

The role of complement in a new mouse model of focal retinal degeneration

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Candidate's Declaration

This declaration certifies that the following work entitled ‘The role of complement in a new mouse model of focal retinal degeneration’ is the author’s own original work, complies with The Australian National University Research Award Rules and has not previously been accepted for award of a degree or diploma to any other university or institution of higher learning. This thesis was supported by an Australian Government Research Training Program (AGRTP) Scholarship.

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“True knowledge exists in knowing that you know nothing”

Socrates

Abstract

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss that poses a major health problem with substantial socioeconomic implications. Vision loss in AMD results from deterioration of photoreceptors and retinal pigment epithelium (RPE) cells in a specialised region in the central retina, the macula. The more prevalent form of AMD is atrophic or ‘dry’ AMD with limited treatment available. In ‘dry’ AMD, the progressive deterioration of photoreceptors and RPE cells in the presence of drusen, leads to a slowly expanding retinal lesion which causes progressive vision loss over time. Despite the multifactorial nature of AMD pathogenesis, a key role for inflammation, which involves the recruitment of retinal microglia and macrophages and activation of the complement cascade, is the most strongly implicated of all factors in the onset of the disease. The chemokine-mediated recruitment of retinal microglia and macrophages and the complement dysregulation in AMD including genetic polymorphisms in complement factor H (*CFH*), component 3 (*C3*) and factor B (*CFB*) are highly associated with the disease.

This thesis explores the contributions of the complement cascade in the manifestation of focal retinal degeneration and further investigates the extent of retinal microglia and macrophage involvement as the key instigators of local complement activation. Firstly, the thesis reports on the design and implementation of a murine photo-oxidative damage model and investigates the extent to which it recapitulates several features of dry AMD, including sequential events of oxidative stress, inflammation and continuous retinal degeneration.

Using this model, I then report that microglia and macrophages are the key cellular source of retinal C3 in both rodent retinal degeneration and human AMD. It is shown that

locally-derived C3, synthesized by retinal microglia and macrophages, contributes to the chronic complement activation and the progression of retinal degeneration.

The effects of complement activation propagated by C3 are diverse in neurodegenerative diseases, including AMD. Here, I report on the possible role of locally-derived C3 in amplifying the involvement of innate immune cells, microglia and macrophages, in focal retinal degeneration. It is shown that the absence of C3 dampens the accumulation, activation and phagocytic activity of retinal microglia and macrophages, and correlates with the reduced progression of retinal degeneration.

Finally, I report on the striking divergence in temporal contributions of two pathways of complement activation – classical and alternative - during photo-oxidative damage. The findings indicate that the alternative pathway, in the form of *CfB*, amplifies the cascade and mediates early retinal damage, while the classical arm, through *C1qa*, exacerbates retinal degeneration through the engagement of NLRP3 inflammasome in myeloid cells. Neutralisation of locally-derived C1q, but not systemic complement, is able to ameliorate the progression of retinal degeneration.

Collectively, this thesis describes the major contributions of complement activation, driven by retinal microglia and macrophages, in the onset and progression of retinal degeneration induced by photo-oxidative damage. The findings suggest that suppressing the capability of microglia and macrophages to elicit complement activation will serve as a potential strategy for reducing their reactivity, mitigating their contribution to retinal inflammation and degeneration.

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List of Publications

A list of publications that were produced during the candidate's PhD are shown below in chronological order (* denotes equal first authorship):

1. Jiao, H., Rutar M, Fernando N, Yednock T, Sankaranarayanan S, Aggio-Bruce R, Provis J and Natoli R (2018) **Subretinal macrophages produce classical complement activator C1q leading to the progression of focal retinal degeneration.** *Molecular Neurodegeneration*. **13**, 45, pp1-18, doi: 10.1186/s13024-018-0278-0
2. Natoli, R., Mason, E., Jiao, H. Chuah, A., Patel, H., Fernando, N., Valter, K., Wells, C., Provis, J., Rutar, M. (2018) **Dynamic interplay of innate and adaptive immunity during sterile retinal inflammation: Insights from the transcriptome.** *Frontiers in Immunology*. **9**, 1666, pp1-17, doi: 10.3389/fimmu.2018.01666
3. Natoli, R.*, Fernando, N.*, Dahlenburg, T.*, Jiao, H., Aggio-Bruce, R., Barnett, NL., de la Barca, J., Tcherkez, G., Reynier, P., Fang, J., Chu-Tan, JA., Valter, K., Provis, J., Rutar, M (2018) **Obesity-induced metabolic disturbance drives oxidative stress and complement activation in retinal environment.** *Molecular Vision*. **24**, pp. 201-217
4. Jiao, H.*, Natoli, R.*, Fernando N.*, Racic, T., Madigan, M., Barnett, NL., Chu-Tan, JA., Valter, K., Provis, J., Rutar, M. (2017) **Retinal macrophages synthesize C3 and activate complement in AMD and in models of focal retinal degeneration.** *Investigative Ophthalmology & Visual Science*. **58**, pp. 2977-90, doi:10.1167/iovs.17-21672.
The candidate's major contribution to this study as first author includes: conducting experiments, data acquisition and analysis for C3^{-/-} mouse study, CVF and intravitreal C3 siRNA treatment, and drafting and revising the manuscript for publication.
5. de la Barca, J., Huang, N., Jiao, H., Tessier, L., Gadras, C., Simard, G., Natoli, R., Tcherkez, G., Reynier, P., Valter, K. (2017) **Retinal metabolic events in preconditioning light stress as revealed by wide-spectrum targeted metabolomics.** *Metabolomics*. **13**. **22**. doi: 10.1007/s11306-016-1156-9.
6. Jiao, H.*, Natoli, R.*, Barnett, NL., Fernando, N., Valter, K., Provis, J., Rutar, M. (2016) **A model of progressive photo-oxidative degeneration and inflammation in the pigmented C57BL/6J mouse retina.** *Experimental Eye Research*. **147**, pp. 114-27, doi: 10.1016/j.exer.2016.04.015.
The candidate made a major contribution to this study as a first author, including conducting the experiments, data acquisition and analysis, and drafting results and discussion, and revising the manuscript for publication.
7. Jiao, H.*, Natoli, R.*, Valter K., Provis, J., Rutar, M. (2016) **Spatiotemporal cadence of macrophage polarisation in a model of light-induced retinal degeneration.** *PLoS One*. doi: 10.1371/journal.pone.0143952

List of Presentations

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XVII International Symposium on Retinal Degeneration – Kyoto International Conference Centre, Kyoto, Japan, September 2016

2nd European Molecular Biology Laboratory (EMBL) Australia PhD Symposium, Bio21 Institute, Melbourne, Australia, November 2015

Platform presentation

Association for Research in Vision and Ophthalmology (ARVO) 2017 Annual Meeting – Baltimore Convention Centre, USA, May 2017

Association for Research in Vision and Ophthalmology – Asia (ARVO-Asia) 2017 Biennial Meeting – Brisbane, Australian, Feb 2017

Canberra Health Annual Research Meeting (CHARM) – The Canberra Hospital, Canberra, Australia, August 2017

Canberra Health Annual Research Meeting (CHARM) – The Canberra Hospital, Canberra, Australia, August 2016

Australian Society for Medical Research – ACT New Investigators Forum – The John Curtin School of Medical Research, The Australian National University, Canberra, Australia, June 2016

Kioloa Neuroscience Colloquium – Kioloa, New South Wales, Australia, April 2016

List of Abbreviations

$\Delta\Delta$ CT – comparative cycle threshold	C3b – complement component 3b
4-HNE – 4-hydroxynonenal	C3 ^{-/-} - C3 knockout
8-OHG – 8 – hydroguanosine	C3d – complement component 3d
ACTB – beta actin	C4 – complement component 4
AEEC – Animal Experimentation Ethics Committee	C4a – complement component 4a
AMD – age-related macular degeneration	C4b – complement component 4b
ANOVA – analysis of variance	C5 – complement component 5
ANU – Australian National University	C5a – complement component 5a
ARVO – Association for Research in Vision and Ophthalmology	C5b – complement component 5b
ATP – adenosine triphosphate	C6 – complement component 6
BRB – blood-retinal barrier	C7 – complement component 7
BSA – bovine serum albumin	C8 – complement component 8
C1 – complement component 1	C9 – complement component 9
C1q – complement component 1q	C57BL/6J – C57 black 6 Jax mouse
C1qa – complement component 1q, subunit a	Cas9 – CRISPR-associated protein-9 nuclease
C1qa ^{-/-} - C1qa knockout	CASP 1 – caspase 1
C1r – complement component 1r	CASP 8 – caspase 8
C1s – complement component 1s	CCL2 – chemokine (C-C motif) ligand 2
C2 – complement component 2	CCL3 – chemokine (C-C motif) ligand 3
C2a – complement component 2a	CCL12 – chemokine (C-C motif) ligand 12
C3 – complement component 3	CCR2 – chemokine (C-C motif) receptor 2
C3a – complement component 3a	CD11b – cluster of differentiation 11b
C3b – complement component 3b	CD68 – lysosome associated membrane protein (LAMP) family

cDNA – complementary DNA	EDTA – ethylenediaminetetraacetic acid
CEP – carboxyethylpyrrole	ERG – electroretinography
CFB – complement factor B	ECL – enhanced chemiluminescence
CFD – complement factor D	FBS – fetal bovine serum
CFH – complement factor H	FGF2 – fibroblast growth factor 2
CFI – complement factor I	GAPDH – glyceraldehyde-3-phosphate dehydrogenase
CNS – central nervous system	GCL – ganglion cell layer
CNTF – ciliary neurotrophic factor	GFAP – glial fibrillary acidic protein
CO ₂ – carbon dioxide	GWAS – genome-wide association study
CRISPR – clustered regularly interspaced short palindromic repeats	GPX3 – glutathione peroxidase 3
Ct – cycle threshold	HBSS – Hank's balanced salt solution
CVF – cobra venom factor	Hmox-1 – Heme oxygenase 1
CX3CL1 – chemokine (C-X3-C) motif ligand 1	IBA-1 – ionised calcium binding adaptor molecule 1
CX3CR1 – chemokine (C-X3-C) motif receptor 1	IgG – immunoglobulin G
CXCR3 – chemokine (C-X-C) motif receptor 3	IHC – immunohistochemistry
DIG – digoxigenin	IL-1 β – interleukin 1 β
DMEM – Dulbecco's Modified Eagle's Medium	IL-6 – interleukin 6
DNA – deoxyribonucleic acid	IL-4 – interleukin 4
dUTP – 2'-deoxyuridine 5'-triphosphate	IL-10 – interleukin 10
EDTA – ethylenediaminetetraacetic acid	IL-18 – interleukin 18
ERG – electroretinography	INL – inner nuclear layer
FACS – fluorescence activated cell sorting	IPL – inner plexiform layer
dUTP – 2'-deoxyuridine 5'-triphosphate	

IS – inner segments	PBS – phosphate-buffered saline
ISH – <i>in situ</i> hybridisation	PD – photo-oxidative damage
JCSMR – John Curtin School of Medical Research	qPCR – quantitative real-time PCR
LED – light-emitting diode	RHOD – rhodopsin
LD – light damage	RNA – ribonucleic acid
LSD – least significant difference	RNAi – RNA interference
MAC – membrane attack complex (C5b-9)	RNP – Ribonucleoproteins
MASP1 – MBL-associated serine protease 1	RPE – retinal pigment epithelium
MASP2 – MBL-associated serine protease 2	RT – reverse transcriptase
MBL – mannose-binding lectin	SD – Sprague-Dawley
MDA – malondialdehyde	SEM – standard error mean
MQ-H ₂ O – Milli-Q water	SERPING1 – serpin family G member 1
mRNA – messenger RNA	SRBC – sheep red blood cells
NBT – nitro blue tetrazolium	siRNA – small interfering RNA
NGS – normal goat serum	SOD – superoxide dismutase
NLRP3 – nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3	SSC – saline-sodium citrate
OCT – optical coherence tomography	Tdt – terminal deoxynucleotidyl transferase
OCT – optimal cutting temperature	TNF – tumor necrosis factor
ON – optic nerve	TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labelling
OPL – outer plexiform layer	VEGF – vascular endothelial growth factor
OS – outer segments	VBS – veronal buffer solution
	WT – wild-type

1. INTRODUCTION

1.1 General overview

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss worldwide. It costs the Australian economy 5 billion dollars and the global economy 300 billion dollars per year. AMD is a degenerative disease of the neural retina which contains light-sensitive photoreceptors for converting wavelengths of light to electrical signals and a network of neurons for processing and relaying the signals to the brain. The onset of AMD involves photoreceptor degeneration in the macula, a region that encodes high acuity and colour vision. Although the aetiology of AMD involves multiple genetic and environmental factors, it is well-established that dysregulation of the innate immune system is highly implicated in the pathogenesis of this disease [1, 2]. Part of our innate immune response, the complement system, and its dysregulated activation at the site of degeneration, is associated with photoreceptor loss and further progression of AMD [3-5]. Unbalanced complement activation in the retinal microenvironment, intertwined with other contributing factors such as oxidative stress and inflammation, is a key feature of AMD. However, the functional significance of complement activation is yet to be explored. My research aims to explore the role of key complement components that propagate deleterious effects of local complement activation in a new mouse model of focal retinal degeneration.

Drawing on current knowledge and findings, this thesis is focused on pinpointing key roles of the complement system in propagating the onset and progression of retinal degeneration induced by photo-oxidative damage. In this chapter, I will first review literature in respect of the complement system, then I will introduce the pathogenesis of AMD, followed by the immunological aspects underlying the progression of AMD, such as oxidative stress and inflammation.

1.2 The eye and retina

Vision is regarded as the most important sense for our daily lives. The eye consists of an outer protective layer, the sclera, and cornea of the anterior surface, which forms a continuous external barrier around the globe [6]. Light passes through the vitreous chamber and enters the eye through the pupil. The lens focuses the light onto the neural retina lying in the posterior surface of the eye [6].

The retina is composed of distinct layers that differ in structure and function (Figure 1.1). Separated by the inner limiting membrane from the vitreous chamber, the innermost retina consists of a ganglion cell layer (GCL), an inner plexiform layer (IPL) and an inner nuclear layer (INL) housing amacrine, bipolar and horizontal cell bodies, as well as Müller cells. Müller cells are radial glial cells that maintain retinal structure and regulate the metabolism and function of neurons and photoreceptors [7, 8] as well as the formation of an inner retinal barrier [9, 10]. In cases of chronic and progressive damage, hypertrophic Müller cells translocate from their usual position in the INL and undergo proliferative gliosis resulting in neuronal disorganisation and chronic retinal damage [11, 12]. Resident retinal immune cells, microglia, also reside in the inner retina where they provide immune surveillance for the retina.

The outer layers of retina consist of an outer nuclear layer (ONL) that is highly populated with photoreceptor cell bodies, rods and cones whose synaptic pedicles are in contact with bipolar cells in an outer plexiform layer (OPL). The outermost layer adjacent to the subretinal space, is separated by the outer limiting membrane and is made up of protrusion of photoreceptor inner and outer segments (IS/OS) that contain chromophore-lined membrane discs for light detection. Retinal pigment epithelium (RPE) is a monolayer of cuboid cells located between the neural retina and the underlying Bruch's membrane. The tight junctions between the neighbouring RPE cells form the blood-

retinal barrier (BRB) that maintains the immune privilege of the eye [13, 14]. The choroid vasculature is a rich and dense network of blood vessels that provide oxygen and nutrients to the outer retina and remove waste from the retina through diffusion across Bruch's membrane.

Photoreceptors

Photoreceptors are light-sensitive cells of the visual system that absorb and convert wavelengths of light into electrical signals in a process known as phototransduction [15]. Each photoreceptor consists of an outer segment (OS), an inner segment (IS) packed with mitochondria, a nucleus, and a synaptic/axonal terminal [16]. They are tightly packed into the outer layers of the retina where they comprise the ONL of cell bodies and an outermost layer of photoreceptor segments. The outer segments contain tightly packed membranous discs containing the photopigment opsins, covalently bound to the light-sensitive chromophore 11-cis-retinal [17, 18]. The inner segments are highly enriched with mitochondria that produce adenosine triphosphate (ATP) by oxidative phosphorylation, acting as a powerhouse to the photoreceptors [18].

There are two types of photoreceptors, rods and cones, with distinct functions and distribution across the retina. The human retina consists of rods and cones in a 20:1 ratio [19-21]. Rods are extremely sensitive to light, while cones are activated only in daylight. Cones are most numerous at the *fovea* and adjacent macula, whereas rods outnumber cones in the *parafovea* and elsewhere in the retina [22]. The fovea is thus responsible for high acuity and colour vision, mediated by cones.

Regardless of their differences, both rods and cones are well known for their very high rate of oxygen consumption [23]. Oxygen for the photoreceptors is supplemented by choroid and retinal vasculature [24, 25]. Photoreceptors are highly depolarised in darkness, whereas phototransduction results in a graded de-polarisation [18, 26]. The

transduced signals are relayed via interneurons to ganglion cells and then to the brain through the optic nerve.

Retinal Pigment Epithelium/ Bruch's membrane/ choroid

Photoreceptor segments are structurally and functionally integrated with RPE cells. They are also dependent on the choroid and Bruch's membrane. Besides the formation of the blood-retinal barrier (BRB) [27], RPE cells also participate in phototransduction due to their contribution in recycling opsins [28]. They also contain lysosomal compartments that reflect their role in phagocytosis of shed photoreceptor outer segments [29]. The apical surface of the RPE interacts with photoreceptor outer segments in the ONL, while the basolateral side interacts with the choroid, acting as a barrier to the highly perfused and permeable choriocapillaris [28]. Moreover, tight junctions between neighbouring RPE cells confer highly selective properties on the blood-retinal barrier function, which in non-inflammatory conditions limits infiltration of monocytes from the circulation into the subretinal space [30].

The choriocapillaris is structurally adapted for a high blood flow and a high rate of diffusion in order to provide photoreceptors with the necessary glucose and oxygen through diffusion across Bruch's membrane. The most obvious source of impaired nutrition of the photoreceptor cells is occlusion and death of choriocapillaris, which is associated with inflammation and leucostasis [31]. Over time, thickening of the Bruch's membrane, RPE cell damage and loss of choriocapillaris reduce appropriate metabolic support to the photoreceptors and disrupt the homeostasis in the subretinal space [32]. This further contributes to retinal degeneration in AMD [33, 34].

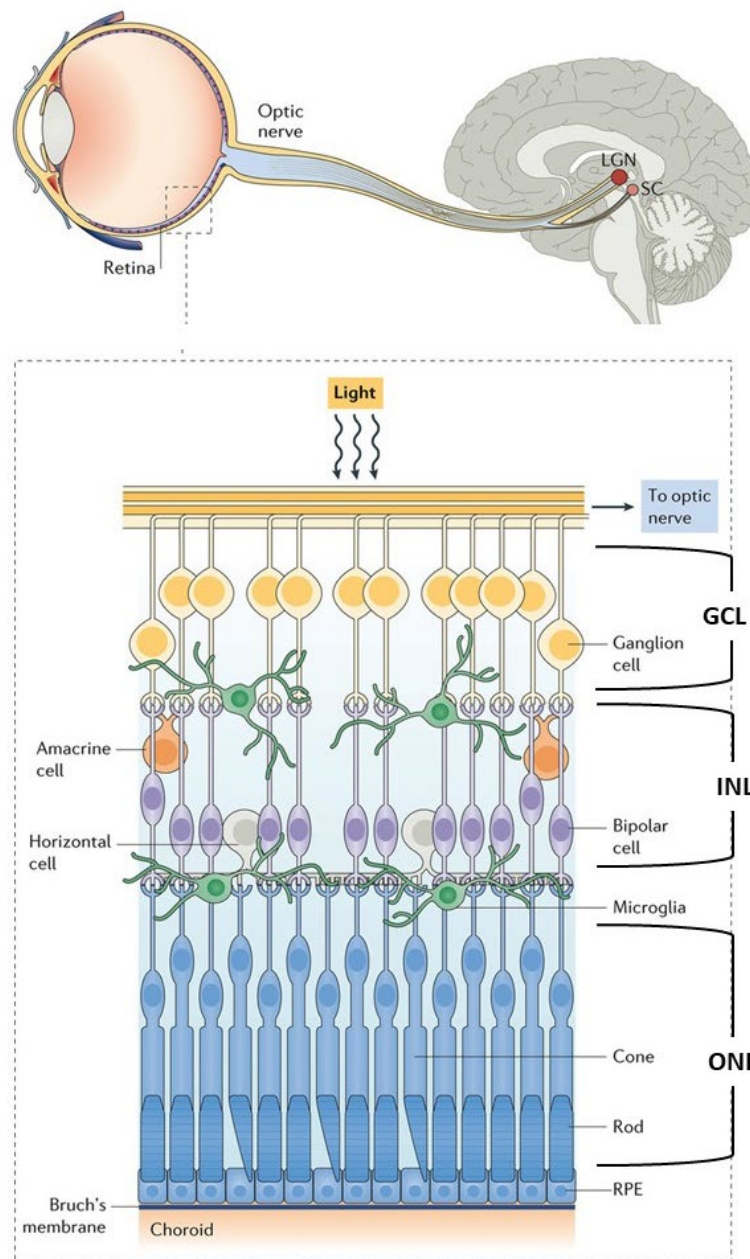


Figure 1.1 Stylized depiction of retinal anatomy and cell types (adapted from Reyes 2017 [35]). Light passes through the pupil and lens of the eye to the retina, which is a multilayered structure. Each layer houses retinal cells that are essential for the visual circuit and for relaying signals to the visual cortex of brain (via LGN: later geniculate nucleus; SC: superior colliculus). Photoreceptor cells (rods and cones), which are important for phototransduction, are in the outer nuclear layer (ONL). Retinal immune cells such as microglia reside in the synaptic region of the retina. The retinal pigment epithelium (RPE) maintains the visual function and aids the maintenance of retinal immune surveillance and privilege.

Immune-privilege and blood-retina barrier

The retina has anatomical and physiological barriers that suppress the immune response and protect the neural retina from the ‘side effects’ of immune activation [36]. The term ‘immune privilege’ was first coined by Sir Peter Medawar who observed that foreign tissue grafts placed in the anterior chamber of the eye were not rejected [37]. Currently, the term immune privilege is associated with a repressed innate and adaptive immune response and inflammation. Multiple mechanisms combine to achieve the retinal immune privilege including physical barriers and an inhibitory ocular microenvironment [38].

The BRB comprises an inner barrier at the level of the retinal vessels and an outer BRB at the level of the RPE [14, 39, 40]. The inner BRB is at the level of the vascular endothelium, where adjacent cells are joined by tight junctions, establishing an intercellular barrier. Retinal vascular endothelial cells allow only very low levels of trans-cellular transport, further limiting cross-endothelial transport and diffusion [30]. The outer BRB is formed by tight junctions between the neighbouring RPE cells so as to limit and regulate the entry of large molecules or immune cells (>300kDa) into the retina [41]. In this way, the BRB protects the retina from the circulating leukocytes as well as maintaining the retinal environment to ensure optimal neural function in healthy conditions.

Under pathological circumstances, disruption to the BRB makes the retina vulnerable. Breakdown of the BRB is associated with recurrent inflammatory activity and oxidative stress, such as histamine release and pro-inflammatory cytokines [42], further promoting the invasion of immuno-competent cells into the retina. In AMD, drusen in the subretinal space disrupt both the tight junctions in the RPE and the Bruch’s membrane [33, 43]. Chronic exposure to light leads to an accumulation of oxygen radicals, which

induce RPE cell damage such as junctional proteins disruption and subsequent breakdown of the BRB [44, 45].

1.3 Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of visual impairment worldwide [46, 47]. It affects more than 150 million people in the world [46, 47], and its prevalence is set to rise as a consequence of mankind's increasing longevity [48, 49]. AMD is a progressive and degenerative condition of the cells and structures of the macula, which has the highest density of photoreceptors [50]. The macula, which lies centrally in the retina, consists of a cone-dominated region responsible for high acuity and fine spatial resolution, surrounded by a rod-dominated *parafovea* and peripheral retina [48]. The vision provided at the macula enables reading, driving and facial recognition. Early/intermediate AMD is commonly asymptomatic, and is characterised by drusen deposits in the central retina.

Drusen consists of lipoproteinaceous debris in the basal surface of RPE and Bruch's membrane [33, 51, 52]. In early/intermediate AMD, drusen appear as either yellow deposits of debris or ill-defined large subretinal deposits protruding to the photoreceptors [53, 54]. Their presence hinders the transport of fluid and nutrients from choroid to retina [55-57], and their composition includes inflammatory components [58-61]. In this way, drusen may be causally associated with the central thinning of the photoreceptor cell layer of the central retina and progressive loss of vision sensitivity [62, 63]. While disruption of the photoreceptor cell layer by drusen is associated with oxidative stress and chronic inflammation, the direct connections between these events are not fully understood. This thesis aims to deepen our understanding on the relationship between photo-oxidative stress and retinal inflammation with a strong focus on the role of complement activation in focal retinal degeneration.

AMD can either progress to atrophic ‘dry’ or neovascular ‘wet’ AMD (Figure 1.2). Neovascular ‘wet’ AMD can lead to sudden vision loss resulting from choroidal neovascularisation [64]. In neovascular ‘wet’ AMD, choroidal capillaries penetrate through the Bruch’s membrane and spread within the sub-retinal space amongst the photoreceptors. Unlike normal retinal vessels, these blood vessels have no barrier properties. Prolonged leakage of serum and leukocytes into the outer retina promotes fibrovascular scarring and vision loss [65]. Progression of the choroidal neovascularisation may be halted by anti-angiogenic therapy, which decreases the permeability of vessels and inhibits the formation of further blood vessels [66, 67].

Atrophic stage of AMD is characterised by a geographic atrophy, which is a continuous, regional loss of RPE and photoreceptors in the macula [68, 69]. RPE cell death, and degeneration of photoreceptor cells, are the hallmarks of a degenerative focal retinal lesion [69, 70]. The loss of outer retinal cells can progressively expand to the *parafovea* and adjacent regions, accompanied by reduced blood flow in the choroid owing to the dropout of choriocapillaris [71]. As the atrophic lesion develops up to 2mm beyond the foveal region, a severe drop in visual acuity becomes evident [72]. The periphery adjacent to the focal lesion can further develop neovascularisation in the choriocapillaris [73].

Although AMD is a multifactorial disease, the outcome is photoreceptor degeneration leading to vision loss [51, 69, 74]. Photoreceptors often degenerate in regions of complete atrophy of RPE and thickened Bruch’s membrane [69, 75], owing to a compromised metabolic and functional support such as an impaired translocation of retinoids for photoreceptor regeneration [76, 77]. An additional loss of photoreceptors also occurs in the absence of RPE cell death, and this is primarily due to the preferential degeneration of rods over cones, in retinal lesions outside the atrophic region [70, 72].

Dysregulated immune activation and inflammation are involved in inducing and advancing AMD pathology [78-80]. The retina and RPE are in contact with multiple innate immune activators that could potentially contribute to vision loss in AMD. Among these immune activation pathways, the complement system is widely accepted as contributing to AMD [3].

Current treatment options for AMD are limited, consisting of AREDS, which may slow the progression of early/intermediate AMD by modulating the complement activity [81-83]. Intravitreal anti-vascular endothelial growth factor antibodies (VEGF), which have anti-angiogenic properties, are available to ameliorate the symptoms of neovascular AMD in clinical practices [84]. The absence of a more effective and universal therapy has been a driver for the continued pursuit of novel therapeutic targets. In particular, there is no effective therapy available to treat non-neovascular disease. Directly targeting the complement system could provide exciting new therapeutic options with the potential to slow AMD progression before the development of RPE atrophy or CNV [85, 86].

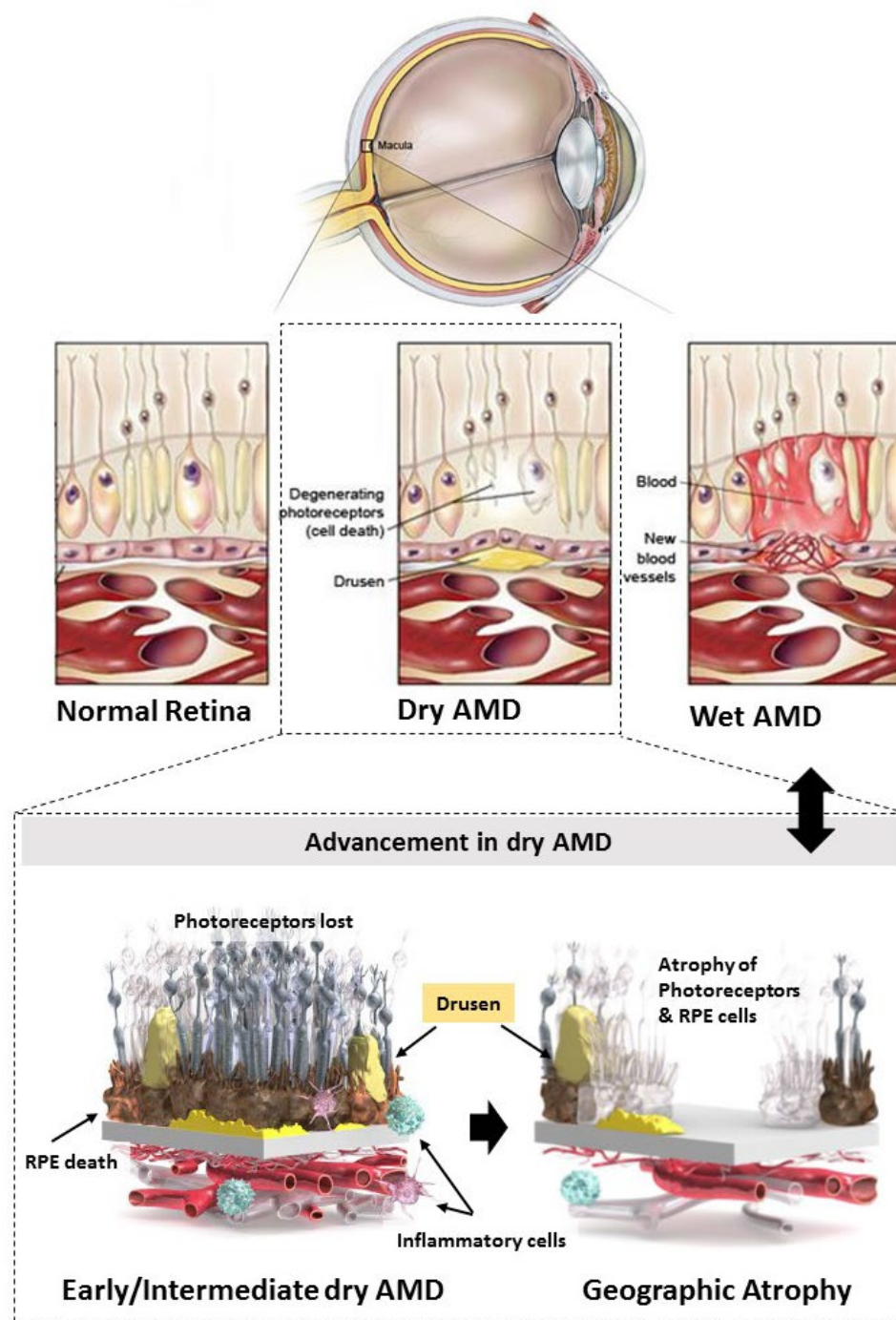


Figure 1.2 Disease progression in AMD (modified from Brightfocus Foundation and Geographic Atrophy - Angiogenesis Foundation). There are two types of AMD, dry AMD, which is characterised by subretinal drusen, and wet AMD, which is characterised by the invasion of abnormal choroidal blood vessels. In dry AMD, the early/intermediate stage is associated with subretinal drusen, degenerating photoreceptors and RPE cell deterioration and invasion of inflammatory cells. Two types of drusen deposits are associated with ageing and AMD: drusen that are sub-RPE, and the less common drusenoid deposits in the subretinal space. The late-stage of dry AMD is geographic atrophy, which is characterised by clustered regions of photoreceptors and RPE cell death. Geographic atrophy sometimes progresses towards wet AMD in patients.

1.4 Complement and AMD

The complement system, first discovered in 1920, is a cascade of more than 30 plasma and cell surface proteins, providing innate immune defence against pathogens and foreign bodies [87]. It is also a modulator of immune cell function in both innate and adaptive immunity, through complement receptor-protein recognition/signalling pathways [88, 89]. The complement system comprises a cluster of activation pathways that are triggered in differing ways, and progress through a series of proteolytic enzymes that lyse the complement proteins to form a terminal cell-killing complex against pathogens and foreign bodies (Figure 1.3). The activation of complement is tightly monitored by regulators to avoid improper inflammation [90]. The complement system consists of a classical pathway, lectin pathway and alternative pathway [88, 91].

Complement pathways

The classical pathway of the complement system is activated through the binding of the pattern recognition molecule, C1q, to immunoglobulin (IgG or IgM) on pathogen surfaces and foreign bodies. C1q also binds to C-reactive proteins on surfaces of apoptotic cells and debris for clearance [88, 92]. Upon binding with surface proteins, C1q forms a complex with C1r and C1s, which cleaves the downstream C2 and C4 into C2a, C2b, C4a and C4b through the formation of a serine protease (Figure 1.3). C3 convertase (C4b2a) of the classical pathway is subsequently formed to cleave C3, a central component of the complement system (Figure 1.3).

The lectin pathway is independent of antibody binding and is activated through recognition of mannose-binding lectins (MBL, L-ficolin, H-ficolin or M-ficolin) on pathogenic cell surfaces [88, 93]. These pattern recognition molecules bind to a serine protease of the lectin pathway. Although the lectin and mannose pathways have different

triggers, their downstream activation promotes the lysis of C2 and C4, which forms the same C3 convertase (C4b2a) as the classical pathway (Figure 1.3).

The alternative pathway can be activated directly on pathogen surfaces without protein-protein interaction through the formation of C3 convertase (C3bBb) [94, 95]. This can be facilitated by complement factor B (CFB), which is then cleaved by factor D (CFD), amplifying the formation of C3 convertase of the alternative pathway (Figure 1.3). The resulting C3 convertase of the alternative pathway converges with C3 convertases of the classical and lectin pathways to ultimately promote the lysis of C3. The lysis of C3 produces inflammatory mediators such as bioactive anaphylatoxin C3a, opsonin C3b and long-lived component C3d [96] (Figure 1.3). The larger fragment C3b promotes the downstream formation of C5 convertase, which lyses C5 into bioactive anaphylatoxin C5a and opsonin C5b (Figure 1.3). C5b accelerates the formation of cell killing membrane attack complex (MAC) on the pathogen's surface and on apoptotic cells, leading to the formation of a membrane pore and cell lysis (Figure 1.3). The second way of activating the alternative pathway involves the spontaneous hydrolysis of the thioester bond in C3 to form C3(H₂O). Steady low-level production of C3(H₂O) can bind CFB, which is then cleaved by CFD, producing a short-lived fluid-phase C3 convertase. This fluid-phase C3 convertase cleaves more C3 into C3a and C3b. This response is known as 'tick-over' response.

Regardless of the pathways, complement activation converges at the generation of three broad effector pathways necessary for host defence: (1) direct lysis of targeted surfaces by MAC assembly; (2) alerting and priming the immune system by the generation of potent pro-inflammatory anaphylatoxins that increase capillary permeability to promote extravasation of infiltrating leukocytes; and (3) opsonisation and clearance of target surfaces by the generation of complement opsonins (C4b, C3b, C3bi)

and engagement of complement receptors on phagocytic cells, such as macrophages and neutrophils [94, 97].

Complement regulators

There is an abundance of fluid phase and membrane-bound regulator proteins that moderate the powerful cell killing activities of complement [90] (Figure 1.3). One fluid phase regulator, complement factor H (CFH) acts on the C3 convertase, tightly regulating the proteolytic cleavage of C3 and complement activation [90, 91] (Figure 1.3). Excessive complement activation and insufficient complement regulation, for example, genetic polymorphism of *CFH* in AMD [98, 99], can cause immune imbalance resulting in a vicious cycle of complement, inflammatory cells and tissue damage.

Complement activation in innate and adaptive immunity

The contributions of complement activation are not only shown in the innate immune response but also in the adaptive immune response. The effector functions of complement activation involve the recruitment of leukocytes and the shaping of their immune response, for example, increasing their chemotaxis, migration and phagocytosis. The innate immune cells such as microglia and macrophages are capable of recognizing, ingesting and eliminating apoptotic cells [100-104] and defective neuronal synapses, through the generation of potent anaphylatoxins/opsonins and the engagement with complement receptors (CRs) on microglia [105-107]. The complement-mediated microglial activation also participates in synaptic pruning during retinal and brain development [108-110]. This is particularly so in the C3a/CR3 and C5a/C5aR signalling pathways, which alert the phagocytic cells to opsonise defective synapses [111-116]. Under pathological circumstances such as an ongoing tissue insult, complement activation can lead to uncontrolled activation of the innate immunity thus further aggravating the degeneration of neurons [117, 118].

In addition to these important contributions to the innate immunity, complement plays a vital role in the adaptive immune responses: functionally integrating it into the ability of the host to combat cellular damage [89]. Classical complement components provide a link to the adaptive immune response through the binding of antibodies to antigens [89]. In addition, the activation of complement may increase the efficiency of the adaptive immune system [89], as observed in human eyes with AMD [119] and in the serum of AMD patients [120]. In retinal degenerative diseases, adaptive immune cells, such as T-cells, may contribute to RPE degeneration [121, 122]. Inadequate control of complement-driven inflammation results in the generation of C5a and C3a, which recruit IL-17 producing $\gamma\delta$ -T cells into the eye [123].

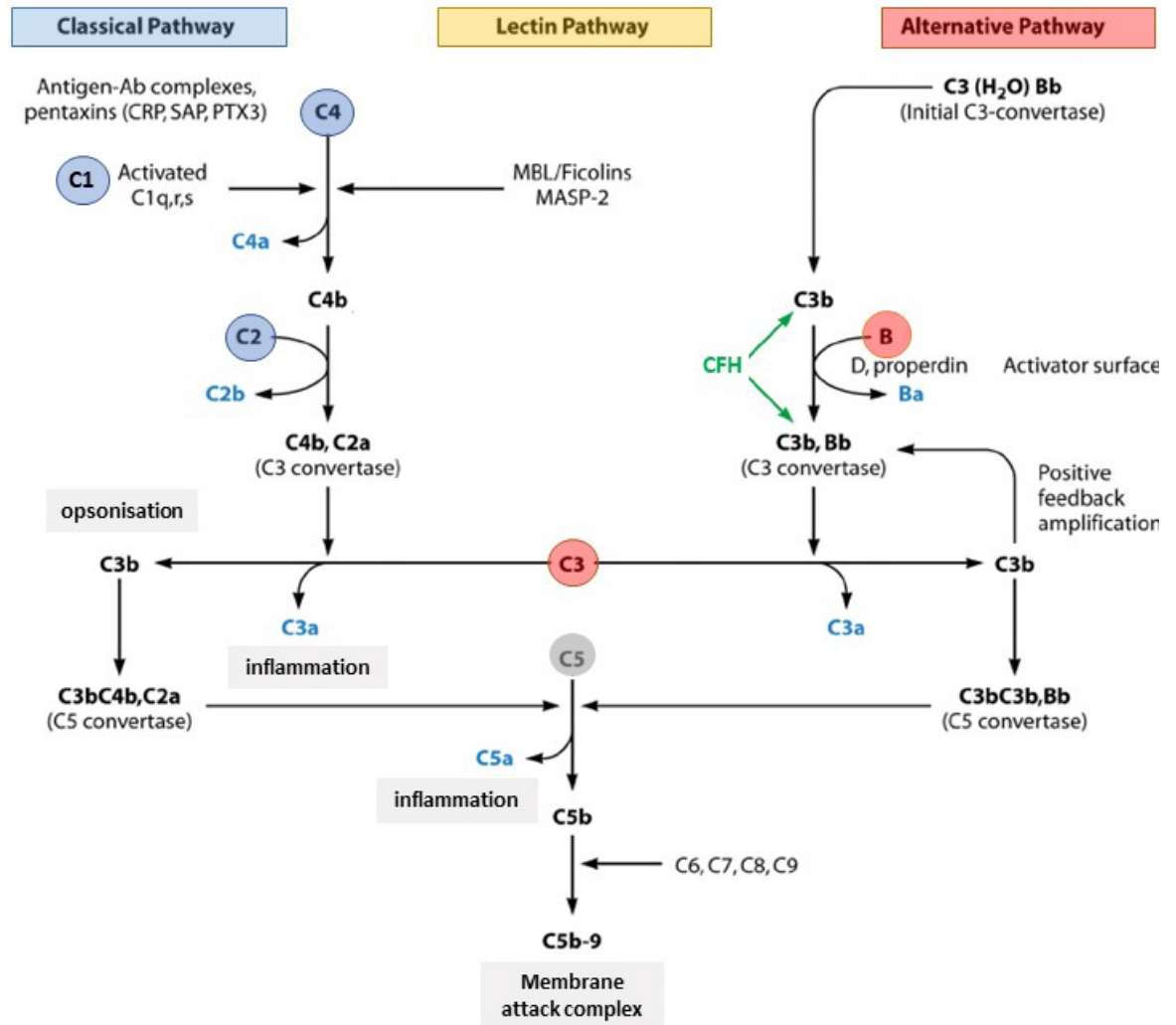


Figure 1.3 Schematic representation of the complement system. There are three pathways of complement activation – classical, lectin and alternative. These pathways lead to the hydrolysis of the central complement component, C3, which triggers the downstream complement activation and the formation of the membrane attack complex (MAC). The activation of complement is tightly monitored by complement regulators such as factor H (CFH), negative regulator of the alternative pathway. The effective functions of complement activation include inflammation/recruitment of immune cells, opsonisation and cell lysis, which assist with the destruction of pathogens, apoptotic cells and debris.

Complement activation and AMD

Genetic association studies have linked the complement system to increasing AMD risk. Y402H sequence variant in complement factor H (*CFH*) was identified to associate with AMD [98, 99, 124, 125]. Susceptible variants in other complement-related genes associated with AMD include complement component 2 (*C2*), complement factor B (*CFB*) [126-129] and complement component 3 (*C3*) [130-132]. More recent evidence shows the presence of rare and highly penetrant mutations in complement genes *CFH*, *C3* and complement factor I (*CFI*) [133-136]. In addition to the human genetic studies on complement dysregulation and AMD, complement localisation has been implicated in human AMD eyes [79, 137, 138]. Newman et al (2012) showed a close correlation between complement levels in human RPE/choroid and neural retina with pathology development and progression in dry AMD, geographic atrophy and wet AMD [138]. Complement-related proteins including C1q, CFH, C3 and C5a are highly concentrated in drusen deposits and in the choroid but are absent from the Bruch's membrane [57, 60, 61, 139]. The association of complement activation with neurodegeneration has also been implicated in models of glaucoma, diabetic retinopathy, uveoretinitis, retinitis pigmentosa and photo-oxidative damage [140-146].

The alternative pathway is primarily activated in AMD and neurodegenerative diseases [114, 147-149]. Increase in the terminal membrane attack complex (MAC) deposition in the choroid is age-dependent [150] and associated with the drop-out of choroidal endothelial cells and the compromised supply of nutrient and oxygen to the photoreceptors and RPE [71, 151, 152]. C3, C5, MAC complex and C1q complex are found in drusen [3]. Complement activation is associated with the accumulation and activation of microglia and macrophages, which further deposit inflammatory mediators in the retina [153-155]. Dysregulated complement activation is linked to inflammation

induced by the oxidative damage in AMD, as evident by the inability of CFH to neutralise the pro-inflammatory property of malondialdehyde (MDA) [156]. CFH polymorphism has recently been implicated in the subretinal macrophage accumulation and in non-resolving subretinal inflammation [157]. Despite the recent evidence on the involvement of complement dysregulation in AMD, our understanding of the cellular events that promote aberrant complement activation and the contribution of other key complement components in retinal pathology remains incomplete. This thesis explores the contributions of key complement components and further dissects the cellular sources that promote the deleterious effects of local complement activation in focal retinal degeneration.

The current status of complement-based therapies is promising, however, still facing many hurdles. Studies have taken aim at regulating complement system activation in AMD patients, including drugs targeting C5 (ARC1905; Clinical Trial Identifier NCT00950638 and Eculizumab; Clinical Trial Identifier NCT00935883), C3 (POT-4/Compastatin; Clinical Trial Identifier NCT00473928) and CFD (Lampalizumab; Clinical Trial Identifier NCT02247479 and NCT02247531). Despite the promise of these ongoing drugs, many hurdles still exist in the therapeutic interventions of inflammation and complement in AMD patients, including delivery method, dose, and disease stage at time of treatment. For example, systemic administration of Eculizumab did not improve retinal drusen in patients or decrease the rate of GA growth in the COMPLETE trial [158]. Recently, intravitreal delivery of Lampalizumab did not slow the progression of GA in the Chroma and Spectri trial [159].

1.5 Oxidative damage and AMD

Prolonged oxidative stress is a key contributing factor in AMD pathogenesis as a result of its ability to induce cellular damage and trigger inflammation in the retina [160].

The vulnerability of the retina to oxidative damage is primarily due to the high proportion of polyunsaturated fatty acids (PUFA) in the outer segments and whose metabolism generates reactive oxygen species [161]. Age-dependent accumulation of subretinal drusen and environmental factors such as ageing and smoking could increase the load of oxidative stress and contribute to oxidative damage in the retina [162]. Early outcomes of oxidative damage include DNA/protein damage, lipid peroxidation and lipofuscin accumulation in AMD [163, 164].

Lipid peroxidation

Lipid peroxidation, the oxidative degeneration of lipids, proceeds through a complex free-radical chain reaction involving the reactive oxygen species and PUFA [165]. PUFA are responsible for the stabilisation of rhodopsin proteins and account for about 50% of the lipid bilayer of rod outer segment membranes. This makes photoreceptors vulnerable for lipid peroxidation [165]. Since PUFAs are susceptible to reactive free radical attack, lipid peroxidation often occurs in the PUFA-containing photoreceptors [166]. Environmental factors such as photo-oxidative damage and ageing trigger the oxidation of photoreceptors [167] and increase the number of oxidised phospholipids in AMD [168].

Oxidisation-specific epitopes such as malondialdehyde (MDA), carboxyethylpyrrole (CEP), 4-hydroxynoneneal (4-HNE) and other oxidised phospholipids accumulate in AMD [160, 167, 169]. Their accumulation is associated with pathological changes in RPE and photoreceptors [170]. In AMD, CEP is shown to arise predominately from oxidation of docosahexanoic acid [171], which is the most abundant fatty acid in photoreceptors. 4-HNE derives from oxidation of arachidonic acid and linoleic acid, commonly found in photoreceptors and RPE [172]. These oxidation-specific epitopes are further localised in lipofuscins, Bruch's membrane and in drusen of

AMD retinas [61, 168, 173]. Over time, the accumulation of oxidised by-products results in retinal oxidative damage. The consequences of the oxidative damage include decreased proteolysis of modified proteins, unwanted protein aggregation and disrupted protein stability and cell apoptosis [174, 175]. They act as upstream immune stimulators of innate and adaptive immune cells, upon identification by pattern recognition receptors [176-178]. In ageing, persistent accumulation of the oxidation-specific epitopes is associated with inflammation [160]. Dysregulation of the immune response as occurs in complement dysregulation (*CFH* polymorphism) reduces neutralisation of the oxidation-specific epitopes (MDA), promoting retinal inflammation and degeneration [156].

Lipofuscins

Lipofuscin is an autofluorescent lipid-protein aggregate that can be excited with short wavelength light. It accumulates in metabolically active non-dividing cells such as photoreceptors and RPE [27]. Light exposure can induce the formation of bis-retinoid N-retinylidene-N-retinylethanolamine (A2E), which begins in the photoreceptor outer segments from condensation reactions between phosphatidylethanolamine and all-trans-retinal [179]. The accumulation of A2E is identified in degenerating RPE cells and in subretinal drusen deposits. It can facilitate deleterious effects in the retina, for example, membrane disruption [180], lysosomal dysfunction [181] and photo-toxicity [182], immune dysregulation through complement activation [183] and inflammasome activation [184]. In addition, accumulation of A2E triggers microglia reactivity and complement activation by increasing both *CfB* and *CfH* expression, leading to photoreceptor degeneration [185]. Excessive lipofuscin accumulation promotes RPE degeneration and damages the photoreceptors and choriocapillaris, resulting in the progression of retinal atrophy in AMD patients [43, 186].

