INNATE IMMUNE RESPONSE POLARIZATION AND TREATMENT POTENTIAL IN A PRECLINICAL MODEL OF DRY AGE-RELATED MACULAR DEGENERATION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Laboratory Medicine and Pathobiology
University of Toronto

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Innate immune response polarization and treatment potential in a preclinical model of dry AMD

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Doctor of Philosophy
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Abstract

Age-related Macular Degeneration (AMD) is the leading cause of blindness in the Western world, depriving older individuals of their central vision and their autonomy. At present, no treatments exist for dry AMD, nor are there animal models that reproduce the complex clinical fundus autofluorescence (FAF) findings of end-stage blinding complications, such as geographic atrophy (GA) - patches of tissue loss of the RPE and overlying neuroretina. The objective of this study was to characterize the immune response and macrophage polarization in a rodent model of GA using clinically relevant imaging modalities, and determine whether modulation of this response would alter atrophy progression. We administered sodium iodate to Sprague Dawley rats to induce loss of the RPE and subsequently the photoreceptors, resulting in retinal atrophy, and monitored the changes using a clinical confocal scanning laser ophthalmoscope (cSLO). Using the FAF channel typically employed for patients in the clinic, we have characterized five stages of atrophy progression that occurs over the period of acute RPE loss and chronic tissue response. This was further observed with our newly developed Delayed Near-Infrared Analysis (DNIRA) technique in the near-infrared channel. This novel finding also led us to detect macrophages in the areas of bright FAF, and using modified DNIRA techniques we have been able to identify and track macrophages directly in vivo. By using inflammatory markers and
quantitative PCR for polarized macrophage phenotypes on retinal and eyecup tissue samples, we identified that inflammatory M1 macrophages can be detected during the stages of atrophy progression, and that the overall tissue profile is heavily skewed towards M1 polarization. By employing intraocular gadolinium chloride and subsequently an M1-blocking drug bindarit to prevent the macrophage response, we have shown that atrophy can be prevented and that the in vivo and tissue phenotypes can be virtually normalized if inflammation is blocked. On the other hand, by further propagating the inflammatory response by combining NaIO₃ with nitroglycerin, the atrophy is expanded, which can also be subsequently blocked by bindarit. These unprecedented findings have strong implications for translation to the clinical setting for the diagnosis, monitoring and treatment of late stage dry AMD.
Acknowledgments

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Chapter 2:

Additional Publications Arising During the Course of Graduate Studies


- This was the first report of Fundus Autofluorescence used in the sodium iodate model of toxin-induced retinal atrophy. It has allowed us to further these findings to apply them as a model of geographic atrophy.


- This study reported that in primary endothelial cells oxidized and native LDL stimulates autophagosome formation, and that transient knockdown of the autophagy gene ATG7 resulted in higher LDL accumulation, suggesting that autophagy may be important in regulating exogenous lipids. This was demonstrated in the retinal pigment epithelium and choroidal endothelium in the rodent eye.


- This was the first study done in rats to show that pre-existing retinal abnormalities exist in these animals prior to any treatments, and that these abnormalities can be observed using confocal scanning laser ophthalmoscopy. Investigators are now aware of these findings, preventing them from attributing these to treatments.
### Abbreviations

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<td>Antibody</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette subfamily A member 1</td>
</tr>
<tr>
<td>AFM</td>
<td>Autofluorescent microscopy</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>AREDS</td>
<td>Age-related eye disease study</td>
</tr>
<tr>
<td>ARMS2</td>
<td>Age-related maculopathy susceptibility 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BM</td>
<td>Bruch’s membrane</td>
</tr>
<tr>
<td>BRB</td>
<td>Blood retinal barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C2</td>
<td>Complement factor 2</td>
</tr>
<tr>
<td>C3</td>
<td>Complement factor 3</td>
</tr>
<tr>
<td>C5</td>
<td>Complement factor 5</td>
</tr>
<tr>
<td>CC</td>
<td>Choriocapillaris</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C Motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine (C-C Motif) receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CEP</td>
<td>Carboxyethylpyrrole</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterylester transfer protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>CFB</td>
<td>Complement factor B</td>
</tr>
<tr>
<td>CFD</td>
<td>Complement factor D</td>
</tr>
<tr>
<td>CFH</td>
<td>Complement factor H</td>
</tr>
<tr>
<td>CFI</td>
<td>Complement factor I</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNV</td>
<td>Choroidal neovascularization</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony Stimulating Factor</td>
</tr>
<tr>
<td>cSLO</td>
<td>Confocal Scanning Laser Ophthalmoscope</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CX3CL</td>
<td>Chemokine (C-X3-C Motif) ligand</td>
</tr>
<tr>
<td>CX3CR</td>
<td>Chemokine (C-X3-C Motif) receptor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DKO</td>
<td>Double knockout</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNIRA</td>
<td>Delayed Near-Infrared Analysis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinography</td>
</tr>
<tr>
<td>FAF</td>
<td>Fundus autofluorescence</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GA</td>
<td>Geographic atrophy</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GDCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Gadolinium chloride</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione s-transferase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HRA-2</td>
<td>Heidelberg retina angiograph-2</td>
</tr>
<tr>
<td>HTRA1</td>
<td>HtrA serine peptidase 1</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICG</td>
<td>Indocyanine green</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IVT</td>
<td>Intravitreal</td>
</tr>
<tr>
<td>IκBα</td>
<td>I kappa B alpha</td>
</tr>
<tr>
<td>KLF</td>
<td>Kruppel-like factor</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LIPC</td>
<td>Lipase C</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1</td>
<td>Classically activated macrophage</td>
</tr>
<tr>
<td>M2</td>
<td>Alternatively activated macrophage</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MRC</td>
<td>Mannose receptor C type</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NaIO₃</td>
<td>Sodium iodate</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR family, pyrin domain containing 3</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>ONH</td>
<td>Optic nerve head</td>
</tr>
<tr>
<td>OxLDL</td>
<td>Oxidized LDL</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>POS</td>
<td>Photoreceptor outer segment</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PPIH</td>
<td>Peptidyl-prolyl cis-trans isomerase H</td>
</tr>
<tr>
<td>RF</td>
<td>Red-free</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPD</td>
<td>Reticular pseudodrusen</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>SD</td>
<td>Sprague dawley</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour associated macrophage</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP3</td>
<td>Tissue inhibitor of metalloproteinases-3</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona occludens protein 1</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

1.1 Structure and Components of the Eye

The posterior pole of the eye is comprised of the sensory retina, including the macula where the fovea is located, and the optic nerve head (Figure 1.1). The neural retina is made up of the outer retina and inner retina. The inner retina is the sensory part containing ganglion cells and bipolar cells which transduce visual information to the brain; the outer retina contains cone and rod photoreceptors where light is converted into chemical signals. The macula is the central portion of the retina primarily responsible for vision, and the fovea has the highest density of cone photoreceptors, and is responsible for high acuity vision. External to the photoreceptors are the retinal pigment epithelium (RPE) cells, which support the photoreceptor layer by recycling by-products of photo-transduction via phagocytosing the photoreceptor outer segments (POS), secreting factors required for photoreceptor health, and maintaining blood-retinal barrier structure and function via tight junctions. There are two blood supplies to the retina, the intra-retinal vasculature which comprises part of the blood retinal barrier through endothelial tight junctions, and the choroidal vasculature which is a dense network of fenestrated blood vessels that supply oxygen and nutrient molecules directly to the RPE through their shared basement membrane (McCaa 1982).

Figure 1.1. Anatomy of the human eye.
1.2 Dry Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a degenerative disorder of the central nervous system (CNS), and the leading cause of blindness in the elderly in the developed world. It is a debilitating progressive degeneration of the retinal pigment epithelium (RPE) and subsequently the neural retina with largely unknown etiology. The disease is characterized by drusen at the early stage, which are deposits of diverse composition formed in the outer retina posterior to the RPE layer (Figure 1.2 (left)). Progression to the later stage of dry AMD is characterized by geographic atrophy (GA) in the macular region of the retina, which results in severe loss of vision. GA appears clinically on imaging modalities such as fundus photography and fundus autofluorescence (FAF) as patches of missing RPE and photoreceptors, which are responsible for the demise of central vision (Figure 1.2 (right)). Unlike the exudative (wet) form of AMD (Figure 1.2 (central)), which can be treated with anti-Vascular Endothelial Growth Factor (anti-VEGF) therapy, there are currently no treatments for dry AMD, which accounts for approximately 90% percent of all cases of late stage disease (FFB 2016). Substantial further increases in prevalence are predicted over the next two decades due to the increasing lifespans and aging populations worldwide (Eye Diseases Prevalence Research Group 2004). Efforts to remedy this are limited by the absence of a suitable preclinical model of dry AMD, and little information regarding the mechanisms of disease. There is a multitude of theories about the etiology of disease, including a substantial genetic component, with abnormalities of the complement system identified in patient populations (Weber, Charbel Issa et al. 2014). Other factors for disease progression include environmental risk factors such as age and smoking (Smith, Assink et al. 2001, Willeford and Rapp 2012). However, due to limited availability and difficulty in obtaining relevant ocular samples, there is a lack of information about specific human mechanisms that lead to this disease. As such, animal models are crucial for elucidating its underlying processes. In this thesis I will evaluate a rodent model of dry AMD using novel in vivo imaging techniques, focusing on the cellular innate immune component of the disease, and targeting macrophage polarization.

The following subsections provide a more in depth description of the stages of dry AMD.
1.2.1 **Early Disease - Drusen**

Drusen are yellowish deposits with rounded borders that are the first visible changes in the eyes of patients. Drusen are located between the RPE and Bruch’s membrane (BM), and consist of diverse protein and lipid components that can vary substantially between patients (Buschini, Piras et al. 2011). Drusen are believed to originate from either the RPE or the choroidal blood flow. Since drusen contain a substantial amount of RPE components, this suggests that the deposits are primarily of RPE origin, likely the products of metabolic waste accumulation. However, a number of drusen deposits are also known to show features of atherosclerotic plaques such as cholesterol deposits, suggesting a systemic component and pointing to a vascular origin (Malek, Li et al. 2003, Pikuleva and Curcio 2014). Drusen do not necessarily indicate AMD disease and don’t typically cause changes in vision, however the likelihood in progression to the late stages is substantially increased with drusen type and amount (Chew, Clemons et al. 2014).

In addition to drusen, early disease can also be characterized by hyperpigmented and hypopigmented areas of the RPE. This may be related to cell death or proliferating RPE cells. Finally, a subtype of drusen called reticular pseudodrusen (RPD) is now known to be strongly associated with late AMD (Zweifel, Spaide et al. 2010). These are typically observed best using a confocal Scanning Laser Ophthalmoscope (cSLO) or Optical Coherence Tomography (OCT), as they tend to be smaller than regular drusen and present in the subretinal space amongst photoreceptor segments rather than below the RPE (Schmitz-Valckenberg, Steinberg et al. 2010).
1.2.2 Late Disease – Geographic Atrophy

Progression to late AMD consists of two broad subtypes. Neovascular, or wet AMD results when choroidal blood vessels break through BM and leak into the RPE and retinal layers, and is known as choroidal neovascularization (CNV). This typically results in substantial damage to the macula which can include scarring and severe vision loss (Jager, Mieler et al. 2008). Wet AMD is now partially treatable with intraocular anti-VEGF injections (Rosenfeld, Brown et al. 2006). The second subtype is atrophic or dry AMD, also known as GA. This is characterized by primary RPE atrophy, followed by atrophy of the choroidal vasculature and the sensory retina. Photoreceptor degeneration is believed to follow RPE degeneration. Unlike in wet AMD, the BM remains intact and no new neovascularization takes place in dry AMD (Sarks, Sarks et al. 1988). Following the death of RPE, retinal cells can undergo apoptosis ultimately causing a reduction in vision. GA occurs in up to 90% of the cases of late stage AMD, and there is currently no available treatments for this subtype of the disease (FFB 2016).

Consensus lies in the observation that soft drusen and pigment abnormalities are the key features that increase the risk to developing late stage AMD (Klein, Klein et al. 2007, Wang, Rochtchina et al. 2007). Interestingly, drusen often regress during development of GA. GA can also follow RPE detachments (Cukras, Agron et al. 2010). Atrophy manifests as a single patch or multiple focal areas, usually in the perifoveal region first (Maguire and Vine 1986). These areas can progress and coalesce, with eventual foveal involvement in the later stages. Typically, if one eye develops GA, the fellow eye is likely to develop it as well, often symmetrically (Bellmann, Jorzik et al. 2002). The initiating event in progression to GA is unknown. Furthermore, patients with GA can often develop CNV in the same eye, with reasons for this switch also unknown (Sunness 1999).

1.3 Clinically Relevant Imaging Modalities

The eye is a unique organ in that it is optically clear, allowing for direct visualization of ocular components using optical techniques. As such, imaging methods are key components of clinical diagnosis and are now becoming important in translational research. In fact, the only way to diagnose, monitor and treat a number of ocular diseases relies heavily on the use of in vivo ophthalmic imaging modalities. While imaging allows for relative ease of diagnosis and
monitoring of treatment outcomes in patients, there is still ample potential for development and improvement of better imaging techniques and equipment.

This section provides an overview on some of the most common imaging methods used to observe the fundus and diagnose dry AMD.

1.3.1 Fundus Photography

High resolution colour fundus photos are the standard in the clinic (Figure 1.2), allowing for easy recording of baseline imaging and following patients through multiple visits, with minimal discomfort. Color photos are acquired using red, green and blue wavelengths that can be separated for analysis if required. Often certain retinal conditions can be observed better in a specific wavelength. Green, or red-free imaging for example, is especially useful for imaging vasculature, which appears dark in the photos (Abràmoff, Garvin et al. 2010). RPD on the other hand, are especially visible in the blue channel of a colour photo (Cohen, Dubois et al. 2007).

1.3.2 Confocal Scanning Laser Ophthalmoscopy and Fundus Autofluorescence

Recent advances in imaging technology have allowed for the development of higher sensitivity imaging modalities, such as the cSLO, which uses laser light to capture images in a number of channels. These include infrared (IR) with reflectance in 830 nm, red-free (RF), with excitation in 488 nm using a red barrier filter, fundus autofluorescence (FAF), with excitation/emission wavelengths of 488/500 nm, and near-infrared (NIR) fluorescence with excitation at 795 nm and emission at 810 nm. It is well documented that the RPE cells of the retina accumulate lipofuscin as they age, a toxic metabolic by-product associated with both cellular senescence and the high metabolic rate of active phagocytosis of the POS that are shed daily. Significant to clinical and preclinical examination of the eye are the optical properties that allow lipofuscin to be viewed using FAF (Delori, Dorey et al. 1995). Light of 488 nm wavelength shone into the retina excites lipofuscin fluorophores including A2E, which emit at a slightly longer wavelength, and permits viewing the RPE monolayer in the posterior eye (Boulton, McKechnie et al. 1989). This endogenous FAF appears as a light glow that can be detected using the cSLO optical system that captures images in vivo in a manner similar to a confocal microscope (Figure 1.3). Since the cSLO uses confocal technology it allows for imaging in multiple planes of focus, capable of separately viewing layers of the inner retina, outer retina, RPE, and choroid.
Abnormalities in the macula can appear as hypo or hyperfluorescent on FAF. Specifically, clinical GA or other RPE loss can be viewed as patches of hypofluorescence against the normal FAF of the RPE cells (Schmitz-Valckenberg, Fleckenstein et al. 2009). In other cases, increased fluorescence surrounding GA, at the border or junctional zones, can be observed to precede development of new areas of GA (Holz, Bellman et al. 2001). Increased hyperfluorescent signal is associated with higher rates of disease progression (Schmitz-Valkenberg, 2006). Furthermore, different patterns of FAF have been identified as different subtypes of GA, and correlate to differences in disease progression (Figure 1.4). Patterns of FAF such as banded and diffuse are associated with much higher rates of progression than focal or abnormal pattern classifications (Schmitz-Valckenberg, Alten et al. 2011). This makes FAF a useful tool for monitoring RPE in vivo over extended periods of time and during disease progression, allowing for observation and measurement of RPE atrophy over time. Recently, the Food and Drug Administration (FDA) has announced that GA progression rate has been approved as a primary outcome measure in clinical trials for the disease (Csaky, Richman et al. 2008). However, to date the source of the hyperfluorescent signal that shows a link to increased disease progression has not been fully elucidated.
1.3.3 Angiography

Angiography is a medical imaging technique that allows for visualization of the vasculature in real time during physiological and disease states. In the eye, two sets of circulation supply the blood to ocular tissues. Retinal vessels comprise part of the blood retinal barrier (BRB) through endothelial tight junctions and occupy the inner and mid retina. Choroidal vasculature is a dense network of blood vessels that supply oxygen and nutrients to the posterior cells including the RPE. The choriocapillaris (CC) endothelium is highly fenestrated where it meets the RPE cells, enabling for molecules to easily pass to the RPE. Angiography allows investigating both retinal and choroidal vessel abnormalities in the eye using two dyes – fluorescein and indocyanine green (ICG) respectively.
1.3.3.1 **Fluorescein Angiography**

Fluorescein has excitation/emission wavelengths of 490/520 nm, and is used to readily visualize the retinal vasculature in patients. The cSLO uses FAF channel wavelengths to detect fluorescein angiography in patients in real time. The dye is a sodium salt and highly water soluble, allowing for easy clearance from the systemic circulation. Following systemic injection, fluorescein can be visualized in the fundus within seconds, which initiates the rapid series of image acquisitions (**Figure 1.5**). The angiography lasts only about 5 minutes, after which the dye typically leaves the vasculature. Normally no leakage of the dye occurs through the blood-retinal barrier, however in conditions where blood vessels are damaged, or during CNV where new blood vessels grow and leak, diffuse fluorescein fluorescence can be observed in the fundus and is indicative of abnormalities.

![Figure 1.5. Clinical fluorescein and ICG angiography](image)

**Figure 1.5. Clinical fluorescein and ICG angiography.** Early phase fluorescein (left) and ICG (right) angiography shows the retinal and choroidal vasculature respectively. Used with permission from Wolters Kluwer Health, Inc. (Jorzik, Bindewald et al. 2005).

Fluorescein angiography may show abnormal choroidal perfusion in AMD patients, and may indicate progression to geographic atrophy (Holz, 1994). It may also better show certain types of drusen, especially smaller ones (Arnold, Quaranta et al. 1997, Spaide and Curcio 2010). GA appears as regions of late hyperfluorescence on fluorescein angiography, resulting from staining of deeper layers of the eye without the normal blockage of overlying RPE, also known as a “window defect” (Bennett and Barry 2009).

1.3.3.2 **Indocyanine Green Angiography**

ICG dye is a protein bound conjugated fluorophore with excitation/emission wavelengths around the 785/810 nm range. Commercially available cSLO devices have been designed with a special
channel to image the ICG fluorescence emission at this wavelength. It is an amphiphilic molecule that consists of polycyclic lipophilic moieties, and hydrophilic sulfonate groups (Desmettre, Devoisselle et al. 2000). Imaging with ICG allows to bypass the melanin of the RPE and choroid more readily than fluorescein, and allows for better visualization of the choroidal vasculature (Herbort, Mantovani et al. 2012). ICG is 98% protein bound in the vasculature, and can be observed to slowly flux through the vascular fenestration in the CC. Proteins that bind ICG include albumin, low density lipoproteins (LDL) and high density lipoproteins (HDL). This is contrary to the small sodium fluorescein molecule that does not bind proteins, passes through vasculature quickly and gets cleared rapidly (Desmettre, Devoisselle et al. 2000, Herbort, Mantovani et al. 2012).

The advent of ICG angiography is relatively new compared to fluorescein, and not all the ICG findings have been well established in the clinical setting. Recent insights using ICG have allowed for differentiation of different types of CNV lesions. Typically images are taken in early phase (5-10 min), and late phase (30 min) following injection. Unlike fluorescein, ICG dye results in some background staining as it leaks out of choroidal capillaries (Figure 1.5). It is also recognized that when the CC is inflamed, it can result in nonperfusion with areas of hypofluorescence on ICG. Localized inflammation can also prevent diffusion of ICG to certain areas resulting in hypofluorescence. These types of observations are typically seen in disease such as uveitis (Herbort, 2012). On the other hand, GA is believed to appear dark on ICG angiography due to lack, rather than obstruction, of CC.

1.4 Risk Factors for Dry AMD

AMD is a highly multifactorial disease, similar to other diseases of aging such as heart disease. It encompasses a number of risk factors, including aging, smoking, diet, exercise and genetics.

This section serves to provide a more in depth look at some of the most important risk factors associated with developing AMD.

1.4.1 Genetics

The most significant risk for developing AMD has been identified to be the complement factor H (CFH) gene and the HtrA Serine Peptidase 1 (HTRA1) gene, which are evident in over 60% of severe cases (Haines, Hauser et al. 2005, Yang, Camp et al. 2006). CFH can act to inhibit
activation of the complement cascade thereby limiting the amplification of inflammation. The Y402H allele, which has been identified as the susceptibility allele in AMD, causes an impairment in the inhibitory function of CFH (Edwards, Ritter et al. 2005, Hageman, Anderson et al. 2005, Klein, Zeiss et al. 2005). Consequently this results in sustained complement activation. CFH is known to be expressed in RPE cells, and has been identified in drusen deposits (Chen, Forrester et al. 2007, Clark, Bishop et al. 2010). HTRA1 and Age-Related Maculopathy Susceptibility 2 (ARMS2) are also significantly associated with AMD susceptibility, however the roles of these genes are not well understood (Jakobsdottir, Conley et al., Ross, Bojanowski et al. 2007). Other important gene associations include variants encoding for complement factor B (CFB) and C2, both located on the major histocompatibility complex III region chromosome, C3 – the convergence point of the complement pathways, and complement factor I (CFI), another complement regulatory protein (Gold, Merriam et al. 2006, Yates, Sepp et al. 2007, Fagerness, Maller et al. 2008). In addition to these, other genes including those for apolipoprotein (Apo) E, tissue inhibitor of metalloproteinases-3 (TIMP3), Lipase C (LIPC), cholesterylester transfer protein (CETP), and ATP-Binding Cassette Subfamily A Member 1 (ABCA1) have also been identified as risk factors for development of AMD, though not to the same extent as the aforementioned ones (Yu, Reynolds et al. 2011, Yu, Reynolds et al. 2012). This suggests that the pathways involved in AMD are primarily those for innate immunity and lipoprotein metabolism.

