells were significantly increased in both mouse strains, which were significantly impaired by the absence of galectin-3 (Fig. 13c).

Subsequently, mRNA expression of pro- inflammatory microglia markers were analyzed by qRT-PCR. Markers such as inducible *nitric oxide synthase* (*iNos*), *interleukin* (Il)- 6, *caspase 3* (*Casp*3) and *CC- chemokine- ligand- 2* (*Ccl2*) were examined at all three analysis time points. RNA levels were normalized to *Atp5b* (ATP synthase), and induction was calculated compared with untreated healthy naïve WT mice. Normalized quantification of mRNA levels of *iNos*, *Il-6* and *Ccl2* showed a strong increase on the first day post light expression in WT mice. This expression decreased after the first day but remained significant until day three (Fig. 13d). The mRNA expression of *iNos* remained elevated on the fourth day post light exposure in WT mice compared to the naïve mice four days post light exposure. In contrast, the expression of *iNos* was reduced in *Lgals3* mice, despite a slight increase compared to naïve mice, there was no significantly increased expression. The strong increase of *iNos* in WT mice on the first day post light exposure is significantly reduced by the absence of galectin-3 in the *Lgals3*-KO mice.

Furthermore, the expression levels of *Il-6* were significantly reduced in *Lgals3*-KO compared to light exposed WT mice four and three days post light exposure. The expression level of *Ccl2* could be attenuated significantly at day one post light damage by the lack of

Lgals3 in mice. In contrast, the expression level of *Casp3* remained unchanged compared to naïve mice, regardless of the time point or mouse genotype (Fig. 13d).

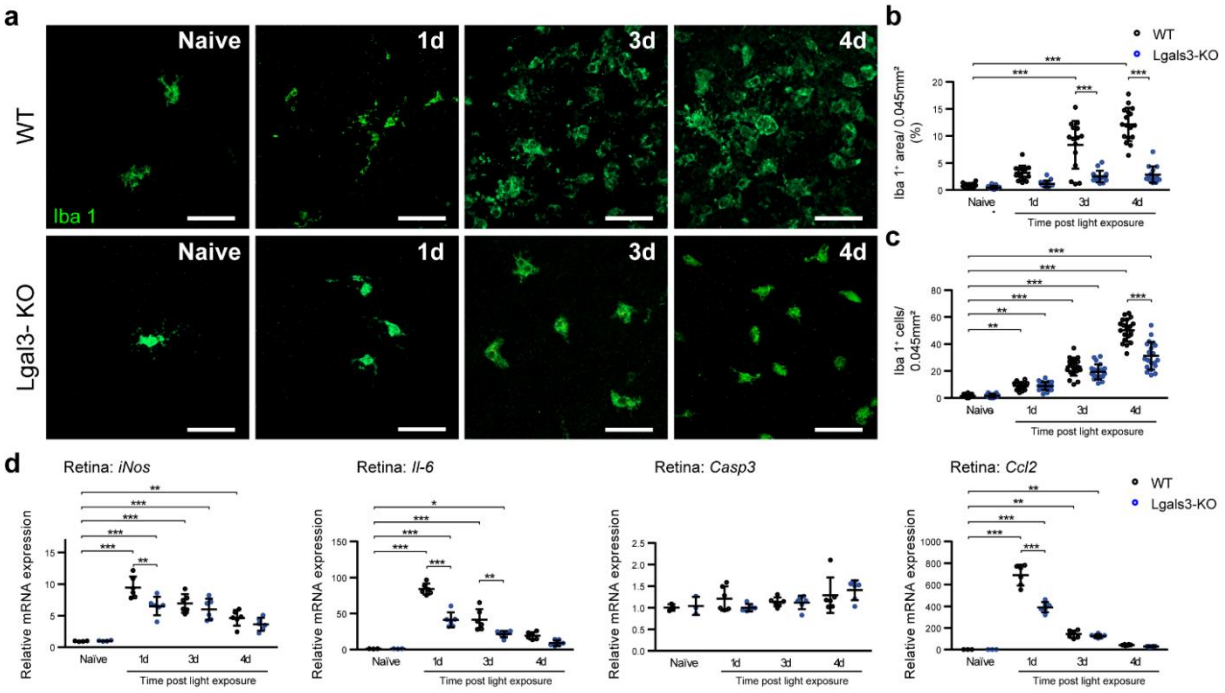

Figure 13: Microglia localization and morphology in retinas of *Lgals3*-KO and WT mice. **a** Immunofluorescence analysis of Iba-1 $^{+}$ cells in subretinal space of retinal flat mounts from light exposed *Lgals3*-KO and WT without light exposure, one, three and four days post light exposure. Scale bar: 50 µm. **b** Quantification of Iba1^{+} area in 0.045 mm² of the retina. (WT/ *Lgals3*-KO n= 15). **c** Number of microglia in the subretinal space. (WT/ *Lgals3*-KO n= 15). **d** Quantification of relative mRNA expression. *iNos*, *Il-6*, *Casp3* and *Ccl2* (WT/ *Lgals3*-KO n= 6, retinas from individual mice). Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.2.3 The deficiency of galectin-3 in *Lgals3*-KO mice reduces microglia reactivity in RPE

We further performed an immunohistochemical staining with microglia marker Iba-1 in the outermost layer, the retinal pigment epithelium (RPE), flat mounts from WT and *Lgals3*-KO mice one, three and four days post light exposure (Fig. 14a). The Iba-1^{+} microglia demonstrated a more amoeboid shape in the RPE of WT mice one day post light damage but the Iba-1^{+} area did not change at this time point, in contrast the number of Iba-1^{+} cells was

slightly higher compared to naïve mice (Fig. 14b, c). The Iba-1$^+$ area and the number of microglia cells were significantly increased three and four days post light exposure in WT mice compared to control. The number of microglia in *Lgals3*-KO mice is also significantly advanced compared to naïve mice at day three and four post light exposure, as well as the Iba-1$^+$ area four days post light exposure. Nevertheless, the reactivation of microglia could be attenuated by the deficiency of galectin-3 in *Lgals3*-KO mice (Fig. 14b, c). The quantification of normalized *iNos*, *Il-6*, *Casp 3* and *Ccl2* expression levels in the RPE implied a similar pattern to the levels in the retina. The highest expression of *iNos*, *Il-6* and *Ccl2* take place on day one post light exposure, whereas the expression of *Casp3* did not change in our model of light- induced degeneration (Fig 14d). In the RPE, it is noticeable that the expression levels of all genes were reduced compared to the expression levels in the retina (Fig. 13d/ Fig. 14d). However, the expression of *iNos*, *Il-6* and *Ccl2* was reduced with deficiency of galectin-3 in *Lgals3*-KO mice.

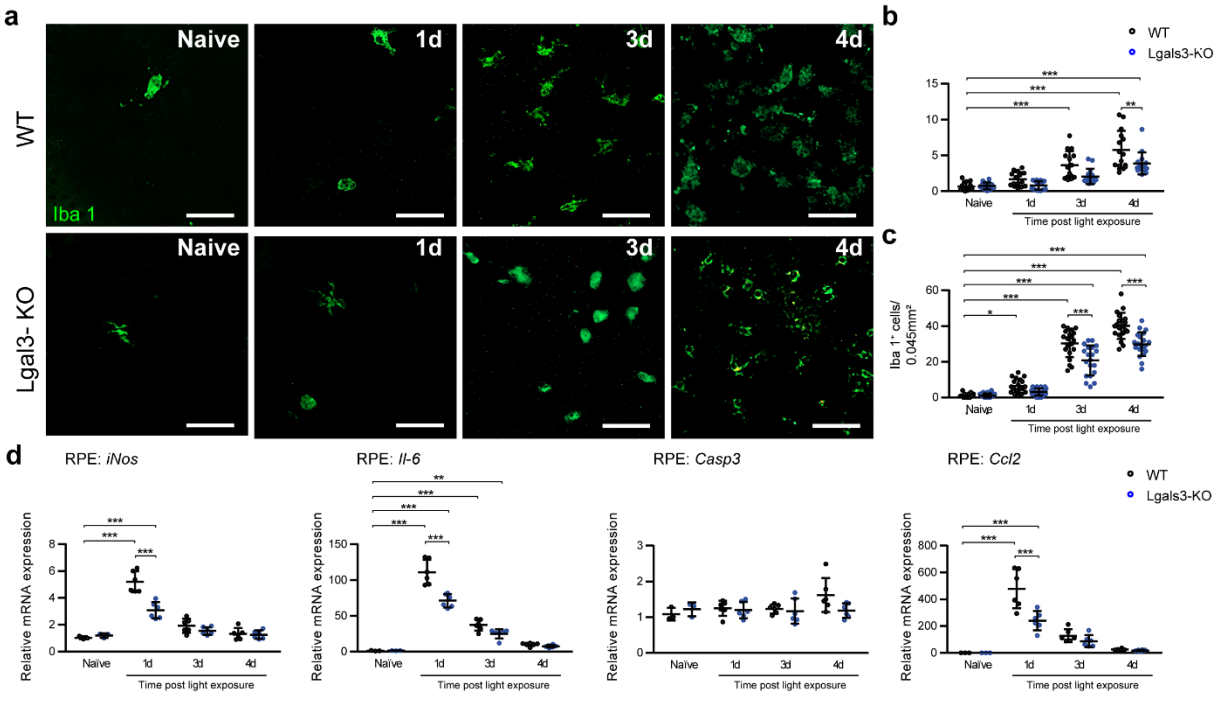

Figure 14: Microglia localization and morphology in RPE of *Lgals3*-KO mice. a Representative images of Iba-1$^+$ cells in RPE from light exposed *Lgals3*-KO and WT mice. Scale bar: 50 µm. **b** Quantification of Iba1$^+$ area in 0.045 mm² in the RPE. (WT/ *Lgals3*-KO n= 15). **c** Number of Iba-1$^+$ cells in the RPE (WT/ *Lgals3-KO* n= 15). **c** Iba-1$^+$ area in 0.045 mm² of the RPE. **d** Quantification of relative mRNA expression of *iNos*, *IL-6*, *Casp3* and *CCL2* (WT/ *Lgals3*-KO n= 6, RPEs from individual mice). Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.2.4 Retinal degeneration is reduced in *Lgals3-KO* mice

