Macrophages, cytokines and complement as therapeutic targets in retinal degeneration

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June 2017

A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

The John Curtin School of Medical Research
The Australian National University

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Candidate Statement

This declaration certifies that the following work entitled ‘Macrophages, cytokines and complement as therapeutic targets in retinal degeneration’ is the author's own original work, complies with The Australian National University Research Award Rules and has not been previously 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 (AGRT) Scholarship.

Word Count: 43,006

Signed: ___________________

Date: ___________________
Acknowledgments

During my PhD candidature over the last four years, I have had the privilege of working with some of the best and brightest researchers in vision science. The production of this thesis would not have been possible without the immense support from the Provis and Valter laboratories, for which I am truly grateful. First, I would like to thank Associate Professor Krisztina Valter, who took me under her wing as an Honours student in 2012, and then encouraged me to continue as a PhD student. Your enthusiasm and passion for science and teaching is inspirational. Thank you for your supervision and guidance over the years. Secondly, I would like to thank Dr Matt Rutar for imparting your remarkable research skills and wealth of knowledge, helping me become a better researcher. I have enjoyed learning and working with you on many projects that you are passionate about, and I thank you for trusting me with these projects so that I too could grow as a scientist.

Third, I thank Dr Riccardo Natoli, whose guidance and mentorship has been invaluable to my career. Your ability to bring people together under a common vision is inspirational in both good times and bad, motivating me to keep going even when the road seems long. Your support has been a turning point in my research career going forward. Fourth, I would like to thank Professor Jan Provis, whose encouragement and wisdom has allowed me to develop as a researcher. Your presence as a prominent leader in this field, your strong support for early-career researchers, and your immense breadth of knowledge has given me something to aspire towards.

My heartfelt thank you goes out to my 'lab family' - Angel, Tanja, Helen, Josh, Riemke, Yvette, Dina, Kaz, Nunzio, Kartik and Zan-Min, for making the lab the best place to work every day. Thank you all for many years of friendship and support. I would also like to thank Dr Aude Fahrer and Dr Anne Bruestle for their advice and mentorship during my PhD. Lastly, I am truly grateful for the support of my family and friends throughout the many years of my studies, especially Mum, Dad and Dilini. Your unwavering support and confidence in my abilities has been greatly appreciated.
“Words are, in my not-so-humble opinion, our most inexhaustible source of magic…”

Albus Dumbledore
Age-related macular degeneration (AMD) is the leading cause of blindness amongst the Western population, with an annual cost of 350 billion dollars worldwide. This disease affects a specialised region in the central retina, the macula, where photoreceptors and the retinal pigment epithelium (RPE) cells begin to degenerate upon the onset of AMD. In the more prevalent atrophic or ‘dry’ form of AMD, gradual photoreceptor and RPE cell death leads to the formation of an expanding retinal lesion, which causes irreversible blindness over time. Although AMD pathogenesis involves multiple genetic and environmental factors, it is well understood that inflammation is highly implicated in disease progression. The recruitment of retinal microglia and macrophages, the production of cytokines and chemokines, and the deposition of complement system components are key features of atrophic AMD.

This thesis identifies retinal microglia and macrophages as therapeutic targets for slowing the progression of retinal degenerations such as AMD. In these studies, a rodent model of photo-oxidative damage is used, which recapitulates features of atrophic AMD including oxidative stress, inflammation and photoreceptor cell loss. This thesis first provides a further understanding of the role of microglia and macrophages in contributing to chronic complement activation and photoreceptor death in the degenerating retina. It is shown that locally-derived complement component 3 (C3) is a major contributor to the progression of retinal degeneration, and that microglia and macrophages are the primary source of retinal C3, in both rodent retinal degeneration and human AMD.

The recruitment of microglia and macrophages in retinal diseases is caused by increased chemokine signalling. It is demonstrated in this thesis that the use of a broad spectrum chemokine inhibitor (NR58-3.14.3) is able to ameliorate the accumulation of
microglia and macrophages in the outer retina, protecting the photoreceptors from further damage. Finally, it is shown that interleukin-1β (IL-1β), a strong pro-inflammatory cytokine, is primarily produced by microglia and macrophages in retinal degeneration. Using therapeutic strategies to neutralise or inhibit IL-1β signalling, it is found that there is a decrease in both macrophage recruitment and photoreceptor loss. Collectively, these three studies implicate a major role for retinal microglia and macrophages in contributing to pro-inflammatory cytokine production and complement synthesis. This thesis demonstrates the therapeutic value of targeting microglia and macrophages as a strategy for reducing inflammation and photoreceptor loss in retinal degenerations including AMD.
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A list of publications that were produced during this PhD are listed below (* denotes equal first author):


Data from this thesis was presented at the following international and national conferences:

Chapter 3:
"Chronic accumulations of complement-expressing microglia/macrophages accompany the progressive expansion of the retinal lesion in an animal model of atrophic AMD"

**Global Young Scientists Summit (GYSS) 2017** - Singapore University of Technology and Design, Singapore, 15-20 January 2017


**6th Neuroimmunology Australia Workshop 2016** - St Vincent's Centre for Applied Medical Research, Sydney, Australia, 19 August 2016 (platform presentation)

**Canberra Health Annual Research Meeting (CHARM)** - The Canberra Hospital, Canberra, Australia, 10 August 2016 (platform presentation)

**Australian Society for Medical Research - ACT New Investigators Forum** - The John Curtin School of Medical Research, The Australian National University, Canberra, Australia, 8 June 2016

Chapter 4:
"The broad spectrum chemokine inhibitor NR58-3.14.3 protects the retina from light-induced degeneration"

**2nd European Molecular Biology Laboratory (EMBL) Australia PhD Symposium** - Bio21 Institute, Melbourne, Australia, 25-27 November 2015 (platform presentation)

**Canberra Health Annual Research Meeting (CHARM)** - The Canberra Hospital, Canberra, Australia, 11-14 August 2015 (platform presentation)

**Australian Society for Medical Research - ACT New Investigators Forum** - The John Curtin School of Medical Research, The Australian National University, Canberra, Australia, 2 June 2015 (platform presentation)

Chapter 5:
"IL1B inhibition reduces chemokine-mediated inflammation in retinal degeneration"

**Invited Speaker** at the **XXII Biennial Meeting of the International Society for Eye Research (ISER)** - Keio Plaza Hotel, Tokyo, Japan, 25-29 September 2016 (platform presentation)


**Australian Society for Medical Research - ACT New Investigators Forum** - The John Curtin School of Medical Research, The Australian National University, Canberra, Australia, 7 June 2017
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<th>Description</th>
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<td>ΔΔC&lt;sub&gt;t&lt;/sub&gt;</td>
<td>comparative cycle threshold</td>
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<td>ACTB</td>
<td>beta actin</td>
</tr>
<tr>
<td>AEEC</td>
<td>Animal Experimentation Ethics Committee</td>
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<td>AMD</td>
<td>age-related macular degeneration</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>ANU</td>
<td>Australian National University</td>
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<td>APOE</td>
<td>apolipoprotein E</td>
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<tr>
<td>ARPE-19</td>
<td>ATCC CRL-2302 (human RPE cell line)</td>
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<td>ART</td>
<td>automatic real time</td>
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<td>ARVO</td>
<td>Association for Research in Vision and Ophthalmology</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
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<td>BRB</td>
<td>blood-retinal barrier</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>BSCI</td>
<td>broad spectrum chemokine inhibitor</td>
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<td>C</td>
<td>choroid</td>
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<td>genome-wide association study</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft-versus-host disease</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>hr(s)</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HNPP</td>
<td>2-hydroxy-3-naphthoic acid-2’-phenylalanilide phosphate</td>
</tr>
<tr>
<td>I</td>
<td>inferior</td>
</tr>
<tr>
<td>IBA-1</td>
<td>ionised calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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xvi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL-1α</td>
<td>interleukin 1α</td>
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<tr>
<td>IL-1β</td>
<td>interleukin 1β</td>
</tr>
<tr>
<td>IL-1r1</td>
<td>interleukin 1 receptor type I</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>interleukin 1 receptor antagonist</td>
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<tr>
<td>IL-1rap</td>
<td>interleukin 1 receptor accessory protein</td>
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<tr>
<td>IL-4</td>
<td>interleukin 4</td>
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<td>IL-6</td>
<td>interleukin 6</td>
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<td>IL-22</td>
<td>interleukin 22</td>
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<tr>
<td>ILM</td>
<td>inner limiting membrane</td>
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<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
</tr>
<tr>
<td>IR</td>
<td>ischemia-reperfusion</td>
</tr>
<tr>
<td>IS</td>
<td>inner segments</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridisation</td>
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<tr>
<td>IVC</td>
<td>individually ventilated cage</td>
</tr>
<tr>
<td>JCSMR</td>
<td>John Curtin School of Medical Research</td>
</tr>
<tr>
<td>LED</td>
<td>light-emitting diode</td>
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<tr>
<td>LD</td>
<td>light damage</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LSD</td>
<td>least significant difference</td>
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<tr>
<td>MAC</td>
<td>membrane attack complex (C5b-9)</td>
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<tr>
<td>MASP1</td>
<td>MBL-associated serine protease 1</td>
</tr>
<tr>
<td>MASP2</td>
<td>MBL-associated serine protease 2</td>
</tr>
<tr>
<td>MBL</td>
<td>mannose-binding lectin</td>
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<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MIO-M1</td>
<td>Müller 1 Moorefields Institute of Ophthalmology (human Müller cell line)</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MQ-H₂O</td>
<td>Milli-Q water</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
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<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFL</td>
<td>nerve fibre layer</td>
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<tr>
<td>NGS</td>
<td>normal goat serum</td>
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<tr>
<td>NHS-fluorescein</td>
<td><em>N</em>-hydroxy-succinimidyl-ester fluorescein</td>
</tr>
<tr>
<td>NLRP3</td>
<td>nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3</td>
</tr>
<tr>
<td>OCT</td>
<td>optical coherence tomography</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
</tr>
<tr>
<td>OLM</td>
<td>outer limiting membrane</td>
</tr>
<tr>
<td>ON</td>
<td>optic nerve</td>
</tr>
<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
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OPL – outer plexiform layer
OS – outer segments
PBS – phosphate-buffered saline
PCR – polymerase chain reaction
PD – photo-oxidative damage
qPCR – quantitative real-time PCR
RHOD – rhodopsin
RNA – ribonucleic acid
RNAi – RNA interference
RNP – ribonucleoprotein
ROS – reactive oxygen species
RPE – retinal pigment epithelium
S – superior
SD – Sprague-Dawley
SEM – standard error mean
SERPING1 – serpin family G member 1
siRNA – small interfering RNA
SOD – superoxide dismutase
SSC – saline-sodium citrate
Tdt – terminal deoxynucleotidyl transferase
TNF – tumor necrosis factor
TNFa – tumor necrosis factor α
TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labelling
UV – ultraviolet
V – vasculature
VEGF – vascular endothelial growth factor
VEGFA – vascular endothelial growth factor A
VEGFR1 – vascular endothelial growth factor receptor 1
VIM – vimentin
WT – wild type
1. INTRODUCTION
1.1. General Introduction

Age-related macular degeneration (AMD) is a disease of the neural retina, which includes the light-sensitive photoreceptors that transduce light into electrical signals, which can be interpreted by the brain. With the onset of AMD, photoreceptors in a localised region of the retina begin to degenerate and a lesion develops. This form of retinal degeneration is the leading cause of blindness, costing the Australian economy 5 billion dollars and the global economy 350 billion dollars annually [1, 2]. 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 [3]. A key feature of AMD is the activation and recruitment of resident microglia and blood-borne macrophages to the site of degeneration, which are associated with photoreceptor loss and further progression of the disease [4, 5].

In this chapter, I will provide an overview of the current understanding of the pathogenesis of AMD, and will introduce the immunological aspects of AMD progression, including the role of chemokines. I will also highlight the use of therapeutic molecules that target the innate immune system as a strategy for slowing the progression of retinal degenerative diseases such as AMD.

1.2. The eye and retina

1.2.1. The eye – gateway to vision

Most people, when questioned, regard vision as being the most important of their special senses. The eye (Figure 1.1) comprises an outer protective layer, the sclera, which together with the transparent cornea at the anterior surface, form a continuous external
Figure 1.1. Anatomy of the eye and retina (adapted from Kolb, 1995 [6]). Light passes through the pupil of the eye and is focused onto the retina, a multi-layered structure at the posterior of the eye. The photoreceptors are contained in the outer nuclear layer (ONL).
barrier around the globe [6]. Internally there is an anterior and a posterior chamber. Light passes through the anterior chamber and into the eye through the pupil, the size of which is adjusted by the iris, to allow more or less light to enter the eye. The lens focuses the light onto the retina, a sensory structure that lines the posterior surface of the eye. The light passes through the vitreous chamber, is absorbed by opsins in the photoreceptor outer segments, and is transduced into electrical signals. Photoreceptors feed this signal via the bipolar cells, to ganglion cells whose axons project to the brain via the optic nerve [6, 7].

1.2.2. Organisation of the retina

The human retina is made up of ten distinct layers which differ in both structure and function [8]. The inner retina consists of the inner limiting membrane (ILM), nerve fibre layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), and outer plexiform layer (OPL). The INL houses multiple cell types including bipolar cells, amacrine cells and horizontal cells, as well as the Müller cell bodies [8]. Müller cells are radial glial cells that span the whole retina providing structural support [9]. They also functionally support neurons by recycling neurotransmitters, and promote the survival of photoreceptors through a range of homeostatic signalling mechanisms to control neuronal activity, metabolism and outer segment recycling (reviewed in [9-12]). Müller cells also function as ‘light guides’, directly contributing to vision [13]. Upon retinal injury, Müller cells can undergo gliosis and proliferation to fill gaps in the photoreceptor layer [10, 14, 15], and may even proliferate to generate retinal neurons [16, 17] and photoreceptors [18, 19].

The outer retina is made up of the outer nuclear layer (ONL), the outer limiting membrane (OLM), and the inner and outer segments (IS and OS) of the photoreceptors in the sub-retinal space [8]. A monolayer of retinal pigment epithelium (RPE) cells - which are part of the neural retina - form the outer blood-retinal barrier (BRB), between the retina
and the underlying Bruch’s membrane. Transporters and tight junctions of the RPE cells allow for the selective movement of ions, proteins and water in and out of the retina, whilst maintaining the immune privilege of the eye by inhibiting the passage of immune cells into the retina [20, 21]. The choroid is a network of blood vessels under the Bruch’s membrane which delivers nutrients and oxygen to the outer retina, and removes waste and heat from the retina [22].

1.2.3. Photoreceptors

Photoreceptors are made up of three major parts: cell bodies which make up the ONL, inner segments (IS), which act as the powerhouse for the photoreceptors, and outer segments (OS), which contain one of four different light-sensitive opsins (in humans) that absorb light of specific frequencies to provide for phototransduction [6, 23]. There are two types of photoreceptor cells: rods and cones. The outer segments of rod cells comprise membranous disks of opsin and retinal, which combine to form rhodopsin. Rods are very light sensitive, and are useful only in dim light conditions. Cone photoreceptors contain one of three different opsins which combine with retinal to form cone opsins, having a particular range of spectral sensitivities (short, medium and long wavelengths) that both broadens the range of visible frequencies as well as allows for the processing of ‘colour-coded’ signals, and colour vision. Cone opsins do not saturate in bright light, and mediate virtually all of our useful vision [6, 23].

The human retina comprises approximately 120 million rods and 6 million cones [24]. Cones are dominant in the macular region of the retina, the centre of the visual field. Cones, but not rods, provide input into retinal circuits which are highly adapted to extract information that provides for high spatial resolution (acuity) as well as colour. The fovea centralis within the macula is a rod-free and vessel-free area where light scattering is minimal, allowing for the maximum amount of light to be absorbed by the cones [6].
However, most likely due to the unique and highly specialised design and microenvironment of the macula, where blood supply is limited and there is high density of oxygen-demanding photoreceptors, the macula is subject to degeneration over time [25].

1.3. Degeneration of the retina

1.3.1. Ageing and retinal disease

Tissue homeostasis is a key element to preserving the health of the retina. Under basal conditions, the immune system in the retina can maintain homeostasis, and low levels of tissue stress are regulated by the microglia, the resident immune cells of the retina [5]. However, constant exposure to noxious conditions, such as overload with reactive oxygen species (ROS) over time may lead to chronic tissue stress, in which para-inflammation may occur [26]. Para-inflammation is an intermediate inflammatory response that returns the stress levels in the retina back to homeostasis [27]. If para-inflammation persists, such as in ageing, this may result in release of pro-inflammatory cytokines, that can propagate a full inflammatory response [26]. Ageing is defined as the accumulation of temporal changes that increase susceptibility to disease with increasing lifespan [28]. Para-inflammation in ageing can contribute to the onset of many retinal degenerative diseases later in life, including diabetic retinopathy, glaucoma and age-related macular degeneration (AMD) [26]. Onset and progression of retinal degenerative diseases including AMD, glaucoma, retinitis pigmentosa, diabetic retinopathy and retinal detachment share several features in common, including increases in both oxidative stress and inflammation [8, 29-31].

1.3.2. Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in the Western World, primarily affecting the population aged over 65, with the projected number
of people with the disease estimated to be 288 million worldwide by 2040 [32]. AMD poses a significant economic burden, with an annual global cost of 350 billion dollars for the treatment and healthcare of visually impaired patients [2]. Early/intermediate AMD is clinically diagnosed by the presence of drusen surrounding the macular region (Figure 1.2). Drusen are yellow deposits of debris that form between the RPE and Bruch’s membrane [33, 34]. The presence of drusen, which may coalesce over time, can lead to the degeneration of RPE cells overlaying the drusen deposits [35, 36]. At this stage, the photoreceptor layer is still largely intact, and there is an absence of neovascularisation.

There are two types of late-stage AMD (Figure 1.2). Neovascular or “wet” AMD, accounts for 10% of all cases, and can lead to sudden loss of vision due to choroidal neovascularisation (CNV). In wet AMD, new blood vessels derived from the choroid enter the subretinal space near the photoreceptors. Prolonged leakage of blood, serum and lipids into the outer retina from these fenestrated, or non-barri ered blood vessels disrupts photoreceptor alignment and alters the neural microenvironment, promoting Müller cell gliosis, scarring and visual impairment [34, 37].

Atrophic or “dry” late-stage AMD is characterised by the emergence of geographic atrophy (GA), in which a lesion in the RPE, photoreceptors and choriocapillaris of the macula develops and progressively expands over time [34]. This is in part due to drusen deposition around the macula, which may cause the degeneration of RPE and photoreceptor cells in these areas, leading to the development of a focal outer retinal lesion [38]. This atrophy is usually accompanied by reduced blood flow in the choroid and thinning of the choriocapillaris [39, 40]. The progressive loss of outer retinal cells and the expansion of the atrophic lesion results in a large drop in visual function in patients with the disease [41]. Development of a two millimetre lesion in the foveal region can result in legal blindness [25].
Figure 1.2. The pathogenesis of AMD (from Ambati et al. 2013 [3]). There are two forms of late-stage AMD that can develop from early or intermediate dry AMD; geographic atrophy (dry AMD), where photoreceptors and RPE deteriorate, and neovascular AMD (wet AMD), where the growth of new blood vessels can cause oedema and cell death.
1.3.3. Pathogenesis of AMD

The pathogenesis of AMD is multifactorial and the precise causes of onset and progression of this disease remain unclear. However, several factors are strongly implicated in increasing the risk of developing AMD, including a range of genetic and environmental factors [34, 42]. Advancing age remains the highest risk factor for all forms of the disease [43-46]. Genetic predispositions regarding the immune system are heavily linked to the development of the disease, especially the dysregulation of complement system genes, which strongly increases the risk of developing AMD [47, 48]. There is some evidence suggesting that autoimmunity may also contribute to the progression of the disease, as autoantibodies have been detected in the blood of AMD patients [49, 50].

A key component of AMD onset and progression has been linked to mechanisms that cause oxidative stress and retinal damage, which can be initiated by a number of environmental factors [29, 30]. The retina is subject to oxidative stress over time, due to the generation of ROS from high light exposure and oxygen consumption, as well as the continual inhibitory mechanisms of the RPE against immune cells in the choroid [22, 51, 52]. Environmental factors including cigarette smoking [45, 53-56] and systemic hypertension [57, 58] have also been linked to the prevalence of AMD.

Additionally, photo-oxidative damage resulting from excessive exposure to light causes lipid peroxidation of the outer segments, and may be a factor in AMD pathogenesis [59]. Continual phagocytic activity of the RPE cells generate lipofuscins, which are pigment granules containing remnants of lysosomal digestion [60]. Lipofuscin accumulation in the RPE is also thought to cause RPE cell degeneration and photoreceptor loss adjacent to the degenerating RPE [61].

Analysis of drusen composition has identified over 100 different proteins [62], many of which were covalently cross-linked with carboxyethylpyrrole (CEP), a by-product
of docosahexaenoic acid (DHA) oxidation, in which fragmentation occurs under oxidative stress conditions [63]. The generation and accumulation of CEP-adducted proteins in the outer retina is heavily implicated in the pathogenesis of AMD [64, 65]. Another oxidative stress by-product found in drusen is malondialdehyde (MDA), produced through the oxidation of phosphatidylcholine [66]. MDA is known to interact with complement factor H (CFH), a critical regulator of the complement system [67]. Induction of the innate immune system following oxidative stress is known to contribute to the progression of AMD [68-70].

Although there are a wide range of factors that may contribute to AMD pathogenesis, the significance of immunological processes in AMD has been well established. Dysregulation of the immune system is critically linked to the development of geographic atrophy, including the recruitment of activated resident microglia to the outer retina, and the persistent accumulation of subretinal macrophages [4, 5, 71-75]. Under normal conditions, the eye has an ocular immune privilege, and is protected from inflammation by separation from the choroidal vasculature by the BRB, as well as through the production of immune system inhibitors in the eye [76, 77]. However, once damage occurs to the RPE and photoreceptors in retinal diseases such as AMD, the BRB is breached which may remove this immune privilege and allow inflammation to occur [31].