1.6 Inflammation and AMD

Inflammation is strongly implicated in AMD pathogenesis [80, 187-189]. The physiological role of inflammation is primarily involved in providing a defensive biological response of the body as to maintain the tissue homeostasis upon damage [190]. In response to the constant stress associated with ageing this low-grade inflammation intensifies with age, triggering persistent chronic inflammation within the damaged tissue [78]. In atrophic AMD, triggers for chronic inflammation include the accumulation of drusen [3], resulting in a locally increased burden of oxidative stress [160, 188], retinal cell death and reduced immune privilege [191]. The inflammatory response in AMD has been associated with dysregulated complement activation, microglial accumulation and chemokine-mediated macrophage invasion [192-194]. These concepts will be expanded on in the following sections.

Microglia

The involvement of inflammatory processes in the histopathology of AMD includes the incursion of phagocytic infiltrates in association with drusen of patients with early AMD [192, 195-198]. These cells engulf membraneous particles suggesting that the focal concentration of deposits in drusen attracts microglia and macrophages [195, 199]. Chronic involvement of macrophages and multinucleated giant cells has been shown both in and on the expanding edges of atrophic AMD lesions [195, 200]. Human retinas from geographic atrophy patients show the presence of bloated microglia within the nuclear layers, thus demonstrating their capability of phagocytosing photoreceptor and autofluorescent debris [201]. Moreover, additional evidence has shown changes in parenchymal microglia in association with early AMD, including increased antigen presentation [192, 197]. In advanced AMD, activated amoeboid microglia infiltrate the ONL and subretinal space where they seem to ingest lipid and cellular debris by phagocytosis, resulting in a lipid-bloated subretinal microglia [196, 201]. However,

whether these giant cells are from the resident microglia or monocytes-derived macrophages remains unclear. Recent efforts to delineate the populations of myeloid cells show the phenotypic differences between resident microglia and macrophages [202], and their possible origins through utilisation of cell fate mapping and immunohistochemical markers [203-206].

Retinal microglia are resident immune cells that normally reside in the inner and outer plexiform layer with antigen-presentation markers for recognition of apoptotic cells [207-209] (Figure 1.4). Their primary function is to provide immune surveillance and maintenance of neuronal homeostasis [210, 211]. They are a long-lived, self-renewing population derived solely from yolk-sac progenitors [202, 205]. The possible sources of microglial replenishment in adult retina include from optic disc and retinal vasculature [207]. They normally exist in a ramified resting state and are characterised by static cell bodies with radial protrusions (Figure 1.4). However, the engagement of microglia can be neurotoxic and is implicated in the aggravation of retinal degeneration [212]. Once the retina has been damaged, the activated retinal microglia acquire the capability to proliferate and migrate to the outer retina where they secrete inflammatory mediators and phagocytose photoreceptors [201, 213] (Figure 1.4). During retinal ageing, microglia accumulate in the outer retina where the subretinal debris and deposits may elicit an age-related dysfunction [214, 215] and produce an inflammatory response from the microglia [198, 211, 216]. This subsequently fuels a vicious cycle of microglial activation, RPE damage and photoreceptor degeneration [210, 212, 217].

Macrophages

In contrast to the ontogeny of microglia, macrophages consist of fetal liver-derived monocytes and adult bone marrow-derived monocytes [35, 218]. Macrophages infiltrate the retina and accumulate in the choroid and in the Bruch's membrane adjacent to drusen

deposits [119, 200, 219]. They are usually circulating in the choroid, and are absent from the neural retina owing to the BRB. However, thickening of Bruch's membrane and subsequent disruption of the barriers promotes the entry of these macrophages into the neural environment of the retina. The functions of subretinal macrophages include secretion of pro-angiogenic factors and phagocytosis of oxidised lipoproteins [220, 221]. The dual nature of macrophage plasticity [222] demonstrates that M1-shifted pro-inflammatory macrophages are associated with photoreceptor degeneration [223]. Therefore, the balance of macrophage plasticity and the complexity of macrophage heterogeneity are crucial for tissue homeostasis.

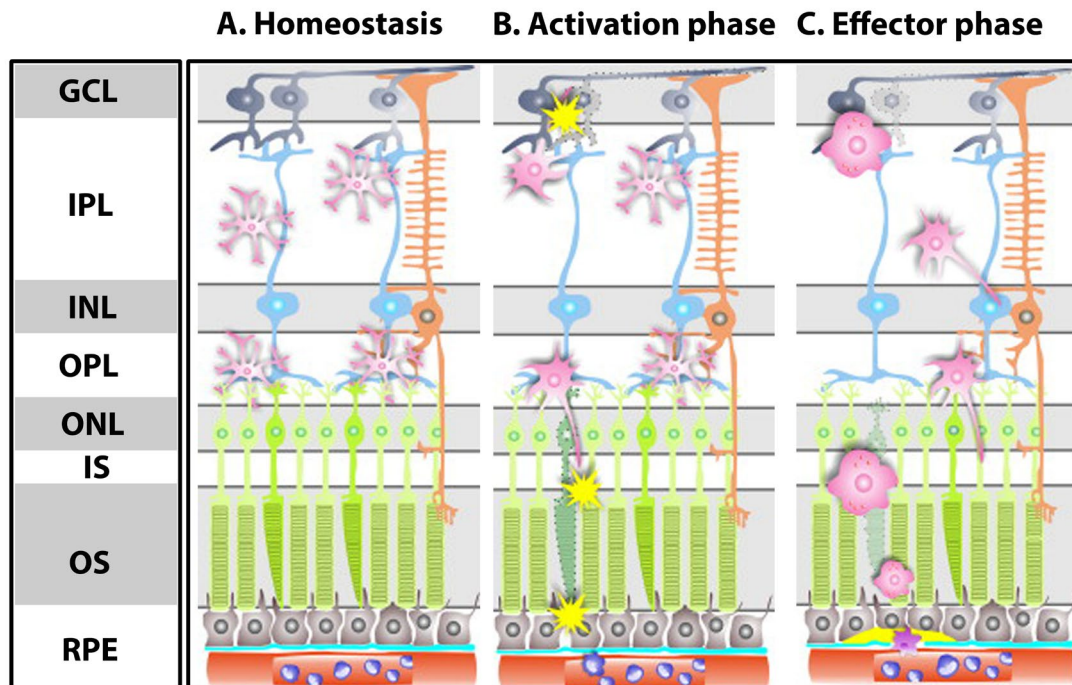


Figure 1.4. Schematic representation of microglial activity in the retina (adapted from Karlstetter 2015 [210]). **A:** Under homeostasis, resident microglia populate in the plexiform layers. **B:** Cellular damage in the photoreceptors and RPE cells alert the resident microglia to shift their homeostasis dynamic to the active phase. **C:** In the effector phase, resident microglia migrate to the retinal lesion site where they adopt an amoeboid morphology as phagocytes.

Cytokines

Cytokines are a large family of small proteins secreted from immune cells. They play a pivotal role in cell signalling. One key group of cytokines, the interleukins (IL), are associated with the inflammatory responses in AMD [224-226]. Sera of AMD patients contain elevated levels of the pro-inflammatory cytokines, including IL-1 β , IL-6 and IL-17 [225]. High levels of other interleukins such as IL-4, IL-10 and IL-18 have also been reported in sera of AMD patients [225, 226].

Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine whose maturation is mediated by inflammasome activation [227]. IL-1 β is secreted by microglia in photo-oxidative damage [228, 229], neovascular AMD [230] and retinitis pigmentosa [217]. Its accumulation induces neuronal apoptosis as well as degeneration in the retina [198, 230] and brain [231]. Interleukin-6 (IL-6) is a pro-inflammatory cytokine whose accumulation is associated with AMD genetic polymorphism (*CFH* polymorphism) [232]. Higher IL-6 levels have been associated with progression of AMD in patients of geographic atrophy [80, 233]. Interleukin-18 (IL-18) is strongly implicated in the development of choroidal neovascularisation [234, 235]; however, its role remains to be determined [236]. Interleukin-17 (IL-17), secreted by Th17 subset of CD4 T helper cells, is also elevated in the serum of AMD patients [237], and gene variations of IL-17 are associated with increased risk of AMD [224]. The role of IL-17 in retinotoxicity has been shown through local genetic inhibition of IL-17 receptors, which result in an improved function of RPE and photoreceptors in mice [238] as well as in *in vitro* studies [239, 240].

Chemokines

Chemokines are a large family of molecules with chemoattractant properties that guide leukocyte migration to a site of retinal damage and mediate subsequent activation through interaction with corresponding chemokine receptors on microglia and macrophages [41, 241]. Chemokines have been implicated in the inflammatory

mechanisms of AMD pathogenesis [196, 242, 243], such as the expression of chemokine (C-C motif) ligand 2 (CCL2) and its corresponding chemokine receptor 2 (CCR2) as well as chemokine (C-X3-C motif) ligand 1 (CX3CL1) and its receptor (CX3CR1) in retinas of dry and wet AMD [138, 244]. *Cx3cr1*^{-/-} mouse models have impaired migration of microglia, increasing subretinal microglia/macrophage accumulation with age [196], and after light injury [245]. In these animals, accumulation of microglia/macrophages increases excessive phagocytosis of healthy photoreceptors, as well as ingestion of intracellular lipids [196]. This results in drusen-like products and further photoreceptor degeneration and atrophy [196, 246]. CCL2 is another chemokine shown to locally increase in expression in AMD patients [244]. *Ccl2*^{-/-} or *Ccr2*^{-/-} mouse models have reduced accumulation of subretinal microglia/macrophages and subsequent choroidal neovascularisation [247]. In aged mice, the absence of chemokine signalling also leads to autofluorescent lesions that are claimed to resemble drusen deposits [221]. Overexpression of *Ccl2* promotes subretinal inflammation and photoreceptor degeneration after light injury [193, 194, 248]. Inhibition of *Ccl2* suppresses the inflammatory responses of microglia, macrophage accumulation and subsequent photoreceptor degeneration [193, 249]. Overall, the evidence suggests that the chemokine signalling axes assist microglia/macrophage activation, and subsequent photoreceptor degeneration at an early stage of AMD (reviewed in [250]).

Inflammasome

The inflammasome is a key pro-inflammatory signalling pathway, initially described in immune cells of the innate immune system of the central nervous system (CNS) [251]. It promotes maturation of the cytokines IL-1 β and IL-18, leading ultimately to apoptosis or pyroptosis [227, 252]. It is an intracellular multiprotein complex that consists of pro-caspase-1, the adapter apoptosis-associated, speck-like protein containing

a CARD (ASC), and pattern-recognition receptors such as nucleotide-binding oligomerization domain-like receptors (NOD-like) [252].

Inflammasome activation is operated by a two-signal mechanism. The first signal is inflammasome priming, which involves the transcription of pro-IL-1 β and NLRP3. Their transcription is induced by activation of the transcription factor nuclear factor- κ B (NF- κ B), by NF- κ B agonists such as lipopolysaccharide, and the cytokines IL-1 α and IL-1 β [253]. The second signal triggers inflammasome activation, which culminates in oligomerisation of the inflammasome complex, recruitment of the adaptor protein ASC and pro-caspase-1, finally leading to cleavage of pro-IL-1 β and pro-IL-18 into its mature cytokine forms [254]. Some triggers responsible for NLRP3 inflammasome activation include oxidative stress [255, 256], ionic disruption by pore-forming complex [257, 258] and disruption of the lysosomal membrane [259].

In AMD pathogenesis, inflammasome activation has been implicated in RPE cell loss [260, 261]. Its activation has been linked with destabilisation of lysosomes [260], accumulation of lipofuscin and drusen components such as C1q and amyloid-beta, and lipofuscin component A2E [234, 262-264]. Upon photo-oxidative damage, photoreactive lipofuscin is more capable of activating NLRP3 inflammasome than non-photoreactive A2E [184]. Accumulation of *Alu* RNA transcripts, which are secondary to DICER1 deficiency, is shown to prime and activate NLRP3 inflammasome in geographic atrophy [261, 265, 266]. Moreover, lipid peroxidation by-product 4-HNE promotes IL-1 β and IL-18 release mediated by inflammasome activation in ARPE-19 cells [256]. Lysosomal destabilisation and factors released from the reactive microglia can also activate inflammasome in RPE cells [260, 267].

Inflammasome activation has been found in macrophages under pathological conditions in the CNS (reviewed in [251]). Oxidised products, such as CEP, prime the

NLRP3 inflammasome [234]. Drusen extracts isolated from AMD donor tissues also activate the NLRP3 inflammasome in macrophages *in vitro* [234]. More importantly, complement component C1q in human drusen [60, 61] activates the NLRP3 inflammasome in peripheral myeloid cells [234]. In addition, complement components such as C3a and terminal membrane attack complex (MAC) mediate activation of inflammasome production in monocytes and macrophages *in vitro* [258, 268, 269]. Mononuclear phagocytes isolated from *Cx3cr1*^{-/-} mice promote inflammasome signalling in an ATP/P2X7-dependent manner and exacerbate photoreceptor toxicity [229]. Cholesterol such as 7-Ketocholesterol (7KCh) that accumulate in the choriocapillaris and in RPE/Bruch's membrane complex also activate NLRP3 inflammasome in microglia and macrophages *in vitro* [270]. The cytokines that are products of inflammasome activation, IL-1 β and IL-18, are associated with the development of AMD [265, 271].

1.7 Photo-oxidative damage

The retina, owing to its photoreceptors, is the only neural tissue that has direct and frequent exposure to light [18]. Moreover, the retina consumes more oxygen and generates a higher rate of reactive oxygen species than other organs [272]. These conditions favour the photo-oxidation of lipids, which can become extremely toxic to the retinal microenvironment [168], further rendering the photoreceptors vulnerable to high oxidative stress and inflammation [167]. It has long been recognized that with prolonged and chronic exposure to sunlight and an oxygen-rich environment, the photoreceptors are extremely susceptible to photo-oxidative damage [273, 274].

Light exposure and AMD

Visible and ultraviolet light require oxygen to enhance the ionising effect on most biomolecules, such as lipofuscins and photopigments, in a process of photo-oxidation.

This promotes the release of reactive oxygen species, which can induce further cellular damage and inflammation if the photo-oxidation events accumulate [164, 256, 275]. Because the anatomical features of the central macula make it vulnerable to degeneration [22], prolonged exposure to light striking the macula can induce further cellular damage.

Sunlight, as the main source of visible and ultraviolet light, must be considered a risk factor for AMD, although there are conflicting findings and opinions on the subject. Most of the epidemiological evidence has reported ambiguous results in humans worldwide, with some demonstrating a positive relationship between AMD development and sunlight exposure, while others have failed to do so [276, 277]. This is most likely due to the difficulty of controlling for individual / population variation, light levels, degree of exposure, and ocular concentration of retinoids. Among the positive results, excessive sunlight exposure is reported to be associated with both early AMD and the development of geographic atrophy in patients [278, 279]. The Beaver Dam Eye Study reported a correlation between early signs of AMD and excessive exposure to sunlight [280]. The European Eye Study found a significant association between blue light exposure and AMD, in patients with a low intake of anti-oxidants [278]. A cumulative effect of light exposure has been associated with elevated oxidative stress, disruption to the retinoid transport system and inflammation [273].

Mechanisms of photo-oxidative damage

Photo-oxidative damage by rhodopsin

Werner Noell (1966) first discovered a rapid and irreversible loss of photoreceptor cells, resulting from the exposure to bright light, in an albino rat model [281]. Longer duration, and specific wavelength and intensity of light have since been shown to cause impairment in retinal function and damage to the photoreceptors and RPE cells [281, 282]. The nature of such damage has been shown to be associated with the type of

photosensitizers activated by certain wavelengths of the action spectrum. The action spectrum of light at wavelength of ~500 nm overlaps with the absorption spectrum of rhodopsin, triggering the rhodopsin activation. Upon activation by light, rhodopsin undergoes photoisomerisation of 11-cis-retinal to all-trans-retinal. After the isomerisation events, the regeneration of 11-cis-retinal for the photoreceptors is known as the retinoid cycle, which is facilitated by RPE cells. Disruptions in the retinoid cycle caused by mutations have been shown to result in an increased susceptibility to light damage [283], owing to an over accumulation of all-trans-retinal in the photoreceptors [284].

Compelling evidence has shown that the presence of retinoids, such as 11-cis-retinal and all-trans-retinal, mediate oxidative stress, while the absence of all-trans-retinal protects against light damage [284]. Variations in RPE65 proteins or deletion of the RPE65 gene, which encodes a protein responsible for the photoisomerisation of all-trans-retinal to 11-cis-retinal, showed protection against light damage in rodents [285, 286]. Furthermore, higher rhodopsin content increases the susceptibility to light damage in rats [287]. This supports an independent finding that dark-reared rats, with increased levels of rhodopsin, are more susceptible to light damage than rats reared in normal cyclic light [288].

Photo-oxidative damage by lipofuscins

Another effect resulting from the accumulation of all-trans-retinal in the photoreceptors is the formation of lipofuscin in the RPE [289]. The lipofuscins are not readily degraded and are capable of oxidising proteins and lipids in the outer segment membrane upon photo-oxidation, thereby increasing the susceptibility to light damage. Above certain wavelengths (~400nm), photo-oxidation of lipofuscins, such as A2E, has deleterious effects. These include increasing levels of reactive oxygen species [290], photoreceptor and RPE cell damage [179], immune dysregulation through complement [291] and microglial activation in the subretinal space [185].

Photo-oxidative damage and inflammation

Constant light exposure has been shown to indirectly result in complement activation and microglial activation [146, 248, 292, 293]. This is thought to depend on the level of photo-oxidative stress and lipid peroxidation by-products in the photoreceptors and RPE cells [156]. Prolonged photo-oxidative damage is also known to increase subretinal microglial accumulation through chemokine expression [294, 295]. Upon photo-oxidative damage, the microglial cells phagocytose apoptotic cells coated with complement-receptors [296]. Chemokines expressed by Müller cells are upregulated as a result of photo-oxidative damage and act as chemoattractants to the microglial cells [193, 248]; while inhibition of chemokine expression reduces the light-induced microglial recruitment [249]. Overall, there is compelling evidence that photo-oxidative damage causes photoreceptors and RPE dysfunction, resulting in increased oxidative stress and reactive changes in the microglia and Müller cells in rodents.

1.8 Animal models for studying AMD

The features of light-induced damage to the retina resemble many of the features of retinal degenerative diseases observed in humans, including AMD [297]. The damaging effect of light appears to accelerate photoreceptor death and RPE damage, which are the principle events in retinal degeneration [298]. Despite the availability of genetic models where photoreceptor health may be hindered for studies on oxidative damage [160, 299, 300], inflammation [196, 221, 245] and lipid metabolism [301, 302], models of light-induced photoreceptor cell death [274, 281] have been widely used to investigate the mechanism of retinal injuries in AMD. The advantages of light exposure models over genetic animal models include, (1) a more rapid photoreceptor degeneration, (2) synchronised and larger scale of photoreceptor cell death and (3) a focal area of degeneration that expands over time, after removal from damaging levels of light. Furthermore, light-induced damage can be used to mimic different aspects of disease by

using different light wavelengths and durations of exposure. Laser-induced choroidal neovascularisation is used to model choroidal neovascularisation of wet AMD [303], while acute or chronic light damage models are used to mimic features of dry AMD [304].

A unique feature of photo-oxidative damage is the localised area of photoreceptor degeneration in the superior hemisphere of the retina. While rodent retinas do not have a macula, they do have an *area centralis* in which the neural elements and photoreceptor densities are highest. Lesions resulting from photo-oxidative damage start at the *area centralis*, in the superior retina at a focal point, 1-2mm from the optic nerve head [305]. Over a longer timeframe, the lesion expands in size and retinal damage progresses to the periphery resulting in destabilisation of the retinal environment [304]. In the inferior retina, there is significantly less photoreceptor cell death owing to the shorter outer segments and lower rhodopsin level [306]. Photoreceptor loss is associated with reactive changes in the microglia and Müller cells, while chemokine expression and complement deposition are present in the region of the focal lesion [146, 193, 248, 294]. For this reason, the photo-oxidative model of retinal degeneration most closely resembles atrophic AMD.

The most common rodents used in photo-oxidative damage models have been albino rats and mice, because of their sensitivity to light [304, 307]. However, the albino animals are inherently unusual, and the generation of albino transgenic mice requires more time as compared to the common C57BL6 background. In this thesis, I describe the design and implementation of a new mouse model for focal retinal degeneration induced by photo-oxidative damage using pigmented mice. I also explore the role of complement activation and the extent to which microglia/macrophages are key instigators of local complement dysregulation in focal retinal degeneration.

1.9 Aims

Complement and accumulation of microglia / macrophages are highly implicated in the pathogenesis of retinal degenerative diseases such as AMD, although many questions are yet to be addressed. In this thesis, the central hypothesis is that **locally-derived complement by microglia/macrophages propagates the early onset and progression of focal retinal degeneration**. Firstly, I aim to better understand the roles of microglia / macrophages in accumulation of complement in the retina, and in the death of photoreceptors. Following this, I aim to dissect the effects of different pathways of complement activation on photoreceptor death and lesion expansion.

To reach the aim, in Chapter 3, I report on the co-authored published work that characterises a pigmented mouse model of photo-oxidative damage, and provides evidence of oxidative stress, photoreceptor degeneration and immune activation. Chapter 4 reports on the published work that explores the cellular sources of the complement component 3 (C3), including the significance of the roles of resident versus systemic sources of C3 and studies of C3 knockout mice. In Chapter 5, I report on new work looking at the impact of C3 knockout on Müller cell activation and phagocytic activity of microglia/macrophages in the retina and subretinal space. My unpublished studies described in Chapter 6 dissect the distinct contributions of the classical and alternative pathways. I show that these two pathways have distinct roles in the onset and progression of retinal degeneration.

2. METHODS

In this chapter, I address the details of the methods and materials used in the thesis including animal experiments, histological analysis and molecular analysis followed by statistical analysis.

2.1 Animal experiments

All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for use of animals and the Australian National University (ANU) ethics protocols outlined by the Animal Experimental Ethics Committee (Ethics ID: A2014/56 and A2017/41). C57BL6-Jax black mice were obtained from the Australian Phenomics Facility and housed at the John Curtin School of Medical Research (JCSMR) animal holding facility. Animals were born and raised in dim (5 lux) cyclic light conditions in 12:12hr cycle and housed in individually vented cages. Food and water were provided in constant supply and cages changed on a weekly basis. Animals aged between postnatal day (P) 60-90 were used for photo-oxidative damage. Equal numbers of male and female mice were used throughout the study to avoid any gender biases.

Genetic knockout animals

Genetic knockouts of the complement system used in this study were bred on the C57BL6 strain. Three strains of mice lacking components of complement system were analysed as shown in Table 2.1. Each complement knockout strain was compared to its corresponding wild-type (WT) for analysis under both photo-oxidative damage and dim-reared conditions.

Table 2.1 Gene knockouts deficient of complement system

Strain	Allele Symbol	Allele Type	Target gene	Molecular Note
ASD509	C3tm1Crr/J (<i>C3^{-/-}</i>)	Targeted (Null/Knockout)	Complement component 3 (C3)	Insertion of a PGK-neomycin resistance cassette into an exon of the C3 gene deleted sequences that code for the C-terminal region of the beta chain and the N-terminal region of the alpha chain, including the site for processing the pro-C3 molecule. ELISA testing did not detect C3 protein in serum of homozygous mutant mice. A C3 haemolytic assay did not detect functional C3 activity.
ASD511	C1qatm1 (<i>C1qa^{-/-}</i>)	Targeted (Null/Knockout)	Complement component 1, q subcomponent, alpha polypeptide	A neomycin selection cassette was inserted into exon 1 of the gene. Northern blot analysis on samples derived from spleen and liver of homozygous mice demonstrated that no transcript is produced from this allele. Western blot and ELISA analysis confirmed that no protein was detected in samples derived from homozygous mice.
ASD693	CfB Knockout (<i>CfB^{-/-}</i>)	Targeted (Null/Knockout)	Complement component factor B; alternative-complement pathway C3/C5 convertase	A neomycin resistance cassette replaced exons 3 through 7 of the gene. Northern blots of liver from homozygous mutant mice showed no detectable full-length transcript, but did show a smaller, truncated transcript when probes encompassing segments 5' or 3' to the deleted segment were used. Small amounts of truncated, but not intact proteins were detected in peritoneal macrophages from homozygous mutant mice. However, sera from homozygous mutant mice showed no detectable protein activity.

Photo-oxidative damage

Photo-oxidative damage has been used as a model to induce retinal degeneration in rodents for over 50 years. The use of photo-oxidative damage models has been reported throughout this thesis (Chapter 3). The floors of the cages were coated with a reflective Perspex surface and illuminated by a 100W 65000k natural white LED (COLDF2, 2x36W, Thorn lighting, UK) mounted 18 cm above the plastic boxes. In order to regulate illumination, each box was equipped with a dimmer and adjusted to 100k lux using a light meter data logging device (HD450; Extech MA, USA). The LED light has an emission spectrum width more closely resembles daylight than halogen or incandescent bulbs. The temperature in the cages was maintained at $\sim 23 \pm 2$ °C with a dual exhaust system to help remove any heat generated by the LED, with one exhaust fan mounted next to the LED light source, and another one on the side of the cage. Animals were provided with bedding, food and water during the time course of light exposure, and their behaviour was monitored daily. The animal behavioural scoring sheet was used to monitor their consumption of water, signs of inactivity/hunched back so as to confirm whether they are dehydrated or stressed during light exposure. This was completed every day to determine if the experiment should proceed or cease.

Experimental paradigm

Duration of photo-oxidative damage was set for 1, 3, 5 and 7 days with food and water provided. Following photo-oxidative damage, mice were then returned to 5 lux of dim-cyclic light conditions for post damage or immediately euthanized for analysis (Figure 2.1). During the course of photo-oxidative damage, each animal was administered with pupil dilator eye drops twice daily, morning and evening (Minims Atropine Sulfate 1% w/v eye drops; Bausch and Lomb). Following photo-oxidative damage, animals were euthanized for tissue collection. Age-matched, undamaged animals were used in all

mouse experiments as dim-reared controls. For each complement knockout strain, mice lacking components of the complement system were placed with corresponding WT for photo-oxidative damage for appropriate comparison.

Intravitreal injections

Intravitreal injections were performed as previously described in detail [308], and animals were anaesthetized using an intraperitoneal injection of ketamine (100 mg/kg; Troy Laboratories, NSW, Australia) and xylazil (12 mg/kg; Troy Laboratories, NSW, Australia). Injections consisted of either siRNA or antibody-based inhibitors. Intravitreal injections into mouse eyes were performed under a dissecting microscope using a Hamilton micro-injector (World Precision Instruments, FL, USA), with mice pupils dilated with 0.1% atropine sulfate (Minims atropine sulfate 1% w/v eye drops, Bausch and Lomb, NSA, Australia) for visualisation of the vitreous. A 30-gauge needle (Becton Dickinson, NJ, USA) was first used to make a punch incision 0.5mm posterior to the temporal limbus, and the Hamilton needle was then inserted through the incision, approximately 1.5mm deep until the tip of needle was visualised in the vitreous. Using Leica M125 stereo microscope (Leica Microsystems, Wetzlar, Germany), visualisation was performed to aid the intravitreal injection process. Antiseptics (Betadine; Faulding Pharmaceuticals, SA, Australia) and antibiotic cream (Aspen Pharma, NSW, Australia) were applied to the injection site afterwards.

RNA-interference (RNAi)

RNAi was conducted using complement gene-specific siRNA (C3 siRNA; Thermo Fisher Scientific, Waltham, USA), while a scrambled negative siRNA (Stealth RNAi Med GC; Thermo Fisher Scientific) served as a control. C3 siRNA and the negative siRNA were encapsulated using a cationic liposome-based formulation (InvivoFectamine 3.0 Reagent; Thermo Fisher Scientific) according to the manufacturer's instructions. To

purify and concentrate the siRNA formulation, the samples were centrifuged at 4000g through an Amicon Ultra-4 Centrifugal Filter Unit (Merck Millipore, MA, USA). The final concentration of the encapsulated siRNA formulation was 1 μ g/ μ l in endotoxin-free 0.1 M phosphate-buffered saline (PBS). For injection, animals were anaesthetised in the same fashion as the antibody neutralisation series. Three microlitres of either gene-specific siRNA or negative siRNA was then delivered intravitreally to both eyes of each animal, which equated to a final dosage of 3 μ g siRNA per eye. Animals were then exposed to photo-oxidative damage for the indicated time periods, during which time corneal hydration was maintained by application of a synthetic tear gel (GenTeal Gel; Novartis, NSW, Australia) until the animals awoke.

Antibody neutralisation

A neutralising antibody to C1q, which has the highest binding affinity to the functional C1q protein, was provided by Annexon Biosciences (ANX-M1; San Francisco, USA). A one microlitre solution containing either ANX-M1 or IgG isotype control antibody was administered intravitreally to mice prior to the commencement of photo-oxidative damage and immediately after 7 days of photo-oxidative damage. The animals were immediately transferred to dim-cyclic light for 7 days of post damage before being used for tissue collection and retinal assessment.

Electroretinogram

Electroretinogram (ERG) was used to measure mouse retinal function in response to full-field flash stimuli under scotopic and photic conditions in dim-reared control and test animals after photo-oxidative damage. Mice were dark-adapted for 12 hours overnight prior to the assessment of retinal function in a dark room with minimal red light sources. Animals were anesthetized by intraperitoneal injection of Ilium Xylazil (10mg/kg body weight; Troy laboratories) and Ketamine (100mg/kg body weight; Troy

laboratories), and the pupils were dilated with a single drop of 0.5% tropicamide (Minims Tropicamide; Bausch and Lomb) and phenylephrine (Minims Phenylephrine; Bausch and Lomb) while resting on a heat mat. The animal was set up on a homeothermic blanket to maintain core body temperature at 37°C using a rectal probe (Harvard Apparatus, MA, USA). To allow access to the eye, a cotton loop was tied around the eye. The head of the animal was placed centrally into a Ganzfeld sphere, containing an LED light source (Photometric Solutions International, Melbourne). An earth probe was placed on the hind foot of the animal. A reference probe was placed in the mouth of the animal while a corneal probe was placed on the corneal surface of the mouse's eye with eye gel administered. To ensure minimal noise in the recordings, a bioamp was run at 2mV prior to taking retinal recordings, with signal adjustment performed using a 50Hz filter.

A single- or twin-flash paradigm was used to elicit mixed or isolated cone responses. Flash stimuli for mixed responses were provided by an LED-based system (FS-250A Enhanced Ganzfeld, Photometric Solutions International, Melbourne), over a stimulus intensity range of increasing flash stimulus (range -4.4 - 1.9 log cd·s·m⁻²). The interval between stimuli was increased for the highest intensities to allow complete recovery of the b-wave between stimuli. Isolated cone responses were obtained at 1.6 log cd·s·m⁻² following a rod-saturating stimulus of 1.9 log cd·s·m⁻². This short interval after a rod-saturating flash does not allow recovery of rod function, thereby revealing cone-only responses. The a-wave amplitude was measured from the baseline to the trough of the a-wave response, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

A-wave and b-wave response data were analysed using Lab Chart 8 (AD Instruments) and expressed as the mean wave amplitude $\pm$ SEM (μ V). The a-wave and b-wave data were fitted with a Naka-Rushton equation [$R/R_{\text{max}} = I/(I + K)$] using the

Solver function in Microsoft Office Excel 2013 to determine Rmax (maximum amplitude) and K (semisaturation constant) from the response amplitude (R) and the flash intensity (I) over the range of flash stimulus (-4.4 to 1.9 log cd·s·m⁻²). Statistics were performed using Prism (GraphPad Software V5; GraphPad Software, Inc., La Jolla, CA, USA) and either a two-way ANOVA for mixed a-wave and b-wave or Student t-test for isolated cone b-wave.

Complement haemolysis

To assess serum complement activity, a haemolytic assay [309] was performed on blood collected from anaesthetised animals at the terminal stage, using sub-mandibular bleeding or cardiac puncture using 25G Insulin Syringe (Terumo, ASP Healthcare, Australia). Blood was then placed on ice for 10 minutes, and centrifuged (15,000g, 15 minutes, 4°C). The supernatant was collected as serum. Serum samples were stored at -80°C prior to haemolytic assay which consists of preparing 5x veronal buffer solution (VBS) using solution 1, solution 2 and solution 3 with the recipes for each indicated below:

Preparation of VBS buffer

Solution 1: 21.25g NaCl and 0.9g sodium barbitone in 350ml of distilled H₂O

Solution 2: 1.44g sodium barbitone in 125ml of distilled H₂O at 60°C

Solution 3: 44.32g magnesium chloride hexahydrate and 6.47g calcium chloride dehydrate in 100ml distilled H₂O

Solutions 1 and 2 were mixed at room temperature, then 1.25ml of solution 3 was added with pH at 7.3-7.5. A final volume of 500ml was obtained with distilled H₂O. For the haemolytic assay, the stock of 5x VBS was diluted to 1x VBS. Sheep red blood cells

(SRBC, Alsever's, Applied Biological Products, Australia) were prepared with 1x VBS for sensitisation with haemolysin.

Sensitisation of sheep red blood cells with haemolysin

Sheep red blood cells (SRBC) and VBS were gently mixed and centrifuged (600g, 5 minutes, 4°C). The supernatant was discarded and washed with up to 10ml of 1x VBS, then centrifuged again – this step was repeated twice. The supernatant from the final wash was discarded, and the pellet containing VBS-bound SRBC centrifuged (900g, 5 minutes, 4°C) to pack the cells. SRBCs were resuspended in 1x VBS to prepare 10% solution. Sheep Red Blood Cell Stroma antibody (Sigma Aldrich) was prepared in VBS (1:50) through gentle inversion. Haemolysin was added to SRBC, while swirling continuously, in equal volume to VBS-bound SRBC, then incubated for 30 minutes at 37°C, with gentle inversion every 15 minutes. The sensitised SRBC was stored only overnight at 4°C. Haemolytic assay was performed by setting up tubes of spontaneous lysis, total lysis and unknown serum samples. These comprised sensitised SRBC and VBS, distilled H₂O and serum samples, respectively, in 1:1 ratio. The tubes were incubated for 30 minutes at 37°C with gentle mixing every 15 minutes, and centrifuged (1500g, 5 minutes, room temperature). Supernatant from each tube was transferred in duplicate to a clear Nunc MicroWell 96-well Flat Bottom plate (Thermo Fisher Scientific). Sample absorbance was determined at 540nm (Tecan, Switzerland). Percentage of SRBC lysis was calculated as

$$\% \text{ lysis} = \frac{OD(\text{Sample } x) - OD(\text{Spont.})}{OD(\text{Total lysis} - OD(\text{Spont.}))} \times 100$$

Tissue collection

Animals were euthanized with CO₂. The left eye from each animal was enucleated with the superior surface marked, immersed in 4% paraformaldehyde for 3 hours, washed in 0.1M PBS and preserved in 15% sucrose solution overnight for cryoprotection. The eyes were embedded in optimal cutting temperature (OCT) medium (Tissue Tek, Sakura, Japan), and frozen in acetone with dry ice. Sections of 12µm were cut in parasagittal plane (superior-inferior) on a vertical axis on Leica CM 1850 Cryostat, placed onto superfrost⁺ glass slides (ThermoScientific), dried overnight at 37°C and stored at -20°C. To enable comparison of histological sections across animals, only sections containing the optic nerve (ON) head were used for analysis. The retina from the right eye of each animal was excised through a corneal incision and placed in RNAlater solution (Ambion Biosystems, Austin, TX, USA), stored at 4°C overnight to allow penetration of the preservatives, and then stored at -80°C until required. Total RNA was extracted from the retinal samples in micro-scale, according to the manufacturer's protocol (RNAqueous-Micro, Biosystems, Austin, TX, USA). The concentration and purity of the RNA samples were determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). Only samples with a 260/280 ratio between 2.0 and 2.2 were considered for analysis. The quality of the RNA samples was examined with an RNA analyser (model 2100 Bioanalyser; Agilent Technologies, USA). RIN values above 8.0 are considered for the downstream analysis such as qPCR. The RNA samples were stored at -80°C.

2.2 Histological analysis

Tissue processing

After incubating with 15% sucrose, eyes were placed into plastic moulds filled with OCT medium (Tissue Tek, Sakura, Japan). For orientating, the superior marked side

on the eyeball was placed parallel to the wall of the mould, which was opposite to the tag on the mould. The mould was then snap frozen in a solution of Acetone (Merk Millipore, Bayswater, VIC, Australia) cooled by dry ice. The embedded blocks were stored at -20°C. The embedded eyes were cryosectioned using Leica CM1850 Cryostat at the 12 µm thickness and mounted directly onto superfrost⁺- Ultra Plus glass slides (Menzel-Glaser, Braunschweig, Germany). Sections were cut in the sagittal plane and those containing the optic nerve head were used in experiments to maintain consistency of location within tissues and different animals. Sections were oven-dried at 37°C overnight, and then stored at -20°C until required.

TUNEL assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End labelling (TUNEL) assay (Roche Diagnostics, Switzerland) was used to detect cell death. Cryosections were thawed at room temperature for 15 minutes, placed in 70% ethanol for dehydration and washed in H₂O washes and PBS for 10 minutes for rehydration. Mouse retina sections were then permeabilised with 0.1% Triton-X100 (Sigma Aldrich) for 5 minutes, and washed with PBS for 5 minutes. Next, sections were immersed in 1xTdT buffer for 10 minutes, and covered in 200ul of reaction mixture containing 1.26 M biotinylated dUTP, 0.5 units/µl terminal transferase (TdT) in 10x TdT buffer in H₂O. The sections were incubated at 37°C in a humid container for 1 hour. The reaction was terminated by a 15-minute immersion in 2x saline-sodium citrate (SSC) solution following the recipe (0.3M sodium chloride, 30mM sodium citrate). The sections were then incubated in 10% normal goat serum (NGS; Sigma Aldrich) in 0.1M PBS for 10 minutes, to block non-specific binding of the streptavidin conjugated fluorophore. Streptavidin-Cy3 conjugate in 0.1M PBS (1:500; Thermo Fisher Scientific) was pipetted evenly over the sections, and then incubated for 1 hour in a humid container at 37°C. Sections were washed twice in 0.1M

PBS before being counterstained with the DNA-specific dye bisbenzimidazole (Bisbenzimidazole, 0.1 µg/ml; Sigma Aldrich) for 2 minutes at room temperature. Sections were then thoroughly washed for 5 minutes before coverslipping with aquamount glycerol (Polysciences, PA, USA).

Toluidine blue

Staining was performed to assess histological changes in different layers of the retina. Sections were dehydrated by immersing in 70% ethanol for 5 minutes, then rinsed with H₂O for 5 minutes. Sections were stained with a few drops of 1% toluidine blue for 2 minutes, followed immediately by a 5-minute wash in H₂O, and coverslipped with Aquamount for visualisation. Toluidine-stained sections were visualised on a laser-scanning A1+ confocal microscope (Nikon A1, Tokyo, Japan) using standard light microscopy. Whole retina image was captured using the stitching function in the NIS Elements C control software (Nikon A1, Tokyo, Japan) on the A1 Confocal Microscope.

Immunohistochemistry

Retinal cryosections were thawed for 5 minutes at room temperature, with a hydrophobic border drawn around the sections using a pap pen to avoid leakage of reagents. Table 2.2.1 and 2.2.2 list the primary and secondary antibodies used in the thesis. Prior to the addition of antibodies, sections were immersed in 70% ethanol for 10 minutes, followed by a 5-minute wash in MQH₂O and two 5-minute washes in 0.1M PBS. To retrieve intracellular antigens, sections were immersed in heat-induced reveal-it antigen retrieval solution (ImmunoSolutions, QLD, Australia) for 1-3 hours at 37°C depending on the antigens, then washed in 0.3% PBSR (0.3% reveal-it antigen retrieval solution in 0.1M PBS). The sections were permeabilised and blocked with 10% normal goat serum in 0.1M PBS containing 0.1% Trion X-100 for 1 hour at room temperature, then incubated

with primary antibodies (Table 2.2.1) overnight at 4°C. The following day, the sections were washed three times in 0.1M PBS for 15 minutes and then incubated with the secondary antibodies (Table 2.2.2) diluted in 0.1M PBS for 4 hours at room temperature. Following incubation with the secondary antibodies, sections were washed in 0.1M PBS for 1-2 hours depending on the required secondary antibody. The sections were counterstained with bisbenzimidazole (0.1 µg/ml) for 2 minutes at room temperature. Sections were then washed thoroughly in 0.1M PBS for 5 minutes before being coverslipped with aquamount/glycerol.

In order to detect complement proteins, the sections were blocked and permeabilised with 3% BSA in 0.1M PBS containing 0.1% Triton X-100 and 0.12% sodium azide. Following the blocking, the primary antibodies were applied to the sections, followed by secondary antibodies and counterstaining, as described in the protocol above. Immuno-labelled cells were quantified along the full length of retinal cyrosections (superior-inferior) in duplicate.

Table 2.2.1 Primary antibodies used for immunohistochemistry

Antibody	Target	Source	Catalog #	Dilution
Rabbit α -Iba1	Ionised calcium binding protein 1	Wako Chemicals	019-19741	1:500
Rat α -CD68	CD68:Biotin Clone FA-11	AbD Serotec	MCA1957BT	1:400
Rabbit α -Gfap	Glial fibrillary acidic protein	Dako	Z0334	1:500
Mouse α -Rhodopsin	Rhodopsin, C-terminus, clone Rho 1D4	Millipore	MAB5356	1:500
Rabbit α -L/M opsin	Red/green opsin	Millipore	AB5405	1:500
Rabbit α -C3	Complement Component 3	Abcam	AB11887	1:50
Rabbit α -C1q	C1q, clone 4.8	Abcam	AB182451	1:400
	C1q complement isolated from human plasma	Dako	F0254	1:1000
Goat α -C3d	Complement Component C3d	R & D systems	AF2655-SP	1:100
Rabbit α -4HNE	4-hydroxy-2-nonenal antiserum	Alpha Diagnostics	HNE11-S	1:200
Mouse α -8OHG	8-hydroxyguanosine (8OHG) IgG	Alpha Diagnostics	8OHG11-M	1:500
Rabbit α -NALP3 /NLRP3	1-50 amino acids of human NLRP3	Novus Biologicals	NPB2-12446SS	1:500
Rat α -F4/80	F4/80 clone A3-1	Abcam	AB6640	1:400

Table 2.2.2 Secondary antibodies used for immunohistochemistry

Antibody	Source	Catalog #	Dilution
Goat α -rabbit Alexa Fluor 488/594	Thermo Fisher Scientific	S31623	1:500
Goat α -mouse Alexa Fluor 488/594	Thermo Fisher Scientific	S31619	1:500
Streptavidin Alexa Fluor 488/594	Thermo Fisher Scientific	S32354	1:1000
Donkey α -Goat IgG (H+L), F(ab') fragment, highly cross adsorbed-Biotin	Sigma-Aldrich	SAB3700318	1:500
Donkey α -Rabbit IgG (H+L), F(ab') fragment, highly cross adsorbed-Biotin	Sigma-Aldrich	SAB370966	1:500

Confocal analysis

Thickness of photoreceptor layer

Retinal cryosections were stained with the DNA-specific dye (Bisbenzimidazole, 0.1 μ g/ml of a 10 mg/ml stock) or toluidine blue to visualise the cellular layers. For each section, digitised images of the entire retina were captured with A1+ confocal microscope (Nikon A1, Tokyo, Japan) at 10x magnification. To quantify photoreceptor loss in retinal cryosections, the number of rows of photoreceptor nuclei were counted at increments of 0.5mm along the full length of a retinal cryosection. Within the retinal lesion site, five

measurements were taken in the superior retina, 1mm away from the optic nerve head. The procedure was repeated for total number of animals, and collected as an average.

Quantification of cell counts

TUNEL, IBA1 and other immuno-labelled cells were quantified along the full length of retinal cryosection, in duplicate. For photoreceptor cell death, only TUNEL⁺ cells in the ONL were counted. IBA1⁺ cells in the outer retina (including subretinal space, ONL-RPE) were taken across the cryosection. For each section, number of TUNEL⁺ or IBA1⁺ cells was recorded (superior-inferior) in increments of 0.5mm across the full length of retinal cryosection. Total cell counts were averaged from at least 2 sections per animal with 6 animals for each experimental group.

Fluorescence intensity

Fluorescence in retinal cryosections was visualised and imaged using a laser-scanning A1+ confocal microscope (Nikon A1, Tokyo, Japan). Images were acquired using NIS-Elements AR software (Nikon A1). Negative control slides were imaged with each different immune-labelled section to determine specificity of staining and to set up threshold for fluorescence intensity. Threshold analysis was performed and repeated on at least 4 negative control sections per animal. The fluorescent intensity of positively-stained retinal cryosections was measured in duplicate and compared between each experimental group. All slides were imaged using the same microscope, laser and gain settings for each section. This allowed for comparison of positive staining between experimental groups. The intensity of fluorescence staining was determined using the confocal software, and images were assembled into figures using Photoshop CS6 software (Adobe Systems, CA, USA).

2.3 Molecular analysis

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was utilised to isolate CD11b⁺ cells from mouse retinas. For each sample, a total number of 2-4 retinas were pooled. Retinas were removed from the eye cup via an incision in the cornea using a scalpel and forceps, and placed in chilled Hank's balanced salt solution (HBSS with 0.1% glucose, no phenol red, Ca²⁺ or Mg²⁺; Thermo Fisher Scientific). Using a 1ml transfer pipette, retinas were transferred to sterile petri dishes and crosshatched with a sterile scalpel blade. Fragments of retina were transferred to an Eppendorf tube containing 0.5ml chilled digestion mix, containing 2mg/ml papain (Worthington Biochemicals, NJ, USA), 660 µg/ml L-cysteine (Sigma Aldrich), 10µg/ml gentamycin (Sigma Aldrich), 100U/ml DNase I (Roche Diagnostics), 5µg/ml superoxide dismutase (SOD; Worthington Biochemicals) and 5µg/ml catalase (Sigma Aldrich) in HBSS with 5mM MgCl₂. Once the retinas were in the digestion mix, the samples were incubated for 7 minutes at 37°C, followed by 30 minutes at 8°C. During the process, the samples were homogenised thoroughly every 15 minutes using a clean cut pasteur pipette. Cells were centrifuged (1350rpm, 5 minutes, 4°C), and the supernatant removed. The pelleted cells were resuspended in 0.5ml of chilled neutralisation mix, containing 50µg/ml antipain (Roche Diagnostics), 10µg/ml gentamycin, 100U/ml DNase 1, 5µg/ml SOD, and 5µg/ml catalase in HBSS (with 0.2% glucose, 5mM MgCl₂ and 4% bovine serum albumin, BSA; Roche Diagnostics). Once the samples were in the neutralisation mix, they were incubated for 10 minutes at room temperature, then centrifuged (1350rpm, 5 minutes, 4°C). The supernatant was removed, and the pellet was resuspended in chilled staining buffer containing HBSS, 1mg/ml sodium azide (Sigma Aldrich) and 10mg/ml BSA for blocking (for 10 minutes at room temperature). Cells were divided into multiple tubes for antibody staining as necessary

(including an unstained control), and centrifuged (1200rpm for 5 minutes at 4°C). Following the removal of supernatant, cells were resuspended with anti-mouse CD11b-PE conjugated antibody (Biolegend, CA, USA) diluted in staining buffer, followed by an incubation at 4°C for 30-45 minutes. The unstained control sample was resuspended in staining buffer with antibody.

Samples were then centrifuged (1200rpm, 5 minutes, 4°C) and the supernatant removed. The pellet was resuspended and centrifuged twice in washing buffer, containing 1mg/ml sodium azide, 10mg/ml BSA and 2mM EDTA. Upon the last centrifugation of the cells, samples were resuspended in chilled staining buffer containing 200U DNase-1 and filtered through 0.7µm mesh into the flow tubes for FACS sorting. CD11b-stained cells were sorted through FACS Aria II (BD Biosciences, NJ, USA) at the Imaging and Cytometry Facility at JCSMR. The isolated CD11b⁺ cells were collected in 48 well culture plate of DMEM F12 with 1ng/ml mouse GM-CSF (Stemcell Technologies, VIC, Australia) for downstream culturing or tubes of PBS for immediate RNA extraction.

Gene expression analysis

Primary microglia (CD11b⁺) were directly sorted into PBS for downstream qPCR. Cells were centrifuged (1300rpm, 10 minutes, 4°C), and the supernatant removed. The resulting cell pellet was processed for RNA extraction using TRIzol/ chloroform and the RNAqueous Total RNA Isolation Micro Kit as described below. The volume of TRIzol and chloroform was half of the volume used for the retinal samples, followed by the addition of 10-15µl of elution solution. Samples were then used for cDNA synthesis and qPCR run.