This light damage model mimics several features of dry AMD, including the degeneration of the photoreceptor cells. Thus, we performed *in vivo* optical coherence tomography (OCT) of mice to detect structural changes of the retina after light exposure in WT and *Lgals3*-KO mice (Fig. 15a). The OCT images showed clear changes in ONL reflectance in retinas of light- exposed animals, indicating a strong degeneration of the photoreceptor layer in animals with an intact galectin-3 gene (WT). The represented heat maps of WT and *Lgals3*-KO mice showed degeneration of the retina (Fig. 15a). Quantification of the retinal thickness in all analyzed animals demonstrated a significant reduction in the outer nuclear lay was after light exposure on day one, three and four in WT mice, which could be rescued by the knockout of galectin-3 (Fig 15b). The thinning of the retina was also significant in the central area and there the thickness is generally a little less. Moreover, the attenuated retinal thickness in WT mice post light exposure was significantly reduced in *Lgals3* mice at all measured time points.

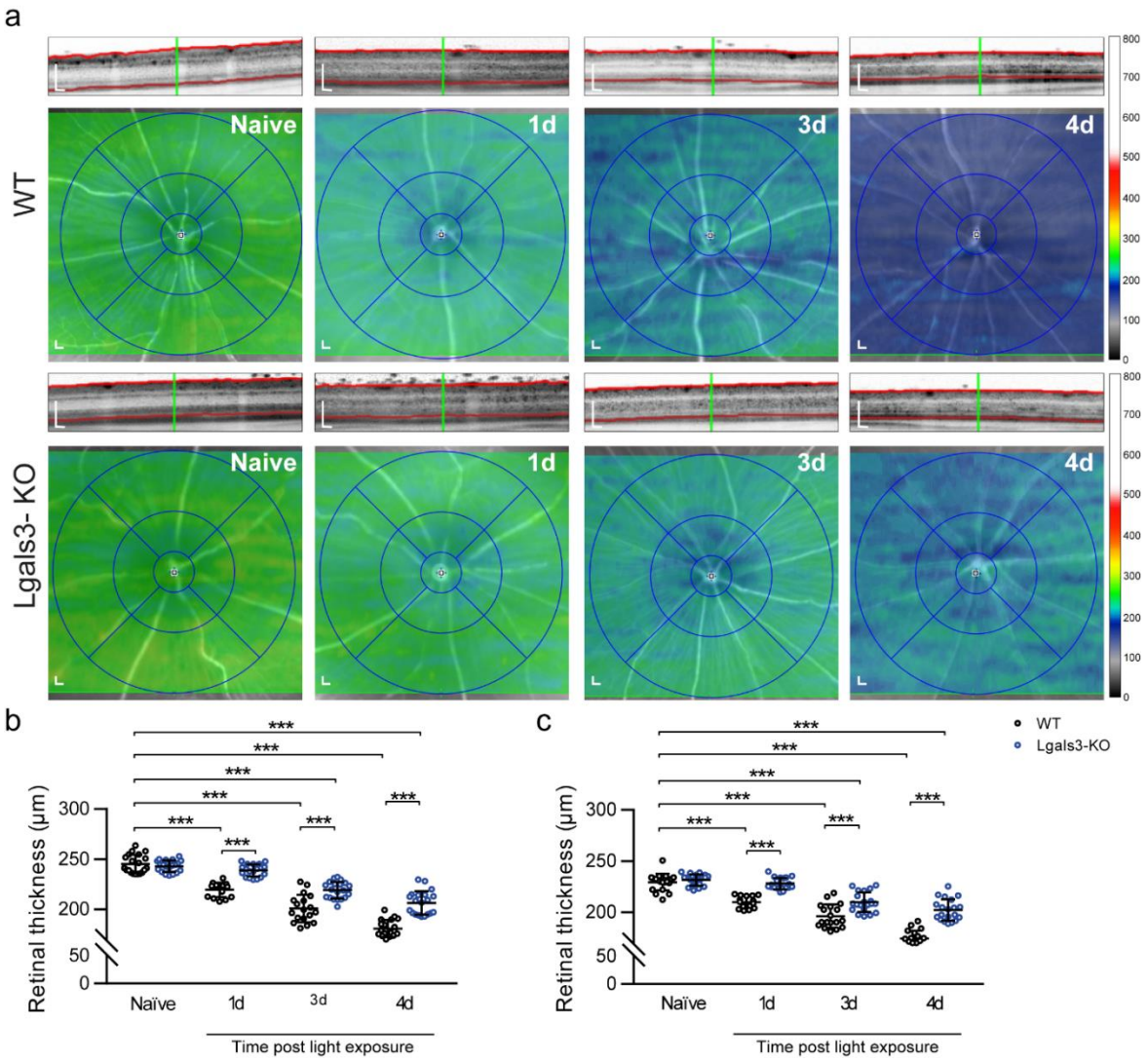

Figure 15: TD139 prevents light- induced retinal degeneration. a SD-OCT was performed in naïve mice, one, three and four days post light-exposure to analyze changes in retinal structure. Represented heat maps show the average retinal thickness light exposed vehicle and TD139 treated mice one, three and four days post light exposure. **b** Quantification of the averaged thickness of the outer (6 mm) retina and **c** inner (3 mm) retina. (WT/ *Lgals3*-KO; naïve n= 20; 1d n= 15/19; 3d n= 18/20; 4d n= 19/20.) Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.3 Immunomodulatory effect by galectin-3 inhibition, via TD139, in light-induced degeneration model

3.3.1 TD139 inhibition as a potential treatment option for dry AMD

In the model of light induced degeneration, we were able to determine that the absence of galectin-3 reduces the degeneration. Since a knockout is out of question as a treatment method in human, we were looking for a suitable inhibitor of galectin-3. TD139 has already been tested in several studies and is therefore a potential galectin-3 inhibitor (Hirani *et al.*, 2021). However, to see whether TD139 has no influence on other regulatory proteins in the eye, we first analyzed the effect of TD139 inhibition in *Lgals3*-KO mice in our light- induced degeneration model. We performed a small study with *Lgals3*-KO mice that received daily injections of TD139 (Figure 16a). This data demonstrates that the TD139 treatment has no influence on the veneration of the retina in *Lgals3*-KO mice post light exposure (Fig. 16). Thus, the retinal degeneration was unaffected in TD139-treated *Lgals3*-KO mice, suggesting that TD139 binds specifically to galectin-3 in the eye and therefore TD139 is a good potential option to inhibit galectin-3 in further studies.

