Characterisation of the neuroinflammatory response in a rat model of atrophic AMD

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of The Australian National University
Declaration

This declaration certifies that the following work entitled ‘Characterisation of the neuroinflammatory response in a rat model of atrophic AMD’ is the authors own original work, complies with The Australian National University Research Award Rules 4.4(2), and has not previously been accepted for award of a degree or diploma to any other university or institution of higher learning.

Signed: 

Date: 29. 8. ii
Acknowledgements

In my experience there is no such thing as a self-made individual; rather, our success and achievements represent in no small part the kindness, wisdom, and support of those around us. As such, there are many I wish to thank who have made this experience as enjoyable as it has been.

First, I would like to give my sincere thanks to everyone in my group, I could not have hoped for a better group of colleagues to have undertaken my PhD with. Particular thanks go to Riccardo Natoli for his invaluable advice in matters both scientific and worldly, and for teaching me almost everything I know about scientific technique; this work would not have been possible without his patience, dedication, and skill, for which I will be forever grateful. Additionally, a big thank you to my friends and officemates Owen ‘Old Man’ Carr, Faran ‘Saffron’ Sabeti, Peter Kozulin, and Rizsa Albarracin, whose advice – of which I hold in very high regard – and good company have made this a thoroughly enjoyable and memorable experience.

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I would like especially to thank my all friends, including those I worked with at the Tea Centre. The good times provided by you guys were welcome distraction from the turbulence of a PhD; I owe a great deal to you all.

Finally, and most importantly of all, I give my deepest thanks to my wonderful family. Those who know me best would know that family is everything to me, and that life is much more enjoyable with their care and dedication. In particular, thanks Mum and Dad for your unwavering love and support throughout the year. Special thanks also to Scott, Josh, and Michelle for maintaining my sanity over the past three years! Last but not least, a big thanks to my Nanna for the ever-present love and support!

This PhD has meant far more to me than merely an academic pursuit. The science is in the words, but the experience – the adventures, friendships, struggles, and perseverance – lies between the lines; and that for me is what life is all about. Thank you for the memories.
'Everything that has been achieved is merely a preliminary exercise for the achievements to come, and no one, not even one who has reached perfection, can say he has reached the end.'

– Eugen Herrigel
Abstract

Age-related macular degeneration (AMD) affects millions of individuals worldwide, and is the leading cause of blindness in the industrialised world. AMD is a multifactorial disorder, involving complex interaction between environmental and genetic factors. While the advent of anti-vascular endothelial growth factor (VEGF) therapy has allowed for the effective treatment of neovascular AMD, no effective treatments are available to mitigate 'dry' forms of AMD, including geographic atrophy. A role for inflammatory processes in the progression AMD has been postulated over a period of many years, mainly through observations of leukocyte infiltrates within AMD-affected eyes in traditional histological examinations and by electron microscopy. Recently however, rigorous genome-wide screening for short nucleotide polymorphisms (SNPs) has identified a significant association of a number of complement system gene variants with AMD, which together have firmly placed inflammation – particularly the involvement of innate immune pathways – as a key factor in the pathogenesis of AMD. Despite this, a number of key aspects of the inflammatory process remain to be clarified, including the spatiotemporal cellular events leading to local complement activation, and inflammatory cell recruitment in the macular region. In the investigations presented in this thesis, I aimed to investigate the suitability of light-induced retinal degeneration in rats as a model for atrophic AMD, and to explore the spatiotemporal emergence of inflammatory events in the retina in relation to light damage, with an emphasis on the recruitment of macrophages/microglia and the complement system.

Light-mediated retinal degeneration was induced in albino Sprague Dawley (SD) rats by exposure to bright continuous light (BCL) at 1000 lux for incremental periods between 0 and 24hrs, followed by a post-exposure period under standard dim-light (5 lux) conditions ranging from 0 to 56 days. Features of light-damage induced changes to
the retina were compared to the histopathology of AMD using histological analyses, immunohistochemistry for markers of retinal stress (glial fibrillary acidic protein (GFAP) and fibroblast growth factor 2 (FGF-2)), and macrophage (ED1) markers, in relation to quantitative assessment of photoreceptor apoptosis, using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Following exposure to BCL, the spatiotemporal profile of monocyte-chemoattractant gene chemokine (C-C motif) ligand 2 (Ccl2) in the retina was investigated using a combination of quantitative real time polymerase chain reaction (qPCR), *in situ* hybridisation, and immunohistochemistry. Expression of Ccl2 was assessed in relation to photoreceptor death using TUNEL, and the infiltration of monocytes, quantified though counts of ED1-immunoreacitive (IR) macrophages/monocytes.

The expression of complement system genes in the retina following BCL was determined by microarray analysis, in which gene expression in animals reared in dim light conditions was compared with those exposed to 24hrs BCL. Genes of interest were validated using qPCR analysis on samples collected at 24 hours exposure, and over a protracted timecourse in the post-exposure period. Because it was highly expressed in the post-exposure period, and plays a pivotal role in the complement cascade, the spatiotemporal expression of complement component 3 (C3) in retinal sections was assessed using *in situ* hybridisation coupled with immunohistochemistry.

Exposing albino rats to BCL induced a rapid and prolonged emergence of focal histopathology at the *area centralis* – a homologue of the *fovea centralis* – in the superior retina. Features of degeneration included breakdown of the blood retinal barrier, photoreceptor death, the prevalence of surviving cones over rods, macrophage recruitment, and reactive gliosis in Müller cells. Additionally the expression of stress factors GFAP and FGF-2 and photoreceptor degeneration continued at the margin of the lesion up to 56 days post-exposure. Progressive expansion of the lesion in the post-
exposure period – a feature of atrophic AMD – was a prominent aspect of the degeneration. The emergence of these features was accompanied by a focal recruitment of macrophages from the choroid and retinal vasculature at the lesion area. Each of these features is also observed in an atrophic AMD lesion, indicating that this light-damage model is a suitable model for atrophic AMD.

To understand the mechanisms that recruit macrophages into the light-damaged region, the spatiotemporal expression of Ccl2 was assessed. It was observed that Ccl2 is synthesised by Müller cells following BCL exposure, in spatiotemporal coincidence with the subsequent emergence of photoreceptor death at the area centralis. Following the expression of Ccl2, a localised recruitment of macrophages from both retinal and choroidal vascular supplies was observed at the area centralis, correlating with the spatial distribution of Ccl2-positive Müller cells. Recruited macrophages were also observed to express Ccl2.

Expression of complement-related genes following exposure to BCL was investigated by microarray analysis. A suite differentially expressed complement genes was identified following exposure to 24hrs BCL, including opsonin mediators from classical and lectin pathways (C1s, C2, C4, Ficolin B), complement receptors (C1qR1, C3aR1, C5r1, CR3, CR4) and regulators (CD46, CD55, SERPING1, C4bp, a2m). A number of these were found by qPCR to positively correlate with levels of photoreceptor apoptosis, both during and after exposure to BCL. Most significantly the study shows by in situ hybridization that C3 deposited in the ONL and outer segments in the damaged region originates from recruited microglia/macrophages.

These findings pinpoint macrophages/microglia as key factors mediating activation of the complement system in the degenerating retina, and whose focal recruitment to the area centralis may be facilitated in part by chemotactic signals
originating from the neural retina. These studies point to the retina as the primary site for initiation of degenerative mechanisms, rather that the supporting layers, including the RPE and choroid. Therapeutic attenuation of microglial/macrophage recruitment may be a useful strategy to control detrimental propagation of complement in the retina, particularly in retinal degenerations such as AMD.
Publications and Abstracts

This work is presented as a thesis by publication, encompassing the following peer-reviewed papers. Journal formatting – including references and abbreviations – is preserved with respect to the papers in chapters 2, 3, and 4; a complete, amalgamated list of references is displayed in appendix 1. The findings in this thesis were also featured in the abstracts listed below.

Peer reviewed papers


Abstracts


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Animals and light exposure

Tissue collection and processing

Analysis of cell death

Outer segment measurements

Immunohistochemistry

Results

The early phase post-exposure: Emergence of a ‘hotspot’ of degeneration

Recruitment of ED1-positive macrophages to the hotspot

The late phase post-exposure: Persistence of degeneration around the hotspot

Recovery of photoreceptors outside the hotspot

Discussion

Comparison with AMD

Conclusions

References

Chapter Three: Early focal expression of the chemokine Ccl2 by Müller cells during exposure to damage-inducing bright continuous light

Abstract

Introduction

Methods

Animals and light exposure
Chapter Four: Analysis of complement expression in light-induced retinal degeneration: Synthesis and deposition of C3 by microglia/macrophages is associated with focal photoreceptor degeneration

Abstract

Introduction

Methods

Animals and light exposure

Tissue collection and processing

Microarray experimentation and analysis

Quantitative real time polymerase chain reaction (qPCR)

In situ hybridisation

Analysis of cell death

Immunohistochemistry

Results

Microarray analysis

Differential expression of complement genes in the retina following BCL

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<td>retinal pigment epithelium</td>
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<td>RT</td>
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<td>Sprague Dawley</td>
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<td>Serping1</td>
<td>serine (or cysteine) peptidase inhibitor, clade G, member 1 (C1NH)</td>
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<td>SNP</td>
<td>short nucleotide polymorphism</td>
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<td>SOD1</td>
<td>Cu, Zn superoxide dismutase</td>
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Chapter One

Introduction
General Introduction

The retina is a laminated neural structure lining the posterior chamber of the eye (Figure 1A). In the outermost layer, photoreceptors convert a range of wavelengths of electromagnetic radiation into electrical signals through the process of phototransduction, and relay the resulting signals to the vision centres of the brain, allowing us to ‘see’ the world. Human retinas and those of other primates include a central macula lutea, situated around the highly specialised ‘fovea centralis’ (or fovea). Together, the macula and fovea mediate high acuity and colour vision (Figure 1B), and are necessary for almost all of our ‘useful’ vision in day-to-day life, including reading, driving, and face recognition.

Age-related macular degeneration (AMD) is a disease of the retina where there is region-specific degeneration of the macula and fovea, resulting in visual impairment or blindness, and considerable loss in quality of life including inability to lead an independent lifestyle. Although AMD is the most common form of retinal dystrophy in the industrialised world, the mechanisms involved in its pathogenesis are only now beginning to be understood. Over the last decade, however, there has been an accumulation of evidence pinpointing inflammatory processes as key factors in the progression of AMD.

In this chapter I will review literature encompassing the current knowledge of retinal immunity in relation to the pathogenesis of AMD, and discuss the animal models that are currently used to facilitate better understanding of inflammatory processes in AMD.
Figure 1. Basic structure and topography of the retina. A: The retina is composed of an organized, laminar network of neurons lining the posterior portion of the eye. Second order neurons are housed in the ganglion cell layer (GCL) and inner nuclear layer (INL) comprising ganglion cells, and amacrine, bipolar and horizontal cells, respectively. The photoreceptor population occupies the outer nuclear layer (ONL), while their light-receptive outer segments (OS) are located below. Outer plexiform (OPL) and inner plexiform (IPL) layers, composed of synaptic connections, are found between the cellular layers. The monolayer of cuboidal retinal pigment epithelium (RPE) is situated beneath the outer segments, and directly above Bruch’s membrane and the choroidal vasculature. B: The macula is named in reference to the central fovea, comprising the fovea itself, the parafovea and perifovea. The fovea is a highly specialised portion on the macula, featuring a pit structure which recedes with increasing eccentricity. Figure 1A adapted from Bird et al. 2010 and Swaroop et al. 2002, Figure 1B adapted from Provis et al. 2005.
Immune competence in the retina

The blood retinal barrier (BRB)

Inflammation in the retina is regulated by a combination of anatomical, physiological and immune-regulatory mechanisms, collectively referred to as the blood-retinal barrier (BRB) \(^8\), which act to preserve the distinctive physiological environment of the neural retina \(^2\). The BRB is a key part of the specialised ocular microenvironment which confers protection or ‘immune privilege’ on the retina by suppressing or minimizing inflammatory processes that might interfere with neuronal function, and hence vision \(^9\). The BRB barrier limits, but does not exclude, normal immune surveillance of the retina by leukocytes from the circulation, which if present can initiate deleterious immune responses in the retinal environment \(^10\). In more general terms, ‘immune privilege’ is thought to be an evolutionary adaptation, which restricts inflammatory processes that might damage tissues with limited capacity for regeneration, including the brain and retina \(^11\). The price of this privilege is the low tolerance of the peripheral immune system to sequestered antigens that become exposed when the retina or central nervous system (CNS) is damaged, leaving it vulnerable to autoimmune inflammation \(^12\). This deficiency is illustrated in sympathetic ophthalmia, a rare granulomatous uveitus associated with an ocular trauma or surgical procedure affecting the fellow eye \(^13\text{-}18\). That is, loss of BRB integrity in one eye leads to an autoimmune reaction in the uninjured fellow eye, against retinal antigens exposed from the injured eye \(^19\).

Structure

The BRB is described as having ‘inner’ and ‘outer’ components \(^2,20,21\). First, is the inner barrier that acts at the level of vascular endothelial cells within the neural retina, and is supplemented by an ensheathment of cellular processes \((\text{glia limitans})\) –
comprising astrocytes, microglia, and Müller cells – which appears to contribute factors that regulate transcellular and paracellular transport across vessel walls. Second is the outer barrier, composed of the retinal pigment epithelium (RPE) monolayer that is situated between the basal lamina of the choroid, Bruch’s membrane, and the photoreceptors. The barrier afforded by the endothelial cells and RPE is due to the nature of the intercellular junctions present. In both cases, tight junctions (zonula occludens) consisting of multilammellar strands of occludins and claudins, seal adjacent endothelial and RPE cells and prevent paracellular transport of ions, proteins and other cells, between adjoining cells. Zonule adherens junctions are also present, though are less restrictive, and formed mainly by pericellular cadherins which interact with intracellular catenins that bind the cytoskeleton.

**Function in normal and pathological states**

An important role of the BRB is to regulate leukocyte traffic from the circulation into the retinal parenchyma in the physiologically normal retina. However, the mechanisms that control this surveillance in the absence of inflammation are poorly understood. In non-inflamed conditions, activated leukocytes induce a localised and transient breakdown of the BRB to gain entry into the neural environment. Circulating leukocytes in the physiologically normal BRB are thought to interact with vascular endothelial cells, secreting cytokines to up-regulate adhesion molecules such as intracellular cell adhesion molecule 1 (ICAM-1), and achieve focal permeability of the BRB.

In many pathological conditions of the retina there is substantial modification of the BRB resulting in altered vascular permeability, ionic imbalance in the extracellular space, leukocyte infiltration, and extensive tissue damage resulting from cytokine mediated activity. BRB dysfunction has been characterised in AMD, as well as in uveoretinitis, diabetic retinopathy, and light-induced retinopathy. A number of
signalling events have been shown to contribute to BRB breakdown in retinal disease. In diabetic retinopathy for example, ischemia-induced expression of vaso-active factors such as vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) promotes increased vascular permeability, disrupting the integrity of the BRB. Studies in experimental autoimmune uveoretinitis (EAU) have demonstrated that retinal endothelial cells up-regulate expression of adhesion molecules (including ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1)) following stimulation by cytokines such as tumour necrosis factor α (TNFα), interleukin 1β (IL1β), facilitating adherence and passage of leukocytes through the BRB and into the parenchyma (reviewed in).

**Microglia and macrophages**

Microglial cells are a major glial constituent derived from the mononuclear phagocyte cell lineage, and play a critical role as the principle resident immunocompetent and phagocytic cell of the CNS, including the retina. Through persistent surveillance of their microenvironment, microglia act as sensors which help maintain homeostasis in the CNS during development and adulthood through a variety of functions. These include facilitating phagocytosis of debris and apoptotic cells, antigen presentation, and secretion of neuroprotective factors. Activation and recruitment of microglia to damaged areas is apparent in almost every pathological condition in the CNS, and is a common response in the retina to ocular infections, autoimmune mechanisms, neuronal injury, ischemia, and hereditary retinopathies.

**Origin**

Despite the considerable amount of research over the last few decades, the origin of microglia and macrophages in the CNS, and their functional roles has been – and remains – somewhat controversial. Despite this it is broadly agreed that these cells are derived from mononuclear phagocytes of haemopoietic origin. In the retina,
microglia arise from haemopoietic cell precursors during development which infiltrate the retina from the retinal margin and optic disc via the ciliary body and retinal vasculature during the late embryonic and early postnatal period\textsuperscript{44,45}. Upon entering the retinal tissue they eventually differentiate to become resting and mature microglia\textsuperscript{42}.

**Types and distribution**

The adult microglia/macrophage population consists of a heterogeneous assortment of cells, distinguished mainly by their localisation and morphological characteristics in the CNS\textsuperscript{46} and retina\textsuperscript{47,48}. It includes CNS-resident macrophages such as ‘perivascular’ and ‘meningeal’ types, a variety of choroid plexus macrophages/monocytes, as well as parenchyma-associated microglia\textsuperscript{46}. Detailed description of these populations in the CNS is beyond the scope of this literature review. For relevance and brevity, I will review the two distinct and best-characterised microglial/macrophage populations in the retina; the ‘parenchymal’ (ramified) microglia, and perivascular macrophages/monocytes – otherwise known as ‘perivascular microglia’\textsuperscript{42,48-50}.

Parenchymal microglia emerge from precursors entering the retina via the ciliary margin prior to vascularisation\textsuperscript{42,49}, and reside in the retinal parenchymal tissues mainly within the plexiform layers and ganglion cell layer (GCL) in their mature resting state\textsuperscript{42}. The outer nuclear layer (ONL) is typically devoid of these microglial cells in the healthy retina\textsuperscript{45}. In the resting state, parenchymal microglia have a characteristic ramified morphology, with dynamic, elaborate processes outstretched in close-contact with neurons to survey and monitor their microenvironment\textsuperscript{29,51}. Microglia and macrophages monitor changes in their surroundings through various cell-surface and nuclear receptors for immune constituents which include, but are not limited to, complement components, cytokines, chemokines, antibodies and adhesion molecules\textsuperscript{38,51}. Stimulation of microglia through signalling events mediated by these receptors may
result in their progression to an activated state. When activated, microglia undergo a
series of morphological changes, which result in hypertrophy of the microglial cell body,
retraction of its ramifications, and alteration of cell surface markers. These fully
activated parenchymal microglia – otherwise known as amoeboid microglia – are able
to effectively migrate to the site of injury, phagocytose dead cells and debris, and
secrete neuroprotective and inflammatory mediators.

Perivascular macrophages/monocytes are non-ramified cells, similar to
tissue macrophages, which originate from the optic nerve head and
vasculature during development. In the adult retina, they are associated with the
perivascular space surrounding blood vessels of the retinal vasculature. Perivascular
macrophages patrol the retinal vasculature in low numbers in the healthy retina,
serving as constitutive phagocytes, which scavenge and accumulate blood-borne
constituents in maintenance of the BRB. When activated through photoreceptor injury
and BRB breakdown, perivascular monocytes leave the vicinity of the vasculature and
migrate into the retinal parenchyma as tissue macrophages, where they participate in the
phagocytosis of dead cells and debris at the site of injury.