RNA extraction

For mice retina, RNA isolation was performed using TRIzol reagent and an RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific). Retina was homogenised in 200µl TRIzol and combined with another 440µl TRIzol. The sample was vortexed thoroughly and incubated for 7 minutes at room temperature. Homogenised retina was then added with 1/5 volume of chloroform, followed by 20 seconds of vortexing. Chloroform-RNA mixtures were centrifuged (10 minutes, 13000g, 4°C) to separate the layers into RNA, DNA and protein phases. The RNA was contained in the top phase and was collected into new sample tubes, along with 100% ethanol in half of the volume. Samples were vortexed for 5 seconds. The RNA-ethanol mixture was purified using wash solution 1 and 2/3 in spin columns, and finally eluted in a pre-heated elution buffer (75°C) from micro RNAqueous Kit. Final RNA concentration was quantified on a ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA). RNA quality was assessed on a 2100-Bioanalyser (Agilent Technologies, Santa Clara, USA). Only RNA samples with A260/280 above 1.8 and RNA integrity greater than 8.0 were used for the study. Final RNA samples were stored at -80°C before undergoing complementary DNA synthesis.

Complementary DNA synthesis

Complement DNA (cDNA) was prepared with Tetro cDNA Synthesis Kit (Bioline, London, UK). Purified RNA samples were primed in a sterile 0.2ml tube by mixing 1µg RNA with 1µl Oligo (dT)18 primers (500ug/ml), 1µl dNTP (10mM) in a 14µl RNase free H₂O. This mixture was incubated at 70°C for 5 minutes and placed on ice for 5 minutes. Samples were briefly centrifuged and added to 6µl of reverse transcription mix containing 200 U Tetro Reverse Transcriptase, 4µl 5x RT buffer and 10 U Ribosafe RNase Inhibitor. The tubes were then mixed by vortexing and the 20µl reaction mixture

centrifuged and placed into a Verti 96-Well Thermal Cycler (Applied Biosystems, CA, USA). The cycling program was set up to run for 30 minutes at 45°C for the reaction to occur, followed by 5 minutes at 85°C to terminate the reaction and held at 4°C. The resulting cDNA samples was then stored at -20°C.

Quantitative Real-Time PCR

Changes in gene expression were determined using quantitative real-time PCR (qPCR) with Taqman hydrolysis probes (Applied Biosystems), Taqman Gene Expression Mastermix (Thermo Fisher Scientific) and synthesized cDNA. Table 2.3 lists the Taqman probes used in the thesis. qPCR was performed on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). The final qPCR data were analysed using the QuantStudio 12K Flex Software (Applied Biosystems). For each gene, technical duplicates were run across a biological triplicate to account for individual sample and animal variability. For each sample, a 10µl final reaction volume was used in a 384 well clear PCR plate (Applied Biosystems), with 4.5µl cDNA mixture in RNase free water (0.5µl cDNA) pipetted first into the well, following a 5.5µl Taqman gene expression master mix (0.5µl Taqman primer probe assay). Thorough mixing at least 5 times and brief centrifugation at 200g were conducted thereafter to collect the mixtures. Samples were then run on the QuantStudio 12K Flex qPCR machine according to the manufacturer's programs. The fluorescence level of each sample's Taqman probe was measured automatically at the end of each cycle for establishment of a critical threshold cycle. Following the qPCR run, fold change was determined using the comparative cycle threshold method ($\Delta\Delta C_t$). The QuantStudio 12K Flex Software generated the values for critical threshold cycle (C_t) and the delta-delta C_t values ($\Delta\Delta C_t$). The final gene expression change for each experimental sample was normalised to the corresponding

control samples and expressed as a percentage change with the control sample. Two reference genes were used in these studies, *Gapdh* and *ActinB*.

Table 2.3 Taqman Hydrolysis probes used for qPCR

Gene Symbol	Name	Accession #	Catalog #
<i>Ccl2</i>	Chemokine (C-C motif) ligand 2	NM_011333	Mm99999056_m1
<i>Ccl12</i>	Chemokine (C-C motif) ligand 3	NM_011331	Mm00441258_m1
<i>Cxcl13</i>	Chemokine (C-X-C motif) ligand 13	NM_018866	Mm01617100_m1
<i>Socs-1</i>	Suppressor of cytokine signalling 1	NM_001271603	Mm00782550_s1
<i>Hmox-1</i>	Heme oxygenase 1	NM_010442	Mm00516005_m1
<i>Gpx3</i>	Glutathione peroxidase 3	NM_008161	Mm00492427_m1
<i>C3</i>	Complement component 3	BC043338	Mm00437858_m1
<i>Cfh</i>	Factor H	NM_009888	Mm01299248_m1
<i>Clqa</i>	Component 1, q subcomponent, alpha polypeptide	NM_007572.2	Mm00432142_m1
<i>Cfb</i>	Factor B	NM_001142706.1	Mm00433918_g1
<i>Cfd</i>	Factor D	NM_001291915	Mm01143935_g1
<i>C2</i>	Component 2	NM_013484	Mm00442726_m1
<i>Serping1</i>	Serine peptidase inhibitor member 1	NM_009776	Mm00437834_m1
<i>C1s</i>	Component 1, s subcomponent 1	NM_144938	Mm01625167_g1
<i>C4</i>	Component 4	NM_011413	Mm01132415_g1
<i>Cntf</i>	Ciliary neurotrophic factor	NM_170786.2	Mm0044373_m1
<i>Fgf2</i>	Fibroblast growth factor 2	NM_008006.2	Mm01285715_m1
<i>Gfap</i>	Glial fibrillary acid protein	NM_001131020.1	Mm01253033_m1
<i>Rho</i>	Rhodopsin	NM_145383.1	Mm00520345_m1
<i>Nlrp3</i>	NLR Pyrin Domain Family 3	NM_145827	Mm00840904_m1
<i>Casp1</i>	Caspase-1	NM_009807	Mm00438023_m1
<i>Casp8</i>	Caspase-8	NM_001080126	Mm01255716_m1
<i>IL-18</i>	Interleukin 18	NM_008360.1	Mm00434226_m1
<i>IL-1β</i>	Interleukin 1-beta	NM_008361	Mm00434228_m1
<i>IL-6</i>	Interleukin 6	NM_031168	Mm00446190_m1
<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	NM_001289726	Mm99999915_g1
<i>Actin b</i>	Actin-beta	AK078935	Mm01205647_g1

Western blot

Protein extraction

For protein analysis, western blots were performed on retinas collected from the euthanized animals. Retinas were snap frozen in dry ice before the processing. Each frozen retina was added to 50µl CellLytic MT Cell Lysis Reagent (for mammalian tissue; Sigma Aldrich), containing 1% Protease Inhibitor Cocktail (Sigma Aldrich). The sample containing lysis/extraction reagent was transferred to a pre-chilled pestle for thorough homogenization, then incubated on ice for 10 minutes. The lysed sample was centrifuged (10 minutes, 12,000g, room temperature) for pellet collection, and the supernatant containing proteins transferred to pre-chilled Eppendorf tubes. The final samples were subsequently stored at -80°C. The concentration of each protein sample was determined using a Bradford Protein Assay (Bio-Rad, NSW, Australia), which was measured via absorbance at 595 nm using a TECAN Infinite 200 PRO plate reader (TECAN, Switzerland).

Bradford Protein Assay was run according to the manufacturer's protocol. In brief, 5µl BSA standards (0mg/ml to 2mg/ml) were set up in a 96 well plate with 245µl 1 x Bradford Dye Reagent (warmed to ambient temperature and inverted before use). Each protein sample was then set up at dilutions of 1:10, 1:20, 1:50 and 1:100 with 245µl 1 x Bradford Dye Reagent (Bio-Rad), thoroughly mixed and pipetted to a clear Nunc MicroWell 96-well Flat Bottom plate (Thermo Fisher Scientific). For each BSA standard and protein sample, duplicates were run to ensure accuracy. Once all BSA standards and dilutions of all proteins samples were added to the plate, the absorbance was measured at 595nm under the 'Bradford assay' program. Once the absorbance values were obtained, the protein concentration was determined using the following template for calculation:

- The absorbance value for each BSA standard concentration was measured, and plotted against each BSA standard concentration on the x-axis (0mg/ml to 2mg/ml).
- The line of best fit generated a formula: $y = m(x) + b$ and R^2 values. The formula was used for calculating the concentration from the absorbance values of the protein samples. The concentration of each protein sample (x) was derived as follows:
- $X \text{ (concentration)} = (Y \text{ (absorbance for each protein sample)} - (b)/ m) * \text{dilution factor}$
- The final protein concentration expressed in mg/ml, was then used for gel electrophoresis.

Gel electrophoresis

Before running the gel, the following steps were performed:

- 2 x sample loading dye: 50 μ l of 2-Bromophenol blue (Sigma Aldrich) in 950 μ l 2x Laemmli Sample Buffer (Bio-Rad) in the fume hood.
- 1x Tris-Glycine-SDS running buffer: 100ml of 10x Tris-Glycine-SDS stock (TGS; Bio-Rad) with 900ml MQH₂O, and pre-chilled at 4°C.
- Protein samples (20-25 μ g) were diluted in PBS and 2x sample loading dye, and then denatured at 95°C for 5 minutes.
- Gradient gels 4-20% Precast- TGX Mini-Protean (Bio-Rad) were rinsed in MQH₂O.

Once prepared, the electrophoresis chamber (Bio-Rad) was set up with inner chamber filled with 1x TGS buffer so that at least the bottom 1cm of gel was covered. The denatured protein samples were then loaded in 20 μ l using a gel loading tip. To aid visualisation of proteins, 10 μ l of Precision-Plus Western C Blotting Standards (Bio-Rad) were added on either side of the gel. The electrophoresis chamber was connected to

PowerPac Universal Power Supply (Bio-Rad), and run at 200V for 30 minutes or until the dye front was 0.5cm from the bottom. The gels were then stained with Coomassie Blue (Bio-Rad) for protein visualisation, and rinsed thoroughly for membrane transfer.

Membrane transfer and antibody labelling

The transfer was run on Trans Blot Semi-Dry System (Bio-Rad). Immun-Blot PVDF or nitrocellulose membrane was cut to size, and placed along with the gel in 1 x Tris-Glycine Buffer. Pre-soaking of the PVDF membrane in 100% methanol for 5 minutes was required. The assembly of the blotting sandwich followed this order: Filter paper, pre-soaked membrane, gel, filter paper and cathode lid. To avoid bubbles, a blot roller was used after adding the first filter paper. The transfer was then run at 20V for 1 hour (protein size <50kDa) and up to 2 hours (protein size >80kDa). To visualise the proteins on PVDF or nitrocellulose blot, Ponceau S solution was used (Sigma Aldrich) for 5 minutes followed by 10 minutes of MQH₂O washes. For antibody labelling, the blot was then placed into 3% BSA in 1x PBS-Tween20 (PBS-T) for phosphorylated proteins or 5% skim milk in PBS-T for 2 hours at room temperature. Table 2.4.1 and 2.4.2 list the primary and secondary antibodies used for the western blot. The blot was placed in a falcon tube containing 5ml of diluted primary antibody (Table 2.4.1) and incubated on the roller at 4°C overnight. Next day, the blot was washed thoroughly three times in PBS-T for 5 minutes before incubating in 5ml of HRP-conjugated secondary antibody (1:4000-8000; Table 2.4.2) for 1 hour at room temperature. The blot was then washed three times in PBS-T for 5 minutes, and placed in a clean dish containing 4ml of enhanced chemiluminescence (ECL) reagent (Bio-Rad) with 1ml pipette continuously covering the blot with ECL for 1 minute. Chemiluminescent images were taken on Chemidoc™ MP Imaging System (Bio-Rad), while the ladder was imaged on the colorimetric setting.

For labelling another protein, PVDF blots were stripped for reprobing for 10 minutes, with 500ml stripping buffer containing 7.5g glycine, 0.5g SDS, 5ml Tween20 adjusted pH to 2.2, and again with fresh stripping buffer. The stripped PVDF blots were then washed in PBS for 20 minutes, and 10 minutes in PBS-T before blocking with 3% BSA in PBS-T for 2 hours at room temperature. After blocking, the blots were incubated with a second primary antibody followed by HRP-conjugated secondary antibody as previously described.

Table 2.4.1 Primary antibodies used for western blot

Antibody	Target	Source	Catalog #	Dilution
Rabbit α -C3	Mouse C3 alpha chain	Abcam	11887	1:100
Goat α -IL1b	Mouse IL-1 β / IL-1F2	R & D systems	AF401-NA	1:1000
Goat α -C3d	Mouse complement component C3d	R & D systems	AF2655-SP	1:2000
Rabbit α -GAPDH	Mouse GAPDH	Sigma Aldrich	G9545	1:4000

Table 2.4.2 Secondary antibodies used for western blot

Antibody	Source	Catalog #	Dilution
Goat α -Rabbit IgG (H + L)-HRP Conjugate	Bio-Rad	170-6515	1:3000
Chicken α -Goat IgG-HRP Conjugate	Millipore	AP-163P	1:3000

2.4 Statistical analysis

All statistical analysis was performed using Prism 6 (GraphPad Software, CA, USA). Unpaired Student t-test, one-way analysis of variance (ANOVA) or two-way ANOVA were run with suitable dataset. Multiple comparisons were run with appropriate post-hoc tests to determine the statistical significance for the interactions between each experimental group ($P < 0.05$). Graphs are all displayed as mean $\pm$ SEM.

3. A model of progressive photo-oxidative degeneration and inflammation in the pigmented C57BL/6J mouse retina

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Haihan Jiao*, Riccardo Natoli*, Nigel Barnett, Nilisha Fernando, Krisztina Valter, Jan Provis, Matt Rutar (2016) A model of progressive photo-oxidative degeneration and inflammation in the pigmented C57BL/6J mouse retina, *Experimental Eye Research*, v147, pp114-127 (*denotes equal first author)

The candidate made a major contribution to this study as a first author, including designing and conducting the experiments, data acquisition and analysis, and drafting results and discussion, and revising the manuscript for publication.

3.1 Abstract

Background: Light-induced degeneration in rodent retinas is an established model for the study of outer-retinal degeneration, in particular for the roles of oxidative stress and neuroinflammatory activity. In these models photoreceptor death is elicited via photo-oxidative stress and is exacerbated by recruitment of subretinal macrophages and activation of immune pathways including complement propagation. Existing light damage models have relied heavily on albino rodents and mostly with the use of acute light stimuli. These models have proven valuable in uncovering the pathogenic mechanisms of such pathways in the context of retinal disease. However, their inherent albinism hinders their comparability to normal retinal physiology and also makes gene technology analyses time-consuming due to the predominance of the pigmented C57BL/6J mouse strain in these applications.

Methods: In this study, we characterised the development of a new light damage model utilising C57BL/6J mice over a 7 day period of chronic light exposure. These experiments used high-efficiency LED technology to deliver a sustained intensity of 100k lux with negligible change in ambient temperature.

Results: We showed that exposure elicited the cardinal features of light damage including photoreceptor degeneration, atrophy of the choriocapillaris, and increased in oxidative stress markers 4-HNE and 8-OHG, which emerged progressively over the 7 day period of exposure. These changes were accompanied by robust recruitment of IBA1⁺ and F4/80⁺ macrophages to the ONL and subretinal space, followed by the strong up-regulation of monocyte-chemoattractants *Ccl2*, *Ccl3*, and *Ccl12*, as well as increases in expression of complement component *C3*.

Conclusions: These findings are in agreement with a prior damage model conducted in albino rodents such as Balb/c mice, and support the use of this new model in further investigating the causative features of oxidative stress and inflammation in retinal disease.

3.2 Introduction

The capacity for exposure to bright light to induce retinal degeneration has been known in a laboratory context for nearly 50 years. In 1966, a seminal study by Noell has demonstrated that exposure to light of a sufficient intensity and duration induces degeneration of photoreceptors and the retinal pigment epithelium (RPE) resulting in severe vision loss in rats [281]. Since this initial discovery, a vast amount of literature has been produced regarding the histological and functional implications of exposure to bright light on the retina (reviewed in [273, 310, 311]). Light-induced degeneration induces photoreceptor apoptosis through oxidative damage in the photoreceptor outer segments, which stems from excessive photo-bleaching of the rhodopsin chromophore [274, 285, 286, 312, 313]. These features are accompanied by atrophy of the RPE and choriocapillaris [293, 304, 314], as well as the induction of inflammatory responses such as macrophage recruitment [249, 315, 316] and complement activation [145, 146, 212].

In recent decades, light damage in albino rodents has become a well-established model in the study of outer retinal degeneration, where it affords high reproducibility, rapid induction, ease of use, and flexibility through modulation of light duration and intensity (reviewed in [274]). Many investigations – including our own – have utilised the model in the study of immunological responses including subretinal macrophage recruitment [192-194, 248, 249, 317, 318], chemokine signalling, and complement activation [145, 146, 212], which are involved in the propagation of inflammation in human diseases such as age-related macular degeneration (AMD) [2, 3, 195, 319].

Despite the advances gleaned from these light-damage models, a recognized disadvantage is the reliance on albino strains. Albino rodents have been preferred because the lack of melanin means that damage is elicited relatively easily [320, 321]. Additionally, albino strains such as Balb/c mice are more susceptible to light damage than pigmented C57BL/6J mice because they express lower levels of RPE65, resulting in lower levels of recycling of photo-bleached rhodopsin chromophores [274, 285, 322, 323]. Thus, albinism induces alterations in retinal physiology and incorporates other abnormalities including delayed neurogenesis and altered retinal ganglion cell projections (RGC) [324-326]. Moreover, the widespread prevalence of the C57BL/6J background among knockout strains necessitates time consuming backcrossing onto albino mice strains (such as BALB/c) in order for them to be utilised in current light damage paradigms.

We recognize the need to develop a light damage paradigm using pigmented mice to address those concerns and to enable further investigation of outer retinal degeneration and inflammation. Here, we report the development of a new light damage apparatus using high efficiency LED technology to induce chronic retinal degeneration in C57BL/6J retinas. We find substantial degeneration of the outer retina after 7 days of light exposure, correlating with robust recruitment of subretinal microglia/macrophages, and up-regulation of potent inflammatory and oxidative damage markers, which are known to be associated with retinal inflammation, including AMD. With the use of pigmented mice, this model is more closely aligned with human physiology and greatly cuts down on the time frame of knockout experiments which may offer clues to mechanisms that underpin retinal disease.

3.3 Additional Methods

Animal Experimentation

All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The study was approved by the Australian National University Animal Experimentation Ethics Committee (Application ID: 2014/56). C57BL/6J mice were born and raised in 12:12 hrs light:dark cycle of 5 lux. They were raised in individually vented cages, with free access to food and water. Age-matched adult mice (8-10 weeks) were randomly assigned to light damage and dim-reared control (non-light damage) groups. Animals of the light damage group were continuously exposed to 100k lux white LED light for 1, 3, 5, and 7 days (Figure 3.1E). Pupils were dilated twice daily at 10am and 6pm with a single drop of 1% atropine sulfate (8.3 mg of atropine). Dim-reared control animals were also pupil dilated twice each day, but returned to dim cyclic light (12:12 hrs light:dark, 5 lux light).

Light Exposure Device

During bright-light exposure, animals were housed in plastic boxes (two per box), with free access to food and water. The floors of the cages were coated with a reflective Perspex surface and illuminated by a 100-W 65000k natural white LED (high CRI LED, Yuji, Beijing) mounted 18 cm above the plastic boxes (Figure 3.1A-C). In order to regulate accurate illumination, each box was equipped with a dimmer and adjusted to 100k lux (Figure 3.1G) using a light meter data logging device (Extech, HD450). The LED light has an emission spectrum which more closely resembles daylight than halogen or incandescent bulbs (Figure 3.1D). The temperature in the cages was maintained at $\sim 23 \pm 2$ °C (Figure 3.1E) with a dual exhaust system to elevate any heat generated from the LED: one exhaust fan was mounted next to the LED light source, and the other on the

side of the cage (Figure 3.1A-C). Animals were provided with bedding, food and water during the time course of light exposure, and their behaviour was monitored daily.

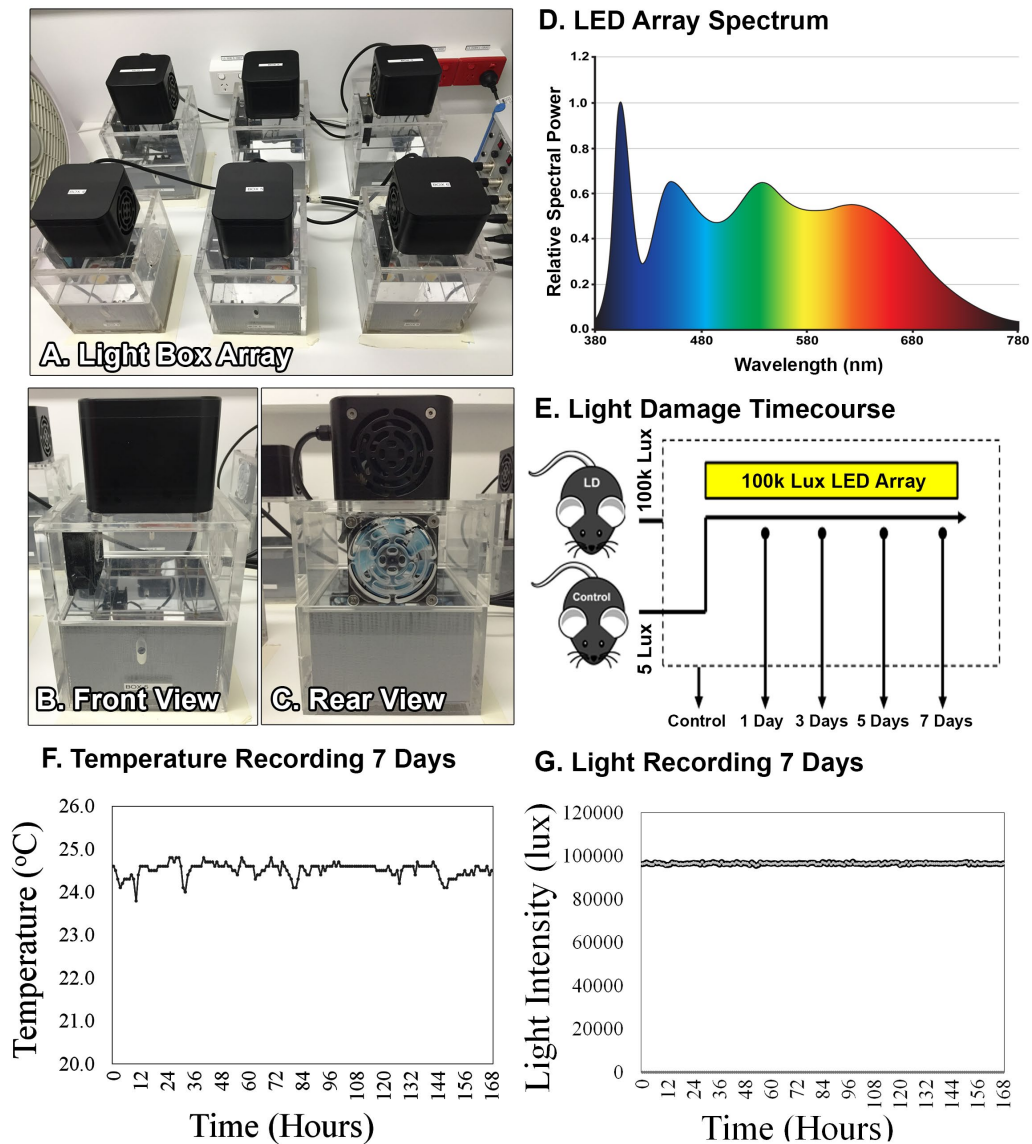


Figure 3.1 The light damage paradigm used for C57BL/6J mice. **A-C**: The design of the light boxes included the array of boxes (A), the front view with hole for the water bottle (B), and the rear view with venting for reducing heat (C). **D**: The LEDs produced a spectrum of light that is similar to that of daylight. **E**: The light damage paradigm with animals either being exposed to 100k lux constant light or 5 lux cyclic light (12:12 hrs, light:dark) over a time course of 7 days. Each animal was administered a single drop of atropine in each eye twice daily in order to dilate the pupils. **F-G**: Data logging over a 7 day period showed stable recordings for both temperature (min/max 23.8- °C) (F) and light (G).

Tissue collection and preparation

Animals were euthanized with CO₂. The left eye from each animal was enucleated with the superior surface marked, then immersed in 4% paraformaldehyde for 3 hrs, washed in 0.1M PBS and preserved in 15% sucrose solution overnight for cryoprotection. The eyes were embedded in OCT medium (Tissue Tek, Sakura, JP) and frozen in acetone with dry ice. Sections of 12 µm were cut in parasagittal plane (superior-inferior) at a vertical axis on Leica CM 1850 Cryostat, placed onto superfrost+ glass slides (ThermoScientific), dried overnight at 37°C and stored at -20°C. To enable comparison of histological sections across animals, only sections containing the optic nerve (ON) head were used for analysis. The retina from the right eye of each animal was excised through a corneal incision and placed in RNAlater solution (Ambion Biosystems, Austin, TX, USA), stored at 4°C overnight to allow penetration of the preservatives, and then stored at -80°C until required. Total RNA was extracted from the retinal samples in micro-scale according to the manufacturer's protocol (RNAqueous-Micro, Biosystems, Austin, TX, USA). The concentration and purity of the RNA samples was determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). Only samples with a 260/280 ratio between 2.0 and 2.2 were considered for analysis. The quality of the RNA samples was examined with an RNA analyser (model 2100 Bioanalyser; Agilent Technologies, CA). The RNA samples were stored at -80°C (n=6 per experimental group).

TUNEL staining and quantification of TUNEL⁺ cells

TUNEL immunolabelling was used to quantify photoreceptor apoptosis during bright light exposure, and performed on retinal cryosections using a protocol published previously [293]. For negative control experiments, the terminal deoxynucleotidyl transferase (TdT) enzyme was omitted. A retinal histological section containing the ON was taken to compare all regions of the retina in the superior and inferior regions. In each region, the number of TUNEL⁺ cells was quantified in increments of 500 µm along the

full length of the retina, starting at the ON head and extending toward the periphery in the superior and inferior regions. In addition, the average number of TUNEL⁺ cells was calculated for the superior and inferior regions of each retina. To obtain the total number of TUNEL⁺ cells from each retina, the process of quantification was performed on two retinal sections, and calculated as the average. The values for the dim-reared control and each light damage group (LD 1, 3, 5 and 7 days) were compared by one-way ANOVA followed by Tukey's multiple comparison post-test (TUNEL counts across time points) and two-way ANOVA (TUNEL counts for eccentricity and superior-inferior analysis) followed by Sidak's multiple comparison post-test, with a n=6 per experimental group.

ONL thickness measurement

Retinal cryosections were stained with the DNA-specific dye, bisbenzimidazole (Calbiochem, US, 0.1 µg/ml of a 10 mg/ml stock), to visualise the cellular layers. For each section, digitised images of the entire retina were captured with a NIKON A1 Confocal Microscope (Nikon, USA) at 10x magnification. Thickness of the outer nuclear layer (ONL) for the dim-reared control and each light damage group was measured at increments of 600 µm along the full length of a retinal cryosection containing the ON head. ONL thickness was counted as the ratio of ONL thickness to the distance between the outer limiting membrane and inner limiting membrane. The total ONL ratio from each retina was the average of two retinal sections at comparable locations. The procedure was repeated in six animals (n=6), and an average value calculated. Statistical significance was determined using one-way ANOVA (ONL thickness ratio across time points), followed by Tukey's multiple comparison post-test and two-way ANOVA (eccentricity and superior-inferior analysis) and Sidak's multiple comparison post-test.

Immunohistochemistry

Sections that were adjacent to those used for TUNEL assay were selected for immunohistochemistry. Details of antibodies used are displayed in Table 3.1. Sections were incubated in 10% normal goat serum (Sigma Aldrich, Australia) for 1 hr at room temperature, followed by overnight incubation in primary antibody at 4°C. Antigen retrieval was performed only for antibody IBA1 and F4/80. With these antibodies, sections were incubated in Revealit-Ag Antigen Recovery Solution (ImmunoSolutions, QLD, Australia) for 1 hr at 37°C before the overnight incubation of the antibody. The sections were then washed in 0.1M PBS, and incubated with appropriate secondary antibody, AlexaFluor 488 or 594, for 4 hrs at room temperature. For F4/80, 4-HNE and 8OHG, biotinylated secondary antibodies were incubated for 2 hrs, followed by 1.5 hrs incubation of streptavidin-AlexaFluor 488 or 594 conjugates at room temperature. Sections were then stained with bisbenzimidazole to identify cellular layers, and coverslipped with Aqua-Poly/Mount (Polysciences, PA, USA). To control for non-specific binding, the primary antibody was omitted from some sections. Visualisation of immunofluorescence and image acquisition were performed using the Nikon A1 Confocal Microscope.

Table 3.1 List of Primary antibodies used for immunohistochemistry

Antibody	Dilution	Company	Cat #
Rabbit IBA1	1:500	Wako, Osaka, JP	019-19741
Rat F4/80	1:400	Abcam, Cambridge UK	Ab6640
Rabbit GFAP	1:500	Dako, Denmark	Z0334
Rabbit 4-HNE	1:200	Alpha Diagnostics, TX, USA	HNE11-S
Mouse 8-OHG	1:500	Alpha Diagnostics, TX, USA	8OHG11-M

Quantification of IBA1⁺ microglia

The number of IBA1⁺ cells was counted at 500 μ m intervals across the retinal cryosections for both the superior and inferior regions (n=6 per experimental group). The average IBA1⁺ cell counts for the superior and inferior regions were analysed with two-way ANOVA followed by Sidak's multiple comparison. The total IBA1⁺ cells from each retina was the average of two retinal sections. These were analysed with one-way ANOVA followed by Tukey's multiple comparison post-test.

Quantitative Real-time Polymerase Chain Reaction

Following purification of RNA, cDNA was synthesized using Tetro cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer's protocol. A 20 μ l reaction mixture was used in conjunction with 1 μ g RNA, 500 ng Oligo dT primer and 200U reverse transcriptase. Gene expression was measured using mouse specific Taqman probes (ThermoScientific, MA, USA), as shown in Table 3.2. The Taqman probes, cDNA and Taqman Gene Expression Mastermix (ThermoScientific), were plated in a 384-well transparent plate. The amplification of each sample was performed in technical duplicates, carried out using a QuantStudio 12K Flex RT-PCR machine (ThermoScientific). Relative fold change was expressed as a percentage change compared to the dim-reared control and normalised to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The statistical significance for each gene was determined using one-way ANOVA with Tukey's multiple comparison post-test (n=6 per experimental group). For each analysis, differences with a $p < 0.05$ were considered statistically significant.

Table 3.2 Taqman hydrolysis probes used for qPCR

Gene symbols	Gene Name	Catalog Number
<i>Ccl2</i>	Chemokine (C-C motif) ligand 2	Mm99999056_m1
<i>Ccl3</i>	Chemokine (C-C motif) ligand 3	Mm00441258_m1
<i>Ccl12</i>	Chemokine (C-C motif) ligand 12	Mm01617100_m1
<i>C3</i>	Complement C3	Mn00437858_m1
<i>Hmox-1</i>	Heme oxygenase 1	Mm01536933_m1
<i>Gapdh</i>	Glyceraldehyde-3-Phosphate Dehydrogenase	Mm01536933_m1

Measurement of retinal function using electroretinography

Full-field scotopic electroretinography (ERG) was performed on 12 hour dark-adapted animals to assess the retinal function in dim-reared control and 7 days light damaged animals. Briefly, mice were dark adapted overnight, anesthetized by intraperitoneal injection of xylazine (10mg/kg) and ketamine (100mg/kg) and the pupils dilated with a single drop of 1% atropine sulfate (8.3 mg of atropine). A single- or twin-flash paradigm was used to elicit mixed (rod and cone) or isolated cone responses, respectively. Flash stimuli for mixed responses were provided by an LED-based system (FS-250A Enhanced Ganzfeld, Photometric Solutions International, Melbourne), over a stimulus intensity range of $6.3 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$ (range $-4.4 - 1.9 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$). The interstimulus interval was increased from 2 s for the lowest intensities to 5 min for the highest intensities to allow complete recovery of the b-wave between stimuli. Isolated cone responses were obtained at $1.6 \log \text{cd} \cdot \text{s} \cdot \text{m}^{-2}$ following a rod-saturating stimulus of

1.9 log cd·s·m⁻² given 400 millisecond (ms) before the test stimulus. This short interval after a rod-saturating flash does not allow recovery of rod function, thereby revealing cone-only responses. The a-wave amplitude was measured from the baseline to the trough of the a-wave response; and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Data are expressed as the mean wave amplitude ± SEM (μV). Two-factor repeated measures analysis of variance was performed to compare the responses from control and light damaged mice over the flash stimulus range. The a-wave and b-wave data were fitted with a Naka-Rushton equation [$R/R_{\max} = I/(I + K)$] using the Solver function in Excel (Microsoft Version 2013) to determine R_{max} (maximum amplitude) and K (semisaturation constant) from the response amplitude (R) and the flash intensity (I) over the range of -4.4 to 1.9 log cd·s·m⁻². Statistical analyses were performed using Prism (GraphPad Software V5; GraphPad Software, Inc., La Jolla, CA, USA) and a two-way ANOVA for mixed a-wave and b-wave and students t-test for cone b-wave. For each analysis, differences with a $p < 0.05$ were considered statistically significant.

3.4 Results

Emergence of focal outer retinal atrophy and photoreceptor death following light exposure

The effect of light exposure on photoreceptor cell death was investigated using TUNEL assay (Figure 3.2). No apoptotic cell bodies were found in the ONL following 1 day light exposure (Figure 3.2B). At 3 days TUNEL⁺ labelling was observed only in the ONL. After 3 days of light exposure, a number of TUNEL⁺ cell nuclei were detected in the ONL (Figure 3.2C) with increasing numbers detected after 5 and 7 days of light exposure (Figure 3.2D,E). Both dim-reared control (Figure 3.2A) and negative control retinas (Figure 3.2F) showed no positive cell labelling in any retinal layers.

Superior-inferior regional analysis showed that TUNEL⁺ nuclei were predominantly localised in the superior region at 5 and 7 days ($p < 0.05$, Figure 3.3A). Their distribution in the superior region was focal, showing a significant increase in the number of TUNEL⁺ cell nuclei at approximately 0.5 mm eccentricity from the optic nerve ($p < 0.05$, Figure 3.3B). In addition, the number of TUNEL⁺ cells was detected to a distance of 2.0 mm in the superior retina ($p < 0.05$, Figure 3.3B); whereas, in the inferior retina there was less photoreceptor cell death distributed over a less extensive region (0.5-1.5 mm).

The cumulative effects of photoreceptor cell death were examined by measuring ONL thickness over the time course of the light damage (Figure 3.4A-E). After 1 and 3 days of light exposure, there were no significant changes in the ONL thickness (Figure 3.4A), but reduced ONL thickness was detected after 5 days, with a further reduction after 7 days. At both time points, the ONL was significantly thinner than the dim-reared control ($p < 0.05$, Figure 3.4A-C). Regional analysis demonstrated that ONL thickness in superior retina was significantly thinner than in inferior retina, after both 5 and 7 days exposure. After 5 days of light exposure, the ONL was thinnest at 0.6 mm eccentricity from the ON head; by 7 days, the area of ONL thinning was larger, extending up to 1.8 mm from the ON head ($p < 0.05$, Figure 3.4D-E). In inferior retina, there was no significant change in ONL thickness across the eccentricity points.

Quantification of TUNEL+ cells across the entire outer retina at different periods of light exposure (100k lux)

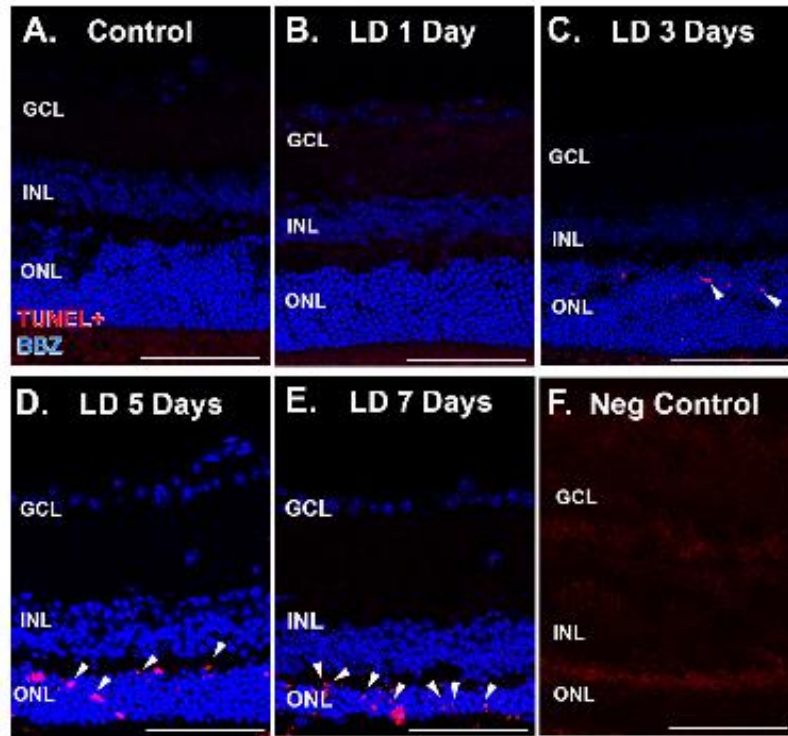
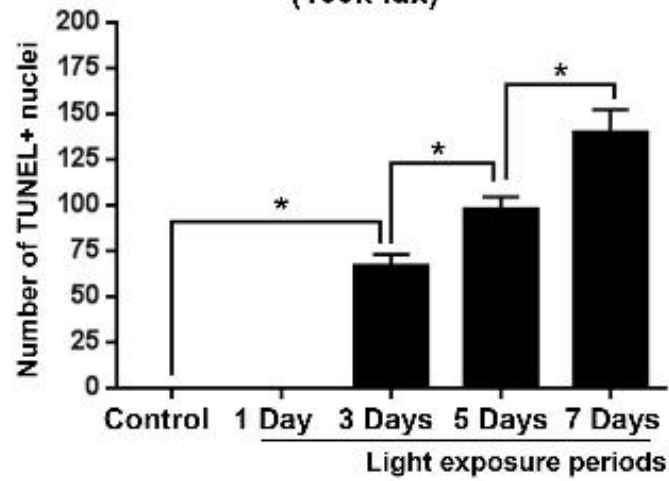
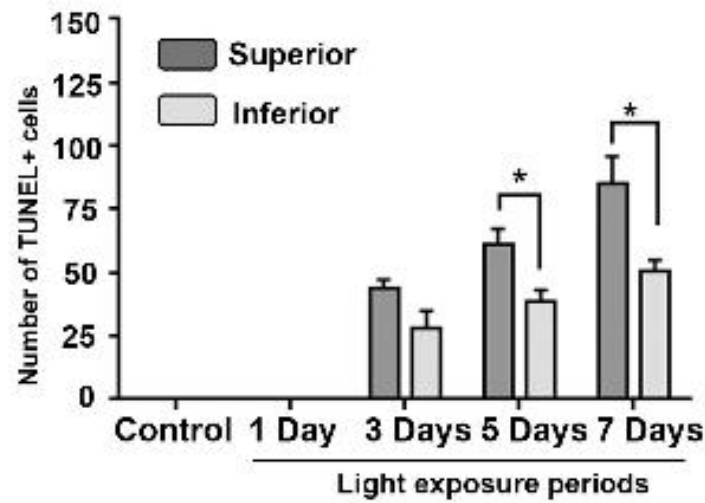


Figure 3.2 Localisation and quantification of light-induced photoreceptor cell death using TUNEL immunolabelling on retinal cryosections. From 3 days there was a significant increase in cell death compared to controls using one-way ANOVA (* denotes $p < 0.05$). Cell death began to increase at 3 days in the ONL, continued at 5 days and was maximal at 7 days (* denotes $p < 0.05$). **A-E**: Representative images were captured for all time points including dim-reared control. There was no TUNEL⁺ cells in the ONL at dim-reared and upon 1 day of light damage (A-B). TUNEL⁺ photoreceptor nuclei (red) appeared at 3 days and increased at 5 days and 7 days (C). Their distribution was sparse but focal at the ONL at 3 days, and became dense at 5 days and 7 days (arrows, C-E). **F**: Negative control confirmed that the TUNEL labelling was specific and not due to background staining. Scale bars represent 100 μ m. INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer.

A. TUNEL: Superior vs inferior



B. TUNEL: Whole length of retina

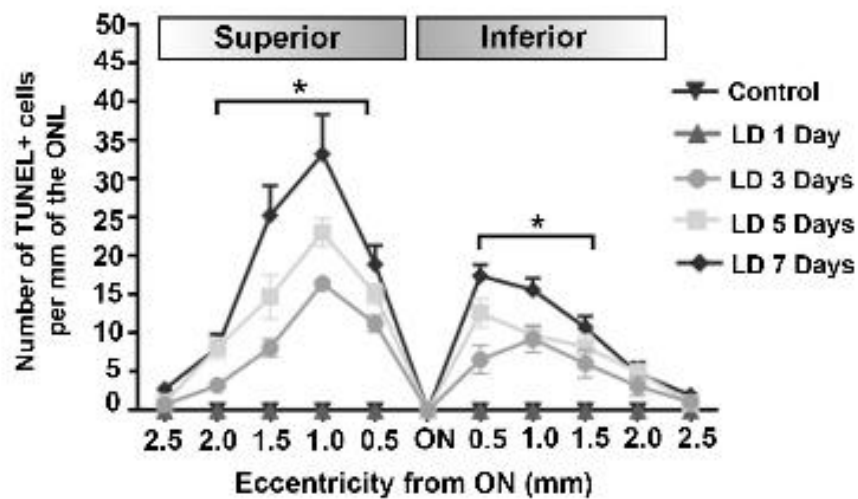


Figure 3.3 Distribution of photoreceptor cell death in the ONL following light exposure. **A:** The total TUNEL⁺ counts across the superior and inferior region of the retina showed that labelling was concentrated in the superior retina, with increasing severity at 5 days and 7 days (* denotes $p < 0.05$, $n = 6$ per group). **B:** TUNEL⁺ counts across the whole length of retina demonstrated a focal localisation of TUNEL⁺ nuclei at 0.5 mm-2.0 mm from ON head in the superior retina at 3 days, 5 days and 7 days. Inferior was only significantly different from control at 0.5 mm-1.5 mm. There was no change in the photoreceptor cell death at 1 day of light exposure (* denotes $p < 0.05$, $n = 6$ per group).

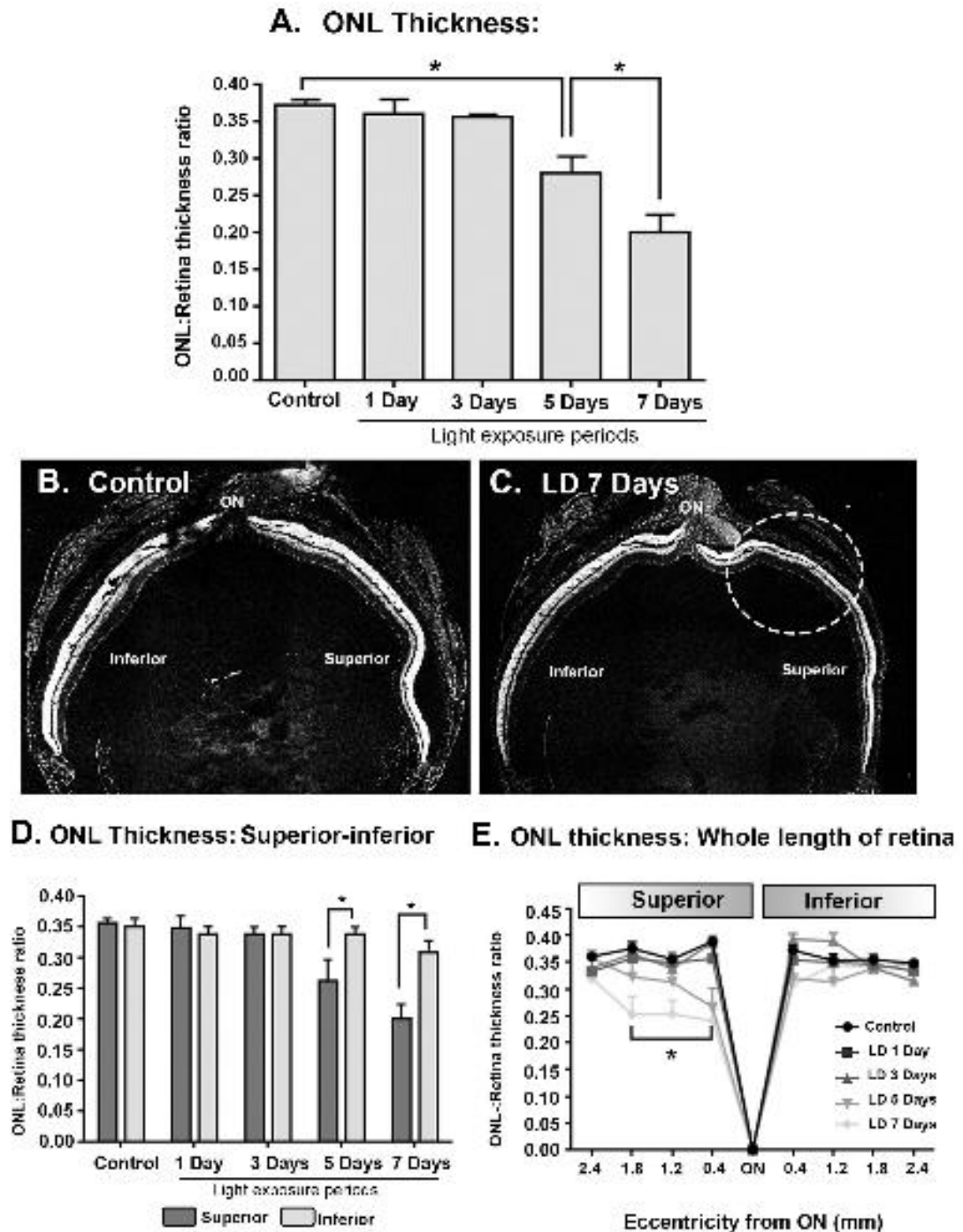


Figure 3.4 Effect of prolonged light exposure on the thickness of outer nuclear layer (ONL). **A:** The ONL thickness ratio was significantly lower at 5 days and 7 days compared to the dim reared control (* denotes $p < 0.05$). **B-C:** Representative retinal images showed a lesion site at 7 days of light exposure with a much thinner ONL thickness, compared to the retina of dim-reared control (white circle). **D:** Superior-inferior regional analysis of ONL:Retina thickness ratio showed a substantially reduced ONL thickness in the superior region than the inferior at 5 and 7 days of light exposure (* denotes $p < 0.05$, $n = 6$ per group). **E:** Eccentricity analysis of ONL:Retina thickness ratio across the length of retina demonstrated a significant reduction of ONL thickness occurred at 5 days, exclusively at 0.6mm superior to the ON head. The reduced ONL thickness persisted up to 1.8mm at 7 days indicating a superior specific progressive cell death (* denotes $p < 0.05$, $n = 6$ per group).

Measurements of retinal function using electroretinography

We used flash ERG to assess the functional consequences of bright light exposure (Figure 3.5). Representative traces from animals which had been dark-adapted for 12 hours, following 7 days light exposure, and from control animals show a clear decrease in response in light damaged animals (Figure 3.5A). Experimental light damaged mice showed a significant reduction in the a-wave response at flash intensities greater than $-0.4 \log \text{cd.s.m}^{-2}$ ($p < 0.0001$, Figure 3.5B) and of the b-wave response at flash intensities greater than $-1.1 \log \text{cd.s.m}^{-2}$ ($p < 0.0001$, Figure 3.5C). With the maximum stimulation intensity ($1.9 \log \text{cd.s.m}^{-2}$), the amplitudes of both a- and b-waves in light damaged mice were significantly reduced to 22.6% (Rmax Control 721.2 μV ; light damage 163.2 μV) and 24.3% (Rmax Control 1604.6 μV ; light damage 389.5 μV) of the values in controls, respectively. The semisaturation constant of the a-wave was $0.002 \log \text{cd.s.m}^{-2}$ for control and $0.39 \log \text{cd.s.m}^{-2}$ for light damage. The semisaturation constant of the b-wave was $-0.61 \log \text{cd.s.m}^{-2}$ for control and $-1.13 \log \text{cd.s.m}^{-2}$ for light damage. Using a twin-flash paradigm, we isolated the cone-derived waveform and found that cone-derived b-waves were significantly suppressed by light exposure to 30% of control values at $1.6 \log \text{cd.s.m}^{-2}$ (Figure 3.5D).