1.4.2 Smoking

Smoking is the most consistent modifiable risk factor for AMD, and the strongest epidemiologic risk for developing the disease. The relationship is dose dependent, with patients who smoke more often, and for longer, having a higher chance of developing the disease, which ranges between 2-3 times for enhancing risk (Willeford and Rapp 2012). Smoking promotes oxidative damage of the retina and RPE, and causes ischemia in the retinal and choroidal tissue (Thornton, Edwards et al. 2005). In addition, toxic components delivered through cigarette smoke overwhelm endogenous antioxidants with excess reactive oxygen species (ROS). This results in a diminished capacity of the antioxidants to carry out normal metabolic functions and scavenging of baseline ROS levels under physiological conditions. This points to a significant oxidative stress component in the progression of disease, specifically relating to the RPE cells, and those of BM and CC (Willeford and Rapp 2012). In vitro cigarette smoke components have been
identified to impair RPE function and generate oxidative and nitrosyl stress in RPE cells (Kunchithapautham, Atkinson et al. 2014, Mansoor, Gupta et al. 2014).

1.4.3 Ageing

Age is the number one risk factor for AMD. This is evident in all studies, across all ethnicities and geographic locations, with increases evident after 60 years of age and increasing steeply after 75 years of age (Smith, Assink et al. 2001). There is a prominent decrease in the retinal thickness with age, which is attributable to the number and characteristics of the retinal cell layers (Eriksson and Alm 2009). The RPE layer is especially sensitive to the ageing process as it is the nurse cell for the photoreceptors and thus highly metabolic, performing functions such as daily phagocytosis of shed POS, and is also non-dividing (fully differentiated). Evidence shows there is a decrease of RPE cells with age (Gao and Hollyfield 1992). Ageing can also result in loss of apical microvilli and basal interdigitations, increase in pigment granules, dysfunctional melanosomes, and accumulation of lipofuscin granules in RPE (Docchio, Boulton et al. 1991, Różanowski, Cuenco et al. 2008). Lipofuscin is a byproduct of cellular metabolism, consisting of retinoid derivatives, and a strong generator of ROS (Rózanowska, Jarvis-Evans et al. 1995). It is also a potent photosensitizer, with increased exposure of lipofuscin to light resulting in free radical generation. Free radicals can cause lipid peroxidation, protein oxidation, loss of lysosomal structure and lower lysosomal pH, decreased phagocytosis, inhibition of proton pumps, mitochondrial damage and RPE cell death (Davies, Elliott et al. 2001, Shamsi and Boulton 2001, Godley, Shamsi et al. 2005). Lipofuscin deposits are also found in drusen, the early stages of AMD (Salvi, Akhtar et al. 2006).

Mitochondrial dysfunction has been reported with increased age, and a decrease in mitochondrial respiration correlates with AMD progression, as does an age-related decline of antioxidant activities of enzymes such as superoxide dismutase (SOD) and glutathione s-transferase (GST) (Maeda, Crabb et al. 2005, Justilien, Pang et al. 2007, Lin, Xu et al. 2011). BM, an important component separating the RPE from the choroid, also shows important changes with age. These include accumulation of lipids resulting in increased thickness, calcification and loss of elastin, preventing functional transport of fluid and nutrients between the choroidal vasculature and the RPE (Ugarte, Hussain et al. 2006, Curcio, Johnson et al. 2011). Taken together, all these factors increase the susceptibility to disease with increased age due to the increasingly vulnerable
cellular components and amplified by-product deposition. Ultimately, the ageing retina shows an inflammatory response, including complement and microglial activation that opens the doors for chronic, sustained low grade inflammation that’s believed to play a role in the disease (Xu, Chen et al. 2009).

1.5 Inflammation

Abnormal innate immunity and heterogeneous phagocytic cell responses underlie a number of diseases of the CNS (Kigerl, Gensel et al. 2009, David and Kroner 2011, Durafourt, Moore et al. 2012). In a relatively recent paradigm shift, it is now well recognized that there is a substantial immune component to AMD. As described above, clinical studies have identified genes that confer susceptibility to the disease, such as those for complement activation and components of innate immunity (Klein, Myers et al. 2013). Phagocytic cell types are associated with the disease, but few studies have delved deep enough to properly characterize this response or target it as a viable therapeutic approach (Forrester 2003, Cherepanoff, McMenamin et al. 2010, Cao, Shen et al. 2011, Mettu, Wielgus et al. 2012).

This section provides an in depth look at some of the main components of innate immunity and the role they play in dry AMD.

1.5.1 Humoural Immunity (Complement Cascade) in AMD

The complement cascade is a proteolytic cascade that acts in response to the detection of antigens, pathogen-associated molecular patterns (PAMPs), and danger associated molecular patterns (DAMPs). Three different pathways, classic, lectin or alternative, converge on a central molecule (C3) which results in a common downstream mechanism ultimately leading to the formation of a membrane attack complex (MAC) that causes cells to lyse (Figure 1.6). The majority of evidence supports the involvement of the alternative pathway in AMD that results in a C3bBb amplification loop cleaving more C3, sustaining inflammatory activation and causing tissue damage. This sustained pro-inflammatory state propagates MAC formation and cell lysis. CFH is an important regulator of the complement cascade which helps cleave and degrade C3.

Histological and proteomic identification of elevated complement proteins in AMD donor eyes compared to controls, specifically localized to drusen has been shown (Anderson, Mullins et al. 2002, Crabb, Miyagi et al. 2002, Wang, Clark et al. 2010). This is in concert with the genetic
studies that have identified CFH polymorphisms and other complement protein genes leading to increased susceptibility to AMD. In addition, blood samples from AMD patients show increased systemic complement activation (Scholl, Charbel Issa et al. 2008). The RPE is believed to be an important cell type in regulating complement activation, in particular CFH levels, since RPE cells can synthesize this protein (Chen, Forrester et al. 2007). In vitro and animal studies show that the RPE complement response is also affected by external stimuli such as oxidative stress that comprise other AMD risk factors (Thurman, Renner et al. 2009). Though compelling, thus far, the evidence for complement involvement is only supportive of early disease development. No links have been made to tie complement activation to progression of the late stages of AMD, including GA.

Figure 1.6. Complement cascade. Used with permission from InTech, CC BY-NC-SA 3.0 license (Joszi 2011).

1.5.2 Cellular Immunity (Macrophages) in AMD

Macrophages are terminally differentiated myeloid cells, a class that includes blood monocytes, dendritic cells, and microglia of the CNS (Cassetta, Cassol et al. 2011). Macrophages are known
to be the initial line of defense of the immune system, and are essential for eliminating invading tissue pathogens. Microglia are tissue resident macrophages specifically localized to the CNS, including the retina. These cells are normally quiescent under homeostatic conditions, however can be activated in response to stress and injury. Quiescent resident cells are responsible for functions that maintain homeostasis, including scavenging, clearance of debris, and modulation of the extracellular environment (Davies, Jenkins et al. 2013). Activated amoeboid microglia are phenotypically and functionally indistinguishable from macrophages, and are thus often classified together. Macrophages and microglia have been identified in human AMD samples and are shown to be activated during disease (Cherepanoff, McMenamin et al. 2010, Ma, Coon et al. 2013). In normal tissue, resident macrophages form extensive networks in the choroid that contact the RPE layer, but may contribute to deposit accumulation due to malfunctioning waste disposal mechanisms (Forrester 2003). Macrophages are believed to be drawn to the sub-RPE space due to the expression of monocyte chemotactic protein-1 (MCP-1) by RPE cells, and genetic rodent models where MCP-1 and its receptor are knocked out appear to display a retinal pathology (Ambati, Anand et al. 2003). Furthermore, during pathological change, both macrophages and microglia appear to be recruited to the outer retina and accumulate in the subretinal space, which may stimulate disease propagation (Saraswathy, Wu et al. 2006, Luhmann, Robbie et al. 2009, Ma, Zhao et al. 2009). Macrophages, while extremely important in the progression of the innate immune response, are highly variable cells that interestingly can display opposing functions. Macrophages are capable of both plasticity, via activation from the external environment, and polarization, or change in phenotype (Paletta-Silva and Meyer-Fernandes 2012).

1.5.2.1 **Macrophage Polarization**

Conventionally, macrophages are capable of polarizing towards a classically activated M1 response or an alternatively activated M2 response. In a simplified view, M2 cells provide housekeeping functions such as scavenging debris and are suggestively good, while M1 cells are aggressive and propagate inflammation and are suggestively bad (Murray and Wynn 2011). Macrophages are believed to polarize into these phenotypes based on signals in their environment. Inflammatory M1 macrophages can generate ROS, proinflammatory cytokines such as iNOS, TNFα and MCP-1, and can be destructive to tissue if not tightly regulated (**Figure 1.7(a)**). As such there becomes a need for alternatively activated, M2 cells that are responsible
for wound healing, tissue remodeling and repair (Figure 1.7(b)). Anti-inflammatory, and sometimes proangiogenic M2 macrophages can thus display scavenging receptors such as macrophage mannose receptor (MMR), and CD163, and secrete anti-inflammatory cytokines such as IL-10, and growth factors for tissue repair and fibrosis. M1 macrophages can be stimulated by bacterial lipopolysaccharide (LPS) through toll-like receptor 4 (TLR4) and interferon gamma (IFNγ), to produce ROS and nitrous oxide (NO), TNFα, IL-1, and MMPs for extracellular matrix degradation. Meanwhile, M2 macrophages are generally induced by IL-4 and IL-13, and produce factors such as IL-10, TIMP1, PDGF and TGFβ to assist in wound healing and fibrosis by fibroblast accumulation, angiogenesis and collagen deposition (Murray and Wynn 2011). Normally tissue resident macrophages are believed to be non-inflammatory (M2), they ingest material and can recruit additional macrophages. These recruited cells are generally believed to be pro-inflammatory (M1) (Cassetta, Cassol et al. 2011, Murray and Wynn 2011).

Figure 1.7. Broad description of macrophage polarization and the phenotypes associated with M1 and M2 macrophage responses. Adapted with permission from Macmillan Publishers Ltd (Murray and Wynn 2011).

Macrophages can be described by the transcriptome, specifically transcription factors such as STAT1, STAT3, STAT6, and suppressors of cytokine signaling (SOCS) 1 and 3, which switch the M1 and M2 gene transcription on and off in cells (Figure 1.8). These transcriptional networks induce other M1 and M2 markers including interleukins, chemokines and cytokines that differentiate the two macrophage subtypes (Mantovani, Sica et al. 2007, Sica and Mantovani 2012). Markers of polarization can vary between species and there is still much debate about the
certainty of polarized phenotypes and their specific markers (Cassetta, Cassol et al. 2011). Generally, gene expression points to primarily one phenotype over the other, however as macrophages are highly plastic and have the propensity to switch phenotype with even subtle changes in environment, there is as much a continuum of specific macrophage phenotypes, as there are discrete phenotypes present (Sica and Mantovani 2012). In addition, the conditions that present with opportunities for macrophage polarization vary greatly across disease types and subtypes, making discrete phenotypes often difficult to establish. Polarization occurs both in physiological conditions, such as ageing and development, and in pathological conditions (Sica and Mantovani 2012).

![Figure 1.8](image)

**Figure 1.8. Transcription factors involved in M1 and M2 macrophage polarization.** Modified with permission from The American Society for Clinical Investigation (Sica and Mantovani 2012).
1.5.2.2 M1/M2 in Disease

In cardiovascular disease atherosclerotic lesions show a predominance of the M1 phenotype compared to M2, and it is believed that a model of phenotype switching from M2 to M1 occurs in this case, rather than a direct recruitment of M1 cells (Chinetti-Gbaguidi and Staels 2011). These macrophages secrete proinflammatory cytokines such as IL-6 in patients with coronary artery disease, and predict poor outcomes in these patients (Iwata and Nagai 2012). Similarly, tissue macrophages in lean mice tend to express an M2 profile of polarization, while diet-induced obesity results in a shift to M1 activation and contributes to insulin resistance (Chinetti-Gbaguidi and Staels 2011). In these models M1 macrophages can be suppressed with angiotensin receptor blockers and peroxisome proliferator-activated receptor gamma (PPARγ) agonists, which promote M2 polarization and prevent inflammation (Chinetti-Gbaguidi and Staels 2011, Yamamoto, Yancey et al. 2011, Stoger, Gijbels et al. 2012).

In models of diabetic retinopathy and ocular glutamate toxicity M2 polarized phagocytes promote tissue repair, reduce oxidative stress and modulate inflammation, indicating a protective effect (London, Itskovitch et al. 2011, Marchetti, Yanes et al. 2011). There is also evidence that M1 macrophages displaying TNFα and IL-12 may infiltrate the retina in a model of retinal degeneration (Cruz-Guilloty, Saeed et al. 2013). During CNS injury in mice, an M1 macrophage response is shown to be rapidly induced and maintained, overwhelming the smaller transient M2 response, with M1 macrophages being neurotoxic, while M2 being protective (Kigerl, Gensel et al. 2009). Furthermore, in the Mdx mouse model of Duchenne muscular dystrophy, the M2-associated cytokines IL-4 and IL-10 can deactivate M1 macrophages and promote tissue repair (Villalta, Nguyen et al. 2009). In the RCS rat, transfer of anti-retinal antibodies causes degeneration by disrupting the BRB, upregulating MCP-1 and attracting macrophages. These Iba1+ and CD163+ cells are indicative of primarily an M2 response, however the upregulation of MCP-1 and VEGF suggests M1 activity as well (Kyger, Worley et al. 2013). Another study in RCS rats showed that intravitreal injection of GM-CSF can prevent apoptosis and degeneration of photoreceptors, while also upregulating STAT3, which is believed to be associated primarily with an M2 response in myeloid cells, as well as with neuroprotection (Schallenberg, Charalambous et al. 2012, Sica and Mantovani 2012). Microglia in the CNS display similar markers of M1 and M2 polarization, supporting their resemblance to other macrophage
populations (Durafourt, Moore et al. 2012). This is evident in both rodent and human microglial populations (Michelucci, Heurtaux et al. 2009, Melief, Koning et al. 2012).

On the other hand, tumour associated macrophages (TAMs) tend to be highly M2 polarized, promoting angiogenesis and tissue deposition in tumours and increasing tumour growth. In this case, M1 macrophages and their associated cytokines can antagonize TAMs and exert protective effects (Murray and Wynn 2011). Similarly, the important M2 cytokine IL-10 is deemed vital in vascularization that marks CNV in mice and thereby wet AMD (Apte, Richter et al. 2006, Kelly, Ali Khan et al. 2007). IL-10 KO mice develop more inflammation but reduced CNV, while increased macrophage populations can inhibit CNV (Apte, Richter et al. 2006). In addition, IL-10 KO mice show a decreased CNV response to ischemia, and blocking IL-10 in WT mice can reduce CNV. This supports that IL-10 promotes angiogenesis by polarizing macrophages to a proangiogenic phenotype (Dace, Khan et al. 2008). These anti-angiogenic responses are impaired in older mice, and transfer of macrophages from young mice into older mice can help inhibit CNV. In addition, IFNγ (M1) polarized macrophages can inhibit CNV supporting opposing roles for these polarized phenotypes (Kelly, Ali Khan et al. 2007). It further implicates a separate mechanism of disease in conditions where vascularization plays an important role, compared to other pro-inflammatory and neurological disease where the M2 polarization component is suggested to be protective.

It is evident that the dynamics of macrophage polarization are complex and highly variable across disease types and animal models. While various cytokine and tissue marker profiles have been identified in human and rodent retinal samples, to date studies are inconsistent in identifying specific cellular sources of these markers, and their contributions to disease progression. Herein lies the inherent difficulty in studying macrophage polarization, especially in disease where definitive preclinical models have not been well established.

1.5.2.3 Evidence for M1/M2 in Clinical AMD

There is a small body of evidence for macrophage polarization in AMD. One study measured expression levels of M1 and M2 chemokines and markers in dry vs wet AMD. The findings suggest that during normal aging there is a shift away from pro-inflammatory M1 phenotype towards an anti-inflammatory M2 phenotype. Additionally, gene expression levels suggest more
M1 activation in dry AMD and more M2 in wet AMD, however the sample size and the markers used to obtain this data place significant limits on these findings (Cao, Shen et al. 2011). Another study looked at levels of iNOS, a prominent M1 marker across different stages of AMD progression. It was concluded that there are more macrophages overall in wet AMD, and positive iNOS expressing cells are present in early AMD and wet AMD. There were no iNOS cells identified in late dry AMD eyes, which would point to wet AMD being more M1, while dry AMD potentially being more M2. One major drawback of this study was the inability to co-stain multiple markers, so it is not possible to exclude the possibility that cells other than macrophages may have been expressing the iNOS signal (Cherepanoff, McMenamin et al. 2010). At this point, studies are inconclusive, however with significant limitations in both studies, more evidence is needed to obtain definitive evidence for macrophage polarization in human AMD samples.

1.6 Treatment for Dry AMD

The search for a treatment for dry AMD is a multi-billion dollar industry, due to the ever-increasing aging population, and the so far elusive results. Clinical trials targeting the main genetic factors that play a role in the disease, such as complement have thus far failed to reach the clinic. There is widespread evidence about the importance of oxidative stress and the immune response in pathophysiology of dry AMD, and there are a number of treatments undergoing clinical trials targeting these pathways. Unlike for the wet AMD subtype, which can now be effectively treated with sustained anti-VEGF intraocular injections, VEGF-targeting therapies do not hold any therapeutic potential for dry AMD. The only currently available “treatment” for dry AMD is a steady intake of antioxidants via vitamin supplementation based on the Age-Related Eye Disease Study (AREDS), which has been shown to slow progression from early to late forms of the disease (Age-Related Eye Disease Study Research 2001).

This section serves to describe some of the treatments that have underwent clinical trials for dry AMD and a proposed new treatment to target the inflammatory component of the disease.

1.6.1 AREDS Vitamins

The AREDS/AREDS2 vitamin supplementation is thus far the only formulation that has shown any protection during AMD progression. The first AREDS formula contains a combination of beta-carotene, vitamins C, E, B, zinc, and copper, and can reduce the onset of late AMD, as well
as slow progression of GA (Age-Related Eye Disease Study Research 2001, Age-Related Eye Disease Study Research Group 2007, Coleman and Chew 2007). These vitamins and minerals target oxidative species that pose a risk to the retinal cells. However, these formulations must be taken with caution, as there are correlations to higher risk of the disease with very high doses of vitamins such as zinc and beta-carotene (Tan, Wang et al. 2008). In addition, high dose beta-carotene can increase the risk of other disease, such as lung cancer in smokers (Omenn, Goodman et al. 1996). Overall, the results appear to point to synergistic effects of multiple vitamins, as studies looking at individual vitamins from this formulation have shown variable and inconclusive results (Seddon, Ajani et al. 1994, VandenLangenberg, Mares-Perlman et al. 1998, Tan, Wang et al. 2008). This is logical, since vitamins can act in concert and may depend on one another, for example vitamin C is needed for regeneration of vitamin E (Stoyanovsky, Goldman et al. 1995). Furthermore, patients with the AMD CFH allele Y402H/Y402H appear to have a reduced response to zinc, making high dose supplementation futile in these populations (Klein, Francis et al. 2008).

Currently AREDS supplementation is only recommended to patients who are at high risk for progression to advanced AMD. More recent work points to an increased intake of lutein, zeaxanthin and omega-3 fatty acids, which are important components of macular pigment and retinal membranes, in helping reduce progression of AMD (Chong, Kreis et al. 2008, Age-Related Eye Disease Study 2 Research Group 2013). The macular region contains a very high concentration of carotenoids and xanthophylls including lutein and zeaxanthin which are obtained from the diet, and give the macula its characteristic colour (Loane, Kelliher et al. 2008). These pigments are highly important for quenching ROS and filtering light thereby protecting the retina (Loane, Kelliher et al. 2008). Since the study outcomes, these components have now been added to the AREDS supplementation formula, known as AREDS2, which has a decreased zinc concentration and lutein/zeaxanthin as a replacement for beta-carotene. These changes offer only slight improvements in disease progression, but decrease the risk of lung cancer associated with beta-carotene (Age-Related Eye Disease Study 2 Research Group 2013). This type of vitamin supplementation however, is by no means a definitive treatment for dry AMD.
1.6.2 Clinical Trials

The best way to measure clinical trial outcome for GA is to monitor progression of the patch of atrophy in patients. This has only recently become an accepted clinical trial endpoint for this disease (Csaky, Richman et al. 2008). It is a more relevant measure of late disease than visual acuity, as some patients may retain central acuity despite large areas of extra-foveal GA, while others may suffer early reductions in visual acuity due to central location of a small area of atrophy. Further, changes in acuity can in some cases take years to detect, extending the duration of clinical trials. Conversely, progression of GA can take place in under a year, and is in all cases associated with regions of irreversible vision loss, since it represents permanent atrophy of the RPE and photoreceptors. In addition, GA can be reliably identified and quantified using a number of imaging modalities, most notably FAF, making it a useful parameter for both trial enrollment and therapeutic outcome.

The GATE study (Alcon) measured the impact of a serotonin-1A-agonist for protection of photoreceptors, and while it appeared effective in animal models of light damage, the phase II study was terminated due to ineffectiveness (Collier, Patel et al. 2011). Brimonidine tartrate treatment for protection of photoreceptors showed efficacy for animal models of retinitis pigmentosa, but also failed to reach statistical significance for its primary endpoints in clinical trial (Saylor, McLoon et al. 2009). Treatment with fenretinide, a synthetic retinoid derivative that binds a retinal protein in circulation and thereby downregulates photoreceptor metabolism, showed efficacy in a model of Stargardt’s disease, and has potential to benefit early dry AMD, but full study results were not disclosed (Radu, Han et al. 2005). A study with Pfizer, targeting amyloid beta with an intravenous antibody showed safety and efficacy, but no further trials have been reported (Ding, Lin et al. 2008). OT-551 by Othera Pharmaceuticals was tested as a potent free radical scavenger with anti-inflammatory and antiangiogenic properties as a topical nuclear factor kappa B (NFkB) inhibitor, and while it was readily bioavailable in the retina following topical administration, it failed to meet primary endpoints of reducing GA (Sternberg, Rosenfeld et al. 2010, Wong, Kam et al. 2010).