1.4. Inflammation and AMD

1.4.1. Cytokine production in AMD

Cytokines are a large family of inflammatory molecules that are involved in many aspects of both the innate and adaptive immune system, including leukocyte activation and recruitment, antigen presentation, bone marrow differentiation and adhesion molecule
Cytokines can be classified as either pro-inflammatory (e.g. tumour necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IL-12) or anti-inflammatory (e.g. IL-4, IL-10, IL-13) [79]. A specialised sub-group of cytokines that are primarily involved in leukocyte migration are the chemokines, a group of small chemoattractant molecules that form chemotactic gradients for the activation and recruitment of leukocytes expressing the appropriate chemokine receptors [80]. Chemokines and their receptors are classified into four sub-groups depending on their characteristic cysteine motifs; C (γ chemokines), CC (α chemokines), CXC (β chemokines) and CXC3 (δ chemokines) [81]. In the central nervous system, chemokines are produced by neurons, astrocytes and microglial cells at lower levels [82]. Upon activation of stress or inflammatory signalling pathways, higher levels of chemokines are expressed, leading to recruitment of blood-borne leukocytes to areas of damage [81]. These leukocytes are primarily monocytes (precursors of macrophages), neutrophils, lymphocytes and eosinophils [83].

In AMD, chemokines attract leukocytes to areas of outer retinal tissue damage [84]. A genome-wide association study (GWAS), involving 68 human donor eyes from normal and AMD patients, demonstrated that several chemokines involved in leukocyte recruitment (CCL2, CXCL1 and CXCL10) were upregulated in both wet and dry AMD eyes [47]. Studies involving animal models of retinal degeneration have demonstrated that expression of Ccl2, Cxcl1 and Cxcl10 is coordinated by RPE and Müller cells to activate resident microglia and recruit blood-borne macrophages to the degenerating retina [85-87]. In addition, it has been shown that culturing RPE and Müller cells with lipopolysaccharide (LPS)-stimulated microglia can induce the expression of Ccl2 and Il-6, indicating that prolonged chemokine expression may occur through the continual expression of chemokines by these retinal cells [86, 88]. Upregulation of a suite of chemokines and pro-inflammatory cytokines, which includes CCL2 [85, 89-92], CCL3 [93, 94], CCL4 [95] and IL-6 [96, 97] are strongly implicated in the progression of retinal diseases.
Interleukin-1β (IL-1β) is a pro-inflammatory cytokine primarily produced through the activation of the inflammasome [98, 99]. The inflammasome is an oligomer protein complex that leads to the maturation and secretion of IL-1β and IL-18 into the extracellular environment [100]. The assembly and activation of the inflammasome is stimulated by several mechanisms, including toll-like receptor signalling and purinergic receptor signalling, the latter which is activated by extracellular ATP released by dying cells [100, 101]. Although inflammasome signalling is thought to play both detrimental and protective roles in atrophic and neovascular AMD due to the production of IL-18 [102-109], the synthesis of mature IL-1β is known to be involved in the pathogenesis of central nervous system (CNS) diseases [110], including both forms of AMD [111, 112]. This has also been supported in several studies involving animal models of retinal degenerations, demonstrating that IL-1β is secreted by activated microglia [113-117].

1.4.2. Microglia and macrophages in AMD

Microglia are the resident immune cells of the CNS, existing in a ramified immune surveillance state in the normal brain and retina [118]. Microglia are derived from primitive yolk sac macrophages in development [119], and have a distinct phenotype from blood-borne macrophages [120]. In the healthy retina, microglia are highly motile, which allows them to constantly sense the microenvironment and interact with other retinal cells such as Müller cells and photoreceptors, using highly mobile processes [121, 122]. As well as monitoring the inflammatory environment, microglia have a key role in neurogenesis, neuronal survival and synaptic maintenance in CNS development [123-125], as well as maintaining synaptic function in the mature retina [126]. Under normal retinal conditions, mechanisms to inhibit microglial activity are in place, including the CD200:CD200R signalling axis [127, 128] and the CX3CL1:CX3CR1 signalling axis [129, 130]. Microglia normally reside in the plexiform layers of the inner retina [5], however, upon receiving
inflammatory cues such as chemokine signalling or purinergic signalling from damaged photoreceptors, RPE and Müller cells, microglia can migrate to sites of tissue damage [131]. During ageing, there are higher numbers of microglia in the retina, which begin to accumulate in the outer retina with age [132, 133].

In age-related retinal diseases such as AMD, the accumulation of leukocytes in the outer retina is a well-established feature of disease pathogenesis [4, 71-75]. This outer retinal leukocyte population primarily consists of activated microglia and infiltrating blood-borne macrophages from the retinal vasculature [5]. Microglial activation and macrophage recruitment are strongly implicated in the pathogenesis of several retinal diseases, including AMD [4, 47, 75, 134, 135], glaucoma [136, 137], retinitis pigmentosa [134], and diabetic retinopathy [138]. There are two types of macrophages, M1 (pro-inflammatory) macrophages that are involved in the clearance of cell debris, pathogens and apoptotic cells [116, 134, 139], and M2 (anti-inflammatory) macrophages, which are involved in wound healing and angiogenesis [113]. However, phagocytosis by these cells has also been found to contribute to the degeneration of living photoreceptors adjacent to damaged photoreceptors [116, 134]. Additionally, retinal microglia and macrophages are known to express complement system components [140-144]. The role that these cells play in contributing to prolonged disease progression is not fully understood.

1.5. Complement system and AMD

1.5.1. The complement system

The complement system is a key component of the innate immune system. It comprises a cascade of over 30 proteins circulating in the plasma and on cell surfaces [145], that are sequentially lysed to form a terminal complex initiating the destruction of a
pathogen or foreign body, which has been associated with cell debris [145, 146]. It is an ancient system that is evolutionarily conserved as a critical mechanism for host defence [147, 148]. The complement system consists of three pathways of activation (Figure 1.3); classical, lectin and alternative pathways [149].

The classical pathway of the complement system is activated through the binding of foreign antibodies on pathogenic surfaces, which initiates activation of the complement component 1 (C1) complex. This complex is comprised of a C1q hexamer molecule bound to C1r and C1s, which together when activated form a serine protease which goes on to cleave the next proteins in the pathway, C4 and C2. Two fragments generated during this interaction, C4b and C2a, form the C3 convertase enzyme (C4b2a) that facilitates the hydrolysis of C3, a central molecule of all pathways of the complement system. C3 hydrolysis generates smaller C3 fragments that are used up further downstream for several complement system functions. These C3 convertase enzymes are attached to the pathogenic cell surfaces, largely limiting complement activity to these bound surfaces. The lectin pathway consists of a similar chain of proteins, whereby the C1 complex is replaced with a mannose-binding lectin (MBL) protein, which binds specifically to mannose residues on pathogenic cell surfaces. MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2) cleave the C4 and C2 proteins, generating C4b and C2a fragments necessary for the formation of the C3 convertase [145-147, 149].

The alternative pathway operates under a spontaneous hydrolysis mechanism. The constant hydrolysis of C3 allows for C3 convertases to be formed at lower levels. C3 convertase (C3bBb) formation via this pathway is regulated by a number of factors. Complement factor B (CFB) and complement factor D (CFD) are positive regulators of C3 hydrolysis, whereas complement factor H (CFH) and complement factor I (CFI) negatively
Figure 1.3. Schematic representation of the complement system (adapted from Murphy et al. 2008 [149]). There are three pathways of complement activation; classical, lectin and alternative. These pathways all lead to the hydrolysis of C3, which triggers the downstream formation of the membrane attack complex (MAC). This marks the destruction of pathogens, apoptotic cells and debris.
regulate this pathway and inhibit the hydrolysis of C3. Properdin (factor P) may bind to the C3 convertase and enhance its stability upon binding to pathogenic cell surfaces [149]. Upon C3 hydrolysis by each of the three complement pathways, a number of fragments are produced including C3a, C3b and C3d (inactive component). The larger fragment C3b forms part of a C5 convertase which lyses C5 into C5a and C5b. This C5b fragment combines with C6, C7, C8 and 16 molecules of C9 to form the membrane attack complex (MAC, C5b-9) terminal complement product, which assembles on the pathogenic cell surface and essentially forms a membrane pore in the surface, facilitating the destruction of pathogens, foreign bodies or cell debris via lysis [145-147, 149].

This key function of the complement system not only plays a vital role in host defence against pathogens, but also clears debris as well as dead, damaged and apoptotic cells, which may protect against autoimmunity [149]. Complement may also lead to a prolonged activation of the innate immune system via several mechanisms. Complement contributes to the opsonisation of pathogens for phagocytic destruction, by coating the cells in C3b (short-lived) or C3d (inactive long-lasting) molecules. The hydrolysis of C3 and C5 also generate C3a and C5a anaphylatoxins, which propagate local inflammation by recruiting phagocytic cells to the site of injury [145-147, 149]. Additionally, C1q is thought to activate the inflammasome, another component of the innate immune system, by affecting the phagolysosomal process to trigger inflammasome activation [106]. Increases in MAC are also associated with activation of the inflammasome [150].

1.5.2. Complement activation and AMD

Under normal conditions, the complement system pathways are under tight regulation. However, in neurodegenerative diseases such as age-related macular degeneration (AMD), complement activation is uncontrolled, leading to the continuous production of complement which can contribute to disease onset and progression [3, 151,
Genetic factors are strongly implicated in the pathogenesis of AMD, especially those involving the innate immune system. In 2005, multiple studies revealed that a single nucleotide polymorphism (Y402H variant) in CFH, a negative regulator of the alternative complement pathway, was responsible for the onset of almost 50% of all cases of AMD [48, 153-156]. Additionally, polymorphisms in C2, CFB and C3 have also been associated with disease onset [157, 158]. GWAS analysis indicated that there is a link between complement activation and the progressive expansion of the macular lesion in geographic atrophy, demonstrating that there was a global upregulation of a suite of complement genes in the neural retina, including C3, C4, C1s, CFI and SERPING1 [47].

Numerous histopathological investigations have shown that a large range of complement components and factors (e.g. CFH, CFB, C3, C5, C5b-9) are present in drusen that have accumulated in the outer retinas of AMD retinas [62, 153, 157, 159-162]. Furthermore, although the terminal membrane attack complex (MAC, C5b-9) is present in the choriocapillaris as early as three years of age, the significantly increased presence of MAC in the choriocapillaris of AMD retinas may be indicative of the contribution of complement to outer retinal cell loss [163-165]. However, even though it is well established that complement system dysregulation is linked to chronic lesion expansion in human atrophic AMD, the cellular events mediating this process are unclear.

1.5.3. Complement as a gateway to adaptive immunity

Complement component C1q is often considered to be the bridging molecule between the innate and adaptive immune system [146, 147, 166]. C1q provides the link to the adaptive immune response through the binding of antibodies complexed to antigens, which is part of the classical pathway [167-169]. In addition, the activation of complement may increase the efficiency of the adaptive immune system [146, 147]. In retinal degenerative diseases, adaptive immune system cells including T-cells and neutrophils
may contribute to RPE degeneration [170, 171]. The anaphylatoxin protein fragment C5a mediates recruitment of pro-inflammatory T-cells into the eye in a model of neovascular AMD [172], and C5a increases production of pro-inflammatory cytokines IL-17 and IL-22 by T-cells in AMD patient sera [173]. In neovascular AMD human tissue and animal models, it is thought that IL-17 is produced primarily by γδT-cells [172, 174, 175]. However, further studies are needed to elucidate the role of the adaptive immune system in contributing to the progression of retinal degenerations.

1.6. Therapeutic targeting of inflammation

1.6.1. Strategies to manage inflammation in AMD

Therapeutic strategies involved in the treatment of AMD have so far been only effective in patients with the neovascular ‘wet’ form of the disease. Vascular endothelial growth factor A (VEGFA)-targeted therapies have proven highly beneficial for neovascular AMD, with market compounds available to inhibit abnormal blood vessel growth such as Ranibizumab (Lucentis; Genentech/Novartis) [176], and Aflibercept (VEGF Trap-Eye/Eylea; Regeneron/Bayer) [177]. However, there are currently no effective treatments for atrophic ‘dry’ AMD, the most prevalent form of the disease. Immune-based therapies are being explored as the most likely drug candidates for clinical trials, including a number of complement system inhibitors [3, 178]. Several clinical trials are currently underway involving the use of C5 inhibitors [179, 180] and a complement factor D inhibitor (Lampalizumab, Genentech) [178]. However, the complement system has a high degree of redundancy, comprising more than 30 different protein components [145]. Therefore inhibition of specific complement components, especially those involved in the activation phase of the complement cascade (e.g. C1, C4, C2 components) is likely
to be challenging, since blocking one pathway may result in the activation of other pathways that lead to terminal membrane attack complex formation [178].

Therefore, strategies that inhibit production of complement system components may be more beneficial. Both RPE cells and macrophages are known to synthesise complement in the retina [140, 141, 144, 181] and drug treatments that inhibit the activation and production of complement by these cells may hold therapeutic potential in slowing the progression of AMD. Novel therapeutic strategies such as microRNAs (miRNAs), known as ‘master gene regulators’, are being explored as therapeutic molecules for retinal degenerations, and have been employed experimentally to reduce the expression of inflammatory genes [182]. Chemokine inhibitors have also been used to regulate the activation and recruitment of macrophages in experimental models of retinal detachment, diabetic retinopathy, retinitis pigmentosa, and in a model of atrophic AMD primarily concerning inhibition of the CCL2-CCR2 axis [89, 183, 184]. However, the issue of redundancy again plays a role in chemokine signalling, as the inhibition of specific chemokines or their receptors may actually lead to an increased production of other chemokines to compensate, of which there are over 50 chemokine ligands and associated receptors [78, 93, 185].

1.6.2. Broad spectrum chemokine inhibition (BSCI)

Addressing the issue of redundancy, a novel broad spectrum chemokine inhibitor (BSCI) NR58-3.14.3 has been shown to suppress the entire CCL- and CXCL-signalling pathways. One of the first BSCIs tested was Peptide 3, which was only effective in vitro [186]. As a result, NR-58.3.14.3 was synthesised as a cyclic 13 amino acid retroinverso analogue of Peptide 3, which is 1000 times more potent than Peptide 3 [187]. The new cyclic structure of the BSCI NR-58.3.14.3 does not affect its broad pan-chemokine inhibiting ability [188]. The exact mechanism of action of BSCI is not fully understood,
however, it is known to act as a blocker for chemokine signalling cues so that leukocyte recruitment is hindered [187].

NR58-3.14.3 has been promising in a number of different tissue types and disease models, including skin inflammation [187], cerebral ischemia-reperfusion (IR) injury [189], obliterative bronchiolitis [190], lung ischemia [191], atherosclerosis [192], intraperitoneal adhesions [193], endometriosis [194] and pulmonary graft-versus-host disease (GVHD) [195]. These studies have reported that treatment with NR58-3.14.3 was able to reduce the accumulation of inflammatory cells in the tissue, and ameliorate tissue damage. While BSCIs have proven to be effective in reducing inflammation and tissue degeneration in these disease models, their use in the eye has not been documented.

1.7. In vivo models of retinal degeneration

1.7.1. Animal models of retinal degeneration

Models of wet AMD primarily focus on the use of experimental methods that induce choroidal neovascularisation (CNV) in the retina (reviewed in [196]). However, to model retinal atrophy that occurs in dry AMD, several types of rodent models exist, and many of these models involve increases in oxidative stress and/or inflammation [196, 197]. Knockout models of chemokine genes or complement system genes have been utilised to develop retinal degeneration in mouse models, including CX3CR1-knockout mice, where a deficiency in the fractalkine receptor involved in the inhibition of microglial activity is known to cause retinal pathology [198, 199]. Complement factor H (CFH)-deficient mice also develop increased C3 deposition and abnormalities in the retina [200].

Rodent models that induce oxidative stress in the retina have been developed to model features of dry AMD. A model involving the immunisation of mice with
carboxyethylpyrrole (CEP)-adducted mouse serum albumin induced retinal degeneration, where mice developed C3 deposition in the outer retina, macrophage recruitment and photoreceptor cell death [63, 68, 69]. Retinal photo-oxidative damage (or light damage) models have been utilised for over 50 years [201] to induce photoreceptor degeneration in the rodent retina (reviewed in [202, 203]). Although the effect of photo-oxidative damage in the retina is affected by circadian rhythm and light history of the animals [204, 205], photo-oxidative damage has been used to initiate photoreceptor death and extensive retinal remodelling in the rodent retina [206, 207]. This occurs primarily in the superior retina where the area centralis, an area analogous to the human macula, is located [208].

Our laboratory has demonstrated the use of photo-oxidative damage models of retinal degeneration using both rats and mice, to investigate the immunological mechanisms of atrophic AMD pathogenesis [209, 210]. By producing several features of the disease in the rodent retina, we have demonstrated that oxidative damage and photoreceptor cell death is induced during exposure to photo-oxidative damage [209, 210]. Damaging light induces ROS production and lipid peroxidation in the retina, which can initiate photoreceptor loss and upregulate stress mechanisms. Chemokines such as CCL2 are expressed by RPE, Müller cells and activated microglia [85, 87], leading to the migration of retinal microglia and the recruitment of blood-borne macrophages from the retinal vasculature and underlying choroid to the damaged photoreceptors [113, 141, 209]. Additionally, these microglia and macrophages have been found to express complement activation markers such as C3 in the outer retina [141, 211]. These inflammatory components are associated with the progression of retinal degeneration following exposure to photo-oxidative damage. Substantial focal lesion development occurs in the photoreceptor layer, starting at the area centralis in the superior retina and eventually expanding over time [209, 210], modelling a key feature of atrophic AMD.
1.7.2. **Complement activation in animal models**

Models of photo-oxidative damage show that complement activation is facilitated by microglia and macrophages in the damaged retina, and indicate that complement expression may be associated with further photoreceptor damage [141, 211, 212]. The expression of retinal complement by microglia and macrophages is also observed in models of ageing [143, 144]. However, further studies are needed to elucidate the role of these cells in contributing to complement activation and prolonged retinal remodelling, using a protracted time course of progressive retinal degeneration. Additionally, the cellular contribution of complement-expressing cells in the retina is not fully understood. RPE cells were initially reported to be major producers of complement in the retina [140, 181, 213, 214]. However, emerging evidence indicates that microglia and macrophages are the more likely primary mediators of complement expression in the degenerating retina [140-144]. Investigation of the contribution of these local retinal sources of complement to retinal degeneration is necessitated.

Furthermore, the contribution of each complement pathway upon activation is not fully understood, and it has been suggested that more than one complement pathway may be involved in the progression of retinal degeneration [215]. The activation of the alternative pathway is known to contribute to retinal degeneration in several animal models [216-220]. Recently, CFH has been described to have a non-canonical role in mononuclear phagocyte clearance in the degenerating retina, where CFH mutations may result in the toxic accumulation of these cells, including microglia and macrophages, in the outer retina [309]. Novel roles for the complement pathways are continuing to be uncovered, and so further characterisation of complement activators and regulators in retinal degeneration is necessary to fully understand how complement activation can be targeted therapeutically.
1.8. **Summary and aims**

The activation and accumulation of microglia and macrophages in the degenerating retina are highly implicated in the progression of retinal diseases such as AMD. However, several key questions remain unanswered, regarding their contributions to complement activation and photoreceptor death in progressive retinal degeneration.

The overall aims of this thesis are:

1. To determine the contribution that microglia and macrophages have in propagating the inflammatory response in retinal degeneration, which may lead to further photoreceptor cell death and atrophic lesion development.

2. To utilise novel therapeutic strategies to inhibit the activity of these microglia and macrophages in the degenerating retina.

Chapter 2 outlines the general methodology described throughout this thesis. Chapter 3 characterises the role of complement activation by microglia and macrophages in contributing to retinal degeneration in humans, rats and mice, with a particular focus on the role of C3 in atrophic lesion development. Chapter 4 describes the therapeutic potential of a broad spectrum chemokine inhibitor (BSCI) NR58-3.14.3 in modulating inflammatory-mediated cell death by macrophages in retinal degeneration. Chapter 5 details the role of interleukin-1β (IL-1β) in promoting chemokine production by retinal supporting cells in retinal degeneration, and we demonstrate the use of two inhibition strategies to reduce IL-1β-derived macrophage recruitment. Finally, the significance of these findings is discussed in Chapter 6.
2. METHODS
2.1. In vitro experiments

2.1.1. Cell cultures

Immortalised human cell lines for Müller cells (MIO-M1, Müller 1 Moorefields Institute of Ophthalmology; Dr A. Limb, Institute of Ophthalmology, University College London, UK) and RPE cells (ARPE-19, ATCC CRL-2302; American Tissue Culture Collection, VA, USA) were cultured for experiments presented in this thesis. Cell lines were authenticated by CellBank, Australia. Cell lines stored at -80°C were thawed and grown in a tissue culture flask with Dulbecco's Modified Eagle Medium (DMEM, high glucose with sodium pyruvate; Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA) and 3mM L-glutamine (Thermo Fisher Scientific). Cells were incubated at 37°C in a humidified atmosphere (95% air and 5% CO2). To passage the cells every 3 to 4 days, cells were incubated with trypsin solution (containing 2.5% trypsin (Thermo Fisher Scientific), 1% EDTA and 0.1M phosphate buffered saline (PBS)) for 3 minutes at 37°C to detach the cells from the flask. Cells were centrifuged then split 1:5 into a new flask and incubated at 37°C until needed for experiments, as detailed later in this thesis.

2.2. Animal experiments

2.2.1. Animal rearing and holding

All animal experiments reported in this thesis were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and with approval from the ANU Animal Experimentation Ethics Committee (Ethics ID: A2012/07, A2014/56). Adult Sprague-Dawley (SD) rats were utilised in these experiments, which
were born and reared under cyclic dim light conditions (12 hours at 5 lux, 12 hours at 0 lux) in open-top cages. All rats were aged between 90-120 post-natal days at the time of use. Additionally, adult C57BL/6J mice aged 60-80 days and age-matched homozygous C3-knockout mice (C3\(^{-/-}\), 129S4-C3tm1Crr/J) were used in these experiments. Mice were born and reared in individually ventilated cages (IVCs) in cyclic dim light conditions (12 hours at 5 lux, 12 hours at 0 lux) until needed for further experiments.