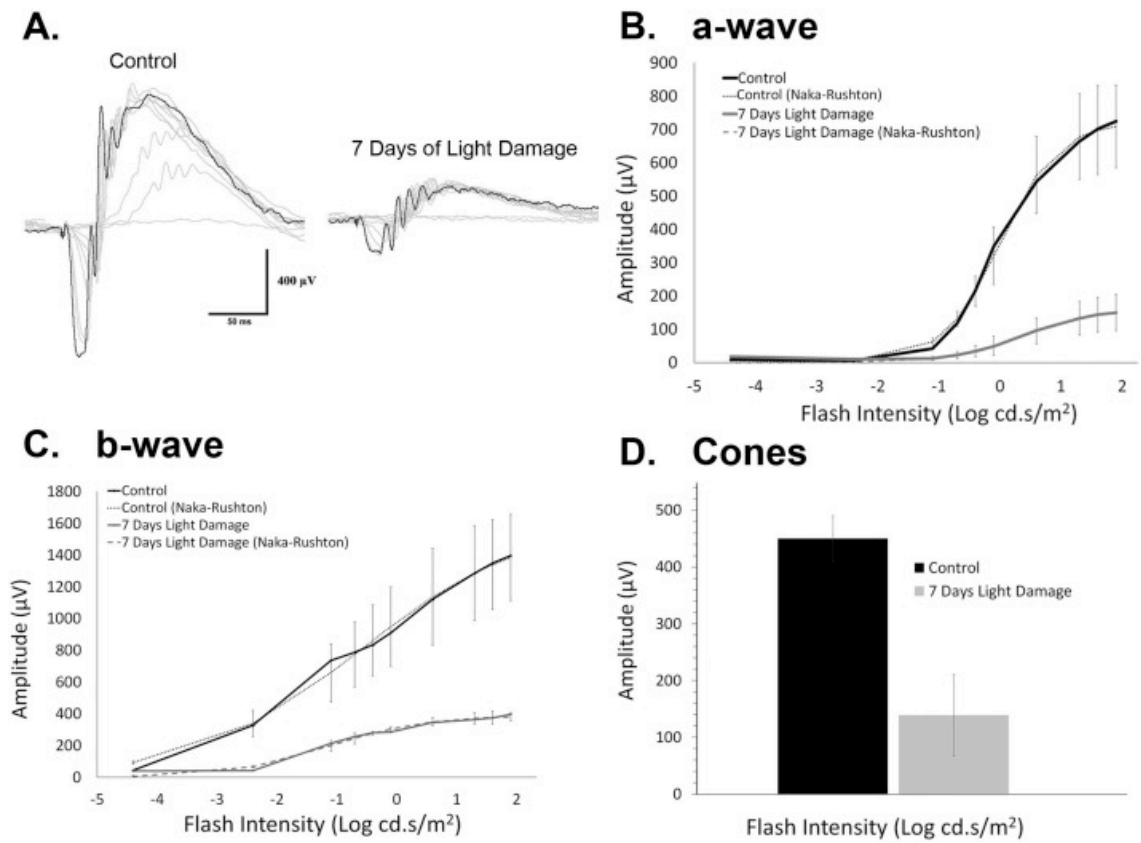


Figure 3.5 Full spectrum scotopic electroretinogram recordings of dark-adapted control and 7 days light damaged animals. **A:** Representative traces of control (dim-reared) and 7 days light damaged intensity-response series covering 6.3 log units in stimulus intensity (log cd.s/m²). **B-C:** Representative traces show reduced a-wave and b-wave which were quantified and fitted to a Naka-rushton curve across the intensity series. Both a-wave and b-wave amplitudes for the 7 day light damages animals were significantly different from dim-reared controls ($p < 0.0001$, two-way ANOVA, $n = 3$). **D:** Cone b-wave in 7 days light damaged animals at a flash intensity of 1.6 log cd.s/m² showed a significant difference from dim-reared control levels ($p < 0.01$, students t-test, $n = 3$).

Change immunoreactivity for the stress marker GFAP during light exposure

Faint immunoreactivity of GFAP was detected in the ganglion cell layer of the dim-reared control retina. Upon 1 day of light exposure, we observed a strong increase in GFAP immunoreactivity in the processes of the GCL (Figure 3.6B, arrows). A stronger GFAP pattern was seen at 3 days and continued until more processes were labelled GFAP⁺ at 5 and 7 days (Figure 3.6C-E, arrows).

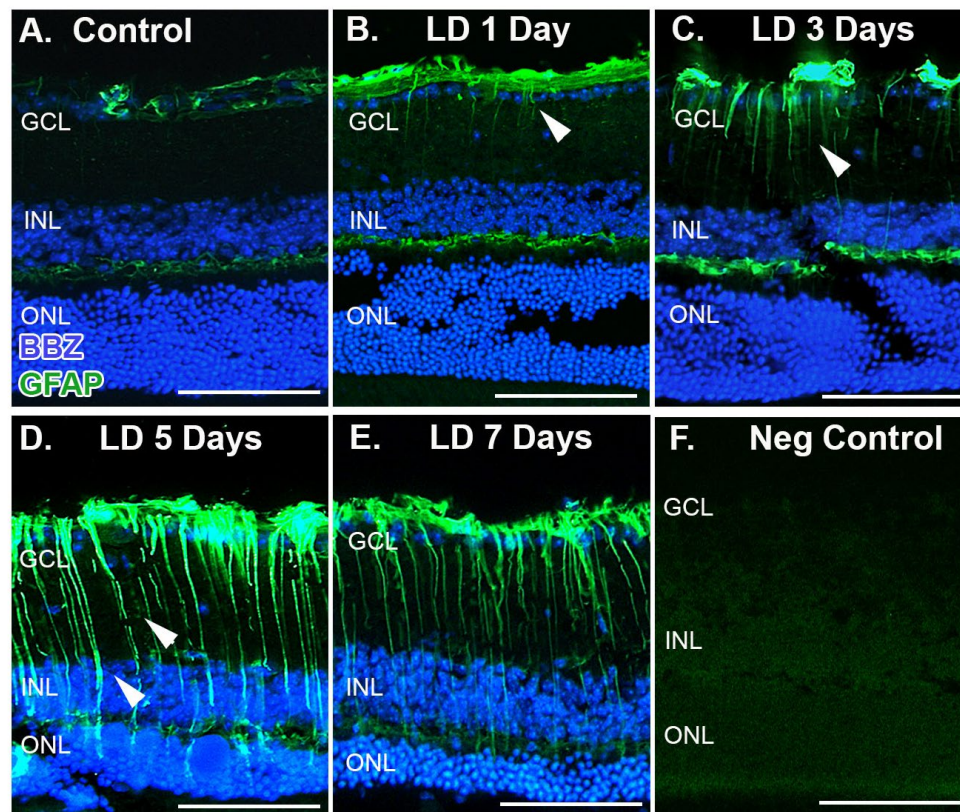
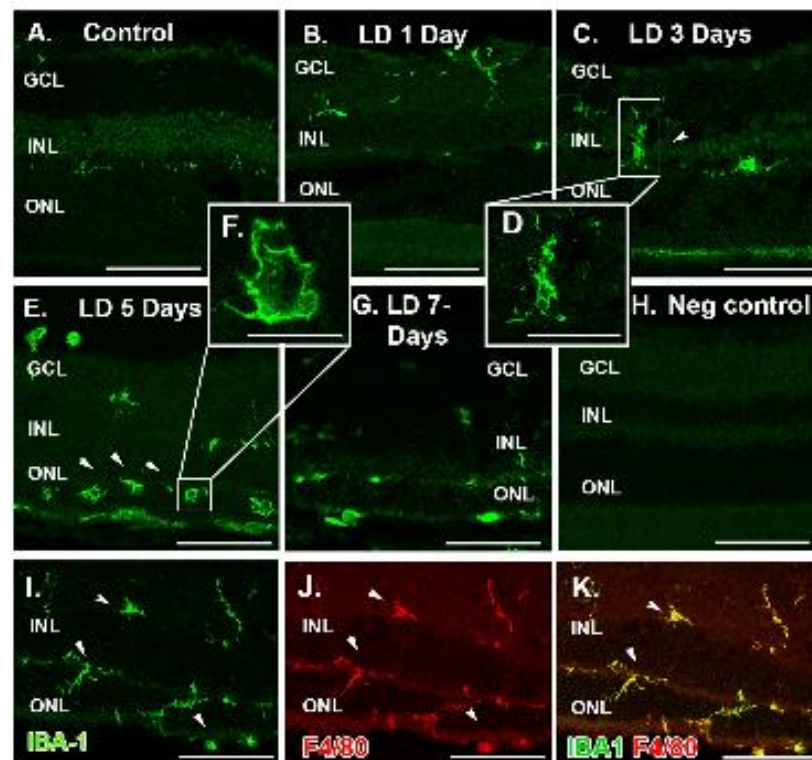


Figure 3.6 GFAP expression following continuous light exposure. **A-E:** Sections immunolabelled with GFAP (green). **F:** Negative control omitting the primary antibody served to confirm the positive immunoreactivity. Normal GFAP expression in the superior retina from a dim-reared animal (A). Immunoreactivity for GFAP was evident in Müller cell processes (arrows, B). GFAP immunoreactivity continued to increase (C). Intense GFAP immunoreactivity featured heavily across the entire retina (D-E). Scale bars represent 100µm.

Mobilisation of IBA1⁺ and F4/80⁺ microglial cells to the outer retina during light exposure

Recruitment of microglia/macrophages to the outer retina following light exposure was assessed using IBA1 immunolabelling (Figure 3.7A-H). In dim-reared controls, IBA1⁺ cells had a ramified morphology, and were localised to the inner and outer plexiform layers (Figure 3.7A). After 1 and 3 days of light exposure, the IBA1⁺ cells remained concentrated in the same retinal layers as the dim-reared control with no IBA1⁺ cells detected in the outer retina (Figure 3.7B-D). By 5 days, a substantial incursion of amoeboid IBA1⁺ cells into the photoreceptor layer and subretinal space was detected (Figure 3.7E-F, arrows). Amoeboid IBA1⁺ cells persisted in the outer retina throughout the light exposure time course (Figure 3.7G, arrows). Double labelling with antibody F4/80 confirmed the identity of IBA1⁺ cells as retinal microglia/macrophage (Figure 3.7I-K, arrows). Quantification of IBA1⁺ cells in outer retina was shown in Figure 3.7L. The number of IBA1⁺ cells present in the outer retina was significantly increased at 5 days of light exposure, compared to dim-reared control ($p < 0.05$) and remained significantly different from control levels at 7 days ($p < 0.05$, Figure 3.7L).

Comparison of IBA1⁺ cell numbers in superior and inferior retina showed that at 5 days of exposure the total numbers of IBA⁺ cells in superior retina were significantly higher than in inferior retina ($p < 0.05$, Figure 3.8A). Comparison of the distribution of recruited cells indicated that, compared to controls, the number of IBA1⁺ cells was substantially higher in superior retina between 0.5mm and 1.5 mm eccentricity, at 3, 5 and 7 days ($p < 0.05$, Figure 3.8B). In inferior retina the numbers of recruited IBA1⁺ cells were significantly different from controls between 0.5 and 1.0 mm, after 5 and 7 days of light exposure.



L. Quantification of IBA1-positive cells across the outer retina following light exposure

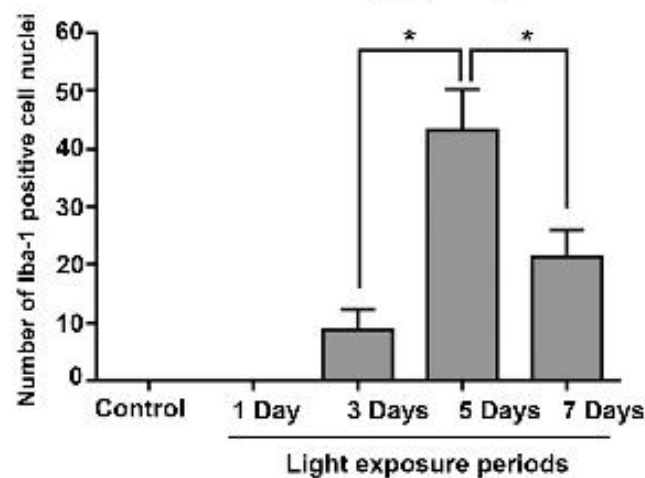
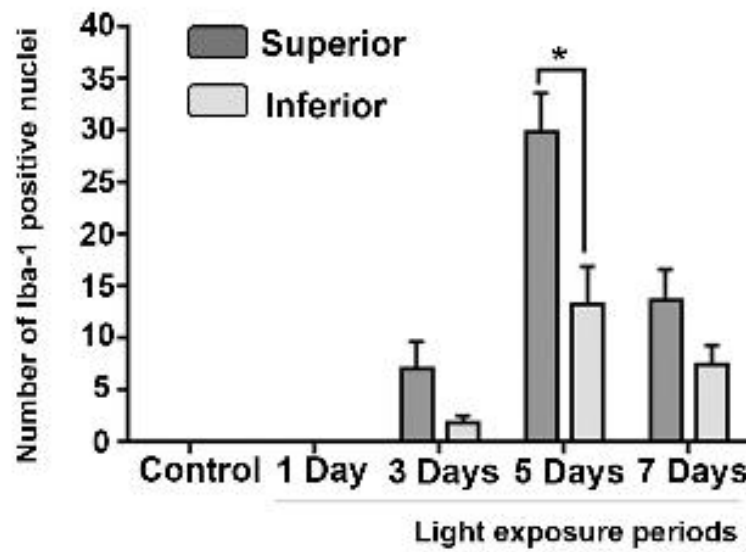


Figure 3.7 IBA1⁺ cells in light-exposed retina. **A:** In normal retinas, scattered IBA1⁺ ramified microglial cells were located in the outer plexiform layer and inner plexiform layer and ganglion cell layer, but not in the outer nuclear layer (ONL) or the subretinal space. **B-G:** Infiltration of IBA1⁺ microglial cells in the ONL was observed at 1 day, 3 days, 5 days and 7 days of light damage. Microglial cells began to migrate to the ONL at 3 days, with an elongated structure (arrow; C, D). By 5 days, these cells accumulated in the ONL with an amoeboid morphology (arrow; E, F), but decreased at 7 days of light damage with some still remaining in the subretinal space. **H:** Negative control confirmed that the staining was specific to IBA1. **I-J:** Co-localisation of IBA1⁺ microglial cells with F4/80 was identified using double labelled immunohistochemistry. **L:** The histogram was the quantitative analysis of IBA1⁺ microglial cells in the ONL, showing a significant increase at 5 days and 7 days of light exposure. (* denotes $p < 0.05$, $n = 6$ per group). The scale bar represents 100 μm in all images, except F and D where the scale bar represents 50 μm .

A. Superior-inferior analysis of IBA1-positive cells



B. Regional analysis of IBA1-positive cells

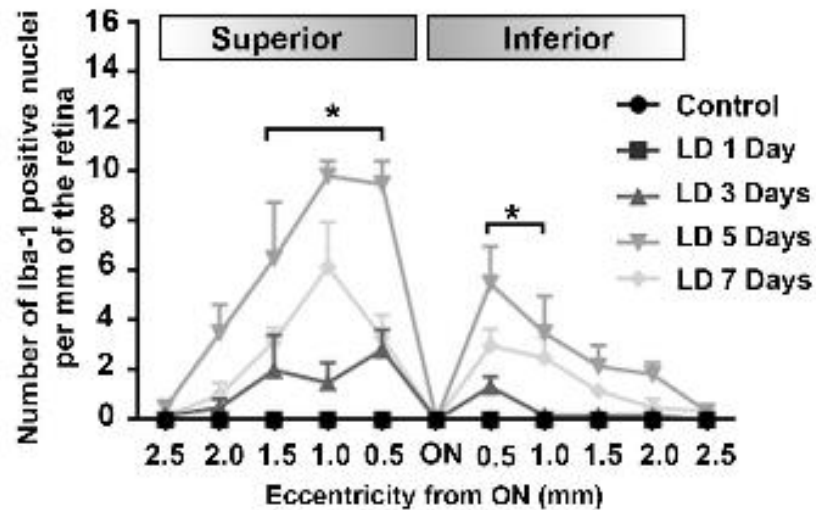


Figure 3.8 Regional analysis of microglial cells in light-exposed retinas. **A:** Superior-inferior regional analysis showed that the superior region of the ONL had more microglial cells than the inferior region (* denotes $p < 0.05$, $n = 6$ per group). **B:** In the superior retina, localisation of microglial cells was significantly different from dim-reared control in a region from 0.5 mm to 1.5 mm at all time points, except for 1 day. The inferior retina was only significantly different from the dim-reared control at 0.5 mm to 1.0 mm at 5 and 7 days ($p < 0.05$, $n = 6$ per group).

Changes in immunoreactivity for 4-HNE and 8-OHG oxidative stress markers during light exposure

Oxidative stress following light exposure was assessed using antibodies to 4-HNE and 8-OHG immunolabelling (Figure 3.9). The control retina showed faint immunoreactivity to 4-HNE in inner/outer segments (IS/OS) of photoreceptors (Figure 3.9A), which was markedly increased at 1 day of light exposure when strong labelling of both inner and outer segments as well as the nuclei of photoreceptors was detected (Figure 3.9B). Increased 4-HNE immunolabelling was detected in photoreceptor segments at both 5 and 7 days (Figure 3.9A-I). Immunoreactivity for 8-OHG was intense in the ganglion cell layer (GCL) following a peak after 1 day of exposure to light and gradually returned to below baseline levels after 7 days of exposure to light (Figure 3.9J-O). Whilst the majority of the 8-OHG staining was seen in the GCL, retinas which had been light-exposed for 1 and 3 days showed some evidence of 8-OHG immunoreactivity in the photoreceptor layer that was not seen at other time points.

Gene expression changes in inflammation and oxidative stress-related markers during light exposure

Marked increases in *Ccl2*, *Ccl3* and *Ccl12* mRNA expression were detected after 1 day of light exposure, and persisted throughout the light exposure period. Peak expression of each of these chemokines was detected after 1 day of light exposure ($p < 0.05$, Figure 3.10A-C).

A significant differential expression in *C3*, compared to dim-reared controls, was also detected over the protracted time course ($p < 0.05$, Figure 3.10D), with expression peaking after 5 days of light exposure ($p < 0.05$).

There was also a significant differential expression of *Hmox-1* over the protracted time course ($p < 0.05$, Figure 3.10E). *Hmox-1* was substantially elevated after 1 day of

light exposure, followed by a decrease in the expression level at 3 days and a steady increase up to 7 days.

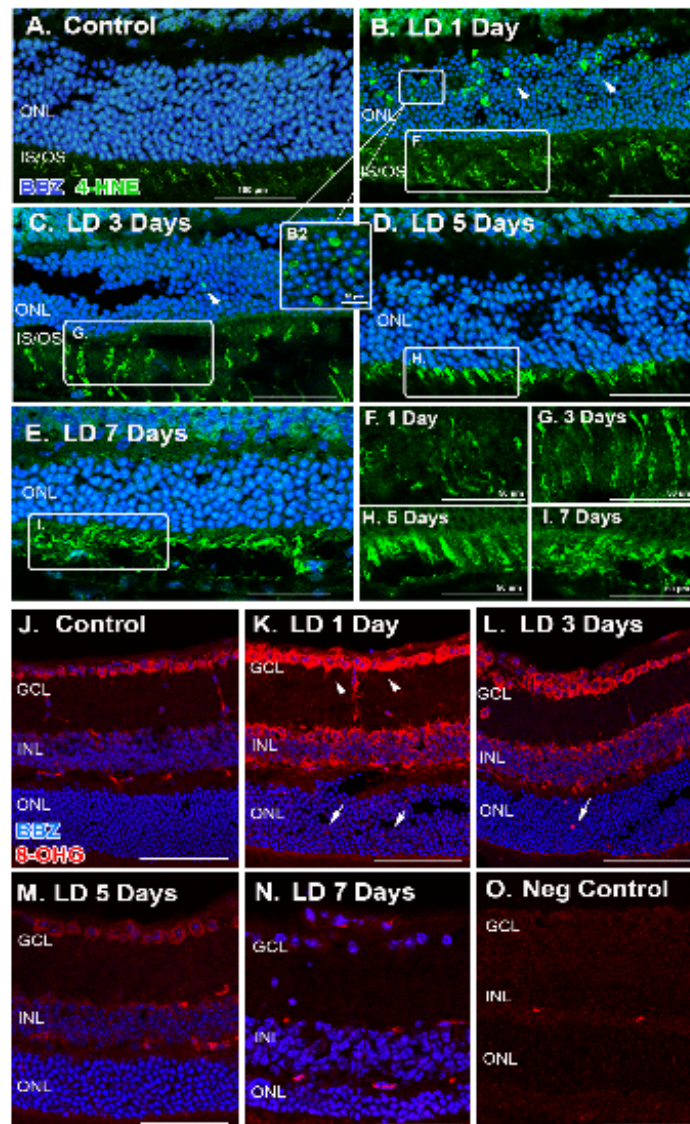


Figure 3.9 Immunoreactivity of oxidative stress markers, 4-HNE and 8-OHG during light exposure periods. **A:** Faint immunoreactivity of 4-HNE was seen in the inner and outer segments (IS/OS) of dim-reared control retinas. **B-C:** Intense 4-HNE immunoreactivity featured heavily in both the ONL and IS/OS upon 1 day of light exposure. 4-HNE immunoreactivity was found in the photoreceptor nuclei (arrows, B2). By 3 days, 4-HNE immunoreactivity was intense in the IS/OS, but reduced in the ONL (arrow). **D-E:** Strong immunoreactivity of 4-HNE persisted and accumulated in the subretinal space at 5 and 7 days continuous light exposure. **F-I:** Magnification of 4-HNE immunoreactivity in the subretinal region. **J:** Control retinas had faint 8-OHG labelling in the GCL. **K-L:** 8-OHG expression was intense at 1 day of light exposure in the GCL and persisted until 3 days. **M-N:** 8-OHG immunoreactivity was less at 5 days and almost diminished in 7 days of light exposure. **O:** Negative control confirmed positive staining seen at the GCL was not due to background. Scale bars represent 100 μ m (A-E, J-O) and 50 μ m (F-I).

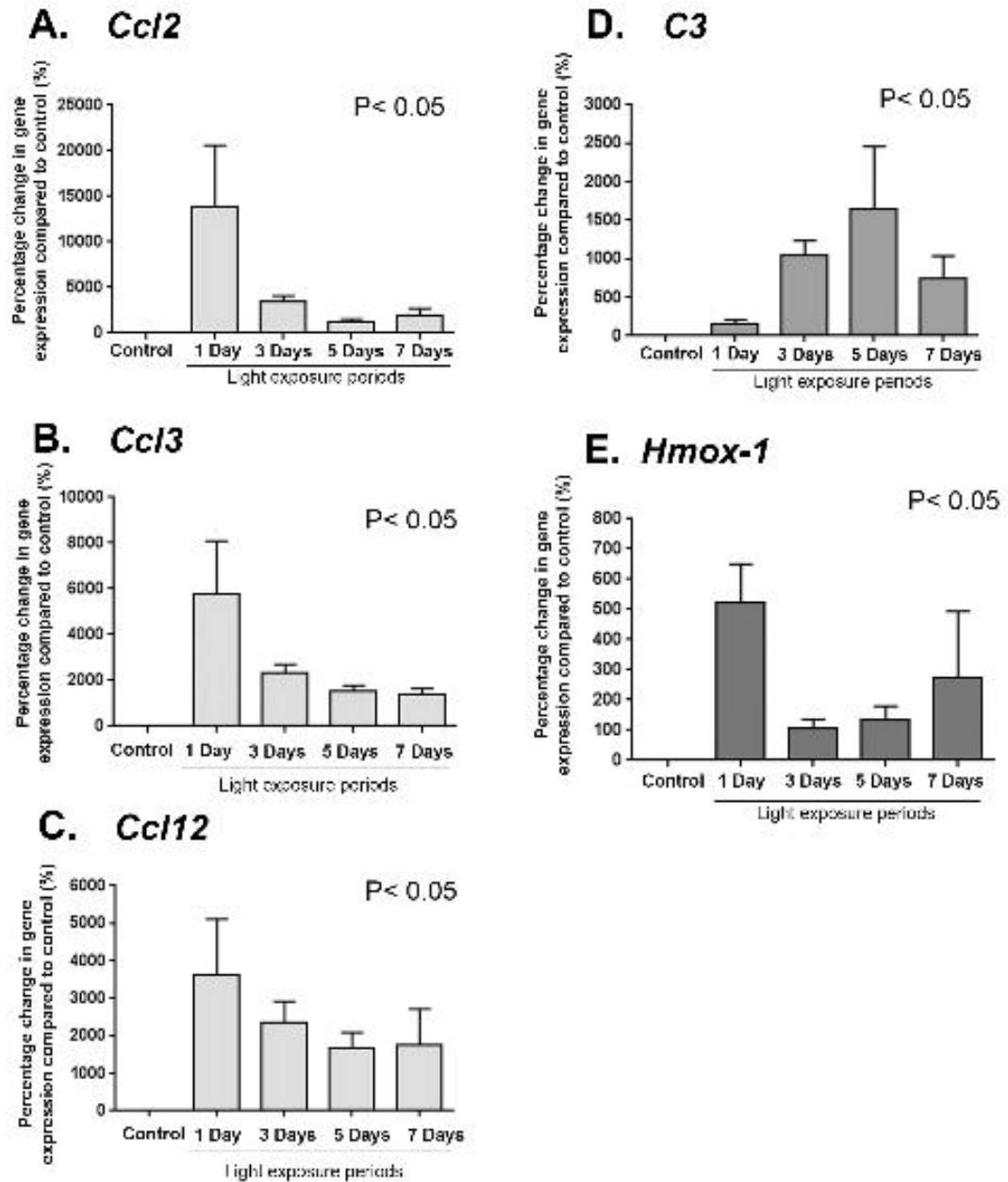


Figure 3.10 Gene expression in light-exposed mouse retinas. **A-E:** Retinas of each light exposure period were extracted and analysed by qPCR for changes in expression of chemokines (*Ccl3*, *Ccl2*, *Ccl12*), complement activation (*C3*) and oxidative stress (*Hmox-1*). Expression trends for all genes were significantly different compared to dim-reared control ($p < 0.05$, $n = 6$ per group). **A-C:** Chemokines, *Ccl2*, *Ccl3* and *Ccl12* showed a significantly peak expression at 1 day of light exposure. **D:** *C3* showed a significantly peak expression at 5 days compared to dim-reared control. **E:** *Hmox-1* showed an initial large increase at 1 day, decreasing at 3 days before gradually increasing up to 7 days ($p < 0.05$, $n = 6$ per group).

3.5 Discussion

Despite the known resistance of pigmented rodents such as C57BL/6J mice to light damage [274, 285, 322, 327, 328], here we define the parameters of a model for light damage in C57BL/6 mice, using LEDs. The outcomes over a 7 day period demonstrate induction of significant chronic outer retinal degeneration, reduced retinal function (mixed a- and b-waves and cone b-waves), and identify markers of oxidative damage and activation of the complement pathway. The changes we describe are comparable with those elicited in albino rat models, which also feature a focal lesion of degeneration and inflammation in superior retina [147, 293, 304, 327].

The key features of the model are photoreceptor and RPE degeneration, and atrophy of the adjoining choriocapillaris, in a localised area in the superior retina [293, 304, 313, 327, 329]. A difference between the albino models and the present one is that induction of a lesion in the C57BL/6J mice requires a 100-fold greater light intensity (100k lux as opposed to only 1000 lux) for more than twice the duration of exposure. The use of high-efficiency LED technology and efficient fans allows this intensity to be maintained without any change in ambient temperature in the light boxes, avoiding the potential of heat stress and its side effects [330, 331].

This is a significant advancement on previous models for pigmented rodents, which have yielded relatively small amounts of cell death with the use of incandescent or fluorescent light damage methodologies [322, 327, 328]. Furthermore, induction of light damage over a longer timeframe, using a chronic stimulus [332, 333] allows for better temporal separation of the features of light-induced degeneration.

We also describe significant indicators of oxidative stress and inflammation in pigmented rodents not characterised previously. Oxidative stress was evidenced by robust

increases in immunoreactivity for oxidative damage by-products 4-HNE and 8-OHG, and up-regulation of the oxidative stress response gene *Hmox-1*, which preceded the progressive onset of photoreceptor death. Inflammation was indicated by robust recruitment of both IBA1⁺ and F4/80⁺ microglia/macrophages to the ONL and subretinal space, coincident with increases in leukocyte-guiding chemokines *Ccl2*, *Ccl3*, and *Ccl12*, as well as the central complement component *C3*. The relatively late peak in *C3* expression (5 days) coincides with the peak of microglial / macrophage numbers in the retina, suggesting that these cells are a key influence on *C3* accumulation in the retina, as described in the albino rat model [146]. Our findings compare favourably with the spatiotemporal emergence of these similar oxidative stress [332-337] and inflammatory [145, 194, 248, 295] markers in light-damage studies conducted with albino rodents. These findings further support the use of our new model as an extension of these previous paradigms.

Conclusions

Models of light-induced retinal degeneration have been used extensively to study the roles of inflammation and oxidative stress, particularly in relation to human retinal diseases where such pathways are heavily implicated [3, 160, 210, 338]. While existing models using albino mice and rats have furthered our understanding of these factors, ongoing investigations using these paradigms are hindered by the inherent albinism. Our new model of chronic photo-oxidative degeneration in C57BL/6J mice overcomes a number of hurdles in existing models, including greater semblance to normal retinal physiology, chronic degeneration elicited via a sustained stimulus, and easier access to gene technology such as knockouts where the C57BL/6J strain is the predominant genetic background. Consequently, our new model has substantial value in furthering our understanding of the causative factors of photo-oxidative damage, as well as that of human dystrophies such as AMD.

4. Retinal macrophages synthesize C3 and activate complement in AMD and in models of focal retinal degeneration

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Haihan Jiao*, Riccardo Natoli*, Nilisha Fernando*, Tanja Racic, Michele Madigan, Nigel L Barnett, Joshua A Chu-Tan, Krisztina Valter, Jan Provis, Matt Rutar (2017) Retinal macrophages synthesize C3 and activate complement in AMD and in models of focal retinal degeneration, *Investigative Ophthalmology and Vision Science*. 58 (7): 2977-2990 (* denotes equal first authors)

The candidate's major contribution to this study as first author includes: conducting experiments, data acquisition and analysis for $C3^{-/-}$ mouse study, CVF and intravitreal C3 siRNA treatment, and drafting and revising the manuscript for publication.

4.1 Abstract

Purpose: Complement system dysregulation is strongly linked to the progression of age-related macular degeneration (AMD). Deposition of complement, including C3, within the lesions in atrophic AMD is thought to contribute to lesion growth, although the contribution of local cellular sources remains unclear. We investigated the role of retinal microglia and macrophages in complement activation within atrophic lesions, in AMD and in models of focal retinal degeneration.

Methods: Human AMD donor retinas were labelled for *C3* expression via *in situ* hybridisation. Rats were subjected to photo-oxidative damage, and lesion expansion was tracked over a 2-month period using Optical Coherence Tomography (OCT). Three strategies were used to determine the contribution of local and systemic C3 in mice: total *C3* genetic ablation, local *C3* inhibition using intravitreally-injected siRNA, and depletion of serum C3 using cobra venom factor.

Results: Retinal C3 was expressed by microglia/macrophages located in the outer retina in AMD eyes. In a rodent model of photo-oxidative damage, C3-expressing microglia/macrophages and complement activation were located in regions of lesion expansion in the outer retina over 2 months. Total genetic ablation of *C3* ameliorated degeneration and complement activation in retinas following damage, although systemic depletion of serum complement had no effect. In contrast, local suppression of *C3* expression using siRNA inhibited complement activation and deposition, and reduced cell death.

Conclusions: These findings implicate C3, produced locally by retinal microglia/macrophages, as contributing causally to retinal degeneration. Consequently,

this suggests that *C3*-targeted gene therapy may prove valuable in slowing the progression of AMD.

4.2 Introduction

The complement system comprises a cascade of proteins that are cleaved sequentially to initiate the destruction of a pathogen, foreign body, or cell debris [88, 97]. Prolonged cleavage of *C3* may lead to membrane attack complex (MAC) assembly and cytolysis [88, 97]. Complement activation is under tight control, including regulation by complement factor H (CFH), a critical inhibitor. However, in neurodegenerative diseases such as age-related macular degeneration (AMD), regulation of complement activation is compromised, contributing to disease onset and progression (reviewed in [1, 3, 339]).

AMD affects photoreceptors and the retinal pigment epithelium (RPE) of the macula, which mediates high acuity vision. Multiple factors contribute to AMD pathogenesis (reviewed in [50]), however, the Y402H mutation in *CFH* is the most highly associated risk factor [98, 125]. Other variants in *C3*, *CFB*, and *C2* genes are associated with AMD susceptibility (reviewed in [3]), with complement gene variants in the population estimated to account for ~70% of the risk for developing AMD [3].

Our poor understanding of the cellular events that initiate and feed complement activation in the retina has been a significant obstacle to the development of innovative approaches for AMD management [1]. Immunohistochemistry analyses have revealed that by-products of *C3* catabolism are deposited in affected areas of RPE/Bruch's membrane, including drusen deposits, from AMD patients [60, 98, 125]. This deposition, however, depends upon the availability of *C3* within the retinal microenvironment, and the sources of *C3* – systemic circulation or local retinal mediators – are unclear. Whole-transcriptome analyses of AMD donor eyes show that *C3* mRNA is significantly

increased in neural retina, but not in RPE/Choroid [138], suggesting that cells in the neural retina may be critically engaged in AMD pathogenesis.

In this study, we seek to identify the retinal modulators of complement in AMD and uncover their significance in the pathogenic activation of the cascade. We identify retinal macrophages as the local producer of C3 in both early and advanced forms of AMD. Using a model of outer-retinal degeneration and inflammation in rodents and Optical Coherence Tomography (OCT), we show that C3-expressing macrophages are associated with the expansion of atrophic lesions. Crucially, we demonstrate that intravitreal delivery of small interfering RNA (siRNA) suppresses this local expression of C3 and ameliorates retinal complement activation and degeneration. On the other hand, inhibition of serum-derived C3 has no effect on retinal complement levels or degeneration. Taken together, these findings inform our understanding of the role of complement in AMD, pinpointing macrophages as the key source of C3 that drives deleterious complement activation, and providing an indication for locally targeted gene therapy to suppress complement activation in retinal degeneration.

4.3 Additional Methods

Experimental animals

All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The study was approved by the Australian National University Animal Experimentation Ethics Committee (Application ID: 2014/56). Sprague-Dawley (SD) albino rats aged 120-150 post-natal days were used in this study. Additionally, C57BL/6J mice and C3-knockout mice ($C3^{-/-}$, 129S4-C3tm1Crr/J), both aged 60-80 days, were obtained from the Australian Phenomics Facility. For the $C3^{-/-}$ experiments, the C3 gene knockout mice were compared to isogenic C57BL/6J littermates. All animals were housed and maintained under standard laboratory

conditions in 12:12hrs light:dark cycle of 5lux (dim-reared). Age-matched dim-reared animals with no photo-oxidative damage were used as controls.

Photo-oxidative damage and tissue collection

SD rats were placed into transparent Perspex open-top boxes and exposed to 1000 lux white light for 24hrs (COLDF2, 2x36W, IHF, Thorn Lighting, Australia), with free access to food and water [293]. Following photo-oxidative damage, animals were euthanized with an overdose of barbiturate (60mg/kg Valabarb; Virbac, NSW, Australia) for tissue collection (0 days) or were held for 3, 7, 14 or 56 days under dim lighting (5 lux), then euthanized.

C57BL/6J and $C3^{-/-}$ mice were housed in Perspex boxes coated with a reflective interior, and exposed to 100K lux of natural white LED for 1, 3, 5 and 7 days, with free access to food and water [340]. Each animal was administered with pupil dilator eye-drops twice daily during photo-oxidative damage (Atropine Sulphate 1%w/v eye-drops; Bausch and Lomb, NSW, Australia). Following photo-oxidative damage, electroretinography (ERG) was used to measure mouse retinal function in response to full-field flash stimuli under scotopic conditions in dim-reared control and 7-day damaged mice as described previously [340]. Animals were euthanized with CO₂ prior to tissue collection. Eyes were collected and processed for cryosections or RNA extraction, as previously described in our publications [293, 340].

Optical Coherence Tomography

Optical Coherence Tomography (OCT) was conducted to assess the lesion progression in rodents after photo-oxidative damage. Cross-sectional and fundus images of live rat retinas were undertaken using a Spectralis™ HRA+OCT device (Heidelberg Engineering, Germany). Animals were anaesthetised with an intraperitoneal injection of Ketamine (100 mg/kg; Troy Laboratories, NSW, Australia), and Xylazil (12 mg/kg; Troy

Laboratories). Following anaesthesia, a pupil dilator was administered to both eyes (Tropicamide 0.5%w/v eye-drops; Bausch and Lomb).

Animals were restrained on a custom-made platform attached to the imaging device, adapted for rat eyes according to manufacturer's specifications. To maintain corneal hydration and improve OCT image quality to the manufacturer's minimum standard (25dB), hypromellose 0.3% eye-drops (GenTeal; Novartis, NSW, Australia) were administered and a rodent contact lens was placed on the eye (PMMA lenses, radius of curvature of the central optic zone of 2.70mm and diameter of 5.20mm; Cantor + Nissel, UK) [341]. Fundus and cross-sectional images were taken from 0-3mm superior to the optic nerve (ON), and 1-2mm inferior to the ON. Eye gel (GenTeal; Novartis) was administered to both eyes for recovery.

Treatment of mice with cobra venom factor

To achieve systemic complement depletion, cobra venom factor (CVF) (Quidel, CA, USA) was dissolved in endotoxin-free PBS to a final concentration of 0.1µg/µl. C57BL/6J mice were administered with 25µg CVF intraperitoneally prior to 7 days of photo-oxidative damage, and on days 3 and 5 during damage to sustain complement depletion. To confirm an inhibition of complement activity, peripheral blood was collected at 7 days for a haemolytic assay. The serum of CVF-treated mice was extracted and incubated with sheep red blood cells (Applied Biological Products Management, SA, Australia), pre-sensitised with haemolysin (Sigma-Aldrich, MO, USA), as described previously [309]. The absorbance at 540nm was read using an Infinite-200-PRO plate spectrometer (Tecan, Männedorf, Switzerland) and the percentage of haemolysis was calculated and compared to serum from PBS-injected mice as a positive control, and compared to *C3^{-/-}* serum as a negative control.

Intravitreal injections of siRNA

In vivo RNA interference was performed using C3 siRNA (s63165; Thermo Fisher Scientific, MA, USA) and negative control siRNA (12935300, Stealth RNAi Med GC; Thermo Fisher Scientific), which were encapsulated using a cationic liposome-based formulation (InvivoFectamine 3.0 Reagent; Thermo Fisher Scientific) as per the manufacturer's instructions. To purify and increase the concentration of the siRNA formulation to a final concentration of 0.3µg/µl in endotoxin-free PBS, the samples were spun at 4000g through an Amicon Ultra-4 Centrifugal Filter Unit (Merck Millipore, MA, USA). Intravitreal injections were performed as described in our previous publication [249]; 1µl of the C3 siRNA or negative control siRNA formulation was injected into both eyes of each C57BL/6J mouse on day 4 of photo-oxidative damage. Animals were recovered, and then returned to photo-oxidative damage for the remainder of the 7-day time-course. Mouse livers were collected 3 days after the siRNA injections for analysis of systemic C3.

Immunohistochemistry and quantification of ONL thickness and microglia/macrophages in cryosections

All histological and immunofluorescent analyses utilised retinal cryosections that were cut along the para-sagittal plane (supero-inferior), described previously [293, 340]. These sections also included optic nerve head, in order to maintain regional consistency between replicates and groups.

To detect and localise specific proteins in the retina, immunohistochemistry was performed using primary antibodies (Table 4.1) as described in our previous protocols with minor modifications [146, 194]. Mouse cryosections were immunolabelled with the α -C3 antibody instead of α -C3d, as it achieved more optimal staining. Fluorescence in retinal sections was visualised under a laser-scanning A1⁺ confocal microscope (Nikon,

Tokyo, Japan), and images were acquired using the NIS-Elements AR software (Nikon). Images were processed using Photoshop CS6 software (Adobe Systems, CA, USA). Immunolabelled IBA1⁺ cells in the outer retina (ONL-RPE) were counted across each whole retinal section and then averaged for each group, consistent with our prior methods [340, 342]. For quantification, only the cell bodies of the IBA1⁺ cells were counted so as to prevent the occasional ambiguity caused by IBA1⁺ processes skewing the dataset.

To assess ONL thinning on cryosections following photo-oxidative damage, the sections were stained with Toluidine Blue and then quantified in accordance with our previous methodology [342]. Briefly, the number of rows of photoreceptor nuclei were quantified within the lesion of the superior retina, and counts were subsequently averaged for each experimental group.

Table 4.1 Primary antibodies used for immunohistochemistry

Primary Antibody	Dilution	Catalogue Number	Company
Rabbit α -IBA1	1:500	019-19741	Wako, Osaka, Japan
Goat α -C3d	1:100	AF2655	R&D Systems, MN, USA
Rabbit α -C3	1:50	AB118877	Abcam, Cambridge, UK
Rabbit α -Rhodopsin	1:500	AB9279	Merck Millipore, MA, USA
Rabbit α -GFAP	1:500	Z0334	Dako, Glostrup, Denmark

***In situ* hybridisation on human and rat retinas**

To localise *C3* mRNA transcripts in retinal cryosections, *C3* was cloned from PCR products derived from human (460bp amplicon) and rat (483bp amplicon) retinal cDNA. These cloned templates were then synthesised into a digoxigenin (DIG)-labelled riboprobe that was specific to human or rat *C3* mRNA, according to our published methodology [146].

In situ hybridisation was performed on cryosections from either human AMD donor tissue or rat retinas subjected to photo-oxidative damage. AMD tissues had been extensively categorised and processed for cryosectioning in an earlier investigation [343]. In brief, human eyes were collected with informed consent through the Lions NSW Eye Bank, Sydney, Australia, and ethical approval from the Human Research Ethics Committee of the University of Sydney and The Australian National University. Grading for the eyes was performed by a team of experienced graders according to published pathological criteria [344] and ranged from normal to early- or late-AMD.

In situ hybridisation was conducted using our established protocol [345]. Both rat and human *C3* riboprobes were hybridised overnight at 57°C and then washed in saline sodium citrate (pH 7.4) at 60°C. The bound probe was visualised with NBT/BCIP and sections were double-labelled using IBA1 immunohistochemistry.

Quantitative real-time polymerase chain reaction (qPCR)

RNA extraction and purification was performed on retinas using a combination of TRIzol reagent (Thermo Fisher Scientific) and an RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific) as described in our previous publication [346]. cDNA was prepared from 500ng of each RNA sample using a Tetro cDNA Synthesis Kit (Bioline Reagents, London, UK) according to the manufacturer's protocol.

Gene expression changes were measured via qPCR using Taqman hydrolysis probes (Table 4.2) and Taqman Gene Expression Master Mix (Thermo Fisher Scientific). Each qPCR was run using a QuantStudio 12K Flex instrument (Applied Biosystems) at the Biomolecular Resource Facility (JCSMR, ANU). Analysis was performed using the comparative cycle threshold method ($\Delta\Delta C_t$) which was normalised to the expression of *Gapdh* and *Actb* reference genes, as established previously [154, 248].

Table 4.2 Taqman hydrolysis probes used for qPCR

Gene Symbol	Gene Name	Catalogue Number	Entrez Gene ID
<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	Rn99999916_s1	24383
		Mm99999915_g1	14433
<i>Actb</i>	Actin, beta	Mm01205647_g1	11461
<i>C1s</i>	Complement component 1, s subcomponent	Rn00594278_m1	192262
		Mm01625167_g1	50908
<i>C2</i>	Complement component 2	Rn00597176_m1	24231
		Mm00442726_m1	12263
<i>C3</i>	Complement component 3	Mm00437858_m1	12266
<i>C4a</i>	Complement component 4A (Rodgers blood group)	Rn00709527_m1	24233
		Mm01132415_g1	625018
<i>Cfb</i>	Complement factor B	Rn01526084_g1	294257
		Mm00433918_g1	14962
<i>Cfd</i>	Complement factor D (adipsin)	Rn01535436_g1	54249
<i>Cfh</i>	Complement factor H	Rn00590326_m1	155012
		Mm01299248_m1	12628
<i>Cfi</i>	Complement factor I	Rn00577333_m1	79126
<i>Serping1</i>	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	Rn01485600_m1	295703
<i>Cntf</i>	Ciliary neurotrophic factor	Mm00446373_m1	12803
<i>Fgf2</i>	Fibroblast growth factor 2	Mm01285715_m1	14173
<i>Rho</i>	Rhodopsin	Mm00520345_m1	212541

OCT analysis

OCT and fundus images were analysed using ImageJ software (NIH, MD, USA). Cross-sectional retinal images were analysed per mm in duplicate, with a minimum of 5 measurements of individual retinal layers taken manually per image. ONL thickness ratios were calculated as the ONL thickness relative to the distance between the outer and inner limiting membranes. Fundus images detecting a lesion (darker area correlated with ONL thinning in OCT scans) were uniformly treated by adjusting the contrast to a set threshold to enhance lesion visibility. ImageJ was used to measure the lesion size manually (arbitrary units). The ONL thickness ratio was calculated at the lesion centre, in comparison to the adjacent left and right sides.

Statistics

All graphing and statistical analyses were performed using Prism 6 (GraphPad Software, CA, USA). Significant trends in time-course datasets were ascertained using the one-way or two-way analysis of variance (ANOVA) to determine statistical significance ($P < 0.05$). Sidak's or Tukey's post-hoc tests were applied where multiple statistical comparisons were desired, and Fisher's uncorrected LSD was used for instances of single comparisons. Student's t-test was utilised for other single comparisons, where noted.

4.4 Results

Localisation of C3 mRNA in human donor retinas: A role for macrophages

We investigated the localisation of *C3* mRNA in normal aged human donor retinas and age-matched AMD-affected retinas using *in situ* hybridisation (Figure 4.1). We detected minimal *C3* mRNA expression in normal retinas, and little-to-none within RPE cells or choroid (Figure 4.1A). However, in early AMD retinas, *C3*-expressing cells were detected in the nerve fibre layer (NFL), superficial retinal vasculature, and subretinal space in regions adjacent to RPE disturbance (Figure 4.1B-D). We detected numerous *C3*-expressing cells in regions of advanced scarring (Figure 4.1E-F), at lesion edges (Figure 4.1G-I), and near the optic nerve head (Figure 4.1J). In contrast, barely any labelling was detected in the choroid of these lesion areas (Figure 4.1E). Counter immunolabelling indicated that most of the *C3*-expressing cells were immunopositive for the macrophage/microglia marker IBA1 (Figure 4.1K-N).