Replenishment in the adult retina

The replenishment of microglia in the adult CNS and retina, under normal and
pathological conditions, has been a subject of controversy. In normal
physiological conditions, pre-existing microglial cells have potential for self-renewal
and proliferation. Additionally, investigations using irradiated chimeric mice have
shown that both perivascular and parenchymal microglia/monocytes are slowly, yet
continuously, replenished by circulating bone marrow derived (BM-D) monocyte
immigrants in CNS (reviewed in) and retina, through their migration and
differentiation into microglial constituents. While accepted previously, doubt has been
cast on the contribution of BM-D cells to microglial renewal under normal conditions as
the use of radiation chimerism to assess microglial turnover has recently been called into question 46, 56, 58. Subsequent findings have since indicated that microglia in the adult CNS are likely maintained independently of circulating BM-D monocytes, by local radiation-resistant myeloid precursors 58. Despite this, it is generally agreed that BM-D monocytes infiltrate the CNS and differentiate into microglia and macrophages in various pathological states 46, 56, which may be mediated by a number of chemotactic signalling mechanisms including chemokine (C-C motif) ligand 2 (Ccl2) 46. In the retina BM-D cells infiltrate the retina from the ciliary body, optic nerve, and retinal vessels following photoreceptor degeneration inflicted through a number of noxious stimuli 55, 59, where they differentiate into microglial cells and participate in phagocytosis of dead cells and debris.

**Role in retinal degeneration**

Despite the beneficial properties of microglia in the maintenance of CNS homeostasis, microglial activity during neuronal stress and injury can damage neuronal cells 60-64. These ‘side-effects’ are most likely due to secretion of pro-inflammatory mediators and cytotoxic factors, such as TNFα, IL1β 39, 65, 66, and nitric oxide 51, 62, 67 which have deleterious effects on neurons and the extracellular environment. As such, microglial activation is thought to contribute to the pathogenesis of several neurodegenerative disorders, including Alzheimer’s disease and multiple sclerosis 39, 40.

In the retina, microglial activation is associated with cell death in several models of retinal degeneration (reviewed in 38), and is directly implicated in models of light damage 60, 68, 69, diabetic retinopathy 70, 71, glaucoma 72, chronic photoreceptor degeneration in rds (retinal degeneration slow) mice 73, laser-induced neovascularisation 74-76, and photoreceptor apoptosis in vitro 61.
Age-related macular degeneration (AMD)

AMD is the leading cause of irreversible blindness among those aged over 65 in industrialised nations. It is a progressive, age-related retinopathy that affects the photoreceptor population, RPE and Bruch’s membrane, and the choroid at various stages of the disease which are most apparent in the central macula region of the retina (Figure 2). The development of early, or ‘dry’, AMD is marked by formation of drusen, basal laminar (BlamD) and basal linear (BlinD) deposits, and RPE abnormalities such as atrophy and pigmentary disturbance. Early AMD may progress to advanced forms such as geographic atrophy and neovascularisation, leading to widespread degeneration and associated vision loss.

Features

Hallmarks of early AMD

AMD is progressive disease, whose features typically emerge gradually, over decades. The onset of early AMD is characterised by the increased presence of extracellular deposits known as drusen in the macula region. Drusen deposits, first documented in independent studies by Wedl and Donders in 1854, form between the basement membrane of the RPE and Bruch’s membrane, and are classified clinically as either ‘hard’ or ‘soft’. Hard drusen appear as small, yellow, punctate deposits typically less than 63 μm in diameter, while soft drusen are paler, larger deposits with indistinct edges. While small numbers of hard drusen are a feature of the normal aging process, numerous hard and soft drusen clustered within the macula area are associated with the progression of AMD. The drusen themselves do not appear to account for the substantial loss of vision in AMD, although they are associated with detachment and atrophy of overlying RPE and photoreceptors. However, the presence of drusen may result in vision deficits in aspects such
Figure 2. Major pathological features of AMD, as viewed through fundoscopy. 

B: Early AMD is typified by large numbers of hard and soft drusen, clustered predominately in the macula region. 

C: Atrophic AMD culminates in a zone of severe atrophy in the macula area, with the near-obliteration of RPE and photoreceptors. 

D: End-stage neovascular AMD results in proliferation of fibrovascular tissue, leading to the formation of a prominent disciform scar. 

Adapted from images obtained from NHS - eyes and vision (http://www.library.nhs.uk/eyes/ViewResource.aspx?resID=262086).
as colour contrast sensitivity and central visual field sensitivity. The significance of drusen in the pathogenesis of AMD remains elusive, though it is understood they are complex deposits that form over a long period of time, and are strong risk factors in the development of advanced AMD. The origin of drusen is also contentious, with both the RPE and the choroidal vasculature considered as sources of drusen biogenesis. Analyses indicate that drusen are a heterogeneous assortment of lipids, carbohydrates, cellular components of the RPE, and inflammatory proteins (reviewed in ). Because drusen include constituents of the immune system, such as those from the complement system, drusen are increasingly regarded as a marker of an inflammatory component in AMD.

BlamD and BlinD are also found in the sub-RPE space, and are amongst the earliest histopathological changes detected in AMD specimens. While these early basal deposits can affect vision, they are not directly visible in fundoscopy and thus may be overlooked in ophthalmoscopic evaluation. BlamD is composed of membrano-granular material and wide-spaced collagen which form between the plasma membrane and basal lamina of the RPE, and is the most prevalent histological hallmark in early AMD eyes. BlamD is also apparent in later, more advanced AMD, correlating with increasing RPE pigmentation abnormalities, and photoreceptor degeneration. BlinD consist of deposits located in the inner collagenous zone of Bruch’s membrane. These are composed mainly of membranous material, and are specific for early AMD. The accumulation of these basal deposits defines a threshold at which aging progresses to early AMD, which may otherwise present as a clinically normal fundus.

**Geographic atrophy**

Geographic atrophy, also known as ‘atrophic AMD’, is an advanced form of dry AMD characterised by widespread atrophy of the RPE and photoreceptors in the
macular region \(^{82, 97}\), with moderate to severe vision loss \(^{103, 104}\). It is associated with degeneration of the choroidal vasculature and reduced choroidal blood-flow \(^{105}\). Geographic atrophy occurs in the absence of neovascularisation and exudation (which are both features of ‘wet’ AMD), and associated with the presence of drusen of all types \(^{97}\). Over time, the area of atrophy widens \(^{103}\), with the progressive apoptosis of photoreceptors and RPE, and atrophy of the choroid at the edges of the lesion \(^{102, 106, 107}\). Rod photoreceptors are particularly vulnerable to degeneration in AMD \(^{108}\), and undergo apoptosis at the edges of the atrophic area, contributing to its expansion \(^{106}\).

Gradual enlargement of the atrophic lesion promotes a significant decline in vision function \(^{103}\). According to one report, geographic atrophy is responsible for approximately 20% of legal blindness resulting from AMD \(^{109}\). The pathogenic mechanisms underlying the progression of atrophic AMD are largely unknown, and no effective treatments are currently available to prevent its onset, or retard its advancement \(^{110}\). However, a recently published investigation has shown that in geographic atrophy, there is down-regulation of the RNA-processing enzyme DICER-1, and aberrant accumulation of the double-stranded RNA Alu \(^{111}\). Significantly, Alu accumulation is associated with stress in \textit{in vitro} models. These findings provide a key mechanistic insight into RPE cell death in geographic atrophy, although further work is needed to determine the relationship between \textit{Alu} and DICER-1 expression.

\textit{Neovascular AMD}

Choroidal neovascularisation (CNV) or ‘wet’ AMD, involves a breach of the outer BRB by the growth of new blood vessels into the retina from the underlying choroid \(^{77, 97}\). Because choroidal vessels are not ‘barrired’, like retinal vessels, this causes leakage – or exudation – of blood and serum into the retina, loss of photoreceptor function, and in the longer term reactive gliosis of Müller cells. While the emergence of CNV is found in only 10% of AMD cases, it accounts for approximately
90% of serious vision loss according to some studies. Neovascular AMD is grouped in two clinical forms, 'classical' and 'occult'. Classical neovascularisation involves the direct intrusion of blood vessels and exudate through Bruch’s membrane and RPE, and into the neural retina. In contrast, the occult form of neovascularisation occurs between the RPE and Bruch’s membrane, resulting subretinal oedema due to the loss of integrity in the RPE. End-stage CNV occurs as a result of recurrent exudation and proliferation of fibrovascular tissue, leading to formation of a ‘disciform’ scar and obliteration of RPE and photoreceptors in the macula.

A number of factors appear to govern the genesis of neovascularisation from the choroid. Developmentally, the RPE and photoreceptor population are dependent on nourishment from the choroidal vasculature. Stress at the AMD-affected macula, possibly arising from the restriction of nutrient flow by increasing deposits on Bruch’s membrane, may therefore generate neovascular signals giving rise to vessel growth from the choroid. Previous research has focused heavily on the modulation of angiogenic factors in neovascular AMD, particularly VEGF, a prominent stimulator of vascular permeability, endothelial growth, and angiogenesis. Early reports demonstrated that VEGF is present in surgically excised CNV membranes. Additionally, VEGF is constitutively expressed by RPE cells, and its expression is increased during CNV. As such, the blockade of VEGF using antagonists has been investigated for therapeutic benefit. Clinical studies using intravitreal injections of anti-VEGF antibodies such as Ranibizumab, or the anti-VEGF aptamer pegaptanib, have shown improved visual acuity in patients with neovascular AMD compared to controls, which together have ushered in a new era of therapeutic options for neovascular AMD.
Key risk factors

Epidemiological studies have identified a number of risk factors associated with the incidence of AMD, which shed light on its pathogenesis. The most consistent and well-established of these is advancing age along with family history of AMD (reviewed in [88]). Other risk factors include lifestyle choices, various systemic factors, environmental influences, and gene polymorphisms; the most prominent and relevant of these are reviewed below.

Lifestyle choices

Cigarette smoking is the most consistent lifestyle choice associated with AMD (reviewed in [129]). The first association of AMD with smoking was reported in 1992, in a small study that examined serum levels of anti-oxidants in individuals affected by AMD, compared with age-matched controls [130]. In that study, a moderate association was observed between cigarette smoking and the prevalence of AMD. The Beaver Dam eye study has been in progress for over 20 years, and shows that smoking is associated with the 10-year incidence of early AMD, including the occurrence of large soft drusen, and pigmentary abnormalities [131]. Epidemiological studies have also implicated smoking in advanced AMD such as neovascularisation and geographic atrophy [129]. Similar associations have been identified in the Australian population, in the Blue Mountains Eye Study [132].

In addition to the likely direct effects of higher levels of carbon dioxide and carbon monoxide in the blood of smokers, a number of metabolic factors are thought to play a pathogenic role in AMD [129]. These include reductions in serum antioxidant levels, macular carotenoid pigments, choroidal blood flow, RPE drug detoxification pathways [133-136] and inflammatory regulators such as complement factor H (CFH) [137].
Dietary intake is another modifiable factor associated with the incidence of AMD. Intake of certain polyunsaturated fatty acids such as Omega-3, particularly through the consumption of fish, has been found to confer protection against early and advanced AMD in a number of studies \(^{138-144}\). Consumption of various vitamin and mineral antioxidants has also been associated with the incidence of AMD. Carotenoids such as lutein and zeaxanthin, primarily obtained from green leafy vegetables, confer a reduced risk of AMD in some studies \(^{145-148}\). Intake of other antioxidants such as vitamins A, C, E, beta carotene, and zinc are also associated with a decreased risk of developing advanced AMD \(^{149, 150}\), although there are a number of contradictory reports \(^{146, 151, 152}\).

The protective effect of various antioxidants in AMD indicates that oxidative damage may play a role in the degenerative process \(^{153, 154}\). The high concentration of oxygen and abundance of the polyunsaturated lipids in photoreceptors, such as docosahexanic acid (DHA), leaves them particularly susceptible to oxidative damage \(^{153}\). This association has been further strengthened by recent findings implicating carboxyethylpyrrole (CEP) – an oxidation fragment of DHA and biomarker of AMD \(^{155-157}\) – in the emergence of AMD histopathology \(^{158}\).

**Systemic Factors**

A range of cardiovascular risk factors are thought to be associated with the pathogenesis of AMD \(^{159, 160}\). Several studies show a link between hypertension and AMD (reviewed in \(^{129}\)). Atherosclerosis, which results in a thickening of arterial walls from accumulations of fatty deposits such as cholesterol, has been shown to increase the risk of AMD, albeit contentiously (reviewed in \(^{1, 88}\)). The composition of atherosclerotic deposits is similar to that of drusen \(^{161}\). Atherosclerosis of the choroidal circulation and deposition of lipids on Bruch’s membrane may increase the risk of AMD \(^{160}\). Furthermore, a history of stroke and other cardiovascular diseases such as myocardial
infarction, angina, and coronary heart disease have been associated with AMD in some studies 162-166.

**Environmental Factors**

Exposure to sunlight has been associated with the incidence of AMD in a number of studies 167-173, and denied in an approximately equal number 174-176. These differences most likely stem from difficulties in accurately assessing a lifetime of light-exposure retrospectively, through questionnaires 177. Using an objective means to gauge light exposure in contrast, an investigation by Hirakawa and colleagues demonstrated a link between increased exposure to sunlight and development of AMD 177. As light damage has been shown to arise from oxidative stress through the rhodopsin-mediated peroxidation of lipids such as DHA 178-180, the link between increased sunlight with incidence of AMD may reflect a role of oxidative damage 153, 154, which is also inferred by the protective effect of antioxidants (as discussed in section 1.3.2.1 – Lifestyle choices).

**Genetic background**

AMD is a multigenic disease, in that a number of genes are reported in association studies to play a role in the pathogenesis (reviewed in 4). Early reports identified a number of AMD-associated genes from diverse gene families such as fibulin 5 (FBLN5) 181 and apolipoprotein E (APOE) 182. Most of these analyses were restricted to particular gene families. More recent investigations have utilised rigorous wide-spread screening for sequence variations in short nucleotide polymorphisms (SNPs) 4. In particular, a genome-wide analysis of SNPs has revealed a highly significant association between AMD and a particular polymorphism (Y402H) in CFH 183, supported by other studies 184-186. Together, CFH and other complement-related gene associations have provided strong support for the role of inflammation –
particularly innate immune pathways – in the pathogenesis of AMD. A more detailed analysis of the findings that associate the complement system with AMD is provided below (1.4.3 – The complement system).

**Inflammation in the pathogenesis of AMD**

Evidence of the significance of immune processes in AMD, including infiltration of inflammatory cells and presence of retinal autoantibodies, has been accruing for some time. The recent discovery of direct involvement of the complement system has firmly placed inflammatory processes as the key factors influencing onset and progression of AMD.

**Autoantibodies**

AMD has been associated with the presence of anti-retinal autoantibodies, observed in the sera extracted from patients with AMD. Various staining specificities of these autoantibodies have been characterised, including photoreceptor outer segments (OS) and astrocytes. The presence of autoantibodies likely reflects a breakdown of the BRB, which in turn exposes retinal ‘self’ antigens. While such findings allude to a role of autoimmune mechanisms in the pathogenesis of AMD, it is unclear whether autoantibodies play a decisive role in AMD, or are merely a secondary effect of BRB breakdown.

**Recruitment of inflammatory cells**

The involvement of inflammatory processes in the histopathology of AMD was first noted almost 100 years ago, and several histological studies since have established the presence of aggregations of choroidal leukocyte infiltrates in association
with disciform macular lesions. Those observations were confirmed and extended in a number of electron microscopical investigations which demonstrated the involvement of a number of inflammatory cells – including macrophages, lymphocytes, and mast cells – in RPE atrophy, and breakdown of Bruch’s membrane. Macrophages and other leukocytes have also been described in excised neovascular membranes. Ultrastructural studies also identified a close relationship between macrophages and the formation of choroidal neovascular membranes in wet AMD.

Multinucleated giant cells – which may form through union of multiple macrophages or microglia – have also been found to correlate spatially with regions of breakdown in Bruch’s membrane and with CNV.

Chronic involvement of macrophages and giant cells has also been shown in atrophic AMD lesions, and on the expanding edges. Other investigations have shown changes in parenchymal microglia in association with early AMD, including increased MHC-II expression and morphological changes suggestive of activation. In advanced AMD, activated ameboid microglia infiltrate the ONL and subretinal space in the degenerating outer retina, where they are associated with neovascular structures, and appear to have a role in the phagocytosis of photoreceptor debris.

Investigations using experimental models have recently demonstrated a direct role for inflammatory cells in the development of AMD-like lesions. These experiments showed that in mice subjected to experimental laser-induced CNV, the use of clodronate liposomes to induce generalised macrophage depletion resulted in a reduction in the size of the lesion, compared to controls. Furthermore, the clodronate-treated mouse retinas showed lower levels of VEGF protein, suggesting that macrophages may induce choroidal neovascularisation by expressing angiogenic factors. Consistent with this, macrophages isolated from retinas following experimental CNV have been found to exhibit angiogenic activity in a dorsal air sac assay in vitro. The findings from
experiments using clodronate liposomes have, however, been met with some controversy, since the liposomes can be taken up by neovascular endothelial cells, possibly inducing side-effects that are difficult to control. Nonetheless, the findings confirm the view that macrophages actively induce neovascularization, and their presence is suggestive of active degenerative processes.

**Role of chemokines**

First discovered in 1987, chemokines are a large, growing family comprising more than 50 molecules interacting with at least 20 chemokine receptors, that play an important role in the chemotactic guidance of leukocyte migration and activation. Chemokines are small molecules grouped according to the relative position of their first N-terminal cysteine residues, comprising C (γ chemokines), CC (β chemokines), CXC (α chemokines), and CX3C (δ chemokines) families.

These may be expressed by endothelial cells, resident macrophages (including microglia), as well as infiltrating leukocytes. Chemokines exert their biological activity through binding cell surface chemokine receptors, which are part of the superfamily of seven transmembrane domain receptors that signal through coupled heterotrimeric G-proteins, consisting of C, CC, CXC, CX3C receptor subclasses. Many of these receptors show a degree of redundancy, as multiple chemokines may bind several receptors; although interactions are mainly restricted to within particular subclasses. Chemokine expression typically generates chemical ligand gradients, which serve as directional cues for guidance of leukocytes bearing the appropriate chemokine receptors to sites of injury, and are also thought to aid in extravasation of leukocytes.

Chemokine signalling is believed to play a role in mediating leukocyte migration in several major CNS disorders. Increased expression of both α and β chemokines have been characterised in multiple sclerosis, Alzheimer’s disease, and
brain ischemia and trauma (reviewed in 207,212-214). The expression of chemokines in the guidance and activation of macrophages has also garnered considerable interest in AMD 215. While direct observations of chemokine expression in various forms of human AMD are lacking 215, studies in experimental CNV have indicated a key of chemokine signalling in AMD.

Investigations using laser-induced CNV in mice have alluded to an important role of β chemokine signalling in neovascular AMD. Ablation of Ccl2 – a potent chemoattractant for monocytes 216, 217 – using target gene knockout inhibits the infiltration of macrophages and results in reduced lesion size following laser-induced CNV compared to controls 74. Increased expression of Ccl2 is also implicated in a number of other CNS pathologies 207,214 including Alzheimer’s disease 218,219, multiple sclerosis 220,221, and brain trauma 222,223. Moreover, a mouse knockout of chemokine (C-C motif) receptor 2 (Ccr2), a receptor for Ccl2 224, exhibits decreased macrophage recruitment and vastly reduced neovascularisation following experimental laser-induced CNV 75. Conversely, other studies suggest that a degree of β chemokine signalling is necessary for the maintenance retinal homeostasis, and prevention of AMD. An investigation in aged, dual Ccl2/Ccr2 knockout mice showed retinal features similar to AMD including formation of lipofuscin, drusen, photoreceptor degeneration, and neovascularisation 225, although one study calls into question the AMD-like phenotype in this model 74. Ccl2/Ccr2 knockout results in the accumulation of hypertrophied subretinal macrophages, possibly because of impaired monocyte trafficking 74.