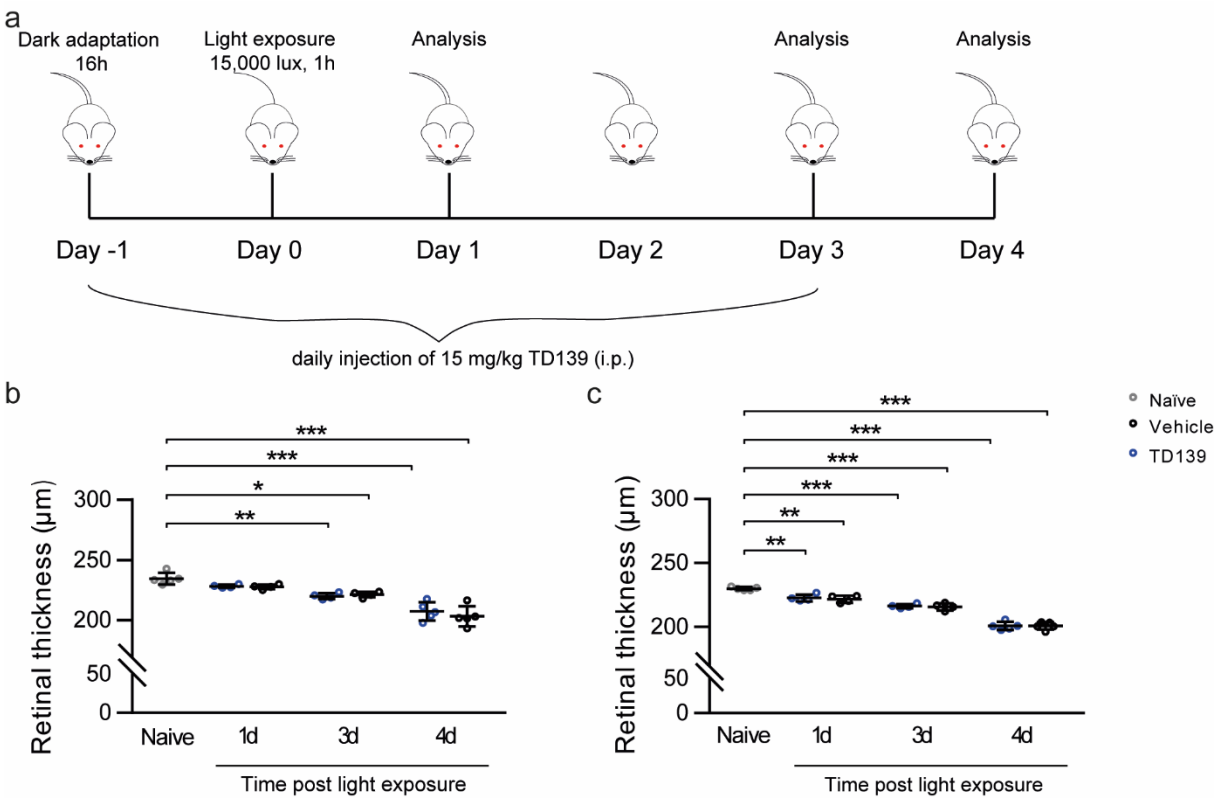

Figure 16: Galectin-3 inhibition via TD139 in *Lgals3*-KO mice. a Experimental strategy of light induced degeneration model in *Lgals3*-KO mice treated with TD139. **b** Quantification of the averaged thickness of the outer (6 mm) retina and **c** inner (3 mm) retina of *Lgals3*-KO mice with vehicle and TD139 treatment. (Vehicle/ TD139 naïve n= 5; 1d n= 4; 3d n= 4; 4d n=5). Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.3.2 Galectin-3 is upregulated in light induced degeneration model

To investigate the expression of galectin-3 in the model of light induced degeneration, we performed *in situ* hybridization of *Lgals3* (gene that is responsible for the protein gal-3) and *Allograft inflammatory factor* (*Aif*)-1. Iba-1 is expressed by microglia and is encoded by *Aif*-1. The specificity of the *Aif*-1- probe was demonstrated by an *in situ* hybridization and immunohistochemical co-staining (Fig. 17). In animal models of healthy, untreated mice, the expression of galectin-3 was very low (Fig. 18a). To validate the results of the genetic loss of galectin-3 and find a potential treatment option for dry AMD, we used the chemical inhibitor TD139 in light- induced BALB/cJ mice. Therefore, 8- 10 weeks old BALB/cJ mice were dark adapted and analyzed according to the same experimental strategy as the other

mice before, the mice were treated with daily intraperitoneal (i.p.) injections of TD139 or vehicle (Fig. 18b).

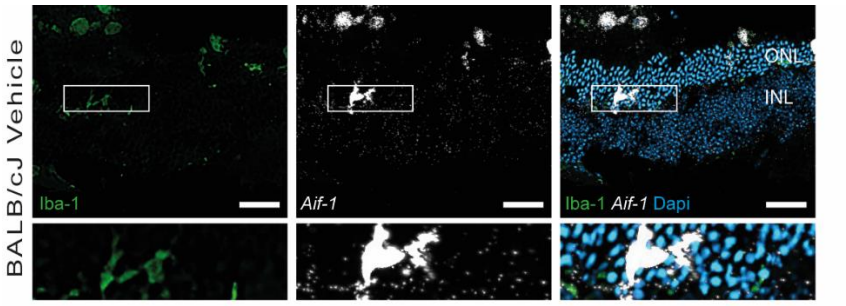

Figure 17: *Aif-1* expression in BALB/cJ. a In situ hybridization of *Aif-1* with Immunofluorescence staining of Iba-1 four days post light exposure. ONL: Outer nuclear layer; INL: inner nuclear layer. Scale bar: 50 µm.

Representative images of cryotome sections from vehicle- treated mice revealed an increased expression of *Lgals3* and *Aif*-1 in the sections, compared to TD139-treated mice (Fig. 18c, d). The pixel intensity of *Lgals3*, *Aif*-1 and the merge of both are increased in a time-dependent fashion from one to four days post light exposure, whereas sections from TD139-treated mice had less *Lgals3* and *Aif*-1, expression (Fig. 18e, f). The expression of *Lgals3* was significantly higher in vehicle group, three and four days post light exposure, compared to TD139-treated group. The ratio of *Aif*-1 and co-expression was also significantly reduced after inhibition of the binding potential of galectin-3. To validate the effect of the expression level of *Lgals3* after pharmacological inhibition via TD139, the mRNA expression of *Lgals3* in the retina and RPE were quantified (Figure 19). Indeed, *Lgals3* transcription levels were reduced in TD139-treated mice. However, the low expression of *Lgals3* in control mice was upregulated post light exposure and could be reduced by inhibiting the binding potential of galectin-3 with TD 139.

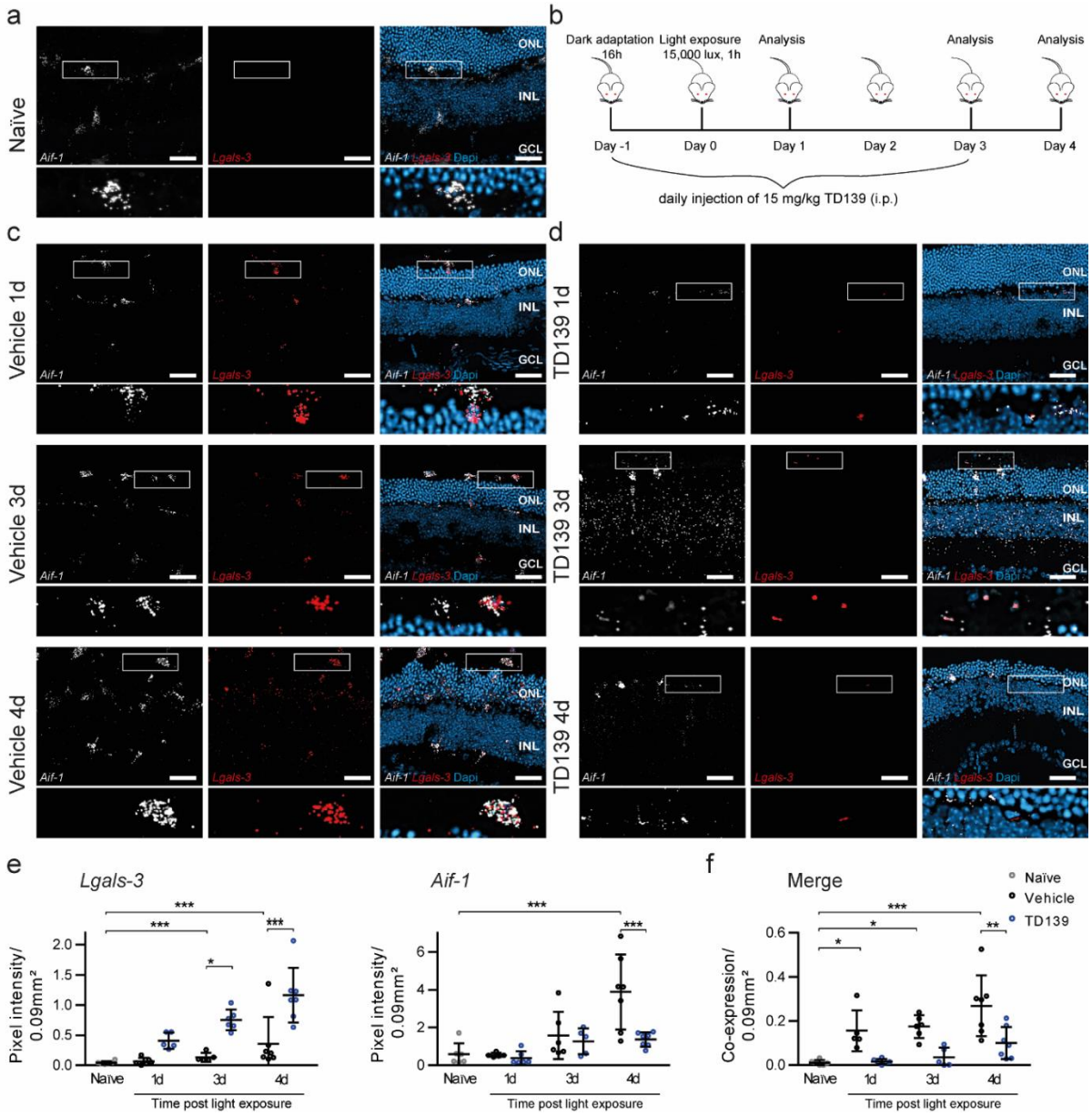

Figure 18: *Lgals3* expression in murine light-induced degeneration model. **a** In situ hybridization of *Aif-1*(white) and *Lgals3* (red) in sections of naïve mice. Scale bar: 50 µm. **b** Experimental design for light induced degeneration model and treatment strategy in BALB/cJ mice. **c** Expression of *Aif-1* and *Lgals3* in light exposed mice one, three and four days post light damage with vehicle and **d** TD139 treatment. Scale bar: 50 µm. **e** Quantification of *Aif-1* and *Lgals3* expression in light-exposed BALB/cJ mice one, three and four days post light damage. **f** Quantification of *Aif-1* and *Lgals3* co-expression using pixel intensity (Vehicle/ TD139 naïve n= 6; 1d n=5/6; 3d n= 6/5; 4d n= 7 sections). ONL: Outer nuclear layer; INL: inner nuclear layer; GCL: Ganglion cell layer. Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