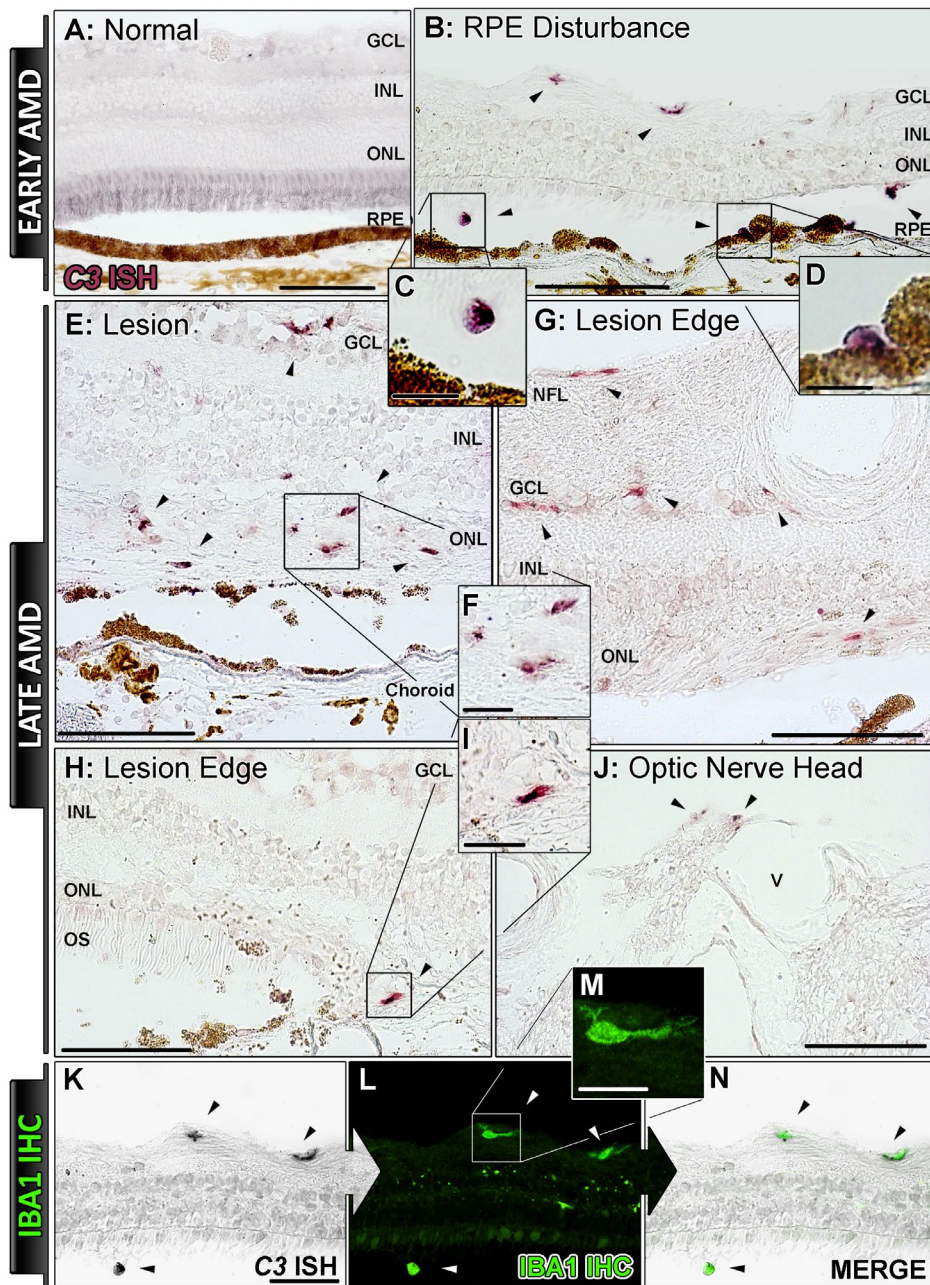


Figure 4.1 C3 expression in human AMD retinas. **A:** No C3-expressing cells were detected in the normal retina. **B-D:** Subretinal C3-expressing cells were present in early AMD retinas exhibiting RPE disturbance. C3 mRNA expression was detected near the inner retinal vasculature. **E-J:** In late AMD retinas, C3-expressing cells were detected within the lesion (E-F), at the lesion edges (G-I), and at the optic nerve head (J). **K-N:** These C3-expressing cells were identified as IBA1⁺ microglia/macrophages. Representative images derived from N=2-3 per group. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; NFL, nerve fibre layer; OS, outer segments; V, vasculature. Scale bars represent 100µm in A,B,E,G,H,J; 10µm in N,P,S-U; 50µm in K; 10µm in C,D,F,I,M.

Complement activation and macrophage recruitment within rat retinal lesions

Progression of lesion expansion in rat retinas following photo-oxidative damage was tracked *in vivo* using Optical Coherence Tomography (OCT) at 0, 3, 7, 14, and 56 days (Figure 4.2). Measurements of outer nuclear layer (ONL) thickness were taken from cross-sectional images along 3 horizontal transects in the superior retina (Figure 4.2A), and 2 in the inferior retina. Graphical representation of those measurements showed a gradual ONL thinning in the superior retina between 0 and 56 days, which was most prominent at 2-3mm superior to the optic nerve head (Figure 4.2B). The data showed a significant lesion detectable by 7 days ($P<0.05$, Figure 4.2B,D), which continued to develop further over the time-course. The thinnest ONL was detected in the 2-3mm transect at 56 days ($P<0.05$, Figure 4.2B,F). As the area of thinning increased over time, the lesion size doubled from 14 to 56 days ($P<0.05$, Figure 4.2G).

Expression of a suite of complement components (*C1s*, *C2*, *C3* and *C4a*) and regulators (*Cfb*, *Cfd*, *Serping1*, *Cfh*, *Cfi*) was analysed across the time-course in whole rat retinas (Figure 4.3). All complement genes showed a significant trend in upregulation across the time-course ($P<0.05$), with *C1s*, *C3* and *C4a* remaining significantly increased at 56 days post-damage, compared to dim-reared controls ($P<0.05$, Figure 4.3B). While the complement regulators *Cfb*, *Cfd*, *Serping1*, *Cfh* and *Cfi* were all highly up-regulated early in the time-course (0-7 Days, Figure 4.3C), none were found to show any significant change by 56 days, compared to controls (Figure 4.3D, $P>0.05$).

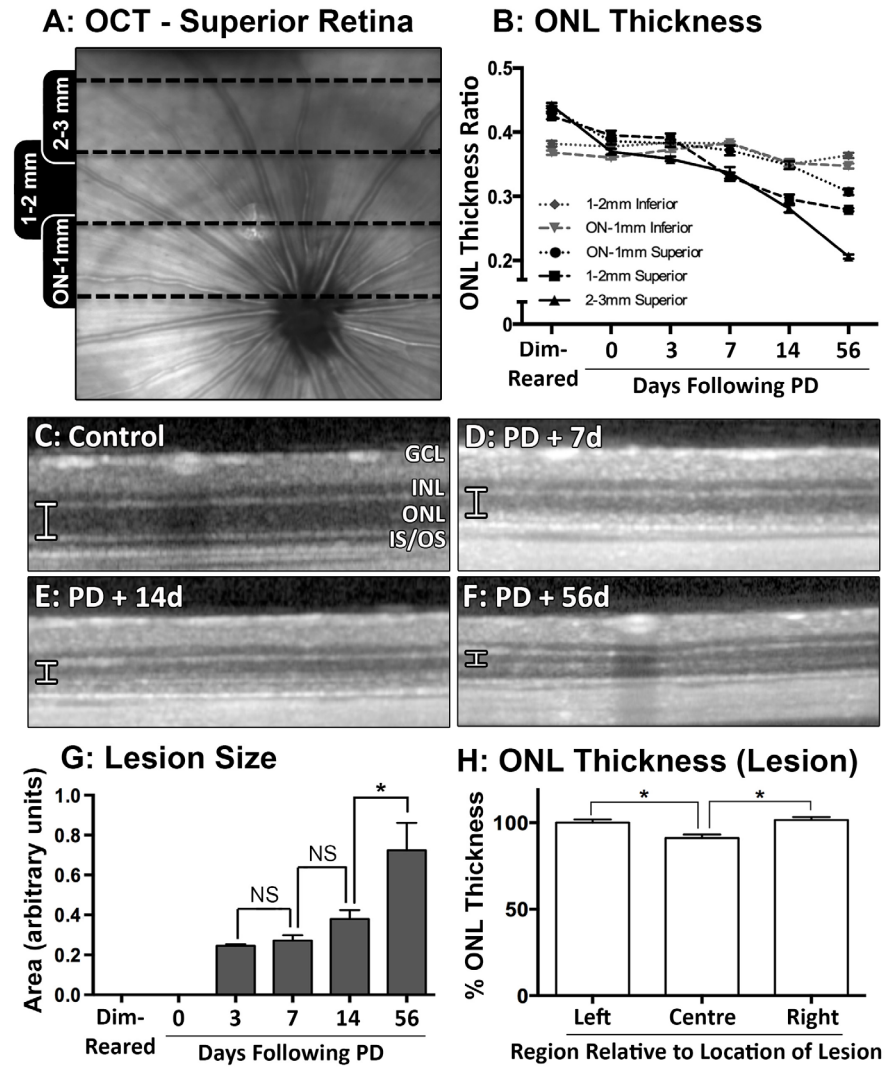


Figure 4.2 Outer nuclear layer (ONL) thickness ratio changes following photo-oxidative damage (PD) in rats. **A:** The regions of the superior retina imaged using OCT were from the optic nerve (ON) to 1mm, 1-2mm, and 2-3mm above the optic nerve. **B:** The ONL thickness ratio from ON-1mm (superior) and ON-2mm (inferior) showed minimal change over the time-course. The trend indicated a decrease in ONL thickness at 1-3mm superior from 0 days onwards ($P < 0.05$). A significant decrease in ONL thickness was observed at 2-3mm (superior) up to 56 days ($P < 0.05$). **C-F:** Representative OCT images of the control (C), in comparison to days 7-56 (D-F) at 2-3mm (superior) illustrate substantial ONL thinning over the time-course. **G:** The area (arbitrary units) of the retinal lesion as observed on fundus images from days 3-56 was quantified using ImageJ. A gradual increase in area over time was observed, which was significant from 14 to 56 days ($P < 0.05$). **H:** Specifically, the ONL thickness significantly decreased within the lesion area, in comparison to the areas adjacent to the lesion. Statistical analysis was determined using one-way ANOVA with Tukey's post-hoc test ($P < 0.05$). $N = 4$ per group.

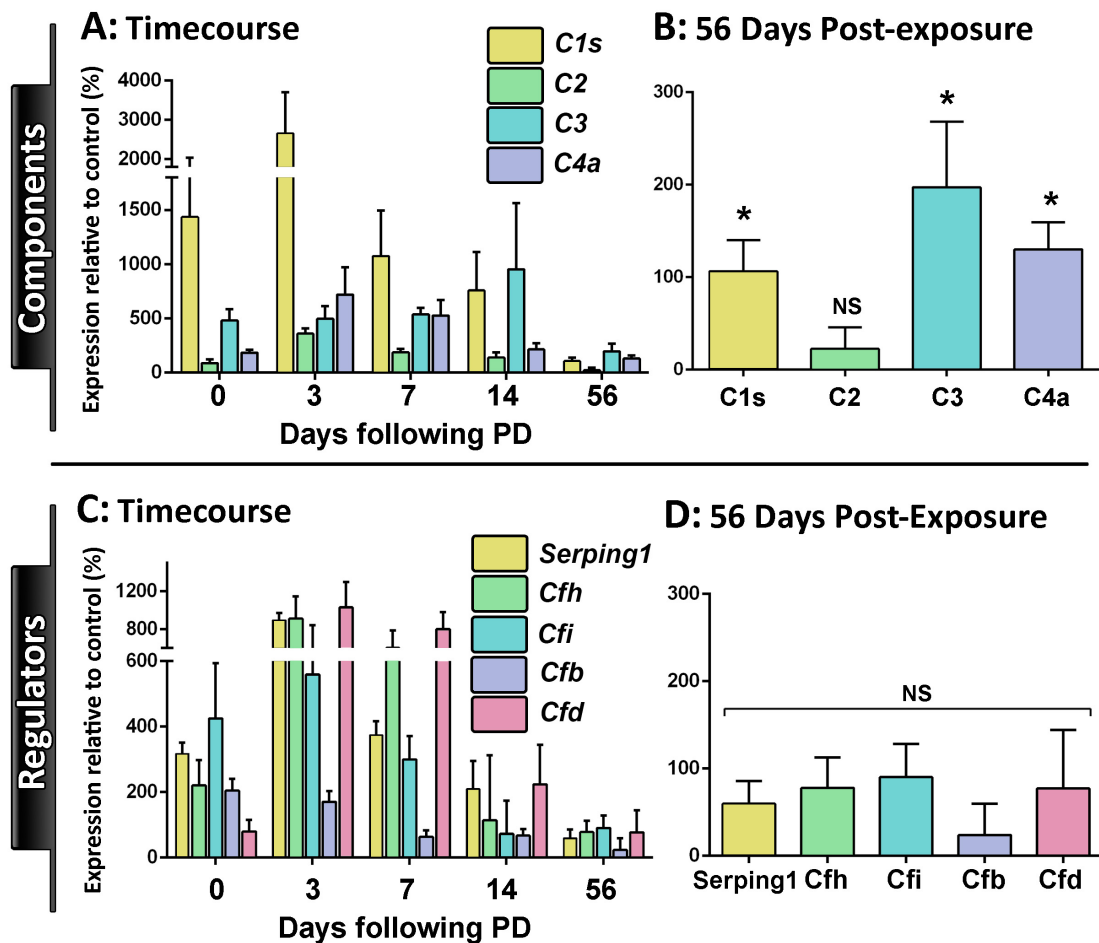


Figure 4.3 Complement gene expression in rat retinas following photo-oxidative damage (PD). All genes investigated were upregulated until 56 days post-damage compared to dim-reared controls. **A:** Complement components *C1s*, *C2* and *C4a* reached peak expression at 3 days. Expression of *C3* was highest at 0-7 days. **B:** *C1s*, *C3* and *C4a* were still significantly increased at 56 days compared to controls ($P < 0.05$). **C:** Expression of the complement regulator gene *Cfb* was highest at 0 days, whereas *Cfd* expression peaked at 3 days. *Serping1*, *Cfh*, *Cfi* expression all peaked at 3 days. **D:** *Serping1*, *Cfh*, *Cfi*, *Cfb*, and *Cfd* showed no statistically significant change after 56 days post-exposure ($P > 0.05$). The trend in expression was determined using one-way ANOVA (A,C, $P < 0.05$), and Student's t-tests was used to compare controls and 56 days (B-D). $N = 4$ per group.

Following photo-oxidative damage, there was an incursion of IBA1⁺ microglia and macrophages into the ONL and subretinal space (Figure 4.4). This persisted within the lesion for up to 56 days (Figure 4.4A). Counts of IBA1⁺ cells in the ONL and subretinal space showed a ~7-fold increase between 0 and 7 days (Figure 4.4A,C), which decreased to approximately half the number by 14 days, but remained significantly higher than control animals in the outer retina at 56 days ($P < 0.05$, Figure 4.4A,D-E).

In situ hybridisation (ISH) was performed to localise expression of *C3* mRNA at 7, 14 and 56 days post-exposure and the results were consistent with previous findings in this model at 7 days [146], showing *C3* mRNA localisation in the neural retina and subretinal space (Figure 4.5A-F). We detected accumulations of *C3*-expressing macrophages predominantly clustered amongst the remnants of the ONL and at the lesion edges, and near the inner retinal vasculature for up to 56 days (Figure 4.5A-F). Co-localisation with IBA1⁺ immunolabelling (Figure 4.5G-I) suggests the identity of these *C3*-expressing cells as macrophages. We did not observe *C3* expression by other cell types in retina, including RPE cells, and this was consistent with our previous reports [146, 154].

We used immunoreactivity for C3d, a by-product of C3 cleavage, to visualise the distribution of C3 following photo-oxidative damage (Figure 4.5J-V). C3d labelling was absent in dim-reared controls (Figure 4.5J). However, C3d immunoreactive deposits were prominent at 7 and 14 days post-damage in the ONL, amongst the photoreceptor segments and in the subretinal space, particularly at the lesion edges (Figure 4.5K, L). These features persisted for up to 56 days (Figure 4.5M-P). Double immunolabelling for IBA1 and C3d showed a close association between macrophages and C3d deposits, situated within the lesion (Figure 4.5Q-V).

A: Migration of IBA1+ cells

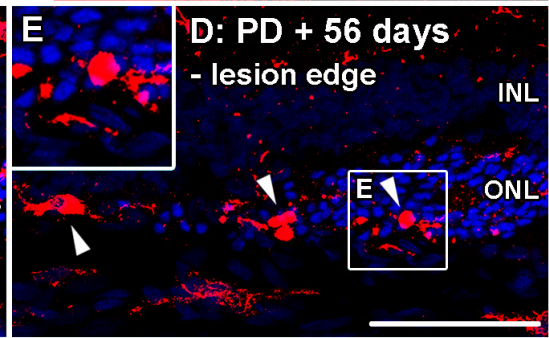
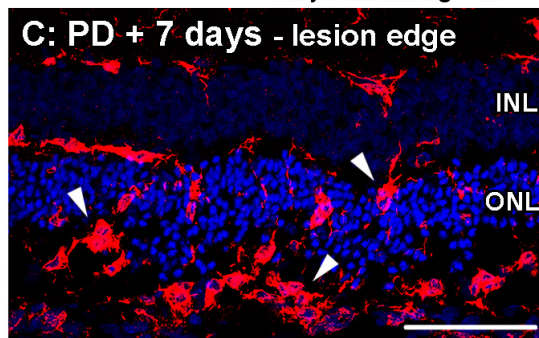
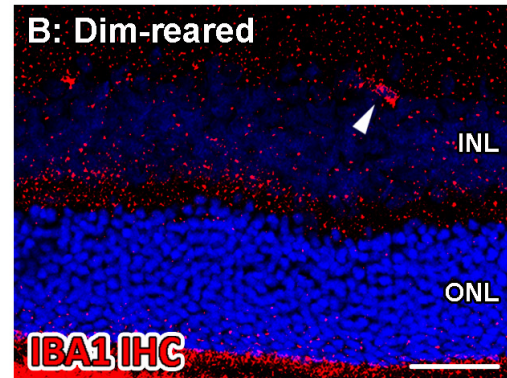
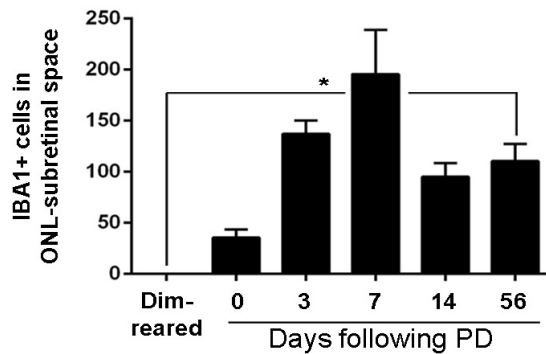


Figure 4.4 Infiltration of IBA1⁺ microglia/macrophages into the outer rat retina following photo-oxidative damage (PD). **A:** In dim-reared controls, there were no IBA1⁺ cells in the outer retina (ONL-RPE). Upon damage, an increase in IBA1⁺ cell numbers in the outer retina reached a peak at 7 days, and remained significantly increased at 56 days ($P < 0.05$). **B:** IBA1⁺ cells were detected only in the inner retinas of controls. **C:** IBA1⁺ cell numbers in the outer retina and subretinal space peaked at 7 days at the lesion edge. **D-E:** At 56 days, there were significant numbers of IBA1⁺ cells in the outer retina at the lesion edges, even though very few photoreceptor cell nuclei remained. Statistical significance was determined using a one-way ANOVA. INL, inner nuclear layer; ONL, outer nuclear layer. N=4 per group. Scale bars represent 50 μ m.

Suppression of local C3 inhibits retinal atrophy - mouse studies

To better understand the role of locally-expressed C3 in complement deposition in the subretinal space and its role in retinal atrophy, we used three different strategies to inhibit C3 in a model of retinal degeneration: C3 knockout ($C3^{-/-}$), local inhibition of C3 using siRNA, and systemic C3 depletion using Cobra Venom Factor (CVF).

C3 knockouts

Previous reports indicate that complete ablation of C3 reduces the ERG function and outer-retinal integrity in 12-month old animals [347]. For this reason we carried out experiments on P60-80 mice, in which no functional or histological deviations were observed [347, 348]. Dim-reared $C3^{-/-}$ animals were assessed for any functional or histopathological changes compared to wild-type (WT). These comparisons showed no reduction in ERG amplitude, changes in rhodopsin (*Rhod*) expression, or up-regulation of the stress/neuroprotective factors GFAP, *Fgf-2*, or *Cntf* (Supplementary Figure 4.1).

C3 gene expression and immunoreactivity for C3 were confirmed as absent in $C3^{-/-}$ animals and in dim-reared WT mice (Figure 4.6A-B). Increased C3 expression in WT retinas was evident after 1 day of photo-oxidative damage and was up-regulated across the exposure time-course, with peak expression on day 5 ($P < 0.05$, Figure 4.6A). Immunoreactivity for C3 deposits in the ONL and subretinal space of WT retinas was evident at 5 and 7 days (Figure 4.6 C-E), and absent in $C3^{-/-}$ retinas over the same period (Figure 4.6F). Over the time-course of photo-oxidative damage, the ONL was better preserved in $C3^{-/-}$ mice compared to WT (Figure 4.6G-M); at 7 days ONL thickness was significantly greater in the $C3^{-/-}$ mice compared to WT ($P < 0.05$, Figure 4.6G, J, M).

The a-wave and b-wave responses of the ERG reflect the differences in retinal morphology of WT and $C3^{-/-}$ animals described above: the ERG a-wave and b-wave intensity response characteristics between groups being significantly different ($P < 0.05$,

Figure 4.6N-O). $C3^{-/-}$ mice had higher a- and b-wave responses compared to WT mice (Figure 4.6N-O): this difference was most pronounced at the highest flash intensity ($P<0.05$, Figure 4.6P).¹

Local C3 inhibition using siRNA

C3 siRNA significantly reduced C3 gene expression in retinas at 7 days photo-oxidative damage ($p<0.05$, Figure 4.7A). Lower levels of C3 protein deposits were observed in C3 siRNA-injected retinas compared with those injected with negative control siRNA (Figure 4.7B-C). In contrast, liver expression of C3 was not affected by intravitreal injection of C3 siRNA, indicating that siRNA injected into the eye was largely localised there (Figure 4.7D).

Quantitative analysis of retinas post-damage showed that the photoreceptor population was protected against cell death in C3 siRNA-injected retinas, compared with negative control siRNA (Figure 4.7E). Comparison of retinal thickness showed that the ONL was significantly thicker ($P<0.05$) in C3 siRNA-injected animals than in negative control siRNA-injected animals (Figure 4.7E). Furthermore, ERG analyses showed that the mean a-wave and b-wave amplitudes of the ERG were significantly greater in C3 siRNA-treated animals compared to negative siRNA controls, which was most pronounced at the highest flash intensity ($P<0.05$, Figure 4.7H). The ERG a-wave and b-wave intensity response characteristics between groups were statistically significant ($p<0.05$, Figure 7F-G).

¹ Additional findings from C3 knockout study are shown in *Chapter 5*

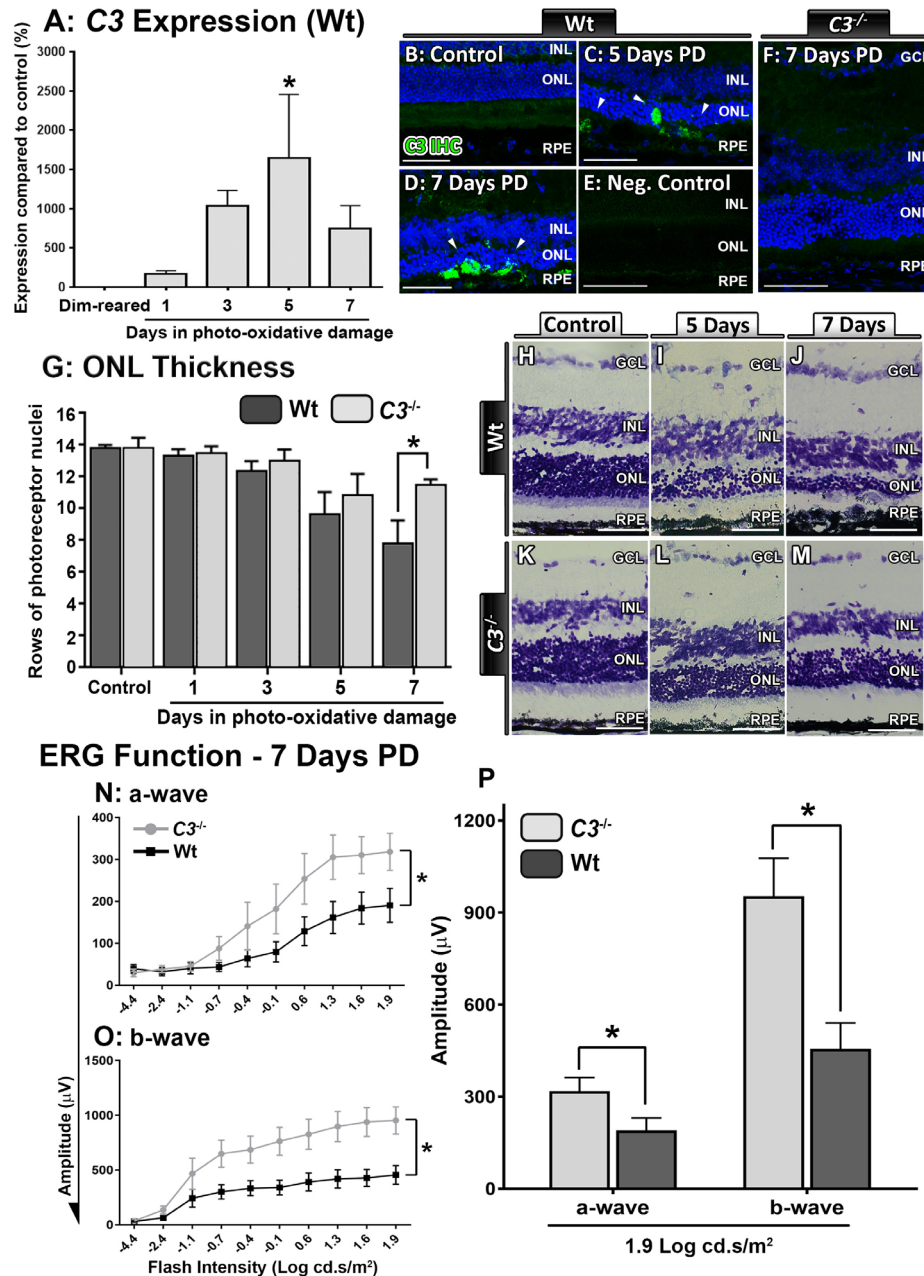


Figure 4.6 *C3* expression in C57BL/6J wild-type (WT) and *C3*^{-/-} mouse retinas. **A:** *C3* gene expression following photo-oxidative damage (PD) increased to a peak at 5 days ($P < 0.05$ compared to control, $N = 8$). **B-F:** *C3* protein deposition was evident at 5 and 7 days in WT retinas (C-D). *C3* detection was absent in *C3*^{-/-} retinas at 7 days (F), which was comparable to WT controls (B). **G:** ONL thinning was significant in WT retinas compared to *C3*^{-/-} retinas at 7 days ($P < 0.05$, $N = 6$). **H-M:** Toluidine blue staining of WT and *C3*^{-/-} retinas showed no ONL disturbance in the controls (H,K), and progressive thinning at 5 days (I,L) and 7 days (J,M), with *C3*^{-/-} retinas having a thicker ONL than WT retinas. **N-P:** *C3*^{-/-} retinas at 7 days demonstrated a significantly higher a-wave (N) and b-wave (O) compared to WT retinas ($P < 0.05$, $N = 10$), which was most pronounced at 1.9 Log cd.s/m² (P , $P < 0.05$, $N = 10$). Statistical significance was determined using one-way (A) or two-way ANOVA (G, Sidak's post-hoc test; P, uncorrected Fisher's LSD for the 1.9 Log cd.s/m² comparison). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars represent 50 μm.

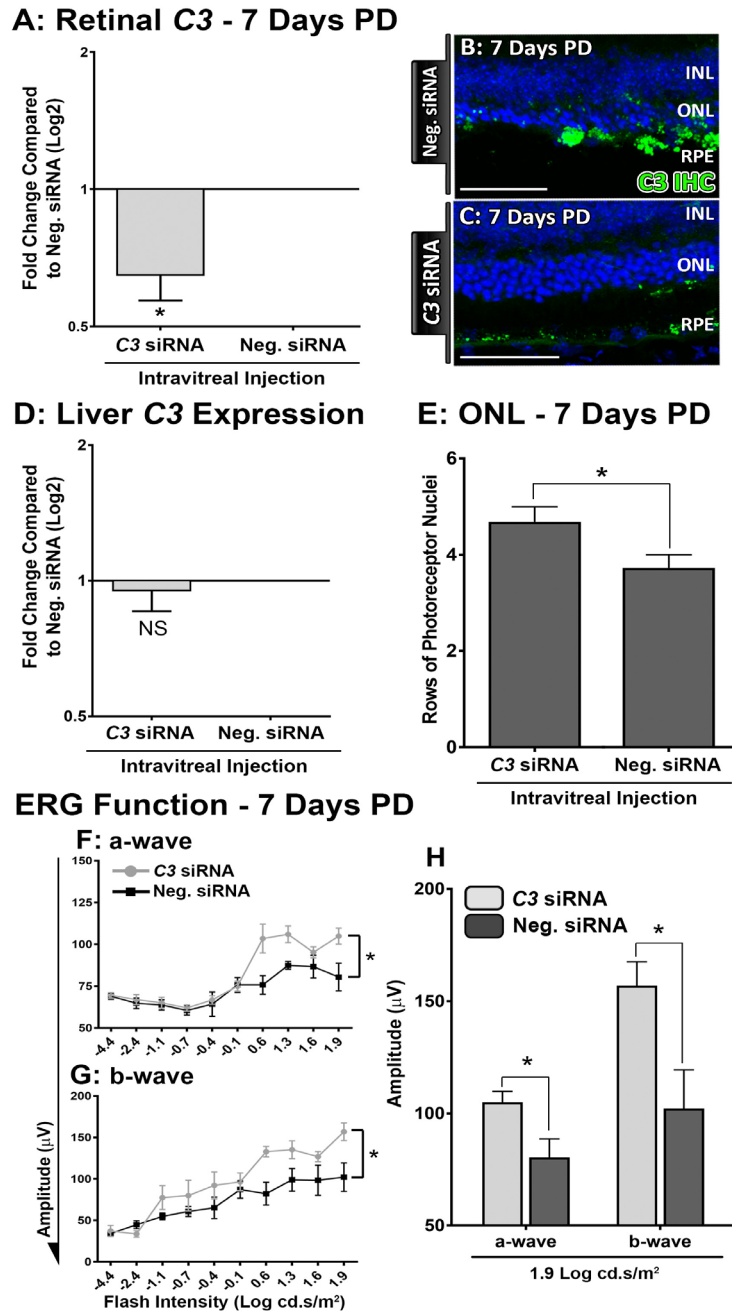


Figure 4.7 Inhibition of retinal C3 expression using C3 siRNA and 7 days of photo-oxidative damage (PD). **A:** Retinal C3 gene expression was significantly decreased following an intravitreal injection of C3 siRNA compared to the negative siRNA controls ($P < 0.05$, $N = 11$). **B-C:** There was a reduction in C3 protein deposition in the C3 siRNA-injected retinas compared to negative siRNA controls. **D:** Liver C3 gene expression was not affected at 3 days after the C3 siRNA intravitreal injection ($N = 3$). **E:** There was a significant reduction in photoreceptor loss in C3 siRNA-injected retinas compared to negative siRNA controls ($P < 0.05$, $N = 13$). **F-H:** C3 siRNA retinas displayed a larger a-wave (F) and b-wave (G) compared to negative siRNA controls, which was most pronounced at 1.9 Log cd.s/m² (H), indicating a significantly improved retinal function ($P < 0.05$, $N = 4$). Statistical significance was determined by an unpaired student t-test (A,D,E) or a two-way ANOVA (F-G, uncorrected Fisher's LSD for the 1.9 Log cd.s/m² comparison). INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars represent 50μm.

Systemic C3 depletion using Cobra Venom Factor

To understand the role of the components of serum complement in the retina, we systemically depleted serum complement activity in WT mice, using cobra venom factor (CVF). A haemolysis assay using serum from *C3*^{-/-} animals, and animals injected with CVF or PBS, indicated that after 7 days of damage, serum complement activity was depleted by CVF to a level comparable to *C3*^{-/-} mouse serum. This was not the case in PBS-injected animals, thus indicating that systemic complement was inhibited by CVF ($P < 0.05$, Figure 4.8A).

To understand the impact of CVF on complement gene expression in the retina, we compared levels of *C3*, *C1s*, *C2*, *C4a*, *Cfb*, and *Cfh* at 7 days in animals injected with CVF, with PBS-injected controls. The elimination of systemic complement by CVF did not significantly alter the retinal expression of those complement components or regulatory genes (Figure 4.8B). In addition, no difference was observed in retinal C3 deposition between CVF- and PBS-injected animals at 7 days (Figure 4.8C-D).

Analysis of retinal morphology showed that depletion of systemic complement did not protect against retinal degeneration. There was no difference observed in ONL thickness in CVF-injected mice compared with PBS-injected mice (Figure 4.8E). Similarly, there were no significant differences in mean a-wave or b-wave amplitudes in CVF- and PBS-injected animals after 7 days damage, and no significant difference in the ERG intensity response characteristics between groups (Figure 4.8F-H).

A: Complement Serum Activity B: Retinal Gene Expression

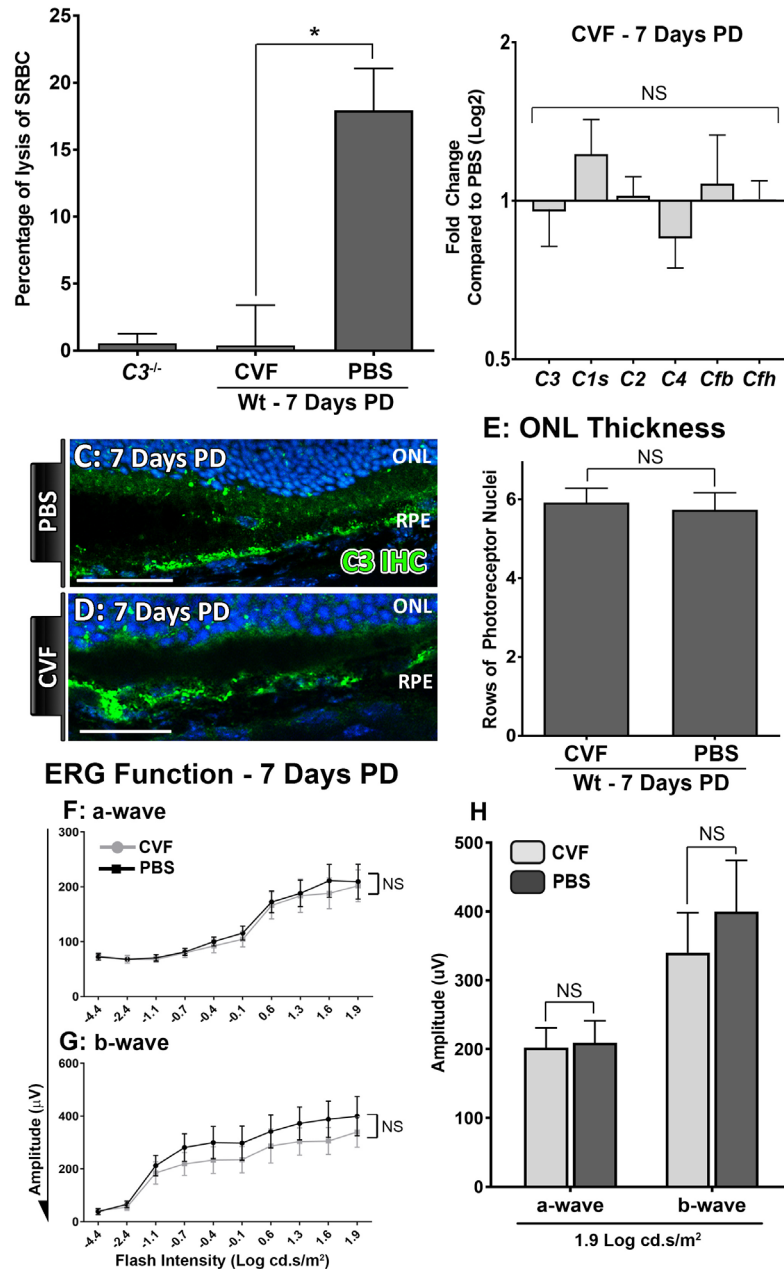
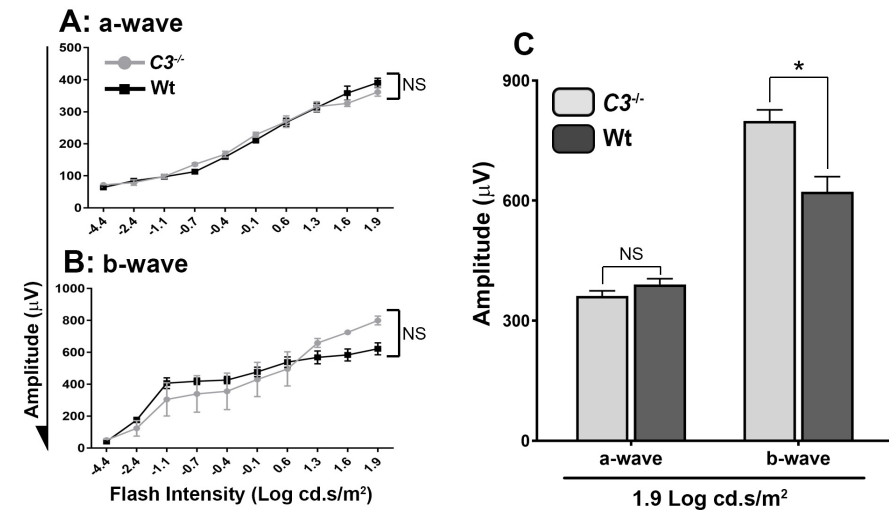
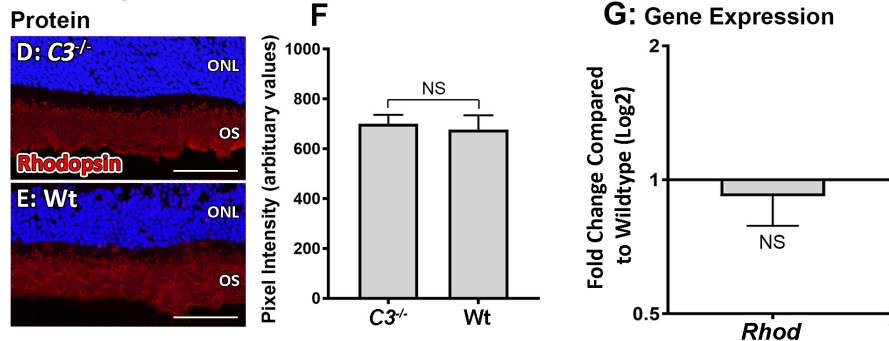


Figure 4.8 Depletion of systemic complement using cobra venom factor (CVF) at 7 days of photo-oxidative damage (PD). **A:** Complement activity in the serum was inhibited in CVF-injected animals compared to PBS controls as determined in a haemolytic assay using sheep red blood cells (SRBC), where complement inhibition in CVF-injected animals was comparable to C3^{-/-} animals (N=4). **B:** Retinal expression of complement genes (*C3*, *C1s*, *C2*, *C4a*, *Cfb*, *Cfh*) were not significantly affected by CVF depletion of systemic complement (N=9). **C-D:** There was no difference in C3 protein deposition between CVF and PBS-injected animals. **E:** No difference in ONL thickness was observed between CVF and PBS-injected animals (N=6). **F-H:** Depletion of systemic complement did not decrease retinal function, as there was no significant difference in the a-wave (F) or b-wave (G) between CVF and PBS groups (N=5). Statistical significance was determined by an unpaired student t-test (A,B,E) or a two-way ANOVA (F-G, uncorrected Fisher's LSD for the 1.9 Log cd.s/m² comparison). ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars represent 50μm.

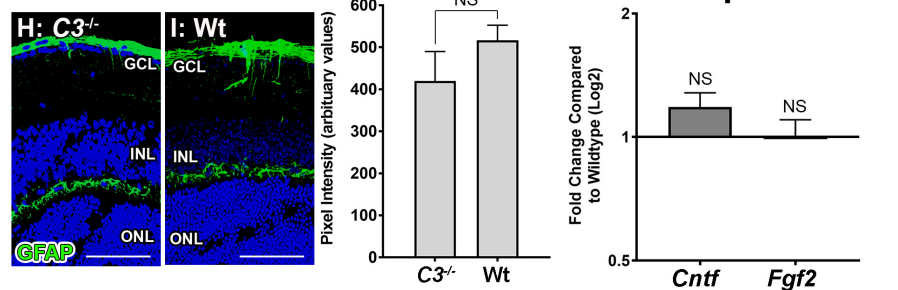
ERG Function - Dim-reared $C3^{-/-}$ vs Wt



Rhodopsin



GFAP



Supplementary Figure 4.1 Characterisation of dim-reared C57BL/6J wild-type (WT) and $C3^{-/-}$ mouse retinas. **A:** When assessing retinal function, there was no change in the a-wave between WT and $C3^{-/-}$ groups. **B-C:** There was no significant difference in the b-wave across the varying flash intensities (B) except at 1.9 Log cd.s/m², where a significant difference was detected (C, $P < 0.05$, $N = 4-5$). **D-G:** Rhodopsin labelling in the outer segments (OS) was similar in the WT and $C3^{-/-}$ retinas (D-F, $N = 5$), and there was no change in *Rhod* gene expression between both groups (G, $N = 3$). **H-J:** GFAP expression in the Müller cells was not significantly different in WT and $C3^{-/-}$ retinas, however, it was slightly higher in the WT ($N = 5$). **K:** There was no significant change in the expression of neuroprotective genes (*Cntf*, *Fgf2*) between WT and $C3^{-/-}$ retinas ($N = 4$). Statistical significance was determined by an unpaired student t-test (F-G, J-K) or a two-way ANOVA (A-B, uncorrected Fisher's LSD for the 1.9 Log cd.s/m² comparison). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

4.5 Discussion

This is the first study to demonstrate the key importance of locally expressed sources of complement in driving complement-induced retinal atrophy. First, we show that *C3* expression by macrophages in the human retina is closely linked to retinal atrophy in AMD. Second, we demonstrate *in vivo* the emergence and progression of a focal atrophic lesion in the rat retina over a period of 56 days, and the modulation of expression of a range of complement-related genes, including the persistent elevation of *C3* and its expression by macrophages over the time-course. Third, using *C3*^{-/-} mice, local *C3* knockdown and systemic complement depletion strategies we demonstrate conclusively that *C3* expressed in the retina, but not serum *C3*, plays a decisive role in complement propagation in retinal degeneration. Taken together, these results suggest that a therapeutic approach targeting *C3* could slow the progression of many neurodegenerative diseases including AMD.

Role of complement in onset of retinal atrophy

Complement dysregulation has been highly implicated in the progression of AMD [3]. Consistent with other similar findings that complement over-activation contributes to retinal degenerations [109, 160, 338, 349-351], our previous studies show an up-regulation of complement genes *C1s*, *C2*, *C3* and *C4a* in retinal degenerations [146, 154, 337, 342]. This study extends those collective findings showing that *C1s*, *C2*, *C3* and *C4a* are up-regulated during progressive lesion expansion, and that accumulation of *C3*-expressing macrophages, at the lesion edges, is a constant feature of lesion progression. This complement gene expression profile is consistent with a broad scope of literature, which has genetically and histologically linked focal retinal atrophy, including AMD pathogenesis, to irregularities in the complement system [61, 79, 98, 128, 132, 138, 139,

352]. In particular, the sustained up-regulation of *C1s*, *C3* and *C4a* demonstrated in this study is consistent with findings from systems-level analysis of AMD neural retinas.

C3 propagation is strongly implicated in AMD through the association of the Y402H variant of *CFH* [98, 125]. We pursued the effects of disrupting local and systemic C3 using a triad of C3 inhibition strategies. The results show that the retina is protected from atrophy in both ablation and local knockdown of C3, but not from systemic CVF depletion of C3. This demonstrates that only locally expressed C3 – not serum C3 – is linked to retinal atrophy in this study. Serum complement components are constitutively produced and replenished by the liver [353], and it has been reported that C3 levels are elevated in the peripheral blood of AMD patients [354]. Because complement components from serum would not be able to cross the intact BRB due to their large size, it is unlikely that they could participate in the onset of retinal atrophy. However, as retinal atrophy progresses and there is breakdown of the outer BRB, there are no barriers to the permeation of serum constituents and this could contribute to further progression of atrophy.

Local C3-expressing macrophages as a therapeutic target for reducing retinal atrophy

Macrophage infiltration of the subretinal space is a feature of AMD histopathology [2, 193, 200], including at the lesion edges in geographic atrophy [200]. Their pathogenic involvement in the disease is well-supported through findings in both laser-induced neovascularization [221, 247] and photo-oxidative damage models [192, 193, 342]. In the present study we show that C3 is expressed only by retinal and subretinal macrophages, and not by RPE cells in AMD-affected retinas. Our current and previous findings [146, 154] are consistent with a transcriptome-wide analysis, combining over 60

donor samples covering all forms of AMD, which demonstrated *C3* upregulation in neural retina, but not RPE/choroid [138].

Despite this, we do not suggest that the RPE/choroid interface plays no role in modulating complement, as these tissues are known to express a range of other complement constituents, and the membrane attack complex is abundant in the choriocapillaris in early AMD [150]. RPE cells have been reported in some studies as a local source of *C3* in mouse retina under homeostasis or in cases of retinal degenerations [153, 355, 356]. These studies relied upon PCR of combined extracts of RPE/Choroid, or used cultured RPE. To date, no study has confirmed that RPE expresses complement genes in the retinal environment *in situ*.

We surmise that complement activation and deposition in the outer retina is dependent on the summative effects of a range of mediators and regulators, and that accumulation of subretinal macrophages is a key component of *C3* synthesis and deposition. We identify retinal *C3*-expressing macrophages as a novel therapeutic target for mitigating the damaging effects of complement in retinal atrophy, and highlight a potential role for *C3*-targeted gene therapies.

An outstanding question is whether the *C3*-expressing macrophages associated with retinal atrophy derive from the pool of resident microglia, or recruited bone-marrow monocytes, or both. O’Koren and colleagues have shown, using genetic lineage tracing, that subretinal macrophages are predominantly resident microglia [203]. On the other hand, the localisation of some *C3*-expressing macrophages adjacent to the superficial retinal vasculature and optic nerve suggest a recruitment pattern of bone-marrow derived macrophages [318]. It is possible that the *C3*-expressing population may also comprise non-resident macrophages.

Conclusions

Through combined observations in human donor tissue and models of retinal degeneration, our study illustrates a novel role of macrophages in priming the pathogenic activation of complement via their secretion of C3. We emphasise that this key contribution of subretinal macrophages does not preclude important roles for the RPE/choroid interface in influencing complement activation. However, our findings do reveal that the orchestration of complement-mediated pathology in AMD involves more elements than surmised previously [3], of which subretinal macrophages are a pivotal element. In demonstrating the efficacy of intravitreal *C3* siRNA in mediating complement-induced retinal atrophy while also negating a contribution of systemic C3, we provide vital proof-of-principle support for locally administered gene therapy to target complement-induced retinal atrophy. This work lays a foundation for testing advanced gene-editing approaches, such as CRISPR/Cas9 technology, and has the potential to revolutionise the therapeutic landscape for complement-mediated retinal degenerations including AMD.

5. *The effect of complement C3 on microglia/macrophage function and photoreceptor death in the mouse photo-oxidative damage model*

5.1 Introduction

As outlined in Chapter 4, there is an incomplete understanding of the cellular events leading to complement activation in the retina. This lack of understanding has been a significant obstacle in development of innovative approaches in the management of AMD. In brief, chapter 4 highlighted a contribution of local complement component 3 (C3), synthesised by retinal microglia/macrophages, to the retinal atrophy after photo-oxidative damage. In this chapter, I present additional data that further illustrate the impact of C3 on the activity of retinal immune cells such as Müller glia and microglia/macrophage in the photo-oxidative damage environment and demonstrate the relationship between the activation of microglia/macrophages and the progression in retinal atrophy following photo-oxidative damage. The data indicate that total genetic ablation of C3 ameliorates progression of retinal degeneration after photo-oxidative damage, and modifies the reactive responses of microglia/macrophages and Müller cells. Together, these data point to a wide-ranging role for complement activation in modulating the function of phagocytes and Müller cells, providing insight into the functional protection of C3 ablation in knockout mice after photo-oxidative damage.