The only δ chemokine receptor characterised, chemokine (C-X3-C motif) receptor 1 (CX3CR1), has also been implicated in maintenance of homeostasis and genesis of AMD-like pathology. CX3CR1 is a chemokine receptor found on microglia, macrophages, astrocytes, and T-cells 215, whose ligand chemokine (C-X3-C motif) ligand 1 (CX3CL1) is constitutively expressed on many cell types in the retina, and
together are thought to mediate the trafficking of microglia and macrophages in the clearance of extracellular deposits. Targeted knockout of CX3CR1 in mice induces progressive degeneration of photoreceptors in correlation with an accumulation of engorged subretinal microglia/macrophages. Moreover, ablation of CXCL1 is associated with an increase in lesion size following experimental neovascularisation. Dual Ccl2/CX3CR1 knockout mice have also been designed. These show AMD-like features such as thickening of Bruch’s membrane, RPE and photoreceptor atrophy, and CNV in some mice, which develops more rapidly than in either Ccl2/Ccr2 or CX3CR1 knockouts. As with the other knockouts, Ccl2/CX3CR1 show increased microglial and macrophage accumulation.

The complement system

The complement system is a component of the innate immune response providing a rapid host defence against a range of immunological challenges, and aiding in the maintenance of homeostasis. The complement system is mediated through a cascade of proteolytic cleavages that generate phagocytosis-enhancing opsonins, chemotactic anaphylatoxins, and membrane-attack complexes, which ‘complements’ the host in a range of immunological functions. These include the initiation of defence against infectious pathogens, and the removal of potentially noxious substances such as extracellular debris, immune complexes, and apoptotic cells.

Pathways and regulation

Three distinct activating pathways – classical, lectin, and alternative – mediate the complement cascade, converging on the enzymatic cleavage of complement component 3 (C3) to generate the opsonin C3b, deposited on activating surfaces, and the pro-inflammatory anaphylatoxin C3a (Figure 3A). This cleavage is mediated chiefly...
A: Complement activation pathways

B: Complement propagation on activating surfaces

Figure 3 Overview of complement activation pathways. A: Complement activation may involve three distinct pathways – classical, mannose binding lectin (MBL), and alternative – which converge in the proteolytic cleavage of complement component (C3). B: Complement may be facilitated on various activating surfaces, mainly through the lectin and classical pathways, which then mediate the sequential assembly of the C3 convertase. The alternative pathway, in contrast, may be activated in the absence of a stimulus, to form an alternative C3 convertase. Prolonged activation of complement leading to the assembly of the membrane attack complex (MAC) on cell surfaces, which may trigger lysis of the target cell. Complement fragments produced through complement activation trigger chemotaxis and phagocytosis by phagocytic cells. Figure 1A and 1B adapted from Janeway et al. 2005 and Lambris et al. 2008 respectively. 251,252
by C3-convertases, which are assembled from complement constituents on activating surfaces (Figure 3B). The classical pathway promotes complement activation on pathogen surfaces \(^{236, 244}\), extracellular deposits \(^{236, 245}\), and apoptotic cells \(^{238, 240-243}\) predominately through binding of the pattern recognition molecule complement component 1, q subcomponent (C1q). The lectin pathway facilitates complement deposition on pathogen surfaces through the recognition of sugar moieties, utilising mannose-binding lectin (MBL) \(^{246}\), as well as on apoptotic cells \(^{247-249}\). In contrast to those pathways, the alternative pathway, may be activated at a low level in the absence of a stimulus through the spontaneous hydrolysis of C3 – known as the ‘tickover’ mechanism \(^{250}\) – and may greatly amplify the generation of complement fragments initiated by the other complement pathways. Prolonged activation of complement may also lead to sequential assembly of the membrane attack complex (MAC) though the initial cleavage of complement component 5 (C5) by the C5 convertase, promoting cytotoxicity and cytolysis of target cells \(^{253}\).

While controlled, low-level activation of the complement cascade is required for the efficient clearance of noxious substances, its powerful pro-inflammatory action also has the potential to exacerbate injury if activated in an excessive or inappropriate manner. This necessitates a balance between activation and inhibition \(^{234, 254}\), which is maintained through a suite of soluble and membrane-bound regulators that act upon various components of the cascade \(^{254}\). These regulators limit complement activation in serum and on host tissue, acting either through accelerating the decay of the C3 convertase or by promoting the degradation of opsonin C3b and C4b. C4 binding protein (C4bp), and CD46 for example, mediate decay of the C3 convertase by binding C4b and presenting it in a cleavable form to complement factor I (CFI), which cleaves and thus degrades C3b and C4b \(^{254}\). Others such as vitronectin inhibit the assembly of alternative pathway constituents such as MAC \(^{254}\). An important and well-characterised
inhibitor, CFH, is a major inhibitor of the alternative pathway, promoting the decay of the alternative pathway C3-convertase while acting as a cofactor for the CFI-mediated degradation of C3b\textsuperscript{254,255}. CFH may regulate complement activation in both fluid-phase and host cell surfaces\textsuperscript{255}.

**Role of complement in disease**

Complement activation and resulting inflammation have been characterised in dystrophies such as Pick’s disease, Huntington’s disease, and Alzheimer’s disease (reviewed in\textsuperscript{234}). Inhibition of C3 in experimental models has directly implicated complement activity in the severity of post-ischemic cerebral injury\textsuperscript{256}, and intracerebral haemorrhage\textsuperscript{257,258}. Moreover, an *in vitro* study using neuronal cell cultures suggests that neurons are deficient in a number of complement regulatory factors, and therefore particularly susceptible to spontaneous activation of complement and formation of MAC, resulting in destruction of affected cells\textsuperscript{259}.

Compelling evidence from gene association studies, serological investigations, and histological observations, suggests a role for complement activation in AMD. The association of the Y402H sequence variant of the regulatory gene CFH with incidence of AMD\textsuperscript{183-186} suggests that a failure to regulate complement activity in the retina is a causative factor in AMD. The Y402H polymorphism confers a lower binding efficiency of CFH to two important ligands – C-reactive protein (CRP) and heparin – and reduced adherence to RPE cells\textsuperscript{260}. These deficiencies are thought to impair the inhibitory function of CFH, resulting in a failure to attenuate complement activation\textsuperscript{260}. Mice deficient in CFH have decreased visual function, subretinal deposits, and accumulations of C3 on RPE, outer segments, and retinal vessels\textsuperscript{261}. In contrast, other variants of CFH have been identified that confer protection against AMD\textsuperscript{262,263}. More recent gene association studies have now characterised other susceptibility variants in complement
pathway genes, including complement component (C2) \(^3, 264, 265\), complement factor B (CFB) \(^3, 264, 265\), and C3 \(^266-270\).

Chronic complement activation in AMD has been shown in histological studies of human post-mortem eyes. The presence of complement constituents - including complement proteins, MAC constituents, and regulatory proteins - in drusen has been shown in a number of studies (reviewed in \(^3, 4, 95\)), and complement constituents have been detected in both basal linear, and basal laminar deposits \(^271-273\). Serological investigations also show an association between increased plasma levels of the complement-related cleavage fragments Bb, C5a, \(^274\) as well as CRP \(^275\) and advanced AMD.

Although a role for complement in the pathogenesis of AMD has been firmly established, some key questions remain unanswered. In particular, identification of the local cellular events leading to complement activation in AMD, and why the macula is the target of these processes, both remain unclear. The role of complement in the manifestation of advanced forms of AMD such as geographic atrophy is also poorly understood, but it is hypothesised that genetic susceptibility to complement dysfunction and environmental factors (such as aging) synergise, causing pathological changes in the RPE and choroid. It is suggested that these changes may promote chronic, local inflammation, leading to alterations in Bruch’s membrane, accumulation of drusen and basal deposits, and eventually the loss photoreceptors \(^3\). Whilst plausible, this proposed mechanism still does not explain why the macula is targeted.

Experimental models offer some insight into the role of complement in AMD, particularly in the neovascular form of the disease. For example, it has been shown that inhibition of complement C3a and C5a anaphylatoxin signalling, through the ablation of complement receptors complement component 3a receptor 1 (C3aR1) and complement
component 5a receptor 1 (C5r1), reduces recruitment of macrophages and neutrophils, VEGF expression, and lesion size following experimental laser-induced CNV \(^{276}\). Rohrer and colleagues have demonstrated that in experimental CNV, the pathogenic involvement of complement requires activation of the alternative pathway, which can be attenuated by treatment with a recombinant form of CFH \(^{277,278}\). Furthermore, it has been shown that in mice immunised with the AMD biomarker CEP \(^{155-157}\) there is copious deposition of C3 on Bruch’s membrane, associated with AMD-like retinal degeneration. It has been suggested that these latter findings indicate that oxidative damage to photoreceptors may induce degeneration through the propagation of complement \(^{158}\).

**Animal models of AMD**

An ideal experimental model of AMD would replicate the full gamut of AMD-related features including the progression of atrophic degeneration in the central retina, and the development of CNV in some animals \(^{279}\). At present, however, there are no animal models that exhibit all the features of clinical AMD \(^1\). This is probably due to the etiological complexity of the disease, and the slow emergence of the pathology, the timecourse of which greatly exceeds the lifespan of experimental animals. Thus, animal models of AMD replicate only one, or at most a few of the clinical features of the disease \(^{279}\). In addition, it is uncertain which of the disease features is an essential component of the degenerative process, and which are secondary factors.

Despite these difficulties, experimental models that mimic specific aspects of AMD have shed some light on the nature of the degenerative process in AMD \(^1\). Experimental CNV induced by laser photocoagulation of the RPE is the best-known and
widely used model to examine the pathogenic mechanisms and test potential therapeutic interventions in AMD. Other models that simulate features of dry AMD have utilised a multitude of transgenic and knockout mice which target genes thought to be involved in AMD (reviewed in ). These include gene knockouts targeting oxidative stress pathways such as mice deficient in Cu, Zn superoxide dismutase (SOD1), a major antioxidant enzyme, and inflammatory pathways in CFH/−, Ccl2/−/Ccr2/−, and CX3CR1/− or Ccl2/−/CX3CR1/− mice. Another prospective model of dry AMD utilises mice immunised with the AMD-biomarker CEP. A major limitation of these dry AMD models, however, is the absence of a macula in rodents. Thus, these models fail to effectively mimic the site-specific nature of AMD.

Light damage-induced retinal degeneration: a model of atrophic AMD?

The capacity for exposure to bright light to induce retinal degeneration has been known for over 40 years. In 1966, a seminal study by Noell demonstrated that exposure to light of a sufficient intensity and duration induces degeneration of photoreceptors and the RPE, resulting in severe vision loss in rats. Since this initial discovery, a vast amount of literature has been produced regarding the histological and functional implications of exposure to bright light on the retina (reviewed in ). It has been established that in these light damage models, photoreceptors die by apoptosis through a mechanism thought to involve oxidative damage in the photoreceptor outer segments, resulting from excessive bleaching of the rhodopsin chromophore. Secondary effects to this process involve atrophy of RPE, and dysfunction of the BRB. Light damage in albino rodents has become a well-established model in the study of retinal degeneration because it affords high reproducibility, rapid induction, ease of use, and flexibility by altering the duration and intensity of light (reviewed in ).
Several lines of evidence have suggested that the light damage model may serve as a useful model of dry AMD. In albino rats exposed to light damage, the region of retina temporo-superior to the optic disc is particularly susceptible to photoreceptor cell death. It has also been noted that degeneration of photoreceptors and RPE cells, and associated changes to the choroid in this focal region, mimics some of the histopathological aspects of dry AMD.

Although the rat retina lacks a macula and its key specialization the *fovea centralis*, it includes a feature that is homologous to the fovea – the *area centralis*, in the superiotemporal portion of the retina. The *area centralis* is a retinal specialization found predominantly in predatory and crepuscular mammals, and originally defined by a peak in ganglion cell density. More recent reports have since indicated other macula-like features in the rat *area centralis*, including longer rod outer segments than elsewhere in the retina, and a peak density of cone photoreceptors. The spatial coincidence of this specialized region with the region of cell loss in the light-damage model of retinal degeneration indicates that this is a suitable model to investigate site-specific onset of retinal degeneration, and its subsequent progression.

Mechanistically, both light-induced degeneration and AMD are thought to involve oxidative damage to photoreceptors. Additionally, evidence has recently emerged for a role of complement activation in light damage, where ablation of the complement alternative pathway gene complement factor D (CFD) attenuates photoreceptor death. Consequently, light damage in albino rats may prove a useful model to characterise the local spatiotemporal emergence of inflammatory mediators such as complement, in the relation to focal degeneration at the *area centralis*.
Summary and Aims

AMD is a complex, multifactorial disease, influenced by the interaction of a range of genetic and environmental factors. While inflammation has been associated with AMD for many years, recent investigations utilising advanced techniques such as genome-wide SNP analysis and targeted gene knockouts have firmly established inflammatory processes as mediators of AMD, including macrophage recruitment and activation of complement. Although effective treatment exists for neovascular AMD with the advent of anti-VEGF therapy, none are currently available for dry forms of AMD such as geographic atrophy. Elucidating the spatiotemporal cellular events leading to local complement activation and inflammatory cell recruitment, particularly in geographic atrophy, may allow for the development of effective measures to inhibit inflammation in AMD. Light damage in albino rats mimics a number of features characterised in atrophic AMD, particularly site-specific AMD-like degeneration in the area centralis, and involvement of the complement system in the degenerative process.

In the studies reported in this thesis, I aimed to assess the suitability of light damage in albino rats as a model of progressive atrophic degeneration in advanced AMD (Chapter 2), and to investigate the spatiotemporal emergence of inflammatory processes in the degenerating retina (Chapters 3 and 4). These investigations encompassed the characterisation of macrophage recruitment in the retina following light damage and their relation to chemotactic signalling pathways (Chapter 3), and the spatiotemporal profile of complement gene expression utilising microarray analysis (Chapter 4).
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Chapter Two

Brief exposure to damaging light causes focal recruitment of macrophages, and long-term destabilization of photoreceptors in the albino rat retina

This chapter is presented as shown in the publication:

Brief exposure to damaging light causes focal recruitment of macrophages, and long-term destabilization of photoreceptors in the albino rat retina

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Abstract

Purpose: To characterize the long-term spatiotemporal features of light-mediated retinal degeneration. Methods: Sprague Dawley rats were exposed to 1000 lx for 24hrs, then kept in dim light (5 lx), for up to 56 days. Animals were euthanized at 0, 3, 7, 28, and 56 days post-exposure, and retinas prepared for immunohistochemistry. Outer nuclear layer (ONL) thickness and TUNEL labelling was used to quantify photoreceptor death. Antibodies to opsins, glial fibrillary acidic protein (GFAP), fibroblast growth factor (FGF) 2 and ED1 were used to assess the retina. Results: At 0 days post-exposure we detected photoreceptor death 2 mm superior to the optic disc (the 'hotspot'), and ED1 positive macrophages in the retinal vasculature and underlying choroid. By 3 days, the ONL was thinner and there was gliosis in the outer retina, where ED1 positive macrophages were also present. Few ED1 positive cells remained at 28 days. At 56 days, there were TUNEL positive nuclei in the penumbra, and increased FGF-2, and GFAP expression by Müller cells (MC). In inferior retina outer segment length was initially reduced, but recovered to near-normal by 28 days. Conclusions: Short exposure to damaging light destabilizes the retina adjacent to a hotspot of degeneration, so that the damaged region expands in size over time. Recruitment of macrophages is associated with the early phase of damage, but not with the longer term photoreceptor loss in the penumbra. Features of the focal and progressive retinal damage in this model are reminiscent of the progression of age-related macular degeneration (AMD).
Introduction

Exposure to light of a sufficient intensity and duration has been shown to cause damage in the outer retina, particularly in the photoreceptor population\(^1\). In these models photoreceptors die by apoptosis\(^2,3\) primarily through a mechanism thought to involve oxidative damage\(^4,5\). In rat models of light-induced photoreceptor degeneration\(^6\), the region of retina superior to the optic disc is particularly susceptible to light-induced photoreceptor cell death\(^7,9\), the main area of damage being localized predominately to the temporal side\(^10,11\). The spatial coincidence of this highly sensitive region with a local area where rod outer segments (OS) are longer\(^12,14\) and ganglion cell density is higher\(^15,16\) suggests that the sensitive area is at the area centralis\(^14\).

There is extensive literature describing the histopathology associated with the rodent light-damage model, including photoreceptor cell death and dysfunction, and associated degeneration of the blood-retinal barrier (BRB)\(^1,17-21\). However, the systematic progression and localisation of retinal changes that follow a single photic insult, and its implications on the longer-term stability of the surrounding retina have not been well characterised. In this study, we show that a relatively short exposure to bright continuous light (BCL) invokes localised, rapid changes in the outer retina of albino rats such that the initial focal damage leads to long-term destabilisation of photoreceptors in the adjacent retina, so that the damaged region increases in size, over time. We suggest that the focal damage in the visual centre of the albino rat retina is reminiscent of the histopathology of age-related macular degeneration (AMD).
Methods

Animals and light exposure

All experiments conducted were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Albino Sprague-Dawley (SD) rats were born and reared in dim cyclic light conditions (12 hours light, 12 hours dark) with an ambient light level of approximately 5 lux. Exposure to BCL was conducted on animals aged between post-natal days (P) 90-150. Prior to BCL exposure, rats were dark adapted for a minimum of 15 hours then transferred to individual cages designed to allow light to enter unimpeded. There were no areas of shadow in the cages; pupillary dilation was not performed. BCL exposure commenced consistently at 9:00 am, and was achieved using a cold-white fluorescent light source positioned above the cages (18W, Cool White; TFC), at an intensity of approximately 1000 lux at the cage floor. BCL exposure was maintained over a period of 24 hours, after which time the animals were immediately returned to dim cyclic conditions for the post-exposure period. Animals were kept in dim light conditions following BCL exposure for a maximum period of 56 days. Retinal tissue was analysed from animals at the end of 24 hrs exposure and at 3, 7, 28, and 56 days. Age-matched, dim-reared animals served as controls.

Tissue collection and processing

Animals were euthanized by overdose of barbiturate administered by an intraperitoneal injection (60 mg/kg bodyweight, Valabarb; Virbac NSW, Australia). The left eye from each animal was marked at the superior surface for orientation then enucleated and processed for cryosectioning. Eyes were immediately immersion fixed in 4% paraformaldehyde in 0.1M PBS (pH 7.3) for 3 hours at room temperature, then washed
in 0.1M PBS before being left in a 15% sucrose solution overnight for cryoprotection. Eyes were oriented and embedded in O.C.T. compound (Tissue-Tek) then snap frozen in liquid nitrogen and cryosectioned at 16 μm. The sections were mounted on gelatin / poly-L-lysine-coated glass slides coated and dried overnight at 50°C. Sections were stained for histological examination using Haemotoxylin and Eosin (H&E), or labeled using either immunohistochemistry or TUNEL.

The right eye from a minimum of 3 animals at each time-point was fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for at least 6 hours at room temperature and embedded for paraffin sectioning at 7 μm using a microtome. Sections were stained with cresyl violet and used to quantify photoreceptor OS length.