2.2.2. Intravitreal injections

Intraocular (intravitreal) injections were performed to deliver compounds of interest (NR58-3.14.3, C3 siRNA, IL-1\(\beta\) siRNA) into the eye. A total volume of 3\(\mu\)l was delivered via intravitreal injection into each rat eye, and a total volume of 1\(\mu\)l into each mouse eye. These volumes were chosen as they minimise disturbance to the pressure of the eye, reducing the risk of cataract formation [221]. All compounds were delivered in ultrapure endotoxin-free 0.1M PBS (Thermo Fisher Scientific).

Prior to all injections, animals were restrained and anaesthetised using a mixture of Ketamine (100mg/kg body weight; Troy Laboratories, NSW, Australia), an anaesthetic, and Ilum Xylazil-20 (12mg/kg body weight; Troy Laboratories), a muscle relaxant, delivered through an intraperitoneal injection with an insulin syringe. Each animal was checked for signs of unconsciousness, including loss of movement (except for breathing), and loss of the corneal and toe-pinch reflexes. A heat mat was used to maintain core body temperature. A pupil dilator was administered to both eyes (Minims Atropine Sulphate 1% w/v eye drops for mice, or Minims Tropicamide 0.5% w/v eye drops for rats; Bausch and Lomb, NSW, Australia). Animals were then transferred to a surgery mat under a Zeiss OPMI 99 examination microscope (Zeiss, West Germany), or a Leica M125 stereo microscope (Leica Microsystems, Wetzlar, Germany), which was used to aid the intravitreal injection process. Following dilation of the pupil, a cotton string loop was tied
around the eye lifting it from the socket. 10% w/v povidone-iodine antiseptic liquid (Betadine; Faulding Pharmaceuticals, SA, Australia) was applied to each eye before injection.

The injectable compound of interest was drawn up into a 10µl Hamilton syringe with an attached 34G bevelled needle (World Precision Instruments, FL, USA). Using a 30G bevelled needle (Becton Dickinson, NJ, USA), a small puncture wound was made into the eye about 1mm from the limbus. The tip of the needle was inserted in a supero-temporal direction and then removed. The 34G needle attached to the Hamilton syringe was inserted into the hole, and 1-3µl of solution was intravitreally injected into each eye. Following the injection, Chlorsig antibiotic cream (Aspen Pharma, NSW, Australia) was applied to the injection site. Eye gel (GenTeal; Novartis, NSW, Australia) was administered to both eyes to prevent dryness, and the animal was recovered on a heat mat.

2.2.3. Photo-oxidative damage

Photo-oxidative damage (or light damage) has been used as a model to induce retinal degeneration in rodents for over 50 years [201]. The use of two photo-oxidative damage models have been reported throughout this thesis as previously published; a rat model [209] and a mouse model [210]. SD rats were placed into transparent Perspex open-top boxes and exposed to 1000 lux white light for 24 hours (C�토, 2x36W, IHF; Thorn Lighting, Spennymoor, UK), with free access to food and water. Lighting levels were set to 1000 lux using a light meter device (DLM2; UEi Test Instruments, OR, USA). Following photo-oxidative damage and post-damage live animal analysis, animals were either euthanized for tissue collection (0 day time point) or were held under dim lighting (5 lux) until required for tissue collection (3, 7, 14 or 56 days).

C57BL/6J and C3/− mice were placed into Perspex boxes coated with a reflective interior surface, and exposed to 100 Klux of natural white light-emitting diodes (LED) for
up to 7 days, with free access to food and water. Lighting levels were set to 100 Klux using a light meter device (HD450; Extech MA, USA). During the course of photo-oxidative damage, each animal was administered with pupil dilator eye drops twice daily, morning and evening (Minims Atropine Sulphate 1% w/v eye drops; Bausch and Lomb). Following photo-oxidative damage and post-damage live animal analysis, animals were euthanized for tissue collection. Age-matched non-damaged animals were used in all rat and mouse experiments as dim-reared controls.

2.2.4. Electroretinography (ERG)

Electroretinography (ERG) was used to measure mouse retinal function in response to full-field flash stimuli under scotopic conditions in dim-reared control and photo-oxidative damaged animals in methods previously published [210]. Mice were dark-adapted overnight prior to the measurement of retinal recordings, and all recordings were performed in a dark room with minimal red light sources. Animals were restrained and intraperitoneally anaesthetised using a mixture of Ketamine (100mg/kg body weight; Troy Laboratories) and Ilium Xylazil-20 (12mg/kg body weight; Troy Laboratories). A heat mat was used to maintain core body temperature. A pupil dilator (Minims Atropine Sulphate 1% w/v eye drops; Bausch and Lomb) and eye gel (GenTeal; Novartis) was administered to both eyes prior to taking retinal recordings.

The animal was setup on a homeothermic blanket to maintain body temperature at 37°C using a rectal probe (Harvard Apparatus, MA, USA). A cotton loop was tied around the eye, and the head of the animal was inserted into the Ganzfeld sphere. An earth probe was placed on the hind foot of the animal. An Ag-AgCl reference probe was placed in the mouth of the animal (SDR Scientific, NSW, Australia) and a corneal probe was placed on the corneal surface of the eye to be examined. Eye drops or gel was administered to both eyes prior to commencing the recordings. A BioAmp (AD Instruments, Dunedin,
New Zealand) set at 2mV was used to ensure there was minimal noise in the setup. A single-flash paradigm was used to elicit mixed (rod and cone) responses. Mice were exposed to flash stimuli over the intensity range of -4.4-1.9 Log cd.s/m² using an LED-based system (FS-250A Enhanced Ganzfeld; Photometric Solutions International, VIC, Australia). The interstimulus interval was increased from 2 seconds for the lowest intensities to 4 minutes for the highest intensities to allow complete recovery of the b-wave between stimuli. Following the retinal recordings, eye gel (GenTeal; Novartis) was administered to both eyes of the animal to maintain hydration upon recovery of the animal.

Measurements of the a-wave and b-wave were performed using Lab Chart 8 (AD Instruments). An assessment of retinal function was made by comparison of the amplitudes of the a-wave and b-wave between experimental groups. The amplitude of the a-wave was measured from the baseline to the trough of the a-wave (minimum value), and the amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave (maximum - minimum value). The a-wave and b-wave data were fitted with a Naka-Rushton equation \[ \frac{R}{R_{\text{max}}} = \frac{I}{(I + K)} \] using the Solver function in Microsoft Excel 2013 to determine \( R_{\text{max}} \) (maximum amplitude) and \( K \) (semi-saturation constant) from the response amplitude (R) and the flash intensity (I).

### 2.2.5. Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT) is a technique used to assess morphology of the retinal layers. Cross-sectional retinal and fundus images were undertaken in live rats using a Spectralis HRA+OCT device (Heidelberg Engineering, Heidelberg, Germany). Animals were anaesthetised with an intraperitoneal injection of Ketamine (100 mg/kg body weight; Troy Laboratories) and Ilium Xylazil-20 (12 mg/kg body weight; Troy Laboratories). A heat mat was used to maintain core body temperature. Following anaesthesia, a pupil dilator (Minims Tropicamide 0.5% w/v eye drops; Bausch and Lomb)
and hypromellose 0.3% eye drops (GenTeal; Novartis) was administered to both eyes. 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, Brackley, UK) [222].

Animals were restrained on a custom-made platform attached to the Spectralis HRA+OCT imaging device, adapted for rat eyes according to the manufacturer’s specifications (Heidelberg Engineering). Eyes were kept well-hydrated using the hypromellose eye drops to maintain corneal hydration and improve OCT image quality. Using the Heidelberg Eye Explorer software (Heidelberg Engineering) with a C-Curve setting of 7.7mm (radius of the curvature of the anterior corneal surface), the camera was switched on and confocal scanning laser ophthalmoscopy (cSLO) was used to take fundus images of the retina. A custom glass 25 diopter lens optimised for rodent cSLO was fitted to the device over the 30 degree lens (Heidelberg Engineering). The filter wheel was set to Position A or R (acquisition mode). The camera was moved left to right to line up with the eye, then was moved up and down until aligned with the eye. The camera was moved slowly towards the eye until an evenly-illuminated cSLO image was displayed. The focus was set to 0.00D prior to image acquisition. For OCT images, the camera was moved back and the custom mouse lens was removed. The camera was slowly moved back towards the eye using the focus knob to keep the retina in the display. Once the retina spanned the length of the display at a minimum imaging quality of 25dB, a line scan or volume scan was obtained with an automatic real time (ART) mean of 100 frames. Cross-sectional OCT images were taken in 1mm increments from the optic nerve (up to 3mm superior, and 2mm inferior to the optic nerve) to assess retinal layer thickness. Following retinal imaging, the contact lens was removed from the eye and eye gel (GenTeal; Novartis) was administered to both eyes of the animal to maintain hydration upon recovery.

OCT and fundus images were analysed using ImageJ software (NIH, MD, USA).
Cross-sectional images of the retina were analysed per millimetre in duplicate, with a minimum of 5 measurements of individual retinal layers manually taken per cross-sectional image. The outer nuclear layer (ONL) containing the photoreceptor cell bodies was measured to determine photoreceptor loss in each experimental group following photo-oxidative damage, and compared to control retinas. ONL thickness ratios were calculated as the thickness of the ONL relative to the distance between the inner and outer limiting membranes (ILM-OLM). Fundus images were used to detect the presence of a retinal lesion in areas of ONL thinning, where the fundus appeared darker. Images of the area of interest (2-3mm superior to the optic nerve) were converted to greyscale (8-bit), and contrast and brightness were optimised using the ‘curves’ function in Photoshop CS6 using a standard approach. Images were then converted to bitmaps using controlled parameters (99 pixels/inch, halftone screen, 53 lines/inch, 45˚, square). ImageJ was used to measure the lesion size manually (arbitrary units).

2.2.6. Tissue collection

Following live animal experiments, rats were euthanized intraperitoneally using an overdose of barbiturate (Lethabarb, 60mg/kg body weight; Virbac, NSW, Australia; 2% Ilium Lignocaine-20; Troy Laboratories), and mice were euthanized using carbon dioxide (CO₂). Tissue collection commenced after confirming death, indicated by the loss of movement, reflexes, breathing and heartbeat. To collect the retina for molecular analysis, a corneal incision was made along the surface of one eye, and the retina was excised from the eye and placed into 150µl RNAlater (Thermo Fisher Scientific) at 4°C to preserve the RNA until extraction. The retinas were kept at 4°C overnight prior to their long-term storage at -80°C until required for RNA extraction.

For histological analysis, the superior aspect of the other eye was marked with a permanent marker pen for orientation. Using dissecting scissors and forceps (World
Precision Instruments), each eye was lifted from the socket, and was enucleated from the connective tissues. The eye was injected with approximately 50μl 4% paraformaldehyde fixative using an insulin syringe and was collected into a vial containing 4% paraformaldehyde for 3 hours at 4°C. Following fixation, the eyes were washed 3 times with 0.1M PBS for 5 minutes each wash. The eyes were then left in 15% sucrose solution overnight at 4°C for cryoprotection prior to embedding the following day.

2.3. Histological and in situ molecular analysis

2.3.1. Embedding

Prior to cryosectioning the eyes for histological analysis, enucleated eyes were embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek USA Inc., CA, USA). Dry ice was slowly added to acetone in a plastic container, until the temperature of the mixture had dropped to approximately -78°C. Truncated plastic embedding moulds (Pro Sci Tech, QLD, Australia) were filled with the Tissue-Tek medium. Each eye was placed into an individual mould, with the marked superior aspect of the eye facing the same direction for orientation. The mould was then lowered into the dry ice and acetone freezing mixture and the eye was snap-frozen for approximately 2 minutes, until the Tissue-Tek medium had frozen into a solid block with a small peak in the centre of the block. The mould was double-wrapped in aluminium foil and transferred to -20°C for long-term storage until cryosectioned.

2.3.2. Cryosectioning

Retinal cryosections of each eye were cut using a Leica CM 1850 cryostat (Leica Microsystems). Cryosections were cut at 16μm for rat eyes, and 12μm for mouse eyes,
where each retinal section contained approximately 2 layers of photoreceptors in the outer nuclear layer (ONL). Each eye was mounted onto the cryostat chuck using a small amount of Tissue-Tek medium, with the superior aspect of each eye facing the same direction for consistent sections. Para-sagittal cryosections were collected from the optic nerve (ON) central region of the eye, where retinal damage is primarily located in the superior retina following photo-oxidative damage [206, 209, 210]. Cryosections contained structures from the whole eye, including the sclera, lens, retina, choroid and optic nerve. For each eye, 20-40 sections were collected onto labelled Superfrost Ultra Plus glass slides in duplicate (Menzel-Glaser, Braunschweig, Germany). A Leica DM 1000 light microscope (Leica Microsystems, Wetzlar, Germany) was used to visualise the sections. Slides were oven-dried at 37°C overnight, and were transferred to -20°C for long-term storage.

2.3.3. TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay is used in situ to detect cells undergoing DNA fragmentation during apoptosis, necrosis and autolysis [223]. The terminal deoxynucleotidyl transferase (Tdt) enzyme adds biotinylated dUTP molecules onto fragmented DNA strands. Upon secondary labelling, cell death is visualised and can be quantified.

In retinal cryosections, photoreceptor cell death was investigated using the TUNEL assay. Retinal cryosections containing the optic nerve were equilibrated to room temperature, and a hydrophobic barrier was drawn around the sections on the slides with a pap pen (Daido Sangyo, Tokyo, Japan). Slides were immersed in 70% ethanol for 10 minutes and then Milli-Q water (MQ H₂O) for 10 minutes. 0.1% Triton X (Sigma Aldrich) was added to sections for 2 minutes for permeabilisation of the membranes, before rinsing in 0.1M PBS for 10 minutes. Slides were equilibrated in 1x Tdt buffer for 10 minutes prior to addition of the Tdt reaction mixture.
The Tdt reaction mixture was prepared using 1060µl MQ H2O, 125µl 10x Tdt buffer, 3µl biotin dUTP (Roche Diagnostics, Basel, Switzerland) and 1.5µl Tdt enzyme (Roche Diagnostics). The mixture was added evenly across all sections, and the slides were incubated at 37°C for 1 hour in a humid chamber. The reaction was then stopped by immersing the slides in 2x saline-sodium citrate (SSC) buffer for 15 minutes. Slides were blocked with 10% normal goat serum (NGS; Sigma Aldrich) for 10 minutes prior to adding the secondary label. Streptavidin conjugated to Alexa Fluor 594 (1:500; Thermo Fisher Scientific) was added evenly across all sections under dim conditions. The slides were incubated at 37°C for 1 hour in a humid chamber. Following secondary labelling, slides were rinsed twice in 0.1M PBS for 5 minutes each. For visualisation of the cellular layers, a DNA label (Bisbenzimide or Hoechst’s stain, 1:10000; Sigma Aldrich) was added to all sections for 2 minutes, followed by another rinse in 0.1M PBS for 5 minutes. Slides were coverslipped with Aqua Poly/Mount (Polysciences, PA, USA) prior to visualisation. Slides were sealed with nail varnish and were stored at 4°C.

2.3.4. Immunohistochemistry

The detection and localisation of specific proteins in situ was determined using immunohistochemistry (IHC), with primary antibodies utilised throughout this thesis detailed in Table 2.1. Retinal cryosections containing the optic nerve were used for histological analysis. For comparison, a negative control slide (no primary antibody) was included with each immunohistochemistry run. Slides were equilibrated at room temperature and a hydrophobic barrier was drawn around the sections with a pap pen. Slides were soaked in 70% ethanol for 10 minutes, MQ H2O for 5 minutes, and 0.1M PBS for 5 minutes. To perform antigen retrieval, sections were incubated in Reveal-It Ag (ImmunoSolution, QLD, Australia) at 37°C for 1 hour. Sections were blocked with 10% normal goat serum (NGS) at room temperature for 1 hour. Modifications to the blocking
procedure are as follows: 3% bovine serum albumin (Roche Diagnostics) was used as the blocking solution prior to the addition of the α-C3d antibody, while Image-iT FX Signal Enhancer (Thermo Fisher Scientific) was used as the blocking solution for the α-C3 antibody. Primary antibodies (Table 2.1) were then added evenly to each section. Slides were incubated at 4°C for approximately 16 hours overnight in a humid chamber.

Slides were washed 4 times, each for 5 minutes in 0.1M PBS. Secondary antibodies, conjugated to either Alexa 594 or 488 (Thermo Fisher Scientific), were added evenly to the sections under dim conditions. Slides were incubated at room temperature for 4 hours or at 37°C for 2 hours in a humid chamber. Following incubation with the secondary antibody, slides were washed 4 times, each for 5 minutes in 0.1M PBS. A DNA label (Bisbenzimide or Hoechst’s stain) was added to the sections for 2 minutes, for visualisation of the cellular layers. Slides were rinsed in 0.1M PBS for 5 minutes before coverslipping with Aqua Poly/Mount. Slides were sealed with nail varnish and stored at 4°C. Note: For double labelling, both primary antibodies (and both secondary antibodies) were added simultaneously.

Table 2.1. List of primary antibodies used for immunohistochemistry

<table>
<thead>
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<th>Primary Antibody</th>
<th>Dilution</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
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<td>AB118877</td>
<td>Abcam, Cambridge, UK</td>
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<tr>
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<td>AF2655</td>
<td>R&amp;D Systems, MN, USA</td>
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<td>Z0334</td>
<td>Dako, Glostrup, Denmark</td>
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<tr>
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<td>AF501</td>
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<td>Merck Millipore, MA, USA</td>
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<td>Sigma Aldrich, MO, USA</td>
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2.3.5. **In situ hybridisation**

*In situ* hybridisation (ISH) is a technique utilised to visualise mRNA expression in tissue sections, to determine the cellular source and/or expression pattern of a specific gene of interest. A riboprobe is synthesised with the complementary sequence to the mRNA being detected. In this thesis, *C3*, *Ccl2* and *Cxcl1* mRNA transcripts were detected in retinal cryosections using riboprobes previously synthesised [87, 141]. *C3* was cloned from PCR products derived from human (460bp amplicon) and rat (483bp amplicon) retinal cDNA. *Ccl2* and *Cxcl1* were also cloned from PCR products (550-bp and 504-bp amplicons respectively) from rat retinal cDNA. These cloned templates were then synthesised into digoxigenin (DIG)-labelled riboprobes. *In situ* hybridisation was conducted using our established protocol [224]. All chemicals were sourced from Sigma Aldrich, unless specified.

RNAses were cleaned from the bench and wash containers with RNase Zap solution (Thermo Fisher Scientific) and MQ H2O. Slides were labelled and air-dried in a fume hood or biological cabinet for 30 minutes. A slide for addition of the ‘sense’ probe was included in the run as a control. For the pre-treatment steps prior to hybridisation, slides were immersed in 0.1M PBS for 5 minutes then in 10% neutral buffered formalin (NBF) for 20 minutes. Slides were rinsed twice in 0.1M PBS for 5 minutes each, before being treated with 20µg/ml Proteinase K in Tris-EDTA (TE) at 37°C for 7 minutes. Slides were rinsed in 0.1M PBS for 5 minutes, and then were soaked in 10% NBF for 20 minutes. Slides were placed in 0.1M triethanolamine (TEA) containing 630µl of acetic anhydride for 10 minutes, followed by a 0.1M PBS 5 minute wash. Slides were washed in 0.9% NaCl for 5 minutes. A series of ethanol solutions were used to dehydrate the sections; 50% ethanol for 1 minute, 70% ethanol for 2 minutes, 90% ethanol for 1 minute, 2 washes with
100% ethanol for 2 minutes. The slides were then air-dried in the fume hood or biological cabinet for 1 hour prior to hybridisation.

The pre-hybridisation (pre-hyb) and hybridisation (hyb) mixes (prepared from NaCl, Tris-HCl, NaH₂PO₄, NaEDTA, Ficoll 400, dithiothreitol, poly ribo A, yeast t-RNA, dextran sulphate, formamide) were heated to 60°C. The pre-hyb mix was added evenly to the sections, and a coverslip was placed on top of the sections. The slides were placed in a hydrated fume box at 55-57°C for 3 hours. Following the pre-hybridisation step, the hyb mix containing the antisense probe was added evenly to the slides; the sense probe was added to the control slide, and a coverslip was placed onto the sections. The slides were incubated in a hydrated fume box at 55-57°C for approximately 16 hours overnight.

Following hybridisation, the slides were washed in a series of saline-sodium citrate buffers (SSC, pH 7.4). Slides were briefly dipped 2 to 3 times into 4x SSC at 60°C to remove the coverslips. Slides were washed in 2x SSC at 60°C for 30 minutes, 1x SSC at 60°C for 30 minutes, twice in 0.1x SSC at 60°C for 30 minutes, and finally in 0.1x SSC at room temperature for 10 minutes. To prepare the slides for antibody detection, slides were rinsed for 1 minute in washing buffer, constituted from maleic acid, Tween-20 (Amresco, OH, USA) and MQ H₂O. Slides were then blocked with 2x blocking solution for 30 minutes (made from Blocking Reagent, maleic acid and MQ H₂O). The anti-digoxigenin (DIG) antibody (1:1000; Roche Diagnostics) was added evenly to the slides, and incubated at room temperature for 30 minutes.

Following addition of the antibody, slides were washed 3 times in washing buffer for 40 minutes each, and then washed in detection buffer (constituting Tris-HCl, NaCl, MQ H₂O, no Mg²⁺) for 5 minutes. Slides were then incubated in a NBT/BCIP colour substrate reaction mixture in detection buffer at 4°C for 1 to 16 hours (variable) under dim conditions until the colour reaction had fully developed with minimal background staining.
as checked under a Leica DM 1000 light microscope (Leica Microsystems), taking care not to overstain the slides. Slides were then rinsed in MQ H$_2$O for 10 minutes to stop the reaction, and then placed into 10% NBF for at least 20 minutes at 4°C. Slides were rinsed twice in 0.1M PBS for 5 minutes. For double labelling of the sections, immunohistochemistry was used following the protocol described above. As an alternative to NBT/BCIP staining, HNPP/Fast-Red (Roche Diagnostics) was incubated for 1.5 hours prior to immersion in MQ H$_2$O to stop the reaction. Slides were coverslipped in Aqua Poly/Mount and stored at 4°C.