1.6.3 Targeting Inflammation

Since the discovery of inflammatory gene associations with dry AMD onset and progression, a number of trials have been initiated targeting this component of the disease, in particular the
complement cascade. A C3 inhibitor POT-4 passed safety and efficacy studies, however no further studies were carried out using the molecule (Zhang, Zhang et al. 2012). The monoclonal antibody against C5 Eculizumab failed to meet its endpoints in efficacy trials (Yehoshua, Filho et al. 2014). A monoclonal Fab fragment antibody to complement factor D (CFD) showed an effect in phase II trials, but only in patients who carried the risk allele (Le, Gibiansky et al. 2015).

Sirolimus, an agent that has been used to suppress host response during transplantation, was tested subconjunctivally and intravitreally in a randomized phase I/II study (NIH), though no treatment benefits were identified in either case (Wong, Dresner et al. 2013, Petrou, Cunningham et al. 2015). Intravitreal Fluocinonlone implant (Alimera Sciences) was tested in a randomized, double-blind GA trial, as a continual corticosteroidal effect to suppress the immune response, however the study was terminated and results showed no difference across study groups (Taskintuna, Elsayed et al. 2016). A study of copaxone (glatiramer acetate), an immunomodulatory agent that downregulates inflammatory cytokines and was shown effective in a model of Alzheimer’s disease was undergoing a phase II/II study, however it was suspended with no posted results (Landa, Butovsky et al. 2008). Collectively, most of the studies targeting dry AMD, and specifically GA have been relatively unsuccessful.

It is unclear why the majority of studies targeting dry AMD have been failing, suspended or terminated. However, it is believed that since the cause of AMD is likely multifactorial, affecting a number of pathways in concert, that treating the disease may require a similar combinatorial approach. In addition, the primary endpoints for some of the earlier studies may have been incorrect, as it was only recently that the FDA has allowed measurement of GA as a valid endpoint. Perhaps previously targeted outcomes such as drusen area is not an effective method for clinical trials, since it is known that drusen can disappear before progression of the disease. In addition, study duration may play a role, since some of the studies were only carried out for a period of 6 months, which may not be sufficient to observe a change in GA. Currently, a major rate-limiting step in advancing therapies for dry AMD is the absence of a suitable preclinical model to test drug targets. This is evident by the testing of compounds in models of other retinal disease including inherited retinitis pigmentosa and Stargardt’s, as well as other disease such as multiple sclerosis and Alzheimer’s disease, which although similar in some aspects, likely have
entirely different fundamental mechanisms. Finally, testing therapies for protection of photoreceptors may not be an effective method, since it is widely suggested that the RPE cells are the primary targets of dry AMD (Kaneko, Dridi et al. 2011, Bhutto and Lutty 2012). As such, even with preservation of photoreceptors and the sensory retina, without a healthy RPE, these cells cannot survive for prolonged periods of time and will eventually degenerate. Ultimately good study design and the use of appropriate preclinical models are critical to the discovery of a successful therapy.

1.7 Bindarit for Inflammatory Disease

Bindarit is a small molecule indazolic derivative originally designed to target inflammation associated with arthritis (Guglielmotti, D’Onofrio et al. 2002). In addition to reducing this type of inflammation, bindarit has proven effective in a number of animal models associated with upregulation of the inflammatory response. For example, it was effective in inhibiting toxin-induced colitis in mice, as well as MCP-1 activity in intestinal epithelial cells (Bhatia, Landolfi et al. 2008). Furthermore, bindarit was shown to reduce neointima formation in animal models of vascular injury by reducing neointimal macrophage content (Grassia, Maddaluno et al. 2009). It can also mitigate myocardial inflammation, fibrosis and vascular remodeling (Lin, Zhu et al. 2009). Most notably, it can reduce MCP-1 expression in all 3 cell types of the neurovascular unit, including astrocytes, microglia and brain endothelia cells, and is effective in diminishing incidence and onset of experimental autoimmune encephalitis in vivo (Ge, Shrestha et al. 2012).

Bindarit downregulates the gene expression of MCP-1, as well as the other MCPs located within the same gene cluster, in humans being MCP-2 and MCP-3 (Mora, Guglielmotti et al. 2012). These molecules are induced by inflammatory stimuli such as bacterial LPS, and result in upregulation of the inflammatory response and sustained inflammation. After an insult, I kappa B alpha (IκBα)-sequestered inactive dimers of NFκB are released due to phosphorylation and degradation of IκBα. This results in activation of the canonical NFκB pathway that leads to translocation into the nucleus and binding of the dimers to the κB consensus sites, specifically on the proximal region of the promoter. This proximal promoter region is where MCP-1 transcription is activated under both physiological and insult-related conditions. However, under physiological conditions, the transcription is controlled primarily by Sp1/AP-1 transcription factor binding, whereas during an insult, NFκB dimers bind to the promoter. One of these is the
p65/p50 dimer, which binds specifically to activate the canonical NFκB pathway. This pathway is related to upregulation of inflammatory mediators including MCP-1, as well as IL-12, and other downstream cytokines.

Bindarit acts by directly reducing the IκBα phosphorylation, and thereby translocation of the p65/p50 subunits to the nucleus and their transcriptional activity (Figure 1.9). The downstream effects result in reduced binding of the subunits to the proximal promoter site and thereby reduced expression of the MCPs. Bindarit has no action on the non-canonical pathway of NFκB, which includes other dimer subunits such as Rel-B. In addition, bindarit is reported to act only on the MCP promoter and not on other promoters (Mora, Guglielmotti et al. 2012). Despite the early descriptions of the CCL-2 KO mouse models that suggested CCL2 elimination may be attributable in some part to development of AMD, due to effectiveness of CCL2 blockade in models of inflammation, it was predicted that this drug can be effective in the retina to reduce inflammation associated with M1 macrophage signaling.
Figure 1.9 Mechanism of bindarit action. Stimulus-induced activation of NFκB pathway is modulated by bindarit pre-treatment. Bindarit causes a reduction of IκBα and p65 phosphorylation, a subsequent significant reduction of the nuclear translocation of p65-constituted dimers and consequently, a reduced recruitment of these transcription factors to the κB sites of MCP promoters. The overall effect of this combined action is the significant inhibition of MCPs that ultimately leads to downregulation of inflammation, particularly at the lesion site. Used with permission from Taylor & Francis (Mora, Guglielmotti et al. 2012).

1.8 Animal Models of AMD

Currently there are a number of animal models used to study the various aspects of the pathology associated with dry AMD. These include cytokine and chemokine modifications, complement, glucose and lipid metabolism and components of the oxidative stress response. According to Cruz-Guilloty et al., animal models of dry AMD are primarily based on findings in human studies, attempting to mimic human histopathology (Cruz-Guilloty, Echegaray et al. 2011). Genetic models may not be ideal in this case, as they often only address only a small component of the overall pathology within this complex and multifactorial disease. As such, it is difficult to discern the specific implications that these types of findings have for the patient population. Another major drawback of the majority of the current models is the requirement for a late age onset, making it difficult to conduct short-term preclinical studies. Overall, no models presently display the FAF patterns of progression nor patches of GA that are observed in patients in the clinic (Figure 1.10).

This section serves to provide an overview of some of the most common animal models that are currently used to study AMD, and the features they typically present with.

1.8.1 Cytokines and Chemokines

The MCP-1, also known as Chemokine (C-C Motif) Ligand (CCL) 2, and Chemokine (C-X3-C Motif) Receptor (CX3CR) 1 murine models of retinal degeneration are some of the earliest model attempts at targeting inflammatory mediators in AMD. Inflammatory monocytes in the blood display both Chemokine (C-C Motif) Receptor (CCR) 2 (usually high) and CX3CR1 receptors (usually low), while non-inflammatory monocytes only display CX3CR1. There is no evidence for CCR2 in normal tissue retinal macrophages and microglia (Raoul, Auvynet et al. 2010). Therefore contribution of these cells in ocular tissue suggests a blood-derived recruitment. In the retina the CX3CL1 chemokine is expressed in retinal neurons and RPE constitutively and only quiescent microglia express the CX3CR1 receptor. There is no receptor for this chemokine
on RPE. Levels of CCL2 cytokine are generally low in the retina and RPE under normal conditions, but can be upregulated during inflammation, aging and oxidative stress. CCL2 can be induced in RPE by subretinal macrophages (Raoul, Auvynet et al. 2010). Therefore it appears that the CX3CR1 axis plays a role in retinal homeostasis and general microglial trafficking, while the CCR2 axis is important during inflammation.

**Figure 1.10 Accepted models of AMD.** Used with permission from Elsevier (Pennesi, Neuringer et al. 2012).

An allele of the CX3CR1 gene is associated with AMD, resulting in a loss of chemotaxis or increased adherence to the ligand, suggesting dysfunctional signaling of this axis may play a role in the disease (Raoul, Auvynet et al. 2010). Clinical drusen are known to contain CX3CR1, suggesting that drusen contain microglial components. Atrophic AMD is associated with increased intraocular CCL2 and subretinal CCR2 positive inflammatory monocyte infiltration
CCL2 is also increased intraocularly in wet AMD (Jonas, Tao et al. 2010). Positive CCL2 and CCR2 cells can also be observed in areas of GA (Despriet, Bergen et al. 2008, Jonas, Tao et al. 2010, Sennlaub, Auvynet et al. 2013). Therefore CCL2/CCR2 appears to have a strong association with AMD in patients - possibly both wet and dry.

The model described by Ambati et al was the first to suggest a role of CCL2 in AMD, however the study aimed to show a protective role for the cytokine in retinal tissue. Only 4/15 CCL2 KO mice, and 3/13 CCR2 KO mice in the study showed any retinal changes, and this was not evident until 18 months of age (Ambati, Anand et al. 2003). The data has not been replicated to date, and a more recent review has dismissed it (Raoul, Auvynet et al. 2010). Luhmann et al supported these contrary findings in their study and suggested that the autofluorescent lesions observed under the KO conditions are not the same as clinical drusen, may be part of a normal ageing process of the mice, or and potentially accelerated in the KO model. The concluding findings were that CCL2 deficiency alone does not lead to any photoreceptor degeneration or spontaneous lesions (Luhmann, Robbie et al. 2009, Raoul, Auvynet et al. 2010, Luhmann, Lange et al. 2012). This is consistent with the theory that CCR2+ cells do not play a large role in the retinal tissue under homeostatic conditions. To date, this model is the only one that suggests that the onset of AMD may be tied to a knockout of CCL2 or CCR2 alone, and these findings have never been reproduced. This is rarely referred to in the literature as a model of AMD.

The CX3CR1/CCL2 double knockout (DKO) mouse model was initially shown to present with lesions on cSLO, FAF, IR and OCT with loss of POS and thinning of the retina (Tuo, Bojanowski et al. 2007, Zhou, Sheets et al. 2011). However, this model was later identified as having the RD8 mutation in the background, and thus not entirely a model of AMD but rather of genetic retinal degeneration. The damage to the RPE was determined to be a secondary feature, and absence of CCL2 and CX3CR1 alone did not significantly affect the structure of the gross retinal architecture (Luhmann, Lange et al. 2012, Vessey, Greferath et al. 2012). Independently generated mice without the RD8 mutation did not present any novel features, and were indistinguishable from CCL2 KO, CX3CR1 KO and WT mice (Raoul, Auvynet et al. 2010).
Out of the chemokine models mentioned in this section, the closest likeness to clinical features appears in the CX3CR1 KO mouse. It is more than anything a model of retinal homeostasis dysfunction, as the result is an accumulation of inflammatory microglia/macrophages that can subsequently lead to dysfunction in the tissue. The CX3CR1 defect appears to further affect the CCL2 associated macrophage population, likely due to release of pro-inflammatory factors that draw these cells in. The migratory deficit may contribute to a reduced trafficking of macrophages/microglia away from the subretinal space, due to their adherence to membrane bound CX3XL1 chemokine, which results in POS phagocytosis by these cells. POS and lipid phagocytosis subsequently results in formation of foam-like cells and appearance of drusen-like deposits on fundus, though the accumulations are subretinal and not sub-RPE. Homeostatic clearance by macrophages is present under normal physiological conditions, however with a trafficking dysfunction it can lead to a photoreceptor degeneration (Combadiere, Feumi et al. 2007, Raoul, Feumi et al. 2008, Raoul, Auvynet et al. 2010). To emphasize, none of the CCL2, CCR2 or CX3CR1 models get primary RPE degeneration, but rather a primary inflammatory cell defect that leads to a secondary photoreceptor degeneration.

1.8.2 Complement

Since the discovery of complement genes as playing a major role in susceptibility to AMD, a number of animal models have emerged targeting the complement pathway. Mice that lack CFH develop retinal abnormalities at 2 years of age. These include decreased visual acuity and electroretinography (ERG) response, subretinal autofluorescence, complement deposition in the retina and disorganized POS (Coffey, Gias et al. 2007). Furthermore, transgenic mice constructed to display the CFH Y402H polymorphism display a greater number of deposits reminiscent of drusen than WT or the CFH KO mice. The mice also have a thickened BM and C3 deposition, however these mice do not appear to have photoreceptor atrophy (at one year of age) (Ufret-Vincenty, Aredo et al. 2010). More recently, it was shown that the Y402H polymorphism results in a decreased binding of CFH to malondialdehyde, which is a product of lipid peroxidation and can be found in AMD (Weismann, Hartvigsen et al. 2011). Loss of the binding results in decreased ability of CFH to downregulate the activation response when in contact with malondialdehyde. This has shed some new light on the way complement activation is tied to the oxidative stress in AMD.
1.8.3 **Glucose and Lipid Metabolism**

Lipid and glucose metabolism are both important components of AMD, with genes that play a role in these functions conferring susceptibility to the disease. Lipid and cholesterol deposition can be observed along BM with age and is believed to interfere with membrane transport between the CC and RPE. These accumulations are known as basal linear and basal laminar deposits. It was shown that older mice (at least 8 months) fed a high fat diet can develop basal laminar deposits (Dithmar, Sharara et al. 2001, Cousins, Espinosa-Heidmann et al. 2002). Similarly, animals fed a high glycemic index diet show increased basal laminar deposits and advanced glycation end products (AGEs), which are known components of drusen, in the RPE (Crabb, Miyagi et al. 2002, Uchiki, Weikel et al. 2012, Weikel and Taylor 2012). ApoE KO, and transgenic ApoE mice with human ApoE2 and E4 alleles show thickened BM and changes in the RPE (Dithmar, Curcio et al. 2000, Malek, Johnson et al. 2005). The ApoE4 mice also display evidence of photoreceptor atrophy, and feeding them a high cholesterol diet results in amyloid beta accumulations, another component of drusen (Anderson, Mullins et al. 2002, Malek, Johnson et al. 2005). Other transgenic mouse models including ApoB100, and LDL receptor KOs also correlate to basal laminar deposits, and thickened BM respectively (Rudolf, Winkler et al. 2005, Fujihara, Bartels et al. 2009). Finally, cluster of differentiation (CD) 36 KO mice accumulate oxidized LDL (OxLDL) even on a regular diet, and show a thickened BM. This receptor is expressed in the RPE and may be important in maintaining lipoprotein homeostasis, with a possible protective effect against AMD development. (Gordiyenko, Campos et al. 2004, Picard, Houssier et al. 2010).

1.8.4 **Oxidative Stress**

Oxidative stress models are an attractive route for studying AMD, since environmental oxidative stress poses a high risk for development of the disease. For example, smoking is known to confer one of the highest environmental risks for AMD, and high rates of disease progression are hypothesized to be due to the already high metabolic demand of the retina, high susceptibility to oxidative stress due to a large concentration of oxidizable fatty acids, and presence of highly phototoxic molecules including lipofuscin (Cai, Nelson et al. 2000). In fact, individuals with AMD have a much higher systemic levels of lipid peroxidation and oxidative stressors, such as NO and MDA compared to healthy controls (Evereklioglu, Er et al. 2003). Furthermore,
antioxidants such as vitamin supplementation are currently the only route of slowing progression of dry AMD, pointing to antagonism of these reactive species as a preventative measure.

Modeling oxidative stress has been carried out by immunization with carboxyethylpyrrole (CEP) adduct proteins to induce retinal degeneration in mice. These mice show C3 deposition, sub-RPE deposits, RPE swelling and lysis, invasion of macrophages and photoreceptor death, in addition to a thickened BM (Hollyfield, Bonilha et al. 2008, Hollyfield, Perez et al. 2010). The model aims to mimic the presence of oxidized fatty acids in the retina that result in CEP-proteins present in drusen of patients. Another model uses iron overload to induce RPE hypertrophy, hypopigmentation, subretinal deposits, photoreceptor atrophy and macrophage infiltration. These mice also show hyperautofluorescence and complement deposition, making it an attractive model. However, due to the knockout of ferroxidases ceruloplasmin and hephaestin, these mice often die young due to other systemic disorders, limiting their usefulness (Hadziahmetovic, Dentchev et al. 2008). Superoxide dismutase 1 (SOD1) KO mice may display signs of drusen-like deposits, oxidative damage to RPE, and thickened BM, suggesting that SOD anti-oxidation plays an important role in AMD (Imamura, Noda et al. 2006). Changes are evident in mice only after 7 months of age. In addition, while SOD2 KO mice die young, partial degradation of SOD2 can demonstrate oxidative damage and presence of CEP-modified proteins in the retina, as well as some RPE degeneration and increased autofluorescence (Justilien, Pang et al. 2007). The OXYS rat model, an inbred strain of Wistar rat is susceptible to hydroxyl radical degeneration and lipid peroxidation, resulting in mitochondrial oxidative damage and accelerated senescence. Still, these rats only develop photoreceptor atrophy, decreased ERG and CC damage around 12 months of age (Markovets, Saprunova et al. 2011). Other models attempted at testing various combinations of cigarette chemical exposure, blue light damage, and high fat diets, but only showed changes in BM and basal laminar deposits (Cousins, Espinosa-Heidmann et al. 2002, Espinosa-Heidmann, Suner et al. 2006). No other significant changes were observed, demonstrating the challenges in using multifactorial approaches to model retinal changes, despite the known multifactorial contributors to pathology.

1.8.4.1 Sodium Iodate

Sodium iodate (NaIO₃) is the sodium salt of iodic acid, a solid white powder, which forms a clear, colourless solution upon dilution in saline. It is a potent oxidizing agent, and given as a
systemic chemical toxin results in selective degeneration of the retinal epithelium and neuroretina. The properties and systemic toxicology of iodate have been well reviewed (Burgi, Schaffner et al. 2001). Early studies determining these effects were carried out by Sorsby in 1941 and Noell in 1953 in rabbits, and showed that a single IV injection results in selective damage to the retina without much effect on other organs and tissues at the dose given (Sorsby 1941, Noell 1953, Sorsby and Reading 1964).

This oxidative stressor has been used for decades to damage RPE cells in vivo, however the majority of studies have never suggested that this may be a potential model of late stage GA, despite its direct effect on the RPE cells. The RPE are highly susceptible to oxidative stress due to the elevated metabolic activity and large number of mitochondria in these cells. The toxin is believed to damage the cells by damaging the RPE membranes, junctions holding the cell monolayer together, and proteins within the cells, including those of the mitochondria and lysosomes (Figure 1.11). RPE damage occurs rapidly, within 24 hours the cells necrose resulting in no support for the photoreceptors, which subsequently apoptose. As such, it is one of the only models that targets the RPE cells primarily, and the photoreceptors secondarily, which is what is believed to occur in dry AMD. In rodents, we and others have shown that NaIO₃ can act in a dose dependent way to damage the RPE and subsequently the neuroretina, making it an attractive model that can be relatively easily controlled to induce a desired response (Kiuchi, Yoshizawa et al. 2002, Enzmann, Row et al. 2006, Franco, Zulliger et al. 2009, Baek, Liang et al. 2015). In addition, unlike the rabbit NaIO₃ model, there is little to no evidence that any regeneration takes place in rodents, making it also an attractive model to study long-term degenerative changes.

There has been considerable debate over how applicable this toxin is as a model of retinal degeneration. Literature on models of AMD does not recognize the NaIO₃ toxin-induced model as a means of studying this disease (Ramkumar, Zhang et al. 2010, Cruz-Guilloty, Echegaray et al. 2011, Pennesi, Neuringer et al. 2012). However, to date there has not been enough information using this model to determine the extent of the inflammatory response that takes place, the gene expression profiles that follow the onset of RPE death, and the in vivo changes that can be observed following NaIO₃. On top of that, much of the literature that addresses the changes in this model utilized limited histological techniques from decades ago, and provided very limited answers to questions even regarding histopathology. As such there were many gaps
in knowledge regarding the suitability of this model for studying the late blinding complications of dry AMD including GA. This study seeks to fill in some of those gaps and confirms its relevance to clinical disease.
**Figure 1.11 Proposed mechanisms of NaIO₃ early action.** Studies of this model collectively identify 6 main early mechanisms that play a role in NaIO₃ toxicity to the RPE. (1) NaIO₃ molecules infuse from the choroidal circulation and come into contact with the basal plasma membrane. This results in inactivation of proteins in zona occludens junctions and adherens junctions (PTEN and other protein SH groups). (2) Rupture of the basal plasma membrane and collapse of microvilli, causing paracellular and intracellular fluid flux, loss of adhesion between POS and RPE microvilli, as well as protein denaturation and potentially a breakdown of the tight junctions of the ELM. (3) Organelle (lysosome, mitochondria, ER) edema and rupture, and NaIO₃ molecules come in contact with RPE organelles resulting in inactivation of protein enzymes in organelles (lactate dehydrogenase, succinate dehydrogenase, acid phosphatase, phosphate dehydrogenase), likely via SH group oxidation, and organelle fragmentation. (4) Mitochondrial depolarization causing accumulation of p62. (5) Increased melanosome conversion of glycine to glyoxylate with H₂O₂ intermediate, causing a further increase in ROS. (6) Melanosome rupture and release of toxic components into the cell, which exacerbate necrosis (necrosis occurs 6-12 hours post NaIO₃).
1.9 Research Objectives

Studies of retinal disease have noted activation of resident microglia and migration of circulating monocyte-derived macrophages into retinal tissue; however few have looked at phenotype distribution in retinal pathology and specifically AMD. There is a small body of work on macrophage polarization and differential macrophage phenotypes associated with dry AMD, however these studies have significant limitations, and provide contradictory evidence. It is necessary to clarify and discern the data in order to gain a better understanding of the current body of work, and more evidence is needed to determine the definitive role that macrophage polarization plays in dry AMD and in GA specifically.