5.2 Additional Methods

TUNEL labelling of retinal sections

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to identify and quantify photoreceptor apoptosis in cryosections during photo-oxidative damage. The retinal sections were permeabilised with 1xPBS, 0.1% Triton-X100 for 10 mins at room temperature, and then labelled using a TUNEL assay (Roche, Indianapolis, IN) according to the manufacturer's specifications. Counts of TUNEL-positive (TUNEL⁺) cells in the outer nuclear layer (ONL) were performed blind to the condition/genotype, and along the full-length of retinal cryosections cut in the superior-

inferior plane (including the optic disc). The final count from each animal was the average of those obtained at comparable locations in two retinal cryosections. Statistical analysis was performed using two-way ANOVA to compare the photoreceptor apoptosis between $C3^{-/-}$ and the corresponding wild-type (WT) animals across the full time course of photo-oxidative damage; differences with a $P < 0.05$ were considered statistically significant.

Immunohistochemistry

Retinal sections were used for immunohistochemical analysis using primary antibodies (listed in Chapter 2 Methods). Some tissue sections were stained with TUNEL prior to immunohistochemistry. Cryosections were first subjected to heat-induced antigen retrieval using 100% Reveal-it (ImmunoSolution, QLD, Australia) for 1 h at 37°C, then washed in 1x PBS containing 0.3% Reveal-it (0.3% PBSR) and blocked and permeabilised (10% normal goat serum in 1x PBS with 0.1% Triton X-100 for 1 h at room temperature). Following an overnight incubation of the primary antibodies (details given in Chapter 2 Methods) at 4°C, the retinal sections were washed in 1x PBS, and then incubated with secondary antibodies (Alexa-488-conjugated goat anti-rabbit for IBA1 and GFAP, 4 h at room temperature; Streptavidin Alexa-594-conjugated anti-biotin for CD68, 1.5 h at room temperature). Sections were then incubated with the DNA-specific dye bisbenzimidazole (0.1 µg/ml, Sigma-Aldrich, MO, USA) for 2 mins, washed and then coverslipped using Aquamount (Polysciences, VA, USA).

Confocal analyses

Fluorescence in retinal sections was visualised under a laser-scanning A1+ confocal microscope (Nikon, Tokyo, Japan), and images were acquired using the z-stack function of the NIS-elements AR software (Nikon) under the same laser settings. This allowed us to make optimal comparisons between sections. Quantification analyses included retinal morphometric analysis and total counts of immunolabelled cells (GFAP,

IBA and CD68). Analyses were based on the average of two sections per experimental group of animals (N = 5-6).

Retinal morphometric analysis

To assess the ONL thinning on cryosections following photo-oxidative damage, the sections were stained with Toluidine Blue and then quantified in accordance with our previous methodology. Briefly, the number of rows of photoreceptor nuclei was counted in six areas of retinal cryosections (superior-inferior) - the central, mid-periphery and peripheral retina, at 500 μ m intervals across retinal cryosections. In at least 2 retinal sections per animal, photoreceptor rows were counted per area and subsequently averaged for each experimental group (N = 5-6 per group).

GFAP Quantification

Response from Müller glia was detected using GFAP immunohistochemistry to localise the proteins upon photo-oxidative damage. Fluorescence intensity and length measurement of the immunolabelled GFAP⁺ cells were quantified in the confocal images of WT and C3^{-/-} retinal sections which were taken under 10x objectives. Using the NIS Elements AR software, the length of the cellular processes immunopositive for GFAP was measured within the lesion region of superior retina, and measurements were subsequently averaged for each experimental group (N = 5 per group).

Confocal images of GFAP-labelled sections were processed and analysed using the NIS Elements AR software. Consistency in the analyses was ensured by employing identical parameters with areas sampled for analysis, camera and laser configuration. Three identical fields of interest were selected in the superior region for each retinal cryosection. During the image quantification, the areas of interest were marked by a rectangle drawn from the apical surface of GCL to the basal surface of ONL, and the fluorescent intensity threshold was set up before the acquisition of the mean fluorescent

intensity for each retina was collected. The mean GFAP fluorescent intensity was measured from each rectangle across the superior retina, and the average of two sections from each animal was taken (N = 5 per group).

Microglial reactivity

After IBA1 immunohistochemistry, three quantification analyses were performed on photo-oxidative damage retinal sections of WT and *C3^{-/-}* to investigate the microglial reactivity (N = 5 per group). The total number of ramified and amoeboid IBA1⁺ cells was separately quantified in the outer retina (ONL-RPE) across the full length of retinal cryosections in duplicate. The total number of phagosomes identified as IBA1⁺ CD68⁺ cells was counted in the outer retina, across the full length of retinal cryosections (superior-inferior) that were double-labelled with IBA1 and CD68.

To assess phagocytosis by microglia/macrophages, the total population of photoreceptor nuclei engulfed by IBA1⁺ cells was determined in the outer retina using retinal sections that were stained positive for IBA1 and TUNEL. TUNEL⁺ and non-TUNEL⁺ cells within the IBA1⁺ cells were quantified in the ONL and subretinal space of the retinal cryosections. These counts were performed at six areas across the full length of retina in duplicate (N = 5 per group).

5.3 Results

Photoreceptor death and retinal function

Photoreceptor apoptosis was assessed in $C3^{-/-}$ and WT retinas by TUNEL assay across the time course of photo-oxidative damage (Figure 5.1). Increasing numbers of TUNEL⁺ cells were identified in the ONL of WT retina across the ensuing days of photo-oxidative damage, with photoreceptor specific cell death evident from 3 days of photo-oxidative damage (Figure 5.1 B, C). However, in $C3^{-/-}$ animals there was negligible photoreceptor cell death observed, comparable to dim-reared retinas (Figure 5.1 A, D). A peak in the numbers of TUNEL⁺ cells was observed at 5 and 7 days, in the $C3^{-/-}$ sections (Figure 5.1 E, F, G).

These observations were also reflected in analyses of retinal function using electroretinogram (ERG) recordings. The amplitudes of scotopic a-wave and b-wave responses were found to be greater in $C3^{-/-}$ animals compared with WT following 7 days of photo-oxidative damage (Figure 5.1 H). The amplitude of cone response was also significantly higher in $C3^{-/-}$ animals compared to WT animals ($P < 0.05$, Figure 5.1 I).

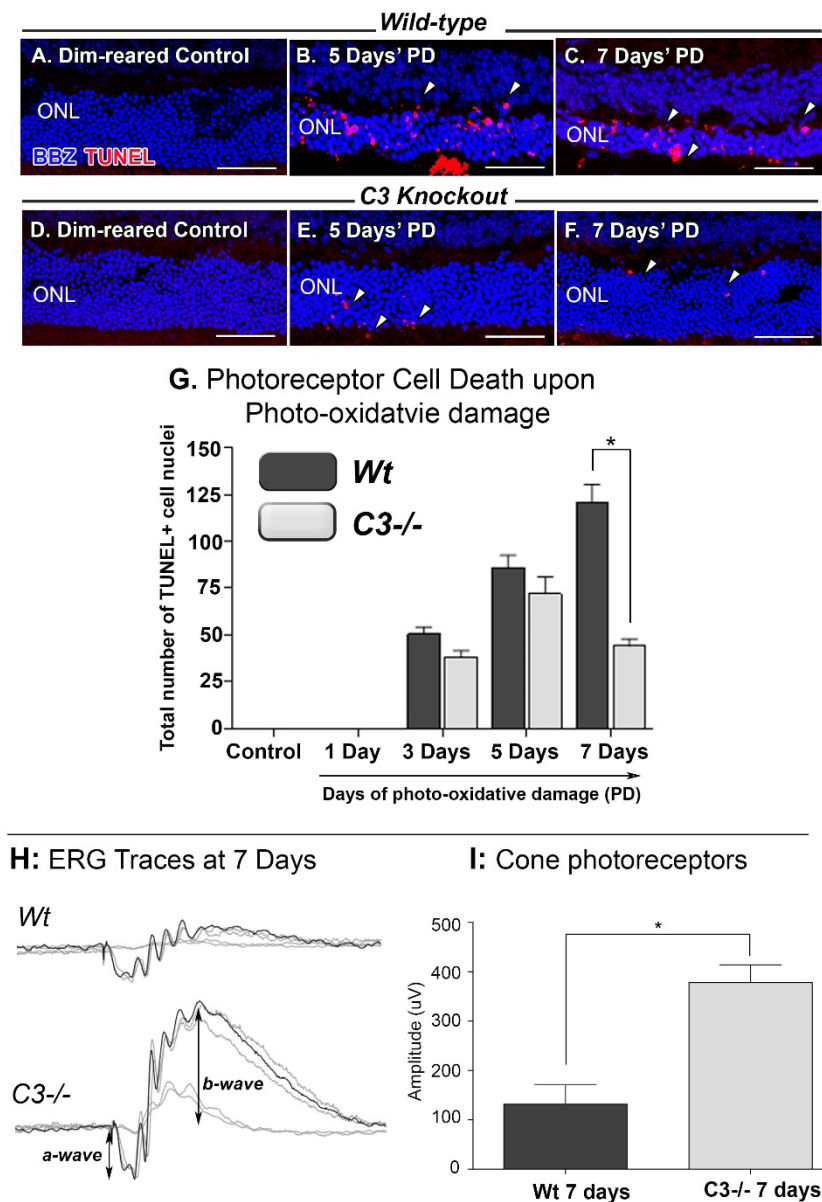


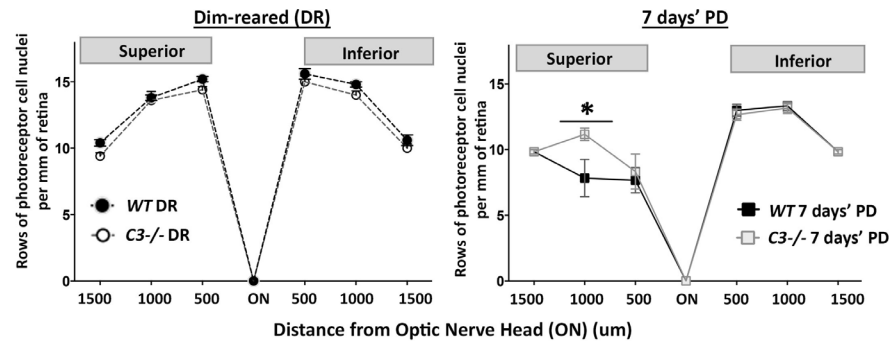
Figure 5.1 Photoreceptor integrity and function in wild-type (WT) (A, B, C) and $C3^{-/-}$ (D, E, F) animals following photo-oxidative damage (PD). **A, D:** Dim-reared WT and $C3^{-/-}$ mice did not show presence of any TUNEL⁺ cells in the ONL. **B-C, E-F:** ONL of WT mice featured many TUNEL⁺ cells after 5 and 7 days of PD (B-C), whereas the ONL of $C3^{-/-}$ mice showed substantially less in comparison (E-F). **G:** Counts of TUNEL⁺ photoreceptors were significantly less in $C3^{-/-}$ compared to WT after 7 days of PD ($P < 0.05$). **H:** ERG traces demonstrated a far more preserved a-wave and b-wave in $C3^{-/-}$ mice compared to WT, after 7 days of PD. **I:** Cone response also displayed a significantly higher amplitude in $C3^{-/-}$ mice compared to WT at 7 days of PD ($P < 0.05$, $N = 6$ per experimental group). Scale bars represent 50um (A-F).

Lesion progression after photo-oxidative damage in $C3^{-/-}$ versus WT

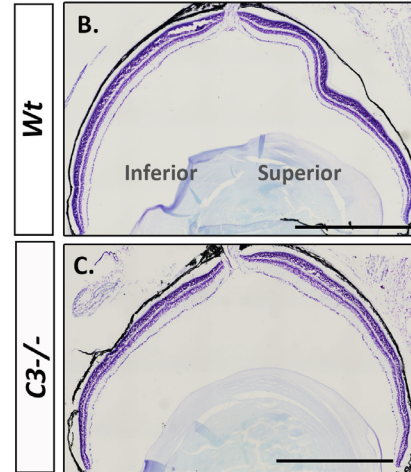
The number of photoreceptor rows in the ONL were counted to determine photoreceptor layer thickness after photo-oxidative damage and to better understand the impact of C3 knockout and photoreceptor death on lesion progression. Both $C3^{-/-}$ and WT had significantly reduced rows of photoreceptors at the focal point of photoreceptor degeneration in the superior retina at 7 days of photo-oxidative damage ($P < 0.05$, Figure 5.2 A). At $\sim 1000\mu\text{m}$ from the optic nerve in the superior retina, there were significantly fewer rows of photoreceptor in WT compared to $C3^{-/-}$ ($P < 0.05$, Figure 5.2 A).

The comparative structural changes in the retina between $C3^{-/-}$ and WT animals at 7 days of photo-oxidative damage are shown in Figure 5.2, B-G. In dim-controls, no perturbation in retinal structure was evident in either WT or $C3^{-/-}$ (Figure 5.2 B, C). At 7 days of photo-oxidative damage, severe thinning of the ONL was more prominent in WT than in $C3^{-/-}$ (Figure 5.2 D, E). The most pronounced disturbances in WT retinas were observed at central ($\sim 500\mu\text{m}$) and more peripheral ($\sim 1000\mu\text{m}$) locations (Figure 5.2 F, G). In $C3^{-/-}$ retinas there was some thinning of the ONL in the central lesion ($\sim 500\mu\text{m}$) following photo-oxidative damage, though the ONL was relatively intact in the peripheral retina ($1000\mu\text{m}$) (Figure 5.2 H, I). In WT, there was also disruption of the RPE monolayer and breaks in the Bruch's membrane while the outer retina featured large, amoeboid cells in the subretinal space at the lesion site (Figure 5.2 J). There was no disruption of the RPE monolayer or Bruch's membrane in $C3^{-/-}$ animals (Figure 5.2 K).

A. Photoreceptor row counts in WT and C3^{-/-} animals



Dim-control:



7 days' PD:

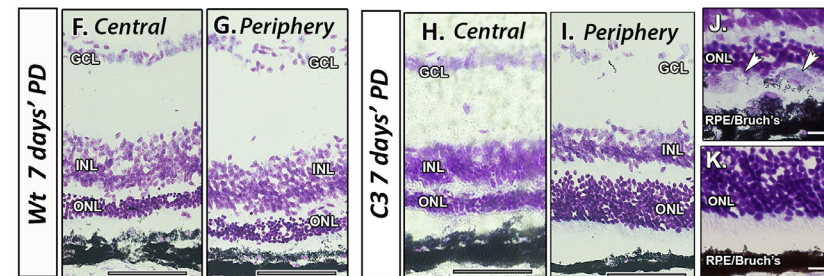
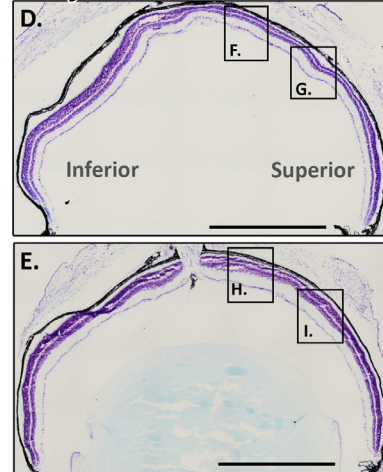


Figure 5.2 Retinal morphology in wild-type (WT) and C3^{-/-} animals following photo-oxidative damage (PD). **A:** ONL thickness was quantified as the number of photoreceptor rows, which showed a significant increase in the number of photoreceptor rows in C3^{-/-} retinas compared to WT after PD. This was at 1000 μm superior to the optic nerve ($P < 0.05$), but not adjacent to the lesion region at 500 μm away from the optic nerve ($P > 0.05$). **B-C:** Toluidine blue images showed retinal cross-sections of WT and C3^{-/-} groups, which both appeared structurally normal. **D-E:** Retinal morphology after 7 days of PD. WT retinas were markedly damaged, as shown by thinner ONL in the peripheral retina, whereas C3^{-/-} retina displayed only a superior retinal lesion and had no further disturbances in the peripheral retina. **F-G:** Both the central retina (~500 μm) and peripheral retina (~1000 μm) of WT showed severe thinning at 7 days of PD. **H-I:** In C3^{-/-}, severe thinning in the ONL was only restricted to the central retina (~500 μm). **J-K:** Higher magnification taken under x60 objective, showed incursion of amoeboid cells in the subretinal space and the disturbances in the RPE/Bruch's membrane of WT retina, which were absent in C3^{-/-} at 7 days of PD. Statistical analysis was determined using two-way ANOVA ($P < 0.05$, $N = 6$ per experimental group). Scale bars represent 100 μm (B, C, D, E), 50 μm (F, G, H, I) and 10 μm (J, K).

Assessment of complement gene expression in $C3^{-/-}$ mice

Expression of select complement components, including those associated with alternative (*CfD*, *CfB* and *CfH*) and classical (*C1s*, *C2* and *C4a*) pathways, was analysed at 1, 3 5 and 7 days of photo-oxidative damage between $C3^{-/-}$ and WT retinas (Figure 5.3). WT retinas showed a significant trend towards upregulation of all complement genes associated with the alternative pathway (*CfB*, *CfD* and *CfH*) across the time course of photo-oxidative damage. In $C3^{-/-}$ retinas however, there were generally lower levels of complement gene expression compared to WT retinas. Significant downregulation of *CfB* and *CfD* was detected at 5 days of photo-oxidative damage, and *CfB* remained downregulated at 7 days ($P < 0.05$, Figure 5.3 A-B); *CfH* was significantly downregulated at 3 and 5 days of photo-oxidative damage in the $C3^{-/-}$ animals ($P < 0.05$, Figure 5.3 C). In the classical and lectin pathways, there was a significant downregulation of *C4a* at 5 days as well as a significant downregulation of *C1s* at 7 days of photo-oxidative damage in $C3^{-/-}$ retinas compared with WT ($P < 0.05$, Figure 5.3 E-F). *C2* expression was not significantly altered in $C3^{-/-}$ retinas ($P > 0.05$, Figure 5.3 D).

Changes in Müller glial reactivity

Immunoreactivity of the GFAP was used to compare the phenotypic differences in the Müller glial cells between WT and $C3^{-/-}$ retina. There was no GFAP expression in Müller cell processes of dim-reared $C3^{-/-}$ or WT retinas (Figure 5.4 A, C). At 7 days photo-oxidative damage, GFAP immunoreactivity in Müller cells was increased markedly in the WT retinas, though comparatively less so in $C3^{-/-}$ animals (Figure 5.4 B, D). The GFAP⁺ processes extended from the inner retina into the outer nuclear layer in WT retina after photo-oxidative damage. In $C3^{-/-}$ retina, the mean intensity of the GFAP labelling was significantly reduced ($P < 0.05$, Figure 5.4 E), and the extent of the processes was reduced ($P < 0.05$, Figure 5.4 F).

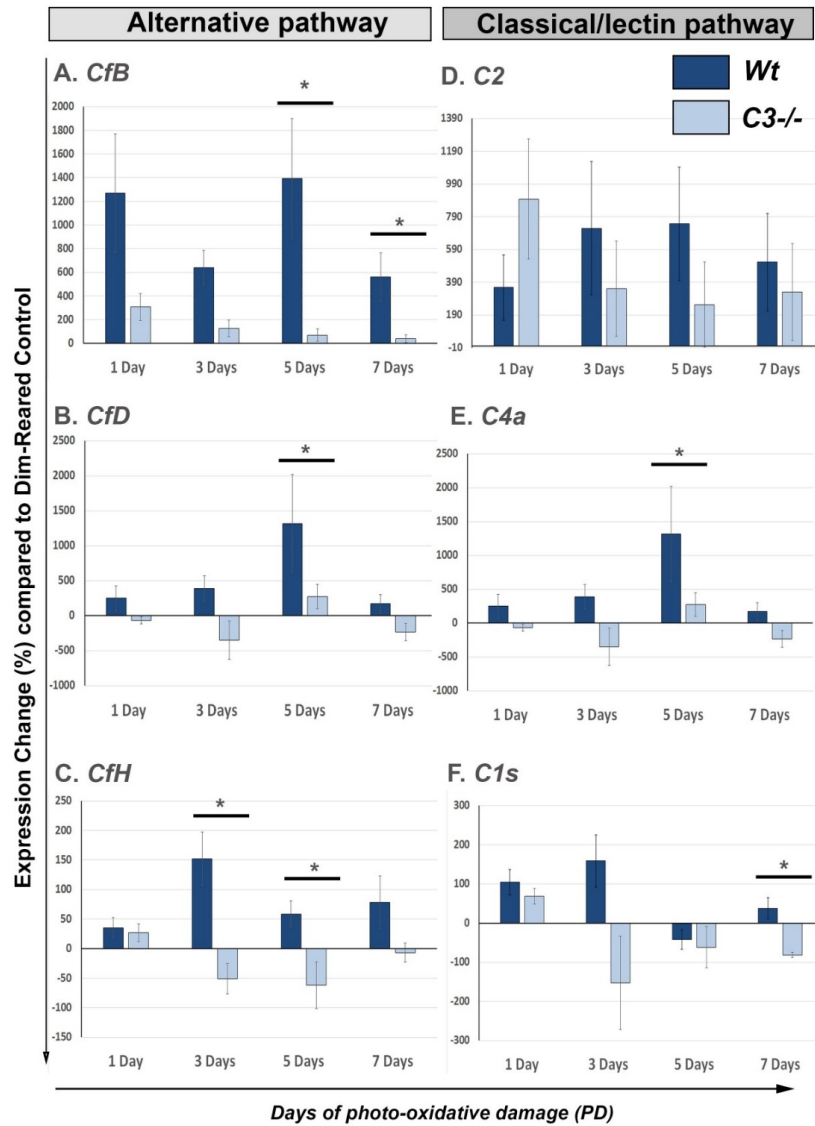
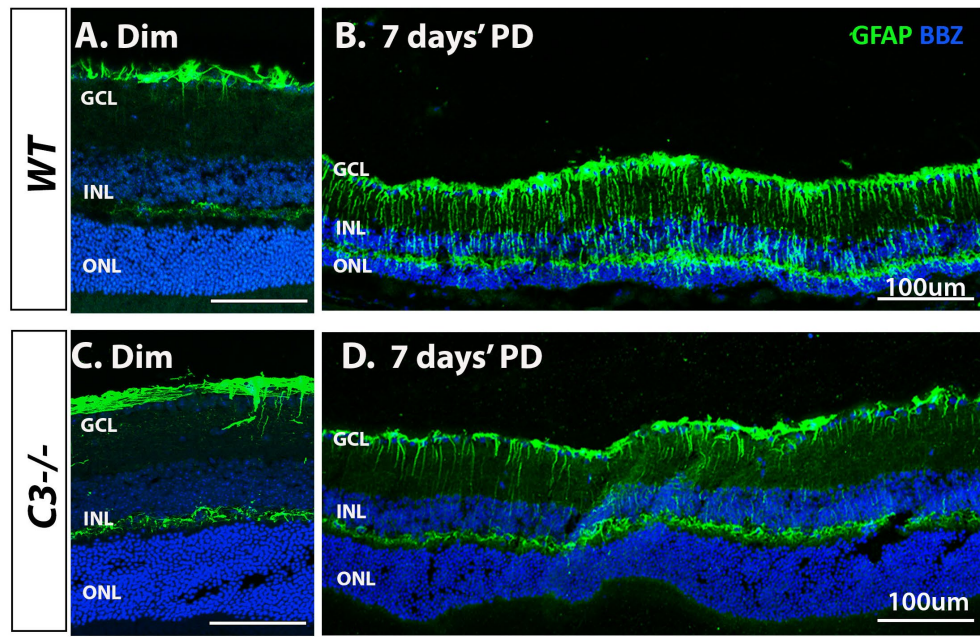


Figure 5.3 Complement gene expression in wild-type (WT) and $C3^{-/-}$ animals over the course of photo-oxidative damage (PD). Changes in gene expression were expressed as a percentage change (%) compared to dim-reared controls. **A-C:** *CfB*, *CfD* and *CfH* were highly upregulated in WT retinas compared to dim-reared control, displaying a significant trend ($P < 0.05$), while $C3^{-/-}$ displayed overall lower levels of upregulation for these genes. **A:** Expression of *CfB* was significantly reduced in retinas of $C3^{-/-}$ compared to WT at 5 and 7 days of photo-oxidative damage ($P < 0.05$). **B:** *CfD* expression level was significantly lower at 5 days ($P < 0.05$). **C:** *CfH*, the negative regulator, showed a decreasing gene expression profile with a significant lower expression level at 3 and 5 days ($P < 0.05$). **D:** *C2* expression remained highly upregulated in $C3^{-/-}$ retina and WT across each time point, and had no significant difference when compared to WT ($P < 0.05$). **E:** *C4a* upregulation reached a peak level in WT retina at 5 days (~1500%), however, its expression level was significantly decreased in $C3^{-/-}$ retina (~200%, $P < 0.05$). **F:** Downregulation of *C1s* was most obvious at 7 days of PD, in $C3^{-/-}$ retina compared to WT ($P < 0.05$). Two-way ANOVA was run for an overall trend analysis, and multiple comparison for statistical difference between $C3^{-/-}$ and WT at each time point ($P > 0.05$, $N = 4-5$ per group and per time point).



E. GFAP mean intensity at 7 days **F. GFAP+ process length (um) at 7 days**

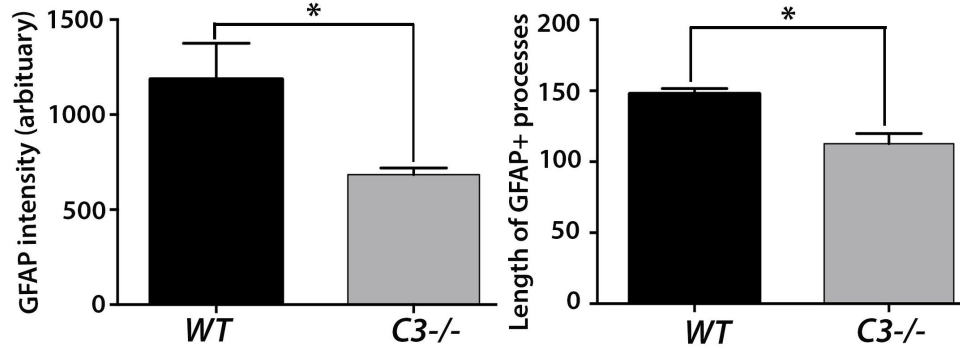


Figure 5.4 Comparative change in GFAP immunoreactivity in Müller glia after 7 days of photo-oxidative damage (7 days of PD). **A:** Retinal sections labelled with glial fibrillary acid protein (GFAP) showing limited GFAP expression in dim reared wild-type (WT) animals. **B:** Following PD, WT retina displayed increased immunoreactivity for GFAP in the Müller cells, indicating gliosis at 7 days of PD. **C:** C3^{-/-} retinal sections labelled with GFAP showing similar level in dim control as WT dim reared retinas. **D:** GFAP immunoreactivity was less prominent in the Müller cells of C3^{-/-} retina at 7 days of PD. **E:** Mean fluorescent intensity of GFAP indicates a significantly reduced expression in C3^{-/-} compared to WT ($P < 0.05$, $N = 5$ per group). **F:** Length of GFAP-positive (GFAP⁺) processes was measured on black-and-white confocal images acquired on x10 objectives using A1 Nikon Confocal Software, and was shown to significantly reduce in C3^{-/-} compared to WT ($P < 0.05$, $N = 6$ per group). Statistical analysis was determined using two-way ANOVA ($P < 0.05$, $N = 6$ per experimental group). Scale bars represent 100um (B, D) and 50um (A, C).

Changes in microglia/macrophage infiltration, activation, and phagocytosis

To assess the effect of C3 in influencing microglia/macrophage function, analyses of their infiltration, morphology and phagocytic activity were undertaken at 1, 3, 5 and 7 days of photo-oxidative damage. IBA1-positive (IBA1⁺) cells were more abundant in the outer retina (ONL-RPE) of WT after 5 days of photo-oxidative damage, than in *C3*^{-/-} retina (Figure 5.5 A-F). Counts of IBA1⁺ cells showed fewer microglia/macrophages localised in the ONL and subretinal space of *C3*^{-/-} retinas at 5 days and 7 days of photo-oxidative damage, compared to WT retinas ($P < 0.05$, Figure 5.5 G). Morphological analysis of microglia showed a more ramified morphology in *C3*^{-/-} retinas, as opposed to the amoeboid microglia engulfing photoreceptors in WT retina (arrows, Figure 5.5 H, I). Counts of amoeboid versus ramified microglia demonstrated that an activated phenotype, characterised by amoeboid morphology, was significantly less prevalent in *C3*^{-/-} retina after photo-oxidative damage, compared to WT retinas ($P < 0.05$ Figure 5.5 J).

The activation of microglia/macrophages was assessed using antibodies against IBA1 and CD68, the latter was commonly used to label lysosome-associated membrane protein (LAMP) and scavenger receptors in microglia/macrophages. After 7 days of photo-oxidative damage, IBA1⁺ microglia/macrophages in WT retinas were highly immunoreactive for CD68; and the majority of subretinal microglia/macrophages contained multiple CD68-positive (CD68⁺) cellular compartments. This indicates the presence of multiple functional phagosomes localising to infiltrating macrophages (Figure 5.6 A). In contrast, there were relatively few CD68⁺ microglia/macrophages in *C3*^{-/-} retina. The majority of IBA1⁺ cells did not express CD68⁺ phagosomes after photo-oxidative damage, demonstrating a reduced activation status (Figure 5.6 B). The total number of CD68⁺ phagosomes expressed by IBA1⁺ microglia/macrophages was markedly less in *C3*^{-/-} retina compared to WT ($P < 0.05$, Figure 5.6 C), and for both groups these phagosomes were mainly observed in the superior retina.

To assess the phagocytic activity of microglia/macrophages in relation to photoreceptor loss, *C3^{-/-}* and WT retinal sections stained with TUNEL were immunolabelled with IBA1. Double-labelling demonstrated that phagocytosis by IBA1⁺ cells was evident in the centre and at the edges of the lesion in WT retina, but was restricted to the centre of the lesion in *C3^{-/-}* retinas at 7 days of photo-oxidative damage (Figure 5.6 D-G). In WT cohort, the subretinal microglia/macrophages contained more engulfed TUNEL⁺ apoptotic cells (Figure 5.6 I). In contrast, microglia/macrophages situated in the ONL, appeared to engulf both TUNEL⁺ and TUNEL-negative (TUNEL⁻) cells (Figure 5.6 H). These observations demonstrate that the contrasting capacity of microglia/macrophages to engulf either apoptotic or viable photoreceptor elements after photo-oxidative damage was notable between the population in either the ONL or the subretinal space of WT and *C3^{-/-}* mice.

Of the microglia/macrophages in the ONL, ~55% of the phagocytosed photoreceptors were found to be apoptotic (TUNEL⁺) in WT retina after 7 days of photo-oxidative damage, while the rest were TUNEL⁻. In the *C3^{-/-}* cohort after photo-oxidative damage, there was a significant reduction in the number of phagocytosed apoptotic photoreceptors, as well as neighbouring viable photoreceptors, by the ONL-situated microglia/macrophages ($P < 0.05$, Figure 5.6 J-K).

Of the macrophages located in the subretinal space, ~90% contained apoptotic TUNEL⁺ cell fragments after photo-oxidative damage, whilst fewer than 10% contained viable (TUNEL⁻) cells. Compared to WT, the total number of macrophages engulfing TUNEL⁺ cell fragments was markedly less in *C3^{-/-}* retinas ($P < 0.05$, Figure 5.6 L). As the data show, very few viable photoreceptors were engulfed by these subretinal microglia/macrophages in either *C3^{-/-}* or WT, and the difference between the two groups was not statistically significant ($P > 0.05$, Figure 5.6 M).

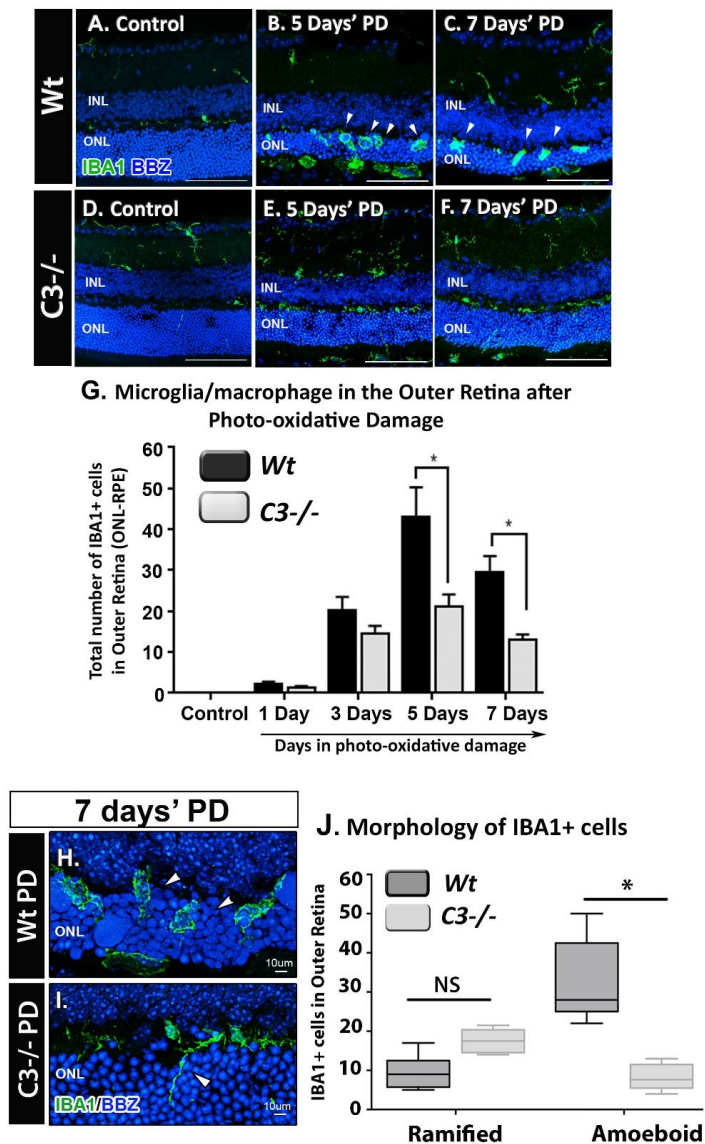


Figure 5.5 IBA1⁺ microglia/macrophages in wild-type (WT) and C3^{-/-} retina after photo-oxidative damage (PD). **A-C:** WT retina did not show any IBA1⁺ cells in the outer retina (ONL and subretinal space) of dim-reared controls (A). But after 5 days, IBA1⁺ cells accumulated in the outer retina (ONL and subretinal space) (B) and the accumulation of IBA1⁺ cells persisted up to 7 days (C). **D-F:** C3^{-/-} dim-reared retina also did not display any IBA1⁺ cells in the ONL (D), and had few IBA1⁺ cells in the ONL by 5 days (E) and 7 days (F). **G:** Total number of IBA1⁺ cells increased in the outer retina of WT from 5 days of PD onwards, whereas the number of IBA1⁺ cells was significantly less in C3^{-/-} compared to WT at both 5 and 7 days. **H-I:** IBA1 immunoreactivity showed amoeboid microglia/macrophages that were highly enriched with photoreceptor nuclei in WT, but the population of amoeboid IBA1⁺ microglia/macrophages were less observed in the outer retina of C3^{-/-} at 7 days of PD. **J:** Ramified and amoeboid microglia were quantified across the full length of ONL for WT and C3^{-/-}, showing a markedly reduced population of amoeboid IBA1⁺ cells infiltrating into the ONL of C3^{-/-} after 7 days of PD ($P < 0.05$). Statistical analysis was run on two-way ANOVA with multiple comparisons for each time point ($N = 6$ per group and per time point, $*P < 0.05$). INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars represent 100 μ m, unless indicated otherwise.

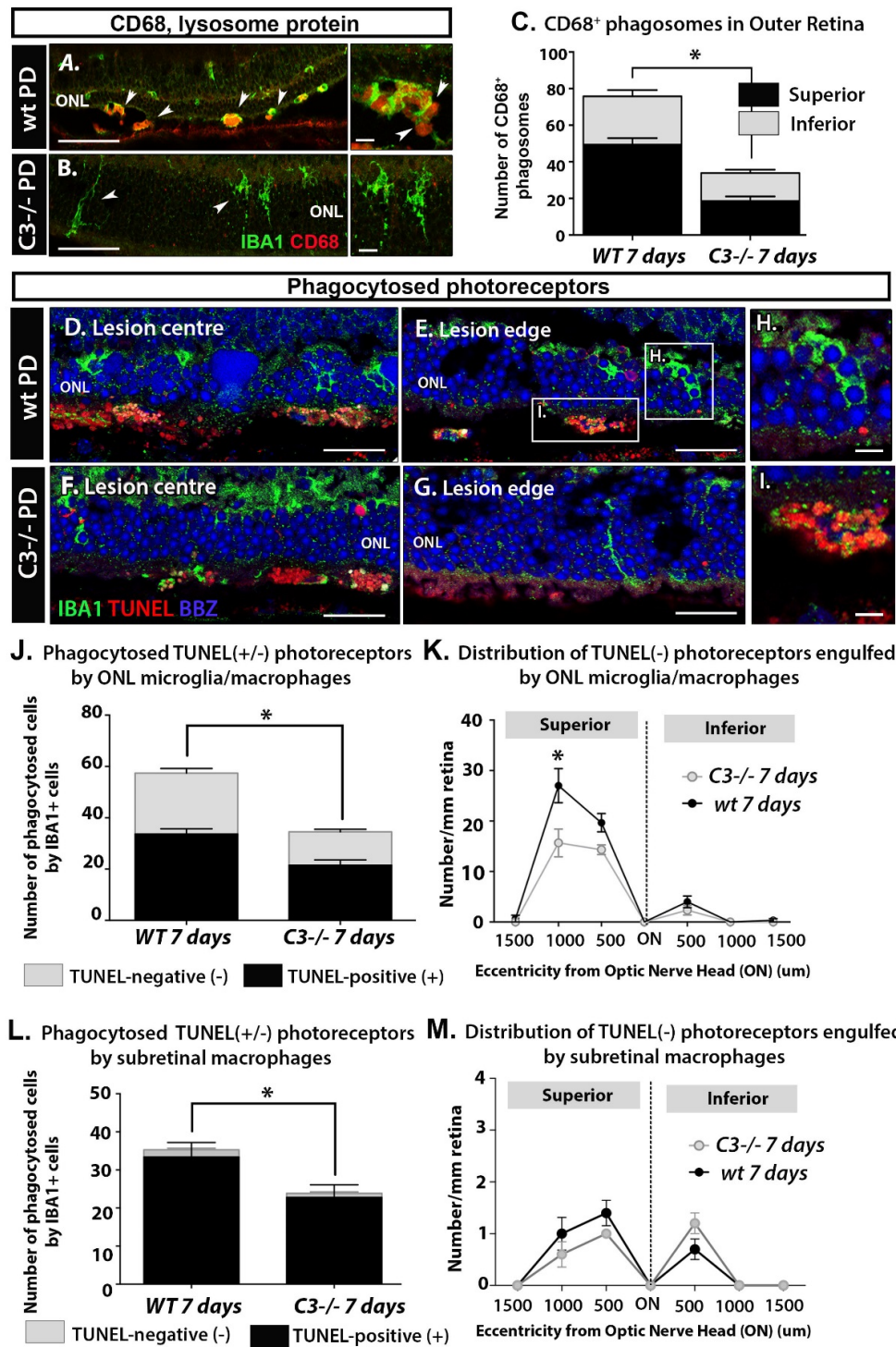


Figure 5.6 Phagocytosis in microglia/macrophages in C3^{-/-} and wild-type (WT) retina at 7 days of photo-oxidative damage (PD). **A-B:** CD68 immunoreactivity was abundant in IBA1⁺ microglia/macrophages of WT but very few were observed in C3^{-/-} after PD. **C:** Total number of CD68⁺ phagosomes as counted on CD68⁺/IBA1⁺ cells in the outer retina showed less phagosomes in C3^{-/-} retina compared to WT ($P < 0.05$). **D-I:** TUNEL and IBA1 double-immunolabelling displayed that microglia/macrophages not only phagocytosed apoptotic but also the viable cells in the ONL and subretinal space after PD. In the subretinal space (I), macrophages contained multiple apoptotic cell debris (TUNEL⁺) but very few viable cells in contrast to ones in the ONL (H). **J:** Of microglia/macrophages in the ONL, fewer photoreceptors (incl. viable cells) were phagocytosed in C3^{-/-} retina compared to WT after PD ($P < 0.05$). **K:** Number of the viable

cells phagocytosed by ONL microglia/macrophages was significantly reduced at the lesion edge (1000um from the optic nerve) in $C3^{-/-}$ mice compared to WT ($P < 0.05$). **L:** *Of macrophages in the subretinal space*, phagocytosis of the apoptotic photoreceptors, not the viable cells was predominant in WT but overall there were fewer in $C3^{-/-}$ ($P < 0.05$). **M:** Number of the viable cells phagocytosed by the subretinal macrophages showed no difference between $C3^{-/-}$ and WT group ($P > 0.05$). Statistical analysis was run on two-way ANOVA with multiple comparisons for each time point ($N = 5-6$ per group and per time point, * represents $P < 0.05$). Scale bars represent 50um (A,B,D,E,F,G) and 10um (H, I).

5.4 Discussion

This study provides further evidence of a correlation between C3 expression, activity of retinal immune cells and progressive photoreceptor degeneration. *First*, the findings show that $C3^{-/-}$ mice have reduced complement activation from both the classical and alternative pathways. *Second*, following photo-oxidative damage there is less cell death and more photoreceptors present in $C3^{-/-}$ retinas compared to WT, particularly on the lesion edge. *Third*, there is less reactivity of Müller glia upon photo-oxidative damage in $C3^{-/-}$ mice; and *lastly*, the findings show lower levels of phagocytic activity by microglia and macrophages in $C3^{-/-}$ mice. This work lays the foundation for exploring the role of complement activation in shaping the innate immune responses and progression of AMD.

Modulation of retinal complement activation

In this study, the decrease in complement activators of the alternative arm (*CfB*, *CfD* and *CfH*), and the classical arm (*C2*, *C4a* and *C1s*) of $C3^{-/-}$ mice suggests that C3 plays a pivotal role in propagating the activation of the complement system. C3 synthesised by retinal microglia and macrophages has been implicated in ageing and degeneration [154, 155]. In $C3^{-/-}$ mice, the down regulation of the classical and alternative complement was associated with the reduced migration of retinal microglia into the outer retina. Further, the differential timing of downregulation between alternative complement *CfH*, which occurred at earlier days of photo-oxidative damage, and classical complement

(*C4a* and *C1s*), which was most effective at later time point, suggest that the alternative pathway is not the only factor involved in complement-mediated retinal damage. Rohrer et al (2011) implicates the interplay between the different pathways of complement cascade in the formation of choroidal neovascularisation [147], however, this area has not been explored in atrophic AMD. The delineation of the role that the alternative and classical pathways play in the timing of disease progression, induced by photo-oxidative damage, is further investigated in Chapter 6.

C2 of the classical pathway was not down regulated in *C3*^{-/-} mice, suggesting that C2 may be regulated independently of C3. In the complement system, C2 is situated upstream of the cleavage of C3 and the lysis of C2 contributes to the formation of the classical/lectin C3 convertase which activates the complement. In AMD, the genetic polymorphisms of *C2* are associated with an increasing risk in the patients [128]. In rodent models of photo-oxidative damage, upregulation of *C2* gene expression is evident in the degenerating retina which is associated with microglia and macrophage migration [146]. However, the precise role of C2 in retinal atrophy is yet to be explored. Future investigations are underway in our laboratory to develop *C2*^{-/-} mice which will enable us to better understand its contribution to retinal degeneration.

Photo-oxidative damage and lesion progression

Local inflammation and oxidative stress are key contributing factors to the pathogenesis of AMD during which the inflammatory stimulus is prolonged in senescence [160]. Data presented here showing reduced retinal atrophy outside the focal lesion in *C3*^{-/-} mice, but not at the lesion centre (Figure 5.2 G, I), suggest that although C3 may not contribute to the initial phase of photo-oxidative damage-induced degeneration, it is prominently associated with the progression of the atrophic retinal lesion after photo-oxidative damage. Oxidative stress and an early inflammatory response

are evident in photo-oxidative damage and manifest as an increase in oxidative stress genes (*Gpx3*, *Hmox-1*), generation of lipid peroxidation by-products [340], chemokine-mediated migration [294, 357] and proliferation [358] of microglia. Trans-retinal photoreceptor proteins which were produced upon photo-oxidative damage can further trigger the activation of microglia/macrophages in the ONL [359]. The activated microglia/macrophages in the ONL and subretinal space are further implicated in complement synthesis and subretinal inflammation [146]. The data here suggest that complement deposition by retinal immune cells, instigated by the early phase of photoreceptor damage, may lead to the second phase of damage by fuelling the activity of microglia/macrophages in photo-oxidative damage. These events in photo-oxidative damage are likely to be replicated in the context of ageing and AMD, during which oxidative damage, inflammatory events and complement activation are known to be active over time [3].

Complement and Müller glia reactivity

An increased level of GFAP immunoreactivity in Müller cells is generally accepted as being indicative of increased levels of retinal stress [360]. In this study, *C3*^{-/-} mice exhibited less Müller glial response, following 7 days of photo-oxidative damage. The present findings correlate with previous evidence demonstrating that adeno-mediated delivery of C3 into the retina via intraocular injection promotes retinal pathology and results in activation of Müller glia [361]. The data suggest an effect on Müller cell reactivity brought about by complement modulation, which merits future investigation.

Complement, microglial activation and phagocytosis

Complement and microglia/macrophages seek out and opsonise cellular debris or apoptotic cells. These two innate responses are associated with the progression of neurodegenerative diseases including AMD [212, 362]. Previous studies report that the C3-dependent phagosomal inflammatory response of microglia contributes to the

degeneration of dopaminergic neurons [118] and synaptic loss in AD [363]. I have demonstrated that $C3^{-/-}$ mice have a reduced number of amoeboid microglia/macrophages in the subretinal space, reduced phagosome expression and dampened phagocytosis of viable photoreceptors, all of which correlate with a reduced severity in the progression of retinal atrophy. This suggests that C3 may amplify the functions of microglia/macrophages in the progression of retinal atrophy induced by photo-oxidative damage. The involvement of microglial phagocytosis in the progressive loss of photoreceptors has been implicated in previous studies which showed their ability to eliminate neighbouring stressed-but-viable rods expressing phosphatidylserine [217]. This further promotes secretion of IL-1 β in the inherited retinal degeneration [217], which in turn propagates cell death and cone segment loss [198, 364]. The current study implicates that C3 may be involved in shaping the activity of microglia/macrophages, suggesting their pivotal contribution to the progression of retinal degeneration. Further investigations should be conducted to validate the functional significance of C3 in shaping microglial activity.

The present data highlight a disparity between the phagocytic capacity of ONL and subretinal microglia/macrophages. The data presents the more specific phagocytosis of TUNEL⁺ cell fragments by the subretinal population versus the less discriminating activity by the ONL population. The genetic ablation of C3 eliminates the accumulation of subretinal microglia/macrophages and their phagocytosis, all of which is associated with the less involvement of the ONL population. This suggests that the accumulation and activation of subretinal microglia/macrophages, possibly amplified by complement C3, may contribute to the migration of activated microglia to the ONL, which is normally devoid of the immune cells [365]. Whilst the answer to the outstanding question rests with the identity of the subretinal population, Ma et al. (2017) suggest that they are predominantly mobilized resident microglia in a mouse model of RPE injury, and this

mobilisation further induces an adaptive response which involves the recruitment of blood-derived monocytes to the inner retina [204]. It would also be interesting to further characterise the functions of resident microglia and blood-derived monocytes that could contribute to the retinal inflammation and damage.

The data presented in this chapter are in agreement with those presented in Chapter 4, specifically, that locally derived C3 is the amplifier of the innate immune response and the progression of retinal atrophy in response to photo-oxidative damage. This suggests that *local* C3 inhibition or receptor blockade may be beneficial to reducing the harmful effects of complement activation in retinal degenerations such as AMD. A caveat is that inhibition of macrophage activation may result in accumulation of toxic debris in and on Bruch's membrane, which is an early sign of AMD [366]. This reinforces the view that timing of interventions to suppress the capacity of microglia and macrophages to elicit their complement-mediated reactions should be strategized to achieve the potential therapeutic benefits.

Conclusions

Genetic ablation of C3 dampens the retinal complement response and reduces the activation of phagocytic microglia/macrophages, further protecting the photoreceptors from progressive degeneration in the murine model of photo-oxidative damage. Local interventions targeting C3 synthesis or CR3 expression which are implicated in retinal microglia/macrophages could be facilitated by CRISPR/Cas9 approach or complement receptor blockade to reduce the deleterious effect of complement on the innate immune response and the progression of focal retinal degenerations.