2.4. **Molecular analysis**

2.4.1. **Fluorescence-activated cell sorting (FACS)**

Fluorescence-activated cell sorting (FACS) was utilised to isolate CD11b+ cells from rat retinas, which were pooled from 2 animals (4 retinas) for each sample. Retinas were removed from the rat eye through a corneal incision and were placed into Hank's balanced salt solution (HBSS with 0.1% glucose, no phenol red, Ca$^{2+}$ or Mg$^{2+}$; Thermo Fisher Scientific) at 4°C. Retinas were transferred onto a sterile petri dish and crosshatched using a razor blade to fragment the retinas. A pipette tip was used to transfer the retinas to an Eppendorf tube containing 0.5ml of chilled digestion mix, containing 2mg/ml (0.2%) papain (Worthington Biochemicals, NJ, USA), 660 µg/ml (5.5mM) 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). Samples were incubated at 37°C for 7 minutes then at 8°C for 30 minutes, during which samples were gently homogenised with a pipette to dissociate the cells in suspension.
Samples were centrifuged for 5 minutes at 1550 rpm at 4°C, and the supernatant was removed. Cells were resuspended and incubated for 10 minutes in 0.5ml chilled neutralisation mix, containing HBSS (with 0.2% glucose and 4% bovine serum albumin (BSA; Roche Diagnostics)), 50µg/ml antipain (Roche Diagnostics), 10µg/ml gentamycin, 100U/ml DNAse I, 5µg/ml SOD and 5µg/ml catalase. Samples were centrifuged for 5 minutes at 1550 rpm at 4°C, and the supernatant was removed. Cells were resuspended in 1ml of chilled staining buffer, containing HBSS, 1mg/ml (0.1%) sodium azide (Sigma Aldrich) and 10mg/ml (1%) BSA. Cells were divided into multiple tubes for antibody staining as necessary (including an unstained control) and centrifuged for 5 minutes at 1200 rpm at 4°C. After the supernatant was removed, the CD11b antibody conjugated to Alexa 647 (Biolegend, CA, USA) was diluted in chilled staining buffer, and was added to the samples for 30 minutes at 4°C. The unstained control sample was resuspended in staining buffer without an antibody.

Samples were centrifuged for 5 minutes at 1200 rpm at 4°C and the supernatant was removed. The cells were resuspended and centrifuged twice in washing buffer, containing 1mg/ml (0.1%) sodium azide, 10mg/ml (1%) BSA and 2mM EDTA. Following the final spin down of the cells, samples were resuspended in chilled staining buffer and were ready for analysis. CD11b-stained samples were sorted through a fluorescence activated cell sorter at the Imaging and Cytometry Facility at JCSMR, ANU (FACS Aria II; BD Biosciences, NJ, USA). The isolated CD11b+ cells were collected in chilled staining buffer and were kept at 4°C for immediate RNA extraction.

2.4.2. RNA extraction

Total RNA was extracted from whole rat retinas using a combination of TRIzol (Thermo Fisher Scientific) and an RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific). For RNA extraction from whole mouse retinas, cultured cells or FACS-isolated
cells, an RNAqueous Micro Total RNA Isolation Kit (Thermo Fisher Scientific) was used. Retinas/cells were homogenised in 200µl TRIZol using a plastic pestle. Following homogenisation, another 400µl TRIZol (660µl for the micro kit; 1ml per 50-100mg tissue) was added to the samples, vortexed for 5 seconds and incubated at room temperature for 7 minutes. 1/5 volume of chloroform was added to the samples, which were vortexed thoroughly for 20 seconds. Samples were centrifuged for 10 minutes at 13000g at 4°C to separate the layers into RNA, DNA and protein phases. Following centrifugation, the top aqueous phase containing the RNA was collected into a new sample tubes. An equal volume of 64% ethanol was added to the RNA (half volume of 100% ethanol for the micro kit). Samples were vortexed for 5 seconds and transferred into the appropriate filter column for the kit, followed by centrifugation for 10 seconds at 13000g to trap the RNA in the filter. The flowthrough was discarded.

Samples were then run through a series of wash steps to purify the RNA. 700µl of wash solution 1 (180µl for the micro kit) was added to the columns and centrifuged for 10 seconds at 13000g. After discarding the flowthrough, 500µl of wash solution 2/3 (180µl for the micro kit) was washed through the columns twice and centrifuged for 10 seconds at 13000g. The flowthrough was discarded, and the filter columns were air-dried in the centrifuge for 1 minute at 13000g. The filter columns were placed in new tubes for collection of the RNA. Pre-heated 75°C elution solution was added onto the filter (30µl for a standard sized filter, 10µl for the small filter in the micro kit) and left for 1 minute to heat the filter to allow the RNA to be eluted through. Columns were spun in the centrifuge for 30 seconds at 13000g. Another 20µl of heated elution solution (10µl for the micro kit) were added to the filters for further elution of the RNA. Samples were analysed for RNA quantity and purity using an ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA) and were stored at -80°C.
2.4.3. cDNA preparation

Using 500-1000ng of extracted RNA, cDNA was prepared using a Tetro cDNA Synthesis Kit (Bioline, London, UK). A 14µl mixture of RNA, Oligo (dT) Primer (500µg/ml), dNTPs (10mM) and RNAse-free H₂O was prepared for each RNA sample in 0.2ml tubes. Using a Veriti 96-Well Thermal Cycler (Applied Biosystems, CA, USA), the tubes were incubated for 5 minutes at 70°C then were chilled at 4°C for 5 minutes. The reverse transcriptase master mix was prepared from 5x RT buffer (4µl per tube), RiboSafe RNAses Inhibitor (10U/µl; 1µl per tube) and Tetro Reverse Transcriptase (200U/µl; 1µl per tube). 6µl of master mix was added to each sample tube. For a negative control tube, no reverse transcriptase enzyme was added. All tubes were incubated for 30 minutes at 45°C on the thermocycler. The reaction was terminated by increasing the temperature to 80°C for 5 minutes, and then tubes were chilled at 4°C. cDNA samples were stored at -20°C.

2.4.4. Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qPCR) was performed using synthesised cDNA. The master mix for each cDNA sample was prepared for each gene to be run in technical duplicate. For each reaction, 0.2-0.4µl of cDNA template (prepared from 500-1000ng RNA) was added to RNase-free H₂O to a total volume of 4.5µl. These master mixes were added to a 384-well plate (Applied Biosystems) for each cDNA sample. To prepare the gene master mixes for each cDNA sample in duplicate, 2x Taqman Gene Expression Master Mix (5µl per reaction; Thermo Fisher Scientific) was mixed with specific Taqman hydrolysis assays (0.5µl per reaction; Thermo Fisher Scientific) dependent on the gene of interest. Table 2.2 is a list of Taqman assays used throughout this thesis. These master mixes were added into the 384-well plate. Plates were covered with a MicroAmp Optical Adhesive Film (Applied Biosystems), and were run on a QuantStudio 12K Flex instrument (Applied Biosystems). The plate was incubated at 50°C for 2 minutes,
and then was heated to 95°C for 10 minutes for AmpliTaq Gold DNA Polymerase activation. In the PCR stage (40 cycles), samples were heated at 95°C for 15 seconds to denature the strands, then at 60°C for 1 minute to anneal and extend; the fluorescence in each well was read on the instrument. The expression of all target genes was compared to the Gapdh and Actb housekeeping genes using the comparative cycle threshold (Ct) method (ΔΔCt). These reference genes do not change expression with retinal photo-oxidative damage [225]. The QuantStudio software was used for qPCR analysis.

**Table 2.2.** Taqman hydrolysis assays used for qPCR (Thermo Fisher Scientific)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Catalogue Number</th>
<th>Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>Actin, beta</td>
<td>Mm01205647_g1</td>
<td>11461</td>
</tr>
<tr>
<td>C1s</td>
<td>Complement component 1, s subcomponent</td>
<td>Rn00594278_m1, Mm01625167_g1</td>
<td>192262, 50908</td>
</tr>
<tr>
<td>C2</td>
<td>Complement component 2</td>
<td>Rn00597176_m1, Mm00442726_m1</td>
<td>24231, 12263</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
<td>Mm00437858_m1</td>
<td>12266</td>
</tr>
<tr>
<td>C4a</td>
<td>Complement component 4A (Rodgers blood group)</td>
<td>Rn00709527_m1, Mm01132415_g1</td>
<td>24233, 625018</td>
</tr>
<tr>
<td>Casp1</td>
<td>Caspase 1</td>
<td>Rn00562724_m1</td>
<td>25166</td>
</tr>
<tr>
<td>Casp8</td>
<td>Caspase 8</td>
<td>Rn00574069_m1</td>
<td>64044</td>
</tr>
<tr>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>Rn01456716_g1</td>
<td>24770</td>
</tr>
<tr>
<td>Ccl3</td>
<td>Chemokine (C-C motif) ligand 3</td>
<td>Rn00564660_m1</td>
<td>25542</td>
</tr>
<tr>
<td>Ccl4</td>
<td>Chemokine (C-C motif) ligand 4</td>
<td>Rn00587826_m1</td>
<td>116637</td>
</tr>
<tr>
<td>Ccl7</td>
<td>Chemokine (C-C motif) ligand 7</td>
<td>Rn01467286_m1</td>
<td>287561</td>
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<tr>
<td>Cfb</td>
<td>Complement factor B</td>
<td>Rn01526084_g1, Mm00433918_g1</td>
<td>294257, 14962</td>
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<td>Cfd</td>
<td>Complement factor D (adipsin)</td>
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<tr>
<td>Cfh</td>
<td>Complement factor H</td>
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<tr>
<td>CfI</td>
<td>Complement factor I</td>
<td>Rn00577333_m1</td>
<td>79126</td>
</tr>
<tr>
<td>Cntf</td>
<td>Ciliary neurotrophic factor</td>
<td>Mm00446373_m1</td>
<td>12803</td>
</tr>
</tbody>
</table>
2.4.5. Standard PCR

Standard PCR was used to detect amplification products in samples following cDNA synthesis. Forward and reverse primers specific to genes of interest (Chapters 4-5) were designed using the Primer3 web-based design program [226]. Oligonucleotides were synthesised by Sigma Aldrich. The PCR reaction mixture was setup using the MyTaq DNA Polymerase kit (Bioline). Components included cDNA template (40ng/µl), MyTaq Reaction Buffer (5x), MyTaq Polymerase, forward and reverse primers (20µM), and DNAse-free H2O. A negative control sample had no cDNA added to the reaction mixture. The PCR was run via a standard amplification protocol on a Veriti 96-Well Thermal Cycler (Applied Biosystems); an initial phase of 95°C preceded the amplification phase. This was followed by 35 cycles of melting (95°C for 15 seconds), annealing (60°C for 15 seconds), and extension (72°C for 15 seconds).
seconds) and amplification (72°C for 10 seconds). To finish, samples were run at 72°C for 1 minute. Following PCR, amplification products were run on a 1% agarose gel via electrophoresis, with Gel Red (Biotium, CA, USA) used for visualisation. Bands on the gel were imaged using a UV light source and camera (Vilber Lourmat, Paris, France).

2.5. Imaging and statistical analysis

2.5.1. Quantification of cell counts and ONL thickness

TUNEL, IBA1 and C3 positive cells were quantified along the full length of retinal cryosections (supero-inferior) in duplicate. Outer retinal counts (ONL-RPE) were taken for IBA1+ and C3+ staining. To determine photoreceptor cell death, only TUNEL+ cells in the ONL were quantified. To quantify the development of an outer retinal lesion, ONL thickness was quantified in OCT images as described earlier. To quantify photoreceptor loss in retinal cryosections, the number of rows of photoreceptor nuclei (visualised using a DNA label) was quantified at the lesion site in the superior retina (approximately 2mm superior to the optic nerve in rats, 1mm in mice). For each section, 5 measurements were taken, and each eye was counted in duplicate.

2.5.2. Confocal imaging

Fluorescence in retinal cryosections was visualised and imaged using a laser-scanning A1+ confocal microscope (Nikon, Tokyo, Japan). Images were acquired using the NIS-Elements AR software (Nikon). Negative control slides were visualised and imaged with each different immuno-label, to determine specificity of staining and the lower threshold of fluorescence detection for comparison to positively-stained slides. All slides were imaged using the same settings for each immuno-label for the comparison of positive staining between experimental groups. The intensity of fluorescent staining was
determined using ImageJ, and images were processed and assembled into figure panels using Photoshop CS6 software (Adobe Systems, CA, USA).

2.5.3. **Statistical analysis**

All graphing and statistical analysis was performed using Prism 6 (GraphPad Software, CA, USA), using unpaired student t-tests, one-way analysis of variance (ANOVA) or two-way ANOVA with the appropriate post-hoc tests to determine statistical significance (P<0.05), as indicated throughout this thesis in Chapters 3-5. Graphs were generated with mean ± SEM values.
3. RESULTS

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

This chapter is presented as the following publication (* denotes equal first author):


The candidate has made a major contribution as a first author to this study (generating 75% of rat data, 50% of mouse data and preparing the manuscript for publication).
3.1. 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 subject 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 rodent 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.
3.2. 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 [145, 146]. Prolonged cleavage of C3 may lead to membrane attack complex (MAC) assembly and cytolysis [145, 146]. Complement activation is under tight control, including regulation by complement factor H (CFH), a critical inhibitor. However, in neurodegenerative diseases including age-related macular degeneration (AMD), regulation of complement activation is compromised, contributing to disease onset and progression (reviewed in [3, 152, 227]).

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 [42]), however, the Y402H mutation in CFH is the most highly associated risk factor [153, 154]. Other variants in C3, CFB, and C2 genes are associated with AMD susceptibility (reviewed in [152]), with complement gene variants in the population estimated to account for ~70% of the risk for developing AMD [152].

Poor understanding of the cellular events that initiate and feed complement activation in the retina has been a significant obstacle in development of innovative approaches in the management of AMD [3]. 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 [62, 153, 157, 159-162]. 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 [47], 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 in the retina. We identified 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.

3.3. Methods

3.3.1. 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<sup>-/-</sup>, 129S4-C3tm1Crr/J), both aged 60-80 days, were obtained from the Australian Phenomics Facility. For the C3 KO experiments, the KO 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.

3.3.2. Photo-oxidative damage and tissue collection

SD rats were placed into transparent Perspex open-top boxes and exposed to 1000lux white light for 24hrs (COLDf2, 2x36W, IHF, Thorn Lighting, Spennymoor, UK), with free access to food and water [209]. Following photo-oxidative damage (PD), 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 (5lux), then euthanized.

C57BL/6J and C3⁻/⁻ mice were housed in Perspex boxes coated with a reflective interior, and exposed to 100Klux of natural white LED for 1, 3, 5 and 7 days, with free access to food and water [228]. Each animal was administered with pupil dilator eye-drops twice daily during PD (Atropine Sulphate 1%/v eye-drops; Bausch and Lomb, NSW, Australia). Following PD, 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 [228]. 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 [141, 228].

3.3.3. Optical Coherence Tomography (OCT)

Cross-sectional and fundus images of live rat retinas were undertaken using a Spectralis™ HRA+OCT device (Heidelberg Engineering, Heidelberg, 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%/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, Brackley, UK) [222]. 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.

3.3.4. **Treatment of mice with cobra venom factor (CVF)**

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 [229]. The absorbance at 540nm was read using an Infinite-200-PRO plate spectrometer (Tecan, Mannedorf, Switzerland) and the percentage of haemolysis was calculated, and compared to serum from PBS-injected mice as a positive control, and C3−/− serum as a negative control.

3.3.5. **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 [89]; 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.

3.3.6. 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 [209, 210]. 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 3.1) as described in our previous protocols with minor modifications [87, 141]. 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
For quantification, only the cell bodies of the IBA1+ cells were counted so as to prevent the occasional ambiguity of IBA1+ processes from skewing the dataset.

Table 3.1. Primary antibodies used for immunohistochemistry

<table>
<thead>
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<th>Primary Antibody</th>
<th>Dilution</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
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<tr>
<td>Rabbit α-IBA1</td>
<td>1:500</td>
<td>019-19741</td>
<td>Wako, Osaka, Japan</td>
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<tr>
<td>Goat α-C3d</td>
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<td>AF2655</td>
<td>R&amp;D Systems, MN, USA</td>
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<td>Rabbit α-C3</td>
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<td>AB118877</td>
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<tr>
<td>Rabbit α-Rhodopsin</td>
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<td>AB9279</td>
<td>Merck Millipore, MA, USA</td>
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<tr>
<td>Rabbit α-GFAP</td>
<td>1:500</td>
<td>Z0334</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

To assess ONL thinning on cryosections following photo-oxidative damage, the section were stained with Toluidine Blue and then quantified in accordance with our previous methodology [228]. Briefly, number of rows of photoreceptor nuclei were quantified within the lesion of the superior retina (1-2mm superior to the optic nerve in mice), and counts were subsequently averaged for each experimental group.

3.3.7. 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 [141].

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 [230]. In brief, human eyes were collected with informed consent through the Lions NSW Eye Bank, Sydney, Australia, with ethical approval from the Human Research Ethics
Committee of the University of Sydney and The Australian National University. Grading for the eyes ranged from normal to early- or late-AMD, and which was assigned by a team of experienced graders according to published pathological criteria [231].

*In situ* hybridisation was conducted using our established protocol [224]. 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 nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), and sections were double-labelled using IBA1 immunohistochemistry.

3.3.8. 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 [232]. 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 3.2) 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 (ΔΔCt) which was normalised to the expression of *Gapdh* and *Actb* reference genes, as established previously [141, 144].

**Table 3.2.** Taqman hydrolysis probes used for qPCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
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<th>Catalogue Number</th>
<th>Entrez Gene ID</th>
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<tr>
<td>Gene</td>
<td>Description</td>
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<td>-------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
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<tr>
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3.3.9. OCT and fundus images

OCT and fundus images were analysed using ImageJ software (NIH, MD, USA). Retinal thickness, and ONL depth were measured in 5 OCT transects (superior and inferior to the optic nerve) spaced at 1mm intervals, with 5 points sampled across each image. ONL thickness ratios were calculated as the ONL thickness relative to the distance between the outer and inner limiting membranes. To detect fundus lesions, images of the area of interest (2-3mm superior to the optic nerve) were converted to grayscale, and contrast was optimised using the ‘curves’ function in Photoshop CS6 using a standard approach. Images were then converted to bitmaps using controlled parameters. ImageJ was used to measure the lesion size manually (arbitrary units).
3.3.10. Statistics

All graphing and statistical analysis was 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, while Fisher’s uncorrected Least Significant Difference (LSD) was used for instances of single comparisons. Student’s t-test was utilised for other single comparisons, where noted.

3.4. Results

3.4.1. 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 3.1). We detected minimal C3 mRNA expression in normal retinas, and little-to-none within RPE cells or choroid (Figure 3.1A). 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 3.1B-D). We detected numerous C3-expressing cells in regions of advanced scarring (Figure 3.1E-F), at lesion edges (Figure 3.1G-I), and near the optic nerve head (Figure 3.1J). In contrast, barely any labelling was detected in the choroid of these lesioned areas (Figure 3.1E). Counter immunolabelling indicated that most of the C3-expressing cells were immunopositive for the macrophage/microglia marker IBA1 (Figure 3.1K-N).
Figure 3.1. 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; 50µm in K; 10µm in C,D,F,I,M.
3.4.2. 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 3.2). Measurements of outer nuclear layer (ONL) thickness were taken from cross-sectional images along 3 horizontal transects in the superior retina (Figure 3.2A), 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 3.2B). The data show a significant lesion detectable by 7 days (P<0.05, Figure 3.2B, 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 3.2B, F). A retinal lesion (2-3mm superior to the optic nerve) was detected in fundus images from 3 days onwards (Figure 3.2G-M). The lesion size steadily increased from 3 to 14 days, and doubled from 14 to 56 days (P<0.05, Figure 3.2G-M).

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 3.3). 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 3.3B). While the complement regulators Cfb, Cfd, Serping1, Cfh and Cfi were all highly up-regulated early in the time-course (0-7 days, Figure 3.3C), none were found to show any significant change by 56 days, compared to controls (Figure 3.3D, P>0.05).

Following photo-oxidative damage, there was an incursion of IBA1+ microglia and macrophages into the ONL and subretinal space (Figure 3.4). This persisted within the lesion up to 56 days (Figure 3.4A). Counts of IBA1+ cells in the ONL and subretinal space showed a ~7-fold increase between 0 and 7 days (Figure 3.4A, C), which decreased to
Figure 3.2. 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 indicates 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 bitmap analysis of the lesion area in ImageJ. A gradual increase in lesion size over time was observed, which was significant from 14 to 56 days (P<0.05). **H-M:** Representative lesion area fundus images from 2-3mm superior to the optic nerve (dashed box in A). No lesion was detected in control (H) and 0 day (I) images. A retinal lesion was observed in fundus images from 3 days (J), which gradually increased in size at 7 days (K), 14 days (L) and 56 days (M). Statistical analysis was determined using one-way ANOVA with Tukey’s post-hoc test (P<0.05). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS/OS, inner and outer segments; S, superior; I, inferior. N=4 animals per group.
Figure 3.3. 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 animals per group.
Figure 3.4. 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 animals per group. Scale bars represent 50µm.
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 3.4A, D-E).

*In situ* hybridisation (ISH) to localise expression of C3 mRNA at 7, 14 and 56 days post-exposure was consistent with previous findings in this model at 7 days [141], showing C3 mRNA localisation in the neural retina and subretinal space (Figure 3.5A-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 up to 56 days (Figure 3.5A-F). Co-localisation with IBA1+ immunolabelling (Figure 3.5G-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, consistent with our previous reports [141, 144].

We used immunoreactivity for C3d, a by-product of C3 cleavage and an indication of complement activation, to visualise the distribution of C3 following photo-oxidative damage (Figure 3.5J-V). C3d labelling was absent in dim-reared controls (Figure 3.5J). 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 3.5K, L). These features persisted up to 56 days (Figure 3.5M-P). Double immunolabelling for IBA1 and C3d showed a close association of macrophages with C3d deposits situated within the lesion (Figure 3.5Q-V).