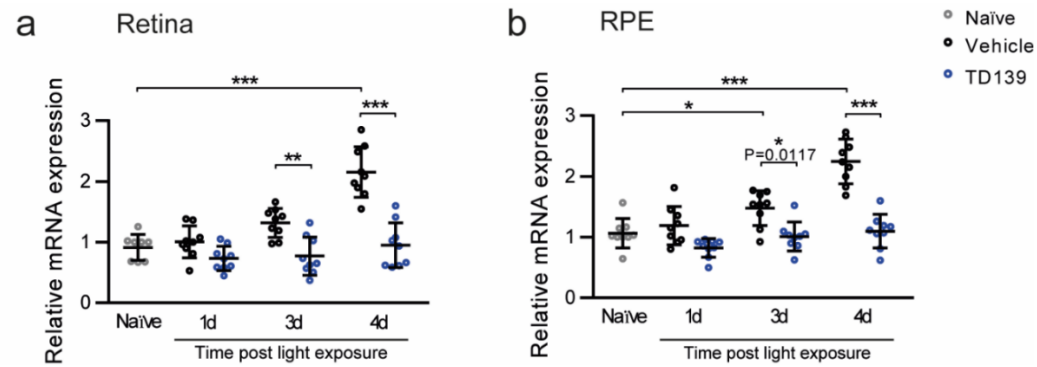

Figure 19: Gene expression of galectin-3 in the **a** retina and **b** RPE in naïve mice and one, three and four days post light exposure. (Vehicle/ TD139 n=9; naïve n= 8 eyes) Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.3.3 The inhibition of galectin-3 via TD139 prevents microglia migration in the retina

To test the effect of the inhibition of galectin-3 by TD139 on microglia migration in the damaged retina, we further analyzed the localization of microglia in cryotome sections of naïve BALB/cJ mice and mice with light exposure and vehicle or TD139- treatment one, three and four days post light exposure (Fig. 20a, b). The ONL of light- exposed mice from vehicle- group was much thinner and demonstrate an accumulation of amoeboid shaped microglia. Retinal immunolabeling with the microglia marker Iba-1 demonstrate microglia in the nuclear layers three and four days post light exposure. BALB/cJ mice of vehicle- group showed Iba-1^{+} cells in the degenerating photoreceptor layer and subretinal space. This degeneration of the photoreceptor layer and migration of microglia in the ONL was much less detectable in retinas of TD139-treated mice. The microglia migration was comparable to the microglia migration in naïve mice. The quantification of microglia in retinal cryotome sections displayed a reduced number of microglia in the ONL in TD139 treated mice, compared to vehicle-group (Fig. 20c). Thus, the treatment with TD139 prevented the migration of microglia to the ONL and subretinal space.

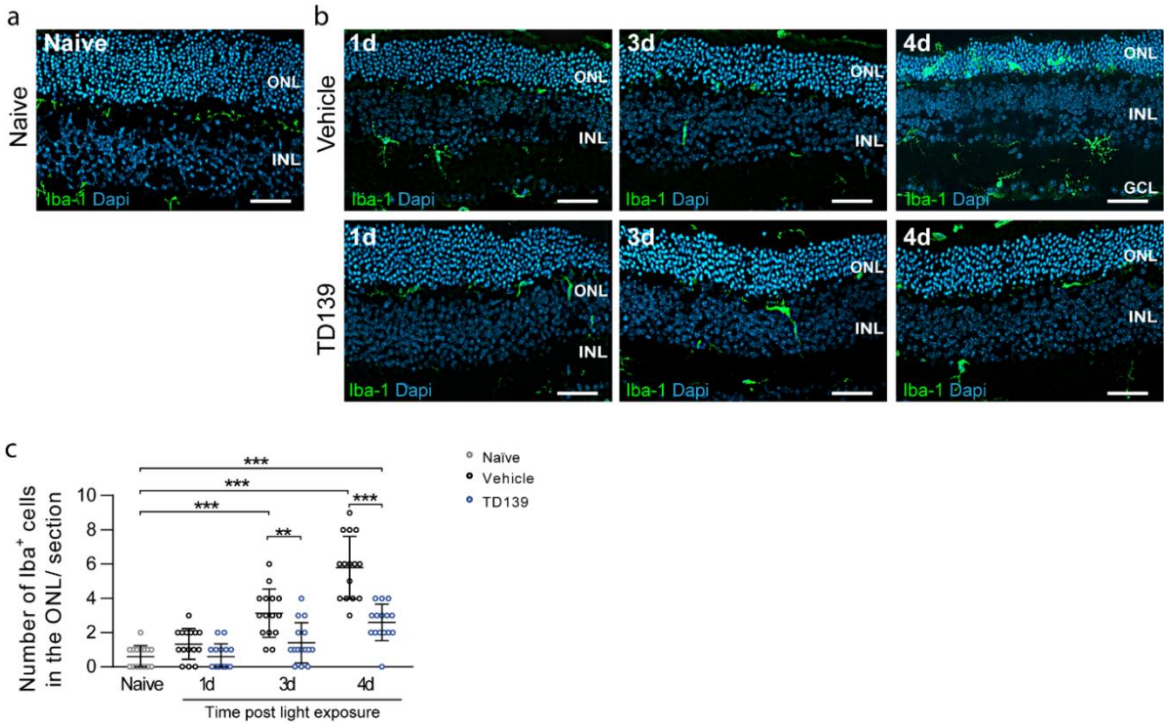

Figure 20: TD139 treatment dampens microglia migration in light induced degeneration. Representative images of Iba-1$^+$ cells migration into the ONL in **a** naïve mice and **b** in light exposed mice one, three and four days post light damage with vehicle and TD139 treatment. Scale bar: 50 µm. **c** Quantification of Iba-1$^+$ cells in the ONL in time-dependent fashion from one to four days post light exposure (Vehicle/TD139 n= 15 sections). ONL: Outer nuclear layer; INL: inner nuclear layer; GCL: Ganglion cell layer. Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.3.4 The inhibition of galectin-3 via TD139 reduces retinal microglia reactivity

We already showed that the lack of galectin-3 reduced the number of microglia in the subretinal space and the RPE. Retina and RPE flat mounts were also stained with Iba-1 in treated BALB/cJ mice. Images of retinal flat mounts from vehicle- treated mice revealed an increased number of Iba-1$^+$ reactivated cells in the subretinal space three days post light exposure. The induction was more strongly after four days, whereas the amount of Iba-1$^+$ cells in the retinas from TD139- treated mice was lower and these cells appeared mainly more ramified (Fig. 21 a- d). Moreover, TD139 treatment significantly reduced the induction of reactivated microglia in the subretinal space post light exposure. We next studied the

mRNA expression of pro- inflammatory microglia marker. Inducible *iNos*, *Il-6*, *Casp3* and *Ccl2* on three different time points. RNA levels were normalized to *Atp5b* (ATP synthase) and the induction was calculated by comparison to naïve controls. The expression levels of *iNos*, *Il-6* and *Ccl2* were increased in retinal tissue of light exposed mice, whereas levels of *Casp3* did not change (Fig. 21e). One day post light exposure, the expression of *iNos*, *Il- 6* and *Ccl2* has reached the highest level. Three days post light exposure, the expression of *iNos* is still significantly higher compared to naïve control, but the expression is rapidly decreasing. From the beginning, the expression of *iNos* much higher in the vehicle group compared to TD139-treated group, so that the increase in the treated group was no longer significant on day four. The ratio of *iNos* expression between vehicle and TD139-treated group was only significant on day one. In addition, the *Il-6* and *Ccl2* expression three and four days post light exposure were also not increased in the same amount, TD139-injected mice had strongly attenuated expression levels comparable to vehicle group (Fig. 21e).