Since macrophages are suspected to play a role in the pathogenesis of AMD and polarization has been identified in other neurodegenerative and immune mediated disease, this study therefore hypothesized that phagocytic immune cell polarization determined the development, phenotype, and rates of progression of dry AMD. This study addressed the hypothesis that elements of macrophage polarization contribute to the inflammatory/anti-inflammatory environment in the posterior eye, and subsequently to the pathogenesis of dry AMD (Figure 1.12). Due to the lack of available effective rodent models to study the macrophage response, a part of this study was dedicated to the characterization of an in vivo model of irreversible atrophy phenotypically mimicking GA. This was carried out using newly developed imaging modalities and a clinical scale cSLO.

It was proposed that (i) differences in macrophage phenotypes can be identified during disease progression over time in a preclinical rodent model of late stage GA, (ii) depletion or manipulation of the M1/M2 response would alter the in vivo and histological features of disease, determined using FAF and other clinically-relevant imaging modalities, tissue analysis, and protein/messenger RNA (mRNA) expression, and (iii) these differences in polarization would lead to increased understanding of the mechanism of the disease, aiding in therapeutic discovery.
1.9.1 Study Aims

**Hypothesis**

M2 = normal house-keeping functions, resting state

M1 = disease, inflammatory state

**Figure 1.12. Hypothesis overview and schematic.** The balance between M2 housekeeping macrophages and the M1 inflammatory macrophages during disease. Not enough clearance by “good” housekeeping resident macrophages during the early stages of disease may result in drusen, which are deposits along BM and indicative of early AMD. Meanwhile too much activation by “bad” inflammatory macrophages during late stage disease may destroy surrounding tissue leading to patches of GA.

The following questions were addressed in this study:

1. Whether using systemic NaIO$_3$ can generate a suitable in vivo rodent model of atrophy that can be characterized using clinically relevant imaging modalities to show evidence of GA and FAF patterns of disease.

2. Whether the model of GA shows evidence for local or systemic macrophage activation that can be identified in vivo in patches of atrophy, using fluorescent techniques and clinical modalities, and whether these spatial and temporal distributions change over time.
3. Whether immune cells expressing M1 and M2 phenotypes are present, whether polarization markers can be identified on activated cells, and whether polarized phenotypes are expressed differentially during disease progression over time in tissue samples.

4. Whether modulation of the inflammatory response can result in changes of polarization profiles, and alter atrophy progression. Specifically, whether decreasing the M1 inflammatory response can prevent disease progression and expansion and result in protection of the RPE and neural retina.
2.1 ABSTRACT

Patches of atrophy of the retinal pigment epithelium (RPE) have not been described in rodent models of retinal degeneration, as they have in the clinical setting using fundus autofluorescence (FAF). We hypothesized that pre-labeling the RPE would increase contrast and allow for improved visualization of RPE loss in vivo. Here we demonstrate a novel technique termed “Delayed Near-Infrared Analysis (DNIRA)” that permits ready detection of rat RPE, using optical imaging in the near-infrared spectrum with aid of indocyanine green (ICG) dye. Using DNIRA we demonstrate a fluorescent RPE signal that is detected using cSLO up to 28 days following ICG injection. This signal is apparent only after ICG injection, is dose-dependent, requires the presence of the ICG filters (795/810 nm excitation/emission), does not appear in the IR reflectance (830 nm) channel, and is eliminated in the presence of sodium iodate, a toxin that causes RPE loss. Rat RPE explants confirm internalization of ICG dye. Together with normal retinal electrophysiology, these findings demonstrate that DNIRA is a novel and safe non-invasive optical imaging technique for in vivo visualization of the RPE in models of retinal disease.

2.2 INTRODUCTION

Blinding eye disease such as non-exudative (dry) age-related macular degeneration (AMD) and inherited retinal degenerations are characterized by patchy loss of the RPE and its overlying photoreceptors. Clinical evaluation of affected individuals identifies the presence of so-called “window defects” in the outer retina/RPE through which an observer gains unobstructed view to the underlying tissue (Bennett and Barry 2009). In patients, these patches of RPE loss appear hypofluorescent when evaluated with FAF imaging, a non-invasive technique that has transformed the ability to locate and quantify disease of the RPE (von Ruckmann, Fitzke et al.
1997). FAF uses blue light to stimulate lipofuscin, a fluorescent byproduct of cellular activity and stress that accumulates in the RPE layer during aging and disease (Boulton, McKechnie et al. 1989, Delori, Dorey et al. 1995). Despite its significant role in the clinic, FAF has only recently been applied to preclinical models of disease, but to date observations of aged or mutant mice have identified punctate hyperfluorescent spots rather than regions of hypofluorescence (Xu, Chen et al. 2008, Luhmann, Robbie et al. 2009). We propose this may be due to insufficient optical contrast between normal and absent RPE in the rodent eye, resulting in no evident differences between areas of normal and absent cells. As such, we asked whether the RPE could be made more visible using a fluorescent dye that was compatible with current in vivo imaging systems such as the cSLO, and whether such pre-labeling would increase contrast and make areas of induced RPE loss evident.

ICG is an FDA-approved dye used for ocular angiography due to its optical properties in the near-infrared (NIR) spectrum, and the resulting ability to better view the deep choroidal blood vessels in addition to retinal vessels. ICG angiography is normally assessed during the transit phase or in the minutes thereafter to detect vascular abnormalities such as leakage or anomalous anatomy (Landsman, Kwant et al. 1976, Dzurinko, Gurwood et al. 2004, Gess, Fung et al. 2011). However in this study we use ICG to observe the RPE layer in vivo in the days and weeks following systemic injection, and suggest that this technique is valuable in the preclinical modeling of disease and for the development of novel pharmaceutical, biological or regenerative treatments.

2.3 METHODS

2.3.1 Animal Studies

Animals were handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the humane use of animals in ophthalmic research, and according to the Canadian Council on Animal Care guidelines. Animals were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg), and pupils dilated with a single drop of 0.8% tropicamide in 5% phenylephrine hydrochloride solution (Diophenyl-T, Sandoz Canada Inc). GenTeal lubricating eye drops (Novartis, Canada), were repeatedly applied to the corneal surface during all procedures.
2.3.2 **Confocal Scanning Laser Ophthalmoscopy**

In vivo images were acquired using a commercially available cSLO (Heidelberg Retinal Angiography, HRA-2, Heidelberg Engineering, Germany). Images were obtained in the RF, FAF (488/500 nm excitation/emission), IR reflectance (830 nm) and ICG fluorescence (795/810 nm excitation/emission) channels.

2.3.3 **ICG and Fluorescein Angiography**

ICG dye (Cardiogreen, Sigma) was freshly prepared prior to experimentation to a final stock concentration of 5.0 mg/ml in sterile water. A 24-gauge catheter was inserted into the tail vein, and ICG dye infused at doses of 0.35, or 5.0 mg/kg. Images were taken prior to injection (baseline), during dye circulation, and at various intervals thereafter out to 20 minutes. In a subset of animals, 200 kD fluorescein-dextran (Sigma) at 5.0 mg/ml, was injected via tail vein catheter to yield a final dose of 5.0 mg/kg. ICG and fluorescein angiography was performed simultaneously in a subset of animals only, otherwise fluorescein was not injected. Angiographic images were obtained in the fluorescein and ICG channels with excitation and emission filters of 488/500 nm and 795/810 nm respectively.

2.3.4 **Delayed Near-Infrared Analysis (DNIRA)**

DNIRA images were obtained in the days and weeks after ICG injection using the ICG angiography settings, with excitation/emission filters in place, but without re-injecting the dye after day 0. Images were taken 2/3, 7/8, 21 and 28 days after angiography. Angiography was not performed again during the time-course of the study.

2.3.5 **Sodium Iodate**

In a subset of animals (n=7) the RPE toxin sodium iodate (NaIO₃, Sigma) was injected systemically at a dosage of 45 mg/kg body weight, via a 24-gauge catheter inserted into the tail vein. In these animals ICG injection (0.35 mg/kg) and angiography were performed at day 0 immediately prior to NaIO₃ injection.

2.3.6 **Ex Vivo RPE Labeling**

Rat eyes were enucleated, and posterior eyecups devoid of overlying retina were isolated in RPMI-1640 cell culture media (Wisent Inc). RPE monolayers were mechanically transferred
from posterior eye cups onto polylysine coated microscope slides by apposition for 1.5 hours at room temperature. Regions of transferred RPE cells were outlined using a hydrophobic barrier pen and their presence confirmed by white light microscopy using a Nikon Eclipse TS100 microscope with DS-Fi1 Digital Sight Capture imaging system. ICG at 0.25 mg/ml was added in cell culture media at 200 µl total volume per slide, and incubated with RPE cells for 30 min at 37°C and 5% CO₂. Cells were washed with PBS, and imaged a second time. RPE layers were thereafter permeabilized with 0.5% Triton X-100 for 15 minutes at room temperature, washed with PBS, and re-evaluated a final time by white-light microscopy. In a subset of animals, RPE monolayers were obtained 24 hours following injection of systemic ICG or saline control, and the slides observed using the cSLO system in the ICG and FAF channels to capture the associated RPE fluorescence.

2.3.7 **Immunofluorescence Microscopy**

Zona occludens protein 1 (ZO-1, 402200, Invitrogen) immunohistochemistry was performed using a standard protocol. Briefly, cells were fixed with 4% PFA/PBS for 15 minutes, and blocked with 1.25% BSA in TBS for 30 minutes at room temperature. Primary rabbit anti-ZO-1 was used at 2.5 µg/ml in 1.25% BSA/TBS and incubated for 1 hour at room temperature. Cells were washed in TBS and incubated with goat anti-rabbit 488 (Invitrogen) at 5 µg/ml for 1 hour at room temperature, washed, and counterstained with To-Pro-3 (Invitrogen). Some RPE monolayers were stained with 647 nm conjugated phalloidin (Invitrogen) for 20 minutes at room temperature and counterstained with Sytox green nuclear stain (Invitrogen). Slides were mounted with fluorescent mounting medium (Dako) and images acquired using a Leica TCS SL confocal fluorescence microscope (Leica Microsystems, GmbH, Wetzlar, Germany), with Leica Confocal Software V 2.61.

2.3.8 **Electroretinography**

The bright-flash ERG response was evaluated in animals using the Espion (DiagnosysLLC, USA) mini-Ganzfeld system following high (5.0 mg/kg) and low (0.35 mg/kg) dose administration of ICG, or saline control. Following anesthesia, animals were placed on an electrically silent heating pad and gold coil electrodes placed at the edge of the cornea after application of GenTeal lubricating drops. Following a short train of dim flashes (0.01 candela
s/m², 1.0 Hz), the photopic b-wave response was evaluated using a single bright flash (3 candela s/m²) that we previously determined consistently approximates the maximum b-wave amplitude.

2.3.9 Statistical Analysis

Three groups of ERG b-wave amplitudes were compared: control (no ICG, n=6), low dose ICG (0.35 mg/kg, n=8) and high dose ICG (5.0 mg/kg, n=10). A two-tailed paired Student’s t-test with a confidence interval (CI) of 95% was performed to compare the initial day 0 and final day 21 b-wave amplitudes for each group. For each eye, t=0 values were subtracted from t=21 values and the change in amplitude over time between groups was then compared. A small downwards trend was noted in all groups including controls, which prompted a comparison of the slopes using analysis of covariance (ANCOVA). Normalized values were plotted against time, and a linear regression was determined for each group using GraphPad Prism 5.0 statistical software. “Compare slopes” function was used to perform ANCOVA using a CI of 95%.

2.4 RESULTS & DISCUSSION

Representative baseline (pre-ICG) cSLO findings in the SD rat eye are shown in Figure 2.1(a), and along with saline-treated animals in Figure 2.2(a), serve as normal control for all experimentation. RF (green dominant) imaging identifies the optic nerve head (ONH), radial blood vessels of the retina and nerve fibre layer. Endogenous IR reflectance (830 nm) also identifies the vasculature with some imaging of the deeper choroidal vessels. Consistent with human studies FAF of the rat eye produces an image characterized by a faint, homogenous glow that is lacking in detail, is obscured by the radial retinal blood vessels, and decreases nearing the ONH (Delori, Dorey et al. 1995, von Ruckmann, Fitzke et al. 1997). Fluorescein and ICG angiography shows normal vasculature of the retina and choroid respectively. Without ICG dye, but with the same NIR excitation and emission filters in place, no or negligible signal is observed in the eye (Figure 2.1(b)). Scan lines are evident and the normally prominent retinal vasculature is barely or not detectable. This lack of NIR signal was observed in over 60 eyes. However similarly-obtained images acquired at days 3, 8, and 28 after a single injection of ICG dye demonstrate a delayed and persistent fluorescence in the NIR channel with the 795/810 nm excitation/emission filters. This persistent, delayed fluorescence is not observed in similar experiments performed following angiography with fluorescein-dextran dye analyzed with the fluorescein 488/500 nm excitation/emission filters in place, where the signal does not differ
between baseline (pre-injection) and any subsequent time point (Figure 2.1(c)). Qualitative analysis of the delayed NIR fluorescence after ICG generally shows a "mosaic" pattern of small speckles in the posterior pole that are external to the retinal vessels and obscure the view to the choroidal vasculature, suggesting its location between the retina and the choroid. Due to the high curvature of the rodent fundus compared against the human eye and the flat confocal plane of the cSLO, the view of the monolayer can vary, with the plane of focus captured slightly above or below the RPE. This can result in blurring of the speckled appearance, and make the retinal or choroidal blood vessels more evident. The ONH and circumpapillary area do not fluoresce. These features are similar to fluorescence patterns obtained using FAF in patients (Delori, Dorey et al. 1995). We determined that the same plane of focus is optimal for ICG-dependent fluorescent imaging as that normally used for FAF, and termed our novel technique Delayed Near-Infrared Analysis (DNIRA).
**Figure 2.1 Fluorescence detected by DNIRA in the 795/810 nm channel of the cSLO in the days following ICG dye administration.** (a) Normal confocal scanning laser ophthalmoscopy (cSLO) of wild-type SD rats obtained using RF, FAF and fluorescein angiography (488/500 nm) IR reflectance (830 nm), and ICG angiography (795/810 nm) wavelengths. (b) Time course of DNIRA at 795/810 nm with a single injection of 5 mg/kg ICG dye at day 0. No detectable signal is seen prior to dye injection. In the days after angiography (lower rows), there is an increased speckled or punctate background fluorescence compared to baseline. The signal remains strong and distributed throughout the fundus out to 28 days after injection. (c) FAF at 488/500 nm shows no change following fluorescein administration compared to prior any dye.

To confirm our hypothesis that DNIRA was dependent on ICG labeling, we performed dose-response experiments. **Figure 2.2** demonstrates that the higher the initial ICG dosage used for angiography, the brighter the DNIRA signal in the days thereafter. The fluorescence does not occur in the absence of previous ICG injection (**Figure 2.2(a)**). The high dose (5 mg/kg) required that the gain (sensitivity) of the cSLO be reduced to obtain suitable quality images, while the low dose (0.35 mg/kg) was less bright, and required that the gain to be set at higher levels. Comparative images taken with the gain set at the same level demonstrated a dose-dependent increase in brightness (**Figure 2.2(a-c)**). In the same experiment, we also show that by day 21 the speckled pattern faded in animals that received low-dose ICG (**Figure 2.2(b)**). By contrast, in animals that received high dose ICG there was little appreciable difference between days 2 and 21 post-injection (**Figure 2.2(c)**). Further, we demonstrate that IR reflectance imaging at 830 nm did not yield a similar dose-dependent and time-dependent signal (**Figure 2.2(d)**). This indicates that the observed fluorescence is not intrinsic to the tissue in the near infrared or infrared wavelengths, but a property of the ICG dye as it is dependent on the dye concentration, and requires the correct excitation and emission settings in place.
Figure 2.2 DNIRA signal intensity is ICG-dose and time dependent, and requires the 795/810 nm ICG excitation/emission filters. (a) Control cSLO images illustrate absence of 795/810 nm fluorescent signal without ICG dye injection. Control animals that do not receive ICG maintain the same level of background fluorescence with negligible signal over the 21 day period evaluated. (b) Using the same gain, DNIRA is readily detectable 2 days after ICG injection when given at the low dose of 0.35 mg/kg. With time there is a gradual fading from 2 to 21 days post-injection. (c) Using the same gain, 5 mg/kg ICG dose results in brighter (higher intensity) DNIRA signal at 2 days than the lower dose. This fluorescence persists largely unchanged out to 21 days. (d) The 830 nm IR reflectance channel which does not utilize the ICG filters does not show similar change despite administration of the dye.

To further address our hypothesis that RPE cells are the source of in vivo fluorescence following systemic ICG injection, we incubated fresh RPE monolayers with ICG dye ex vivo and evaluated them by white light imaging to detect their visible green colour. Figure 2.3 outlines a schematic of the experiment, and confirms ICG internalization by explanted RPE monolayers. Transferred
monolayers were evident using white light microscopy, and stained positive for actin binding phalloidin and ZO-1 protein for tight junctions, confirming RPE integrity following transfer and prior to experimentation (Figure 2.3(a)). ICG was visualized as green using white light microscopy only after dye incubation and not prior (Figure 2.3(b)). The green colour was no longer detectable in the RPE monolayers following permeabilization of their membranes, and reverted from green to colourless, suggesting that internalized dye was released. As it was not possible to perform 795/810 nm microscopy on posterior eye cups due to the lack of instrumentation at this wavelength, we performed low-magnification imaging of RPE monolayers transferred to glass slides using the cSLO, after in vivo investigation (Figure 2.4(a)). Isolated RPE from ICG-injected rats sacrificed at least 24 hours after dye injection show a bright cellular fluorescence in the ICG channel of the cSLO. This fluorescence is absent in RPE from saline treated rats, and similar to baseline in vivo imaging, the ICG channel shows little to no signal. The presence of RPE cells in the areas of ICG fluorescence is confirmed by concomitant faint fluorescence in the FAF channel, which is consistent with FAF emitted by RPE in vivo. This FAF signal is also evident in RPE from saline treated animals and is not dependent on ICG dye. Accompanying white light microscopy confirms the presence of the typical hexagonal pattern of binucleate RPE cells in the areas of fluorescence. These results are consistent with previously published studies reporting that cultured primary RPE cells or RPE cell lines take up ICG in vitro (Chang, Zhu et al. 2005, Hirasawa, Yanagi et al. 2007). Furthermore, it has been shown that injection of ICG for angiography can result in accumulation of dye at the RPE/BM complex in primates (Chang, Morse et al. 1998). Together, these data confirm that RPE monolayers are capable of taking up ICG dye, strengthening our premise that the RPE is the source of the ICG fluorescence observed in vivo using DNIRA.
**Figure 2.3** RPE monolayers are capable of internalizing ICG dye *ex vivo*. (a) Diagram of RPE monolayer transfer shows that naïve rat posterior eyecups can be dissected in cell culture media, RPE transferred onto polylysine coated slides, and incubated with ICG dye at physiological conditions. Morphology and integrity of transferred RPE is confirmed by 647 nm fluorescent-tagged phallodin (red, left) and ZO-1 protein (green, right). Nuclei are shown in blue (left) or red (right). (b) Diagram of RPE labelling and permeabilization shows cells as colourless when viewed with light microscopy prior to ICG incubation (left). RPE incubated with ICG dye internalize green colour (center). Following permeabilization RPE release the ICG into solution and revert to colourless (right).

To further support this hypothesis we proposed that DNIRA could be used to identify areas of RPE loss by coupling this technique with systemic injection of NaIO\(_3\), which is a known RPE toxin (Anstadt, Blair et al. 1982, Kiuchi, Yoshizawa et al. 2002). **Figure 2.4(b)** confirms that DNIRA performed in the days after NaIO\(_3\) injection identifies patches of profound...
hypofluorescence, evident as large dark areas within an otherwise continuous background of speckled fluorescence. Direct viewing through these patches permits clear visualization of the choroidal detail (enlarged box). Choroidal vessels are identified by their complexity, variable size, and non-radial pattern with respect to the ONH. By contrast, the speckled layer in non-NaIO₃ treated animals (refer to Figure 2.2) generally obscures the view of the choroidal vessels. These data are consistent with the notion that DNIRA detects ICG-labeled RPE, with the speckled fluorescent signal lost in areas of RPE loss.

Figure 2.4 The RPE monolayer is the source of in vivo ICG signal detected using DNIRA. (a) Monolayers isolated on polylysine slides from rats injected with ICG and viewed with the ICG channel of the cSLO display a bright cellular fluorescence that is absent in RPE layers isolated from control rats (white box). Similar analysis using the FAF channel of the cSLO displays faint fluorescence in both conditions confirming presence of the cells on the slides.
dependent on their autofluorescent properties at 488 nm. White light microscopy identifies presence of hexagonal binucleate RPE cells in regions of fluorescence (b) Following systemic injection of NaIO₃ the RPE is lost, and the speckled hyperfluorescent signal is also lost showing areas of hypofluorescence with choroidal vasculature readily evident in the ICG channel of the cSLO. Enlarged box (right) shows the junction of absent and present RPE using DNIRA.

Despite its known safety following systemic injection for clinical angiography in patients, previous studies have demonstrated that high-dose ICG dye can be toxic to RPE cells in vitro (Dzurinko, Gurwood et al. 2004, Hsu, Kao et al. 2004, Sato, Tomita et al. 2006). Similar concerns exist during ophthalmic surgery when ICG dye is injected intra-vitreally to help with visualization of diaphanous epiretinal membranes (Rodrigues, Meyer et al. 2007, Rodrigues and Meyer 2008). Given that we now suggest in vivo labeling of the RPE monolayer as a useful tool for investigating the rodent eye, we wished to determine whether it was toxic in vivo to the retina at the intravenous doses given. We therefore evaluated the bright-flash b-wave amplitude following high and low dose administration of ICG, and show that the ERG response does not differ between these two groups, nor between these two groups and saline injected animals (Figure 2.5). A slight downward trend observed in all groups including controls prompted regression analysis using ANCOVA of normalized slopes. ANCOVA showed that there was no statistically significant difference between all three groups. The slight downward trend may be inherent to SD rats at this age and is not considered biologically significant.