### 3.4.3. 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 [228]: C3 knockout (C3<sup>-/-</sup>), local inhibition of C3 using siRNA, and systemic C3 depletion using Cobra Venom Factor (CVF).
Figure 3.5. Infiltration of C3-expressing cells and C3d protein deposition in the outer rat retina following photo-oxidative damage (PD). A: There was no outer-retinal C3 expression in dim-reared controls. B-D: At 7 days post-damage, C3-expressing cells were detected within the lesion and at the lesion edges in the outer retina (B-C), as well as near
the inner retinal vasculature (D). E: C3-expressing cells were present in the outer retina at 14 days. F: At 56 days, C3-expressing cells were still present at the lesion edges. G-I: These C3-expressing cells were identified as IBA1+ microglia/macrophages. J: Low levels of C3d were detected in the inner retinal vessels of controls. K: At 7 days post-damage, C3d deposition was evident throughout the outer retina (ONL and subretinal space) at the lesion edges. L: C3d labelling peaked at 14 days, with large amounts of protein deposition detected at the lesion edges. M-P: At 56 days, there was still C3d labelling present within the lesion (M-N) and at the lesion edges (O-P). Q-U: At 14 days (Q) and 56 days (R-U), there was some co-localisation of the C3d protein with IBA1+ microglia/macrophages at the lesion edges. V: The negative control (no primary antibodies) showed only background staining, with some visible autofluorescence of debris near the outer retina. Representative images derived from N=4 animals per group. INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; C, choroid; IPL, inner plexiform layer. Scale bars represent 50µm in A-M,O,Q-R,V; 10µm in N,P,S-U.
3.4.4. **C3 knockouts**

Previous reports indicate that complete ablation of C3 reduces the ERG function and outer-retinal integrity in 12-month old animals [233]. For this reason we carried out experiments on P60-80 mice, in which no functional or histological deviations were observed [233, 234]. Dim-reared C3⁻/⁻ animals were assessed for any functional or histopathological changes compared to wild types (Wt). These comparisons showed no reduction in ERG amplitudes, changes in rhodopsin (Rhod) expression, or up-regulation of the stress/neuroprotective factors GFAP, Fgf-2, or Cntf (Supplementary Figure 3.1).

C3 gene expression and immunoreactivity for C3 were confirmed as absent in C3⁻/⁻ animals and in dim-reared Wt mice (Figure 3.6A-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 3.6A). Immunoreactivity for C3 deposits in the ONL and subretinal space of Wt retinas was evident at 5 and 7 days (Figure 3.6C-E), and absent in C3⁻/⁻ retinas over the same period (Figure 3.6F). Over the time-course of photo-oxidative damage, the ONL was better preserved in C3⁻/⁻ mice compared to Wt (Figure 3.6G-M); at 7 days ONL thickness was significantly greater in the C3⁻/⁻ mice compared to Wt (P<0.05, Figure 3.6G, J, M).

The a-wave and b-wave responses of the ERG reflects the differences in retinal morphology of Wt and C3⁻/⁻ animals described above, where the ERG a-wave and b-wave intensity response characteristics between groups was significantly different (P<0.05, Figure 3.6N-O). C3⁻/⁻ mice had higher a- and b-wave responses compared to Wt mice (Figure 3.6N-O); this difference was most pronounced at the highest flash intensity (P<0.05, Figure 3.6P).
Figure 3.6. 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 animals). 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 animals). H-M: Toluidine blue staining of Wt and C3−/− retinas show 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 (at the site of the retinal lesion, 1-2mm superior to the optic nerve). 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 animals), which was most pronounced at 1.9 Log cd.s/m² (P, P<0.05, N=10 animals). 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 50µm.
3.4.5. **Local C3 inhibition using siRNA**

C3 siRNA significantly reduced C3 gene expression in retinas at 7 days photo-oxidative damage (p<0.05, Figure 3.7A). Lower levels of C3 protein deposits were observed in C3 siRNA-injected retinas compared with those injected with negative control siRNA (Figure 3.7B-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 3.7D).

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 3.7E). Comparison of retinal thickness shows that the ONL is significantly thicker (P<0.05) in C3 siRNA-injected animals compared with negative control siRNA (Figure 3.7E). 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 3.7H). The ERG a-wave and b-wave intensity response characteristics between groups was significant (p<0.05, Figure 3.7F-G).

3.4.6. **Systemic C3 depletion using Cobra Venom Factor**

To understand the possible role of serum complement components 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 at 7 days damage, serum complement activity was depleted by CVF to a comparable level to C3−/− mouse serum, but not in PBS-injected animals, indicating that systemic complement was inhibited by CVF (P<0.05, Figure 3.8A).
A: Retinal C3 - 7 Days PD

![Graph showing fold change compared to Neg. siRNA (Log2) for C3 siRNA and Neg. siRNA after intravitreal injection.]

D: Liver C3 Expression

![Graph showing fold change compared to Neg. siRNA (Log2) for C3 siRNA and Neg. siRNA after intravitreal injection.]

ERG Function - 7 Days PD

F: a-wave

![Graph showing ERG a-wave amplitude for C3 siRNA and Neg. siRNA at various flash intensities (Log cd.s/m²).]

G: b-wave

![Graph showing ERG b-wave amplitude for C3 siRNA and Neg. siRNA at 1.9 Log cd.s/m².]

B: 7 Days PD

![Image showing INL, ONL, RPE, and C3 INC with Neg. siRNA.]

C: 7 Days PD

![Image showing INL, ONL, RPE, and C3 INC with C3 siRNA.]

E: ONL - 7 Days PD

![Bar chart showing rows of photoreceptor nuclei for C3 siRNA and Neg. siRNA after intravitreal injection.]

H: Amplitude (µV)

![Bar chart showing amplitude for a-wave and b-wave for C3 siRNA and Neg. siRNA at 1.9 Log cd.s/m².]

* indicates statistical significance.
**Figure 3.7.** 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 animals). **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 animals). **E:** There was a significant reduction in photoreceptor loss in C3 siRNA-injected retinas compared to negative siRNA controls (P<0.05, N=13 animals). **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 animals). 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.
A: Complement Serum Activity

B: Retinal Gene Expression

C: 7 Days PD

D: 7 Days PD

E: ONL Thickness

F: a-wave

G: b-wave

H: Amplitude (μV)

ERG Function - 7 Days PD
**Figure 3.8.** 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 animals). **B:** Retinal expression of complement genes ($C3$, $C1s$, $C2$, $C4a$, $Cfb$, $Cfh$) were not significantly affected by CVF depletion of systemic complement (N=9 animals). **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 animals). **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 animals). 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.
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 3.8B). In addition, no difference was observed in retinal C3 deposition between CVF- and PBS-injected animals at 7 days (Figure 3.8C-D).

Analysis of retinal morphology showed that depletion of systemic complement does not protect against retinal degeneration. No difference in ONL thickness in CVF-injected mice compared with PBS-injected mice was observed (Figure 3.8E). 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 3.8F-H).
**Supplementary Figure 3.1.** 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 animals). **D-G:** Rhodopsin labelling in the outer segments (OS) was similar in the Wt and C3⁻/⁻ retinas (D-F, N=5 animals), and there was no change in *Rhod* gene expression between both groups (G, N=3 animals). **H-J:** GFAP expression in the Müller cells was not significantly different in Wt and C3⁻/⁻ retinas, however, was slightly higher in the Wt (N=5 animals). **K:** There was no significant change in the expression of neuroprotective genes (*Cntf, Fgf2*) between Wt and C3⁻/⁻ retinas (N=4 animals). 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.
3.5. 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, knockdown and 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.

3.5.1. Role of complement in onset of retinal atrophy

Complement dysregulation has been highly implicated in the progression of AMD [152]. Consistent with other similar findings that complement over-activation contributes to retinal degenerations [68, 69, 196, 200, 217, 219], our previous studies show an up-regulation of complement genes C1s, C2, C3 and C4a in retinal degenerations [141, 144, 211, 228]. 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 [47, 62, 153, 157, 158, 160-162]. In particular, sustained up-regulation of C1s, C3 and C4a demonstrated in this study, is consistent with findings from systems-level analysis of AMD neural retinas [47].
C3 propagation is strongly implicated in AMD through the association of the Y402H variant of \textit{CFH} [153, 154]. 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 \textit{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 [235], and it has been reported that C3 levels are elevated in the peripheral blood of AMD patients [236]. Because complement components from serum would not be able to cross the intact blood-retinal barrier (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.

3.5.2. \textbf{Local C3-expressing macrophages as a therapeutic target for reducing retinal atrophy}

Macrophage infiltration of the subretinal space is a feature of AMD histopathology [4, 92, 135], including at the lesion edges in geographic atrophy [135], and their pathogenic involvement in the disease is well-supported through findings in both laser-induced neovascularization [237, 238] and photo-oxidative damage [92, 228, 239] models, where their presence is associated with photoreceptor loss. In the present study we show that C3 is upregulated only by retinal and subretinal macrophages, and not by RPE cells in AMD-affected retinas. Areas of lesion development in our experimental model of retinal degeneration are associated with complement activation and deposition by microglia and macrophages, where photoreceptor cell death may occur through membrane attack complex formation. Our current and previous findings [141, 144] 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 [47].

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 [164]. RPE cells have been reported in some studies as a local source of C3 in mouse retina in homeostasis or resulting from retinal degenerations [140, 181, 214]. These studies relied upon PCR of combined extracts of RPE/Choroid, or used cultured RPE. However, 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 from the perspective 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 [120]. On the other hand, their localisation of some C3-expressing macrophages adjacent to the superficial retinal vasculature and optic nerve suggest recruitment pattern of bone-marrow derived macrophages [185], suggesting that the C3-expressing population may also comprise non-resident macrophages.
3.5.3. 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 itself in influencing complement activation. However, our findings do reveal that the orchestration of complement-mediated pathology in AMD involves more elements than surmised previously [152], of which subretinal macrophages are a pivotal element. In demonstrating the efficacy of intravitreal C3 siRNA in mediating complement-induced in 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, with the potential to revolutionise the therapeutic landscape for complement-mediated retinal degenerations including AMD.
4. RESULTS

The broad spectrum chemokine inhibitor NR58-3.14.3 modulates macrophage-mediated inflammation in the diseased retina

This chapter is presented as the following publication (* denotes equal first author):


The candidate has made a major contribution as a first author to this study (generating 80% of all data and preparing the manuscript for publication).
4.1. Abstract

**Background:** The activity of macrophages is implicated in the progression of retinal pathologies such as atrophic age-related macular degeneration (AMD), where they accumulate amongst the photoreceptor layer and subretinal space. This process is aided by the local expression of chemokines, which furnish these cells with directional cues that augment their migration to areas of retinal injury. While these qualities make chemokines a potential therapeutic target in curtailing damaging retinal inflammation, their wide variety and signalling redundancy pose challenges in broadly modulating their activity. Here, we examine the efficacy of the broad spectrum chemokine inhibitor NR58-3.14.3 – a suppressor of Ccl- and Cxcl- chemokine pathways – in suppressing macrophage activity and photoreceptor death, using a light-induced model of outer-retinal atrophy and inflammation.

**Methods:** Photo-oxidative damage was induced in SD rats via exposure to 1000lx of light for 24hrs, after which animals were euthanized at 0 or 7 day post-exposure time points. Prior to damage, NR58-3.14.3 was injected intravitreally. Retinas were harvested and evaluated for the effect of NR58-3.14.3 on subretinal macrophage accumulation and cytokine expression profile, as well as photoreceptor degeneration.

**Results:** We report that intravitreal administration of NR58-3.14.3 reduces the accumulation of macrophages in the outer retina following exposure to light damage, at both 0 and 7 days post-exposure time-points. Injection of NR58-3.14.3 also reduced the up-regulation of inflammatory markers including of Il6, Ccl3 and Ccl4 in infiltrating macrophages, which are promoters of their pathogenic activity in the retina. Finally, NR58-3.14.3 injected retinas displayed markedly reduced photoreceptor death following light damage, at both 0 and 7 days post-exposure.
**Conclusions:** Our findings indicate that NR58-3.14.3 is effective in inhibiting subretinal macrophage accumulation in light-induced retinal degeneration, and illustrate the potential of broad spectrum chemokine inhibitors as novel therapeutic agents in thwarting retinal inflammation. Although broad spectrum chemokine inhibitors may not be appropriate for all retinal inflammatory conditions, our results suggest that they may be beneficial for retinal dystrophies in which chemokine expression and subretinal macrophage accumulation are implicated, such as advanced AMD.

### 4.2. Background

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among those aged over 65 in industrialised nations [34]. It is a progressive retinopathy that affects the photoreceptor population, retinal pigment epithelium (RPE) and Bruch’s membrane, and the choroid at various stages of the disease. The advanced stages of AMD are classified into two forms. Exudative ‘wet’ AMD, induced by sudden choroidal neovascularisation (CNV), and geographic atrophy (GA) marked by a progressive expanding lesion of the RPE, photoreceptors, and choriocapillaris. While AMD has a complex aetiology involving multiple lifestyle and genetic risk factors, the significance of immunological processes has emerged slowly [4, 71-74, 240]. The relatively recent confirmation in genome wide association studies (GWAS) of the importance of the complement system and macrophages has placed inflammation among the forefront of factors influencing both the onset and progression of AMD [152, 241].

The accumulation and activation of macrophages within the retinal tissues is a prominent feature of most retinopathies, and is well documented in all forms of AMD. Microglial cells are the resident macrophages of the retina that are derived from embryonic
yolk sac progenitors during development [119, 242], while non-resident bone marrow-derived macrophages may be recruited into the retina from the vasculature in pathology [5, 120]. In AMD lesions, macrophages form large aggregations in the photoreceptor layer, subretinal space, and RPE [71, 72, 74, 92, 134, 135, 243], which are typically free of these leukocytes in healthy individuals [244]. Despite having some beneficial properties [131], excessive accumulation of these cells is implicated in degeneration in animal models of AMD, retinal detachment [245], diabetic retinopathy [183, 246, 247], and glaucoma [248, 249].

Chemokines are key regulators of macrophage activation and accumulation in AMD [250, 251], as well as degenerations associated with retinal detachment [245] and diabetic retinopathy [183]. The chemokine family comprises more than 50 molecules and associated receptors that activate and recruit leukocytes to sites of damage. Transcriptome-wide analysis of AMD donor retinas reports that the leukocyte chemoattractants Ccl2, Cxcl1, Cxcl10, Cxcl11 are up-regulated in all forms of the disease [47]. Moreover, intraocular Ccl2 protein levels are increased in patients with CNV or GA [92, 252, 253], and infiltrating microglia/monocytes in retinas with GA express the cognate Ccl2 receptor, Ccr2 [92]. Chemokines signalling is implicated in exacerbating the accumulation of macrophages and photoreceptor death in a range of animal models that demonstrate some features in common with AMD, including laser-induced-CNV [3], photo-oxidative stress [89, 91, 92], Abca4−/−Rdh8−/− mice [93], and carboxyethylpyrrole (CEP)-immunized mice [254].

While these findings underscore the importance of chemokines in shaping inflammation in the retina, therapeutic blockade of individual chemokine ligands/receptors may have limited efficacy due to chemokine receptor redundancy, and the tendency toward compensatory increases in expression of other chemokine family members [93, 185].
Broad spectrum chemokine inhibitors (BSCIs) are a class of chemokine modulators with the ability to inhibit signal transduction of a broad swathe of chemokine receptors [187]. Among these, NR58-3.14.3 is a robust cyclic peptide that specifically inhibits chemokine-mediated migration of macrophages by blocking the signalling of Ccl- and Cxcl- pathways [187]. NR58-3.14.3 has been shown to effectively inhibit macrophage accumulation and ameliorate pathology in lung ischemia [191], obliterator bronchiolitis [190], skin inflammation [187], and atherosclerotic plaques [192].

To our knowledge there are no studies which have explored the potential of BSCIs in retinal degeneration models. Here, we investigate the effect of NR58-3.14.3 in modulation of the accumulation of macrophages in the outer retina, using a light-induced model of oxidative stress and retinal inflammation. While acute exposure to light damage does not induce some of the classical features of AMD (including drusen and CNV) it induces focal and progressive atrophy of the outer retina and models very effectively the accumulation of subretinal macrophages and up-regulation of chemokines Ccl2, Cxcl1, Cxcl10, and Cxcl11 [87]. Furthermore, we have shown that the degenerative changes associated with light damage closely mimic naturally occurring degenerative changes in the aging rat retina [144].

In the current study, we show that intravitreal delivery of NR58-3.14.3 is safely tolerated in experimental animals, is long-lasting in the retinal environment, suppresses macrophage accumulation in the outer retina, and reduces retinal pathology following light damage. Additionally, we demonstrate that NR58-3.14.3 reduces the expression of pro-inflammatory factors Il6, Ccl3 and Ccl4 by macrophages, which are implicated in promoting their deleterious activity in retinal dystrophy. These data provide proof-of-principle support of the value of BSCIs in thwarting deleterious macrophage activity in retinal degeneration.
4.3. Methods

4.3.1. Animal handling and rearing

All experiments were in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and with approval from the ANU Animal Experimentation Ethics Committee (Ethics ID: A2014/56). Adult Sprague-Dawley (SD) rats were utilised in all experiments, which were born and reared under dim light conditions (5 lux) and aged between 90-120 post-natal days at the time of use.

4.3.2. Preparation of NR58-3.14.3 and intravitreal injections

Animals were anaesthetised with an intraperitoneal injection of Ketamine (100mg/kg; Troy Laboratories, NSW, Australia) and Xylazil (12mg/kg; Troy Laboratories, NSW, Australia). Intravitreal injections of consisting of 3µL were conducted as described previously [89]; this route was preferred over systemic administration as it minimizes the potential for off-target effects which may confound the data. The 3µL injection volume was chosen as offers good reproducibility, minimal disturbance in vitreal volume, and negligible cataract formation [221]. The broad spectrum chemokine inhibitor (BSCI) NR58-3.14.3 (Auspep, VIC, Australia) was reconstituted in endotoxin-free 0.1M PBS to a final concentration of 66.7µg/µl. In the treatment group, 3µl of NR58-3.14.3 in PBS was intravitreally injected into both eyes of each animal, so that 200µg of NR58-3.14.3 was delivered to each eye. The control group received an injection of 3µl PBS vehicle in each eye. Following intravitreal injections the animals were monitored closely throughout the time course of experiments. As expected, no incidences of cataract formation or other abnormalities were observed.
4.3.3. Light damage

Immediately after administering intravitreal injections, animals were exposed to 1000 lux light for a period of 24 hours, as described previously [209]. During light damage, animals were placed into transparent Perspex open-top cages under a light source (COLDF2, 2x36W, IHF, Thorn Lighting, Australia) with access to food and water ad libitum. After light damage, animals were either euthanized for tissue collection (0 day time point) or were placed into recovery under dim light conditions (5 lux) for 7 days.

4.3.4. Retinal localisation of injected NR58-3.14.3 via fluorescein

The fluorescein-labelled NR58-3.14.3 was prepared using an NHS-Fluorescein Antibody Labelling Kit (Pierce, Thermo Fisher Scientific, MA, USA), applied according to the manufacturer’s protocol. In brief, the NR58-3.14.3 was reconstituted in endotoxin-free PBS to a concentration of 1mg/ml. The NR58-3.14.3 was then incubated with the NHS-Fluorescein for 1 hour at room temperature, before being purified through a column to remove excess dye from the final product. The fluorescein-NR58-3.14.3 was stored at 4°C.

Using the intravitreal procedure described earlier, each animal was injected with 3µl fluorescein-NR58-3.14.3 in one eye only; the contralateral eye was used as either a fluorescein-only injected control, or a non-injected control. Following the injection, animals were placed under dim-light conditions to recover. Both eyes of each animal were collected for cryosectioning at several times after the injection (20 minutes, 3 hours, 24 hours and 7 days). The levels of fluorescein-NR58-3.14.3 in injected retinas were measured on cryosections. The fluorescence was visualised with a laser-scanning A1+ confocal microscope (Nikon, Tokyo, Japan), and images were acquired at consistent regions using the NIS-Elements AR software (Nikon, Tokyo, Japan). From these images fluorescence intensity was quantified with ImageJ software (NIH, MD, USA), using integrated density for a fixed area.
4.3.5. Tissue collection and processing of whole retinas

Animals were euthanized using an overdose of barbiturate, which was administered via intraperitoneal injection (Valabarb; Virbac, NSW, Australia). The left eye from each animal was marked for orientation then enucleated for cryosectioning, while the retina from the right eye was excised through a corneal incision for RNA extraction. Both cryosectioning and RNA extraction of the excised retinas were performed according to our previous methodology [141].

4.3.6. Fluorescence-activated cell sorting (FACS) of retinal macrophages

Rats in each treatment group were euthanized immediately after light damage (0 days) using barbiturate overdose as previously described. Retinas from both eyes were promptly removed through a corneal incision. Retinas from each animal were pooled and immediately placed in chilled Hank's balanced salt solution (HBSS), and then subjected to light mechanical separation using a razor blade. Samples were transferred into a 0.2% papain digestion cocktail as described in our previous protocol [87], with minor modifications. Samples were incubated at 37°C for 7 minutes then 8°C for 30 minutes. After neutralisation and resuspension, the resulting homogenate was incubated in staining buffer containing a CD11b antibody conjugated to Alexa 647 (Biolegend, San Diego, CA) for 30 minutes at 4°C, then washed in HBSS and resuspended in staining buffer. The resultant CD11b-stained samples were run through a fluorescence-activated cell sorter (FACS) (BD FACSARia II; BD Biosciences, Franklin Lakes, NJ). The isolated CD11b+ macrophages were collected in staining buffer and kept chilled on ice until RNA extraction could be commenced. RNA extraction was conducted via a protocol we have previously established [87]. Isolated total RNA was analysed for quantity and purity with a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).
4.3.7. TUNEL assay and quantification of photoreceptor survival

To quantify levels of photoreceptor cell death in the retina, retinal cryosections were stained for apoptosis using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) kit (Roche Diagnostics, NSW, Australia) as previously described [255, 256]. TUNEL+ cells in the ONL were counted throughout the full length of each retinal section along the para-sagittal plane (supero-inferior), and also included the optic nerve for consistency. These counts were subsequently averaged for each experimental group. To quantify photoreceptor survival, sections were stained with bisbenzimide (Sigma-Aldrich Co., MO, USA), and the number of rows of photoreceptor cell bodies in the ONL were counted. Five counts were made for each retinal section, and then were averaged for each experimental group.