**6. *Classical and alternative complement pathways
act on different phases of photoreceptor cell
death following photo-oxidative damage***

6.1 Introduction

The complement cascade comprises 3 pathways – classical, mannose-binding lectin, and alternative pathway – all of which converge at the proteolysis of C3, promoting complement activation and downstream assembly of membrane attack complex (MAC). Activation of the classical pathway is triggered by C1q binding to immune complexes on pathogenic cell surfaces, or atypical activators such as modified lipids, apoptotic cells [367], advanced glycation end products [368] and C-reactive proteins [369]. C1q binding promotes the proteolysis of C3 and the downstream activation of the complement cascade. Activation of the alternative pathway is amplified by complement factor B, a crucial component in the assembly of C3 convertase which promotes the accumulation of C3b/C3d and subsequent assembly of the MAC. The activation of complement via the three pathways propagates the effector functions of complement, such as apoptosis of target cells [370].

I have shown in the model of photo-oxidative damage (described in Chapter 3), that the central complement component, C3 which is expressed locally by retinal macrophages but not by systemically-derived serum complement, plays a critical role in propagating the deleterious downstream effects of complement activation (Chapter 4) [371]. There is strong evidence from animal and human data supporting the involvement of the alternative pathway, but emerging evidence has implicated the classical pathway in human AMD, such as genetic polymorphisms [128] and abundance of autoantibodies that are associated with AMD [169, 372, 373]. Bora et al (2006) demonstrate that the alternative pathway by *CfB* contributes to the development of choroidal neovascularisation in mice [148] which is a rodent model of wet AMD, while the initial complement activation proceeds via other pathways such as the classical pathway [374]. However, we do not know how each pathway contributes to the activation of C3 in dry

AMD, impeding the effective development of therapeutic targets for anti-complement drugs [86, 375].

In this study, I aim to dissect out the respective contributions made by the classical and alternative complement pathways in focal retinal degeneration. By utilising complement knockout mice lacking genes encoding a-subunit of C1q complex (*Clqa*^{-/-}) and factor B (*CfB*^{-/-}), in a photo-oxidative damage model, the data demonstrate that retinal atrophy induced by photo-oxidative damage requires early involvement of *CfB* (alternative pathway), and that involvement of *Clqa* (classical pathway) is delayed. *CfB*^{-/-} mice showed a dampened complement-mediated inflammation, and better preserved retinal function, while *Clqa*^{-/-} mice did not show any protection in the retina against photo-oxidative damage. *Clqa*^{-/-} mice did, however, exhibit preserved retinal function over an extended time-frame, compared to wild-type (WT). In addition, the data suggest that C1q acts in a complement-independent role by promoting IL-1 β secretion in the retina, through engagement with the NLRP3 inflammasome. Furthermore, the subretinal macrophages are shown to express C1q proteins in human AMD and rodent retinas after photo-oxidative damage. Local neutralisation of C1q (provided by Annexon Biosciences; ANX-M1) via an intravitreal delivery at post-photo-oxidative damage, reduces the progression of retinal degeneration while systemic delivery of anti-C1q antibody has no effect on the progression of retinal degeneration. The findings illustrate the interplay of classical and alternative complement in focal retinal degeneration, pinpointing subretinal macrophages as a key target, and suggesting that blocking C1q may be a useful strategy to slow down the progression of retinal atrophy.

6.2 Additional Methods

Molecular analyses on the retinas of *Clqa*^{-/-} and *CfB*^{-/-} mice were performed using immunohistochemistry, qPCR, FACS sorting and western blotting. Histological analyses

and visual function were assessed using TUNEL assay, ONL thickness measurement and electroretinogram (ERG). Details were elaborated in Chapter 2.

C1q neutralisation strategies included local pre-treatment, local post-treatment and systemic treatment following the paradigms listed below. Briefly, a neutralising antibody to C1q, ANX-M1 and a non-specific isotype control IgG monoclonal antibody (mAb) were provided by Annexon Biosciences (San Francisco, USA). Monoclonal antibody M1 binds and neutralises C1q thereby preventing the activation of the classical complement. All experiments were performed on adult C57BL/6J mice under double-blind conditions.

Local inhibition – Pre-treatment: A 1 µl solution containing either ANX-M1 anti-C1q antibody or IgG isotype control antibody was injected into individual animals, prior to the commencement of photo-oxidative damage (day 0). ANX-M1 anti-C1q antibody and IgG control cohort were placed in the same light box for 7 days of photo-oxidative damage. Retina were assessed at 7 days after photo-oxidative damage (day 14) and analysed (N= 10/group).

Local inhibition – Post-treatment: A 1 µl solution of ANX-M1 anti-C1q antibody or IgG isotype control antibody was administered intravitreally immediately after photo-oxidative damage (day 7) and placed in dim-cyclic light until day 14, for retinal assessment (N=10/group).

Systemic inhibition: A 3mg of ANX-M1 anti-C1q antibody or IgG isotype control antibody was administered intraperitoneally on day 0 and on day 4 across the ensuing days of photo-oxidative damage, and on day 8 post damage in order to sustain complement inhibition. Haemolytic assay and retinal assessment were performed at day 12 (N =10/group).

6.3 Results

***Clqa* and *CfB* gene expression following photo-oxidative damage**

The expression of *Clqa* and *CfB* in the murine retina were studied across the time course of photo-oxidative damage (1, 3, 5 and 7 days of photo-oxidative damage) in wild-type (WT) animals using qPCR (Figure 6.1). *Clqa* and *CfB* genes were both upregulated in the retina during the course of photo-oxidative damage, compared to dim-reared control retinas (Figure 6.1A-B). *CfB* expression was significantly increased following 1 day of photo-oxidative damage, and remained significantly increased at 3, 5 and 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.1A). Retinal *Clqa* expression was significantly elevated at 5 and 7 days ($P < 0.05$, Figure 6.1B), but not at 1 or 3 days of photo-oxidative damage. Temporal expression of *Clqa* and *CfB* in the retina coincided with the focal loss of photoreceptors (TUNEL⁺ cells) in the ONL (Figure 6.1C, D, arrows). TUNEL⁺ photoreceptor cell death reached peak numbers at 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.1C). Although *CfB* upregulation at 1 day of photo-oxidative damage preceded the emergence of IBA1⁺ cells in the outer retina (Figure 6.1F, arrows), the sustained upregulation of *CfB* correlated with the increase in the number of IBA1⁺ macrophages at 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.1E).

Knockout of classical and alternative complement components at 1-7 days of photo-oxidative damage

Complement knockout mice (*Clqa*^{-/-} and *CfB*^{-/-}) and WT were exposed to photo-oxidative damage to assess independently the functional significance of *Clqa* (classical pathway) and *CfB* (alternative pathway) on retinal degeneration. To control for any underlying effects, I thoroughly assessed *CfB*^{-/-} and *Clqa*^{-/-} mice raised in low light conditions for any histopathological or physiological changes, compared to WT (Appendix).

There was no significant difference in the amplitude of a-wave, b-wave and cone responses in the *Clqa*^{-/-} and *Cfb*^{-/-} mice compared to WT. Also, there was no significant change in the expression of genes encoding neurotrophic factors (*Cntf*, *Fgf2*), and the stress marker *Gfap*. A reduction in expression was detected in *Fgf2* in *Clqa*^{-/-} retinas (P<0.05), but not in *Cfb*^{-/-} retina (Appendix).

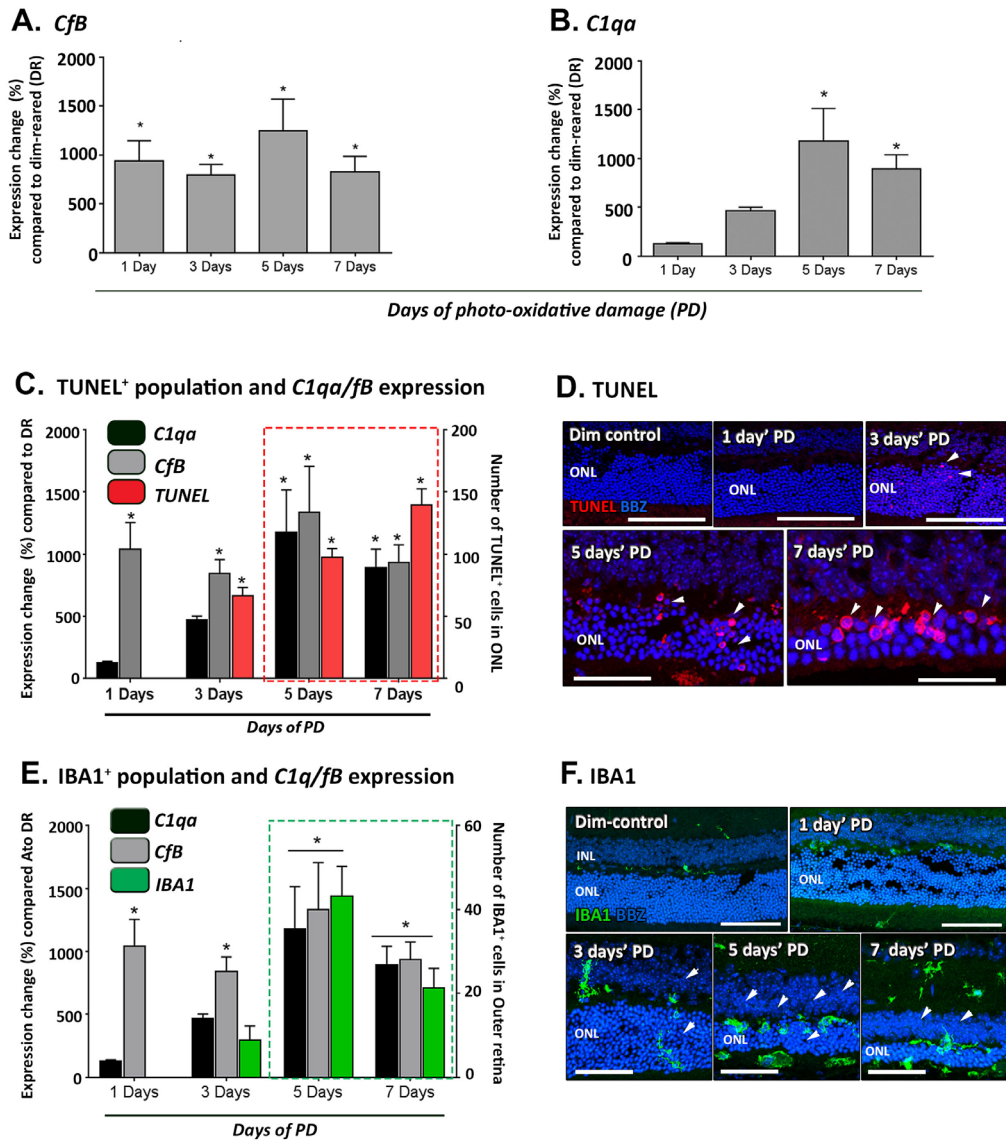


Figure 6.1 *Clqa* and *CfB* gene expression following days of photo-oxidative damage (PD) in wild-type animals (WT). **A:** Upregulation *CfB* was observed throughout 1-7 days of PD, compared to dim-reared (DR) control ($P < 0.05$). **B:** Upregulation of *Clqa* gene expression was significant at 5 and 7 days of PD ($P < 0.05$). **C:** Increasing numbers of TUNEL⁺ cells in the outer retina was temporally associated with changes in expression of *CfB* and *Clqa*. **D:** TUNEL⁺ cells (red; arrows) were most abundant in the ONL at 5 and 7 days, and absent in dim-control. **E:** Increasing number of IBA1⁺ macrophages in the outer retina was significant at 5 and 7 days of PD, and overlapped with upregulation of *CfB* and *Clqa* ($P < 0.05$). **F:** IBA1⁺ cells were prominent in retinas at 5 and 7 days of PD and included the activated / amoeboid cells in outer retina and subretinal space. ONL, outer nuclear layer; INL, inner nuclear layer. For all images, scale bars represent 50 μm . Statistical significance was determined by student t-test and two-way ANOVA accompanied with post-hoc multiple comparison ($N = 5-6$ per group, *represents $P < 0.05$).

***CfB*^{-/-} after 7 days of photo-oxidative damage**

Compared to controls, *CfB*^{-/-} mice had less evidence of retinal pathology, and improved retinal function at 7 days of photo-oxidative damage. Histological analysis of *CfB*^{-/-} and WT retinas showed TUNEL⁺ photoreceptors (Figure 6.2 B) as well as a thinner ONL after 7 days of photo-oxidative damage. However, the number of TUNEL⁺ photoreceptors was reduced by 50% in *CfB*^{-/-} retina compared to WT ($P < 0.05$, Figure 6.2 B-D). There was also a significantly thicker ONL in *CfB*^{-/-} mice compared to WT ($P < 0.05$, Figure 6.2 E). There was no significant difference in the number of photoreceptor rows between the *CfB*^{-/-} and WT retinas at the focal lesion spot, 0.5mm adjacent to the optic disc ($P > 0.05$, Figure 6.2 F). Towards the lesion edge (at mid-peripheral ~1mm and peripheral locations ~1.5mm), there was a significant increase in the number of photoreceptor rows in the *CfB*^{-/-} retina ($P < 0.05$, Figure 6.2 F).

The cumulative effect of photoreceptor cell death on retinal function was assessed in the *CfB*^{-/-} and WT following 7 days of photo-oxidative damage using electroretinography (ERG). *CfB*^{-/-} mice displayed a significantly increased amplitude in a-wave, b-wave, and cone b-wave response, compared to WT following 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.2 G – J). ERG response characteristics were significantly different between *CfB*^{-/-} retina and WT for both the a-wave and b-wave over the range of flash intensities. The differences were most pronounced at highest flash intensity of 1.9 logcd.s/m² ($P < 0.05$, Figure 6.2 G).

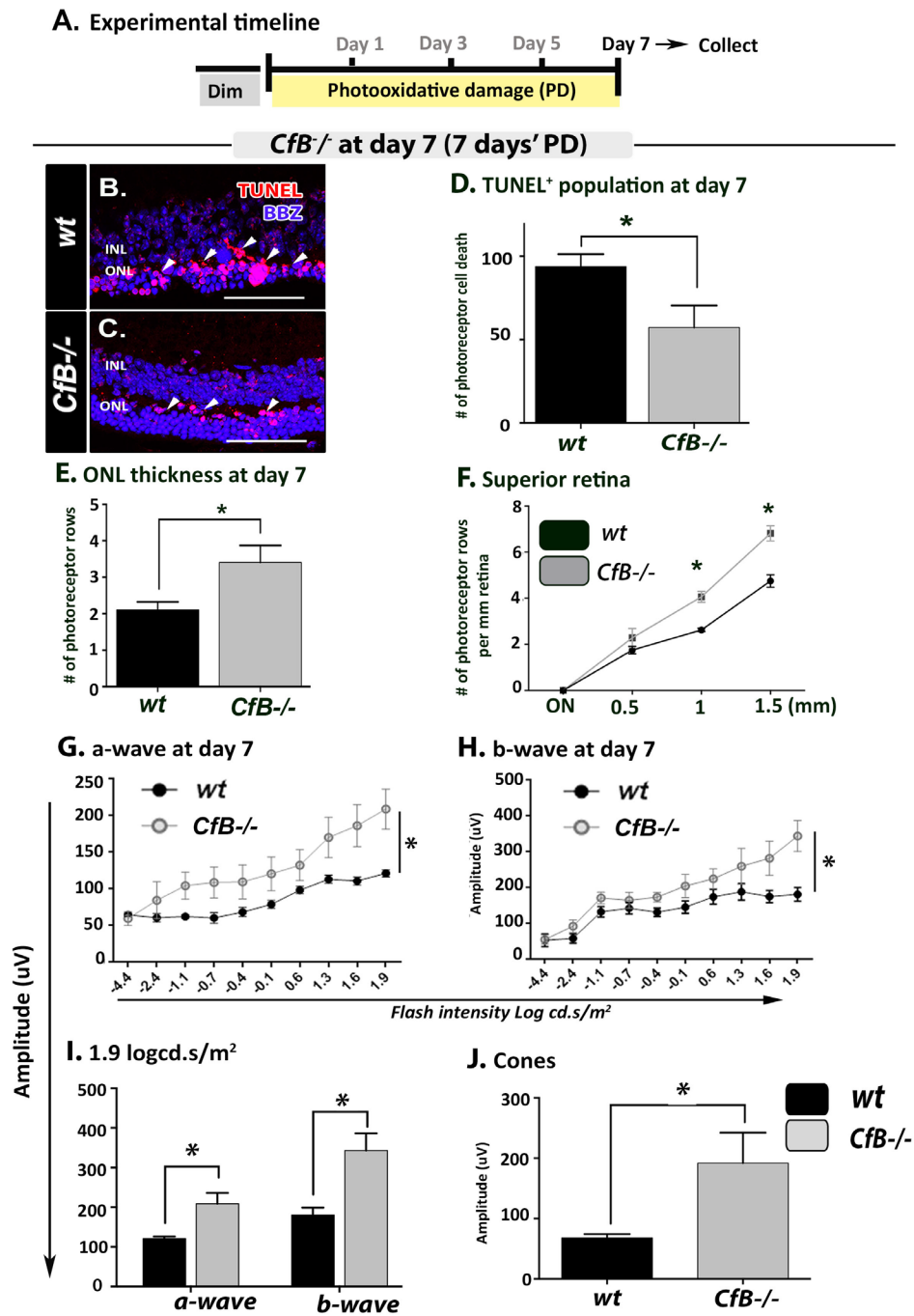


Figure 6.2 Retinal morphology and function in *CfB*^{-/-} and wild-type (WT) mice at 7 days of photo-oxidative damage (PD) (A). **B-D**: TUNEL⁺ cell death was most abundant in WT retina at 7 days of PD, and *CfB*^{-/-} retinas had fewer TUNEL⁺ cells than WT retinas overall. **E-F**: *CfB*^{-/-} retinas had more photoreceptor rows compared to WT ($P < 0.05$, E), particularly away from the central lesion in superior retina (~1 to 1.5mm away from the optic nerve) ($P < 0.05$, F). **G-J**: ERG data indicated that *CfB*^{-/-} animals had significantly higher a-wave (G) and b-wave (H) amplitudes, as well as stronger cone responses (J). The functional differences were most pronounced at the brighter flash intensities ($P < 0.05$; I). Statistical significance was determined by student t-test and two-way ANOVA accompanied with post-hoc multiple comparison ($N = 5$ per group, * represents $P < 0.05$). INL: inner nuclear layer; ONL: outer nuclear layer. For representative images, scale bars represent 50 μ m.

IBA1⁺ macrophages in the ONL and subretinal space of WT retinas were amoeboid, or reactive in morphology, whereas macrophages in *CfB*^{-/-} retinas mainly had a ramified/resting morphology in the ONL, with occasional amoeboid subretinal IBA1⁺ macrophages present (Figure 6.3 B-E). Total counts of IBA1⁺ cell population demonstrated that there were significantly fewer IBA1⁺ macrophages in the ONL and subretinal space of *CfB*^{-/-} mice compared to WT after 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.3 F). To assess the inflammatory status in the retina, expression of pro-inflammatory cytokines *IL-6* and suppressor of cytokine signalling -1 (*Socs-1*) were investigated. *Socs-1* mediates the trafficking and survival of leukocytes in ocular inflammation [376, 377]. Both cytokines were expressed in significantly lower levels in *CfB*^{-/-} retina compared to WT at 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.3 G). Furthermore, complement deposition in the retina was assessed by the abundance of retinal C3d which is a long-lived by products of C3 proteolysis and its accumulation propagates the complement activation [378]. Western blotting for the abundance of the retinal C3d proteins showed a significantly lower intensity of C3d in *CfB*^{-/-} retina compared to WT retina at 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.3 H, I). Expression of the classical complement genes *C4a*, *C1qa*, and *SERPING1* were elevated in *CfB*^{-/-} retina compared to WT ($P < 0.05$ Figure 6.3 J), however, *C2* expression was not significantly different ($P > 0.05$, Figure 6.3 J). Expression of *CfH* was significantly lower in *CfB*^{-/-} compared to WT ($P < 0.05$, Figure 6.3 J).

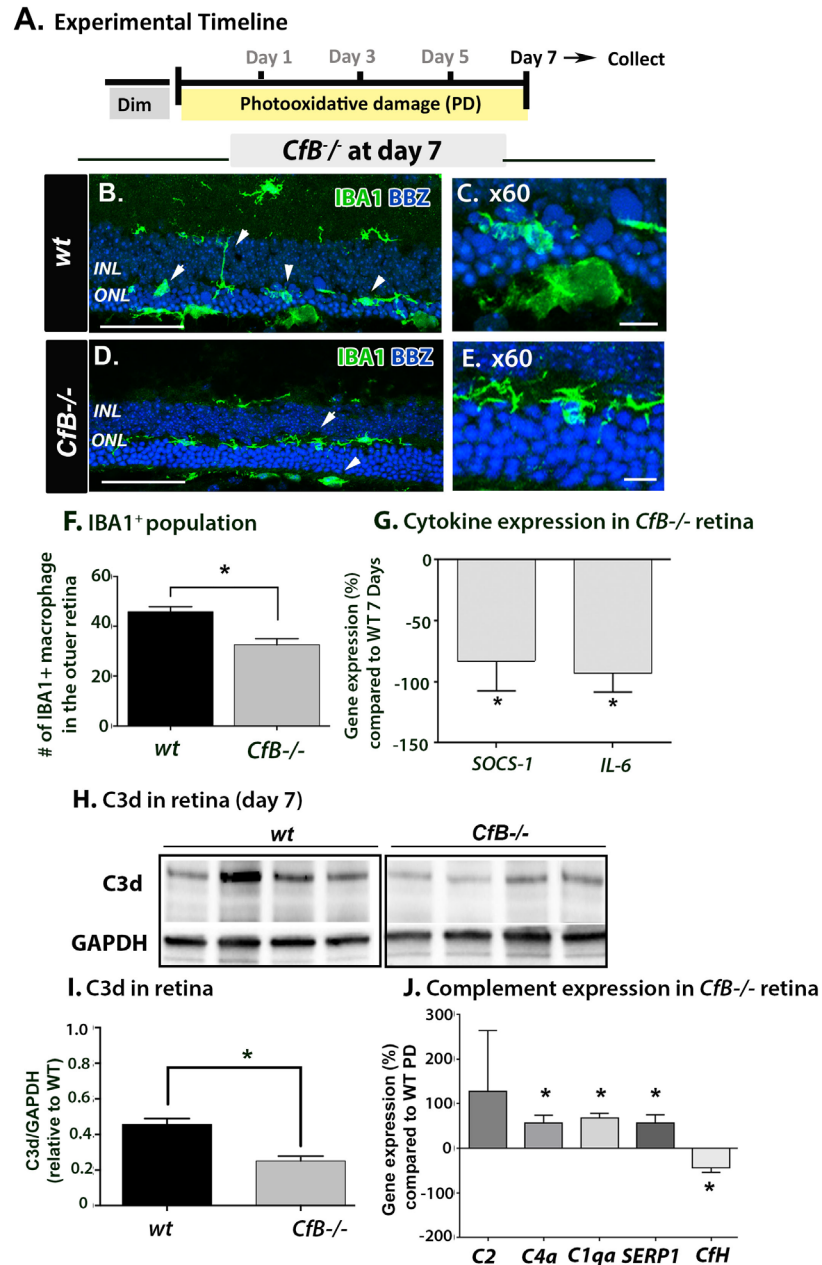


Figure 6.3 Measures of inflammation in *CfB^{-/-}* cf wild-type (WT) retinas after 7 days of photo-oxidative damage (PD) (A). **B-C:** IBA1⁺ cells with a reactive / amoeboid morphology were numerous in the outer retina (ONL and subretinal space) of WT animals. **D-E:** Fewer IBA1⁺ cells were observed in the outer retina of *CfB^{-/-}* mice (arrows) and those IBA1⁺ macrophages in the ONL of *CfB^{-/-}* retina showed a ramified / resting morphology. **F:** Counts of IBA1⁺ cells indicated significantly less cells in *CfB^{-/-}* retinas compared to WT ($P < 0.05$). **G:** Expression of pro-inflammatory cytokines *Socs-1* and *IL-6* were significantly reduced in *CfB^{-/-}* retina compared to WT ($P < 0.05$). **H-I:** Immunoblots for C3d proteins in whole retinas indicated less abundance of C3d proteins in *CfB^{-/-}* retinas compared to WT, when normalised to the loading control (GAPDH). **J:** Classical complement genes (*C4a*, *C1qa*, *SERP1*) were significantly upregulated in *CfB^{-/-}* retina, compared to WT at 7 days of PD ($P < 0.05$); there was no significant change in *C2* gene expression but there was a reduced expression of the negative regulator of the alternative pathway *CfH*, in *CfB^{-/-}* retina compared to WT ($P < 0.05$). Statistical analyses were performed using student t-test and one-way ANOVA

followed by post-hoc multiple comparison (N = 6; *represents $P < 0.05$). For representative images, scale bars represent 50 μ m.

***Clqa*^{-/-} after 7 days of photo-oxidative damage**

After 7 days of photo-oxidative damage similar levels of photoreceptor death, indicated by numbers of TUNEL⁺ cells, were observed in *Clqa*^{-/-} and WT retinas ($P > 0.05$, Figure 6.4 B-D). Measures of retinal morphologies including thickness of the photoreceptor layer at the focal lesion spot and towards the lesion edge were comparable ($P > 0.05$, Figure 6.4 E-F). Comparable numbers of IBA1⁺ macrophages were found in the outer retina of *Clqa*^{-/-} and WT animals following 7 days of photo-oxidative damage ($P > 0.05$, Figure 6.4 G- I). Immunoblots for complement C3d proteins showed no difference in the abundance of C3d proteins in *Clqa*^{-/-} retina and WT at 7 days of photo-oxidative damage (Figure 6.4 J); and C3d protein expression normalised to the loading control, did not significantly change in *Clqa*^{-/-} and WT ($P > 0.05$, Figure 6.4 K).

ERG analysis showed no differences in a-wave, b-wave, or cone responses in *Clqa*^{-/-} mice compared to WT ($P > 0.05$, Figure 6.4 L, M, N). Expression of complement genes in the classical pathway (*C2*, *C4a*, *SERPING1*), however, were significantly lower in *Clqa*^{-/-} retinas compared to WT ($P < 0.05$, Figure 6.4 O). *CfH* expression (alternative pathway) was significantly elevated in *Clqa*^{-/-} retinas at 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.4 O), however, there was no significant change in the expression of *CfB*.

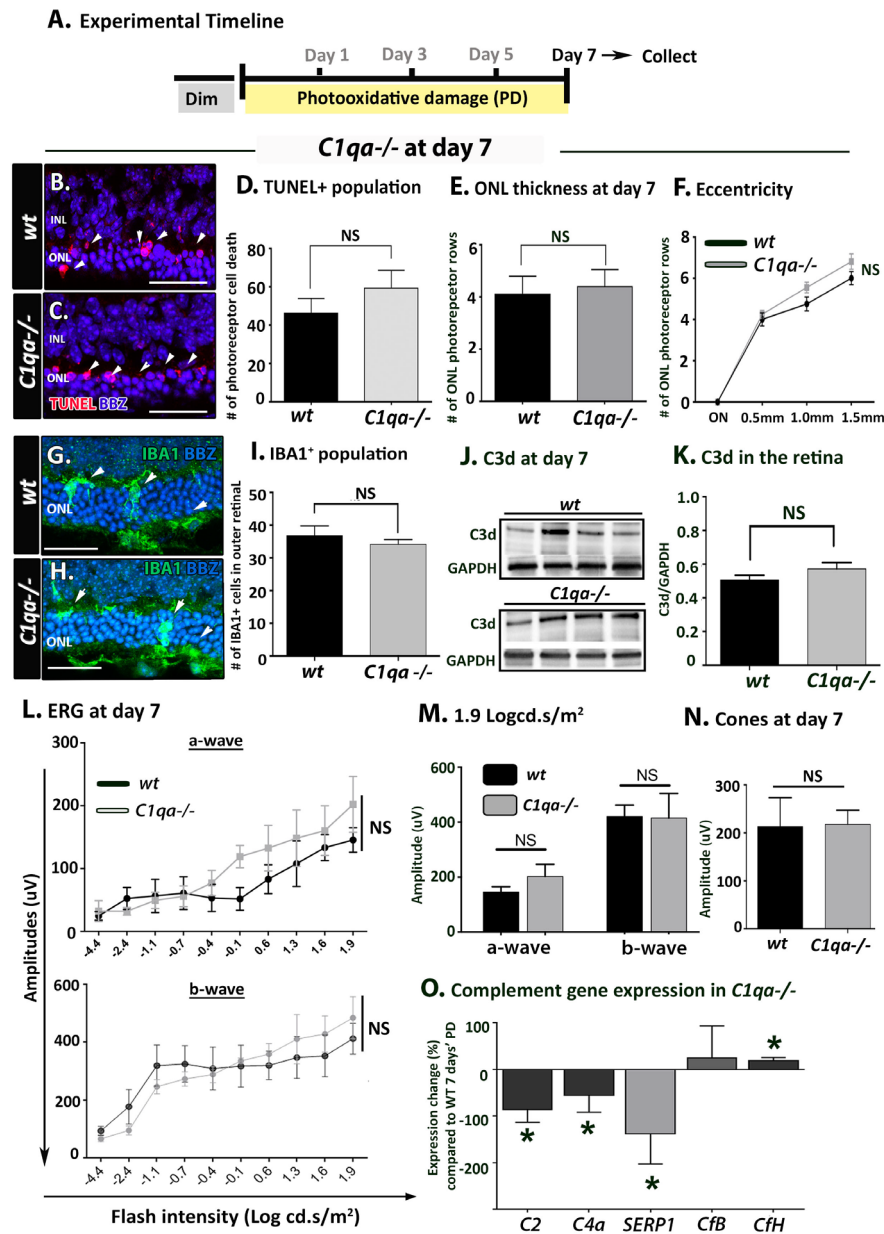


Figure 6.4 Retinal morphology and measures of inflammation in *C1qa*^{-/-} retinas at 7 days of photo-oxidative damage (PD) (A). B-C: Photoreceptor cell death indicated using TUNEL at the focal lesion site in wild-type (WT) and *C1qa*^{-/-} retinas. D-F: Quantitative analyses showed no significant difference between *C1qa*^{-/-} retinas and WT retinas in the total numbers of TUNEL⁺ cells (D), thickness of the ONL at the lesion site (E), or in adjacent retina (E, F) (NS, $P > 0.05$). G-I: There was no difference in the morphology of IBA1⁺ cells in WT and *C1qa*^{-/-} retinas (G,H) or in the numbers of IBA1⁺ cells present after 7 days of PD (I) (NS, $P > 0.05$). J-K: Abundance of C3d proteins by Western blotting appeared comparable in the two groups (J), and confirmed by optical densitometry (K). L-N: No significant differences in retinal function were detected in *C1qa*^{-/-} compared to WT at 7 days of PD. O: Expression of classical complement genes *C2*, *C4a*, and *SERP1* significantly reduced in *C1qa*^{-/-} retinas compared to WT ($P < 0.05$), whereas the *CfH* of the alternative pathway displayed a significant upregulation ($P < 0.05$), but had no significant change in *CfB* ($P > 0.05$). Student t-test, one- and two-way ANOVA followed by post-hoc multiple comparison were used for analysis (N = 6 per group, *represent $P < 0.05$; NS represents no significance). For representative images, scale bars represent 50 μ m.

The effect of *Clqa* knockout: Extended timecourse

To assess an effect of the classical pathway on photoreceptor damage beyond the standard 7-day time course, the impact of *Clqa* gene ablation over an extended period *after* photo-oxidative damage was determined (days 8-14, Figure 6.5A). Retinal expression of *Clqa* was significantly elevated in WT mice between day 8 (one day after the end of photo-oxidative damage) and day 14 ($P < 0.05$, Figure 6.5 B) and accompanied by elevated levels of TUNEL⁺ photoreceptor cell death ($P < 0.05$, Figure 6.5B). The number of TUNEL⁺ photoreceptor cell death was significantly less in the *Clqa*^{-/-} compared to WT at day 14 ($P < 0.05$, Figure 6.5 C, D), and the ONL was significantly thicker ($P < 0.05$, Figure 6.5 E). The ONL of the superior retina in *Clqa*^{-/-} mice was better preserved at the lesion site (0.5mm) and at 1.0mm away from the optic disc at day 14, indicated by the presence of significantly more rows of photoreceptors in the ONL compared to WT ($P < 0.05$, Figure 6.5 F).

The retinal macrophage response in *Clqa*^{-/-} mice was attenuated at day 14 (Figure 6.5 G-H). Fewer IBA1⁺ macrophages were present in the ONL of *Clqa*^{-/-} at day 14 compared to WT (Figure 6.5 G), and the numbers of IBA1⁺ macrophages infiltrating the ONL and subretinal space were significantly less than WT ($P < 0.05$, Figure 6.5 H). Immunoblots of complement C3d in *Clqa*^{-/-} and WT retina showed no difference in the abundance of complement C3d deposition in *Clqa*^{-/-} retina compared to WT at day 14 ($P > 0.05$, Figure 6.5 I-J).

ERG analyses showed clear differences in retinal function at day 14 between *Clqa*^{-/-} and WT ($P < 0.05$, Figure 6.5 K-N). The a-wave rod response and b-wave response amplitudes to flash intensities $\geq 0.6 \log \text{cd.s/m}^2$ were significantly greater in *Clqa*^{-/-} retina compared to WT ($P < 0.05$, Figure 6.5 K, L). Differences in ERG response characteristics between *Clqa*^{-/-} and WT were significant across increasing flash intensities, with the most

pronounced differences observed at 1.9 logcd.s/m² ($P < 0.05$, Figure 6.5 M). There were no differences in the cone responses between the two groups ($P > 0.05$, Figure 6.5 N). Comparison of ERG responses at day 7 (7 days of photo-oxidative damage) and day 14 (7 days after photo-oxidative damage) indicate improved retinal function in *Clqa*^{-/-} animals at 14 days ($P < 0.05$, Figure 6.5 O). At 7 days of photo-oxidative damage, *Clqa*^{-/-} retina had significantly better a-wave responses (~100μV) and b-wave responses (~200μV) than WT at 1.9 logcd.s/m² ($P < 0.05$, Figure 6.5 O). At day 14, even at low flash intensities, the a-wave amplitudes in *Clqa*^{-/-} retinas were significantly greater than in WT retinas.

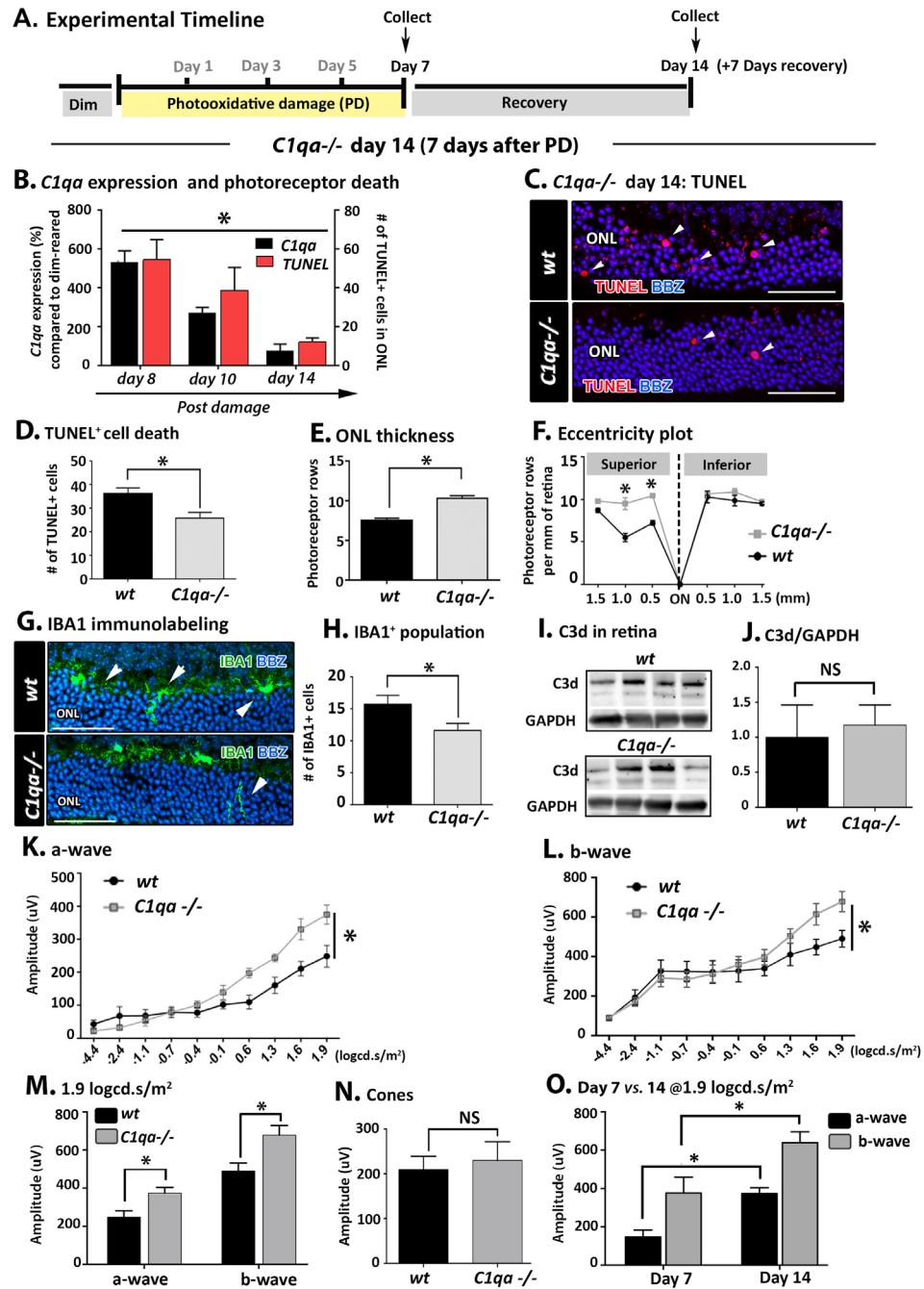


Figure 6.5 *C1qa*^{-/-} and wild-type (WT) retina at day 14 (7 days after the end of photo-oxidative damage) (PD) (A). **B:** *C1qa* expression was significantly elevated compared to WT between day 8 and day 14, and the expression level decreased across the days of recovery, in concert with the wave of TUNEL⁺ photoreceptor cell death ($P < 0.05$). **C-D:** TUNEL⁺ cells were present in the ONL of *C1qa*^{-/-} and WT retinas, although the rate of cell death was significantly lower in *C1qa*^{-/-} retinas ($P < 0.05$). **E-F:** Photoreceptor row counts were significantly higher in *C1qa*^{-/-}, compared to WT, in the superior retina (0.5mm-1.0mm away from the optic nerve) ($P < 0.05$). **G-H:** Fewer IBA1⁺ cells were found in the ONL of *C1qa*^{-/-} retinas compared to WT, and was statistically significant ($P < 0.05$). **I-J:** Immunoblots showed that there was no significant difference in the relative intensity of C3d proteins between *C1qa*^{-/-} and WT retinas ($P > 0.05$). **K-L:** There was a stronger a-wave and b-wave responses in *C1qa*^{-/-} animals compared to WT, particularly at higher flash intensities ($P < 0.05$). **M:** At the highest flash intensities, *C1qa*^{-/-} animals had significantly

higher a-wave and b-wave amplitudes compared to WT ($P < 0.05$). **N:** Cone responses were not significantly different in *Clqa*^{-/-} and WT animals ($P > 0.05$). **O:** Comparison of a-wave and b-wave responses at day 7 and 14 showed significantly higher amplitudes in *Clqa*^{-/-} retina at day 14. Statistical significance was measured using student t-test and two-way ANOVA followed by post-hoc multiple comparison ($N = 6$ per group; * represents $P < 0.05$; NS represents no significance). For representative images, scale bars represent 50µm.

Inflammasome expression in *Clqa*^{-/-} mice

To further assess the potential mechanism involved in modulation of the retinal inflammation observed in *Clqa*^{-/-} mice, I investigated the role of inflammasome activation in photo-oxidative damage. C1Q is known to activate NLRP3 inflammasome and production of IL-1 β in mouse bone-marrow derived macrophages (BMDMs) primed with carboxyethylpyrrole adducts (CEP), which are by-products of oxidative damage in dry AMD [234]. Immunolabelling of NLRP3 demonstrates co-localisation with F4/80⁺ macrophages in the subretinal space of WT mice following photo-oxidative damage (Figure 6.6 A-B). However, NLRP3⁺ or F4/80⁺ cells were not detected in the ONL, or in other retinal layers. The NLRP3⁺ F4/80⁺ macrophages detected in the subretinal space of WT animals were distributed at the focal retinal lesion and towards the lesion edges, with the rounded amoeboid morphology of activated macrophages (Figure 6.6 A, C). No NLRP3-expressing cells were detected in the *Clqa*^{-/-} retinas, although F4/80⁺ macrophages were present in the subretinal space (Figure 6.6 D). Negative control that omitted the primary antibodies showed no specific staining for NLRP3 or F4/80 (Figure 6.6 E).

Expression of inflammasome-related genes were assessed in *Clqa*^{-/-} and WT retinas at 7 days of photo-oxidative damage, using qPCR. *Nlrp3*, *Casp1* and *Casp8* expression significantly decreased in retinas of *Clqa*^{-/-} mice compared to WT at 7 days of photo-oxidative damage ($P < 0.05$, Figure 6.6 F). Additionally, expression of the pro-inflammatory cytokine genes *IL-1 β* and *IL-18* were also significantly lower in *Clqa*^{-/-} mice compared to WT (~1500%, *IL-1 β* ; ~800%, *IL-18*; $P < 0.05$, Figure 6.6 F).

FACS sorting was used to analyse expression of inflammasome-related genes in retinal CD11b⁺ cells, isolated from *Clqa*^{-/-} and WT mice following photo-oxidative damage (Figure 6.6 G). Primary myeloid cells from *Clqa*^{-/-} retinas had a significantly reduced expression of *IL-1β* and *Casp-1* compared to WT retinas, but there was no detectable change in the expression of *IL-18*, *Casp-8* or *NLRP3* ($P > 0.05$, Figure 6.6 G).

The presence of mature IL-1β protein in *Clqa*^{-/-} and WT retinas was used to confirm inflammasome activation at 7 days of photo-oxidative damage (day 7; Figure 6.6 H-I) and post damage (day 14; Figure 6.6 J-K) using Western blot. There was no significant difference in the abundance of mature IL-1β proteins observed, between *Clqa*^{-/-} and WT retinas at day 7 ($P > 0.05$, Figure 6.6 I). At day 14 post-photo-oxidative damage, the IL-1β protein band in *Clqa*^{-/-} retinas was significantly less intense than that for the WT ($P < 0.05$; Figure 6.6 K).

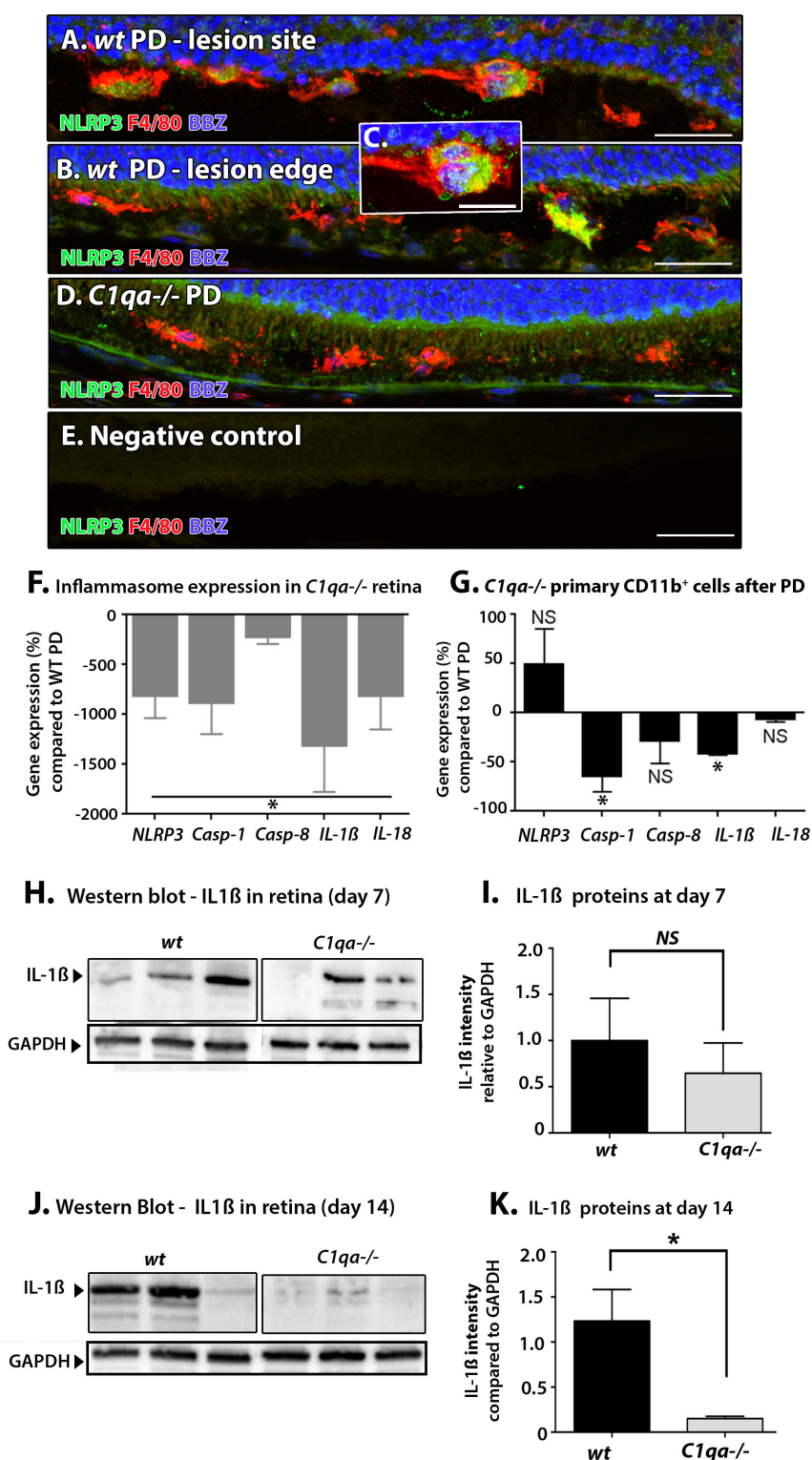


Figure 6.6 Inflammasome activation in wild-type (WT) and *C1qa*^{-/-} retinas. **A-B:** NLRP3-expressing cells (green) localised with subretinal macrophages expressing F4/80 (red) in the subretinal space both at the lesion site and lesion edges at 7 days of photo-oxidative damage (PD). **C:** NLRP3-expressing subretinal macrophages had NLRP3-immunopositive and BBZ-immunopositive nuclei, confirming its localisation within the cell. **D:** In the retina of *C1qa*^{-/-} mice, there were no NLRP3-expressing cells co-localising with F4/80⁺ macrophages. **E:** Negative control omitting the primary antibodies displayed no specific staining for either NLRP3 or F4/80. **F:** *C1qa*^{-/-} retina showed significantly reduced expression of inflammasome-related genes compared to WT following PD ($P < 0.05$). **G:** Primary myeloid cells (CD11b⁺) isolated from

Clqa^{-/-} retina by FACS had significantly reduced expression of *IL-1β* and *Casp-1* ($P < 0.05$), but not *IL-18*, *NLRP3* or *Casp-8* ($P > 0.05$). **H-I:** Immunoblots for IL-1β proteins at day 7 showed similar abundance of IL-1β proteins in *Clqa*^{-/-} and WT retinas; there was no significant change in the relative intensity of IL-1β protein bands between *Clqa*^{-/-} and WT retinas ($P > 0.05$ H). **J-K:** At day 14, there was significantly less abundance of IL-1β proteins in *Clqa*^{-/-} retinas, compared to WT, as confirmed by densitometry ($P < 0.05$). Statistical analyses were performed using student t-test and one-way ANOVA followed by post-hoc multiple comparison (N = 4 per group; * represents $P < 0.05$; NS represents no significance). For representative images, scale bars represent 50μm.