![Figure 2.5](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Slope of Regression</th>
<th>Comparison of Slopes</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 mg/kg ICG)</td>
<td>-0.004571 ± 0.004325</td>
<td>Control vs. Low</td>
<td>P=0.4438</td>
<td>No</td>
</tr>
<tr>
<td>Low Dose (0.35 mg/kg ICG)</td>
<td>-0.01025 ± 0.005730</td>
<td>Control vs. High</td>
<td>P=0.5045</td>
<td>No</td>
</tr>
<tr>
<td>High Dose (5.0 mg/kg ICG)</td>
<td>-0.008768 ± 0.004245</td>
<td>Low vs. High</td>
<td>P=0.8232</td>
<td>No</td>
</tr>
</tbody>
</table>

**Figure 2.5.** ICG administration at low and high doses does not result in retinal toxicity in vivo. ERG response of the b-wave amplitude between t = day 0 and t = day 21 shows no significant decrease in the low (b, 0.35 mg/kg) or high (c, 5.0 mg/kg) ICG dose when compared
to control (a). The amplitude of the dark-adapted bright flash response showed a slight decrease in all groups including control over the course of experimentation. Data was normalized to allow for slope comparison. ANCOVA showed no significance in the downward trend between all three groups.

Extravascular accumulation of ICG dye within choroidal stroma and the RPE/BM complex has been described using histological analysis of the non-human primate eye enucleated during the early, mid and late phases of ICG angio

graphy (Chang, Morse et al. 1998). Such leakage is consistent with the highly fenestrated nature of the choroidal vessels (Bernstein and Hollenberg 1965). In the present study, owing to both the prolonged fluorescence of the RPE and absence of ICG in the extravascular retina, we reason that RPE cells take up ICG through their basal surface, potentially through an ATP-dependent active transport process (Chang, Morse et al. 1998, Chang, Zhu et al. 2005). When injected into the systemic circulation, ICG binds to proteins such as albumin, and lipoproteins such as LDL and HDL (Yoneya, Saito et al. 1998). RPE cells are known to contain receptors for lipoproteins, and the uptake of cholesterol into the retina occurs mainly via the RPE (Hayes, Lindsey et al. 1989, Tserentsoodol, Sztein et al. 2006). It has also been shown that RPE cells can be effectively labelled for post-enucleation study by injection of systemic rhodamine-conjugated LDL (Gordiyenko, Campos et al. 2004). Presently we show that in vivo labeling of RPE can be accomplished by systemic injection of ICG that we propose may be due to the lipoprotein binding characteristics of the dye and its uptake by basolateral RPE receptors.

In summary, we describe a novel technique, DNIRA, for identifying the RPE layer in vivo in the rodent eye. This technique capitalizes on a persistent extra-vascular NIR fluorescent signal that occurs after ICG injection, is dose dependent, persists for at least four weeks, and is eliminated in discrete patches when the RPE layer is lost. DNIRA is distinct from autofluorescence imaging in that it requires prior injection of a fluorescent dye so is not endogenous, and must be performed using the excitation/emission filters normally used for angiography. However unlike angiography, photos are obtained in the days and weeks following dye injection, not during the transit stage or minutes thereafter. We suggest this is a powerful new method for assessing preclinical models of disease, for identifying RPE toxicity during drug or biomaterial toxicity testing, and for evaluating therapeutic outcomes in pharmacological or regenerative studies.
Chapter 3

Evolution of Complex Patterns of Hyperfluorescent Fundus Autofluorescence (FAF) in the Rat Eye Following Acute Retinal Pigment Epithelium Damage

3.1 ABSTRACT

Complex patterns of FAF are observed in multiple chorioretinal disorders, including AMD, and are an important part of their diagnosis and prognosis. Hyperfluorescent FAF correlates with disease activity in patients, but similar complex patterns of FAF have not been described in the rodent eye. This study aimed to identify and describe a rodent model of retinal disease characterized by complex clinically-relevant patterns of hyperfluorescent FAF. Acute damage to the RPE was induced using the toxin sodium iodate (NaIO₃), injected systemically into Sprague Dawley rats, and the fundus observed using the FAF channel (488/500 nm excitation/emission) of the commercially available HRA-2 (Heidelberg, Germany) cSLO at multiple time points out to 3 months. Patterns of FAF were described qualitatively using a grading system, and quantified using a thresh-holding method with ImageJ software. Following toxin injection, five stages of changing FAF are described: (1) induction, (2) early evolution, (3) late evolution, (4) maturation and (5) senescence. The curvilinear patterns of FAF resemble clinical disease such as advanced stages of dry AMD, and quantification of the signal demonstrates time-dependent changes in these patterns. Localized differences between the inferior and superior hemiretina were observed, supporting clinical relevance. In conclusion, acute toxin-induced damage of the RPE induces chronic, complex and changing patterns of hyperfluorescent FAF in the rat eye with notable similarity to clinical disease.

3.2 INTRODUCTION

Diagnosis and classification of multiple blinding eye diseases, most notably AMD and inherited retinal dystrophies routinely includes FAF imaging (von Ruckmann, Fizke et al. 1997, Lois, Owens et al. 2002, Bindewald, Bird et al. 2005, Bindewald, Schmitz-Valckenberg et al. 2005). The autofluorescent signal that results from blue light stimulation of the posterior pole is
presently understood to derive from lipofuscin, an autofluorescent protein aggregate that increases in RPE cells with age, and is a toxic by-product of RPE metabolism (Boulton, McKechnie et al. 1989, Eldred and Lasky 1993, Delori, Dorey et al. 1995). Many diseases are characterized by the presence of complex, two-dimensional (2D) or en face patterns of hyperfluorescent (bright, or white) signal that are not well understood. For instance, inherited outer retinal degenerations can be characterized by a multiplicity of hyperfluorescent FAF patterns that include flecks or parafoveal rings (Lenassi, Troeger et al. 2012). Dry AMD can similarly be stratified into multiple risk groups by its FAF patterns (Holz, Bindewald-Wittich et al. 2007, Schmitz-Valckenberg, Fleckenstein et al. 2009). In the presence of GA, when patches of RPE are lost, FAF is also lost, appearing as defined areas of profoundly hypofluorescent FAF (dark) signal. However, prior to onset and expansion of GA, FAF is characterized by complex patterns of hyperfluorescent signal (Holz, Bindewald-Wittich et al. 2007). In the presence of pre-existent GA, the borders or junctional zones can also demonstrate unique patterns of hyperfluorescent FAF. These patterns correlate with rates of disease progression and visual prognosis (Holz, Bindewald-Wittich et al. 2007). Thus, areas of hyperfluorescence spatially predict areas of future tissue loss during disease progression.

Despite the frequent observation of complex patterns of hyperfluorescent FAF in disease, there are no preclinical animal models that develop similar findings. With normal aging in the wildtype mouse, small discrete hyperfluorescent dots accumulate, particularly in the inferior retina (Xu, Chen et al. 2008). In addition, strains of genetically modified mice can demonstrate an accumulation of small hyperfluorescent dots that occur to a greater extent and at earlier ages than in age-matched wildtype controls (Luhmann, Robbie et al. 2009).

In this study, acute toxin-induced damage to the RPE is shown to lead to chronic and progressive change in the amount and 2D patterns of hyperfluorescent FAF. Further, a grading scale to differentiate the complex stages of progression is presented, and we suggest that these findings are of relevance to clinical disease. We confirm that these FAF changes take place in areas where the RPE is missing due to the action of NaIO₃, and where the retina becomes progressively damaged subsequent to this toxic insult.
3.3 METHODS

3.3.1 Animal Studies

Animals were handled with adherence to the Association for Research in Vision & Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and according to the Canadian Council on Animal Care (CCAC) guidelines. 46 SD rats aged 6-10 weeks, weighing 200-300 g were kept on a 12 hour dark/light cycle, with food and water ad libitum. Animals were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg), and pupils dilated with a single drop of 1% tropicamide and 2.5% phenylephrine hydrochloride solution (Diophenyl-T, Sandoz Canada Inc). GenTeal lubricating eye drops (Novartis, Canada), were consistently applied to the corneal surface during all procedures.

3.3.2 Sodium Iodate

NaIO₃ (Sigma, Oakville, Canada) was prepared fresh weekly at a stock solution of 45 mg/ml in injectable saline (Baxter, Mississauga, Canada). This solution was diluted to final concentrations of 25 mg/ml-45 mg/ml, so that all animals received the same volume to achieve final dosages of 25 mg/kg-45 mg/kg.

3.3.3 Confocal Scanning Laser Ophthalmoscopy

*In vivo* images were acquired using a commercially available cSLO (Heidelberg Retinal Angiograph, HRA-2, Heidelberg Engineering, Germany), in the 488/500 nm excitation/emission channel. Animals were followed serially with repeated in vivo imaging at days 2-4, 6-8, 12-15, 21-28, and 2-4 months. Fluorescein and ICG angiography was performed in a subset of animals as previously described to illustrate the normal vasculature. RPE DNIRA signal was used to determine the area of missing RPE as previously reported in Chapter 2 (Pankova, Zhao et al. 2014). Animals receiving fluorescein injections were not used for FAF analysis. Composite figures of individually acquired images were assembled by exporting captured cSLO frames into Microsoft PowerPoint 2010 followed by manual alignment of vascular and tissue landmarks.

3.3.4 Quantification of Fluorescent FAF

Individually acquired FAF cSLO images from at least 7 eyes per time point were analyzed using ImageJ v 1.48u, Java 1.6.0_24 (64-bit), using an 8-bit image format. Background subtraction
(rolling ball radius 50-150 pixels) was performed to eliminate background noise. Threshold was adjusted (minimum 0, maximum 243) to set a basal level of autofluorescence and measurements were acquired as percentage of hyperfluorescent area over total area, both measured in pixels. The percent area of hyperfluorescent signal per image was then compared between stages. Images over the time course of the study were initially compared to pre-NaIO₃ baseline measurements. However, owing to considerable inter-animal variation at baseline, this method was subsequently adjusted to compare later timepoints against the level of hyperfluorescent signal at the “induction” stage. Inferior retinal images were also compared against superior retina using this method. Statistical analysis was performed using GraphPad Prism v 5.0. An unpaired two-tailed Student’s t-test with a P value of 0.05 was used for all analysis.

### 3.3.5 Tissue Immunofluorescence

Following dissection of retinal and eyecup tissue, samples were blocked with 5% BSA/TBS, 0.1% Triton X-100 for 45 minutes at room temperature. Primary antibodies were added at a concentration of 5 µg/ml (unless specified otherwise) in blocking buffer, and incubated overnight 4°C. Samples were subsequently washed with TBST (TBS, 0.1% tween-20) for 5 x 1hr at room temperature followed by incubation with Alexa-fluor conjugated secondary antibody (1:400) overnight 4°C. Samples were washed with TBST for 5 x 1hr at room temperatures. Tissue was mounted onto 2-well Teflon printed slides using fluorescent mounting medium (Dako) and coverslipped. Primary antibodies used were as follows: mouse monoclonal anti-rat RPE65 (Abcam, ab78036) and rabbit polyclonal anti-ZO-1 (Life Technologies). These were compared against normal mouse IgG (Santa Cruz) or rabbit IgG (Abcam) controls. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (Life technologies) Alexa Fluor 555 goat anti-mouse (Life technologies). Phalloidin staining was done on tissue that was blocked and permeabilized simultaneously for 30 minutes in BSA/TBS + 0.1% Triton-X100, and samples were incubated 1:20 in Alexa-fluor-647 conjugated phallodin (Life Technologies) for 20 minutes. Subsequently samples were washed 2x5 min with PBS, mounted on slides and coverslipped. For nuclear stain of retinal tissue, samples were incubated with To-Pro-3 (Life Technologies) 1:1000 dilution in PBS for 10 min, followed by 2x5 min PBS wash. All images were acquired using a Leica TCS SL confocal fluorescent microscope (Leica Microsystems, GmbH, Wetzlar, Germany), with Leica Confocal Software Version 2.61.
3.3.6 Quantitative PCR

Total RNA was isolated using 8 eyes for each time point following NaIO₃ injection (baseline, induction, early evolution, late evolution, maturation, senescence), from rat retinas and posterior eyecups using Trizol Reagent (Life Technologies, USA). Briefly, after enucleation of rat eye balls, they were dissected in cold RNAlater Solution (Life Technologies, USA). The entire retina was then removed and stored in RNAlater solution, and RPE and choroid layers were scraped with a surgical scalpel blade and combined with the retina. Manufacturer’s protocol was followed for RNA extraction with the Trizol method. Total RNA was further purified with RNeasy MinElute Cleanup kit (Qiagen, USA). Their quantity and quality were checked with a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

cDNA was synthesized using RT² First Strand Kit (Qiagen, USA) with 2 µg of total RNA per reaction. Real-time PCR was performed using Qiagen 384-well Custom RT² Profiler PCR Array and RT² SYBR Green ROX qPCR Mastermix (Qiagen, USA) with 10 ng cDNA per well. The reactions were run on a ViiA 7 PCR system (Life Technologies, USA) with default cycling conditions. Differences in gene expression were analyzed using the Qiagen RT² Profiler PCR Array Data Analysis Software V3.5 Web Portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) based on ∆∆Cₜ method and normalized to Peptidyl-prolyl cis-trans isomerase H (PPIH) as a reference gene. Results are reported as fold-change (2^∆∆Ct) normalized to baseline as 1-fold. P values were calculated based on a Student’s t-test of the replicate 2^∆∆Ct values for each gene in the control group and treatment groups, and P values less than 0.05 were considered significant.

The primer sequences were proprietary. The amplicon size, Qiagen gene catalog number and primer reference positions are listed below.

Table 3.1 RPE65 Qiagen RT² PCR array gene information.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Qiagen Gene Catalog Number</th>
<th>Reference position of amplicon start in gene sequence relative to gene start</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPE65</td>
<td>PPR52080B (NM_053562)</td>
<td>659</td>
<td>125</td>
</tr>
<tr>
<td>PPIH</td>
<td>PPR57387B (XM_001073803)</td>
<td>127</td>
<td>54</td>
</tr>
</tbody>
</table>
3.4 RESULTS

3.4.1 Systemic NaIO$_3$ induces patterns of FAF change distinct from normal fundus features

Figure 3.1 In vivo changes in rat FAF following systemic injection of NaIO$_3$. (a) Representative FAF HRA-2 image of the baseline (pre-NaIO$_3$) rat fundus demonstrates a normal homogenous autofluorescence. In the days following IV injection of 45 mg/kg NaIO$_3$ a distinct curvilinear fluorescent pattern develops. (b) The FAF pattern is distinct from the RF (488 nm barrier filter, left) and IR reflectance (830 nm, right) channels (c) Fluorescein angiography (left) and ICG angiography (right) of the normal rat vasculature shows inner and mid-retinal vessels (left), and choroidal vessels (right), which do not correlate to the pattern observed on FAF.

A representative cSLO FAF image of the baseline (pre-NaIO$_3$) SD rat fundus, centered on the optic nerve head (ONH) is shown and serves as control (Figure 3.1(a)). The associated baseline FAF typically has a dim, homogenous signal throughout the fundus that is blocked by the retinal vessels and absent at the ONH, consistent with its origin in the RPE layer and with findings in
the clinical setting (Delori, Dorey et al. 1995). In the days following NaIO$_3$ injection, marked changes of FAF are readily noted in the circum-papillary area in particular. Initially, these changes include a hyperfluorescent arc or circle that partially or completely encircles the ONH. This arc or circle is adjacent to, but not contiguous with the ONH. Short alternating hyper- and normo-fluorescent curved lines or segments form in proximity to this arc or circle. A complex pattern of alternating hyper- and normo-fluorescent FAF may be more obvious in some areas, and the segments may coalesce. Early FAF patterns are distinctly in the FAF channel and not clearly observed in the RF or IR channels (Figure 3.1(b)). High resolution fluorescein and ICG angiographic images are also shown to identify the normal retinal and choroidal vasculature, and serve to illustrate that NaIO$_3$-induced changes do not mirror any features of these vascular beds (Figure 3.1(c)).

3.4.2  **NaIO$_3$-induced FAF changes can be categorized using a grading system**

Following NaIO$_3$ injection, images obtained in the mid- and far retinal periphery demonstrate that a border of hyperfluorescent arc or circle is the earliest change observed on FAF (Figure 3.2, Stage 1). The area defined within these borders is typically comparable to background or hyperfluorescent relative to background. With time (Figure 3.2, Stage 2), both the proximal and distal arcs/circles develop widening zones of curvilinear patterns of hyper- and normo-fluorescent FAF segments. Over the following weeks, the developing small curvilinear segments of hyperfluorescence coalesce and continue to evolve, forming scalloped arcs, loops, ovals and circles, many of which are contiguous (Figure 3.2, Stage 3). Eventually this mature pattern fills the mid-peripheral retina between the two bands and is more homogenous (Figure 3.2, Stage 4). Out in the weeks and months following NaIO$_3$, the hyperfluorescent signal becomes less intense and more granular until it is barely detectable (Figure 3.2, Stage 5).
Figure 3.2 Progressive changes in FAF observed in the mid- and peripheral fundus over time following induction of RPE damage. (Stage 1) Induction of RPE damage, with homogenous FAF defined by a border of brighter FAF that is located in the mid- or far periphery. (Stage 2) Early evolution of FAF showing heterogeneous areas of complex alternating hyper- and normo-fluorescent signal in the form of short curvilinear segments in proximity to the borders observed at induction. (Stage 3) Late evolution of FAF showing heterogeneous areas of hyper and hypofluorescence with coalescing segments, arcs and loops. (Stage 4) Maturation of FAF results in a more homogenous pattern of FAF with coalesced loops, arcs and segments. The area surrounded by the border present at induction is filled in. (Stage 5) Senescence of FAF shows a homogenous cellular/granular pattern with decreased hyperfluorescent signal.

Based on the location, shape and distribution of these patterns, we termed five stages of FAF change: induction (stage 1), early evolution (stage 2), late evolution (stage 3), maturation (stage 4) and senescence (stage 5). Table 3.2 summarizes the characteristic findings of each stage that were used for grading.

3.4.3 FAF patterns can be quantified using fluorescence thresholding

In addition to the qualitative changes described above, we quantified the amount of hyperfluorescent FAF signal at each stage and compared this signal across stages. High variability in the level of FAF at baseline precluded its use as comparator (data not shown). Overall FAF hyperfluorescence was at a maximum at stage 1 (induction) and was quite similar between animals, so was used as the comparator. This hyperfluorescence did not change significantly between the induction and evolution stages (2 and 3, early and late evolution). However, by the later stages (4 and 5, maturation and senescence), the relative area of hyperfluorescent signal per cSLO image was significantly reduced, consistent with the visual
observation that the signal becomes reduced towards senescence, as the appearance of the pattern becomes more punctate and granular.

Table 3.2 Grading scale summary describing FAF patterns of progression observed in the rodent fundus following induction of RPE damage.

<table>
<thead>
<tr>
<th></th>
<th>Induction (Stage 1)</th>
<th>Early Evolution (Stage 2)</th>
<th>Late Evolution (Stage 3)</th>
<th>Maturation (Stage 4)</th>
<th>Senescence (Stage 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniformity</td>
<td>Homogenous</td>
<td>Heterogeneous</td>
<td>Heterogeneous</td>
<td>Homogenous</td>
<td>Homogenous</td>
</tr>
<tr>
<td>Shape/form</td>
<td>Surrounding arc/circle</td>
<td>Alternating curvilinear hyperfluorescent segments</td>
<td>Coalescing segments, arcs, loops, ovals, circles</td>
<td>Coalescing segments, arcs, loops, ovals, circles</td>
<td>Granular/cellular</td>
</tr>
<tr>
<td>Localization</td>
<td>Peripapillary and peripheral</td>
<td>Proximity to the edges seen in induction phase</td>
<td>Peripapillary and peripheral</td>
<td>Fills in the mid-periphery</td>
<td>Central and peripheral</td>
</tr>
</tbody>
</table>

Figure 3.3 Threshold analysis permits quantification of the amount of hyperfluorescent FAF signal in the central fundus. (a) Representative example of thresholding technique for quantification of the amount of FAF. The region of interest (ROI, yellow box) was set to exclude image borders and applied to each image. A constant threshold intensity was established and the supra-threshold bright region, in pixels, was used to determine the percent area of hyperfluorescent FAF compared against total image area. (b) Compared against Stage 2 (evolution) the percent area of hyperfluorescence shows a decreased amount of FAF at the maturation stage, indicating that hyperfluorescent signal is quantifiable over time. *P<0.05 (P=0.0022), unpaired Student’s t-test; n=12 eyes per stage.

Using thresholding analysis set to the same level to pick up the curvilinear/scalloped pattern observed on FAF, it is evident that the appearance of a distinctive pattern is minimal at
induction, progresses slightly through evolution, and evident during maturation (Figure 3.4), suggesting that the pattern is progressive and does not revert back to a normal phenotype.

![Induction, Evolution, Maturation](image)

**Figure 3.4 Progression of curvilinear FAF is detected using thresholding in ImageJ.** Representative threshold overlays of FAF images show a change in amount of curvilinear pattern captured at the same level of threshold across stages. The pattern begins to emerge at induction and becomes more defined over time.

### 3.4.4 Inferior retina is more susceptible to develop abnormal patterns of FAF and progresses more rapidly than inferior retina

Using both the qualitative grading system and quantification of the hyperfluorescent signal, it was possible to detect differences in fluorescence between the superior and inferior retina. A representative composite FAF image, constructed from a representative animal with a less extensive response to NaIO₃, demonstrates a small patch of abnormal FAF in the inferior half of the fundus only (Figure 3.5(a)). This suggests that the inferior fundus is more susceptible to the toxic challenge, and the superior fundus is more resistant. Threshold analysis of this area from a subset of eyes (n=7) confirms the presence of more hyperfluorescent FAF signal in images taken below the ONH compared against those taken above (Figure 3.5(b)). Examples of these images that were used for analysis are shown in Figure 3.5(c), and it is evident that during the same timepoint (as recorded during the same imaging session of the same animal), when there is extensive damage after toxic challenge that involves both the upper and lower hemispheres, it evolves and matures more rapidly in the inferior fundus compared to the superior fundus.
Figure 3.5. Inferior fundus is more susceptible to damage, and patterns of FAF progress through the stages more rapidly than in the superior fundus. (a) Representative composite FAF image including central and peripheral fundus showing greater distribution of hyperfluorescence in the inferior fundus. (b) Using grading and fluorescent quantification of the amount of hyperfluorescence in pixels, there is a higher amount of FAF in the inferior fundus than superior fundus. *P < 0.05; (P=0.0111), unpaired Student’s t-test; data is presented as means ± SEM from n=7 eyes at evolution stage. (c) Representative FAF images showing late evolution and maturation stages of progression in the inferior fundus, while superior fundus is still indicative of induction and early evolution when observed in the same animal during the same imaging session.