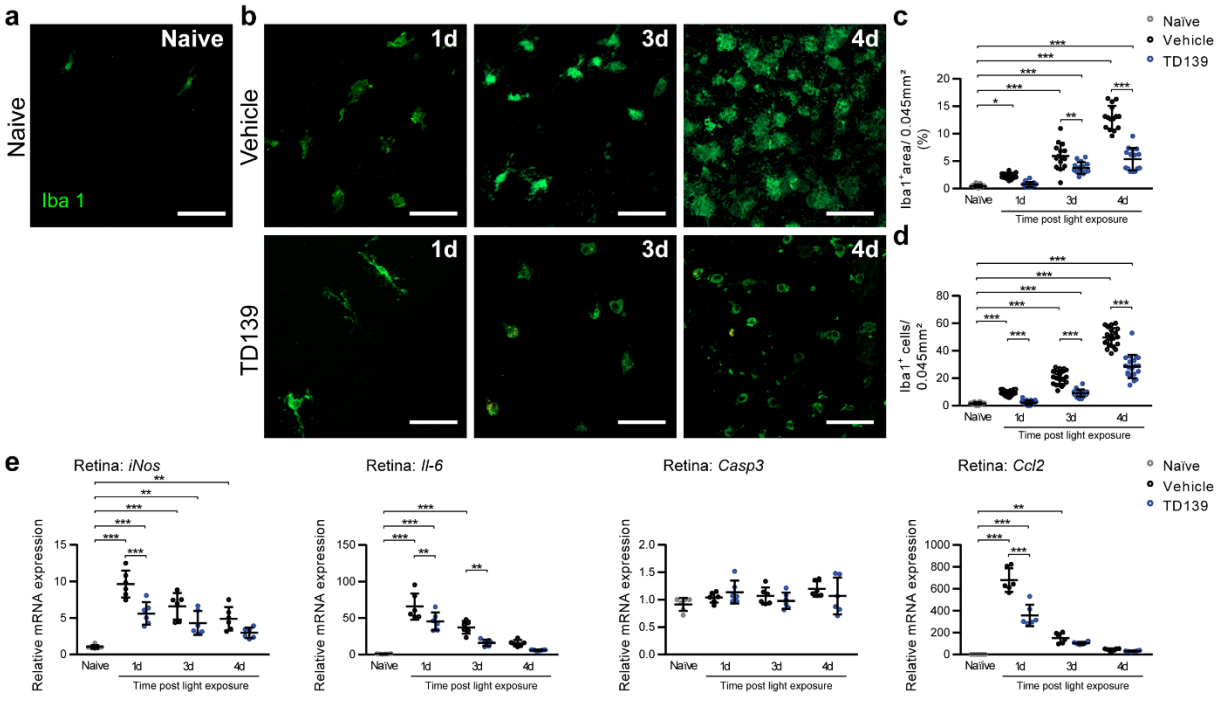

Figure 21: Microglia localization and morphology in murine retinas of light exposed BALB/cJ mice. Immunofluorescence analysis of Iba-1 $^{+}$ cells in subretinal space of retinal flat mounts from **a** naïve and **b** light exposed BALB/cJ mice with TD139- or vehicle treatment without light exposure, one, three and four days post light exposure. Scale bar: 50 µm. **c** Quantification of Iba1^{+} area in 0.045 mm² of the Retina. (Vehicle/ TD139 n= 15). **d** Number of Iba-1^{+} cells in the subretinal space.

(Vehicle/ TD139 n= 15). **e** Quantification of relative mRNA expression. *iNos*, *Il-6*, *Casp*3 and *Ccl2* (Vehicle/ TD139 n= 6, retinas from individual mice). Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.3.5 Microglia in the OPL remains uninfected by inhibition via TD139

To investigate the function of the inhibitor in more detail and its influence on the structural changes of the microglia, the morphological changes of the OPL cells were also analyzed using MotiQ. Immunohistochemical staining in the OPL showed large changes after light damage in both the TD139-treated group and the vehicle control. The images clearly showed already one day after the light damage the microglia branches retract and the cells become rounder, similar to the subretinal space. However, there is no difference between the TD139 and the vehicle group. During the morphological examinations with MotiQ analysis, the impression of the representative images was confirmed. Although the area on the first day post light exposure differs significantly between TD139- and vehicle-treated animals. However, the area remains significantly lower all the time in TD139-treated mice and vehicle group compared with naïve animals. Also in the spanning outline, a small difference is seen one day post light exposure, but this is cancelled on day 3 post light exposure. All other OPL examinations are consistently significantly reduced compared to naïve mice. Not only the area, and the spanning outlines, but also the spanned area and the outlines. The total tree length and the ramification index are also lower compared to the naïve control, and increase again equally in both groups. This suggests that TD139 has no effect on the microglial cells in the OPL.

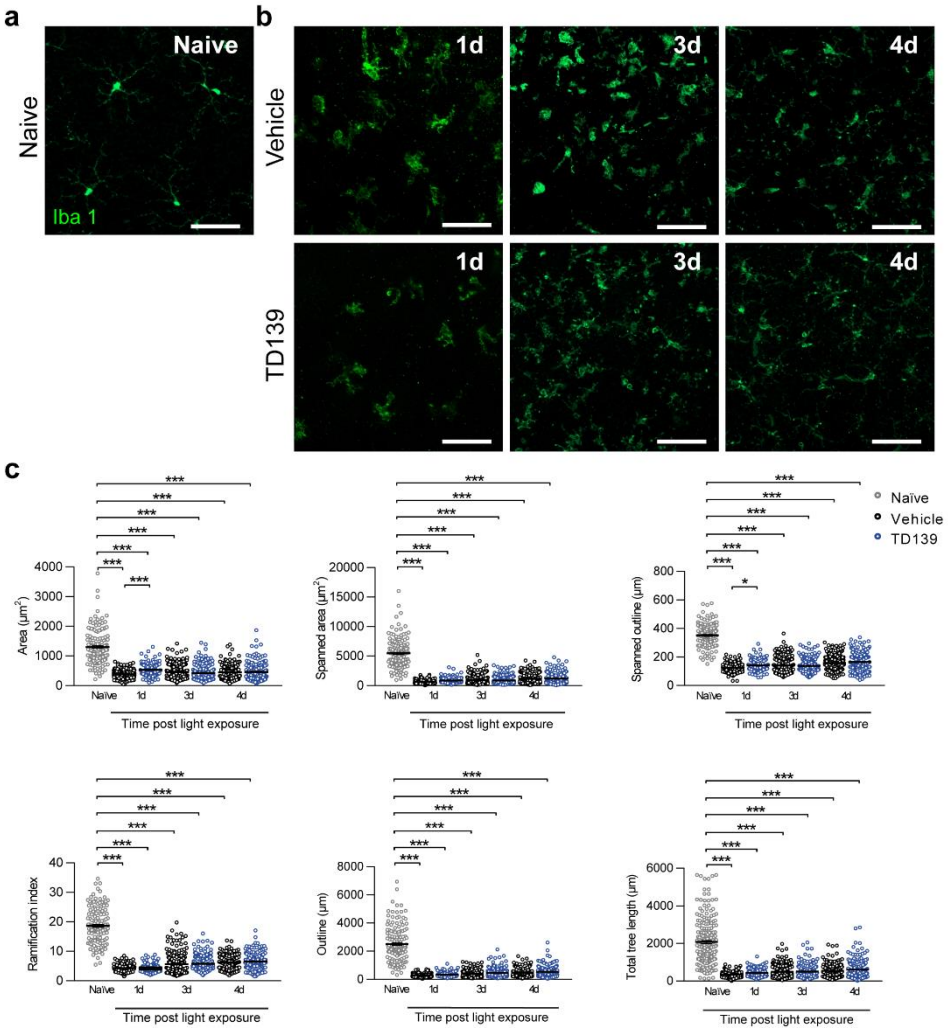

Figure 22: Morphological changes in OPL of BALB/cJ mice post light exposure. a Representative images of the OPL from naïve mice and **b** TD139 and vehicle treated mice. **c** Morphological analysis by MotiQ, including area (µm²), spanned area (µm²), spanned outline (µm), ramification index (µm), outline (µm) and total tree length (µm), in the OPL of mice post light exposure. (Vehicle/ TD139 naïve n= 145; 1d n=212/151; 3d n= 224/205; 4d n= 189/225 Iba-1$^+$ cells). Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.3.6 The inhibition of galectin-3 via TD139 reduces microglia reactivity in RPE

In addition, immunohistochemical staining of Iba-1 in RPE in naïve and light damaged mice were performed. It could be demonstrated Iba1$^+$ cells in an amoeboid shape from day three

post light damage, in vehicle and TD139-treated mice (Fig. 23a, b). The inhibition of galectin-3 via TD139 resulted significantly the reactivity and migration of Iba-1$^+$ cells in RPE compared to vehicle group. Nevertheless, there is a significant induction of reactivated microglia in the RPE in TD139-treated mice three and four day post light exposure compared to naïve mice (Fig. 23c, d). We next analyzed the expression level of *iNos*, *Il-6*, *Casp3* and *Ccl2* in RPE. Similarly, as in the retina, expression levels of *iNos*, *Il-6* and *Casp3* increased in the RPE in a time- dependent fashion from one to four days post light exposure and the TD139 treatment reduced this expression (Fig. 23e). It is noticeable that the expression of *iNos* and *Ccl2* reduced compared to the expression in the retina. Collectively, these data indicate that an inhibition via TD139 reduced migration of Iba-1$^+$ microglia in the subretinal space and RPE and attenuated the expression of pro- inflammatory marker *iNos*, *Il-6* and *Ccl2*. The expression level of *Casp3* did not change in retina and RPE.