4.3.8. Immunohistochemistry

Cryosections were used for immunohistochemical analysis, using antibodies against IBA1 (1:500, Wako, Osaka, Japan) and GFAP (1:500, Dako, Agilent Technologies, CA, USA) as described in our previous study, with minor modifications [87, 209]. Fluorescence in retinal sections was captured with a laser-scanning A1+ confocal microscope (Nikon, Tokyo, Japan). Images panels were analysed and assembled using ImageJ (NIH, MD, USA) and Photoshop CS6 software (Adobe Systems, CA, USA).

4.3.9. Quantification of microglia/macrophages

Macrophages counts were performed on sections immunolabelled with the IBA1 marker, a label for retinal microglia and macrophages. Counts of IBA1+ cells were carried out along the full-length of retinal sections cut in the para-sagittal plane (supero-inferior) within the vertical meridian, and each section also included the optic nerve to ensure consistency. Counts were made of all IBA1+ cells throughout the retina, which were split
into two groups: the inner retina (ILM-OPL), and the outer retina (ONL-RPE) where activated macrophages are known to aggregate during retinal degeneration [131, 245, 257].

4.3.10. Polymerase chain reaction (PCR)

cDNA was prepared from 1µg of RNA of each sample using a Tetro cDNA Synthesis Kit (Bioline Reagents, London, UK). The quantitative real-time PCRs (qPCRs) were conducted using Taqman hydrolysis probes (Table 4.1) and Taqman Gene Expression Master Mix (Applied Biosystems, Life Technologies, CA, USA). These were applied following the manufacturer’s instructions, and run on a QuantStudio Flex 12K instrument (Applied Biosystems), at the Biomolecular Resource Facility, JCSMR, ANU. Data analysis was performed using Expression Suite v1.0.3 software (Life Technologies, CA, USA); the fold change was determined for each gene and sample using the comparative cycle threshold (Ct) method (ΔΔCt). All data was normalised to the GAPDH reference gene, which does not change in expression over the course of retinal light damage [141, 217, 225]. ACTB was utilised as an additional reference gene in qPCRs involving the CD11b+ isolates.

Table 4.1. Taqman hydrolysis probes used for qPCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Catalog Number</th>
<th>Entrez Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>Rn01456716_g1</td>
<td>24770</td>
</tr>
<tr>
<td>Ccl3</td>
<td>Chemokine (C-C motif) ligand 3</td>
<td>Rn00564660_m1</td>
<td>25542</td>
</tr>
<tr>
<td>Ccl4</td>
<td>Chemokine (C-C motif) ligand 4</td>
<td>Rn00587826_m1</td>
<td>116637</td>
</tr>
<tr>
<td>Ccl7</td>
<td>Chemokine (C-C motif) ligand 7</td>
<td>Rn01467286_m1</td>
<td>287561</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
<td>Rn01410330_m1</td>
<td>24498</td>
</tr>
<tr>
<td>IL-1B</td>
<td>Interleukin 1β</td>
<td>Rn00580432_m1</td>
<td>24494</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor α</td>
<td>Rn00562055_m1</td>
<td>24835</td>
</tr>
<tr>
<td>IL-12B</td>
<td>Interleukin 12β</td>
<td>Rn00575112_m1</td>
<td>64546</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Rn99999916_s1</td>
<td>24383</td>
</tr>
<tr>
<td>ACTB</td>
<td>Actin, beta</td>
<td>Rn00667869_m1</td>
<td>81822</td>
</tr>
</tbody>
</table>
Standard PCR was performed on RNA samples purified from FACS-isolated CD11b+ microglia/macrophages, using primers specific to chemokine receptor genes (Ccr1, Ccr2, Ccr5, Cxcr3) which are detailed in our previous investigation [87]. First-strand cDNA synthesis was performed from 50 ng of RNA using the Tetro cDNA Synthesis Kit (Bioline), and standard PCR was then performed using MyTaq DNA polymerase (Bioline); negative controls had cDNA omitted from the reaction cocktail. The presence of PCR product and specificity of the reaction were assessed by gel electrophoresis.

4.3.11. In situ hybridisation

Ccl3 was cloned from a 540bp PCR product using cDNA prepared from rat retinas (as described in the qPCR section), and which was synthesised into a digoxigenin (DIG)-labelled riboprobe according to our previous publication [87]. The In situ hybridisation was performed using our established protocol [224]; the Ccl3 riboprobe was hybridised on retinal cryosections overnight at 55°C, and then washed in decreasing concentrations of saline sodium citrate (pH 7.4) at 60°C. The bound probe was visualised with either NBT/BCIP, or HNPP/Fast-Red (Roche).

4.3.12. Statistical analysis

All graphing and statistical analysis was performed using Prism 6 (GraphPad Software, CA, USA). A two-way ANOVA with Tukey’s multiple comparison post-test, or an unpaired student t-test was utilised to determine the statistical outcome; a P-value of <0.05 was considered statistically significant.
4.4. Results

4.4.1. Stability and localisation of NR58-3.14.3 in injected retinas

Localisation and persistence of NR58-3.14.3 within the retina was determined by intravitreal injections of fluorescein-conjugated NR58-3.14.3 (Figure 4.1). At 20 minutes post-injection, levels of fluorescence increased dramatically in retinas injected with NR58-3.14.3-fluorescein, compared to fluorescein-only and non-injected controls (P<0.05, Figure 4.1A). Fluorescence levels were maintained at 3 hours post-injection, though were reduced by approximately a third after 24hrs – this was not significant however compared to the 20 minutes post-injection group (P>0.05). After 7 days, fluorescence was still present in NR58-3.14.3-fluorescein injected retinas at levels significantly higher than the fluorescein-only and non-injected control groups (P<0.05).

At 20 minutes-post injection, NR58-3.14.3-fluorescein was distributed throughout the retina, spanning the ganglion cell layer (GCL) to the RPE (Figure 4.1B). This distribution was mostly unchanged after 3hrs, although fluorescence appeared more concentrated within the GCL, nerve fibre layer (NFL), inner nuclear layer (INL), and RPE (Figure 4.1C, arrows). After 24hrs incubation, fluorescence was localised in intense clusters encompassing the NFL, retinal vasculature, and RPE (Figure 4.1D, arrows). This localisation was maintained at 7 days post injection (Figure 4.1E, arrows), while fluorescein-only controls showed little fluorescence at all (Figure 4.1F).

4.4.2. Tolerance of NR58-3.14.3 within the retinal environment

NR58-3.14.3 was initially injected into dim-reared retinas to determine whether it induced any change in photoreceptor death, retinal stress, or macrophages alone (Figure 4.2). At 24hrs following intravitreal injection with NR58-3.14.3 analyses showed no significant change in the number of TUNEL+ photoreceptors (P>0.05, Figure 4.2A) or in
Figure 4.1. Stability and localisation of intravitreally injected NR58-3.14.3. **A:** Levels of fluorescence readily increased in retinas injected with fluorescein-conjugated NR58-3.14.3 after only 20 minutes, and remained highly elevated after 3hrs. Fluorescence was significantly lower at 7 days post-injection (P<0.05), but was still far higher than fluorescein-only and non-injected controls (P<0.05). **B:** NR58-3.14.3-fluorescein (green, arrow) was present throughout the retina at 20 minutes post-injection. **C:** Fluorescence
remained consistent in retinas after 3hrs, though was more concentrated within the GCL, NFL, INL, and RPE (arrows). **D-E:** After 24hrs and 7 days post-injection, fluorescence for NR58-3.14.3-fluorescein was markedly reduced, but still readily apparent around the NFL, retinal vasculature, and RPE (arrows). **F:** Retinas injected with fluorescein-only showed only very faint background fluorescence in comparison to NR58-3.14.3-fluorescein injected animals (E). GCL, ganglion cell layer; INL, inner nuclear layer; NFL, nerve fibre layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars represent 100µm. NR58-3.14.3-fluorescein n=3, fluorescein-only n=3, non-injected n=3.
Figure 4.2. Tolerance of NR58-3.14.3 within the retinal environment in relation to stress and macrophage recruitment. A: Injection of dim-reared controls with NR58-3.14.3 did not yield any change in TUNEL+ photoreceptors after 24hrs incubation, compared to non-injected controls (P>0.05). B: NR58-3.14.3 alone did not induce any recruitment of IBA1+ macrophages to the outer retina after 24hrs incubation in dim-reared animals, and there was also no change in the inner retinal population (P>0.05). C-D: The integrity of the ONL was unchanged in animals injected with NR58-3.14.3, compared to non-injected controls. E-F: The morphology of IBA1+ macrophages/microglia (green) was unaltered in NR58-3.14.3 treated retinas, and the ONL was devoid of any of these cells (arrows) in both groups. G-H: Immunoreactivity for stress marker GFAP was restricted to the GCL in both NR58-3.14.3 and non-injected groups (arrows), consistent with a normal physiological state. GCL, ganglion cell layer; IHC, immunohistochemistry; INL, inner nuclear layer; NFL, nerve fibre layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars represent 100µm. NR58-3.14.3 n=3, non-injected n=3.
integrity of the retinal layers (Figure 4.2C-D), compared to control retinas; both parameters were consistent with our previous measurements in control SD retinas [209]. General retinal stress was assessed via immunohistochemistry for GFAP (Figure 4.2G-H), an accepted indicator of retinal pathology [10]. Immunoreactivity for GFAP was confined to the GCL (arrows) in both NR58-3.14.3- and PBS-injected retinas, consistent with a normal physiological retinal state [10, 209, 258]. Finally, there was no change in the distribution of IBA1+ microglia/macrophages in NR58-3.14.3-injected retinas compared to PBS-injected controls (P>0.05, Figure 4.2B, E-F). The outer nuclear layer (ONL) and subretinal space was devoid of macrophages in both groups (Figure 4.2E-F, arrows), while resident microglia/macrophages in the inner retina showed no change in number or morphology.

4.4.3. Effect of NR58-3.14.3 on macrophage accumulation following light damage

The accumulation of macrophages within the outer retina (including the photoreceptor layer and subretinal space) following NR58-3.14.3 injection was assessed using IBA1 immunoreactivity (Figure 4.3) to identify microglia/macrophages [259, 260]. Immediately following light damage (0 days recovery), we observed a substantial incursion of IBA1+ cells into the outer retina of PBS-injected controls (Figure 4.3A, C-D arrows). In animals injected with NR58-3.14.3, this accumulation was substantially less per retinal section (~70% reduction after NR58-3.14.3, P<0.05, Figure 4.3A, E).

In PBS-injected animals 7 days post-light damage, the number of IBA1+ cells increased significantly throughout the retina, particularly in the subretinal space (Figure 4.3A) where accumulations of swollen, amoeboid-like macrophages were observed amongst the degenerating photoreceptor segments (Figure 4.3F-G arrows). In animals injected with NR58-3.14.3 however, the number of IBA1+ cells infiltrating the outer retina was significantly less than in PBS controls (~60% reduction after NR58-3.14.3, P<0.05, Figure 4.3A), and virtually no macrophages were detected in the subretinal space (Figure
Figure 4.3. Macrophage/microglia recruitment to the outer retina following injection of NR58-3.14.3 and light damage. A-B: At 0 days post-LD, there was a substantial incursion of IBA1+ cells and their ramified processes into the outer retina of PBS-injected controls, which was substantially lower in animals injected with NR58-3.14.3 (P<0.05). During the chronic phase of degeneration at 7 days post-LD, the number of IBA1+ cells was further increased in the outer retina, although animals injected with NR58-3.14.3 had far fewer IBA1+ cells than in PBS controls (P<0.05). For the inner retinal counts of IBA1+ macrophages/microglia, there was no change at either 0 or 7 days recovery for both treatment groups (P >0.05). C-E: IBA1+ cells invaded ONL with their extended processes
in PBS-injected animals at 0 days (C-D, arrows), which was not apparent in the NR58-3.14.3 group (E). F-H: Accumulations subretinal IBA1+ macrophages were observed in PBS-injected animals at 7 days (F-G, arrows), while the subretinal space was nearly devoid of these cells in NR injected retinas. GCL, ganglion cell layer; IHC, Immunohistochemistry; INL, inner nuclear layer;NFL, nerve fibre layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. C,E,F,H scale bars represent 100µm, D,G are 25µm. N=11 per group at 0 and 7 days.
Conversely, the numbers of IBA1+ cells in the inner retina was similar in NR58-3.14.3- and PBS-injected animals at both 0 and 7 days recovery (P>0.05, Figure 4.3B).

4.4.4. Change in photoreceptor integrity following NR58-3.14.3 injection and light damage

The effect of NR58-3.14.3 administration on photoreceptor cell death following light-induced damage was investigated using the TUNEL assay (Figure 4.4). Exposure to bright continuous light induced an increase in the number of TUNEL+ photoreceptors in both PBS- and NR58-3.14.3-injected groups (Figure 4.4A). However, the number of TUNEL+ cells in animals injected with NR58-3.14.3 was approximately half that in the PBS-injected control animals at 0 days recovery (P<0.05, Figure 4.4A-C). The effect of NR58-3.14.3 was even more pronounced during the chronic phase of degeneration at 7 days recovery, wherein the number of TUNEL+ photoreceptors was 4 times lower in NR58-3.14.3-injected animals compared with the PBS-controls (P<0.05, Figure 4.4A, D-E). In addition, the cumulative effect of NR58-3.14.3 on the number of surviving photoreceptors following light damage was determined via measurements of ONL thickness. There was no change in the number of photoreceptor rows at 0 days recovery compared to PBS controls (P>0.05, Figure 4.4F). At 7 days recovery however, there was in increased number of photoreceptor rows in NR58-3.14.3 retinas compared to PBS controls (P<0.05, Figure 4.4F-H).

4.4.5. Cytokine and chemokine expression following NR58-3.14.3 injection and light damage

The expression of chemokines (Ccl2, Ccl3, Ccl4, Ccl7) and cytokines (Il6, Il1b, TNFa, Il12) was assessed using qPCR to determine the effect of NR58-3.14.3 on chemokine signalling (Figure 4.5). These were selected based on their up-regulation in our microarray analysis conducted previously using the light damage model [87]. Expression
Figure 4.4. Change in photoreceptor integrity following injection of NR58-3.14.3 and light damage. **A:** At 0 days post-LD, animals injected with NR58-3.14.3 had approximately half the number of TUNEL+ cells per retina compared to PBS-injected controls (P<0.05). After 7 days post-LD, where chronic retinal degeneration is most apparent, the number of TUNEL+ photoreceptors was roughly 3 times lower in animals injected with NR58-3.14.3 than in the PBS-controls (P<0.05). **B-E:** Representative TUNEL images showcase the reduction in TUNEL+ photoreceptors in NR58-3.14.3 injected retinas at both 0 (C) and 7 (E) days post-LD, compared to PBS controls (B,D). **F:** At 0 days post-LD, there was no
change in the number of rows of photoreceptor nuclei in NR58-3.14.3 injected retinas compared to PBS controls (P>0.05). However, at 7 days post-LD, animals injected with NR58-3.14.3 had a larger number of remaining photoreceptor rows than PBS controls (P<0.05). **G-H:** Representative ONL images demonstrate this reduction of photoreceptor loss in NR58-3.14.3 injected retinas compared to PBS controls at 7 days post-LD. INL, inner nuclear layer; ONL, outer nuclear layer. B-E scale bars represent 100µm, G-J are 50µm. N=10-11 per group at 0 days, N=12 per group at 7 days.
Figure 4.5. Expression of chemokines (A, C) and cytokines (B, D) in whole retinas following injection of NR58-3.14.3 and light damage. **A-B:** All genes assessed exhibited significant up-regulation at 0 days post-LD in both treatment groups. However, Ccl3, Ccl4, and Il6 had significantly lower expression (P<0.05) in retinas injected with NR58-3.14.3 compared to PBS-controls. **C-D:** By 7 days post-LD, up-regulation of all genes assessed was only 10-20% of that observed at 0 days, with no difference between NR58-3.14.3 and PBS injected groups (P>0.05). 0 days N=11-12 per group, 7 days N=5 per group.
levels of all genes were substantially upregulated immediately after bright light exposure (0 days) in both PBS- and NR58-3.14.3-injected animals (Figure 4.5A-B). However, expression levels of Ccl3, Ccl4, and Il6 were significantly less in NR58-3.14.3-injected compared to PBS-injected animals (Figure 4.5A-B; P<0.05). At 7 days post-light damage, expression levels of all cytokines/chemokines assessed had dropped by approximately 80-90% (Figure 4.5C-D), and there was no difference between expression levels in the two treatment groups (P>0.05).

4.4.6. Change in expression of inflammatory factors by macrophages following NR58-3.14.3 injection and light damage

Microglia/macrophages were FACS-sorted from retinal suspensions for each treatment group on the basis of CD11b immunoreactivity (Figure 4.6A), which co-localises with IBA1 [261-263]. CD11b was preferred over IBA1 as a microglia/macrophage marker in this instance, because the intracellular staining required for IBA1 would prevent any downstream RNA analysis following FACS. We first sought to confirm whether the CD11b+ microglia/macrophages expressed Ccl- and Cxcl- chemokine receptors which correspond to the ligands up-regulated following light damage [87], as well as in AMD [47]. Using standard PCR (Figure 4.6B), CD11b+ microglia/macrophages from both treatment groups were found to express Ccr1, Ccr3, Ccr5, and Cxcr3 receptors.

To further explore the role of microglia/macrophages in retinal inflammation we compared the expression of Ccl3, Ccl4, and Il6 in isolated CD11b+ microglia/macrophages from NR58-3.14.3-injected and PBS-injected groups using qPCR. These were chosen due to their differential expression in the whole-retina samples (shown in Figure 4.5) and the implicit roles of these genes in promoting pathogenic macrophage activity [93, 239]. After light damage, expression of Ccl3, Ccl4, and Il6 were significantly decreased in CD11b+ cells from NR58-3.14.3-injected retinas, compared to PBS control retinas (P<0.05,
Figure 4.6. Effect of NR58-3.14.3 on the pathogenic phenotype of the retinal macrophage population following light damage. A: Representative FACS plots, with gating strategies for the isolation of CD11b+ microglia/macrophages. Gating methodology was applied equally for all samples. B: Representative images of PCR products via electrophoresis for chemokine receptors Ccr1, Ccr2, Ccr5, Cxcr3, in samples of CD11b-sorted cells. Receptor expression was observed in both NR58-3.14.3 and PBS CD11b isolates. C: Following exposure to LD, expression of Ccl3, Ccl4, and Il6 were all decreased in CD11b+ microglia/macrophages isolated from NR58-3.14.3–injected retinas, compared to those from PBS injected retinas (P<0.05). N=5 per group at 0 days.
Figure 4.6C). *In situ* hybridisation for Ccl3 mRNA showed clusters of ramified microglia in the outer retina of PBS controls (Figure 4.7A-B arrows) following light damage, which were also immunoreactive for IBA1 (Figure 4.7E-F arrows). Ccl3-expressing macrophages were far less numerous in retinas from NR58-3.14.3-injected animals (Figure 4.7C-D arrows).
Figure 4.7. *In situ* hybridisation for Ccl3 mRNA following NR58-3.14.3 injections and light damage. **A-B:** At 0 days LD recovery in PBS-injected retinas, there was staining for Ccl3 mRNA (purple) in among clusters of irregularly shaped nuclei traversing the ONL (arrows). **C-D:** Retinas injected NR58-3.14.3, conversely, displayed very few Ccl3-expressing nuclei. **E-F:** Ccl3-expressing nuclei (red, arrow) were correlated with immunofluorescence for IBA1+ macrophages/microglia (green, arrow). IHC, immunohistochemistry; ISH, *in situ* hybridisation; ONL, outer nuclear layer; OS, outer segments. Scale bars represent 50µm.
4.5. Discussion

The findings of the current study demonstrate the efficiency of the broad spectrum chemokine inhibitor NR58-3.14.3 in modulating the pathogenic activity of macrophages/microglia and ameliorating photoreceptor death, in a model of photo-oxidative damage and outer retinal inflammation. First, our data demonstrate that the intravitreal delivery of NR58-3.14.3 is tolerated within the retinal environment, and its persistence in the retina provides for a generous window of activity. Second, we show that injection of NR58-3.14.3 suppresses the chronic accumulation of macrophages/microglia within the photoreceptor layer and subretinal space following damage. Third, NR58-3.14.3-treated macrophages demonstrated reduced pathogenic activation, indicated by decreased expression of Ccl3, Ccl4, and Il6. Finally, we show that photoreceptor degeneration is reduced across the time course of damage in retinas injected with NR58-3.14.3, which is accompanied by an increase in photoreceptor survival at the site of focal and progressive atrophy.

NR58-3.14.3 is thought to exert its inhibitory effect on Ccl- and Cxcl- families though disruption of intracellular signal transduction, which effectively renders leukocytes blind to directional cues provided by Ccl- and Cxcl- ligands [195, 264]. This broad suppression is desirable, as the blockade of singular chemokine ligands, such as Ccl2, can induce compensatory increases in the expression other chemokines which exacerbate retinal inflammation and degeneration [93, 185]. Up-regulation of Ccl2, Cxcl1, Cxcl10, Cxcl11 are present in all forms of AMD [47], and the Ccl2/Ccr2 axis is implicated in the pathogenic accumulation of macrophages to the outer retina in models that feature aspects of either CNV or geographic atrophy, such as light damage [3, 89, 91-93, 254]. Increases in ocular Ccl- and Cxcl- chemokines are also found in individuals affected by retinal detachment [265, 266], uveitis [267], glaucoma [268], diabetic retinopathy, and retinitis
pigmentosa [269], while blockade of Ccl2 signalling has proven beneficial in thwarting macrophage aggregation and cell death in experimental retinal detachment [245], diabetic retinopathy [183], and retinitis pigmentosa [184]. While the light damage paradigm does not model features of CNV or degenerations such as retinal detachment, our findings allude to the potential of BSCIs in targeting a broad swathe of chemokine activity across a spectrum of retinal degenerations.