Quantifying the total number of eyes imaged following a low-dose toxin administration, it can be observed that none of the animals presented with damage to the superior fundus only (Table 3.3, Figure 3.6). The majority of the eyes presented with damage either in the inferior fundus only (45.9%), or in the inferior fundus encompassing the mid-fundus and optic nerve head (47.5%).
Table 3.3 Summary of FAF damage localization in the rodent fundus. Damage following low dose (25-35 mg/kg) NaIO₃ administration predominantly emerges in the inferior fundus.

<table>
<thead>
<tr>
<th>Localization of Damage</th>
<th># eyes</th>
<th>% eyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inferior retina only</td>
<td>28</td>
<td>45.9</td>
</tr>
<tr>
<td>Superior retina only</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inferior retina and equatorial region of superior retina (optic nerve head encircled)</td>
<td>29</td>
<td>47.5</td>
</tr>
<tr>
<td>Inferior retina and equatorial region of superior retina (optic nerve head not encircled)</td>
<td>4</td>
<td>6.6</td>
</tr>
<tr>
<td>Total number of eyes *from 32 animals</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.6 Quantification of eyes imaged following low dose (25-35 mg/kg) NaIO₃.**
Measuring the percentage of eyes with damage, as observed on FAF after low dose systemic NaIO₃ indicates a 100% preference of damage to the inferior region of the fundus compared to superior.

### 3.4.5 Bright FAF emerges in areas of missing RPE

In order to confirm that areas of bright FAF are associated with atrophy in the model, we confirmed that the RPE cells were missing in these areas. Using the previously described RPE DNIRA technique (Chapter 2), we can confirm that areas of bright FAF correlate to the same areas where DNIRA is dark (signal absent), indicative of missing/atrophied RPE (**Figure 3.7(a)**). To further support these findings, we performed quantitative PCR on samples of excised tissue that include the retina and RPE components at each timepoint/stage of atrophy after NaIO₃ (**Figure 3.7(b)**). PCR confirms that compared to baseline, and compared to expression of reference gene PPIH, RPE65 is significantly downregulated following induction of damage using NaIO₃, and remains so until the late evolution stage. Further analysis using excised RPE/eyecup tissue samples at the evolution stage with markers of RPE expression shows a decreased amount of RPE65, ZO-1 and phalloidin staining in the NaIO₃-treated tissue, compared to IgG controls.
Furthermore, compared to normal controls where RPE show distinct hexagonal shape and normal morphology, RPE cells are broken, damaged and disorganized, confirming their total atrophy in this model.

**Figure 3.7(c).** Bright FAF emerges in areas of missing RPE. (a) Combining the two imaging modalities of DNIRA (as in Chapter 2), and FAF, bright FAF (during early evolution stage) signal appears in areas where the DNIRA signal is dark, and therefore RPE are absent. (b) RPE65 mRNA expression shows decreased RPE65 at induction and evolution stages, confirming damage to the RPE cells very early following NaIO\(_3\). Fold-Change \(2^{\Delta\Delta Ct}\) is the normalized gene expression \(2^{\Delta Ct}\) in the test sample (stage of atrophy progression) divided by the normalized gene expression \(2^{\Delta Ct}\) in the control sample (baseline). Data from n=3 independent samples of chorioretinal extracts was collected for custom qPCR arrays, and generated using Qiagen RT\(^2\) Profiler PCR Array Data Analysis software version 3.5, normalized to reference gene PPIH. *(P<0.05). (c) Tissue immunofluorescence of excised posterior eyecup samples using phalloidin (blue), RPE65 (green) and ZO-1 (red) confirms missing RPE in areas of bright FAF/dark DNIRA signal at the evolution stage of NaIO\(_3\) damage.
3.4.6 Loss in RPE barrier function leads to progressive deformation of outer retinal structure

Following the onset of NaIO$_3$-induced retinal degeneration due to the breakdown of RPE barrier structure and function, as observed by dye leakage on angiography (Supplemental Figure 3.1), a progressive disruption of retinal morphology takes place. This can be observed in Figure 3.8 by the formation of folds in the photoreceptor nuclei of the outer retina, stained with To-Pro-3 nuclear dye and observed using confocal fluorescence microscopy. At baseline prior to NaIO$_3$ administration, confocal microscopy using the nuclear stain confirms that the ONL is flat and uniform. At induction and early evolution, slight curvilinear grooves and creases of the ONL are observed. By late evolution, ONL deformation is more pronounced producing a distinctly interconnected series of grooves. By maturation and senescence these changes are more complex and coalescing, supporting a progressive deformation of the retina following the toxic challenge.

3.5 DISCUSSION

Complex patterns of hyperfluorescent FAF characterize multiple blinding outer retinal diseases including AMD and inherited retinal dystrophies, and often correlate with disease severity or
disease activity (Holz, Bindewald-Wittich et al. 2007). In this study we describe for the first time, similarly complex changes in the rodent eye. In particular we describe patterns of hyperfluorescent FAF that are curvilinear, scalloped, oval or circular. Such forms are reported extensively in clinical disease, for example in subtypes of dry AMD (Bindewald, Bird et al. 2005, Bindewald, Schmitz-Valckenberg et al. 2005, Holz, Bindewald-Wittich et al. 2007). Such hyperfluorescence can be found in association with patches of GA and predict its onset or expansion (Holz, Bellman et al. 2001, Schmitz-Valckenberg, Bindewald-Wittich et al. 2006). In the presence of pre-existent GA, hyperfluorescence is described at its border or in the broader adjacent junctional zones (Holz, Bellman et al. 1999, Brar, Kozak et al. 2009). Hyperfluorescent patterns are also identified in the absence of GA in forms of dry AMD, including reticular pseudodrusen (RPD). We note similarities between the patterns observed here and the most aggressive forms of dry AMD: banded, diffuse trickling, and RPD ((Holz, Bindewald-Wittich et al. 2007, Zweifel, Spaide et al. 2010, Sarks, Arnold et al. 2011, Schmitz-Valckenberg, Alten et al. 2011). Amongst patients with advanced AMD and pre-existent GA, the diffuse trickling pattern of FAF holds the worst prognosis. Longitudinal studies of these patients show that the area of RPE/outer retinal atrophy increases at rates of up to 3 mm$^2$ per year – the fastest described (Holz, Bindewald-Wittich et al. 2007). The diffuse trickling form of AMD is characterized by the formation of oval and curvilinear patterns of greyish rather than black hypofluorescence that are prominent in the mid-periphery and slowly progress to the retinal periphery. This actively “trickling” border is associated with hyperfluorescent FAF (Holz, Bindewald-Wittich et al. 2007).

Based on consistent observations in over 90 eyes, we describe the progressive changes in FAF in our model as sequential stages of induction, early evolution, late evolution, maturation and senescence. Using a subset of the criteria used by Bindewald et al for the clinical classification of AMD, our scheme is based on the location, shape/form and distribution of the pattern. Because of the inherent inability to readily quantify spatial patterns of retinal disease, staging or grading systems are used routinely in clinical ophthalmology. Examples include the description of mild, moderate or severe pre-proliferative diabetic retinopathy and, as noted, the various patterns of FAF associated with GA (Bindewald, Bird et al. 2005, Holz, Bindewald-Wittich et al. 2007, Wu, Fernandez-Loaiza et al. 2013). Use of such a grading system accommodates the inter-individual variation that is not possible to capture using stratification based strictly on the number of years.
with diabetes for example, or by analogy, the number of days, weeks or months post-NaIO$_3$ injection. The results observed in variability of progression of the pattern across animals, even when receiving the same dose and measuring at the same timepoints indicates the power of using a system of stages of disease rather than specific timepoints. Further, for experimental purposes in the rodent, such stratification is useful for downstream correlative studies aimed to determine the cellular or molecular mechanism of disease and to identify new treatments.

Notwithstanding the results of this study, there are significant limitations to quantifying FAF in the rodent eye. We found considerable variation in background autofluorescence of the normal SD rat, possibly due to the use of clinical scale equipment on a much smaller rodent eye (Charbel Issa, Singh et al. 2012, Joshi, Pankova et al. 2016). In particular we suggest that the high radius of curvature leads to patchy or sectoral regions of FAF variability. Once damage is induced, our data suggests that the profound change in FAF and increased contrast overwhelms this baseline variability. Second, we found considerable variation in the overall FAF signal between individual images. Despite these difficulties, the applicability of our qualitative and quantitative methods is supported by their ability to detect differences between the superior and inferior retina, an observation made in multiple animal models of retinal disease (Chen, Simon et al. 1999, Grimm, Wenzel et al. 2001, Tanito, Kaidzu et al. 2007, Xu, Chen et al. 2008).

Though NaIO$_3$ has been known to vision scientists for decades, it fell out of favour as a model of retinal degeneration, in particular AMD (Kiuchi, Yoshizawa et al. 2002, Franco, Zulliger et al. 2009, Hariri, Moayed et al. 2012). However, we are revisiting this model as having potential for studying dry AMD, and suggest the NaIO$_3$ model of RPE damage can be applicable to this disease when employing clinically-relevant FAF imaging alongside our newly developed DNIRA technique. We confirm that despite the acute toxic insult, observed phenotypic changes occur slowly in the weeks and months thereafter. As such, this model may be particularly relevant for studying progressive chronic retinal changes in vivo. The power of identifying animal models with phenotypic similarity to human disease is not to be underestimated, particularly given that the clinical description of ophthalmic patients relies almost exclusively on imaging techniques, such as fundoscopy, cSLO and OCT, rather than the testing of blood samples or tissue biopsy that typify other areas of medicine.
In summary, this study illustrates for the first time, progressive, complex changes in hyperfluorescent FAF in a rodent model of RPE damage. We have thus far described the applicability of this model to studying retinal disease such as dry AMD due to the noted damage to the RPE barrier, atrophy and absence of RPE cells, and subsequent loss of retinal structure. The similarity of these findings to those of clinical diseases, particularly some forms of non-exudative AMD, suggest that further analysis of this model with current and clinically-relevant imaging techniques is warranted, and can provide novel and uniquely translational information. Subsequent work will examine in depth the cellular and molecular correlates of the FAF patterns that have been presented here, and explore the underlying mechanisms responsible for these phenotypes, to help find new and much needed treatments.

**Supplemental Data**

**Supplemental Figure 3.1 Leakage of dye due to breakdown of the RPE barrier during the onset of NaIO₃ atrophy.** ICG angiography at baseline and very early during induction shows typical ICG angiograms that clearly depict choroidal blood vessels around the ONH. During the induction stage blood vessels become obscured by the leakage of dye across the damaged RPE barrier as it fill the surrounding tissue. This leakage is still evident, though not to the same extent in the evolution stage of atrophy, indicative that damage is progressive and RPE cells do not recover.
Chapter 4

Macrophage Activation Can Be Identified In Vivo with the Aid of Immuno-DNIRA and Pulsed-DNIRA Techniques in a Model of Dry AMD

4.1 ABSTRACT

Phagocytic macrophages are found in AMD and in preclinical models of disease, but it is not possible to identify macrophage populations clinically in vivo. However, GA in AMD does correlate to hypofluorescent FAF, and eyes at high risk for GA or with expanding GA demonstrate hyperfluorescent FAF. It was shown that Delayed Near-Infrared Analysis (DNIRA), can be used to view the RPE using ICG dye and cSLO imaging. The purpose of this study was 1) to modify the DNIRA technique to view macrophages in vivo in the eye, 2) to combine DNIRA with FAF to correlate labelled macrophages to patterns of retinal disease 3) correlate the patterns of labelled macrophages to those in excised tissue samples following retinal damage. Rat peritoneal macrophages are shown to take up ICG under white light and 790 nm fluorescence. Retinal damage was subsequently induced in rats by systemic injection of NaIO₃ (vs saline control), and 10⁷ ICG- labelled cells were injected per animal via tail vein. Sequential cSLO images using the ICG excitation/emission parameters in the minutes and days after cell injection show motile fluorescent spots in the fundus. In control animals, cells are found mostly in the vasculature. In toxin-treated animals, they are seen both in the vasculature and extravasating into areas of tissue damage. In vivo imaging shows progressive changes in DNIRA that support migration of macrophages into the damaged outer retina following toxin damage. Patterns of cells using DNIRA imaging correlates to the patterns observed with FAF, and co-localization of the two channels of the cSLO supports these findings. Immunofluorescence confirms CD68+ and Iba1+ cells in the areas of damage that recapitulate the patterns observed in vivo. These data demonstrate that macrophages can be safely labelled with ICG in vitro, and following their systemic injection can be viewed in vivo in both a healthy and damaged rodent fundus. Labelled cells preferentially localize to areas of outer retinal damage, suggesting they may be recruited during disease, which is supported by autofluorescent and immunofluorescent microscopy.
4.2 INTRODUCTION

Macrophages consist of a broad group of cells from the monocytic lineage, and have phagocytic properties that have been widely implicated in mediating a number of diseases both in the eye and in other organs (Geissmann, Manz et al. 2010). Specific to the eye, there are a number of macrophage populations, including resident retinal microglia, choroidal macrophages, and infiltrating monocyte-derived macrophages. For example, macrophages are well described to propagate the late phases of uveitis, and their infiltration into the retina from peripheral circulation is important in progression of this disease (Robertson, Erwig et al. 2002, Forrester, Lumsden et al. 2005, Chen, Copland et al. 2012). More recently, these cells have further been implicated in AMD, both in patient specimens and in rodent models of wet and dry AMD (Cherepanoff, McMenamin et al. 2010, Cao, Shen et al. 2011, Cruz-Guilloty, Saeed et al. 2013, Sene, Khan et al. 2013). They are believed to be responsible for worsened disease prognosis, and are associated with new blood vessel growth into the eye (Kelly, Ali Khan et al. 2007). However, there is a little evidence to suggest that macrophages can be visualized in vivo in a patient eye, and whether this can be a useful method in diagnosing disease and monitoring progression.

The ability to label and follow macrophages in vivo has been studied in areas such as cardiovascular imaging, however most technology is currently limited to low resolution and/or sensitivity in modalities such as positron emission tomography (PET) or magnetic resonance imaging (MRI) (Quillard, Croce et al. 2011). The eye is unique in the ability to view internal features such as the retina non-invasively, and therefore affords the possibility of high contrast high resolution optical imaging via the pupil for the purpose of disease diagnosis and treatment progression (Abramoff, Garvin et al. 2010). Imaging in the eye therefore has distinct advantages and is a routine procedure in the clinical setting. For example, in vivo ocular imaging in the blue spectrum is standard for the diagnosis of AMD where distinct hyper- and hypo-fluorescent areas on FAF imaging are associated with GA, the end stage of the dry subtype of the disease. In particular, advanced and rapidly progressing GA is associated with areas of hyperfluorescent (bright) FAF, the etiology of which is not entirely known (Holz, Bindewald-Wittich et al. 2007, Schmitz-Valckenberg, Fleckenstein et al. 2009).

In recent years the focus of in vivo imaging has shifted towards the Near Infra-Red (NIR) spectrum. NIR imaging is superior to that in the visible spectrum due to the absence of
endogenous tissue autofluorescence, higher sensitivity and better optical properties (Luker and Luker 2008). This holds especially true for the retina, where the autofluorescence of the RPE pigment lipofuscin emits a high background signal at wavelengths 500-750 nm (Delori, Dorey et al. 1995). ICG is the only NIR dye approved by the FDA for use in clinical ophthalmic procedures, such as during epiretinal peel surgery, and systemic angiography to view the choroid. This makes it an ideal compound to further study cellular and tissue function in vivo in preclinical studies. We have previously reported on the uptake of ICG dye by phagocytic RPE cells, and the imaging technique we have developed associated with this phenomenon, Delayed Near-Infrared Analysis (DNIRA). Furthermore, we have reported on the safety and efficacy of ICG dye injection in rodents for serial evaluation of the outer retina (Pankova, Zhao et al. 2014). It is suggested that targeting probes for phagocytosis is a good way to preferentially detect phagocytic cells, such as macrophages, and a small body of evidence suggests that macrophages are capable of internalizing ICG dye in atherosclerotic lesions (Pande, Kohler et al. 2006, Quillard, Croce et al. 2011, Vinegoni, Botnaru et al. 2011). Therefore in this study we have 1) modified our existing DNIRA technique to specifically label macrophages in the eye and allow for their detection in vivo in the NaIO3 model of dry AMD, and 2) correlate patterns of DNIRA to patterns of FAF typically associated with retinal atrophy such as that observed in dry AMD, and 3) correlate in vivo DNIRA patterns to macrophages in excised tissue samples.

4.3 METHODS

4.3.1 Animal Studies

All animal studies were carried out in accordance with the Canadian Council on Animal Care guidelines and with adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For all experiments, SD rats (Charles River) aged 6-10 weeks, weighing 200-300 g were kept at a 12 hour dark/light cycle, with food and water ad libitum. For in vivo evaluation, animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and pupils dilated with one drop of 1% tropicamide and 2.5% phenylephrine hydrochloride solution (Diophenyl-T, Sandoz Canada Inc). Eyes were conditioned repeatedly using GenTeal lubricating eye drops (Novartis, Canada) during the course of the experiments.
4.3.2 Macrophage Isolation

Rat peritoneal cells were isolated using a modified protocol based on that previously described (Davies and Gordon 2004). Briefly, rats weighing 200 grams were sacrificed and sterilized with 70% ethanol. In a biosafety cabinet, a small incision was made in the lower abdomen and skin pulled away from the abdominal cavity. Approximately 20 mL of cold sterile PBS (Wisent Biosciences) was injected into the lower abdominal cavity with a 25 gauge needle. The abdomen was massaged for 10-20 seconds, and opened to reveal the cavity. A 20 gauge needle was used to collect the fluid into 15 ml polypropylene tubes on ice and centrifuged at 1500 x g, 4°C for 10 minutes. After discarding the supernatant, the cell pellet was re-suspended in 5 ml of preconditioned RPMI-1640 medium (Wisent Biosciences) containing L-glutamine, 25 mM Hepes buffer, 10% fetal bovine serum and 50 units Penicillin and Streptomycin. Cells were seeded at 5 x 10^5 per well in 8-well chamber slides, and allowed to adhere for 2 hours.

4.3.3 Cell Immunofluorescence

After washing away non-adherent cells from culture, macrophages were confirmed by immunostaining for CD68 (MCA341R, AbD Serotec) and Iba1 (019-19741, Wako Japan). Briefly, cells were fixed with 4% PFA/PBS for 15 minutes, and blocked with 1.25% BSA in TBS for 45 minutes at room temperature. Primary mouse anti-CD68 or primary rabbit anti-Iba1 was used at 5 µg/ml in 5% BSA/TBS and incubated for 2 hours at room temperature. Cells were washed in PBS and incubated with goat anti-rabbit 488, or goat anti-mouse 555 conjugated secondary antibodies (Life Technologies) at 5 µg/ml for 2 hours at room temperature, washed, and counterstained with To-Pro-3 (Life Technologies). Slides were mounted with fluorescent mounting medium (Dako Canada, Inc) and images acquired using a Leica TCS SL confocal fluorescent microscope (Leica Microsystems, GmbH, Wetzlar, Germany), with Leica Confocal Software V 2.61.

4.3.4 Ex Vivo Macrophage Uptake and Release Assay

ICG permeabilization release assay was performed as previously described (Pankova et al, 2014). Briefly, after allowing macrophages to grow in culture for 2 days, ICG (Cardiogreen, Sigma) at 0.25 mg/ml or sterile water control was added to the wells in 500 ul total fresh culture media (1 in 10 dilution). Cells were incubated for 30 min at 37°C and 5% CO₂. Macrophages were then washed with PBS three times to remove residual ICG dye, and visualized by white-
light microscopy using a Nikon Eclipse TS100 microscope with DS-Fi1 Digital Sight Capture imaging system. Cells were subsequently permeabilized with 0.5% Triton X-100 for 15 minutes at room temperature, washed with PBS, and re-evaluated by white-light microscopy. The pre-permeabilization washes, permeabilized contents, and post-permeabilization washes were collected for both ICG-treated and control cells, diluted 10 times and absorbance at 795 nm was measured in 96-well plates using an M5e spectrophotometer. A standard curve of 0-0.3 mg/ml ICG was used to quantify absorbance of ICG released by macrophages from n=8 animals.

4.3.5 LICOR Odyssey Imaging

To confirm uptake of ICG by macrophages, cells were scanned in chamber slides using the LICOR Odyssey infrared imaging system. Following dye uptake as described above, treated and control cells were kept in 100 microlitres of PBS and scanned using the 800 nm laser excitation. Resolution was set up at 21 microns, intensity was L1.0 and focus offset at 1.5 mm.

4.3.6 Cell Viability and Live Cell Injections

Following isolation cells were incubated with ICG dye (0.25 mg/ml) or sterile water control at 37°C in a shaking incubator for 30 minutes. Prior to injection cells were quantified using Trypan blue exclusion test before and after 30 min incubation with ICG. Cell viability was determined as % viable cells = \[1.00 - \frac{\text{Number of non-viable cells}}{\text{Number of total cells}}\] \times 100. For injection into recipient rats, cells were washed 5 times with PBS by centrifugation at 1500 x g, 4°C for 10 minutes and re-suspended in 300 ul of injection saline. Rats that have previously been injected with NaIO₃ (or saline control) and were at the evolution stage of retinal atrophy, were anesthetized as previously described. ICG-labelled macrophages were infused at 1 x 10⁷ cells per rat IV via a 24 gauge catheter.

4.3.7 Immuno-DNIRA Imaging

In vivo images were acquired using a commercially available cSLO (Heidelberg Retinal Angiography, HRA-2, Heidelberg Engineering, Germany). Baseline imaging was performed using the ICG angiography filters of the cSLO (795 nm excitation/810 nm emission). Live cell injections were done at the evolution stage following systemic NaIO₃ (Sigma) via a 24-gauge catheter inserted into the tail vein at a dosage of 45 mg/kg body weight. Following live cell injections, images acquired using the cSLO ICG angiography filters during infusion and out to
20 minutes, similar to that typically done for ICG angiography. Images were further acquired 1 day – 1 week following live cell injections. At the same time, images were acquired in the FAF channel of the cSLO using 488 nm excitation and 500 nm emission filters. For quantification of cells, Immuno-DNIRA images were imported into image J, set to 8-bit and threshold adjusted to remove background so that individual cells were visible. Analyze particles function was set to analyze fluorescent particles with size of at least 50 pixels. A total of n=5 NaIO₃ treated-eyes (55 images), and n=4 control eyes (57 images) were analyzed.