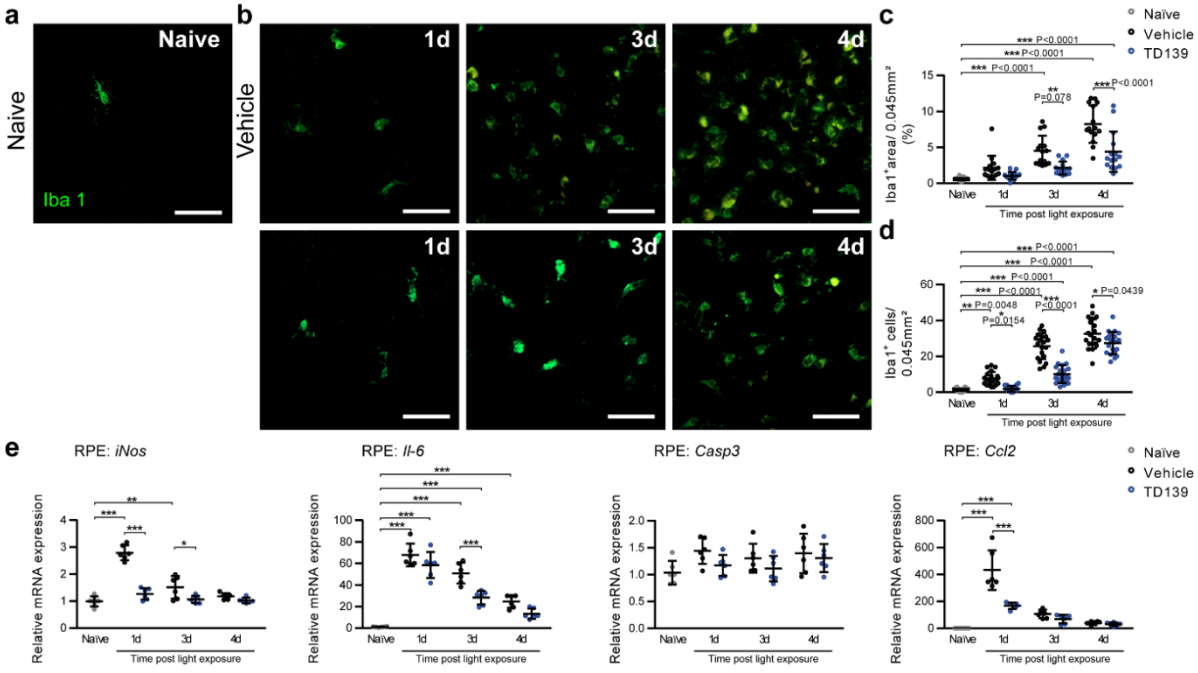

Figure 23: Localization and morphology of microglia in the RPE of BALB/c mice. Representative images of Iba-1$^+$ cells in RPE from **a** naïve and **b** light-exposed BALB/cJ mice with Vehicle and TD139 treatment. Scale bar: 50 µm. **c** Quantification of Iba1$^+$ area in 0.045 mm² in the RPE (Vehicle/ TD139 n= 15). **d** Number of microglia in the RPE. (Vehicle/ TD139 n= 15). **e** Quantification of relative mRNA expression. *iNos*, *Il-6*, *Casp3* and *Ccl2* (Vehicle/TD139 n= 6, RPEs from individual mice). Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.3.7 TD139-treatment rescued reduction of retinal thickness post light exposure

Subsequently, we performed *in vivo* optical coherence tomography (OCT) with vehicle and TD139- treated mice to detect structural changes of the retina post light exposure (1h, 15000 lux) (Fig. 24a, b). Already one day post light exposure, there was a significant thinning of the retina in the vehicle group compared to naïve mice detectable, indicating a strong degeneration of the photoreceptor layer in these mice. The thinning of the retina increased clearly from day one to day four post light exposure. In contrast, TD139-treated mice displayed a normal hyperreflective photoreceptor layer similar to that of non-light-exposed naïve mice, one day post light exposure. But TD139- treated mice also implied an attenuated thickness of the retina three and four days post light exposure compared to naïve mice. In the outer and central area around the optic nerve (Fig. 24c, d). However, volume scans revealed a severe thinning of the retina, especially in the central area around the optic nerve head (Fig. 24d) post light exposure in vehicle-group, which was significantly reduced in the TD139-treated groups (Fig. 24a-d). These indicate a strong neuroprotective effect of galectin- 3 inhibition via TD139 in conditions of acute light damage.

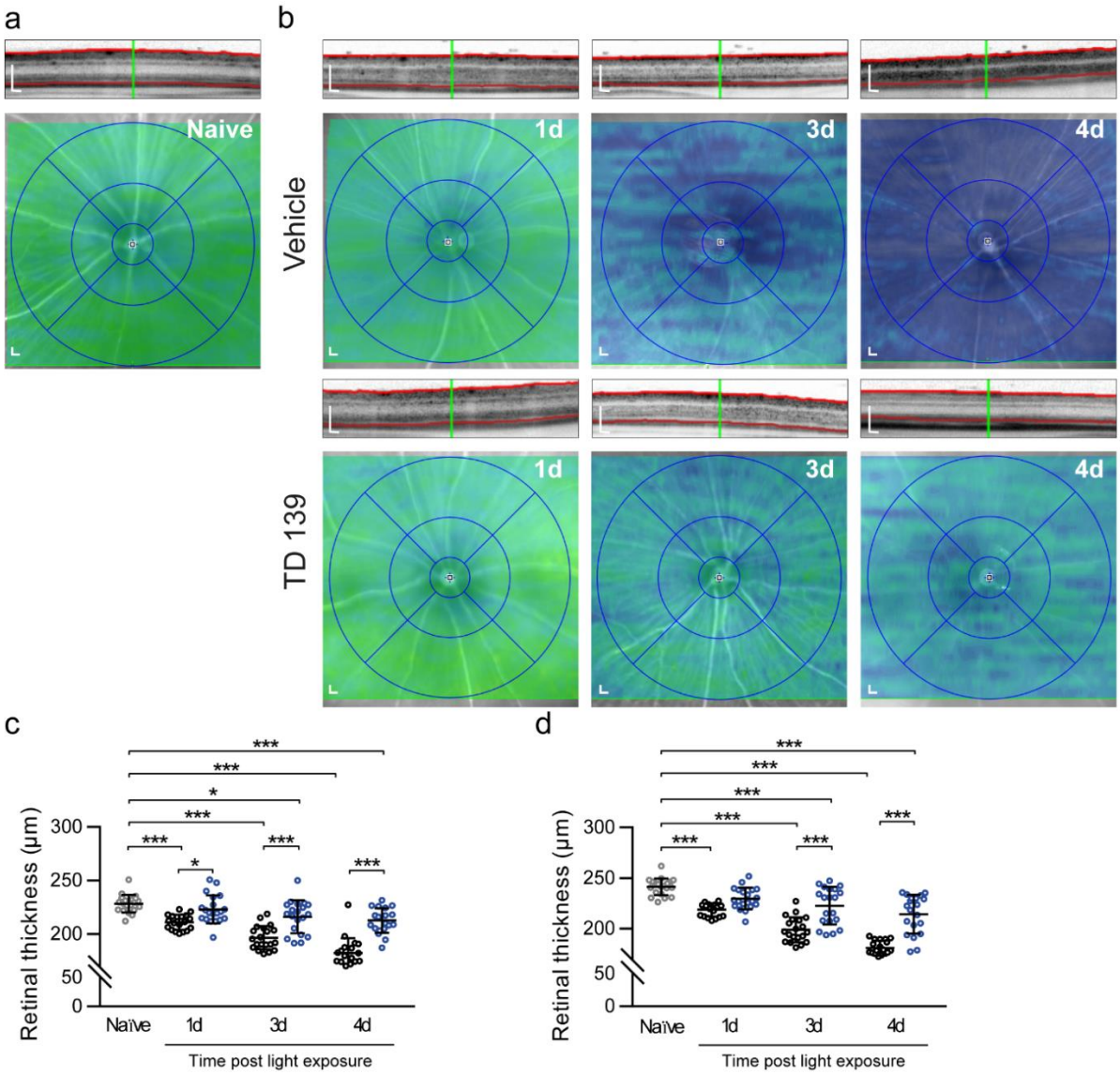

Figure 24: TD139 prevents light- induced retinal degeneration. a Representative heat maps of naïve mice. Scale bar: 200 µm. **b** SD-OCT was performed one, three and four days post light exposure to analyze changes in retinal structure. Represented heat maps show the average retinal thickness light exposed vehicle and TD139 treated mice one, three and four days post light exposure. Scale bar: 200 µm. **c** Quantification of the averaged thickness of the inner (3 mm) retina and **d** outer (6 mm) retina. (Vehicle/ TD139 naïve n= 20; 1d n= 19/20; 3d n= 20; 4d n= 19/20.) Data are presented as mean ± SEM. Linear mixed model was used for statistical analyses; * P< 0.05, ** P<0.01, *** P≤0.001.