Analysis of cell death

TUNEL staining was used to quantify photoreceptor apoptosis following BCL, in frozen sections using a protocol published previously. Counts of TUNEL positive cells in the outer nuclear layer (ONL) were carried out along the full-length of retinal sections cut in the para-sagittal plane (superio-inferior), including the optic disc, in adjacent fields measuring 1000 x 1000 μm. The final count from each animal is the average at comparable locations in 2 non-sequential sections. Statistical analysis was performed using a two-tailed Student t-test. Differences with a P value < 0.05 were considered statistically significant. Thickness of the ONL in these sections was also measured in 1 mm intervals on the same sections. The DNA-specific dye Bisbenzamide (Calbiochem, La Jolla, CA) was used to visualize the cellular layers. ONL thickness was calculated as the ratio of ONL thickness to the distance between the outer- and inner-limiting membranes (OLM-ILM), to take into account any
obliquely cut sections or regions. Statistical analysis was performed using a two-tailed Student’s t-test.

**Outer segment measurements**

Analysis of photoreceptor OS length was performed on paraffin-embedded sections stained with cresyl violet. For each eye, images of 4 specific regions in the inferior and far-superior retina were acquired with an Axiocam MRc5 digital camera (Zeiss). From each of these images, 4 equi-spaced measurements of OS length were performed using AxioVision software (Version 4.2; Zeiss), which were averaged for each group of animals. Statistical analysis was performed using a two-tailed Student’s t-test.

**Immunohistochemistry**

Sections adjacent to those used for TUNEL analysis were used for immunohistochemistry. Details for the antibodies used are displayed in Table 1. Sections were incubated in 10% normal goat serum (Sigma, St. Louis, MO) for 1 hr to block non-specific binding, before incubation in the primary antibody overnight at 4°C. Sections were washed in 0.1M PBS, then incubated in the appropriate secondary antibody overnight at 4°C (1:1000 anti-mouse IgG-alexa 594 or anti-rabbit IgG-alexa 488; Molecular Probes, Eugene, OR). Sections were washed in 0.1M PBS then incubated in Bisbenzamide (1:1000) for 2 minutes and coverslipped in a glycerol/gelatin mixture. Primary antibodies were omitted to control for non-specific binding of the secondary antibody. Immunofluorescence was viewed using a Zeiss laser scanning microscope, and acquired using PASCAL software (Zeiss, v4.0). Images were enhanced for publication using Adobe Photoshop software, which was standardised between images.
### Table 1. Antibodies used:

<table>
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<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>rabbit α L/M Opsin</td>
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<td>Chemicon, Temecula, CA</td>
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<tr>
<td>mouse α Rhodopsin (4D1)</td>
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<td>rabbit α GFAP</td>
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<td>Dako, Carpinteria, CA</td>
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<td>Upstate, Temecula, CA</td>
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<td>1:200</td>
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<td>mouse α Glutamine Synthetase</td>
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Results

The early phase post-exposure: Emergence of a ‘hotspot’ of degeneration

Following exposure to 24 hours BCL an increase in apoptotic profiles was evident along the vertical meridian of the outer nuclear layer (ONL) (Figure 1A), consistent with previous findings. The distribution of dying cells was not uniform along the retina. Rather, a circumscribed area approximately 2 mm supero-temporal to the optic disc was observed where TUNEL-positive (+) nuclei were concentrated. This ‘hotspot’ appeared in the same location in all retinas examined, immediately following BCL. After 3 and 7 days post-exposure, the number of TUNEL+ nuclei had decreased substantially, but of those TUNEL+ cells detected, most were located in the hotspot or the immediately surrounding area.

Evidence of the cumulative impact of cell death on the photoreceptor population was demonstrated by differential changes in ONL thickness in the hotspot compared with other locations (Figure 1C). ONL thickness within the hotspot decreased rapidly so that by 3 days post-exposure, the ONL was 49.3% thinner than in controls (P <0.05) at the centre of the hotspot (2 mm eccentricity from the optic nerve). There was no further reduction in ONL thickness within the hotspot by 7 days post-exposure (51.7%; P >0.05), consistent with the slowing of photoreceptor death over the course of the post-exposure period.

The structural and immunohistochemical changes that occur following light damage, at each time point, are shown in Figures 2 and 3. At 24 hours post-exposure only minor perturbations in retinal structure are evident compared with controls (Fig 2A), including evidence of minor disorganization in the inner portion of the ONL.
Figure 1 Distribution of photoreceptor cell death in the ONL following exposure to BCL. **A**: TUNEL+ cell counts over the length of the retina immediately following BCL reveal a dense localization of TUNEL+ nuclei to a hotspot in the superior retina approximately 2mm from the optic nerve. The number of TUNEL+ cells diminishes following 3 days post-exposure, but remains persistent at a low level within the hotspot after 7 days. (Control n = 6; 0 days n = 6; 3 days n = 6; 7 days n = 6; error bars represent SEM). **B**: Chronic appearance of TUNEL+ nuclei is shown to cluster within and around the hotspot from 7 days post-exposure onward. (7 Days n = 6; 28 Days n = 5; 56 Days n = 6; error bars represent SEM). **C**: ONL thickness measurements across the retina illustrate an emerging depression in ONL thickness developing rapidly after 3 days post-exposure. (Control n = 6; 0 days n = 6; 3 days n = 8; 7 days n = 9; 28 Days n = 4; 56 Days n = 5; error bars represent SEM.)
(Figure 2B; black arrow). At 3 and 7 days post-exposure, however, there are pronounced signs of stress and degeneration evident at the hotspot (Figure 2C, D; 3B). Thinning of the ONL at 3 days post-exposure is accompanied by accumulation of acellular debris in the layers that normally comprise photoreceptor OS (Fig 2C and D). Surviving photoreceptors in the hotspot showed profound structural abnormalities from 3 days post-exposure, culminating in a virtual absence of rod and cone OS by 7 days post-exposure (Figure 3A-B). This loss of photoreceptor OS coincided with a redistribution of opsins into the soma and axon terminal by 7 days (Figure 3E-G), highlighting structural abnormalities in the soma and axon.

We also observed interruption of the RPE monolayer by 3 days post-exposure (Figure 2C; black arrowhead), associated with debris-like deposits (Figure 2C; asterisks) and discontinuities in Bruch’s membrane (Figure 2C; white arrow). Such disruption was also associated with an infiltration of large cells into the subretinal space (Figure 2D; black arrows). By 7 days post-exposure (Figure 2E) the subretinal space was indistinct, so that the remains of the ONL abutted the choroid.

The emergence of major pathological features in the hotspot 3 days following BCL coincided with the formation of a ‘stress margin’, or ‘penumbra’, around the degenerating hotspot (Figure 2F). In the penumbra there were pockets of hotspot-like degeneration (Figure 2F, black arrows) interspersed with regions of relatively normal appearance, where photoreceptor OS were still present and giving rise to rosette-like formations in the outer retina. Beyond the penumbra, while there was pronounced thinning of the ONL, while the retina and Bruch’s membrane appeared to be structurally intact (Figure 2G).
Figure 2 Histological examination using H&E staining of the emerging pathology of the hotspot during 0 to 7 days post-BCL exposure. A: Retinal section of a dim-reared young-adult rat from the superior mid-periphery. B: Section from the hotspot of TUNEL+ cell death immediately after 24hrs BCL, showing a slight destabilization of the inner portion of the ONL (arrow). C: After 3 days recovery a vast disruption in the integrity of the outer retina is depicted, including gross disorganization of the ONL (black arrows), and accumulations of debris (asterisks). The RPE is disrupted amongst clumps of debris (black arrowhead), and Bruch’s membrane appeared discontinuous in parts (white arrow). D: High magnification of a section of the hotspot after 3 days post-exposure reveal large nuclei (arrows) within the subretinal space and ONL in addition to considerable debris (white asterisk). E: Sections from 7 days post-exposure depict the progressive formation of the retinal lesion with the collapse of ONL into the subretinal space, with pockets of debris still apparent. F: Intermediate manifestations of retinal disruption towards the edge of the hotspot after 7 days post-exposure. Rosette structures within the ONL are formed as isolated regions collapse into the OS layer (arrows). G: A region from the inferior mid-periphery 7-day post-exposure representing the maintenance of structural integrity outside the hotspot. BM, Bruch’s membrane; C, choroidal vasculature; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments; S, subretinal space. Scale bars represent 50μm (A-C, E-G), 20 μm (D).
Figure 3 Changes in photoreceptor morphology within the hotspot following exposure to BCL. Sections are immunolabelled with L/M opsin (green) and Rhodopsin (red) in A-D. E-G depicts high gain and magnification of photoreceptor cell bodies, illustrating BCL-induced changes in L/M opsin (green) distribution beyond the OS. A: Photoreceptors from a dim reared animal showing slender, organized OS’s. B: Retinal section of the hotspot after 3 days recovery showing extreme reduction of OS’s in both rods and cones to small disorganized fragments dotting the OS layer. C: 7 days post-exposure section revealing OS’s nearly absent amongst photoreceptors in the hotspot, with immunoreactivity for L/M opsin and rhodopsin becoming redistributed to within the somas of the photoreceptors (arrow). D: Shortening of OS’s is shown in the inferior retina after 7 days, however structural integrity is maintained. E-G: L/M opsin immunoreactivity in the hotspot after 3- and 7-days post-exposure respectively; (F-G) shows an increased distribution of photopigment from the OS to soma and axon terminals in some photoreceptors (arrows) in contrast to controls (E). The redistribution also reveals the distorted structure of surviving photoreceptors. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments. Scale bars represent 50 μm (A-D), and 20μm (E-G).
Localized changes in Müller cells (MC) were also evident following BCL (Figure 4). Changes in immunoreactivity for GFAP were apparent within inner MC processes in the hotspot immediately following BCL exposure (Figure 4B; arrow), in contrast to typical expression predominately within the astrocytes (Figure 4A). By 3 days post-exposure, and at 7 days post-exposure, GFAP immunoreactivity was intense in the inner and outer MC processes (Figure 4C-D; white arrowheads). This extensive labelling coincided with profound structural rearrangement suggestive of reactive gliosis within the hotspot (Figure 4 H-I). Labelling of MCs using anti-glutamine synthetase – a known MC marker\(^{23}\) – showed hypertrophied MC processes in the remnant ONL at 3 and 7 days post-exposure (Figure 4H, I; white asterisks), which infiltrated the sub-retinal space to form a mass of processes (white arrows), suggestive of a fibroglial scar. Despite sustaining substantial visual cell loss, regions of retina in the inferior showed intact MC morphology and OLM integrity at 7 days post- BCL exposure (Figure 4J), with GFAP expression also comparatively attenuated in the outer processes (Figure 4E).

**Recruitment of ED1-positive macrophages to the hotspot**

During the early phase post-exposure to BCL we found evidence of macrophage recruitment into the hotspot, evidenced by ED1 immunoreactivity - a known marker for rat macrophages\(^{24}\) (Figure 5). A preferential clustering of ED1-positive cells was apparent in the choroidal vasculature underlying the hotspot, immediately following BCL exposure (Figure 5B). This recruitment was localized to the hotspot, with virtually no ED1-positive nuclei observed in the choroid outside the hotspot (Figure 5C). Numerous ED1-positive cells were also observed in the retinal blood vessels immediately after BCL exposure (Figure 5D).
Figure 4 GFAP expression and reactive gliosis of Müller cells (MC) in the hotspot during the early post-exposure period. Sections are immunolabelled with GFAP in A-E, and with glutamine synthetase to visualize MC morphology in F-J. A: Typical retinal GFAP expression in the superior mid-periphery from a dim reared animal. B: Immunoreactivity for GFAP becomes evident in the inner processes of the MC (arrow) immediately following exposure to BCL. C-D: GFAP immunoreactivity features heavily throughout the MC in the hotspot at both 3 and 7 days post-exposure, localizing particularly within the rapidly formed glial scar situated below the ONL (white arrowheads). E: Upregulation of GFAP is also evident outside the hotspot in the inferior mid-periphery at 7 days post-exposure, although expression is mainly limited to the inner MC processes. F: Retinal section from a dim reared animal immunolabeled with glutamine synthetase showing normal MC morphology; a dashed line marks the outer limiting membrane. G: The structural integrity of the OLM is maintained in the hotspot immediately following exposure to BCL. H: Sections from 3 days post-exposure show reactive gliosis apparent within the hotspot. The integrity of the OLM is disturbed as MC processes hypertrophy (asterisks) and infiltrate the subretinal space, forming a fibroglial scar beyond the subretinal space (arrows). I: MC processes continue to infiltrate into the subretinal space after 7-days post-exposure J: A retinal section from the inferior mid-periphery demonstrates the retention of OLM integrity outside the hotspot. C, choroidal vasculature; GCL, ganglion cell layer; INL, inner nuclear layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OS, outer segments. Scale bars represent 50µm (A-J).
Figure 5 Preferential recruitment of ED1-positive macrophages to the hotspot following exposure to BCL. Sections are immunolabelled with ED1 for rat macrophages (red) and for nuclei as labeled with bisbenzamide (blue) in A-E. A: ED1-positive nuclei are rare and sparsely localized within the choroidal vasculature of dim-reared animals (arrows). B-C: Sections immediately following BCL show a marked recruitment of ED1-macrophages to the choroidal vasculature underlying the hotspot (B), while showing no such localization to non-hotspot retina (inferior mid-periphery) (C). D: Incursion of ED1-positive nuclei into the superficial retinal vasculature is highly evident in association with the hotspot, immediately following BCL. E: Retinal section at 3 days post-exposure showing ED1-positive macrophages present in the subretinal space and ONL within the hotspot (arrows). C, choroidal vasculature; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars represent 50µm (A-E).
By 3 days post BCL exposure, ED1-macrophages were present amongst the degenerating remains of the OS and in the ONL (Figure 5E; arrows). ED1-positive nuclei in both the ONL and vasculature was largely dissipated by 28 days post-exposure, reaching numbers similar to controls by 56-days post-exposure (data not shown).

The late phase post-exposure: Persistence of degeneration around the hotspot

The stability of BCL-exposed retinas was also assessed at 28 and 56-days post-exposure. TUNEL cell counts in the ONL across the vertical meridian of retinas 28 days following BCL exposure show persistence of TUNEL+ nuclei in a broad region in and around the hotspot even at 56 days post-BCL exposure (see Figure 1B). A cumulative impact of this chronic photoreceptor loss was observed through the persistent decrease in ONL thickness associated with the hotspot. By 56 days post-exposure, the relative reduction in ONL thickness compared to dim-reared animals (86.6%) was found to have increased by 34.9% (P <0.05) over measurements taken after 7 days post-exposure (51.7%) at comparable locations in the hotspot (2mm superior from the optic nerve).

Histologically, at 28 and 56 days post exposure only a few scattered photoreceptors were present in the ONL which appeared compressed between the choroid and INL (Figure 6A-B). In contrast, structural integrity was maintained in the inferior retina as late as 56 days post exposure. Immunoreactivity for L/M opsin and rhodopsin revealed a sparse population of surviving photoreceptors in the hotspot, mainly comprising disorganised cones, with rods present only towards the rim of the hotspot (Figure 6H-I). The remaining cones had lost their normally slender, elongated morphology, lacked inner and outer segments and were immunoreactive to L/M opsin.
Figure 6 Instability and degeneration amongst photoreceptors in-and-surrounding the hotspot at 56-days post-exposure. A-B: H&E stained sections of a hotspot lesion (L) 56 days after BCL exposure show the further reduction of the ONL to a mere few degenerative nuclei compacted within the scar tissue of the retinal lesion (B; arrows). A substantial cluster of degenerative photoreceptors is apparent in the penumbra (black asterisks), in which nuclei appear disorganized while lacking inner and outer segments. C-D: Immunoreactivity for GFAP (red) shows heavy localization to the outer processes of Muller cells within the cluster of photoreceptor nuclei on the edge (asterisk) of the hotspot as visualized with bisbenzamide (blue). E-F: From the penumbra (asterisk), photoreceptor nuclei display preferential labeling (arrow) for the neuroprotective factor FGF-2 (green), which recedes with increasing distance from the lesion. G: TUNEL+ nuclei (red) clustering on the edge of the hotspot (asterisk) at 56 days post-exposure, the blue label is bisbenzamide. H-I: Immunoreactivity for Rhodopsin (red) and L/M opsin (green) in the hotspot at 56 days post-exposure. Photoreceptors are mainly composed of L/M opsin immunoreactive cone remnants (I) toward the centre of the lesion, while degenerative OS-lacking rods predominate from the rim of the hotspot (asterisk) (H). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OS, outer segments; RPE, retinal pigment epithelium. Scale bars represent 100μm (A,C,E,G,H), and 20μm (B,D,F,I).
throughout the somas and axon terminals (Figure 61). On the edge of the lesion there was marked upregulation of GFAP expression by MC (Fig 6C,G), increased immunoreactivity for FGF-2 in photoreceptor nuclei (Figure 6E,F) and the continued presence of TUNEL+ nuclei (Figure 6G) indicative of continued photoreceptor loss on the edge of the hotspot, long after BCL exposure.

**Recovery of photoreceptors outside the hotspot**

Contrasting with the sustained degeneration of photoreceptors associated with the hotspot, we found evidence of photoreceptor recovery in the inferior and far-superior retina following photic insult (Figure 7). Measurement of photoreceptor OS’s in these regions following BCL, showed a shortening of OS’s to 57% the length of dim-reared controls by 7 days post-exposure (P <0.05; Figure 7A), while immunoreactivity for L/M opsin displayed a heavy translocation to the soma and axon (Figure 7C). However, by 28 days post-exposure OS length had recovered to ~90% the length of controls at comparable locations (P <0.05), which was maintained after 56 days post-exposure (Figure 7A). Recovery of OS length at this time was also associated with a reduction in L/M opsin immunoreactivity in the soma and axonal processes of cones, with opsin immunoreactivity confined predominately to the OS (Figure 7D).
Figure 7 Long-term recovery of photoreceptor morphology in non-hotspot regions of retina. **A:** Quantitative measurements of OS length from consistent populations of photoreceptors in the inferior and far-superior show a vast reduction in OS length after 3 days post-exposure. A significant renewal of OS’s however, is demonstrated at 28 days post-exposure (Control n = 8; 0 Days n = 6; 3 Days n = 3; 7 Days n = 4; 28 Days n = 4; 56 Days n = 5; error bars represent SEM; ‘*’ denotes a significant change where P <0.05). **B-D:** Sections immunolabelled with L/M opsin reveal a strong redistribution of L/M opsin to the soma and axon in some cones within the inferior mid-periphery 7 days after BCL exposure (C) compared to controls (B). At 28 days recovery L/M opsin labeling shows a normal distribution (D). ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bars represent 20µm (B-D).
Discussion

The results of the present study confirm in this model the highly focal nature of light-induced photoreceptor degeneration in a 'hotspot', as described previously \(^{10}\), and further emphasize its co-localization with the *area centralis* of the rat retina \(^{14}\). In addition, our findings elaborate on the existing understanding of this light-induced model of retinal degeneration in that we show (i) that photoreceptor degeneration continues in the hotspot and surrounding *penumbra* up to 56 days post exposure, resulting in expansion of the hotspot, while (ii) photoreceptors in inferior and far superior retina show substantial recovery from photic insult between 7 and 28 days post exposure. We also demonstrate (iii) localized recruitment of ED1 positive macrophages to the choroid underlying the hotspot, and in the inner retinal vasculature near the hotspot which is evident 24 hours from commencement of bright light exposure, and (iv) infiltration of the outer retina by ED1 positive macrophages by 3 days post-exposure, associated with breakdown of the outer BRB, and the elaboration of MC processes in the subretinal space.