To our knowledge, this is the first study to examine the potential of BSCIs as a potential therapeutic agent in retinal disease. The findings from our study are consistent with previous investigations using NR58-3.14.3 in inflammatory models ranging from atherosclerotic plaques to obliterative bronchiolitis, wherein an inhibition of macrophage accumulation and amelioration of tissue damage were observed [189, 190, 192, 194, 195]. Our data own show that intravitreal NR58-3.14.3 reduces the accumulation of macrophages within the outer retina, including ONL and subretinal space. The proportion of these subretinal macrophages that are either resident microglia or recruited bone-marrow derived monocytes is unclear, although the effect of intravitreal NR58-3.14.3 on these subpopulations is beyond the scope of this study. Nevertheless, we speculate that intravitreal NR58-3.14.3 could impact the infiltration of both subpopulations to the subretinal space, despite the local nature its administration. This is suggested by the localisation fluorescein-NR58-3.14.3 within the retinal vasculature, where blood borne monocytes are known to recruit into the retina following light-induced injury [185]. Additionally, as NR58-3.14.3 penetrates through to all layers of the retina, it may act on RPE and Müller cells, which are both known to secrete Ccl- and Cxcl- chemokines to recruit leukocytes to the site of injury [85, 87].

Our data indicate that NR58-3.14.3 modulates the transcriptional phenotype of macrophages following light damage. The expression of Ccl3, Ccl4, and Il6 were all down-
regulated in macrophages exposed to NR58-3.14.3, and is consistent with the down regulation of IL6 observed in experimental obliterative bronchiolitis following administration of NR58-3.14.3 [190]. CCL3, CCL4, and IL6 are markers of M1 polarisation, which is typically associated with deleterious pro-inflammatory responses [113]. Although macrophage polarisation is considered a simplification of extremes which form part of a phenotypic spectrum, the suppression of these genes within the macrophage population in NR58-3.14.3 injected retinas do point toward a lower inflammatory state among these cells as a result. How NR58-3.14.3 elicits this transcriptional change is unclear, though we speculate it may occur via its inhibition of macrophage chemotaxis to the outer retina, wherein emergent photoreceptor death may stimulate up-regulation of cytokines such as CCL3, CCL4, and IL6. This is supported by our in situ hybridisation data, wherein CCL4 mRNA is expressed only in macrophages that have migrated to the outer retina.

Several lines of evidence suggest that CCL3, CCL4, and IL6 are important mediators of pathogenic activity by macrophages in retinal degeneration, which may account for the neuroprotective action of NR58-3.14.3. Increased levels of IL6 are associated with the incidence and progression of AMD, and the cytokine is considered a potential therapeutic target [46, 96, 270]. A pathogenic role of IL6 is more recently highlighted by Levy and colleagues, who show that accumulation of the AMD biomarker apolipoprotein E (APOE) in subretinal macrophages up-regulates their expression of IL6, which in turn promotes their increased survival and induction of retinal pathology in light-damaged Cx3cr1−/− mice [239]. Additionally, a deleterious role of IL6 has been characterised in experimental ocular toxoplasmosis via antibody neutralisation, which considerably improved retinal morphology compared to controls [97].

CCL3 is implicated in animal models of AMD/Stargardt disease (Abca4−/−Rdh8−/−), retinitis pigmentosa (MerTK−/−) [93], and oxygen induced...
retinopathy [94]. Ccl3 was expressed by macrophages/microglia as shown by Kohno and colleagues – consistent with our current investigation – and its ablation reduced the extent of photoreceptor degeneration in both aged Abca4−/−Rdh8−/− mice and young Mertk−/− mice [93]. In experimental oxygen induced retinopathy, mice were treated with the combination therapy of Ccl3 and Ccl2 neutralising antibodies, rather than a singular therapy. Regardless the neovascular pathology was reduced by 30% in these animals [94]. The precise role of Ccl4 in retinal degeneration has yet to be examined, although protein levels are increased in the aqueous humour of AMD patients [271], and its expression is up-regulated concomitantly with emergence of retinal degeneration in experimental light damage [87], diabetic retinopathy, and Abca4−/−Rdh8−/− and Mertk−/− mice [93]. Moreover, ablation of Ccl3 induced compensatory increases in Ccl4 expression in light-damaged Abca4−/−Rdh8−/− mice, which was postulated to contribute to the increased pathology observed in this paradigm (rather than the age paradigm where there was protection).

We speculate that NR58-3.14.3 may also confer retinal protection by inhibiting other macrophage processes, such as aberrant phagocytosis. This phenomenon has been recently demonstrated in the rd10 model of retinitis pigmentosa, in which infiltrating microglial cells were found to exacerbate cell death by phagocytosing non-apoptotic photoreceptors [116]. In suppressing the infiltration of macrophages within the outer retina, NR58-3.14.3 may also reduce the erratic phagocytosis of photoreceptors by activated macrophages. This may be a contributing factor to the increases in ONL thickness in NR58-3.14.3-injected retinas, however, additional investigations including longer-term and functional studies are required to firmly establish this in the future.
4.5.1. Conclusion

Our findings showcase the efficacy of BSCIs such as NR58-3.14.3 in modulating macrophage/microglia responses in retinal degeneration. NR58-3.14.3 ameliorated the chronic aggregation of subretinal macrophages in light-induced subretinal inflammation, and suppressed their potentiation of Il6, Ccl3 and Ccl4 signalling. As with all current models, light damage does not encompass every aspect of AMD pathology; nevertheless our findings have implications for the treatment of inflammation in the disease. Chemokines are associated with the aggregation of subretinal macrophages in AMD, while expression of Il6, Ccl3 and Ccl4 are implicated in augmenting their survival and pathogenicity within the subretinal space. Together, these experiments offer proof-of-principle for the potential of BSCIs as therapeutic agents in targeting subretinal inflammation in diseases such as AMD.
5. RESULTS

Microglia-derived IL-1β promotes chemokine expression by Müller cells and RPE in focal retinal degeneration

This chapter is presented as the following publication (* denotes equal first author):


The candidate has made a major contribution as a first author to this study (generating 50% of all data and preparing the manuscript for publication).
5.1. Abstract

**Background:** Chemokine signalling is required for the homing of leukocytes during retinal inflammation, and is associated with pathogenesis of diseases such as age-related macular degeneration (AMD). Here, we explore the role of interleukin-1β (IL-1β) in modulating AMD-associated chemokines Ccl2, Cxcl1, and Cxcl10 during photo-oxidative retinal damage, and the effect on both the accumulation of outer-retinal macrophages, and death of photoreceptors.

**Methods:** Inhibition of retinal IL-1β expression was performed using either siRNA or antibody neutralisation, which was intravitreally injected in SD rats prior to photo-oxidative damage. Changes in the expression and localisation of Il-1β, Ccl2, Cxcl1 and Cxcl10 genes were assessed using qPCR and in situ hybridisation, while the recruitment of retinal macrophages was detected using immunohistochemistry for IBA1. Levels of photoreceptor cell death were determined using TUNEL.

**Results:** Photo-oxidative damage elevated the expression of Il-1β and inflammasome-related genes, and IL-1β protein was detected in microglia infiltrating the outer retina. This was associated with increased expression of Ccl2, Cxcl1, and Cxcl10. Intravitreal IL-1β inhibitors suppressed chemokine expression following damage and reduced macrophage accumulation and photoreceptor death. Moreover, in Müller and RPE cell cultures, and in vivo, Ccl2, Cxcl1 and Cxcl10 were variously upregulated when stimulated with IL-1β, with increased macrophage accumulation detected in vivo.

**Conclusions:** IL-1β is produced by retinal microglia and macrophages and promotes chemokine expression by Müller cells and RPE in retinal degeneration. Targeting IL-1β may prove efficacious in broadly suppressing chemokine-mediated inflammation in retinal dystrophies such as AMD.
5.2. Background

Inflammation plays a key role in the pathogenesis of age-related macular degeneration (AMD), which is the leading cause of blindness in the ageing population of the Western world [34]. One of the characteristics of atrophic or ‘dry’ AMD, is the accumulation of microglia/macrophages in the outer retina and subretinal space [4, 71-74]. Homing of leukocytes, such as macrophages, to sites of neuronal damage is orchestrated in part by the co-ordinated expression of chemokines (reviewed in [27]). The chemokine family consists of numerous ligands and receptors belonging to particular subclasses (such as Ccl- and Cxcl-), which act as guidance cues for leukocytes during homeostasis and injury [78]. Chemokine expression is prominent in many retinal degenerations, including AMD, wherein the up-regulation of genes encoding potent ligands such as Ccl2 and Cxcl10 is a characteristic of the disease [47]. The Ccl2-Ccr2 signalling axis has been well-studied in relation to retinal disease, and genetic ablation or pharmacological inhibition of the ligand or receptor reduces pathology in laser-induced neovascularisation and photo-oxidative damage models [84, 92, 238].

Our previous work has shown that RPE and Müller cells are the mediators of chemokine responses, and up-regulate the expression of Ccl2, Cxcl1 and Cxcl10 in response to damage [87]. Furthermore, pharmacological suppression of the Ccl- and Cxcl-signalling axes ameliorates subretinal macrophage infiltration and photoreceptor/RPE degeneration [228]. However, the factor/s that stimulate expression of these chemokines during retinal inflammation remain unclear. Recent in vitro studies indicate that cytokines such as Il-6 and Ccl2 may be stimulated in RPE or Müller cells when co-cultured with lipopolysaccharide (LPS)-stimulated microglia [86, 88], suggesting that similar interactions may promote chemokine expression by Müller cells and RPE during retinal degeneration.
IL-1β is a pro-inflammatory cytokine whose maturation and secretion into the extracellular environment is mediated by assembly of the NLRP3 inflammasome (reviewed in [98-100]), and is associated with the progression of retinal pathologies including neovascular and atrophic AMD [111, 112]. Several studies have also indicated that IL-1β is secreted by microglia in photo-oxidative damage [113, 114], as well as in models of neovascular AMD [115], retinitis pigmentosa [116], and retinal detachment [117]. In this study, we tested the hypothesis that IL-1β promotes the up-regulation of chemokines in Müller cells and RPE, increasing outer-retinal macrophage accumulation and photoreceptor death, using a model of focal retinal degeneration. In this model, several inflammatory features observed in atrophic AMD are produced, including the expression of chemokines such as Ccl2, macrophage accumulation and outer retinal lesion development [85, 141, 209]. We find that inhibiting IL-1β, either via antibody neutralisation or targeted small interfering RNA (siRNA), suppresses the expression of RPE- and Müller cell-associated chemokines Ccl2, Cxcl1, and Cxcl10, reduces accumulation of macrophages in the outer retina, and mitigates photoreceptor death. We also find that IL-1β protein directly stimulates retinal chemokine up-regulation in vivo, and in cultured RPE and Müller cells. Targeting IL-1β as a therapeutic approach to reduce chemokine synthesis in the damaged retina may be beneficial in slowing the progression of retinal degenerations.

5.3. Methods

5.3.1. Animals and photo-oxidative damage

All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and had approval from the Australian
National University (ANU) Animal Experimentation Ethics Committee (Ethics ID: A2014/56). Adult Sprague-Dawley (SD) albino rats aged 90-120 days were used for all experiments. Animals were born and reared under dim light conditions (5 lux) prior to photo-oxidative damage. For the photo-oxidative damage paradigm, animals were placed into transparent Perspex open-top cages under a light source (COLD F2, 2x36W, IHF, Thorn Lighting, Australia) at 1000 lux for either 3, 6, 12, 17, or 24 hours (hrs), with access to food and water *ad libitum*. Following light exposure, animals were immediately euthanized using an overdose of barbiturate via an intraperitoneal injection (Valabarb; Virbac, NSW, Australia). For each animal, eyes were processed for either cryosectioning or RNA extraction, according to protocols detailed in our previous publications [141].

5.3.2. Intravitreal injections

Intravitreal injections were performed as described in detail previously [273], wherein animals were anaesthetised using an intraperitoneal injection of ketamine (100mg/kg; Troy Laboratories, NSW, Australia) and xylazil (12mg/kg; Troy Laboratories). Injections consisted of either siRNA- or antibody-based IL-1β inhibitors, or IL-1β protein.

A neutralising antibody to IL-1β (Cat# AF-501-NA, R&D Systems, Minneapolis, MN) was administered intravitreally to rats immediately prior to photo-oxidative damage. A 3µL solution containing either anti-IL-1β or an isotype control antibody was injected into individual animals, which equated to a delivery of 0.6µg of antibody per eye. After intravitreal injections, the animals were immediately transferred to individual cages designed to allow light to enter unimpeded. Animals were exposed to photo-oxidative damage for 24hrs, during which corneal hydration was maintained though application of a synthetic tear gel (GenTeal Gel; Novartis, NSW, Australia) until the animals awoke.

RNA-interference (RNAi) was conducted using *Il-1β*-specific siRNA (Cat# s127941; Thermo Fisher Scientific, Waltham, MA, USA), while a scrambled negative
siRNA (Cat# 12935300, Stealth RNAi Med GC; Thermo Fisher Scientific) served as a control, which 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 1µg/µl in endotoxin-free 0.1M PBS. For injection, animals were anaesthetised in the same fashion as the antibody neutralisation series. 3µl of either IL-1β or negative siRNA was then intravitreally delivered to both eyes of each animal, which equated to a final dosage of 3µg siRNA per eye. Animals were then exposed to 24hrs photo-oxidative damage under the same parameters as the antibody neutralisation cohort.

IL-1β protein was administered to rats following the same intravitreal methodology as described in the inhibition experiments. Recombinant IL-1β protein (Cat# AF-501-RL-010, R&D Systems) was injected at final concentration of 10ng per eye, as established in a previous study [274]; injections with only the PBS vehicle served as controls. Animals were euthanized at 3, 6, or 12 days post-injection, with eyes then processed as described in the previous section.

5.3.3. RPE and Müller cell cultures

Immortalised human cell lines MIO-M1 (Müller 1 Moorefields Institute of Ophthalmology; Dr A. Limb, Institute of Ophthalmology, University College London) and ARPE-19 (ATCC CRL-2302, American Tissue Culture Collection, Manassas, VA) were used to study responses to IL-1β stimulation. Cell lines were authenticated by CellBank, Australia. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 3mM L-glutamine (Life Technologies), in a humidified atmosphere
consisting of 95% air and 5% CO₂ at 37°C. The cells were passaged by trypsinization every 3 to 4 days.

The MIO-M1 and ARPE-19 lines were incubated in IL-1β protein to assess its effect on their expression of chemokines. Cells were first seeded to a density of 1 × 10⁵ cells on 24-well plates and left to recover for at least 48hrs. 24hrs prior to experimentation, the cells were placed in serum-deficient DMEM containing 1% FBS. IL-1β protein (Cat# 201-LB, R&D Systems) was then added to the culture medium at a concentrations of 10ng/mL, as per previous literature [275, 276], and then incubated for 12hrs. The cells were then harvested for either RNA extraction and PCR, or an MTT assay to verify cell viability. RNA was extracted from each sample well using a retinal RNA extraction protocol that we have established previously [85], with slight modifications for cell culture. The MTT assay was performed with a kit supplied by Roche (Cell Proliferation Kit I, Roche Applied Science, Penzberg, Germany) following the supplied instructions. Following IL-1β stimulation, MTT reagent was added to the sample wells and left to incubate for 4hrs, after which a solution of 0.04M HCl in isopropanol was then added to each well to dissolve the resulting formazan crystals. The absorbance for each sample was then read at 570nm on a TECAN Infinite 200 PRO (TECAN Seestrasse, Männedorf, Switzerland), and quantified as a percentage relative to unstimulated culture samples.

5.3.4. TUNEL and immunohistochemistry

Retinal cryosections were stained for apoptotic cells using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) kit (Roche Applied Science) and following our previous methodology [255, 256]. To quantify photoreceptor cell death, TUNEL+ cells in the ONL were counted throughout the full length of each section cut in the parasagittal plane (supero-inferior). For each animal, technical duplicates were counted, and these counts were averaged for each experimental group.
Immunohistochemistry was performed on retinal cryosections according to previously described protocols, with minor modifications [87]. A list of primary antibodies used is provided in Table 5.1. Fluorescence in sections was captured using a laser-scanning A1+ confocal microscope (Nikon, Tokyo, Japan). Images were processed using Photoshop CS6 software (Adobe Systems, CA, USA). Immunolabelled IBA1+ microglia/macrophages were quantified across the full length of each section in the parasagittal plane (supero-inferior). The number of IBA1+ microglia/macrophages in the outer retina was quantified by counting the IBA1+ cells in the ONL and subretinal space.

Table 5.1. Primary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Source</th>
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<tbody>
<tr>
<td>Rabbit α-IBA1 (ionized binding calcium adaptor molecule 1)</td>
<td>1:500</td>
<td>#019-19741, Wako Pure Chemical Industries, Osaka, Japan</td>
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<tr>
<td>Goat α-IL-1β</td>
<td>1:500</td>
<td>#AF501, R&amp;D Systems, Minneapolis, MN, USA</td>
</tr>
<tr>
<td>Mouse α-Vimentin</td>
<td>1:100</td>
<td>#V6630, Sigma-Aldrich, St. Louis, MO, USA</td>
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5.3.5. Polymerase chain reaction (PCR)

In preparation for quantitative real-time PCR (qPCR) and standard PCR, the RNA from retina or cell culture samples was synthesised into cDNA using a Tetro first-strand cDNA Synthesis Kit (Bioline Reagents, London, UK), as described in our previous investigation [87].

qPCR was performed on cDNA samples using Taqman hydrolysis probes (Table 5.2; Thermo Fisher Scientific), which were applied according to the manufacturer’s instructions with the Taqman Gene Expression Master Mix system (Thermo Fisher Scientific). The qPCR reactions were run on a QuantStudio Flex 12K instrument (Thermo
Fisher Scientific). The resultant data were analysed according to the comparative cycle threshold \((C_t)\) method (\(\Delta \Delta C_t\)), which was normalised to the expression of both \(Gapdh\) and \(Actb\) reference genes, as established in our previous analyses [85, 144].

**Table 5.2.** Taqman hydrolysis probes used for qPCR

<table>
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<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Catalog Number</th>
<th>Entrez Gene ID</th>
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<tbody>
<tr>
<td>Actb</td>
<td>Actin, beta</td>
<td>Rn00667869_m1</td>
<td>81822</td>
</tr>
<tr>
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<td>Caspase 1</td>
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Standard PCR was conducted from cDNA synthesised from cells cultures or retinal homogenates, using primers specific to \(Il-1\beta\) receptor-related genes in human: \(Il-1r1\) (F: 5’ ATCGTGATGAATGTGGCTGA 3’; R: 5’ TCTCATTAGCTGGGCTCACA 3’), \(Il-1rap\) (F: 5’ CGTTTCATCTCACGACTCC 3’; R: 5’ CCACACCTTATTGCAGAAT 3’), or rat: \(Il-1r1\) (F: 5’ ACATTGAGTCTGCTCCTT 3’; R: 5’ TGTGCTCAGGAGCTTCTCTT 3’), \(Il-1rap\) (F: 5’ TCATACGCAAGTAGTACACA 3’; R: 5’ GGGCTCAGGACAAATCAT 3’). The primers designed using the Primer3 web-based design program [226]; both transverse an intron splice site to avoid genomic amplification. The PCR was performed using MyTaq DNA Polymerase (Bioline) as per
the manufacturer’s instructions, and the presence and specificity of the PCR product were inferred by gel electrophoresis.

5.3.6. **In situ hybridisation**

*Ccl2* and *Cxcl1* were cloned from PCR products (550-bp and 504-bp amplicons respectively) using cDNA synthesised from retinal RNA (as described above). Digoxigenin (DIG)-labelled riboprobes were then prepared as described in our previous publication [87]. *In situ* hybridisation on retinal cryosections was carried out according to our established methodology [224]; briefly, each riboprobe was hybridised to sections overnight at 55°C, then was washed in decreasing concentrations of saline sodium citrate (pH 7.4) at 60°C. The bound probe was visualised with either NBT/BCIP or HNPP/Fast-Red (Roche Applied Science).

5.3.7. **Quantitative and statistical analysis**

Graphing and statistical analysis for this study was performed using Prism 7 (GraphPad Software, CA, USA). Statistical analysis was conducted using a Students t-test for instances of single comparisons. For assessing trends or multiple comparisons over protracted time-courses, a Kruskal–Wallis one-way analysis of variance (ANOVA) with Dunn’s multiple comparison post-test was applied, as per our previous investigation [87]. A *P* value of < 0.05 was considered statistically significant.
5.4. Results

5.4.1. Expression of retinal IL-1β in relation to chemokine up-regulation and macrophage infiltration following photo-oxidative damage

Retinal expression of Il-1β and genes associated with inflammasome assembly and activation (Casp1, Casp8, and Nlrp3) were assessed by qPCR following 24hrs photo-oxidative damage (Figure 5.1A). Il-1β was dramatically up-regulated after 24hrs photo-oxidative damage, consistent with our prior reports [87], and in concert with expression of Casp1, Casp8, and Nlrp3 (P<0.05, Figure 5.1A). Immunoreactivity for IL-1β was not detected in dim-reared control retinas, but after photo-oxidative damage IL-1β immunoreactivity was evident on some ramified IBA1+ microglia/macrophages in the ONL and subretinal space (Figure 5.1B-C, yellow). IBA1+ cells located in the choroid did not exhibit IL-1β immunoreactivity (Figure 5.1C).

Comparison of Il-1β expression with changes in retinal Ccl2, Cxcl1 and Cxcl10 (Figure 5.1D) shows a correlation between Il-1β and chemokine expression. Over the 24hr time-course of photo-oxidative damage (3, 6, 12, 17, and 24hrs), Il-1β expression was markedly upregulated after 6hrs, and increasing Il-1β expression was associated with an upregulation of Ccl2, Cxcl1, and Cxcl10, with all markers reaching peak expression at 24hrs (P<0.05; one-way ANOVA). Consistent with previous reports [85, 141] we also observed incursions of IBA1+ macrophages into the ONL and subretinal space by 24hrs of photo-oxidative damage (P<0.05, Figure 5.1E-G).