3.4 Model of galectin-3 inhibition via TD139

Based on the data presented in this thesis, we described that the migration of microglia is initiated by galectin-3. As well as the expression of pro-inflammatory cytokines and degeneration of the retina. Targeting galectin-3 by gene knockout or using a specific galectin-3 inhibitor the reactivation of microglia is reduced. In response to a light-induced damage, resident microglia starts to change their morphology and migrate to the lesion site of the damage. The reactivated microglia cells phagocytose death photoreceptor cells and the degeneration of the photoreceptor layer begins. The dysregulation and chronical activation of microglia is responsible for phagocytoses of healthy photoreceptor cells. The deficiency and inhibition of galectin-3 with TD139 decreased the reactivation of microglia and reduced the expression of pro-inflammatory cytokines, thereby limits it the photoreceptor cell death (Fig. 25).

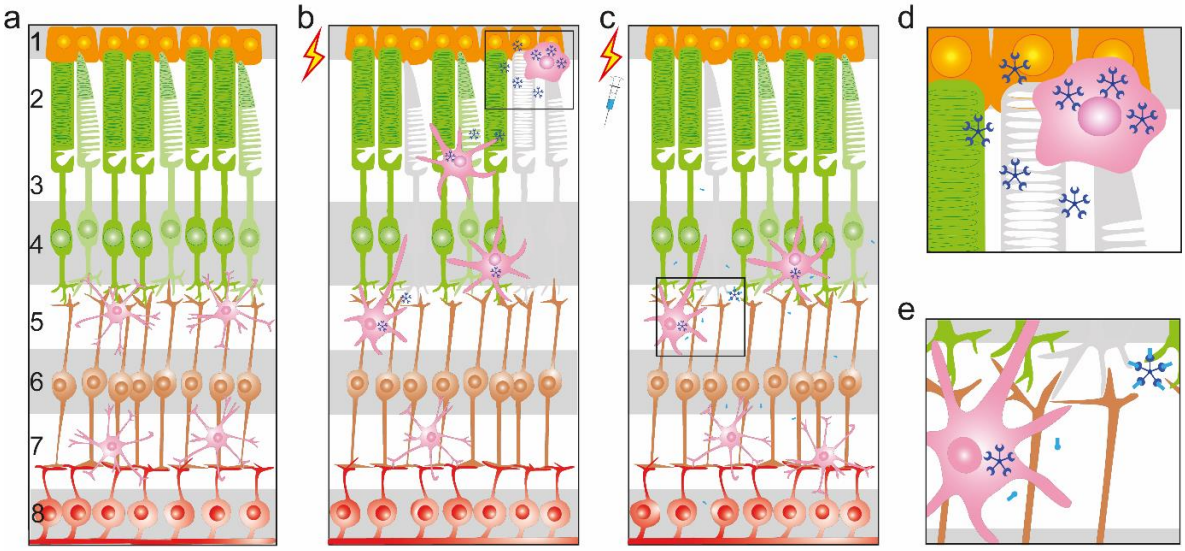

Figure 25: Schematic representation of the retina under healthy, damaged, and inhibited conditions. a Schematic overview of microglia in homeostasis state, **b** in damaged retina and **c** in retina treated with TD139. **d** Represents an activated microglia cell with galectin-3 (dark blue) expression extra- and intracellular **e** demonstrate a typical microglia in light-induced degeneration model treated with TD139 (light blue) which bind galctin-3 and inhibits the immunoreaction caused by galectin-3. The mammalian retina is distributed in three cellular layers and two synaptic (plexiform) layers. 1: RPE; 2: outer segment; 3: inner segment; 4: outer nuclear layer; 5: outer plexiform layer; 6: inner nuclear layer; 7: inner plexiform layer; 8: ganglion cell layer. Modified from Karlstetter, Marcus *et al.*, 2010 and Wolf *et al.*, 2020.

4. Discussion

4.1 Galectin-3 expression in AMD

The innate immune system is one of the relevant mechanisms in the development and progression of neurodegenerative diseases, such as age-related macular degeneration (AMD). Chronically activated microglia and their dysfunctional regulatory system contribute to neurodegenerative retinal diseases (Gupta, N. *et al.*, 2003). Therefore, the regulation of the immune system, including reactivated microglia, represents promising target structures for new therapeutic options of AMD (Karlstetter, Marcus *et al.*, 2010). Neurodegenerative diseases were often associated with increased expression of galectin-3 (Ashraf *et al.*, 2018; Caridi *et al.*, 2021; Trompet *et al.*, 2012; Yazar *et al.*, 2021; Yip *et al.*, 2017). Its expression is increased in glial cells during an inflammatory response and regulates migration by promoting extracellular matrix adhesion and cell survival (Dhirapong *et al.*, 2009; Dumic *et al.*, 2006; Sirko *et al.*, 2015). The exact mechanism of galectin-3 regulation has not yet been explored and is not elucidated in this study. Since there is no treatment option for dry AMD available, it is essential to do research on this field. In the present study, we have shown that galectin-3 is increasingly expressed in the retina from AMD patients. In the healthy retina, galectin-3 is also expressed, but only in small amounts. This is in line with proteomics analyses from 2012, where it was shown that galectin-3 is expressed in AMD patients dependently of the developed form the patients are affected. However, the expression is highest in patients with geographic atrophy, which is the worst form of dry AMD (Fleckenstein *et al.*, 2018; Yuan *et al.*, 2010). Considering, AMD patients with choroidal neovascularization (NCV) also shown two to three times higher galectin-3 expression in the RPE compared to donors of the same age (An *et al.*, 2006). Another study investigating human and mouse brain samples of Alzheimer's disease displayed galectin-3 expression in Iba-1^+ cells around Aß- plaques (Boza-Serrano *et al.*, 2019). Consistent with this, we demonstrated that the expression of galectin-3 is produced by microglia, using *in situ* hybridization of cryotome sections from an AMD patient. Remarkably, although galectin-3 is expressed by microglia, some neurons, particularly in the ganglion cell layer, also express galectin-3 (Yoo *et al.*, 2017).

4.2 Immunological effect of galectin-3 deficiency and pharmacological inhibition on light-induced degeneration

In this study, the function of galectin-3 was investigated in the light-induced degeneration model of the retina. This model is an established animal model for dry AMD because it reflects different points of AMD (Grimm *et al.*, 2000; Wenzel *et al.*, 2005). In the light-induced degeneration model in mice, as in human AMD, a reactivity of microglia in the ONL and subretinal space can be observed as well as the death of photoreceptors. (Grimm *et al.*, 2000). In many studies this model has already been applied and the comparability is given (Scholz *et al.*, 2015b; Wenzel *et al.*, 2005). Nevertheless, there are clear differences to the disease in humans, which differ in the formation of a macula and drusen in comparison to mice. In the present study, the effects of genetic deficiency of galectin-3 and the pharmacologic inhibition of this protein were investigated. The microglia reactivation is dependent on various factors (Biber *et al.*, 2007; Chen, M. *et al.*, 2019; Hickman *et al.*, 2013; Xu *et al.*, 2009). Photoreceptor cell degeneration is promoted by dysregulated and chronical activated microglia. Photoreceptors are often stressed by dying cells in their immediate environment and microglia phagocytose the stressed but healthy photoreceptors (Zhao *et al.*, 2015). It has been reported that microglia increase galectin-3 expression, particularly under stressful conditions (Lalancette-Hébert *et al.*, 2012). Therefore, microglia were stimulated in cell culture and shown strong gene expression and secretion of galectin-3 (Cockram *et al.*, 2019). This could also be demonstrated in mouse models (Siew et al., 2019; Yip et al., 2017). Using the mouse model of light-induced retinal degeneration, we also found an increase in gene expression of galectin-3 in WT mice after light exposure.