To our knowledge, the spatiotemporal relationship of these characteristics following light damage to the retina in the albino rat has not been explored previously. Our findings are consistent with earlier reports showing that in the albino rat retina 24 hours exposure to BCL invokes localised, rapid changes in the outer retina that result in photoreceptor death by apoptosis. In addition we show that the hotspot - the region most affected in the short-term - is surrounded by a *penumbra* where photoreceptor death continues at levels substantially higher than background, until at least 56 days post-exposure. Persistent photoreceptor death and stress in the penumbra results in a gradual increase in the size of the hotspot over this period, consistent with observations from another phototoxic model, using Lister hooded and RCS rats \(^{11}\). We also find
death of rod photoreceptors ahead of cone photoreceptors, and persistence of opsin-expressing cone somata within the degenerating region at 56 days. In contrast, we find that, remote from the hotspot in far-superior and in inferior retina, there is evidence of recovery from light damage over the 56-day period, demonstrated by the recovery of near-normal OS length in photoreceptors in these regions \textsuperscript{17,25-27}. Together our findings indicate that there are region-specific factors that predispose the more central regions of the albino rat retina to the prolonged effects of retinal damage, which are not in effect in inferior and far-superior retinal locations.

The regional nature of photoreceptor cell death in this model has been well documented, however, the notion that the hotspot of degeneration may result from specializations associated with the \textit{area centralis} has received relatively little attention. Specializations of the rat \textit{area centralis}, which may render this region more susceptible to light-induced degeneration, have been alluded to previously \textsuperscript{14}. At the \textit{area centralis} the OS are more elongated and have higher rhodopsin content \textsuperscript{12,14,28}, and other studies show a correlation between rhodopsin content and the severity of light-damage \textsuperscript{8,28,29}. One possibility is that regional responses of the retina to light damage over the 56 day period may be due to the relative severity of the initial photic insult on the retina in the different regions, the \textit{area centralis} being more severely affected due to local photoreceptor specializations. However, further studies that include controls for rhodopsin content are required to specifically address this hypothesis.

We also detected region-specific recruitment of activated macrophages to the incipient hotspot, early in the post exposure period. Recruitment of macrophages in light-damage models of retinal degeneration has been shown previously, albeit in a more delayed fashion. Gordon and colleagues (2002) showed that 4-5 hours exposure to high intensity white light (1800 lux) resulted in DNA fragmentation that was associated with monocyte recruitment into the ONL by 42 hours post-treatment \textsuperscript{30}. 
Similarly, in a high-intensity model utilizing blue light, ED1 positive macrophages were detected in the ONL 7 days after exposure to damaging light. Activated microglia have also been implicated in progression of photoreceptor degeneration in a light-induced model, as well as in rd mice. However, those studies did not report detection of ED1 positive cells in the choroid, nor did they report regional variation in the recruitment of the activated cells. The present findings show early, site-specific recruitment of ED1 positive cells 24 hrs from commencement of bright light exposure, in both the choroidal and retinal vascular supplies, in the region of the hotspot, but not elsewhere in the retina. By 3 days post-exposure we found that many ED1 positive cells had migrated into the degenerating ONL of the hotspot, consistent with previous reports, and by 28 days post-exposure the invasion of ED1 positive cells had largely abated. The precise role played by the ED1 positive macrophages in the progress of photoreceptor degeneration remains unclear. One interpretation is that macrophages are recruited to the site of retinal damage to facilitate clearance of cellular debris resulting from cell death. However, it has also been shown that suppression of activation of the retina's resident macrophages, the microglia, significantly reduces photoreceptor death, suggesting that activated macrophages may have an active role in the progress of light-induced photoreceptor loss. In support of this hypothesis, in vitro studies show increased levels of death amongst cultured photoreceptors exposed to basal medium conditioned by activated microglia.

Comparison with AMD

The progress of retinal degeneration described here over the 56-day period post-exposure to damaging light bears many similarities with the progression of AMD in the absence of neovascularization, albeit in a considerably compressed timeframe. First, photoreceptor loss in this model is strictly centered on the area centralis, a
retinal specialization that is a homologue of the *fovea centralis*, and found predominantly in predatory and crepuscular mammals \(^{37}\). Both *foveae* and *areae centrales* are adapted to mediate best spatial resolution, and include a range of specializations which in humans may predispose the *fovea* and surrounding *macula* to degeneration \(^{38,39}\). Second, loss of the RPE \(^{40}\), breaks in Bruch's membrane \(^{41-44}\), MC gliosis and GFAP up-regulation \(^{45}\), and changes to the choroid \(^{46-49}\), are all features of early AMD. Consistent with findings from previous light-damage studies \(^{36,50}\), we find a similar range of features emerging following exposure to BCL, albeit in different time frames, post exposure.

The present findings also demonstrate new features of the light-damage model of retinal degeneration that support the analogy with AMD. First, the focal accumulation of ED1 positive macrophages to the choroid underlying the incipient hotspot following BCL bears striking similarity to the increased infiltration and activation of leukocytes characterised in the macular choroid in both early and late AMD \(^{42,51-54}\). Such accumulations of macrophages are known to be associated with the progression and severity of AMD histopathology. Studies involving experimental models of choroidal neovascularization have shown that macrophage inhibition induced through liposomal clodronate reduces the resulting lesion size \(^{55,56}\), while deficiency in the fractalkine chemokine receptor (CX3CR1) induces microglial accumulation in conjunction with increased lesion size \(^{57}\).

Second, our long-term analysis of photoreceptor death and immunoreactivity for stress-related proteins in MC shows the spread of a focal lesion into adjacent retina over time, resembling the expansion of retinal lesions in the 'dry' form of AMD \(^{40,58,59}\). Third, the changes detected in photoreceptors in and around the emerging hotspot are similar to those seen in photoreceptors in and around AMD lesions \(^{60}\). These include an initial redistribution of opsin from the OS to the soma and pedicles \(^{60-63}\), followed by
hypertrophy of the soma and axon terminal in cones, then loss of OS and axon terminals in cones in the centre of the lesion, where islands of surviving cone somata can be detected 60.

Two features of AMD not observed in the light damage model, however, are druse and neovascularization. While the precise origins and significance of drusen remain elusive, it is understood that they are complex deposits that form over a long period of time 64, possibly in response to pro-inflammatory signals 65. Furthermore it has been suggested that C3a and C5a present in drusen may promote choroidal neovascularization in AMD 66. We suggest, therefore, that the light-induced retinal degeneration seen in the albino rat model differs from AMD in that it develops over a very short time frame, and the characteristics of AMD that develop long term - drusen and neovascularization - are not observed.

Light exposure has been associated with the incidence of AMD in a number of studies previously 67-73, although the association has remained somewhat contentious 74-76 and may stem from the difficulty of accurately assessing a lifetime of light-exposure through questionnaires 77,78. Indeed, a recent investigation by Hirakawa and colleagues (2008) which utilised an objective means to gauge light exposure, has also demonstrated a link between increased sunlight and AMD 77. Several lines of evidence also support an association between the casual events both of AMD and light damage, specifically through oxidative damage. Light-induced oxidative damage has been shown to arise through rhodopsin-mediated peroxidation of lipids such as docosahexanic acid (DHA) 4,5,79, and a role for oxidative damage has long been suggested in the pathogenesis of AMD 80,81. This association has been further strengthened by recent findings implicating carboxyethylpyrrole (CEP) – an oxidation fragment of DHA and biomarker of AMD 82-84 – in the emergence of AMD histopathology 85.
Conclusions

Our findings demonstrate that a rapid and prolonged development of AMD-like histopathology occurs in albino rats exposed for a relatively short period of BCL, which features a strong localisation to the apparent visual centre of the rat retina. Building on previous findings\textsuperscript{36,50}, our data offer compelling histological evidence for a common pathway underpinning the process of photoreceptor degeneration in this model of light damage and AMD, and demonstrate the potential of this model for analysis of mechanisms of retinal degeneration.

References


81. Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. Mol Vis 1999;5:32.


Chapter Three

Early focal expression of the chemokine Ccl2 by Müller cells during exposure to damage-inducing bright continuous light

This chapter is presented as shown in the publication:

Early focal expression of the chemokine Ccl2 by Müller cells
during exposure to damage-inducing bright continuous light

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Abstract

**Aim:** To investigate the time-course and localisation of Ccl2 expression and recruitment of inflammatory cells associated with light-induced photoreceptor degeneration.

**Methods:** Sprague Dawley (SD) rats were exposed to 1000 lux light for up to 24hrs, after which some animals were kept in dim-light (5 lux) to recover for 3 or 7 days. During and following light exposure, animals were euthanized and retinas processed. Ccl2 expression was assessed by qPCR, immunohistochemistry, and *in situ* hybridization at each time-point. Counts were made of perivascular monocytes/microglia immunolabeled using ED1, and photoreceptor apoptosis was assessed using TUNEL. **Results:** Up-regulation of Ccl2 expression was evident in the retina by 12hrs exposure, and correlated with increased photoreceptor death. Ccl2 expression was maximal at 24hrs, coinciding with peak cell death. Immunohistochemistry and *in situ* hybridization showed that Ccl2 is expressed by Müller cells from 12hrs exposure, most intensely in superior retina, in the region of the incipient light-induced lesion. Following the Müller cell-driven expression of Ccl2, there was a substantial recruitment of monocytes to the local retina and choroidal vasculature. This coincided spatially with the expression of Ccl2 in the superior retina. Peak monocyte infiltration followed maximal Ccl2 expression by up to 3 days. Furthermore, Ccl2 immunoreactivity was observed in many infiltrating monocytes following 24hrs exposure. **Conclusions:** Our data indicate that photoreceptor death promotes region-specific expression of Ccl2 by Müller cells, which facilitates targeting of monocytes to sites of injury. The data suggest that recruitment of monocytes to developing lesions is secondary to signalling events in the retina.
Introduction

Microglia are the principle immune cells of the central nervous system (CNS) \(^1\). Through persistent surveillance of their microenvironment \(^2\), microglia act to maintain homeostasis in the CNS parenchyma, including the retina, facilitating the phagocytosis of debris and apoptotic cells \(^3-5\), antigen presentation \(^6, 7\), and the secretion of neuroprotective factors \(^8\) following neuronal stress and injury. However, activated microglia may also promote the secretion of pro-inflammatory mediators tumour necrosis factor α (TNFα) and interleukin 1β (IL1-β) \(^9-11\), in addition to cytotoxic factors such as nitric oxide \(^12-14\), shown to harm neuronal cells \(^12, 15-18\). In the retina, activated microglia respond to degeneration elicited from a range of human retinal pathologies including age-related macular degeneration (AMD) \(^4, 19-22\), retinitis pigmentosa \(^4\), and late-onset retinal degeneration \(^4\), as well as in many experimental models of retinal degeneration \(^8\). In the light-induced model of photoreceptor degeneration, recruitment and activation of microglia in the retina has been particularly well characterised \(^23-28\), and involves the infiltration of both parenchymal microglia and perivascular monocytes/microglia to the outer nuclear layer (ONL) and subretinal space following the damaging stimulus. Moreover, it has been demonstrated that attenuating the microglial response results in reduced photoreceptor death and IL1-β production following light-damage \(^27\), indicating that the extensive aggregation and over-activation of microglia plays a role in propagating the neurodegenerative process.

We have shown previously that light damage-induced microgliosis of perivascular monocytes is site-specific to the region of peak photoreceptor death, at the area centralis of the rat retina \(^24\). However, the process by which microglia are recruited into the retina following light-damage remained to be clarified. Chemokines have been shown to have potent chemoattractant properties in the trafficking of leukocytes in immune
surveillance and inflammation in the CNS \cite{29,32}. Chemokine expression results in the establishment of chemical ligand gradients, that serve as directional cues for the guidance of certain leukocytes to sites of injury, and are also thought to aid in their extravasation into tissues \cite{29}. Studies have shown that a number of chemokines are expressed at high levels following light-damage \cite{26,33}; the most well-characterised of these being chemokine (C-C motif) ligand 2 (Ccl2) \cite{34}. Ccl2 (also known as monocyte chemoattractant protein 1 (MCP-1)) is a strong chemoattractant for monocytes \cite{35,36}, whose up-regulation is implicated in a number of CNS pathologies \cite{31,32} including Alzheimer’s disease \cite{37,38}, multiple sclerosis \cite{39,40}, and brain trauma \cite{41,42}.

We aimed to investigate the site and cellular localization of Ccl2 expression in the retina following photoreceptor death induced by exposure to bright continuous light (BCL), and to relate this expression pattern to infiltration of perivascular monocytes/microglia (hereby referred to as monocytes). Our findings show that in this model, Müller cells located in the region of the incipient lesion are the early source of Ccl2, which is up-regulated in concert with the onset of photoreceptor degeneration. These findings demonstrate the role of the neural retina in the active initiation of an inflammatory response, as a result of light injury. This Müller cell-driven expression is closely correlated with the spatial and temporal infiltration of monocytes from both the retinal and choroidal vascular supplies, which in turn were found to express Ccl2.

**Methods**

**Animals and light exposure**

All experiments conducted were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sprague-Dawley (SD) rats were
born and reared in dim cyclic light conditions with an ambient level of approximately 5 lux. Exposure to bright continuous light (BCL) was conducted on animals aged between post-natal day (P) 130 and 160. Prior to BCL exposure, animals were dark adapted for a minimum of 15 hours, then transferred to individual cages designed to allow light to enter unimpeded. BCL exposure, which was achieved using a fluorescent light source (18W, Cool White; TFC) positioned above the cages, commenced consistently at 9:00 am and kept at an intensity of approximately 1000 lux at the cage floor. Animals were exposed to BCL for a period of 1, 3, 6, 12, 17, or 24 hours, after which time retinal tissue was obtained for analysis. Some animals were returned to dim-light conditions immediately following 24hrs BCL for a period of 3 or 7 days, to assess post-exposure effects. Age-matched, dim-reared animals served as control samples.

**Tissue collection and processing**

Animals were euthanized by overdose of barbiturate administered by an intraperitoneal injection (60 mg/kg bodyweight, Valabarb; Virbac, NSW). The left eye from each animal was marked at the superior surface for orientation then enucleated and processed for cryosectioning, while the retina from the right eye was excised through a corneal incision and prepared for RNA extraction.

Eyes for cryosectioning were immediately immersion fixed in 4% paraformaldehyde in 0.1M PBS (pH 7.3) for 3 hours at room temperature, then washed in 0.1M PBS before being left in a 15% sucrose solution overnight for cyroprotection. Eyes were oriented and embedded in O.C.T. compound (Tissue-Tek) then snap frozen in liquid nitrogen and cryosectioned at 16 μm. The sections were mounted on gelatin / poly-L-lysine-coated glass slides coated and dried overnight at 37°C, which were subsequently stored at -20°C until use.
Retinas for RNA extraction were immediately deposited in RNAlater solution (Cat# 7024; Ambion, Austin, TX) pre-chilled on ice. The samples were incubated at 4°C overnight to allow adequate penetration of the preservative, and then stored at -80°C until required. Samples were processed in batches encompassing the entire time-course, in order to ensure comparability. Upon extraction, the retinal samples were thawed on ice and the RNAlater solution removed. RNA extraction was performed with a combination of TRIzol Reagent (Cat# 15596-026; Invitrogen, Carlsbad, CA) and an RNAqueous-small scale kit (Cat# 1912; Ambion) utilised in tandem to extract and purify the RNA respectively, as described previously. Isolated total RNA was analysed for quantity and purity with a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE); samples with a 260/280 ratio greater than 1.90 were considered sufficient. The RNA quality in each sample was assessed using a 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA) where only samples with an integrity number (RIN) of ≥ 8 were used.

**Quantitative real time polymerase chain reaction (qPCR)**

First-strand cDNA synthesis was performed using a SuperScript® III Reverse Transcriptase kit (Cat# 18080–044; Invitrogen) applied following the manufacturer’s instructions; a 20μL reaction mixture was utilised in conjunction with 1μg RNA, 500ng of oligo (dT)₁₈ primer, and 200U SuperScript® III Reverse Transcriptase. Gene amplification was measured using commercially available TaqMan® hydrolysis probes (Applied Biosystems, Foster City, CA), the details of which are provided in Table 1. The hydrolysis probes were applied following the manufacturer’s instructions using the Gene Expression Master-Mix (Cat# 4369514; Applied Biosystems), with the fluorescence measured on a FAM 510nm detection channel by a StepOnePlus™ qPCR system (Applied Biosystems). The samples in each well were normalised using a
Table 1: Taqman® probes used

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ROX™ passive reference dye – included in the Gene Expression Master-Mix – to account for well-to-well discrepancies. The amplification for each biological sample was performed in experimental duplicate, with the mean Cₗ (quantitation cycle) value then used to determine the fold change in expression. The fold change was determined using the ΔΔCₗ method, where the expression of the target gene was normalised relative to the expression of two reference genes – glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Actin-β (Actb). Amplification specificity was assessed using gel electrophoresis.

**In situ hybridisation**

To investigate the localisation of Ccl2 mRNA transcripts in the retina following BCL, a riboprobe to Ccl2 was generated for in situ hybridisation on retinal cryosections. Ccl2 was cloned from a PCR product (550bp amplicon) using cDNA prepared from rat retinas (as described above), the pGEM® -T DNA vector system (Cat# A3600; Promega, Madison, WI) and One Shot® TOP10 competent cells (Cat# C4040-10; Invitrogen). A DIG RNA Labeling Kit SP6/T7 (Cat# 1175025; Roche, Basel, Switzerland) was used to transcribe linearised plasmid and generate DIG-labeled anti-sense and sense riboprobes. The in situ hybridisation was performed using a protocol described previously⁴⁴; the Ccl2 riboprobe was hybridised overnight at 55°C, and then washed in saline sodium citrate (pH 7.4) at 60°C.

**Analysis of cell death**

TUNEL labeling was used to quantify photoreceptor apoptosis during and following BCL, in cryosections using a protocol published previously⁴⁵. Counts of TUNEL positive cells in the outer nuclear layer (ONL) were carried out along the full-length of retinal sections cut in the para-saggital plane (superio-inferior), including the
Table 2: Antibodies used

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optic disc, in adjacent fields measuring 1000 x 1000 µm. The final count from each animal is the average at comparable locations in 2 non-sequential sections.

**Immunohistochemistry**

Cryosections adjacent to those used for TUNEL analysis were used for immunohistochemistry. Details for the primary antibodies used are shown in Table 2. Following antigen retrieval (RevealIt Ag; ImmunoSolution, QLD, Australia), the sections were incubated in 10% normal goat serum (Sigma) for 1 hr at room temperature (RT), then incubated in the primary antibody overnight at 4°C. The sections were washed in 0.1M PBS then incubated in the appropriate biotinylated secondary antibody for 4 hours at RT (1:200 anti-hamster IgG-Biotin; Biolegend / 1:200 Biotinylated anti-mouse IgG+A+M; Molecular Probes, Eugene, OR). After an additional wash in 0.1M PBS, the sections were incubated with a Streptavidin – Alexa Fluor® 594 conjugate (1:1000; Molecular Probes) for 1.5 hours at RT. To block autofluorescence, an incubation using 0.05% Sudan Black B was performed, as described previously. Sections were then stained with either Bisbenzamide (1:1000; Calbiochem, La Jolla, CA) or Syto12 (1:1000; Molecular Probes) to visualize the cellular layers, then coverslipped using Aquamount (Cat# 18606; Polysicences, Warrington, PA). Primary antibodies were omitted to control for non-specific binding of the secondary antibody. Immunofluorescence was viewed using a Zeiss laser scanning microscope, and acquired using PASCAL software (Zeiss, v4.0). Images were enhanced for publication using Adobe Photoshop software, which was standardised between images.