5.4.2. Effect of IL-1β suppression on photoreceptor death, macrophage accumulation, and chemokine expression

Inhibition of IL-1β and its effect on photo-oxidative retinal damage was ascertained using both siRNA and antibody neutralisation approaches (Figures 5.2 and 5.3). Intravitreal
Figure 5.1. Temporal relation of IL-1β to chemokine expression and macrophage infiltration following photo-oxidative damage (PD). A: After 24hrs of light exposure, a number of inflammasome activation markers were significantly upregulated (Casp1, Casp8, Nlrp3, P<0.05), in addition to IL-1β (P<0.05). B-C: Representative images show
immunoreactivity for IL-1β (red) in the outer retina following 24hrs of photo-oxidative
damage, particularly in the ONL, subretinal space and RPE (B; arrows), and which co-
localised to IBA1+ microglia (green) (C; arrows). **D:** Up-regulation of IL-1β was
documented over a 24hr time-course of photo-oxidative damage (P<0.05), which was
found to align closely with up-regulation of *Ccl2, Cxcl1* and *Cxcl10* over the same period
(P<0.05). **E:** A large increase in the number of IBA1-immunoreactive macrophages was
quantified within the outer retina (ONL and subretinal space) following photo-oxidative
damage (P<0.05) compared to dim-reared controls. **F-G:** Representative images showcase
the infiltration of IBA1-immunolabelled macrophages (green) within the ONL and
subretinal space (arrows) in retinal sections following photo-oxidative damage (G), in
contrast to the absence of these cell in sections from dim-reared retinas (F). C, choroid;
INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments. N=4-6 per group.
Asterisks denote a significant change, where P<0.05. Scale bars equate to 50µm.
injection of the *Il-1β*-specific siRNA induced a 1.8 fold reduction in the expression of retinal *Il-1β* at 24hrs photo-oxidative damage, compared to negative control siRNA (P<0.05, Figure 5.2A). Animals injected with *Il-1β* siRNA had ~60% fewer TUNEL+ photoreceptors 24hrs post-exposure to photo-oxidative damage compared to controls (P<0.05, Figure 5.2B). In experiments where IL-1β was neutralised using the antibody, injected intravitreally prior to photo-oxidative damage, there was an 80% reduction in the number of TUNEL+ photoreceptors in the retina, compared to those injected with an isotype antibody control (P<0.05, Figure 5.2C-E). Counts of IBA1+ macrophages confirm that inhibition of IL-1β with either siRNA or neutralising antibody reduces the number of IBA1+ macrophages in the outer retina (ONL and subretinal space), compared with the respective controls (P<0.05, Figure 5.2F-H).

We then sought to determine the effect of IL-1β suppression on the retinal expression of chemokines *Ccl2, Cxcl1*, and *Cxcl10* (Figure 5.3A). In both modes of IL-1β inhibition, there was a significant reduction in the expression of *Ccl2* and *Cxcl1* compared to controls (P<0.05, Figure 5.3A). While *Il-1β*-specific siRNA did not modify expression of *Cxcl10*, antibody neutralisation of IL-1β was effective in reducing *Cxcl10* expression (P<0.05). By use of *in situ* hybridisation, we also confirmed that *Ccl2* mRNA was present in vimentin-immunoreactive Müller cell processes after 24hrs photo-oxidative damage (Figure 5.3D-E; arrows), and that *Ccl2* mRNA labelling was reduced in IL-1β-inhibited retinas compared to controls (Figure 5.3B-C; arrows). *Ccl2* mRNA was not detected in RPE cells (Figure 5.3B-C), consistent with our previous findings [85, 87]. We detected *Cxcl1* mRNA labelling in the INL (Figure 5.3F; arrows) and RPE layer (Figure 5.3H; arrows) after photo-oxidative damage, which was reduced in retinas where IL-1β had been inhibited via neutralising antibody (Figure 5.3G, I). INL staining for *Cxcl1* mRNA correlated with vimentin-immunoreactive Müller cells (Figure 5.3J-K; arrows), consistent with our previous report [87].
**Figure 5.2.** Effect of IL-1β inhibition on photoreceptor apoptosis and macrophage infiltration following photo-oxidative damage (PD). **A:** A significant knockdown of IL-1β expression in the retina was achieved in retinas injected with an IL-1β-specific siRNA, compared to the negative control siRNA group (P<0.05). **B:** The number of TUNEL+ cells in the ONL was reduced in IL-1β siRNA-injected retinas after photo-oxidative damage, compared to controls (P<0.05) **C:** A reduction in the number of TUNEL+ cells in the ONL was also documented in animals that had been intravitreally injected with an IL-1β
neutralising antibody prior to photo-oxidative damage, in comparison to an isotype control antibody (P<0.05). **D-E:** Representative images showcase a decrease in TUNEL+ profiles (red) in retinal section from the IL-1β neutralisation group (E), compared to a section from the isotype control group (D). **F:** The infiltration of IBA1-immunolabelled macrophages into the outer retina (ONL and subretinal space) following photo-oxidative damage was significantly decreased in animals injected with either *Il-1β*-specific siRNA or an IL-1β-specific neutralising antibody, compared to their respective controls (P<0.05). **G-H:** Representative images of IBA1-immunolabelled macrophages demonstrate the reduction in IBA1+ cells in the outer retina in the IL-1β antibody neutralisation retinas compared to controls (green). INL, inner nuclear layer; ONL, outer nuclear layer. N=4-5 per group. Asterisks denote a significant change, where P<0.05. Scale bars equate to 25µm.
**Figure 5.3.** Effect of IL-1β inhibition on chemokine expression following photo-oxidative damage (PD). A: In animals that were intravitreally injected with Il-1β-specific siRNA, there was significantly lower expression of Ccl2 and Cxcl1 compared to control siRNA after photo-oxidative damage (P<0.05). In the group injected with IL-1β neutralising antibody however, the expression of Ccl2, Cxcl1 and Cxcl10 were all significantly down-
regulated in comparison to the isotype control group (P<0.05). B-K: *In situ* hybridisation was used to examine the localisation of *Ccl2* and *Cxcl1* mRNA transcripts following IL-1β inhibition and photo-oxidative damage, as shown in representative images. Staining for *Ccl2* mRNA (purple/red) was decreased in IL-1β antibody-injected retinas compared to control IgG retinas (B-C; arrows), and was co-localised to vimentin–immunoreactive Müller cells (green) (D-E; arrows). Staining for *Cxcl1* mRNA was observed within INL (F-G) and RPE layers (H-I), which was decreased in the IL-1β neutralising antibody group. The INL staining correlated with Müller cell processes that were immunolabelled with vimentin (J-K). INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments. N=5 per group. Asterisks denote a significant change, where P<0.05.
5.4.3. Effect of IL-1β stimulation on chemokine expression in Müller and RPE cell cultures

The capacity for IL-1β to stimulate chemokine up-regulation in Müller cells and RPE cells was assessed in MIO-M1 and ARPE-19 cell cultures, respectively (Figure 5.4). Both cultures were incubated with 10ng/mL IL-1β protein for 12hrs, at which point we observed dramatic increases in the expression of Ccl2, Cxcl11 and Cxcl10 in MIO-M1 cells compared to unstimulated control wells (P<0.05, Figure 5.4A). To discount the possibility that the IL-1β-induced up-regulation of chemokines was a result of cell stress/death, we conducted an MTT assay on the cultures after IL-1β stimulation (Figure 5.4B). We determined that both cultures exhibited no reduction in viability as a result of IL-1β stimulation compared to controls (P>0.05). We also found that both MIO-M1 and ARPE-19 cells express Il-1r1 and Il-1rap receptor genes necessary for IL-1β signal transduction (Figure 5.4C-D).

5.4.4. Changes in chemokine expression and macrophage infiltration following intravitreal delivery of IL-1β protein

Finally, we investigated the effect of IL-1β protein administered intravitreally on the expression of chemokines and the accumulation of macrophages over a 24hr period (Figure 5.5). The data show a broad up-regulation of retinal Ccl2, Cxcl11, and Cxcl10 (Figure 5.5A), up to 24hrs post-injection compared to PBS-injected controls. This was particularly evident for Cxcl11, which by 6 hrs-post-injection increased ~22 fold (P<0.05), although was somewhat reduced by 12 and 24hrs. The localisation and number of IBA1+ macrophages at 24hrs after the injection of IL-1β protein (Figure 5.5B-F) demonstrated a significant increase in the total number of retinal macrophages present compared to the PBS-control group (P<0.05; Figure 5.5B). IBA1+ cells were observed predominantly in the GCL and optic nerve head (Figure 5.5E-F; arrows), and comprised a population with a
Figure 5.4. Chemokine expression in RPE and Müller cell cultures stimulated with IL-1β.

A: MIO-M1 and ARPE-19 cells were incubated with IL-1β protein for 12hrs, after which MIO-M1 cultures were found to up-regulate expression of Ccl2, Cxcl1 and Cxcl10 (P<0.05), while ARPE-19 cells had significantly increased expression of Cxcl1 and Cxcl10 (P<0.05), compared to unstimulated controls. B: MTT assays were conducted on MIO-M1 and ARPE-19 cultures, which showed no difference in cell viability between IL-1β and control groups. C-D: Representative images of Il-1r1 and Il-rap PCR products indicate that MIO-M1 and ARPE-19 express the receptor genes necessary for Il-1β signal transduction. N=5 per group. Asterisks denote a significant change, where P<0.05.
Figure 5.5. Change in retinal chemokine expression and macrophage infiltration in retinas following intravitreal injection of IL-1β protein. A: Injection of IL-1β protein increased the expression of Ccl2, Cxcl1 and Cxcl10 in retinas over a time-course of 6, 12, and 24hrs post-injection, compared to PBS-injected controls (P<0.05). B: There was a significant increase in the number of IBA1+ cells throughout retinal sections in the IL-1β group at
24hrs post-injection, compared to the PBS control group (P<0.05). **C-F:** Representative images of IBA1-immunolabelled retinal sections show increased clusters of IBA1+ macrophages amongst the GCL and optic nerve head of the IL-1β-injected group (E-F; arrows) compared to PBS controls (C-D). **G-H:** Representative images show positive expression of PCR products for *Il-1r1* and *Il-rap* in control rat retinas. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. N=3-6 per group. Asterisks denote a significant change, where P<0.05. Scale bars equate to 50µm.
rounded (activated) rather than ramified (resting) morphology. PCR conducted on isolates of control rat retinal tissue confirmed the presence of \textit{Il-1r1} and \textit{Il-1rap} genes (Figure 5.5G-H).

5.5. Discussion

These findings describe for the first time a key role for IL-1\(\beta\) in mediating the accumulation of outer-retinal macrophages by modulating the expression of chemokines by Müller cells and RPE. First, we demonstrate that \textit{Il-1\(\beta\)} is up-regulated in concert with the Müller and RPE cell-expressed chemokines (\textit{Ccl2}, \textit{Cxcl11} and \textit{Cxcl10}), and with the influx of macrophages into the outer retina following photo-oxidative damage. Second, we showed that therapeutic suppression of retinal IL-1\(\beta\) using either siRNA or antibody neutralisation curtails chemokine expression, accumulation of outer-retinal macrophages and photoreceptor degeneration. Finally, we show that Müller and RPE cell cultures up-regulate \textit{Ccl2} (Müller cells only), \textit{Cxcl11} and \textit{Cxcl10} in response to IL-1\(\beta\) stimulation, and that intravitreal delivery of IL-1\(\beta\) induces up-regulation of these same chemokines in the retina, coinciding with increases in the population of retinal macrophages. The data from this study supports the use of IL-1\(\beta\) inhibition strategies as a therapeutic approach to reduce chemokine synthesis, and subsequent macrophage accumulation and photoreceptor death in retinal degenerations.

Previous studies using injections of recombinant IL-1ra, an endogenous antagonist for IL-1r1, have suggested a role for IL-1\(\beta\) in propagating retinal degeneration, using models of photo-oxidative damage in Cx3cr1-deficient mice [114, 277], laser-induced CNV [115], and retinitis pigmentosa [116]. However, a short-coming of that approach is that IL-1ra does not suppress IL-1\(\beta\) signalling specifically, as the inflammatory cytokine
IL-1α also competes for binding and is equally as effective in activating Il-1r1 as IL-1β [98, 278, 279]. In this study, we have used Il-1β-specific siRNA, as well as IL-1β antibody inhibition methodologies to target IL-1β directly, circumventing any possibility of off-target inhibition of IL-1α-mediated signalling. Our data clearly show that decreased IL-1β correlates with decreased chemokine production by RPE and Müller cells, reduced macrophage recruitment and increased photoreceptor survivability. Excluding the effect of IL-1α signalling is an important consideration, as IL-1α released from dying cells promotes sterile inflammation and leukocyte recruitment [279, 280], and ablation of IL-1α alleviates inflammation in myocardial infarction [281]. Moreover, our previous microarray analysis (gene expression omnibus GSE22818) indicates that IL-1α is up-regulated following photo-oxidative damage [141].

Our previous investigations have indicated that RPE and Müller cells are potentiators of Ccl- and Cxcl- expression during photo-oxidative damage [85-87]. Several of these chemokines, including CCL2, CXCL1 and CXCL10 are involved in leukocyte recruitment in CNS diseases and in retinal degeneration [84, 85, 87, 272]. The significance of the expression of such chemokines is underscored by our finding that the broad spectrum chemokine inhibitor NR58-3.14.3 – a suppressor of Ccl- and Cxcl- signalling – ameliorates macrophage recruitment and photoreceptor degeneration resulting from photo-oxidative damage [228]. The involvement of IL-1β signalling in chemokine expression shown in the current study is consistent with its known influence outside the retina, including its ability to induce CCL2 in pancreatic β-cells [282, 283], and spur up-regulation of CXCL1 in intestinal tissue during infection with Clostridium difficile [284]. Precisely how IL-1β induces the up-regulation of chemokines in RPE and Müller cells is uncertain, though it has been demonstrated in pancreatic β-cells that IL-1β signalling mediates nuclear localisation of the transcription factor NF-κB, which then promote the expression of chemokines such as CCL2 [285].
While the data generated in these investigations was generally consistent between the IL-1β inhibition strategies employed, it is noted that expression of Cxcl10 was not inhibited by Il-1β siRNA, in contrast to the findings using antibody neutralisation. This difference may be due to delayed efficacy of the siRNA resulting from the time required for adequate transfection and mRNA suppression. Consistent with this idea, comparison of the data indicate that the neutralising antibody had a more potent effect on Ccl2 and Cxcl1 expression compared with siRNA. However, given the reduction in macrophage infiltration identified in both treatment groups (siRNA and antibody), despite the discordant suppression of Cxcl10, it is possible that Cxcl10 does not play a crucial role in macrophage recruitment in the retina compared to Ccl2 and Cxcl1. While indeed plausible, other studies have shown that CXCL10 specifically elicits macrophage recruitment in experimental nonalcoholic steatohepatitis [286], and is also implicated in macrophage infiltration in kidney during puromycin aminonucleoside nephrosis [287].

Though our investigation focused on the effect of IL-1β on expression of RPE/Müller cell-associated chemokines and macrophage recruitment, the potential contribution of other leukocyte populations should not be overlooked. Peripheral neutrophils and T-cells, as well as macrophages, express the receptors for CXCL1 and CXCL10 [288-291]. These leukocyte populations are poorly characterised in sterile retinal inflammation, although are understood to comprise a small proportion amongst the predominantly macrophage-led response in AMD and models such as photo-oxidative damage [3, 170]. Nonetheless, the contribution of these cell types to pathology in sterile retinal inflammation is unclear, and identifies neutrophils and T-cells as candidates for investigation in future studies.
5.5.1. Conclusions

Our study identifies a key role for IL-1β in orchestrating the infiltration of macrophages to the outer retina, by inducing the up-regulation of chemokines in RPE and Müller cells following retinal damage. Moreover, we confirm the potential of specific IL-1β inhibitors in dampening inflammation and ameliorating photoreceptor degeneration. Consequently, their application may have value in the treatment of retinal dystrophies in which chemokine expression and subretinal macrophage accumulation are implicated, such as AMD.
6. SUMMARY
These studies collectively build upon previous findings in our research group that C3 is expressed by macrophages in models of retinal degeneration and ageing [141, 144] as well as the finding that Ccl2 siRNA reduces macrophage recruitment in the retina [89]. This thesis presents data which strongly indicates that retinal microglia and macrophages contribute to photoreceptor degeneration, and have a sustained effect on atrophic lesion development in the degenerating retina. This supports the use of therapeutic molecules to target inflammatory activation by retinal microglia and macrophages, as a strategy for slowing the progression of retinal degenerative diseases.

The work described in this thesis provides a deeper understanding of how microglia and macrophages propagate the inflammatory response in retinal degeneration. First, it is demonstrated that microglia and macrophages play a critical role in activating complement, leading to increased photoreceptor loss and further accumulation of inflammatory cells in the damaged retina [292]. It is shown, in both human atrophic age-related macular degeneration (AMD) and in rodent photo-oxidative damage, that complement component 3 (C3), a central component of the complement system, is synthesised and deposited in the photoreceptor layer by retinal microglia and macrophages. Using intravitreally-delivered C3 siRNA to inhibit retinal sources of C3, it is demonstrated that locally-derived C3, not systemic C3, contributes to the onset of retinal degeneration implicating complement-expressing retinal microglia and macrophages in prolonged disease progression.

The findings from Chapter 3 using C3 siRNA demonstrates a potential role for gene therapies to knockdown the expression of specific inflammatory genes, particularly C3. The strong association between polymorphisms in complement genes, and the prevalence of AMD gives further weight to the potential use of gene therapies to combat retinal diseases. Using locally-delivered C3 siRNA via lipid-based transfection, we demonstrated that C3 expression in the retina was knocked down efficiently. However, the
delivery of siRNA to the retina may only be for a short duration, as other research from our group suggests it may be cleared within 3-4 days of delivery. Gene therapies that exert a prolonged effect within the retina are therefore warranted, such as the use of vectors including AAV2, which have proven useful in the treatment of Leber Congenital Amaurosis (RPE65 mutation) [310]. The use of clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9 technology (reviewed in [293]) would allow sustained C3 knockout to occur within the retina, and has been performed in several models of retinal disease [294-296]. Most recently, one group demonstrated the use of Vegfa Cas9 ribonucleoproteins (RNPs) in a choroidal neovascularisation (CNV) model of wet AMD to reduce Vegfa-associated retinal damage [297, 298]. Alternatively, conditional ablation of C3 from retinal microglia using Cre-loxP could be useful in further determining the contribution of the local microglia/macrophages to complement activation in retinal degeneration. Utilisation of these technologies may lead to valuable insights into the reduction of deleterious complement gene activation in AMD.

Second, the work highlights the therapeutic potential of broad spectrum chemokine inhibition as a strategy to prevent retinal degeneration [228]. It was demonstrated that an intravitreal injection of NR58-3.14.3 reduced the recruitment of microglia and macrophages into the outer retina, and ameliorated photoreceptor cell death in rodent photo-oxidative damage. This indicates its potential for the treatment of retinal degenerations such as atrophic AMD, where chemokine secretion and macrophage recruitment to the outer retina are key features of disease progression. This study also shows that targeting chemokine production to inhibit macrophage recruitment and activity may be an optimal therapeutic strategy for slowing the progression of retinal degeneration.

Third, it has been shown that the pro-inflammatory cytokine interleukin-1β (IL-1β) plays a key role in contributing to retinal degeneration in rodent photo-oxidative damage
This study shows that retinal microglia and macrophages are the primary expressers of IL-1β, promoting the expression of chemokines by Müller cells and RPE, leading to the further recruitment of macrophages to the degenerating retina. This study also demonstrates the value of targeting cell signalling mechanisms upstream of complement, further highlighting the key roles of microglia and macrophages in retinal degeneration.

Here we demonstrate that Il-1β knockdown is also an effective method for reducing chemokine-mediated macrophage recruitment in the retina. However, the inflammasome signalling pathway is largely responsible for Il-1β production, which is primarily expressed by macrophage cells [100]. The role of the NLRP3 inflammasome has been controversial in the literature, with research suggesting that blocking NLRP3 inflammasome activation is beneficial in dry AMD [103], but detrimental in wet AMD [106]. Current work in our research group is being undertaken to understand the role of the NLRP3 inflammasome in the photo-oxidative damage model. Additionally, as we have shown that gene therapies to target Il-1β and inflammasome activation may be useful, our research group is focused on the use of microRNAs (miRNAs), ‘master gene regulators’ [300], to regulate inflammation in retinal degeneration [301]. By identifying miRNAs that may regulate Il-1β and inflammasome signalling [302-305] in the retina, this may lead to the development of novel gene therapies to target macrophage-derived inflammation in retinal degeneration.

The next critical question, beyond the scope of the studies reported here is do retinal microglia and blood-borne macrophages contribute similarly to the progression of retinal degeneration? A limitation of the studies presented in this thesis is that thus far it has not been possible to differentiate these two cell populations. However, two recent advances now make this distinction possible. First, studies indicate that a TMEM119 antibody is able to differentiate resident microglia from blood-borne macrophages in the brain, using flow cytometry and immunohistochemistry [306-308]. Second, a fate mapping
technique to endogenously label the two cell types with fluorescent reporters can distinguis

sh cells as either resident or recruited, using tamoxifen-inducible Cre recombinases [120], and suggests that these populations are phenotypically distinguishable by a specific set of markers. Characterising the inflammatory profiles of these two separate populations will assist in understanding how microglia and macrophages each contribute to pro-inflammatory cytokine production, complement activation and photoreceptor cell death. This will allow for further development of therapeutic interventions for retinal diseases including AMD.

The photo-oxidative damage model recapitulates many features of atrophic AMD, including oxidative stress, pro-inflammatory cytokine production, the recruitment of microglia and macrophages, the synthesis of complement system components and the progression of an expanding photoreceptor lesion. However, further investigation to identify the translational potential of the therapeutic molecules described in this thesis is warranted, using additional models that reproduce other features of AMD not modelled in photo-oxidative damage including the CEP model [63, 68, 69] and the laser-induced CNV model [196, 197]. Additionally, a model of early AMD, such as ageing, could be used to further investigate the thickening of Bruch’s membrane, drusen and RPE disruption.

This thesis provides further understanding of the role of microglia and macrophages in amplifying the inflammatory response and their contribution to the progression of retinal degeneration. It also demonstrates that the inhibition of factors that drive the recruitment of these cells into the degenerating retina reduces their accumulation and ameliorates photoreceptor damage. Further studies that delineate resident microglia and blood-borne macrophages to elucidate their individual contributions will be valuable in developing more effective therapeutic strategies to manage photoreceptor loss in AMD.
7. REFERENCES
7.1. Complete Reference List


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120. O’Koren, E., Mathew, R., and Saban, D. (2016) Fate mapping reveals that microglia and recruited monocyte-derived macrophages are definitively distinguishable by phenotype in the retina. Scientific Reports. 6, 20636.