Intravitreal C1q inhibition using ANX-M1 anti-C1q antibody

To determine whether local inhibition of C1 activation could mitigate the progression in retinal atrophy, I used an antibody against C1q (anti-C1q) (ANX-M1, Annexon Biosciences), to block the classical complement cascade. Intravitreal ANX-M1, but not its immunoglobulin G (IgG) isotype control, administered immediately after photo-oxidative damage (day 7), protected the photoreceptors against continuous degeneration at day 14 (Figure 6.7). Post-treatment, ANX-M1 significantly reduced the number of TUNEL⁺ cells in retinas at day 14, compared to IgG control antibody ($P < 0.05$; Figure 6.7 B). Comparison of retinal thickness showed that the ONL was significantly thicker ($P < 0.05$; Figure 6.7 C) in the ANX-M1 anti-C1q antibody group. Negligible effects on the number of IBA1⁺ microglia/macrophages in the outer retinal space and ONL were observed in mice treated with anti-C1q antibody, compared to IgG control ($P > 0.05$; Figure 6.7 D). ERG analyses showed that the response characteristics for rod a-wave and b-wave were significantly better in the ANX-M1 treated animals ($P < 0.05$; Figure 6.7 E, F), with the ANX-M1 cohort evidencing significantly higher amplitudes (a- and b-wave) at the highest flash intensities compared to IgG controls ($P < 0.05$; Figure 6.7 G). Cone responses were also significantly better in the post-treatment ANX-M1 groups ($P < 0.05$; Figure 6.7 H).

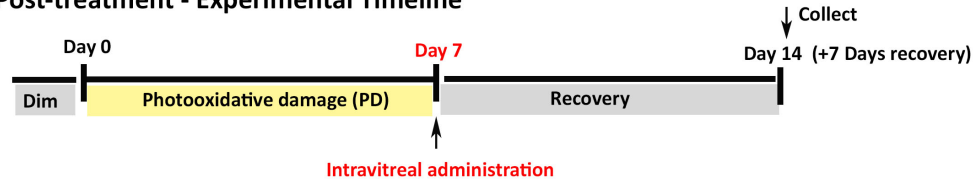
ANX-M1 administered before photo-oxidative damage showed no major protection nor morphological damage in the retinas, between the groups at day 14 (Figure 6.8). Retinal morphology (Figure 6.8 B-C), IBA1⁺ microglia/macrophage numbers (Figure 6.8 D) and ERGs (Figure 6.8 E-H) showed no significant differences between the treatment and control groups at day 14 ($P > 0.05$; Figure 6.8 B-H), indicating that intravitreal pre-treatment to neutralise C1q was ineffective.

Systemic C1q inhibition using ANX-M1 anti-C1q antibody

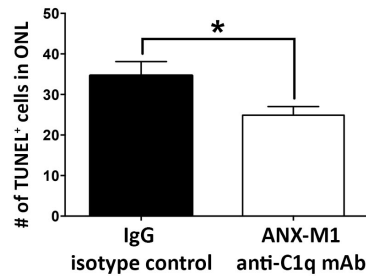
The results from animals treated systemically are presented in Figure 6.9. Haemolytic assay on mouse serum showed that by day 12, serum complement activity was reduced by $> 50\%$ compared to IgG control group, following the systemic delivery paradigm of ANX-M1 anti-C1q antibody ($P < 0.05$; Figure 6.9 B).

The impact of systemic C1q neutralisation on the retinal morphology was not significant. No differences in the number of TUNEL⁺ cells, ONL thickness or IBA1⁺ microglia/macrophage numbers were observed in mice treated systemically with ANX-M1 anti-C1q antibody ($P > 0.05$; Figure 6.9 C-E). Similarly, there were no significant functional differences in a-wave, b-wave or cone response at day 12, between ANX-M1 anti-C1q antibody and IgG control antibody ($P > 0.05$; Figure 6.9 F-I).

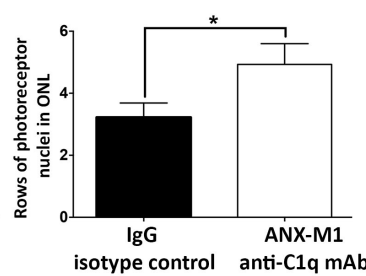
A. Post-treatment - Experimental Timeline



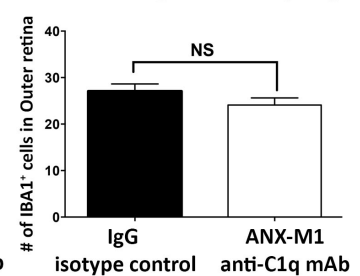
B. TUNEL⁺ cell death at day 14



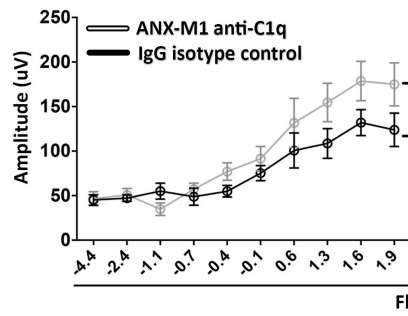
C. ONL thickness



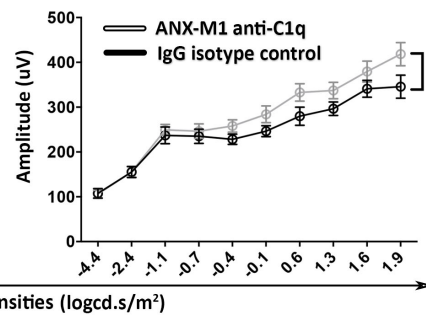
D. IBA1⁺ microglia/macrophages



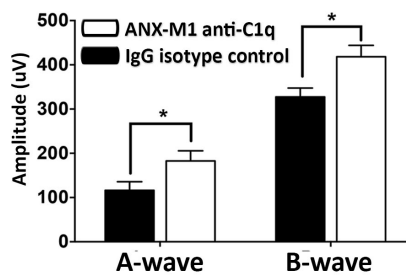
E. A-wave



F. b-wave



G. 1.9 logcd.s/m²



H. Cones

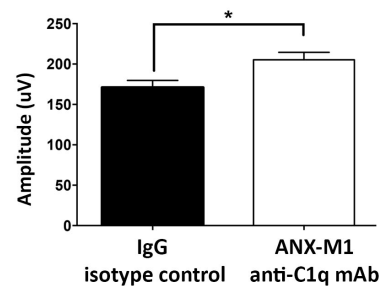
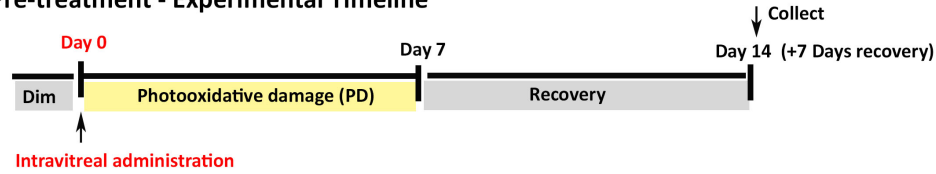
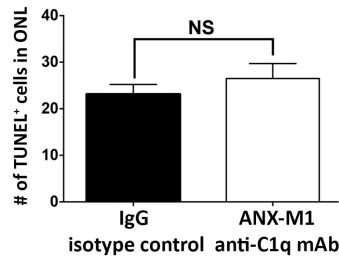


Figure 6.7 Post-treatment with local neutralisation of C1q and assessment of the retina at day 14 (**A**). **B**: Post-treatment with anti-C1q monoclonal antibody (mAb), ANX-M1, immediately after 7 days of photo-oxidative damage (PD) (day 7) reduced the number of TUNEL⁺ cells at 7 days of post damage (day 14), versus IgG control at day 14. **C**: There was a significant reduction in photoreceptor loss in mice treated with ANX-M1 anti-C1q antibody. **D**: There was minimal effect on the IBA1⁺ microglia/macrophages in ANX-M1 anti-C1q antibody cohort compared to IgG control antibody ($P > 0.05$). **E-G**: Post-treatment of retina with ANX-M1 anti-C1q antibody displayed a larger a-wave (**E**) and b-wave (**F**) compared to IgG control cohort, which was most pronounced at 1.9 logcd.s/m² (**G**), indicating a significantly improved retinal function ($P < 0.05$). **H**: Cone response demonstrated a significant improvement in ANX-M1 anti-C1q antibody cohort, compared to IgG control ($P < 0.05$). Statistical analyses were determined by an unpaired student t-test or two-way ANOVA followed by post-hoc multiple comparison (* denotes $P < 0.05$; NS denotes no significance).

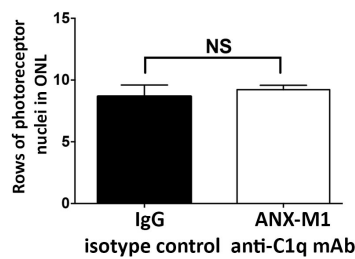
A. Pre-treatment - Experimental Timeline



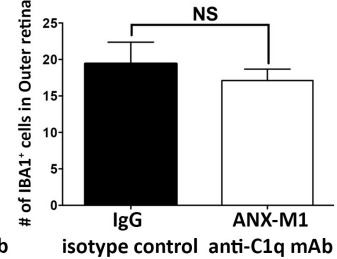
B. TUNEL⁺ cell death at day 14



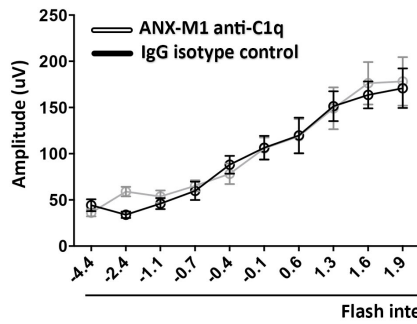
C. ONL thickness



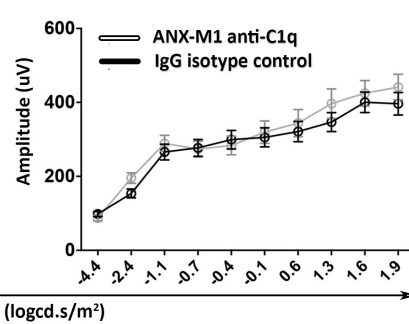
D. IBA1⁺ microglia/macrophages



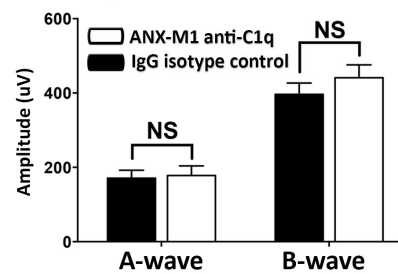
E. A-wave



F. b-wave



G. 1.9 logcd.s/m²



H. Cones

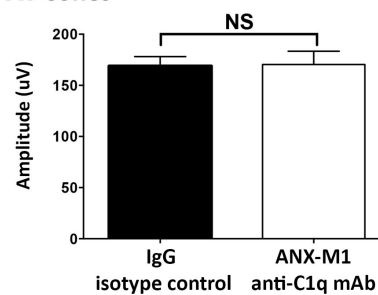


Figure 6.8 Pre-treatment with local neutralisation of C1q and assessment of retina at day 14 (A). **B:** Pre-treatment with ANX-M1 anti-C1q antibody prior to the start of photo-oxidative damage (PD) (day 0) showed no significant change in the number of TUNEL⁺ cells at day 14, versus IgG control. **C:** There was no difference in ONL thickness observed between ANX-M1 anti-C1q antibody and IgG control. **D:** Minimal effect was observed in the number of IBA1⁺ microglia/macrophages between two cohorts. **E-G:** Pre-treatment with local inhibition of classical complement did not decrease the retinal function as observed in a-wave (E), b-wave response characteristics (F) and mean amplitudes at 1.9 logcd.s/m² (G). **H:** No change in the cone response was also observed between mice treated with ANX-M1 anti-C1q antibody and IgG control. Statistical analyses were determined by an unpaired student t-test or two-way ANOVA followed by post-hoc multiple comparison (NS denotes no significance).

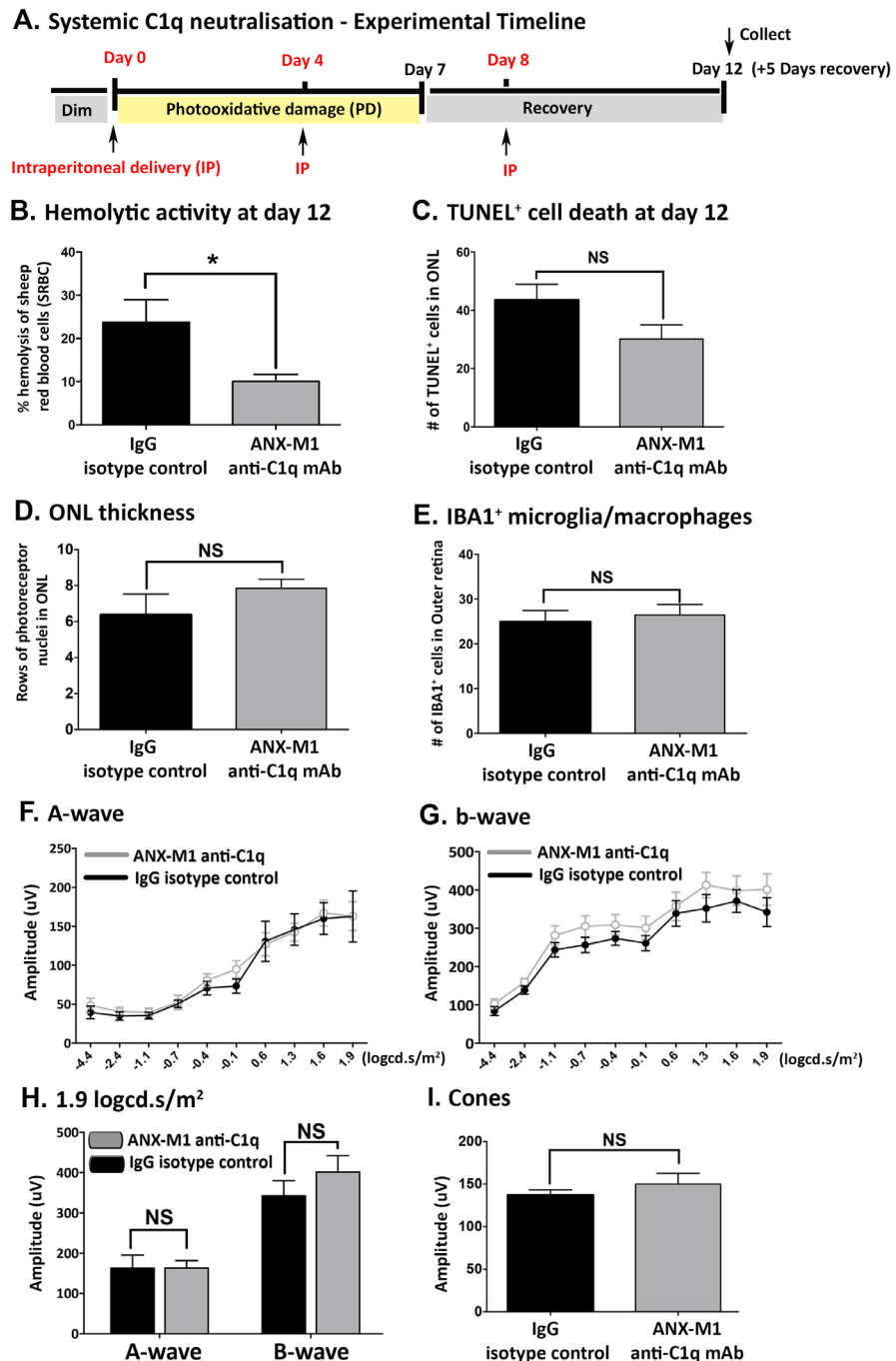


Figure 6.9 Neutralisation of systemic classical complement using ANX-M1 anti-C1q antibody during the time course of photo-oxidative damage (PD) and assessment of the retina at day 12 (**A**). **B**: Complement activity in the serum was inhibited (>50% less) in mice treated with ANX-M1 anti-C1q antibody compared to IgG control as examined in the haemolytic assay using sheep red blood cells (SRBCs). **C-E**: There was no difference in number of TUNEL⁺ cells, ONL thickness and IBA1⁺ microglia/macrophages observed between ANX-M1 anti-C1q antibody cohort and IgG control cohort. **F-H**: Inhibition of systemic classical complement did not change retinal function, as evident in no significant difference in a-wave (**F**), b-wave (**G**) response characteristics and mean amplitudes at highest flash intensities between two cohorts (**H**). **I**: No significant change in cone response was detected between two cohorts. Statistical analyses were determined by an unpaired student t-test or two-way ANOVA followed by post-hoc multiple comparison (*denotes significance; NS denotes no significance).

Localisation of complement in rodent and human donor retina: A role for macrophages

Immunohistochemistry for C1q and F4/80 in AMD donor retinas was used to gain a better understanding of how the findings in mouse retinas after photo-oxidative damage relate to human pathology. I first examined the expression of C1q in mouse retinas with negative control slide included (Figure 6.10 A-H). In dim-reared control mouse retina, C1q protein or F4/80⁺ macrophages were not detected (Figure 6.10 A). In mouse retina exposed to photo-oxidative damage, C1q protein was co-localised with F4/80⁺ subretinal macrophages which appeared in an amoeboid and activated state at the focal retinal lesion (Figure 6.10 B-D) and at the margins of the photoreceptor lesion (Figure 6.10 E-G). Negative control showed no specific staining for either C1q or F4/80 in the mouse retinal sections (Figure 6.10 H). Absence of C1q immunoreactivity was confirmed in F4/80⁺ macrophages in the outer retina after 7 days of photo-oxidative damage in C1qa^{-/-} mice (Figure 6.10 I). Immunohistochemistry for the C1Q protein in normal retina and AMD-affected retinas (Figure 6.10 J-R) showed no C1q-expressing cells in the normal retina (Figure 6.10 J). In the AMD-affected retinas, C1Q proteins were detected adjacent to the subretinal drusen (Figure 6.10 K), and in the subretinal space at the lesion edges (Figure 6.10 L-N). Double immunolabelling demonstrated that most of C1Q-expressing cells were immunoreactive for macrophage/microglia marker IBA1 (Figure 6.10 O-Q). Negative control showed no specific staining for C1Q or IBA1 in the human AMD section (Figure 6.10 R).

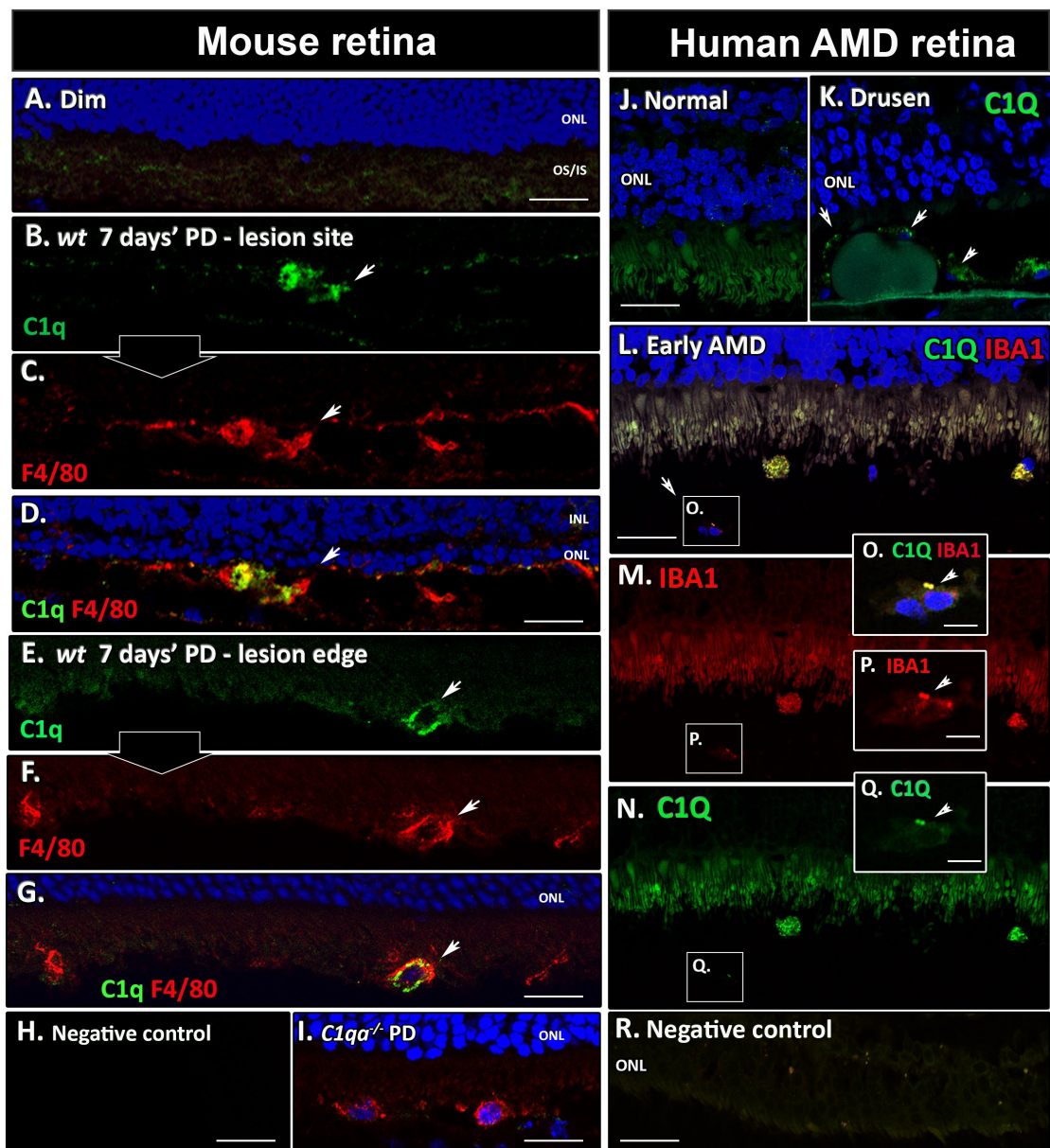


Figure 6.10 C1q localisation in the mouse retina after photo-oxidative damage (PD) and human AMD retina. **A:** No immunoreactivity to C1q or F4/80 was detected in retinas from dim-reared/control animals. **B:** At 7 days of PD C1q⁺ cells were detected in the subretinal space at the lesion site. **C:** At 7 days of PD, F4/80⁺ cells were also detected at the lesion site. **D:** Co-localisation of C1q and F4/80 was observed in cells in the subretinal space at the lesion site. **E-G:** At the lesion edge C1q⁺/F4/80⁺ macrophages were present in the subretinal space. **H:** Negative control slide for the mouse retinal immunohistochemistry showed no specific staining for either C1q or F4/80. **I:** C1Q immunoreactivity was detected adjacent to the subretinal drusen of early AMD retinas (arrows). **J:** C1q localisation in the normal human retina. No C1q-expressing cells were detected in the normal retina. **K:** C1q immunoreactivity was detected adjacent to the subretinal drusen of early AMD retinas (arrows). **L-N:** At the lesion edge, C1Q-expressing cells were present in the subretinal space of AMD retina where IBA1⁺ cells were predominant (arrows). **O:** C1Q⁺ immunoreactivity co-localised in IBA1⁺ macrophages. **P, Q:** C1Q⁺/IBA1⁺ cells at higher magnification. **R:** Negative control slide for the human AMD immunohistochemistry showed no specific staining for either C1Q or IBA1. Representative images derived from N =3 per group. INL, inner nuclear layer; ONL, outer nuclear layer; OS/IS, outer/inner segments. Scale bars represent 10um in O-Q; elsewhere 50um.

6.4 Discussion

This study directly compares the contributions of the alternative (*CfB*) and classical complement (*Clqa*) pathways in the timing of disease progression induced by photo-oxidative damage. This study further illustrates the role of locally derived retinal *Clqa* complement in driving progression of retinal atrophy.

The findings indicate that: **First**, genetic ablation of *CfB* dampened activation of the alternative pathway and attenuated the retinal inflammation such as a reduced macrophage infiltration and pro-inflammatory cytokine expression. This ultimately protects the retinal morphology and function against degeneration at 7 days of photo-oxidative damage. **Second**, genetic ablation of *Clqa* indicated variable and inconclusive effects on retinal function during the 7 days of photo-oxidative damage. Over the longer term however, in the week after photo-oxidative damage, the data indicate that absence of C1q protects retinal structure and is associated with improved retinal function. **Third**, the data demonstrate a possible role for the classical complement in the engagement of NLRP3 inflammasome and secretion of IL-1 β in the degenerating retina, which may promote retinal damage during this post-exposure period. **Last**, by neutralising the classical complement C1q in the retina, I demonstrate that local C1q, expressed by subretinal macrophages, and not serum complement, plays a vital role in the progression of retinal degeneration. Together, the results show that the alternative pathway is not the sole contributor to the progression of retinal degeneration. This indicates that a therapeutic approach targeting the classical complement initiator C1q may be the most useful option to slow progression of focal retinal degeneration, and by implication, AMD.

Our previous studies have shown upregulation of a range of complement pathway genes, including *CfB*, *C3* and *CfH* after photo-oxidative damage [371] and have demonstrated a critical role for C3-expressing retinal macrophages using *C3*^{-/-} mice [371].

Other investigators utilising the knockdown of specific complement pathway genes have demonstrate the involvement of complement pathways in the development of experimental choroidal neovascularization in *CfB*^{-/-}, *C1qa*^{-/-} and *MBL*^{-/-} mice [374], and retinal degeneration induced by photo-oxidative damage [145]. Furthermore, local *CfB* expression by RPE cells is demonstrated to facilitate experimental choroidal neovascularization [382].

The alternative complement pathway in retinal atrophy

The findings presented in this chapter suggest that CfB of the alternative pathway is an instigator of complement-mediated inflammation and may facilitate processes associated with the onset of retinal atrophy induced by photo-oxidative insults.

To our knowledge this is the first study to elucidate the functional significance of *CfB* in complement activation in photo-oxidative damage. Evidence has shown the sufficiency of *CfB* to promote laser-induced choroidal neovascularisation [148, 382], as well as retinal detachment [350]. The present data extends the collective findings on the role of CfB, by showing that genetic ablation of CfB prevents excessive complement activation such as the accumulation of C3d in the photo-oxidative damage-exposed retina. This explains the reduced severity of the retinal atrophy in *CfB*^{-/-} animals at day 7.

The robust upregulation of classical pathway genes (*C1q*, *C4a* and *SERPING1*) in *CfB*^{-/-} retina suggest that the absence of one pathway (alternative) may lead to a heightened involvement of the classical pathway, compensating for the loss of the alternative pathway amplification. The findings presented in this chapter suggest that CfB, as the necessary part of the amplification loop of the alternative pathway, could be a potential target for intervention to dampen severity of the early events, and mitigate the onset of degeneration. *CfB* was identified as expressed by IBA1 cells by qPCR analysis [155]. The identification of CfB protein is not included in the study because the CfB

response was short-lived and appears not relevant to the progression of the disease. As shown by the data, CfB dysregulation settles quickly, therefore inhibition of CfB alone is not likely to be helpful in preventing the progression of retinal atrophy.

The classical pathway in retinal atrophy

C1Q is associated with pathological features of age-related neurodegenerative diseases such as amyloid- β accumulation in Alzheimer's Disease (AD), glaucomatous damage in the DBA/2J spontaneous glaucoma model and drusen deposition in AMD [140, 383, 384]. In AMD, C1Q of drusen deposits and IgG deposition in the choriocapillaris both associate with inflammation in the retina [385]. One possibility is that downstream complement activation is triggered by C1Q sequestered in drusen, where it detrimentally affects the photoreceptor population and enhances the progression of retinal atrophy. Others, however, have reported a neuroprotective influence of C1q on the survival of cone photoreceptors in *Rho*^{-/-} mice and elucidated on the maintenance of C1q for cone cell preservations in hereditary retinopathies [386].

In this study, I demonstrate that *Clqa*^{-/-} mice did not change the local deposition of complement C3d at day 14 although the retinal inflammatory profile was dampened. Evidence from other studies indicates that non-canonical functions of C1q influence local inflammation in the central nervous system (CNS), such as by promoting the dynamic shift of astrocytes to a more pro-inflammatory state which is associated with AD and Huntington's Disease [387]. Moreover, C1q is involved in the elimination of defective synapses which significantly preserved the functional neurons in transgenic mouse model of AD deficient for *Clq* [388]. The ablation of *Clqa* further prevented the progressive loss of retinal ganglion cells in a mouse model of glaucoma [106].

The present findings suggest that C1q of the classical pathway facilitates the progression of late stage retinal atrophy, but not the onset upon photo-oxidative damage.

Studies in our laboratory have previously shown upregulation of classical complement components *C1s*, *C4a* and *C2* in retinas following photo-oxidative damage [371], but their specific roles have not been described. Other studies on the pathological effects of *C1qa* in neurodegeneration have been implicated in mouse models of retinal ischemic injury [389], and glaucoma [106]. The findings presented in this chapter show that initially (days 1-7 of photo-oxidative damage) the absence of *C1qa* has no definitive impact on retinal damage induced by photo-oxidative damage, but over an extended timeframe sustained expression of *C1qa* is a driver of progressive loss of photoreceptors. Rohrer et al. (2010) suggest an involvement of the activation of the classical complement in the development of choroidal neovascularisation, in the late stage of AMD in a laser-induced rodent model of choroidal neovascularisation, since the alternative complement is not sufficient to produce the pathology [374]. Consistent with this concept, the data here demonstrates that localised post-treatment with ANX-M1 C1q inhibition provides protection against retinal degeneration during the progression phase of damage (7-14 days), but not earlier during the onset of retinal degeneration. The findings suggest the potential role of C1q in mediating the progression of retinal degeneration. Furthermore, the data demonstrating no impact from the pre-treatment with ANX-M1 C1q inhibition reinforces the concept that the elimination of late-involvement of C1q protects against degeneration.

C1Q and inflammasome activation by subretinal macrophages in photo-oxidative damage

C1Q stimulation activates the NLRP3 inflammasome and the secretion of IL-1 β in CEP-primed BMDMs [234]. Therefore, it is plausible that the classical pathway triggers other inflammatory mechanisms to further promote progressive photoreceptor degeneration and retinal atrophy.

NLRP3 inflammasome activation in AMD has been proposed to occur in response to several stimuli including drusen components, RPE and complement proteins, oxidative stress, oxidative by-products and DNA [234, 260, 390]. In this study, in the absence of classical pathway activation, there was a reduced NLRP3 inflammasome expression in a specific population of cells - the subretinal macrophages. This has resulted in protection of the retina from the progressive retinal atrophy. This finding is consistent with previous studies showing that C1Q can activate NLRP3 in bone-marrow derived macrophages of humans, and in mice after immunization with CEP-adducted mouse serum albumin, to model a dry AMD-like pathology [234]. Other evidence has demonstrated the capability of RPE cells to induce NLRP3 inflammasome expression and propagate photoreceptor toxicity in the presence of blue-light photo-oxidative damage [184], although NLRP3 expression in RPE cells was not detected in this model. The findings presented in this chapter also show significant down regulation of IL-1 β in isolated retinal macrophages from *Clqa*^{-/-} retinas compared to wild-type controls. This was associated with a better-preserved photoreceptor population at day 14. The findings indicate that the reduced activity in the classical pathway dampens NLRP3 inflammasome expression along with pro-inflammatory IL-1 β secretion, resulting in protection of the photoreceptor population. Further studies are underway to confirm the role of NLRP3 inflammasome activation in photo-oxidative damage, and clarify the mechanism of C1q in the inflammasome activation.

Local complement-expressing macrophages as potent mediators of the onset and progression of retinal atrophy

Retinal microglia/macrophages are shown to express complement components, *CfB* and *C3* in rodent retinas in response to aging and photo-oxidative damage [155]. Chapter 4 implicates the locally expressed *C3* in mediating the onset of retinal atrophy. Here, the data demonstrate that subretinal microglia/macrophages which are the cellular

source of *C1q* play a crucial role in focal retinal degeneration. This finding positively correlates with findings from human AMD retinas (Figure 6.10). Resident microglia have been shown to express many-fold higher levels of *C1qa*, *-b* and *-c* which encode the classical complement protein C1q complex, compared to macrophages and other myeloid cells in normal and diseased brain tissues [206, 391]. The capability of microglia to synthesize *C1qa* has also been confirmed in rodent brain [392]. However, in the context of AMD, the close interdependence and proximity of the retina with Bruch's membrane/RPE/choroid may convolute the argument of microglia as the key synthesizer of C1q. The findings presented in this chapter shows that C1q proteins are localised near drusen and in the subretinal microglia/macrophages of human AMD patients whose RPE atrophy was prominent. The delayed appearance of these C1q-expressing subretinal microglia/macrophages, co-incident with RPE and choriocapillaris breakdown [31] suggests this may be a cell population derived from the choriocapillaris. However, a recent study investigating models of RPE breakdown suggests that subretinal populations are predominantly resident microglia, owing to their rapid mobilisation from the inner retina to the site of injury [204]. The findings presented in this chapter suggest that only locally-derived C1q contributes to the progression of retinal atrophy. Further investigations are required to explore the mechanisms underlying the protective effect of local C1q neutralisation. Coupled with the association of C1q expressed by myeloid cells with neuroinflammation and age-related neurodegeneration [393], the findings presented in this chapter, linking C1q with the progression of focal retinal degeneration, supports the idea that locally administered immunotherapy targeting the classical complement may be effective in slowing the progress of retinal degeneration.

Conclusions

The present study illustrates the strikingly differential contribution of the alternative complement pathway and classical complement pathway in facilitating the

retinal atrophy induced by photo-oxidative damage. The contribution of the alternative pathway, in the form of *CfB*, is to amplify the cascade and early photoreceptor damage, while the classical pathway, through *Clqa*, appears to exacerbate photoreceptor damage through engagement of the inflammasome. The efficacy of intravitreal C1q-targeted inhibition in ameliorating the late stage of photo-oxidative damage-induced retinal atrophy, reinforces the concept that targeting macrophage capability to express complement may be effective in slowing AMD progression. Future utilisation of immunohistochemical markers, or fate-mapping techniques that can elucidate the exact origins of complement-expressing macrophages – microglia or monocytes - are warranted. Clarifying the identity and function of the complement-expressing myeloid cells will enable the design of precise therapeutics that limit their capacity to elicit deleterious reactions.

7. GENERAL DISCUSSION

Previous studies conducted in our laboratory showed that *Ccl2* signalling by Müller glia governs the spatiotemporal localisation of macrophages recruited into photo-oxidative damaged retina [194, 248, 249], and that subsequent C3 deposition is mediated by retinal macrophages [146]. However, the contributions of local *versus* systemic macrophages and other complement components had not been explored. This thesis first describes the features of our new mouse model of photo-oxidative damage (Chapter 3), and then dissect the differential roles of complement component 3 (C3), factor B (CfB) and C1q complex (C1q) in focal retinal degeneration utilising the new mouse model (Chapter 4-6). Chapter 4 provides the insights into the functional significance of C3, which is synthesised by retinal macrophages in facilitating local complement activation and further promoting retinal atrophy. Chapter 5 elaborates on the contribution of C3 in shaping the microglia/macrophages activities and its association with the progression of focal retinal degeneration. Chapter 6 illustrates the significant contribution of the alternative pathway (CfB) and the classical pathway (C1q) in mediating the progress in retinal atrophy. Beneficial effects brought by the local neutralisation of C1q implicates the involvement of local C1q in promoting the progression of retinal atrophy. The thesis overall underpins the subretinal microglia/macrophages as the key sources of local complement activation which amplify the progression of focal retinal degeneration in rodent model of photo-oxidative damage.

Since the outcomes of my studies pinpoint subretinal microglia/macrophages as the potent producers of complement proteins, such as C3, C1q and CfB, targeting these locally-derived complement proteins reduces the subretinal inflammation and progression in this new mouse model of focal retinal degeneration. Furthermore, the experiments demonstrate that local administration of complement inhibition strategies, such as C3 siRNA and C1q ANX-M1 antibody have higher efficacy in slowing the progression than the systemic administration in a mouse model of focal retinal

degeneration, supporting the practice of local administration. Further, the findings highlight the classical complement activator C1q as key driver in the progression of retinal degeneration, and thus a new candidate for treatment at late stage of disease.

The findings from my studies lay foundation for the use of gene therapy to target the cellular sources of complement proteins and inflammation in AMD patients. Early studies examining the gene therapy-mediated treatments are underway in mice (CR2-FH fusion protein [394], CFH over-expression [395] and other agents inhibiting complement activation [396]) and human clinical trials (AAV-CAGsCD59; Clinical Trials Identifier NCT03144999). Additionally, the use of CRISPR/Cas9 technology is recently attempted in several models of retinal degeneration, such as *Vegfa* Cas9 ribonucleotides (RNP) to reduce choroidal neovascularisation in mice [397, 398]. The use of Cas9 RNPs may be a promising approach to modulate the complement expression in retinal microglia/macrophages and reduce inflammation.

Pivotal to these studies has been the development of a photo-oxidative damage model using pigmented C57BL/J mice, which has enabled the access to complement gene knockout animals (Chapter 3). In this model centralised focal degeneration of the outer retina, such as photoreceptor, takes place after 5-7 days of exposure to 100k lux white light. Retinal responses include upregulation of inflammatory and oxidative damage markers, such as chemokine signalling (*Ccl2*, *Ccl12* and *Ccl3*), complement upregulation and oxidation markers (4HNE, 8-OHG). Concurrently, the model also demonstrates reactive gliosis of Müller glia and microglia/macrophage activation, and atrophy of the adjoining choriocapillaris. These features commonly occur with atrophic AMD histopathology, highlighting the utility of this model for studying oxidative and inflammatory mechanisms in the retina, and how they mediate retinal atrophy. Nonetheless, we cannot exclude the utility of other arising models mimicking dry AMD,

such as the experimental NMDA model that displays signs of inflammation and complement though it lacks strong evidence of oxidative stress [399].

Chapter 4 shows that retinal macrophages synthesize C3 locally to propagate local complement activation and initiate focal retinal degeneration using genetic knockout, local knockdown of C3, and systemic complement depletion. Genome wide deletion of C3 protects the retina from the onset of degeneration after photo-oxidative damage, while local knockdown of C3 in the retina by an intravitreal delivery of C3 siRNA effectively suppresses the retinal sources of C3, and mitigates photoreceptor loss after photo-oxidative damage. Systemic depletion of C3, however, has no effect on the onset of photoreceptor degeneration. The findings indicate that sustained C3 modulation, which may be achievable using a CRISPR/Cas9 approach, is likely to have a significant beneficial impact on photoreceptor survival in the mouse model of photo-oxidative damage. The primary target for CRISPR/Cas9 would be retinal microglia/macrophages that are the major element to the complement response. CRISPR/Cas9 can be utilised to target complement expression in retinal microglia/macrophages using C3-targeted Cas9/gRNA ribonucleoproteins (RNP), which generate robust C3 modification or C3 knockdown within the cell, and with minimal off-targeted effect [400]. Lentiviral-mediated delivery or adeno-associated virus delivery [401, 402] of C3 Cas9 RNP would together ensure a prolonged knockdown effect on complement expression in retinal microglia/macrophages. However, it is still plausible that the subretinal C3-expressing macrophages infiltrate the degenerating retina from the systemic/choroidal circulation as the RPE breaks down. Clarification of the roles of recruited *versus* retinal monocytes is needed to better understand the pathogenesis. This will require accurate identification of myeloid cell subpopulations using genetic lineage fate mapping techniques in animals exposed to photo-oxidative damage and/or mouse chimeras.

The study in Chapter 5 elaborates the role of C3 in mediating retinal pathology, by comparing the responses of C57BL/6J wild-type (WT) and $C3^{-/-}$ animals, to photo-oxidative damage. The results indicate that the genetic absence of C3 has the effect of dampening the reactive gliosis of Müller glia and reducing the phagocytic activity of microglia/macrophages. Microglia / macrophages in WT retinas mediated higher levels of phagocytosis than those in $C3^{-/-}$ retinas; but in both WT and $C3^{-/-}$ retinas, approximately 50% of photoreceptors ingested by microglia/ macrophages were not TUNEL⁺. This indicates that ingestion of viable photoreceptors amongst patches of apoptotic ones may contribute to the retinal atrophy. Most interestingly, the study indicates that phagocytic capacity of subretinal macrophages neighbouring the ONL lesion is higher than that of those in the ONL, suggesting differing functional roles for subretinal macrophages.

The investigations reported in Chapter 6 examine the differing contributions of classical (C1q) and alternative complement pathways (CfB) in photo-oxidative induced retinal degeneration using knockout mice. Both C1q and CfB trigger cleavage of C3, propagating downstream complement activation. Genetic ablation of C1q and CfB, in mice exposed to photo-oxidative damage indicates that both classical and alternative pathways contribute to retinal degeneration, but on different timescales of activation. The contribution of CfB, expressed by senescent microglia/macrophages as reported previously, is demonstrated to have an immediate and early onset effect on downstream complement activation, including macrophage infiltration, subretinal inflammation and retinal atrophy. The contribution of C1q, also expressed by subretinal microglia/macrophages in retinas exposed to photo-oxidative damage and in retinas of human AMD, is shown to have a relatively minor role during onset of complement activation, since the differences in retinal gene expression, microglial activation and photoreceptor death are detected at day 14, but not at day 7, after commencement of photo-oxidative damage.

The pivotal contribution of C1q involves NLRP3 inflammasome activation in subretinal macrophages and secretion of pro-inflammatory cytokines, IL-1 β in the degenerating retina. The absence of C1q reduces the retinal inflammation and protects rodent retinas against the progressive loss of photoreceptors over an extended time course. The findings localise C1Q protein expression by retinal macrophages in the subretinal space of atrophic AMD patients, and in analogous locations in mouse retinas affected by photo-oxidative stress.

The data show that subretinal macrophages are a key cellular source of classical pathway complement activation, through the synthesis of both complement components C3 and C1q. *In situ* hybridisation for C1q mRNA expression is required to confirm that subretinal macrophages synthesise, and not simply transport C1q. These findings suggest yet another distinctive feature of subretinal macrophages which may indicate a distinct population. A hypothesis is that complement-expressing, subretinal macrophages are derived from the monocytes, and are distinct from resident microglia. In view of recent findings in RPE degeneration [204], further studies are warranted using recent advances that allow us to delineate the identity of resident microglia and monocyte-derived macrophages, and to assess the precise changes in their metabolism and /or epigenetics that control their inflammatory functions in retinal degeneration. The first method is the use of TMEM119 antibody, which is able to differentiate resident microglia from blood-derived monocytes in brain using flow cytometry and immunohistochemistry [206]. Because the antibody is commercially available, this option would be a promising approach to test in human AMD patients alongside *in situ* hybridisation for inflammatory markers, including complement genes. The second method is fate-mapping technique, which endogenously label the two cell types with fluorescent reporters and distinguish resident microglia from blood-derived monocytes using tamoxifen-inducible Cre recombinase [203]. Lastly, other established methods include and depletion of bone-

marrow derived macrophages using clodronate liposomes [403], and bone-marrow chimera, which is designed to pinpoint the contribution of blood-derived population in mice [318]. The above approaches would accurately identify the responsible cell population for inflammation and complement and tailor the design of future treatment.

The data suggest that there may be therapeutic efficacy in inhibiting the classical pathway for slowing progression of retinal degeneration. C1q is considered as the bridging molecule between the innate and adaptive immune responses. Upstream stimulators for classical complement activation include retinal autoantibodies which are evident in human AMD - as well as binding cells during late-stage apoptosis. Further investigations are required to identify the mechanism that activates the classical pathway, and which underlies the protective effect occurring as a result of local C1q neutralisation.

Overall, this thesis begins to unravel the distinct roles of local complement activation expressed by subretinal macrophages in shaping the innate immunity, amplifying the inflammatory response and contributing to the onset and progression of focal retinal degeneration in a mouse model of photo-oxidative damage. The findings place emphasis on subretinal macrophages as the potent source of locally-derived complement, and provide an indication for locally targeted therapy to suppress aberrant complement activation in retinal degeneration. Since the multifactorial nature of advanced AMD pathology includes oxidative stress, a multi-targeted approach using antioxidants in combination of complement gene editing or drug therapies targeting the retinal microglia/macrophages, could resolve the current shortfalls and provide a promising option for improving visual acuity in AMD patients.

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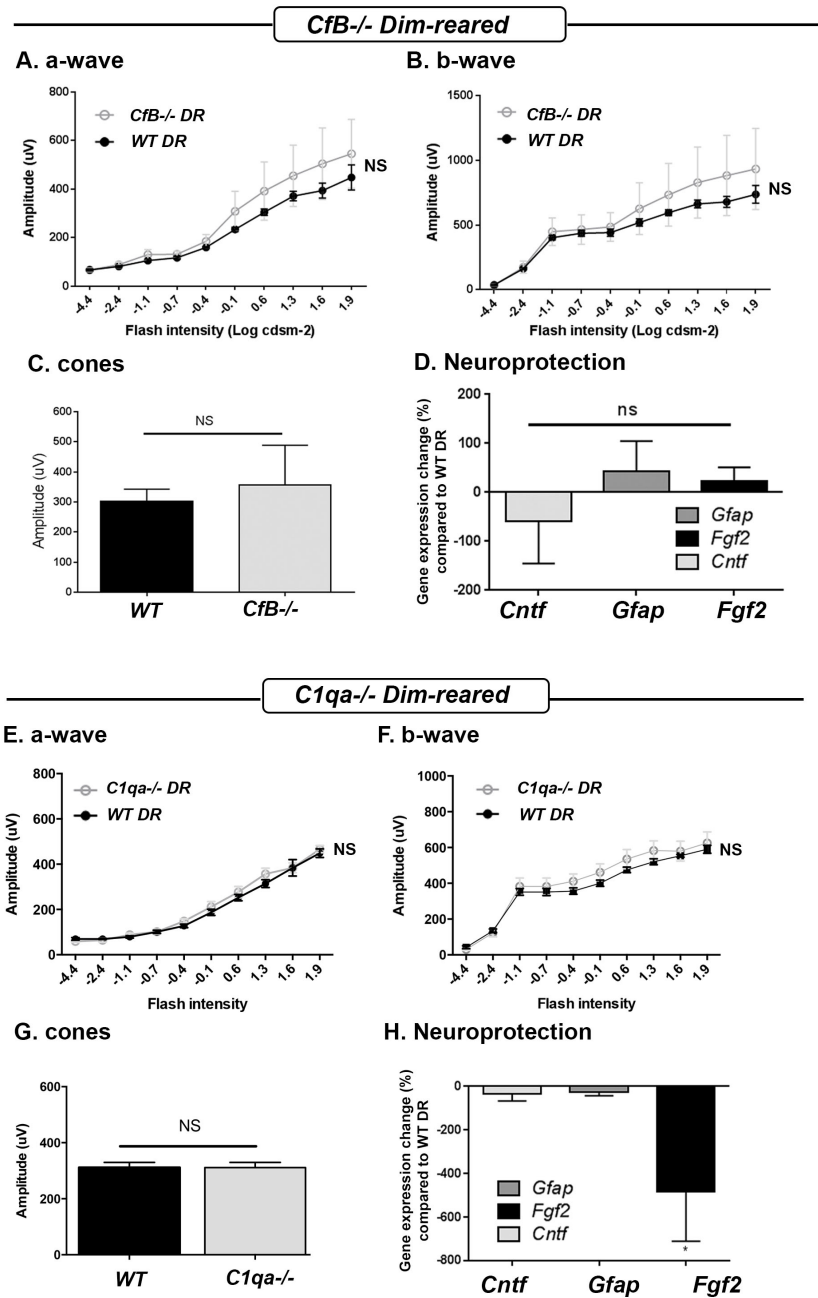
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9. APPENDIX



Appendix. Characterisation of dim-reared C57BL/6J wild-type (WT), *CfB^{-/-}* and *C1qa^{-/-}* mouse retinas. **A-C:** When assessing retinal function, there was no change in the a-wave and b-wave between WT and *CfB^{-/-}*. Cone response also did not change significantly among WT and *CfB^{-/-}*. **D:** There was no significant change in the expression of neuroprotective genes (*Cntf*, *Gfap*, *Fgf2*) between WT and *CfB^{-/-}* retinas (N=5 animals per group). **E-G:** The a-wave and b-wave responses also did not change significantly between WT and *C1qa^{-/-}*; cone response also displayed no difference between two groups. **H:** No difference was shown in the expression of neuroprotective genes except for *Fgf2*, which showed a significantly downregulation compared to WT (P<0.05; N = 5 animals per group). Statistical significance was determined by an unpaired student t-test or two-way ANOVA with multiple comparison post hoc (NS represents no significance; * represents P<0.05).