**Monocyte quantification**

Monocyte counts were performed on sections immunolabeled with ED1 – a marker for perivascular monocytes/microglia and bisbenzamide (as per the
methodology described above). Counts of ED1-positive nuclei carried out along the full-length of retinal sections cut in the para-sagittal plane (supero-inferior), including the optic disc, in adjacent fields measuring 1000 x 1000 μm. The numbers of monocytes in the superficial vasculature, deep vasculature, ONL and choroidal vasculature were assessed, where the graphs showing the numbers of ED1 positive cells include the total number in each field of view.

**Statistical analysis**

Statistical analysis was performed using the one-way ANOVA, two-way ANOVA, or the unpaired Student’s t-test. For each analysis, differences with a P value < 0.05 were considered statistically significant. The one-way ANOVA, and unpaired Student’s t-test were used for the quantification of TUNEL and monocytes, while qPCR analysis utilised both the one-way and two-way ANOVA.

**Results**

**Time-course of TUNEL labelling, GFAP and Ccl2 expression**

During the first 12hrs exposure to BCL the average number of TUNEL positive (+) photoreceptors increased from near 0 to 10 (P < 0.05, Student’s t-test), and reached a peak of 470 by 24hrs exposure (P < 0.0001, Student’s t-test). During the post-exposure period the number of TUNEL+ nuclei decreased to 213 by 3 days and then to 33 after 7 days (P < 0.01, Student’s t-test). Using qPCR we also detected modulation of GFAP expression over the experimental period (P < 0.0001, One-way ANOVA). GFAP expression reached peak levels at 24 hrs (2446% relative to control levels), and was somewhat reduced by 7 days post exposure. These data are shown in Figure 1, where
Figure 1 Expression of Ccl2 in the neural retina by qPCR in relation to (A) photoreceptor cell death and (B) GFAP expression, following exposure to BCL. A/B: Exposure to BCL induced a significant increase in the expression of Ccl2 (dark grey) relative to dim-reared animals (P < 0.0001, one-way ANOVA), which was detected at 12hrs exposure. Ccl2 expression continued to rise significantly, reaching a peak differential expression of 196.479% by 24hrs. Following 7 days post-exposure, relative expression had fallen sharply to 2092%. Large increases in TUNEL positive nuclei in the ONL (A) and GFAP expression (B) were also observed from 12hrs exposure (P < 0.0001, one-way ANOVA), correlating with the rapid up-regulation of Ccl2 expression over the same period (Interaction 43.60% and 42.82% of total variance respectively, P < 0.0001; two-way ANOVA). (Ccl2 qPCR n = 4, GFAP qPCR n = 4, TUNEL n = 5 per timepoint; error bars represent SEM).
the expression levels of Ccl2 are plotted alongside both the time-course of photoreceptor death (TUNEL) (Figure 1A), and expression levels of GFAP (Figure 1B).

Exposure to BCL induced a significant differential expression of retinal Ccl2 mRNA relative to dim-reared animals over the experimental period (P < 0.0001, One-way ANOVA) (Figure 1A-B). By 12hrs exposure qPCR showed a 3499% increase in Ccl2 expression relative to dim-reared controls. Levels of Ccl2 mRNA reached a peak of 196,479% at 24hrs. In the post-exposure period, Ccl2 expression fell to 5547% of control levels by 3 days, and 2092% by 7 days post-exposure. The progressive increase in Ccl2 expression observed in the 24 hr exposure period correlated significantly with TUNEL labelling over the same period (interaction 43.60% of total variance, P < 0.0001, Two-way ANOVA) (Figure 1A). Expression of GFAP and Ccl2 were also significantly correlated in the 24 hr exposure period (interaction 42.82% of total variance, P < 0.0001, Two-way ANOVA) (Figure 1B). However, GFAP expression in the recovery period did not show a clear downward trend, in contrast to a progressive reduction in expression of Ccl2 post-exposure.

Localisation of Ccl2 Expression

Ccl2 mRNA was not detectable by in situ hybridisation in retinas of dim-reared animals (Figure 2A). However, in experimental animals at 12 hrs BCL exposure we detected expression of Ccl2 mRNA in isolated cells within the inner nuclear layer (INL) of the superior retina (Figure 2B); by 24 hrs BCL we detected robust expression of Ccl2 in many cells and processes in the INL (Figure 2C), consistent with the qPCR expression profile (Figure 1). We also observed that Ccl2 expression was distributed in a supero-inferior gradient following BCL, with peak expression observed within the superior mid-periphery (Figure 2E-G). Using fluorescent markers the Ccl2 mRNA (Figure H-I; white arrows) appeared to co-localise with vimentin-immunoreactive
**Figure 2** *In situ* hybridisation for Ccl2 mRNA following exposure to BCL. **A:** In retinas from control, dim-reared animals, expression the Ccl2 was not detectable. **B:** Ccl2 mRNA expression was detected in isolated cells of the INL in the superior retina by 12hrs BCL (black arrowhead). **C:** Following exposure to 24hrs BCL, Ccl2 mRNA was observed in cells with radially oriented processed throughout the INL (black arrowheads). **D:** Straining was not observed in retinas following 24hrs BCL using the sense Ccl2 riboprobe. **E-G:** The distribution of Ccl2 expression was detected in a supero-inferior gradient, following BCL, with the most intense labelling detected in superior retina. **H-J:** Ccl2 expression (red) in sections counter immunolabelled with anti-vimentin, showing double-labelled Müller cell processes (green) (white arrows, white arrowheads) indicating that Ccl2 in the INL is expressed by Müller cells. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RPE, retinal pigment epithelium.
Figure 3 Immunoreactivity (IR) for Ccl2 in Müller cells following exposure to BCL. **A:** IR for Ccl2 was not detected in control dim-reared retinas. **B, C:** Faint Ccl2-IR (red) was detected in thin, radial processes (white arrowheads) in the INL of the superior retina at 12hrs BCL exposure. **D:** By 24 hrs exposure Ccl2-IR was present in a large number of radial processes in superior retina. **(D).** **E-F:** Lower levels of Ccl2-IR were evident during the post-exposure period. **G-H:** Ccl2-IR processes were more numerous in superior retina (**H**), compared with inferior retina (**G**). **I-M:** Dual immunolabelling for Ccl2 (red) and the Müller cell-specific protein S100β (green) shows the co-localisation of Ccl2 IR with S100β in Müller cell processes. **L,M:** Field with inset showing co-localization of Ccl2 and S100β-IR in the inner processes of Müller cells (white arrows). INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer.
Müller cell processes (Figure H-J; white arrows), suggesting that Ccl2 is initially expressed by the Müller cells.

There was no immunoreactivity (IR) for Ccl2 protein in dim-reared animals (Figure 3A), but in experimental animals at 12 hrs exposure some Ccl2-IR was present in radially-oriented processes in superior retina (Figure 3B, C), and in increasing numbers of processes at 24 hrs exposure (Figure 3D) respectively. Double immunolabelling with the Müller cell-specific protein S100β (Figure 3I-K) showed Ccl2-IR localised within the inner processes of Müller cells, consistent with the in situ hybridisation results (Figure 2). Ccl2-IR Müller cell processes were most prominent at 24 hrs BCL exposure (Figure 3D), and declined markedly in the post-exposure period (Figure 3E-F), so that relatively few Ccl2-IR processes were present by 7 days post-exposure (Figure 3F). As with the distribution of Ccl2 mRNA (Figure 2E), Ccl2-IR was predominant in Müller cell processes in the superior retina following BCL (Figure 3 G,H).

**Quantification of ED1-positive monocytes**

Histograms showing the numbers of monocytes in the retina and choroid are shown in Figure 4. The total number of monocytes in the retina and choroid remained unchanged in the first 17 hrs of BCL exposure, but increased significantly by 24 hrs exposure, compared with dim-reared controls (P < 0.01, Student’s t-test) (Figure 4A). Peak numbers of monocytes were detected at 3 days post-exposure (196 cells; P < 0.05, Student’s t-test), which were significantly diminished by 7 days (123 cells; P < 0.05, Student’s t-test). The overall trend in monocyte infiltration was found to highly significant by One-way ANOVA (P < 0.0001). The numbers of recruited cells in different locations (retinal vasculature, choroid, or ONL) are shown in Figure 4B. The data show significant monocyte recruitment following BCL in both the choroid and
Figure 4 Recruitment of monocytes from the choroid and neural retina following BCL. 
A: Increased numbers of monocytes are detected in the retina and choroid between 12 and 24 hrs exposure; monocyte numbers continue to accumulate in the retina and choroid post-exposure, reaching peak numbers at 3 days (P < 0.001, One-way ANOVA).
B: Following the onset of BCL exposure, significant monocyte recruitment was observed in all locations of the tissue examined, including the retinal vasculature, choroid and ONL (P < 0.05, One-way ANOVA). (n = 3 per time-point; error bars represent SEM; ‘*’ denotes a significant change using the Student’s t-test – compared to dim-reared animals unless otherwise noted – where P < 0.05).
retinal vasculature, reaching peak numbers in the retinal vasculature by 24 hrs exposure, and in the choroid at 3 days (P < 0.001, One-way ANOVA). Peak numbers of monocytes were also detected in the ONL by 3 days (P < 0.01, One-way ANOVA). While larger numbers of ED1 positive cells were detected in the choroid (cf retinal vessels), the data indicate that recruitment of monocytes from the retinal vessels occurred earlier than from the choroid, the numbers rising acutely from a baseline of near-zero to 16 cells per retina, between 12 and 17 hrs BCL exposure (P < 0.01, Student’s t-test).

The distribution of the infiltrating monocytes along the vertical axis of the retina is shown in Figure 5. In dim-reared animals, circulating monocytes were evenly distributed across superior and inferior retina (Figure 5A). By 24hrs BCL exposure monocytes were more numerous in superior retina by a ratio of about 2:1 (P <0.05, Student’s t-test) (Figure 5A). By 3 days there were more than 6 times as many monocytes in superior retina than in inferior retina, and the numbers remained elevated in superior retina at 7 days post-exposure (P < 0.01, Student’s t-test). A more detailed analysis of the distribution of monocytes in adjacent fields along the vertical axis of the retina, at different times of BCL exposure and recovery, is shown in Figure 5B. The data indicate that monocyte numbers reach a peak at 3 days post exposure, in the region of superior retina that corresponds to the incipient 'hot-spot' of degeneration, characterised in our previous investigation 24.

We also found that many infiltrating monocytes in superior retina expressed Ccl2 following exposure to BCL (Figure 6). After 24hrs BCL, many infiltrating monocytes within the choroid were Ccl2-IR (Figure 6A-C). Extravasated tissue macrophages located within the outer segment layer (Figure 6A) and the ONL (Figure 6D) were also strongly Ccl2-IR. Ccl2 positive monocytes were observed predominately at the 24hrs BCL exposure period; none were observed in the early exposure period (12hrs BCL)
Figure 5 Distribution of monocyte recruitment in relation to the expression and immunoreactivity for Ccl2 following BCL exposure. **A**: The total number of monocytes in superior and inferior regions of the retina. There is a significant preferential recruitment of monocytes in the superior retina following 24hrs BCL, which persists into the post-exposure time points. **B**: The distribution of monocytes across the vertical meridian of the retina. The graph shows that at 24hrs BCL and 3 and 7 days post-exposure, monocytes are recruited primarily into superior retina, and concentrate in a region approximately 2 mm superior to the optic nerve. (n = 4 per timepoint; error bars represent SEM; '*' denotes a significant change using the Student’s t-test – compared to the inferior retina – where P <0.05).
Figure 6 Ccl2-immunoreactivity (IR) (red) and ED1-IR (green) monocytes at 24hrs BCL. **A, B:** Many infiltrating ED1-positive nuclei were also Ccl2-IR, in the choroid and retina. **C:** An extravasated monocyte in the choroid, double labelled for Ccl2 and ED1. **D:** A cluster of extravasated Ccl2 and ED1-IR monocytes in the ONL. ONL, outer nuclear layer; OS, outer segments; RPE, retinal pigment epithelium.
coinciding with the emergence of Ccl2 expression by Müller cells. By the post-exposure period, relatively few Ccl2-IR monocytes were detected (data not shown).

Discussion

The results of this study confirm the early and robust up-regulation of Ccl2 in conjunction with the light-mediated induction of photoreceptor death and the expression of the stress-factor GFAP, consistent with findings from previous studies. Our data also elaborate on the role of Ccl2 in light-mediated retinal dystrophy through several key findings. First, utilizing both in situ hybridization and immunohistochemistry, we verify that Ccl2 is expressed early by the Müller glia following exposure to BCL, whose expression – predominately in the superior retina – is consistent with both the temporal and spatial onset of photoreceptor apoptosis. Second, we demonstrate a preferential recruitment of monocytes to both retinal and choroidal vascular supplies in the superior retinal following the expression of Ccl2, which correlates with the spatial distribution of Ccl2-positive Müller cells. Third, our data show that extravasating monocytes provide additional stimulatory expression of Ccl2 after 24hrs BCL following the initial expression driven by the Müller cells.

While the expression of Ccl2 in light-damaged retinae has been documented previously through PCR, the precise distribution and cellular localization of Ccl2 expression following the damaging stimulus has not, to our knowledge, been investigated previously. Zhang and colleagues hypothesized that Müller cells or retinal pigment epithelial cells (RPE) may be the source of chemokines following BCL and several in vitro studies have shown RPE cell cultures express Ccl2, including in response to the introduction of stimulatory cytokines. This investigation, however, is the first to show the preferential distribution of Ccl2 mRNA in Müller cells in the
region of the incipient lesion, as early as 12 hours exposure to BCL. The findings pinpoint a response by Müller cells – not RPE cells – as the primary source of local Ccl2 expression which promotes the monocyte infiltration that is associated with light-mediated photoreceptor apoptosis. The present findings are consistent with a previous study of experimental retinal detachment, which showed a rapid up-regulation of Ccl2 expression in micro-dissected sections of INL, and the localization of Ccl2-IR in Müller cells that was correlated with detachment-induced photoreceptor death.

Our data also demonstrate an important role for chemokines in mediating local neuroinflammatory responses driven by the neural retina. Ccl2 is a potent chemokine for monocytes in vitro and is induced in a range of CNS pathologies. Its rapid site-specific expression by Müller cells, as demonstrated in the present study, appears to serve as an early pro-inflammatory signal that targets monocytes to sites of degeneration. We propose that Ccl2 expression is stimulated in Müller cells as a result of regional photoreceptor apoptosis, since increased levels of apoptotic photoreceptors are detected around 6 hours before increased levels of Ccl2 are detected (Figure 1A). Following Ccl2 up-regulation, our data also shows preferential infiltration of monocytes from retinal and choroidal vascular supplies into superior retina – consistent with our previous findings – which correlates with the spatial distribution of Ccl2-positive Müller cells.

Perivascular monocytes express the Ccr2 chemokine receptor, of which Ccl2 is a known ligand, and ED1-postive monocytes specifically have been shown to express Ccr2. Our findings are consistent with previous investigations which have established Ccl2 as a key factor in the chemotactic guidance of monocytes following injury. An investigation in experimental retinal detachment using Ccl2-deficient knockout mice, and Ccl2-specific antibody neutralisation, noted a substantial decrease in monocyte recruitment following detachment in conjunction with reduced photoreceptor death.
compared to controls. Similar deficiencies in monocyte recruitment have also been reported following Ccl2 inhibition in a variety of models, including skin inflammation, thioglycollate challenge, experimental autoimmune encephalomyelitis, pulmonary granuloma, and peripheral endotoxin insult. In spite of these findings however, a recent study by Joly and colleagues did not observe a difference in a population of F4/80-positive macrophages in the subretinal space following light-induced damage to Ccl2 knockout mice. However, those authors did not assess the expression of other microglial markers such as ED1, nor did they determine whether the F4/80-positive cells examined expressed the Ccr2 receptor. Indeed, a study in lung infection showed that <10% of F4/80 bright cells express Ccr2, suggesting that they are resident, rather than recruited, macrophages.

Our findings also show that many extravasating monocytes in the superior retina display strong immunoreactivity for Ccl2 following 24hrs BCL, contributing to the vast increase in Ccl2 mRNA observed at 24hrs BCL through qPCR. Such expression is consistent with the altered responsiveness in monocytes as they differentiate to form tissue macrophages, resulting in a concomitant down-regulation of the Ccr2 receptor and an enhancement of Ccl2 secretion following endothelial adherence. This modulation of Ccr2 may act as a regulatory mechanism for controlling the extent of macrophage activation, while the up-regulation of Ccl2 could serve to refine and amplify the Ccl2 gradient initially laid forth by the Müller cells.

**Relevance to AMD**

Our previous findings – in conjunction with other studies – suggest that the light-mediated model of retinal degeneration has a number of features in common with the pathogenesis of AMD. This model, like the established laser-induced model of neovascular AMD, uses an acute damaging stimulus to evoke long-term, site-specific
retinal degeneration. We have recently shown recruitment of monocytes/macrophages to incipient lesion on the visual axis (area centralis) of the rat retina following BCL injury. Activated macrophages are associated with the progression and severity of AMD pathology, and several studies show macrophage/microglial inhibition to reduce lesion size in the laser-induced model of neovascular AMD. The light-damage model used here involves localized macrophage recruitment to the site of retinal damage and endogenous Ccl2 expression in neural retina (present study). These findings provide insight into the mechanisms that guide macrophages to regions of incipient degeneration, which may be common to AMD.

While direct studies of Ccl2 expression in the human retina are lacking, it has been shown that both Ccl2- and Ccr2- knockout mice exhibit reduced lesion size and macrophage infiltration following laser-induced choroidal neovascularisation compared to controls. It has been suggested that aging Ccl2/Ccr2 -knockout mice develop AMD-like retinal degeneration, indicating that Ccl2 is also required for retinal homeostasis; although the AMD-like phenotype in the knockout has been questioned recently. Despite this, the findings suggest a role for Ccl2-induced monocyte trafficking in the pathogenesis of AMD, and point to a possible role of the retina in localization of the lesions to the macula.

**Conclusion**

While the recruitment of microglia has been thought to serve a beneficial function following retinal injury, such as promoting the removal cellular debris and apoptotic cells, recent evidence has suggested that the extensive microglial activation induced in BCL is detrimental. Our data demonstrate that monocyte recruitment following light-mediated cell death correlates with both the temporal and spatial expression of Ccl2 by Müller cells, which supports a role of the neural retina in guiding neuroinflammatory...
responses of microglia following damage. The potential modulation of such endogenous chemokine responses may provide a powerful means to control excessive microglial recruitment and activation, which has relevance in the treatment of human pathologies such as AMD.