Here, two mouse lines were used, the first experiments were performed with galectin-3 knockout (*Lgals3*-KO) mice. Using this mouse line, we were able to determine the influence of a loss of galectin-3 in mice in our model of dry AMD. Furthermore, it is investigated that galectin-3 deficiency in the whole body of the animals shows some differences between *Lgals3*-KO and WT mice. On the one hand, there is the assumption that galectin-3 deficient animals be obese (Pang *et al.*, 2013; Pejnovic *et al.*, 2013). This could cause an increased light damage in our light induced degeneration model, because obesity is a risk for a more severe progression of AMD (Fritsche *et al.*, 2014; Parekh *et al.*, 2009). On the other hand, studies show that weight gain is reduced in galectin-3 deficient animals compared to WT mice (Pejnovic *et al.*, 2013). However, galectin-3 is also secreted more in mouse models with a high fat diet (Yilmaz *et al.*, 2015). Another important point is that galectin-3 plays an

essential role of the immune system and controls many functions of immune cells. It is essential for cell-cell and cell-matrix interaction (Delacour *et al.*, 2008; Jiang *et al.*, 2014). Furthermore, it is involved in cell growth, proliferation, differentiation and inflammatory processes (Nakahara *et al.*, 2005; Panjwani, 2014; Ruvolo, 2016; Sciacchitano *et al.*, 2018). In the second part of the experiment, we were able to investigate a promising inhibitor targeting galectin-3, which could be administered directly in case of disease. Galectin-3 is defined as a target molecule for various diseases (Burguillos et al., 2015; Dhirapong et al., 2009) and new immunomodulatory molecules against galectin-3 were constantly being developed. Other studies are crucial to the development of such immunomodulatory inhibitors against galectin-3. The inhibition of galectin-3 is already described to reduced fibrosis in the lung and decreases cardiac function in the doxorubicin (DOX)-induced cardiac function model in rats (Hirani *et al.*, 2021; Mackinnon *et al.*, 2012; Tian, Y. *et al.*, 2020). There are a several galectin-3 inhibitors identified. Some of these inhibitors are already in clinical trials (Chalasani *et al.*, 2020; Curti *et al.*, 2021; Harrison *et al.*, 2016; St-Gelais *et al.*, 2020). TD139, which binds to the CRD of the galectin-3 protein and is in clinical trial for the treatment of idiopathic pulmonary fibrosis, is one of these promising candidates (Chan *et al.*, 2018). Additionally, it is described that TD139 binds mouse galectin-3 (Kumar *et al.*, 2021), which leads to the decision to perform our experiments with this inhibitor.

In this study, galectin-3-deficient (*Lgals3*-KO) mice and those with pharmacological inhibition of the protein were exposed to a bright white light with an intensity of 15,000 lux. This allowed us to trigger an immune response very similar to dry AMD (Grimm *et al.*, 2000; Scholz *et al.*, 2015b). In response to this light, microglia migrate to the nucleolar layers of the retina and the RPE (Karlstetter, M. *et al.*, 2015; Langmann, 2007; Nowak, 2006; Scholz *et al.*, 2015b). The effect of the loss of galectin-3 in *Lgals3*-KO mice already led to a reduction of the immune response of the microglia in previous studies (Yin *et al.*, 2020). The deficiency of galectin-3 may reduce the number of proliferating microglia cells in the ischemic brain (Lalancette-Hébert *et al.*, 2012) and reduces the Alzheimer's disease phenotype by making galectin-3 responsible for the reactivity of microglia in Aß plaques (Boza-Serrano *et al.*, 2019). By the absence or inhibition of galectin-3, we demonstrated that microglia reactivity and migration in the lesion area of the retina and RPE was significantly decreased post light exposure compared to WT and vehicle treated mice. Nevertheless, microglia continue to migrate in the subretinal space and RPE, and the migration is not completely stopped. Microglia not only respond to galectin-3 secreted by themselves, the activation of microglia is very complex and is influenced by many different components.

Membrane associated and cytosolic receptors interacts with classes of PAMPs or DAMPs in the environment of microglia and promote the reactivation (Colton, 2009; Lively *et al.*, 2013; Luo *et al.*, 2012). One of the prominent pathways to induce a microglia response is probably the signal pathway of TLR-4 and Nf-κB. In cell culture experiments, BV-2 microglia cells were stimulated with PBS and microglia directly starts with inflammatory response (Meng *et al.*, 2020). This stimulation leads to activate the Nf-κB- pathway (Bao *et al.*, 2019). It is known that galectin-3 as a ligand of TLR-4 influences the activation of the cells via the Nf-κB-pathway (Zhou *et al.*, 2018). However, galectin-3 is not the only ligand of TLR-4 and reactivation of microglia still occurs (Eisenstein, 2019; Zusso *et al.*, 2019). Interestingly, a study with primary microglia showed that knockdown of galectin-3 led to an almost complete transformation of microglia morphology from an amoeboid to a branched state (Reichert et al., 2019).

Surprisingly, in our mouse model, even a daily intraperitoneal injection of this inhibitor leads to reduced migration of microglia. Previously, it was only shown that systemic inhibition affects galectin-3 binding in the liver region (Yu *et al.*, 2021). In human tissue studies, the application is via the medium directly to the tissue (Shochet *et al.*, 2020). An injection straight into the eye would possibly result in an even better effect. Due to the solubility of the inhibitor TD139, dissolved in DMSO, it is not possible. However, one possibility would be to try another inhibitor (GR-MD-02) which is soluble in water (Chalasani *et al.*, 2020) and has so far only been tested in other diseases, such as NASH (Harrison *et al.*, 2016), to investigate a local effect.

Reactivation of microglia not only leads to migration and morphological change of these cells. The production of pro-inflammatory cytokines and chemokines begins with reactivation more strongly (Scholz *et al.*, 2015a; Yoshimura *et al.*, 2009). According to previous studies, we have shown that the production of pro-inflammatory cytokines was reduced in galectin-3 deficient mice (*Lgals3*-KO). Studies have shown that galectin-3 increases oxidative stress and pro-inflammatory cytokine production in cell culture experiments (Yin *et al.*, 2020). This also confirms treatments with galectin-3 resulting in increased cytokine secretion of Tnf-α, Il- 1ß, Il-6, and Inf-γ (Jeon *et al.*, 2010; Lalancette-Hébert *et al.*, 2012). Consistent with this, other studies reported that epithelial cell-associated galectin-3 activates dendritic cells to produce Tnf-α and Il-6, resulting in the increase of the expression of activation markers (Schroeder *et al.*, 2020) and induces the expression of JAK/STAT1 and other (Nita-Lazar *et al.*, 2015). In AMD, increased microglia activity is

associated with faster disease progression (Wolf *et al.*, 2020). Cytokine expression of the pro inflammatory markers *iNos*, *Il-6*, and *Ccl2* is reduced in galectin-3 deficient mice compared with WT mice. This is also be observed in other studies, showing that have shown that iNos is increased in stressed and reactivated microglia cells (Alliot *et al.*, 1999; Santos *et al.*, 2008). Experimental data show a correlation of IL-6 in AMD patients and the severity of AMD. Thus, patients in the early AMD stage show only a slightly increased production of IL-6, but patients with GA show a strong induction of IL-6 (Nahavandipour *et al.*, 2020). However, we could observe the highest *Il-6* expression on day 1 post light damage, which may cause due to the design of the model. AMD does not usually develop from one day to the next. In our mouse model, we induce light damage within a very short time, so the response is much faster. Other studies show that the Ccl2 level may be directly related to AMD (Ambati *et al.*, 2003; Combadière *et al.*, 2007; Fujimura *et al.*, 2012). Thus, reduction of Ccl2 levels by loss of galectin-3 may lead to a reduction of damage in AMD patients. We could also confirm the reduced expression of pro-inflammatory cytokines with our second study in Balb/cJ mice receiving the inhibitor.

Besides the positive effect on microglia reactivation, reduced migration and decreased expression of pro-inflammatory cytokines, the deficiency of galectin-3 and the pharmacological inhibition also affects retinal degeneration. Therefore, we demonstrated that retinal degeneration measured by OCT appears more slowly in galectin-3 knockout mice and TD139-treated BALB/cJ mice. This could have a positive impact on vision. Degeneration of photoreceptors is a hallmark of AMD disease (Curcio, 2001; Curcio *et al.*, 1996). Consistent with previous studies, a lower immune response of microglia affects the development of retinal degeneration (Scholz *et al.*, 2015b). This is confirmed with other ocular studies show that inhibition of galectin-3 by a small chemical inhibitor reduces pathologic corneal neovascularization and fibrosis (Blanda *et al.*, 2020; Chen, W. S. *et al.*, 2017).

Thus, our data demonstrated that galectin-3 is a potential starting point for therapy in AMD patients because whole-body knockout of galectin-3 significantly reduced the retinal innate immune response, number of reactivated microglia in the subretinal space and RPE of light-exposed mice, and inhibited photoreceptor degeneration. The same could be determined in the second part of our mouse studies. Our study also revealed that inhibition of galectin-3 reduced the microglia-reactivation, pro-inflammatory cytokine expression and retinal degeneration in the model of light-induced degeneration.

4.3 Conclusion

In this study, we investigated the effects of genetic knockout of galectin-3 and immunomodulation by pharmacological inhibition on microglia-induced retinal degeneration in the light-induced degeneration model of dry AMD. There we could determine that microglia in AMD patients represent the major part of galectin-3 production, which occur in a lower level in a healthy retina. Furthermore, using our mouse models, we could observe that galectin-3 is a key factor in the reactivation of microglia. Loss or inhibition of this protein leads to reduced migration of microglia and lower production of pro- inflammatory cytokines due to attenuated activation compared to control animals. At the same time, light-induced retinal degeneration was attenuated. Overall, this work reflects that galectin-3 plays an important role in retinal microglial activity and confirms that inhibition of this protein is a potential immunomodulatory target for therapy against AMD.

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7. Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten 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.

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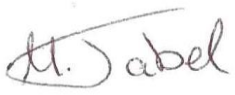

(Mona Tabel)