4.3.8 **Pulsed-DNIRA and Control cSLO/Angiography Imaging**

Rats were injected with systemic NaIO₃ as above. At the evolution stage of atrophy following NaIO₃ injection, freshly prepared ICG (2 mg/ml) was injected systemically via tail vein based on rat body weight to a final dose of 2 mg/kg, and images were taken during dye circulation as in angiography, as well as 24-48 hrs following injection using the ICG settings of the cSLO. The Pulsed-DNIRA images were also compared to the standard angiography done during the dye injection phase, to the IR reflectance (830 nm) channel of the cSLO, and to the FAF channel (488 nm excitation/500 nm emission). To observe the effect of Pulsed-DNIRA over time, ICG was injected systemically via tail vein, and imaged within 24-48 hours following injection at t = -48 hrs, t = 0, t = day 3, t = day 7, t = d 14, t = d 28. No dye re-injections were carried out during imaging at consecutive timepoints. For false colour overlay of FAF and Pulsed-DNIRA images, grayscale cSLO images were imported into Photoshop CS4, colour set to green for FAF and red for DNIRA, and images were merged to observe yellow overlay.

4.3.9 **Autofluorescent Microscopy of Excised Retina and RPE**

For autofluorescent analysis of posterior eye tissue, eyes were enucleated and retina and eyecup dissected out separately in PBS with no fixative and no staining of any kind. Retinal and eyecup (with RPE layer) tissue was mounted onto a glass slide and coverslipped with PBS. Autofluorescent microscopy was performed using an upright epifluorescent microscope (Olympus BX50) with 488 nm excitation, 520 nm emission filters.

4.3.10 **Tissue Immunofluorescence**

Following dissection of retinal and eyecup tissue, samples were treated as described in Chapter 3. Primary antibodies were added at a concentration of 5 µg/ml (unless specified otherwise).
Primary antibodies used were as follows, mouse monoclonal anti-rat CD68 (AbD Serotec, MCA341R), rabbit polyclonal anti-Iba1 (Wako Pure Chemical Industries Ltd, 019-19741), mouse monoclonal anti-rat CD163 (Hycult biotech, HM3025). These were compared against normal mouse IgG (Santa Cruz) or rabbit IgG (Abcam) controls. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (Life technologies) Alexa Fluor 555 goat anti-mouse (Life technologies). Images were acquired using a Leica TCS SL confocal fluorescent microscope (Leica Microsystems, GmbH, Wetzlar, Germany), with Leica Confocal Software Version 2.61.

4.3.11 Statistical Analysis

For all statistical analysis unpaired, two-tailed Student’s t-test was performed using GraphPad Prism 5.0 statistical software.

4.4 RESULTS

4.4.1 Macrophages are capable of taking up ICG dye in vitro

Since the wavelength of the ICG dye is outside the range of most microscope systems, in order to confirm internalization of ICG dye by macrophages in vitro, an ICG permeabilization assay was developed to capture the dye by white light microscopy and 795 nm absorbance using a spectrophotometer. Figure 4.1(a) illustrates the assay, showing incubation of macrophages with ICG, followed by washing and permeabilization steps to release the dye into solution. Figure 4.1(b) shows that isolated primary rat peritoneal macrophages in their native state stain positive for macrophage markers CD68 and Iba-1, confirming their in vitro phenotype. Following incubation with ICG dye and subsequent wash steps, these cells take on the green colour of the dye which can be captured using white light microscopy (Figure 4.1(c)). This is not evident for control cells. Similarly, when scanned using the NIR fluorescent scanner, a punctate cellular pattern can be visualized at low resolution in the sample incubated with ICG dye, which is not visible for the control population. Following permeabilization using Triton-X100 cells that have been previously treated with ICG dye can be observed to release a prominent green colour into the permeabilized solution (Figure 4.2(a)). This characteristic colour of ICG is not evident before permeabilization, or for the control treated cells. Measuring absorbance of these solutions using a spectrophotometer results in a quantifiable difference between treated and control cells. Figure 4.2(b) depicts linearity of the assay, showing a direct relationship between absorbance at
795 nm and concentration of ICG dye. Measuring absorbance of wash solutions obtained from cell samples before permeabilization, permeabilization, and post-permeabilization steps, there is a significant increase in the readings for permeabilized cells that have been incubated with ICG (Figure 4.2(c)). A P<0.0001 was obtained using an unpaired Student’s t-test for treated compared to untreated permeabilization steps, as well as treated cells before permeabilization compared to during permeabilization, confirming that macrophages internalized the dye, which was subsequently released into solution following breakdown of the cell membrane.

Figure 4.1 Peritoneal macrophages can be visualized with white and near-infrared light after taking up ICG dye. (a) Overview of permeabilization assay using white light properties of ICG dye to observe the green colour in macrophage cells. (b) Immunofluorescence of rat peritoneal cells showing positive macrophage markers CD68 (green) and Iba1 (red), nuclei are counterstained with To-Pro-3 (blue). (c) White light microscopy (top) and LICOR Odyssey fluorescent scan (bottom) showing macrophage that have taken up ICG dye (left), compared to cell that incubated with vehicle control solution (right).
Figure 4.2 Permeabized macrophages release ICG dye which can be quantified using a spectrophotometer, confirming that ICG was internalized. (a) ICG labelled cells release green dye into solution following permeabilization that can be observed visually. This colour is not observed in cell solution before permeabilization, nor in control cells. (b) By measuring the ICG wavelength on a spectrophotometer there is a direct relationship between ICG dye concentration and absorbance confirming assay linearity. (c) Significant difference in ICG dye release between ICG labelled and control cells as well as ICG labelled cells before and after permeabilization can be observed. *P < 0.0001, Unpaired Student’s t-test, data is presented as means ± SEM from n=8 individual cell samples.

4.4.2 ICG labelled cells are viable and Immuno-DNIRA visualizes them in the rat bloodstream and retinal tissue

In order to ensure that ICG labelling was safe to macrophages, viability was confirmed using a trypan blue exclusion assay. Figure 4.3 demonstrates that following incubation with ICG dye, cell viability did not differ for cells before incubation with ICG compared to following incubation, as well as between ICG and control treated cells. This was confirmed with an unpaired Student’s t-test with a P value of > 0.05 showing no statistical significance. Thus it was determined that labelled cells can be injected into recipient rats.

SD rats typically display no signal in the ICG channel of the cSLO at baseline (Figure 4.4(a)). Following systemic injection of ICG-labelled macrophages the cells can be seen in the recipient rat retinal vasculature and tissue, as bright fluorescent dots (Figure 4.4(b)). This is true for both naïve rats, and those injected with NaIO₃ to damage the RPE and induce atrophy. At least 24 hrs following systemic injection labelled cells can still be visualized moving through the vasculature in the inner retina (Figure 4.5(a)). They appear elongated due to the effect of rapid motion, and can be observed in both control and to a lesser extent in the NaIO₃ treated rats. However moving the focus to the outer retina, labelled cells can be visualized in the NaIO₃ treated rats only, and
appear static (Figure 4.5(b)). The outer retina of control rats shows little/no punctate fluorescent dots. Quantification of n=5 NaIO3 treated eyes demonstrates significantly more labelled cells in the outer retina of NaIO3 rats compared to control (Figure 4.5(c)).

**Figure 4.3 ICG dye does not affect macrophage viability.** No significant difference in viability is observed between ICG-labelled and control macrophages. P > 0.05. Unpaired Student’s t-test, data is presented as means +/- SEM. Viability was performed using trypan blue exclusion assay across control and ICG-treated cells, prior to, and 30 min following 0.25 mg/ml ICG incubation.
Figure 4.4 Labelled macrophages are observed on Immuno-DNIRA in vivo in the eye immediately following their systemic injection. (a) At baseline no signal is evident with the ICG settings in both the control and the NaIO₃ treated animals. (b) During injection of ICG-labelled cells, bright punctate, fluorescent cells can be observed in the vasculature of both control and NaIO₃-treated animals using the 795/810 nm excitation/emission settings of the cSLO that are typically used to view ICG angiography.

Figure 4.5 Macrophages observed using Immuno-DNIRA localize to areas of damage in rats with atrophy. (a) In the inner retina of control animals, labelled cells can be visualized in the vasculature. They are observed as elongated due to rapid motion effects, indicating movement of the cells. In NaIO₃-treated animals, these cells are present but to a lesser extent. (b) In the outer retina of control animals, none/few cells can be observed localized to the tissue. In NaIO₃-treated animals a multitude of fluorescent, punctate, stationary cells is evident in the tissue. (c) Quantification confirms a significantly higher number of fluorescently-labelled cells in the outer retina of NaIO₃-treated animals. *Unpaired Student’s t-test P < 0.0001, data is presented as means ± SEM from n=5 NaIO₃ treated-eyes, and n=4 control eyes.

4.4.3 Pulsed-DNIRA detects labelled immune cells directly in vivo

Having been able to detect macrophages in vivo after systemic cell injection, we elected to label cells in vivo directly by systemically injecting ICG dye alone. Following a single systemic ICG injection, during dye injection the typical vascular patterns associated with angiography can be observed (Figure 4.6(a)). A clear image of the retinal and choroidal vasculature during the transient phase is evident. This is in stark contrast to the pattern previously shown in Figure 4.4 during injection of individually labelled cells. Imaging an animal at the evolution stage of NaIO₃ atrophy, and ICG at least 24 hours prior, a cellular pattern of NIR signal can be observed using
the ICG filters of the cSLO (**Figure 4.6(b)**). This pattern shows individual small punctate fluorescent dots, analogous to those seen using Immuno-DNIRA. This pattern is not evident prior to dye injection, or using the IR reflectance (830 nm) channel of the cSLO (**Figure 4.6(c)**). Furthermore, it is distinctly different from the ICG angiography observed in **Figure 4.6(a)**. Together this suggests that this imaging is dependent on injection of ICG dye following the induction of damage with NaIO₃, and the NIR fluorescence filters of the cSLO to observe these cells. We have termed this technique Pulsed-DNIRA and have adapted it to viewing activated inflammatory cells in vivo in the fundus.

**Figure 4.6 Punctate fluorescent immune cells are observed in vivo in the eye using Pulsed-DNIRA.** (a) Direct systemic injection of ICG immediately after NaIO₃ using ICG angiography settings of the cSLO shows the typical rodent vasculature. (b) At least 24 hours post-ICG dye injection, individual punctate fluorescent cells can be identified in the fundus of animals that have previously received NaIO₃. (c) No signal is observed in the ICG channel without dye injection (left), and no punctate fluorescence is visualized in the IR reflectance (830 nm) channel of a similar, but not dye-exciting wavelength (right).
4.4.4 **Macrophage DNIRA signal is persistent and dynamic over stages of atrophy and cells are observed to have migratory properties**

Using pulsed-DNIRA imaging by injecting ICG at different time points it is possible to observe dynamics in fluorescent cell populations that change over time, with different cell populations taking up the fluorescent dye at different stages of atrophy (Figure 4.7; Supplemental Figure 4.1). Imaging at least 24 hours following systemic ICG injection was determined to afford sufficient clarity from background fluorescence to identify individual immune cells in the degenerating rodent retina. This signal persists in the days and weeks following a single systemic ICG injection in animals that have been treated with NaIO₃ to induce retinal degeneration. Imaging from one timepoint to the next, differences in location of individual fluorescently labelled cells in the retinal tissue are detectable (Figure 4.8(a)). In particularly, where cells appear to migrate from the central optic nerve head towards the periphery of the fundus over time following systemic NaIO₃, ICG labelling identifies the migratory properties of these cells, further suggesting that they are activated phagocytes. Confirmation by fluorescent confocal microscopy using macrophage marker CD163 at the maturation stage of atrophy, numerous cells can be observed to surround the optic nerve head region in the excised rat retina (Figure 4.8(b)).

4.4.5 **Macrophage DNIRA signal correlates to bright FAF**

Next we aimed to determine whether the signal associated with DNIRA labelled cells would be relevant to areas of bright FAF that are observed in rats at the evolution stage of atrophy following NaIO₃, as reported in Chapter 3, and those typically observed in patients with GA. Comparing the cellular ICG fluorescence observed with DNIRA to the fluorescence observed in the FAF channel, there is an apparent correlation of the fluorescent signal in the two channels during atrophy evolution (Figure 4.9). This can be viewed both using Immuno-DNIRA imaging (Figure 4.9(a)), and Pulsed-DNIRA imaging (Figure 4.9(b)). This points to both signals coming from the same source and suggests that macrophages are associated with complex patterns of FAF, and with disease such as GA.
Figure 4.7 Dynamics of immune cell populations throughout progression of atrophy can be identified using Pulsed-DNIRA. ICG dye injected systemically at stages of baseline, induction, evolution, maturation, senescence, and imaged 2 days after dye injection shows fluorescence of different cell populations over time. ICG injected at baseline depicts RPE cell fluorescence. This fluorescence is eliminated when RPE cells are destroyed with NaIO₃. When the dye is injected subsequent to RPE atrophy, immune cells in the retina take up the ICG. This points to multiple active cell populations in this model.

Analyzing further using false colouring of the grayscale cSLO images to green and red, and overlaying the two signals, it is evident that the signals are localized to the same source by observing the yellow overlapping signal (Figure 4.10). This strengthens the argument that macrophages that have taken up ICG dye and are responsible for the in vivo DNIRA signal are the same cells responsible for autofluorescence observed on FAF using the cSLO.
Figure 4.8 Pulsed-DNIRA immune cells display migratory properties around the optic nerve head suggesting they are inflammatory macrophages. (a) Enlargement from figure 4.7 shows migratory properties of the labelled cell populations around the optic nerve head across the stages of retinal atrophy, suggesting that pulsed-DNIRA labels dynamic and not stationary cell populations. (b) Confocal fluorescent microscopy of excised retina using macrophage marker CD163 confirms numerous retinal macrophages in the region of the optic nerve head at the maturation stage following RPE damage.

Figure 4.9 DNIRA macrophages are localized to areas of bright FAF. Imaging macrophages in vivo using both (a) Immuno-DNIRA, and (b) Pulsed-DNIRA techniques reveals that cells that have taken up ICG correlate to patterns of bright FAF signal in the 488 nm channel of the cSLO, suggesting both signals originate from the same source.
4.4.6 Localization of labelled macrophages correlates to immune cells on microscopy

DNIRA signal of labelled immune cells has a likeness to autofluorescence signal observed in tissue using 488 nm AFM and fluorescent confocal microscopy (Figure 4.11; Supplemental Figure 4.2). Small fluorescent cells can be seen to line the choroidal blood vessels using pulsed-DNIRA in areas devoid of RPE, where choroidal vasculature is readily visible (Figure 4.11(a)). Following dissection of the eyeball and removal of the overlying retina, AFM of the posterior eyecup, prior to any fixation and staining procedures, demonstrates similar patterns of bright autofluorescent immune cells lining the choroidal vasculature (Figure 4.11(b)). These small autofluorescent cells have single nuclei and inflammatory cell morphology, suggesting they are choroidal inflammatory cells. Following immunofluorescent staining and confocal microscopy, macrophage markers Iba1 and CD163 confirm that cells lining the choroidal blood vessels are macrophages (Figure 4.11(c)).
Figure 4.11 Choroidal macrophages are evident using DNIRA and correlate to patterns observed in excised tissue. (a) DNIRA imaging of eyes devoid of RPE shows labelled DNIRA+ cells lining the choroidal blood vessels. (b) Multiple small punctate fluorescent cells can be detected by 488 nm AFM of excised posterior eyecup tissue and do not present with RPE morphology (top). Higher magnification confirms that these cells line the choroidal blood vessels (bottom). (c) Confocal microscopy using macrophage markers Iba1 and CD163 confirms presence of macrophages lining along the blood vessels of the choroidal vasculature.

4.4.7 Macrophages trapped in deformed retinal folds contribute to FAF

Having observed that the bright pattern observed using FAF correlates to inflammatory cells on DNIRA imaging, and that DNIRA cells correlate to autofluorescent cells on AFM, and to macrophage markers, we next aimed to determine whether these cells can directly correlate to curvilinear patterns associated with bright FAF. As such, the retina was excised and analyzed under AFM to determine whether a similar cellular pattern could be observed in the retina using 488 nm autofluorescence. In chapter 3 we report that the retina deforms and presents with curvilinear folds during evolution and subsequent stages of NaIO3-induced atrophy. Staining for macrophage markers Iba1 and CD68 in this folded retinal tissue, we can observe positively-stained macrophages in these folds (Figure 4.12). Figure 4.12(a) displays the curvilinear FAF hyperfluorescence associated with NaIO3-induced damage in the maturation stage (as reported in Chapter 3). Under 488 nm autofluorescence a similar curvilinear pattern can be observed in the retinal tissue (Figure 4.12(b)), and upon closer observation can be seen to be composed of
round, autofluorescent cells with inflammatory cell morphology, analogous to those observed in the eyecup in Figure 4.11(b), and in Supplementary Figure 4.3. When the tissue is immunostained for macrophage markers CD68 and Iba1, a similar pattern can be observed in the folds of the deformed retina (Figure 4.12(c)). The labelled macrophages are interspersed within the folds made up of photoreceptor nuclei. Iba1+ and CD68+ cells are arranged in a lacy, curvilinear pattern when viewed en face in wholemount retina. This pattern of Iba1+ and CD68+ cells corresponds with the pattern of hyperfluorescent FAF imaging, and with the pattern of autofluorescent cells in excised retina, suggesting that macrophages trapped in the folds of the deformed outer retina are responsible for the in vivo patterns of FAF during atrophy progression following NaIO₃-induced damage.

![Figure 4.12](image)

**Figure 4.12** Macrophages trapped in the folds of deformed retina are responsible for autofluorescent patterns of damage observed in vivo. (a) Curvilinear in vivo FAF in the maturation stage of atrophy (b) Curvilinear pattern of autofluorescent cells observed in excised retina using 488 nm AFM (c) Confocal fluorescent microscopy of rat retina in the maturation stage of atrophy, stained for Iba1 (red) and CD68 (green) shows macrophages interspersed among the folds and grooves of the retina made up of photoreceptor nuclei (blue).

### 4.5 DISCUSSION

The objectives of this study were to develop a method to observe macrophages in vivo in the eye, and to correlate these cells to patterns of FAF in the NaIO₃ model, and those typically associated with GA in AMD patients. To make these techniques translationally-relevant, and to be able to better study the roles of these cells, their numbers, localization, and migration, we developed a method that uses the clinically approved dye ICG and a commercial HRA-2 cSLO. Using the
techniques developed in this study, we confirm that rat macrophages are 1) capable of taking up ICG dye in vitro, 2) this labeling method is safe and the cells can be re-injected into recipient rats and visualized in the eye, 3) labelled cells localize to areas of retinal damage during evolution of atrophy, analyzed both using FAF and tissue staining, and 4) inflammatory, migratory macrophages can take up systemic ICG dye directly in vivo and be viewed in the eye. We have termed the two associated techniques Immuno-DNIRA and Pulsed-DNIRA for imaging macrophages in vivo in the rodent eye. Since this wavelength is outside the range of most microscope systems we confirmed the internalization of ICG by macrophages in vitro by developing a permeabilization assay and measuring internalized fluorescence using a spectrophotometer. Since ICG dye has been reported to cause toxicity in other cells such as RPE, particularly during epiretinal peel surgery, prior to injection we measured viability of cells before and following treatment with ICG. Supporting our previous report that ICG dye is safe at low doses even for RPE cells, no macrophage toxicity was observed, and cells remained viable after short treatments with ICG dye for the purpose of labelling. Other studies observed similar results, where it was determined that high viability can be maintained even with high ICG concentrations, provided the ICG dye is used in the dark so as to not induce its phototoxic properties (Varriale, Crescenzi et al. 2000).

Applying these modified DNIRA techniques to view macrophages in vivo to the NaIO₃ model of retinal atrophy, we have been able to localize macrophages in areas of bright FAF that are typically associated with GA in AMD patients. The fluorescence associated with ICG dye is distinct from autofluorescence, as it is absent without the dye injection in both normal and damaged tissue. Dry AMD is currently the leading cause of irreversible blindness in North America, without an available treatment alternative. Methods to study dry AMD are hindered by the unavailability of proper in vivo models of the disease, and low number of methods for monitoring the disease in patients in the clinic. It is imperative that better methods are determined to track patient progression of the disease from early stages to late stages and to differentiate between subtype progressions. Many studies have implicated cells of the innate immune system, notably macrophages in the progression of the disease by analyzing histological patient samples (Forrester 2003, Sennlaub, Auvynet et al. 2013). However, it is not yet certain which specific roles these cells play in the disease, and no in vivo studies have been able to definitively link macrophages to dry AMD. Using ICG-aided techniques such as Pulsed-DNIRA
enables for simple, non-invasive detection of macrophage-rich retinal lesions in vivo, and therefore applicable to direct detection of the inflammatory response associated with dry AMD. In this manner, it is feasible to track macrophages in the retina of patients with AMD using the cSLO equipment available in clinics, which can improve monitoring of the disease and warn physicians of potential progression or expansion of geographic atrophy.