References


Chapter Four

Analysis of complement expression in light-induced retinal degeneration: Synthesis and deposition of C3 by microglia/macrophages is associated with focal photoreceptor degeneration

This chapter is presented as shown in the publication:

Analysis of complement expression in light-induced retinal degeneration: Synthesis and deposition of C3 by microglia/macrophages is associated with focal photoreceptor degeneration

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Abstract

**Purpose:** To investigate the expression and localisation of complement system mRNA and protein in a light-induced model of progressive retinal degeneration. **Methods:** Sprague Dawley (SD) rats were exposed to 1000lx of bright continuous light (BCL) for up to 24hrs. At time-points during (1-24 hrs) and following (3 and 7 days (d)) exposure, animals were euthanized and retinas processed. Differential expression of complement genes at 24hrs exposure was assessed using microarray analysis. Expression of complement genes was validated by qPCR, and expression of selected genes was investigated during and post BCL exposure. Photoreceptor apoptosis was assessed using TUNEL and C3 was further investigated by spatiotemporal analysis using *in situ* hybridisation and immunohistochemistry. **Results:** Exposure to 24hrs BCL induced differential expression of a suite of complement system genes including, classical and lectin components, regulators, and receptors. ClqR1, MCP, Daf1 and ClqTNF6 all modulated in concert with photoreceptor death and AP-1 expression, which reached a peak at 24hrs exposure. C1s and C4a reached peak expression at 3d post-exposure, while expression of C3, C3aR1 and C5R1 were maximal at 7d post-exposure. C3 mRNA was detected in ED1- and IBA1- positive microglia/macrophages, in the retinal vessels and optic nerve head, and in the subretinal space particularly at the margins of with the emerging lesion. **Conclusion:** Our data indicate that BCL induces the prolonged expression of a range of complement genes, and show that microglia/macrophages synthesize C3 and deposit it in the ONL following BCL injury. These findings have relevance to the role of complement in progressive retinal degeneration, including atrophic AMD.
Introduction

The complement system is a component of the innate immune system, which provides a rapid host defence against a range of immunological challenges \(^1,2\). Through a cascade of proteolytic cleavages that generate phagocytosis-enhancing opsonins, chemotactic anaphylatoxins, and membrane-attack complexes \(^3,4\), the activity of this system 'complements' the ability of the host to initiate humoural defences against infectious pathogens \(^5\), and promotes the removal of potentially noxious substances including extracellular debris \(^1,3,4,6\), immune complexes \(^2,7-9\), and apoptotic cells \(^8,10-13\).

Despite this beneficial function, a pathogenic role of complement in age-related macular degeneration (AMD) has been uncovered through gene association studies. These identify a significant association between the Y402H sequence variant in the regulatory gene complement factor H (CFH) with incidence of AMD \(^14-17\), along with other susceptibility variants in complement pathway genes such as C2 \(^18,19\), CFB \(^18,19\), and the central component C3 \(^20-24\). These findings firmly establish complement activation and inflammation as factors that influence the onset and progression of AMD. Furthermore, histological analyses of post-mortem AMD eyes show that complement components and regulatory proteins are present in drusen \(^18,25,26\), suggestive of chronic complement activation. However, a number of key aspects of the disease process remain unclear, including the cellular events that promote complement activity in the retina \(^18\).

A number of investigations have shown that the light-mediated model of retinal degeneration in rats has pathogenic features in common with atrophic AMD \(^27-31\), and it has been shown that ablation of the alternative pathway gene complement factor D (CFD) in this model attenuates photoreceptor death \(^32\). To better understand the role of complement in the degenerative process, in this study we aimed to investigate the transcriptional profile and spatiotemporal distribution of complement gene expression in...
relation to areas of photoreceptor loss in the retina following light damage. We find that photoreceptor death is accompanied by robust expression of complement-related genes, many of which are associated with degeneration that takes place sometime after the damaging stimulus. Further, we find synthesis of C3 in the retina by infiltrating microglia/macrophages, in spatiotemporal coincidence with photoreceptor degeneration in the atrophic lesion.

**Methods**

**Animals and light exposure**

All experiments conducted were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sprague-Dawley (SD) rats aged from 130-160 post-natal days were exposed to bright continuous light (BCL) at 1000 lux following protocols described previously. Animals were exposed to BCL for a period of 1, 3, 6, 12, 17, or 24 hours, after which time retinal tissue was obtained for analysis. Some animals were returned to dim-light (5 lux) conditions immediately following 24hrs BCL for a period of 3 or 7 days, to assess post-exposure effects. Age-matched, dim-reared animals served as control samples.

**Tissue collection and processing**

Animals were euthanized by overdose of barbiturate administered by an intraperitoneal injection (60 mg/kg bodyweight, Valabarb; Virbac). The left eye from each animal was marked at the superior surface for orientation then enucleated and processed for cryosectioning, while the retina from the right eye was excised through a corneal incision and prepared for RNA extraction.
Eyes for cryosectioning were immediately immersion fixed in 4% paraformaldehyde in 0.1M PBS (pH 7.3) for 3 hours at room temperature, then processed as previously described \textsuperscript{28} and cryosectioned at 16 µm. Retinas for RNA extraction were immediately deposited in RNAlater solution (Cat# 7024; Ambion, Austin, TX) pre-chilled on ice, then stored according to the manufacturer’s instructions. RNA was then extracted from each sample following methodology established previously \textsuperscript{33}. Isolated total RNA was analysed for quantity and purity with a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE); samples with a 260/280 ratio greater than 1.90 were considered sufficient. The RNA quality in each sample was assessed using a 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA) where only samples with an integrity number (RIN) of $\geq 8$ were used.

**Microarray experimentation and analysis**

Microarray analysis was performed utilizing raw microarray data derived from a previous study conducted using Rat Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA) by members of our group \textsuperscript{34}; the full set of microarray data has been deposited in the NCBI Gene Expression Omnibus repository under accession number GSE22818. The analysis compared samples from dim-reared and 24hrs BCL experimental groups (n=3 for each). The microarray data was analyzed using Partek® Genomics Suite™ 6.4 software (Partek Inc., St. Louis, MO, USA). Affymetrix CEL files were imported into Partek with background correction, normalization, and summarization, using the robust multi-array average (RMA) algorithm adjusted for probe sequence and GC content (GC-RMA). The processed values were displayed as individual probe sets representing exonic coding sequences, which were log-transformed using base 2. Differential expression analysis was performed using the analysis of variance (ANOVA) statistic with significance level of $P < 0.05$. 
The heterogeneity of the resulting differential expression data was evaluated with agglomerative hierarchical clustering, utilizing the Euclidean distance metric, and principle component analysis (PCA), both of which were provided by the Partek® Genomics Suite™. The differential expression data was then clustered according to biological process as described by the Gene Ontology Consortium 35, utilizing functional analysis with Gene Ontology (GO) enrichment provided by the Partek GS Genomics Suite™ 36. Following this, the list of differentially expressed genes was screened for those relating to the complement cascade, using a differential expression cut-off of >50% and aided by pathway information summarized from the Gene Ontology Consortium 35, and gene grouping from the HUGO Gene Nomenclature Committee 37.

Quantitative real time polymerase chain reaction (qPCR)

First-strand cDNA synthesis was performed as described previously 33. Gene amplification was measured using either commercially available TaqMan® hydrolysis probes (Applied Biosystems, Foster City, CA) or SYBR® Green with custom designed primers, the details of which are provided in tables 1 and 2 respectively. The hydrolysis probes were applied following a previously established qPCR protocol 33. The primers for SYBR® Green qPCR (table 2) were designed within a coding domain sequence transversing an intron using the Primer3 web-based design program 38. SYBR® Green qPCR was performed using the StepOnePlus™ qPCR system (Applied Biosystems). The amplification for each biological sample was performed in experimental triplicate, with the mean Cq (quantitation cycle) value then used to determine the fold change in expression. For both Taqman® and SYBR® Green qPCR, the percentage change compared to dim-reared samples was determined using the ΔΔCq method. The expression of the target gene was normalised to the expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which showed no differential
Table 1: Taqman® probes used

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<td>Reverse primer (5'-3')</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>CD46</td>
<td>NM_019190.1</td>
<td>CTCTTGGGAGCCCTCTATCC</td>
<td>ATTCCTTACGGGACTAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008.3</td>
<td>CCTGGAGAAACCTGCAAG</td>
<td>CCTCAGTGTAGCCAGGATG</td>
</tr>
</tbody>
</table>
expression in the current study or in previous retinal light damage investigations \(^{32, 39}\). Amplification specificity was assessed using gel electrophoresis. Statistical analysis was performed using the one-way ANOVA, to assess the significance of the trend in expression. Differences with a P value < 0.05 were considered statistically significant.

**In situ hybridisation**

To investigate the localisation of C3 mRNA transcripts in the retina following BCL, a riboprobe to C3 was generated for *in situ* hybridisation on retinal cryosections. C3 was cloned from a PCR product (483bp amplicon) using cDNA prepared from rat retinas (as described above), the pGEM® -T DNA vector system (Cat# A3600; Promega, Madison, WI) and One Shot® TOP10 competent cells (Cat# C4040-10; Invitrogen). A DIG RNA Labeling Kit SP6/T7 (Cat# 1175025; Roche, Basel, Switzerland) was used to transcribe linearised plasmid and generate DIG-labeled anti-sense and sense riboprobes. The *In situ* hybridisation was performed using a protocol described previously \(^{40}\), the C3 riboprobe was hybridised overnight at 57°C, and then washed in saline sodium citrate (pH 7.4) at 60°C. Following hybridisation, some sections were further stained using immunohistohchemistry (see section below).

**Analysis of cell death**

TUNEL labeling was used to quantify photoreceptor apoptosis during and following BCL, in cryosections using a protocol published previously \(^{41}\). Counts of TUNEL positive cells in the outer nuclear layer (ONL) were carried out along the full-length of retinal sections cut in the para-saggital plane (superio-inferior), including the optic disc, in adjacent fields measuring 1000 x 1000 \(\mu\)m. The final count from each animal is the average at comparable locations in 2 non-sequential sections. Statistical
analysis was performed using the one-way ANOVA. Differences with a P value < 0.05 were considered statistically significant.

**Immunohistochemistry**

Cryosections from each timepoint were used for immunohistochemical analysis, using primary antibodies for complement C3 (1:50, Cat# ab11887; Abcam, Cambridge, MA), C3d (1:100, Cat# AF2655; R&D Systems, Minneapolis, MN), ED1 (1:200, Cat# MAB1435; Millipore, Billerica, MA), and IBA1 (1:1000, Cat# 019-19741; Wako, Osaka, Japan). Immunohistochemistry was performed using methodology previously described. Immunofluorescence was viewed using a Zeiss laser scanning microscope, and acquired using PASCAL software (Zeiss, v4.0). Images were enhanced for publication using Adobe Photoshop software, which was standardised between images.

**Results**

**Microarray Analysis**

Analysis of microarray data compared gene expression in retinas of animals reared in dim-light conditions with those exposed to 24hrs BCL. We compiled a list of differentially expressed gene probe sets (P < 0.05) following BCL. Hierarchical clustering dendrograms (Figure 1A) generated from the resulting microarray data showed strong homogeneity among biological replicates for their respective conditions (dim-reared or BCL). Principle component analysis (PCA) (Figure 1B) showed that 79% of variance in gene expression amongst the 6 animals analyzed by microarray was due to light conditions (dim-reared or BCL), showing high reproducibility in the microarray data.
Figure 1 Hierarchical clustering and principle component analysis (PCA) of the processed microarray gene expression data. **A:** Hierarchical clustering data show that sample replicates clustered closely according to their condition, either dim-reared or 24hrs BCL. **B:** PCA analysis, where each circle represents a sample replicate, showed a strong aggregation of replicates for their condition across PC1 (x-axis), which explains the majority of the variance (79%).
Table 3:

<table>
<thead>
<tr>
<th>Gene Ontology ID</th>
<th>Biological Process</th>
<th>Definition</th>
<th>Enrichment Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0050896</td>
<td>Response to stimulus</td>
<td>73.0</td>
<td></td>
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<tr>
<td>GO:0009987</td>
<td>Cellular process</td>
<td>50.0</td>
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<tr>
<td>GO:0032502</td>
<td>Developmental process</td>
<td>26.0</td>
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<tr>
<td>GO:0065007</td>
<td>Biological Regulation</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>GO:0016043</td>
<td>Cellular component organization</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td>GO:0051234</td>
<td>Establishment of localization</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>GO:0022610</td>
<td>Biological adhesion</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>GO:0032501</td>
<td>Multicellular organismal process</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>GO:0008152</td>
<td>Metabolic process</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>GO:0040007</td>
<td>Growth</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>GO:0002376</td>
<td>Immune system process</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>GO:0044085</td>
<td>Cellular component biogenesis</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>
A broad perspective on the differential gene expression in these animals was achieved using Gene Ontology (GO) enrichment clustering – according to ‘biological process’ – from which a list of the top 12 highly represented gene ontology clusters was generated (Table 3). The cluster with the largest enrichment score included genes involved in ‘response to stimulus’ (GO: 0050896). Within this cluster (data not shown), differentially expressed genes include those involved in stimulus detection (GO:0051606), response to external, chemical, and abiotic stimuli (GO:0042221, GO:0009628, GO:0009605), stress response (GO:0006950), and immune response (GO:0006955) – which in turn includes a cluster for complement activation (GO:0006957).

**Differential expression of complement genes in the retina following BCL**

Screening of the list of differentially expressed genes (P < 0.05) for those involved in the complement cascade resulted in the identification of a total of 17 complement-related genes, with a broad range of functional roles (table 4). Of the complement activators, complement components C1s, C2, C3, and C4 (C4b, and C4-2) were up-regulated as a result of exposure to BCL, as well as the carbohydrate recognition molecule ficolin B of the lectin pathway. Increased expression of complement receptors was observed, including integrin genes encoding C3 receptors CR3 (CD18/CD11b), and CR4 (CD18/CD11c), the C3 anaphylatoxin receptor C3aR1, and C1qR1. A number of complement regulators were also differently expressed after exposure to BCL, including increased expressed of cell-surface inhibitors CD55, and CD46, soluble inhibitor SERPING1 and a2m, while C4bp expression was reduced. Additionally, we observed modulation in complement-related genes C1qTNF3, and
Table 4: List of Differentially Expressed Complement System Genes following 24hr BCL exposure

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>% DE (Light Damage vs Dim-Reared)</th>
<th>Affymetrix Probe Set ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement Activators (GO:0006956, GO:0006958, GO:0001867)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>complement component 1, s subcomponent</td>
<td>C1s</td>
<td>170.55</td>
<td>10865444</td>
</tr>
<tr>
<td>complement component 2</td>
<td>C2</td>
<td>93.51</td>
<td>10828172</td>
</tr>
<tr>
<td>complement component 3</td>
<td>C3</td>
<td>138.55</td>
<td>10931755</td>
</tr>
<tr>
<td>complement component 4, gene 1</td>
<td>C4b</td>
<td>203.91</td>
<td>10828234</td>
</tr>
<tr>
<td>complement component 4, gene 2</td>
<td>C4-2</td>
<td>117.93</td>
<td>10828255</td>
</tr>
<tr>
<td>ficolin B</td>
<td>fcnb</td>
<td>152.44</td>
<td>10844013</td>
</tr>
<tr>
<td>Complement Binding Receptors (GO:0001848) and Integrins (GO:0008305)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD93 molecule (C1qr1)</td>
<td>CD93</td>
<td>96.85</td>
<td>10850534</td>
</tr>
<tr>
<td>complement component 3a receptor 1</td>
<td>C3ar1</td>
<td>51.62</td>
<td>10865370</td>
</tr>
<tr>
<td>integrin beta 2 (CD18)</td>
<td>Itgb2</td>
<td>84.13</td>
<td>10832317</td>
</tr>
<tr>
<td>integrin alpha M (CD11b)</td>
<td>Itgam</td>
<td>122.52</td>
<td>10711273</td>
</tr>
<tr>
<td>integrin alpha X (CD11c)</td>
<td>Itgax</td>
<td>282.41</td>
<td>10711305</td>
</tr>
<tr>
<td>Regulators of Complement Activation (GO:0045916, GO:0001869, GO:0001971)</td>
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</tr>
<tr>
<td>alpha-2-macroglobulin</td>
<td>a2m</td>
<td>121.17</td>
<td>10858410</td>
</tr>
<tr>
<td>CD46 molecule, complement regulatory protein (MCP)</td>
<td>CD46</td>
<td>64.55</td>
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<tr>
<td>CD55 molecule (Decay Accelerating Factor)</td>
<td>CD55</td>
<td>94.87</td>
<td>10767402</td>
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<tr>
<td>complement component 4 binding protein, beta</td>
<td>C4bpb</td>
<td>-63.15</td>
<td>10767442</td>
</tr>
<tr>
<td>serine (or cysteine) peptidase inhibitor, clade G, member 1 (C1NH)</td>
<td>Serpin1</td>
<td>54.07</td>
<td>10846859</td>
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<table>
<thead>
<tr>
<th>Complement Related Genes</th>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>% DE (Light Damage vs Dim-Reared)</th>
<th>Affymetrix Probe Set ID</th>
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<tr>
<td>C1q and tumor necrosis factor related protein 3</td>
<td>C1qtnf3</td>
<td>-112.29</td>
<td>10813676</td>
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<td>C1q and tumor necrosis factor related protein 6</td>
<td>C1qtnf6</td>
<td>62.45</td>
<td>10905309</td>
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</table>
Validation of differentially expressed microarray genes

Figure 2 Comparative qPCR validation of differentially expressed genes at 24hrs BCL. A number of significant (denoted '*' for each probeset, where P < 0.05) differentially expressed microarray genes were selected for comparative expression analysis using qPCR. Consistent increases were detected by qPCR for each gene, which confirmed the increases in microarray gene expression. The trends in qPCR expression were generally higher than those in the microarray, particularly for C1qR1 and C3aR1.
ClqTNF6, which belong to the C1q/TNF-related protein (CTRP) family of adiponectin paralogs. We tested the veracity of this data by analysing the expression of 8 of the complement genes listed in table 4, using comparative qPCR. Expression of all 8 genes corresponded well with the data obtained from the microarrays (Figure 2).

Modulation of complement-related gene expression and cell death

We examined the temporal expression of these same 8 complement genes (Figure 2) in relation to TUNEL-positive cell number in the ONL, and expression of the apoptosis-related gene (Jun (AP-1)) (Figure 3). We also included the anaphylatoxin receptor C5r1, in addition to the receptor C3aR1, even though this gene was not annotated in the microarray at the time of analysis. The temporal analysis spanned time points during BCL exposure (1, 3, 6, 12, 17, and 24hrs), as well as post-BCL exposure (3 and 7 days). Our previous analysis shows that the post-exposure time points coincide with emergence of the lesion in the area centralis.

Consistent with our previous data, we observed large increases in the number of TUNEL-positive nuclei in the ONL after 12hrs BCL, progressing to a prominent peak in cell death at 24hrs exposure (Figure 3A). Numbers of TUNEL-positive nuclei decreased substantially in the post-exposure period, at 3 and 7 days. We observed a significant upregulation in AP-1 expression after 3hrs BCL exposure, which reached peak expression at 24hrs co-incident with peak cell death and declined rapidly during the post-exposure period (Figure 3A).

Expression of complement components C1s, C3, and C4b (Figure 3B) increased significantly between 12 and 24hrs BCL, and all three components reached peak expression in the post-exposure period. C1s and C4a reached peak expression at 3
Figure 3 Expression of validated complement genes (from figure 2) over a protracted BCL time course in relation to cell death markers. **A:** The frequency of TUNEL-positive nuclei in the ONL, and expression of AP-1 were used as markers of apoptosis. Increases in TUNEL were observed after 12hrs BCL, which progressed to a peak at 24hrs. The number TUNEL-positive nuclei decreased substantially in the post-exposure period. AP-1 increased rapidly from 3hrs BCL onward to reach a peak at 24hrs, and then tapered off rapidly by 3 days post-exposure. **B:** Up-regulation of complement components C1s, C3, and C4b occurred between 12 and 24hrs BCL, and remained substantially up-regulated in the post-exposure period, particularly C3. **C:** Expression of complement receptors C1qR1, C3aR1, and C5r1 increased substantially by 17hrs BCL. C3aR1, and C5r1 continued to up-regulate into the post-exposure period, while C1qR1 reached a considerable peak at 24hrs before falling rapidly. **D:** MCP up-regulated rapidly from 12hrs BCL to reach a peak at 24hrs, while Dafl also increased over a similar time course. The complement-related gene C1qTNF6 was found to increase slightly after 24hrs BCL, which decreased slightly over the post-exposure period. The trend in expression for all genes, as well as TUNEL, was significant by one-way ANOVA (P < 0.05).
days; the data do not show what happens to C3 expression after its apparent peak at 7 days. The receptor C1qR1 reached a peak expression in conjunction with the maximal cell death and AP-1 expression, at 24hrs BCL (Figure 3C). In contrast, expression of complement receptors C3aR1 and C5R1 reached peak expression at 3-7 days post-exposure (Figure 3C); the present experiments do not show how prolonged the expression of these receptor might be. Expression of complement inhibitors MCP (CD46) and Daf1 (CD55) reached peak expression at 24hrs BCL (Figure 3D), coincident with the peak in photoreceptor apoptosis, with CD46 showing the earlier and more robust expression between 12 and 24 hours exposure. C1qTNF6 showed modest up-regulation by 24hrs, followed by a gradual decrease during the post exposure period (figure 3D).

**Spatiotemporal analysis of C3 expression and immunoreactivity following BCL**

Due to its pivotal role in the activation of all three complement pathways and robust long-term up-regulation following BCL (Figure 3B), we selected C3 for further characterization. Spatiotemporal analysis of C3 expression was conducted using *in situ* hybridization (Figure 4). In dim-reared animals, C3 expression was observed in sparsely distributed nuclei associated with the superficial retinal vasculature (Figure 4A). After 24 hrs BCL, we detected a substantial increase in these C3-positive cells in the retinal vasculature, which were preferentially recruited into superior retina (Figure 4B-C), where a 'hotspot' in photoreceptor death has been described previously. C3-expressing nuclei showed continued recruitment to the retinal vasculature in spatial correlation with the developing lesion at 3 days post-exposure (Figure 4D), and were also detected in vessels at the optic nerve head (Figure 4E-F). By 7 days post-exposure, C3-expressing cells were still present in large numbers in the retinal vasculature and
Figure 4 In situ hybridisation for C3 mRNA in the retina following exposure to BCL. A: In retinas from dim-reared animals, expression of C3 was observed infrequently in nuclei associated with the superficial vasculature (arrowhead). B-C: After 24hrs BCL C3-positive nuclei were preferentially recruited to the retinal vasculature in the superior retina (B: arrowheads); no such infiltration was found in the inferior (C). D: The recruitment of C3-positive nuclei in the retinal vasculature continued 3 days after exposure, in association with the developing lesion (arrowheads). E-F: Robust infiltration of C3-positive nuclei was observed in association with the optic nerve head after 3 days post-exposure. G-I: By 7 days post-exposure, C3-positive nuclei had infiltrated the ONL and subretinal space within the lesion (G-H), while non-lesion areas in the inferior retina remained comparable to dim-reared animals (I). J-L: 7 days after exposure C3-positive nuclei aggregated in large numbers at the edges of the lesion. M-P: C3 expression (dark-grey) in sections counter-immunolabelled with anti-IBA1 (green), showing immunoreactivity in many C3-expressing nuclei within the degenerating ONL (white arrowheads); some of these cells were ramified (asterisks). Q-U: C3 expression (dark-grey) in sections counter-immunolabelled with anti-ED1 (green), showing immunoreactivity in a number of C3-expressing nuclei associated with the edge of the lesion (white arrowheads). C, choroid; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; S, subretinal space.
Figure 5 Immunoreactivity (IR) for whole-C3 (red) and C3d (green) in the retina following exposure to BCL. A: IR for C3 was faintly detected in the choroid and retinal vasculature of dim-reared retinas (arrows and arrowheads). B-C: At 3 days post-exposure C3-IR was observed in the layer of degenerating outer segments in superior retina (B, arrowheads); inferior retina was largely unreactive to C3 (C). D-E: At 7 days post-exposure C3-IR was intense in the ONL and subretinal space in association within the lesion. F-I: At the edges of the lesion after 7 days, C3-IR was found deposited within the ONL (F, arrowheads) and in the layer of outer segments (G-H, arrowheads). J: Portions of superficial retinal vasculature showed strong IR for C3 at 7 days post-exposure. IR for C3d (K-M) showed a similar spatiotemporal distribution to C3. K: C3d IR was faintly detected in the choroid and retinal vasculature (data not shown) of dim-reared retinas. L-O: In the lesion at 7 days post-exposure, C3d-IR was widespread in deposits in the ONL (arrowheads) and subretinal space (L-M); on the lesion edge, C3d-IR was detected on photoreceptor outer segments (N-O). Blood vessels are indicated by asterisks. P-Q: Negative controls showed no specific staining for either C3 or C3d. BV, blood vessel; C, choroid; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OS, outer segment layer; RPE, retinal pigment epithelium; S, subretinal space.
optic nerve head (data not shown), and were also observed in association with the
degenerative remains of photoreceptors in the superior retina (Figure 4G-H).
Accumulations of C3-expressing cells were particularly abundant within the ONL and
subretinal space at the margin of the lesion by 7 days post-exposure (Figure 4J-L). Few-
to-none of these were present in non-lesion areas of retina at 7 days (Figure 4I). The
identity of these C3-expressing cells was investigated using microglia/macrophage
markers IBA1 (Figure 4M-O) and ED1 (Figure 4Q-U). Most C3-expressing cells were
immunoreactive (IR) for IBA1, mainly within the ONL and subretinal space associated
with the lesion (Figure 4M-O), and some with ramified morphology (Figure 4N-O,
asterisks). IR for ED1 was also shown by some C3-expressing nuclei, such as those
associated with the subretinal space in-and-around the lesion (Figure 4Q-U).

IR for C3 protein was performed using antibodies against whole-C3 (Figure
5A-J), and the long-lived cleavage fragment, C3d (Figure 5K-M). C3 was detected
faintly within the retinal vasculature and choroid of dim-reared animals (Figure 5A).
Similar levels of expression were detected in animals exposed to 24hrs BCL (data not
shown). However, at 3 days post-exposure we detected emerging C3-IR in the layer of
outer segments at the site of the developing lesion (Figure 5B); areas outside the lesion
were only faintly C3-IR (Figure 5C). By 7 days post-exposure C3-IR was more intense
(Figure 5D-I) and formed multiple deposits throughout the ONL within the lesion area,
in the subjacent subretinal space (Figure 5D-E), and in segments of the retinal
vasculature (Figure 5J). On the margins of the lesion, we observed intense C3-IR in
ONL deposits (Figure 5F-G), and on photoreceptor outer segments (H-I), coinciding
with aggregations of C3-expressing macrophages (Figure 4J-L). IR for C3d showed
very similar spatiotemporal distribution to whole-C3 following BCL (Figure 5K-O). In
dim-reared animals, C3d-IR was faintly detected in the choroid and retinal vasculature
(Figure 5K). By 7 days post-exposure, C3d had accumulated in numerous deposits
situated in the ONL and subretinal space within the lesion area (Figure 5L-M) in close association with photoreceptor cell bodies (Figure 5M), and on photoreceptor outer segments at the lesion edge (Figure 5N-O)

Discussion

The results of this study confirm robust differential expression in the neural retina of a number of complement system genes following light-induced retinal degeneration. These data are consistent with the findings from a previous microarray study. In addition, we demonstrate several key findings which elaborate on the role of complement in retinal damage induced by BCL. First, by microarray analysis and qPCR we show modulation of a suite of complement genes including opsonin mediators from classical and lectin pathways (C1s, C2, C4, Ficolin B), complement receptors (C1qR1, C3aR1, C5r1, CR3, CR4) and regulators (CD46, CD55, SERPING1, C4bp, a2m); many of which have not been reported previously. Second, we have shown the time-course of modulation of a number of these genes in relation to increasing levels of photoreceptor apoptosis, demonstrating persistence of up-regulation beyond 24 hrs exposure - the peak in cell death (C1s, C3, C4b, C3aR1, C5r1). Third, we show that microglia/macrophages synthesize C3 which is preferentially deposited in the ONL and layer of outer segments in the damaged region, and at the margins of the lesion in the post-exposure period.

Microglia synthesize C3 and mediate photoreceptor death in damaged retina

To our knowledge, this is the first investigation to specifically identify cells expressing C3 in the degenerating retina, and to determine the spatiotemporal profile of
C3 deposition in relation to the degenerating region. In this study we demonstrate that C3 is expressed by microglia/macrophages IR for IBA1 and ED1, in spatiotemporal correlation with degeneration of the superior retina and C3 deposition following exposure to BCL \(^{28}\), suggesting that these aggregating cells are responsible for the local propagation of complement in the retina following light damage. The emergence of C3-expressing cells from within the retinal vasculature and optic nerve head – rather than the parenchyma – following BCL suggests that C3 is expressed initially by recruited perivascular macrophages/monocytes \(^{43,45}\). This also supported by immunoreactivity of some cells for the monocyte/macrophage marker ED1 \(^{46}\), and with other studies describing the synthesis of C3 by macrophages \textit{in vitro} \(^{47-50}\). Expression of C3 was observed in both ramified and non-ramified IBA1-positive cells in the subretinal space at 7 days post-exposure. This late expression of C3 by ramified cells suggests that parenchymal microglia also synthesize C3 \(^{46}\), but are relatively slow to respond to BCL in this synthesis, compared to recruited monocytes. Our data strongly suggest that photoreceptors are a target of C3 deposition by infiltrating C3-expressing cells following BCL, as both C3 and C3d immunoreactivity was closely associated with photoreceptor cell bodies and outer segments in relation to the lesion.

The present findings suggest that retinal degeneration associated with the developing lesion, is mediated at least in part by C3 deposited by C3-expressing microglia/macrophages. While complement activation has beneficial properties such as aiding debris clearance by recruited phagocytes \(^4\), complement propagation has been shown to exacerbate photoreceptor death in light-damage \(^{32}\), of which C3 is a vital component that drives activation of all three complement pathways. C3 is also crucial to assembly of the C5 convertase, a mediator of the MAC complex, which may induce apoptosis or necrosis of host cells \(^{51}\). Moreover, a number of studies have shown that microglial activation and aggregation are associated with photoreceptor degeneration in
light-damage\textsuperscript{52, 53}. The association of microglia/macrophages at the edge of lesion also suggests a role in chronic expansion of the lesion characterised previously\textsuperscript{28}, although the localisation of C3-expressing microglia past 7 days post-exposure is currently unknown.

**A transcriptional profile of complement activation following BCL**

The transcriptional profile of complement gene expression following BCL provides insight into the breadth of complement activation in this model, and its likely roles in the degenerative process. First, up-regulation of macrophage receptors that recognize cleavage products of C3 (C3aR1, CR3, and CR4) and C5 (C5r1)\textsuperscript{54} after exposure to BCL, indicate extensive activation of complement in the neural retina with the involvement of phagocytic cells. Second, there is an apparent compensatory up-regulation of complement inhibitor genes (CD46, CD55, SERPING1, a2m) associated with the peak in photoreceptor death. Of these, MCP (CD46) attenuates all three complement pathways\textsuperscript{55} and is expressed by photoreceptors\textsuperscript{56}. Increased expression of CD46 suggests activation of a protective mechanism by stressed photoreceptors following BCL in an attempt to safeguard from deleterious complement activation. Third, we also find increased expression of many genes involved in the clearance of apoptotic cells. Classical pathway complement components (C1s, C2, and C4), as well as C3-fragment receptors CR3, and CR4 were expressed strongly following 24hrs BCL. This pattern of expression is consistent with a role in the removal of apoptotic cells\textsuperscript{1, 10-13}, including uptake of apoptotic cells by macrophages\textsuperscript{57, 58}. However, the ablation of C1qa in rd1 mice did not appear to delay the removal of apoptotic cells, indicating that the classical pathway – or at least C1qa – alone may not be sufficient to initiate their clearance\textsuperscript{59}. We also showed differential expression of the carbohydrate recognition
molecule Ficolin B, which promotes the removal of late-stage apoptotic cells through binding to DNA, mediated by the lectin pathway. Up-regulation of these genes suggests that a role of complement activation in this model is the efficient removal of apoptotic cells following BCL.

Conversely, our findings implicate complement activation in exacerbating light-mediated retinal damage, where complement components (C1s, C3, and C4) and anaphylatoxin receptors (C3aR1, and C5r1) showed persistent up-regulation in the post-exposure period, despite the significantly lower levels of photoreceptor death. These findings are indicative of chronic propagation of complement, long after its initial activation by the damaging stimulus, and correlate with the detrimental effect of complement demonstrated by Rohrer and colleagues. This dichotomy in the effects of complement activation has been documented previously, and likely reflects a disruption of the balance between complement activation and inhibition. In light-damage, this may be spurred by the abundance of activating surfaces (apoptotic cells, debris) that arises following the damaging stimulus. Given that alternative pathway activation is implicated in mediation of light-induced degeneration, chronic synthesis of classical components detected in this study may stimulate further activation of complement through the amplification loop initiated by the alternative pathway, as demonstrated by Rohrer and colleagues in experimental choroidal neovascularisation.

Relevance to the role of complement in AMD

Previous studies have shown that light damage in rats has features in common with the pathogenesis of atrophic AMD. While the long-term features of atrophic AMD – like pigmentary disturbance and drusen – are absent from this model, the development of a lesion aligned to the visual axis, the characteristics of the lesion (which affect the photoreceptor layer, RPE and Bruch's membrane), and its progressive
nature, occur in common with the atrophic AMD lesion. Like the widely used laser-induced model of neovascular AMD, this model employs an acute damaging stimulus to evoke long-term, site-specific changes in the retina and adjacent tissues.

The cellular events that lead to propagation of complement in the human retina are not well understood. Nonetheless, it is well established that polymorphisms in a range of complement-related genes is associated with risk of AMD, and the high levels of association between the Y402H polymorphism of the CFH gene and risk of AMD implicate failure to regulate the alternative pathway of complement activation with onset and progression of AMD. Experimental findings using the present model of retinal degeneration implicate activation of complement pathways in onset and progression of the retinal lesion, and show conclusively that microglia/macrophages express C3, which is deposited in the photoreceptor layer. In these conditions photoreceptors may become the target for activation of the alternative pathway, which would culminate in the formation of MAC, cytolysis and cytotoxic cell death. Consistent with this as a mechanism in AMD, the MAC complex has been identified in drusen of donor eyes from individuals with and without the at-risk Y402H genotypes. Moreover, accumulation of microglial cells in the macula is strongly associated with AMD pathology. In the atrophic form of AMD photoreceptor and RPE degeneration on the edges of the lesion results in progressive expansion of the lesion, and a recent study has shown that macrophages accumulate on the edges of AMD lesions. The present findings suggest that these aggregations of macrophages are focal points of C3 synthesis and deposition, which might actively progress the elimination of photoreceptors in the vicinity, if left unchecked. Indeed, several studies which have shown an association in polymorphisms of C3 with the progression of AMD to geographic atrophy. Defects in regulatory genes, including CFH, may therefore
predispose the photoreceptors to progressive degeneration through aberrant complement propagation induced by activated microglia/macrophages.

Conclusion

Our findings demonstrate that exposure to damaging light induces prolonged spatiotemporal changes in the expression of a suite of complement-related genes, and further clarify the role of complement in the progression of retinal degeneration. Our evidence suggests that complement activation in the retina has both positive and negative effects, being beneficial to the maintenance of homeostasis following injury, yet harmful because of sustained propagation of components beyond the immediate injury and clean-up period. The synthesis of C3 by microglia/macrophages implicates their recruitment in the local activation of complement following retinal damage. The findings therefore suggest that therapeutic attenuation of microglial recruitment may be useful strategy to control detrimental propagation of complement in the retina.

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Chapter Five

Summary and Conclusion
The investigations presented in this thesis provide insight into the mechanisms that govern the spatiotemporal localisation of macrophages recruited into the retina following light damage, and subsequent deposition of complement. The findings pinpoint macrophages/microglia as the cells that mediate activation of the complement system in the degenerating retina, whose focal recruitment appears to be facilitated by chemotactic signals originating from Müller cells at the area centralis.

The results presented in Chapter 2 elaborate on aspects of the focal degeneration induced by light damage, including its co-localisation with the area centralis of the rat retina, showing the emergence of an AMD-like histopathology in the post-exposure period. Outside the area centralis, the study shows recovery of the retina, with down-regulation of stress-markers, in the post-exposure period.

The data show that the focal lesion has several histological features in common with AMD, including breakdown of the blood retinal barrier, photoreceptor death, the prevalence of surviving cones over rods, macrophage recruitment, as well as reactive gliosis in Müller cells – all features of atrophic AMD. The data also show that expression of stress factors (GFAP and FGF-2) and photoreceptor degeneration continues at the margin of the lesion, up to 56 days post exposure (and possibly beyond), resulting in progressive expansion of the lesion. These findings demonstrate the potential of this model for the analysis of inflammatory mechanisms in retinal degenerations.

The paper presented in Chapter 3 shows that Ccl2 is expressed by Müller cells at the area centralis as a result of exposure to damaging light. Expression of Ccl2 is spatiotemporally coincident with the subsequent emergence of photoreceptor death at the area centralis. The data show preferential recruitment of macrophages from both retinal and choroidal vascular supplies to the site of Ccl2 expression by Müller cells.
Furthermore, these extravasating macrophages were found to provide additional stimulatory expression of Ccl2 following the initial expression driven by the Müller cells, thus mediating further macrophage recruitment.

These findings indicate a role for the neural retina in directing a localised neuroinflammatory response to retinal damage. Although macrophage and microglial activation may initially serve a beneficial function following retinal injury, extensive accumulation these cells is known to be detrimental. It is suggested that, given chemotactic processes govern the recruitment of macrophages in AMD, down-modulation of endogenous chemokine responses may be a useful therapeutic strategy to ameliorate retinal damage.

The study presented in Chapter 4 explores the relationship between macrophage recruitment, and the synthesis of complement-related genes in the retina in this model. Complement genes modulated by light damage were identified by microarray analysis and confirmed by qPCR. Those identified included opsonin mediators from classical and lectin pathways (C1s, C2, C4, Ficolin B), complement receptors (C1qR1, C3aR1, C5r1, CR3, CR4) and regulators (CD46, CD55, SERPING1, C4bp, a2m). A number of these were found to positively correlate with levels of photoreceptor apoptosis. Most significantly, the study shows by in situ hybridization that C3 deposited in the ONL and outer segments in the damaged region originates from recruited microglia/macrophages.

The findings show that exposure to damaging light induces changes in the expression of complement-related genes, including an increase in C3 expression in the post-exposure period. The demonstration of C3 synthesis by microglia/macrophages suggests that macrophage-recruitment to the site of retinal damage results in local deposition of C3, and subsequently, the death of photoreceptors and RPE cells. These
findings clarify the roles of macrophages and complement in the progression of retinal degeneration, and have direct implications for the management of atrophic AMD. That is, therapeutic attenuation of microglial/macrophage recruitment may be a useful strategy to control detrimental propagation of complement in the retina.

Future experimentation will explore the extent that these recruited macrophages contribute to complement activation and retinal degeneration in the current light damage paradigm. This may be achieved through their depletion in the retina prior to BCL exposure, utilizing injections of clondronate liposomes. Alternatively, or perhaps concurrently, a C3\(^{-}\) knockout rat could be engineered to assess the direct role of C3 expression in light-induced degeneration, and determine whether such a role is dependent on microglia/macrophage recruitment. Additionally, future directions could involve an investigation into whether modulation of Ccl2 expression following BCL alters the recruitment of C3-expressing macrophages, and thus complement activation. This could be facilitated by technologies such as RNA-interference (RNAi), whereby the expression of Ccl2 could be knocked down in Muller cells using intravitreal injections of specific short-interfering RNA (SiRNA).