Macrophages are phagocytic and have previously been suggested to take up ICG in a model of atherosclerosis, likely bound to albumin or LDL. The study also noted that macrophages were the only cells in atherosclerotic lesions that took up the dye (Vinegoni, Botnaru et al. 2011). It is possible that ICG is internalized via the LDL receptor, since it can bind to LDL particle among other hydrophobic moieties of proteins in the blood (Varriale, Crescenzi et al. 2000). While it is difficult to directly correlate the dye uptake to a specific cell in this study due to the absence of imaging systems capable of carrying out confocal microscopy in the 795 nm wavelength, the experiments combined above suggest that macrophages do indeed take up ICG dye in vivo and that this can be a feasible tool for imaging macrophages in the eye. In addition, where it was previously thought that RPE cells are the only contributors to autofluorescence in the eye due to the accumulation of lipofuscin pigment in these cells, it is now evident that there are two sources of autofluorescence that contribute to this signal (Supplemental Figure 4.3). Lei et al have shown that macrophages fed photoreceptor debris in vitro rapidly, in approximately 4 days, become autofluorescent. They describe this 488 nm autofluorescence as lipofuscin-like autofluorescence (LLAF) (Lei, Tzekov et al. 2012). As such, seeing as hyperfluorescent FAF is such a prevalent characteristic during the progression of GA, it is important not to discount the potential contribution of macrophages to this signal. The data above suggests that the macrophage response in the NaIO₃ model is robust, and linked to the presence of atrophy, and therefore may also be a factor in GA progression. Subsequent work will aim to discern the mechanisms of macrophage action in this model, and determine whether these can be targeted for potential therapy of this disease.
Supplemental Data

**Supplemental Figure 4.1** Individual phagocytic cells with migratory properties can be distinguished in the degenerating retina over time using Pulsed-DNIRA. ICG dye injected systemically following induction of RPE and retinal damage, and imaged at least 2 days after dye injection shows fluorescent cells that have taken up ICG in vivo. Imaging from one timepoint to a subsequent timepoint in the same area using the same settings on the cSLO (ICG wavelengths 795 nm excitation/810 nm emission) allows to capture dynamic cells that change spatially. This suggests they can be migratory immune cells that take up ICG.

**Supplemental Figure 4.2** Patterns of DNIRA macrophages correlate to 488 nm autofluorescent signal in excised tissue. In vivo imaging of macrophages that have taken up ICG dye in vivo and observed using DNIRA imaging (left), shows patterns of fluorescence similar to those that can be observed in 488 nm autofluorescent microscopy in excised posterior eyecup tissue samples (right).
Supplemental Figure 4.3 Two sources of fluorescence contribute to the 488 nm autofluorescent signal. Autofluorescent microscopy using 488 nm excitation confirms presence of two types of cells. *Large damaged and dying multinucleate RPE remnants, and # Smaller fluorescent cells with single nuclei and inflammatory cell morphology that line choroidal vasculature and infiltrate into areas of atrophy.
Chapter 5

Dynamics of M1/M2 Polarization during the Course of RPE and Retinal Atrophy in a Model of Dry AMD

5.1 ABSTRACT

Inflammatory M1 and anti-inflammatory M2 macrophages play a role in a number of neurodegenerative and immune-mediated disease. The purpose of this study was to identify whether M1 and M2 phenotypes play a role in the NaIO₃ model of dry AMD, and to determine whether these phenotypes change throughout model progression. We used in vitro validation studies on rat peritoneal macrophages to confirm several markers of M1 and M2 polarization, and then used those markers in excised tissue samples of retina and RPE/eyecup of rodents that had atrophy induced with NaIO₃, compared to control animals. We further confirmed our findings using qPCR arrays to analyze a variety of M1 and M2 transcripts and determine the changes of these transcripts that take place over time in this model. Our findings show that macrophages play an important role in this model due to the tight interactions of these cells with the RPE monolayer, which gets immediately broken down by NaIO₃ treatment. Following this insult, macrophages take on an M1 profile, drastically different from the baseline M2 marker expression. The M1 expression is robust, compared to the baseline M2, and remains sustained throughout the stages of atrophy progression. This points to an important role that M1 macrophages play in promoting inflammation in the NaIO₃ model, which was previously believed to be only an acute model of apoptosis and necrosis, and suggests that the M1 response may be a key feature of the formation and expansion of the patches of GA that accompany late dry AMD.

5.2 INTRODUCTION

AMD of the central retina, the macula, is the leading cause of irreversible blindness in the Western world. Early AMD is characterized by the accumulation of small deposits known as drusen, however progression to the late dry form of AMD, or GA, is associated with a regression of drusen, and patches of atrophy or tissue loss in the layers of the RPE and the overlying retina.
GA is progressive, as the patches of atrophy expand over time, and the etiology of this expansion is not entirely known. Human histopathological specimens and genetic testing suggest AMD is highly associated with innate immunity. Over 20 genes, including those for the innate immune system, alone and in combination confer susceptibility to the disease (Ratna Priya, Chew et al. 2012). The innate immune system is composed of a non-cellular arm, the complement cascade, and a cellular arm made up of phagocytic cells including macrophages and microglia (the tissue macrophages of the CNS). To this date, clinical trials for dry AMD targeting the complement cascade predominantly either failed to achieve their endpoints or have not yet reached the clinic (Zhang, Zhang et al. 2012, Yehoshua, Filho et al. 2014).

A simplified model of macrophage activity suggests polarization into two broad subtypes - M1 and M2. M2 macrophages are believed to be involved in normal tissue homeostasis and to have a non-inflammatory or house-keeping role. They typically present with scavenger receptors such as MMR and CD163, and release modulatory cytokines such as IL-10. M2 macrophages are also activated during tissue healing and fibrosis. M1 macrophages, on the other hand, are highly pro-inflammatory and respond to tissue stressors such as foreign stimuli and oxidative species, with increased phagocytosis, migration and activation of inflammatory cytokines, such as iNOS, MCP-1 and TNFα (Murray and Wynn 2011). Transcriptional networks of M1/M2 chemokines, cytokines and transcription factors are suggested to amplify the appropriate response and down-regulate the opposing one (Sica and Mantovani 2012). Macrophages with markers of M1 and M2 polarization are reported in pathological specimens of AMD, but the findings are limited to one or two markers, and their significance is not yet understood (Cherepanoff, McMenamin et al. 2010, Cao, Shen et al. 2011).

Having identified macrophages in the tissue samples in the NaIO₃ model of dry AMD, we conducted this study to better understand the macrophage dynamics that play a role in atrophy progression by looking at markers of polarization in this model. Since macrophages are observed to infiltrate the damaged tissue, we used tissue samples to look for markers of macrophage phenotypes in this model using immunofluorescent staining and quantitative PCR, to determine whether these phenotypes change throughout the stages of progression and whether they may be associated with the progressive RPE loss observed in this model.
5.3 METHODS

5.3.1 Animal Studies

All procedures were performed in accordance with the Canadian Council on Animal Care and with adherence to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. For all experiments, SD rats aged 6-10 weeks were kept at a 12 hour dark/light cycle, with food and water ad libitum. For evaluation, rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and pupils dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride. Eyes were conditioned repeatedly using GenTeal lubricating eye drops (Novartis, Canada) to prevent desiccation. Following completion of in vivo work, animals were humanely sacrificed by intracardiac injection of T61 (Intervet Canada, Whitby, Canada).

5.3.2 Sodium Iodate Injection

Sodium iodate (Sigma) was prepared fresh at a concentration of 45 mg/ml in 0.9% saline for each set of experiments, and injected to a final concentration of 45 mg/kg body weight using a 24 gauge catheter inserted into the tail vein.

5.3.3 Macrophage Isolation and In Vitro Polarization

Primary peritoneal macrophages were isolated from SD rats as described in Chapter 4. Cells were seeded in 8-well chamber slides at $5 \times 10^5$ cells/chamber, and kept in culture for up to 1 week. For in vitro confirmation of polarization markers, cells were polarized towards M1 using LPS (1 ug/mL, Sigma)/IFNγ (20 ng/mL, PeproTech), or towards M2 using dexamethasone (250 nM, Sigma) for 24 hours in RPMI-1640 macrophage media. A subset of cells were also polarized to M2 using IL-4/IL-13 (20 ng/mL each, PeproTech) for 24 hours (Supplementary Table 1). Following polarization, cells were fixed with 4% PFA for 10 minutes and used for immunofluorescent staining.

5.3.4 Cell Immunofluorescence

Cells were permeabilized with 0.1% Triton-X 100 for 15 minutes and blocked with 5% BSA/PBS for 45 minutes at room temperature. Following this, cells were incubated with primary antibody (or isotype control) at a concentration of 5 µg/ml unless noted otherwise overnight at
4°C, washed with PBS, and followed by secondary antibody (1:400) 2 hours at room temperature. Cells were then counterstained with To-Pro-3 nuclear stain (Life Technologies), mounted with fluorescent mounting medium (Dako), coverslipped and viewed using a Leica TCS SL confocal fluorescent microscope. Antibodies used to validate phenotypes were as follows, mouse monoclonal anti-rat CD68 (AbD Serotec, MCA341R) and rabbit polyclonal anti-Iba1 (Wako Pure Chemical Industries Ltd, 019-19741) were used to confirm a neutral phenotype prior to polarization. Mouse monoclonal anti-rat CD163 (Hycult biotech, HM3025), rabbit polyclonal anti-rat MCP-1 (PeproTech, 500-P76), rabbit polyclonal anti-iNOS (Abcam, ab15323), rabbit polyclonal anti-TNFα (Millipore, AB1837P). See Supplementary Table 2 for full list of antibodies tested. These were compared against normal mouse IgG (Santa Cruz) or rabbit IgG (Abcam) controls. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (Life technologies) Alexa Fluor 555 goat anti-mouse (Life technologies).

5.3.5 Cell Quantification

For a subset of experiments cells were stained for macrophage polarization markers iNOS and CD163, and counted using Image J software. Ten fields each from n=4 experiments were imported into Image J and threshold adjusted to Min = 100, Max = 255 for each image. Particle analysis with particle size cutoff = 25 was used to obtain number of particles staining positive for iNOS and CD163 in each field. Statistical analysis was performed using GraphPad Prism v5.0 on the percent (%) positive cells/field of view, and data is reported as mean +/- SEM using an unpaired Student’s t-test.

5.3.6 Cryopreservation of Isolated Rat Retina

Enucleated rat eyes were fixed with 4% PFA/PBS for 20 minutes at room temperature, then anterior portion of the eye was dissected, leaving posterior section including retina and eyecup. The tissue was subsequently fixed in 4% PFA/PBS for 1 hour at room temperature, and 4 radial cuts were made to flatten the eyecup. Samples were incubated in 30% sucrose/PBS at 4°C approximately 1 hour until they sunk to the bottom of the well. They were then transferred to OCT compound in a cryomold and covered completely with OCT. Frozen OCT blocks were stored at -80°C until use. Sections (20 µm) were prepared using a Leica CM 1900 cryostat at -20°C, positioned onto lysine-coated slides, allowed to air dry overnight at room temperature, and stored at -80°C until further use.
5.3.7  **Immunofluorescence of Cryopreserved Sections**

Samples were removed from -80°C, air dried for 20 min, outlined with PapPen and dried further for 10-30 minutes. Following a 1 minute rinse and 2 x 10 minute washes in TBS, the sections were hydrated for 20 minutes in TBS. Blocking was carried out in 5% BSA in TBS at room temperature for 1 hour with rotating. Sections were incubated with primary antibody (5 µg/ml unless specified otherwise) overnight at 4°C while rotating, followed by an hour of washing with TBS. For sections stained with fluorescently-labelled isolectin Ca2+ was added to the secondary antibody solution to a final concentration of 0.5 mM in 5% BSA/TBS. Samples were incubated in secondary antibody for 2 hours at room temperature, light-protected, followed by a hour of washing with TBS. Nuclei were counter-stained with To-Pro-3 (1:1000) and incubated at room temperature, light-protected for 5 minutes, followed by washing with TBS. Tissues were mounted with Dako, coverslipped until solid, and stored at 4°C.

5.3.8  **Whole Tissue Immunofluorescence**

Following dissection of retina and eyecup (RPE) tissue, samples were blocked with 5% BSA/TBS, 0.1% Triton X-100 for 45 minutes at room temperature. Primary antibodies were added at a concentration of 5 µg/ml (unless specified otherwise) in blocking buffer, and incubated overnight 4°C. Samples were subsequently washed with TBST (TBS, 0.1% tween-20) for 5 x 1hr at room temperature followed by incubation with Alexa-fluor conjugated secondary antibody (1:400) overnight 4°C. Samples were washed with TBST for 5 x 1hr at room temperature and nuclei counterstained with To-Pro-3 (Life Technologies). Tissue was mounted onto 2-well Teflon printed slides using fluorescent mounting medium (Dako) and coverslipped. Images were acquired using a Leica TCS SL confocal fluorescent microscope (Leica Microsystems, GmbH, Wetzlar, Germany), with Leica Confocal Software Version 2.61. Primary antibodies used were as listed above for cell immunofluorescence. Mouse monoclonal anti-rat RPE65 (Abcam, ab78036) was used to label RPE. These were compared against normal mouse IgG (Santa Cruz) or rabbit IgG (Abcam) controls. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (Life technologies) Alexa Fluor 555 goat anti-mouse (Life technologies).
5.3.9 RNA Isolation, QIAGEN RT\(^2\) Profiler PCR Array and Data Analysis

Total RNA was isolated using 8 eyes for each stage of atrophy progression following NaIO\(_3\) injection (baseline, induction, early evolution, late evolution, maturation, senescence), from rat retinas and posterior eyecups using Trizol Reagent (Life Technologies, USA). Briefly, after enucleation of rat eye balls, they were dissected in cold RNALater Solution (Life Technologies, USA). The entire retina was then removed and stored in RNALater solution, and RPE and choroid layers were scraped with a surgical scalpel blade and combined with the retina. Manufacturer’s protocol was followed for RNA extraction with the Trizol method. Total RNA was further purified with RNeasy MinElute Cleanup kit (Qiagen, USA). Their quantity and quality were checked with a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

cDNA was synthesized using RT\(^2\) First Strand Kit (Qiagen, USA) with 2 µg of total RNA per reaction. Real-time PCR was performed using Qiagen 384-well Custom RT\(^2\) Profiler PCR Array and RT\(^2\) SYBR Green ROX qPCR Mastermix (Qiagen, USA) with 10 ng cDNA per well. The reactions were run on a ViiA 7 PCR system (Life Technologies, USA) with default cycling conditions. Differences in gene expression were analyzed using the Qiagen RT\(^2\) Profiler PCR Array Data Analysis Software V3.5 Web Portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) based on \(\Delta\Delta C_T\) method and normalized to Peptidyl-prolyl cis-trans isomerase H (PPIH) as a reference gene. Results are reported as fold-change (\(2^{-\Delta\Delta C_T}\)) normalized to baseline as 1-fold. P values were calculated based on a Student’s t-test of the replicate \(2^{-\Delta\Delta C_T}\) values for each gene in the control group and treatment groups, and P values less than 0.05 were considered significant. The primer sequences are proprietary. The amplicon size, Qiagen gene catalog number and primer reference position found below.

**Table 5.1 Qiagen RT\(^2\) PCR array gene information**

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<tr>
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<td>PPR57387B (XM_001073803)</td>
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### 5.4 RESULTS

#### 5.4.1 Macrophages change spatially and temporally in the damaged retinal tissue

Staining the retinal tissue with inflammatory markers during the stages of atrophy progression identifies numerous inflammatory cells following the toxic insult compared to baseline ([Figure 5.1](#)). Lectin staining, which identifies activated inflammatory cells as well as blood vessels, shows that these cells increase in number initially during the early stages of damage. The cells can then be observed to decrease gradually over the course of progression ([Figure 5.1(a-b)](#)). It is believed that macrophages migrate from the inner to the outer retina and subretinal space during an insult or disease, and this has been previously shown in other studies, including earlier studies using the NaIO₃ model (Mendes-Jorge, Ramos et al. 2009). Therefore, cross-sections were
stained with general macrophage markers Iba1 and CD68 to determine whether evidence of migration across the retinal layers can be identified in this model under our conditions. **Figure 5.1(c)** clearly shows that following damage to the RPE and retina, CD68+ and Iba1+ macrophages migrate not only from the inner retinal layers, but also from the choroidal layers and move into the damaged sub-retinal space. This supports the gradual decrease in inflammatory cell populations in the inner retina observed in **Figure 5.1(a-b)**.

**Figure 5.1** Macrophages change spatially and temporally in the NaIO₃ model of atrophy (a) Lectin staining of inflammatory cells in the inner retina at stages of atrophy progression (b) Quantification of lectin-stained cells shows a significant increase in the amount of cells in the retina following induction of atrophy, followed by subsequent decreases in the later stages. All timepoints were statistically significant compared to baseline values. Each time point was statistically significant compared to the previous time point. *P < 0.0001, Unpaired Student's t-test. (c) Macrophage markers Iba1 and CD68 can be identified on cells in the damaged tissue. Inner retinal and choroidal cells migrate to the damaged outer retina (atrophy) where they are not typically found (baseline).

### 5.4.2 Macrophages are tightly interspersed beneath the RPE layer and progressively infiltrate following its damage

Reconstructions of confocal microscopy Z-stack images using IMARIS software show the tight interactions between RPE cells (as determined by RPE65 staining) and Iba1+ macrophages under normal conditions as observed in the baseline image (**Figure 5.2**). The macrophages are located directly beneath the RPE cells and their processes appear to reach the junction areas between individual cells. With atrophy induction, these cells begin to infiltrate into the damaged RPE layer through the broken RPE barrier. With time, macrophages accumulate in the area where the RPE cells typically reside (**Figure 5.2, Supplementary Figure 5.1**), and the RPE65 signal
diminishes over time. This points to the possibility that macrophages may be responsible for the progressive demise of the RPE.

**Figure 5.2** Tight interactions between RPE and macrophages are observed using 3D reconstructions of RPE and choroidal tissue. Perspective view of reconstructed RPE and its underlying choroidal tissue z-stacks observed from above and from below, shows the separate but close interactions between RPE and Iba1+ macrophage processes under normal conditions, as observed in baseline. Following the toxic insult and with atrophy induction, the infiltration of Iba1+ cells from the choroidal space through the broken RPE barrier can be observed. With time, the RPE layer atrophies as more macrophages are observed interspersed in the tissue. Image analysis done using IMARIS Software Version 8.2.

### 5.4.3 Markers of macrophage polarization can be elicited in primary rat peritoneal macrophages

To determine whether M1 polarized macrophages may be responsible for the demise of the RPE in the NaIO₃ model, a number of markers were first tested in vitro on primary rat peritoneal macrophages to determine whether the cells from these species can be polarized. The complete list of markers tested can be found in **Supplementary Table 1**. The markers were tested under a number of conditions that favour either M1 or M2 polarization, which are identified in **Supplementary Table 2**. Reliable macrophage polarization markers for rat macrophages were determined to be iNOS, TNFα, and MCP-1 for M1, and CD163, IL-10 and MMR for M2 (**Table 5.2**).

<table>
<thead>
<tr>
<th>Reliable Macrophage Markers</th>
<th>Phenotype</th>
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<tr>
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</tr>
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<td>M1</td>
</tr>
<tr>
<td>MCP-1 (CCl2)</td>
<td>M1</td>
</tr>
<tr>
<td>CD163</td>
<td>M2</td>
</tr>
<tr>
<td>IL-10</td>
<td>M2</td>
</tr>
</tbody>
</table>
In the examples of in vitro polarization results in Figure 5.3, it is clear that only under conditions targeting M1 polarization cells display the M1 marker iNOS, and only under conditions targeting M2 polarization cells display the M2 marker CD163. The neutral (PBS control) cells and negative antibody controls show no expression of polarization markers. Quantification of % positive cells polarizing towards M1 and towards M2 phenotypes following M1 and M2 conditions is shown in Supplementary Figure 2. Overall 89% of M1 polarized cells were iNOS positive, while less than 1% were positive in the control condition (*P<0.0001). Overall 46% of M2 polarized cells were CD163 positive, while less than 1% of the control cells were positive (*P<0.0001), supporting the validity of the M1 and M2 antibody markers.
Figure 5.3 **In vitro polarization of rat peritoneal macrophages.** (a) Macrophages targeted for M1 polarization using LPS/IFNγ display the M1 marker iNOS (red) along with the general macrophage marker CD68 (green). Neutral (PBS control) and M2 polarized cells only display the general marker CD68 under these conditions. (b) Macrophages targeted for M2 polarization using IL-4/IL-13 display the M2 marker CD163, along with the general macrophage marker Iba1. Neutral and M1 polarized macrophages only display the Iba1 under these conditions. (c) Cells polarized to M2 with dexamethasone also display the M2 marker CD163, and do not display the M1 marker iNOS. (d) Negative antibody controls confirm antibody selectivity.

5.4.4 **M1 and M2 cells can be identified in all layers of the retina and choroid**

Having successfully validated markers of M1 and M2 polarization in vitro, we stained excised retinal and choroidal tissue samples for some of these markers to determine whether markers of polarization can be identified on macrophages in this model. **Figure 5.4** shows that markers for M1 and M2 cells can be observed in tissue from the onset of atrophy, in all the layers of the retina, RPE and choroid. In particular, M1 inflammatory markers TNFα, MCP-1 and iNOS are prominent in the inner retina, outer retina and choroid during atrophy, pointing to an important role that these inflammatory cells may play in the model.

**Figure 5.4** Polarized macrophages are present in all layers of the retina and choroid during NaIO₃-induced atrophy. Cells showing markers for both M1 (TNFα, MCP-1, iNOS), and M2 (primarily CD163) polarization can be identified in both retinal and choroidal tissue. Negative antibody controls confirm no marker expression.
5.4.5 **M2 markers present in healthy tissue, M1 markers upregulated with atrophy progression**

Having observed the presence of M1 and M2 markers in the damaged tissue on a general scale, we next determined whether these markers are expressed differentially over time with model progression. Using a time course of iNOS (M1) and CD163 (M2) expression as an example, it can be observed that during baseline only CD163+ cells are present in the retina (**Figure 5.5**). With atrophy induction, iNOS+ expression is evident, and remains so throughout atrophy progression. During later stages, iNOS appears to be subsequently downregulated, while CD163 remains expressed comparatively to baseline. This suggests that M2 is the prominent phenotype in this model under normal conditions. Once the toxic insult is given, M1 is strongly expressed and remains so for the large part of atrophy progression. Very late stages of atrophy appear to show some decrease in M1 and potentially an increase in M2 expression. A similar timecourse of expression can be observed with another M1 inflammatory marker, TNFα (**Figure 5.6**), and is observed not only in the retina, but also in the choroidal tissue (**Supplementary Figure 5.4**). In the choroid resident cells also display an M2 phenotype at baseline, and an increase in M1 expression can be observed during NaIO₃-induced atrophy.
Figure 5.5 Retinal M1 iNOS expression over course of progression in NaIO₃ RPE and retinal atrophy. During baseline, only M2 CD163+ cells (green) are observed in the inner retina, with no inflammatory cell presence in the outer retina, which is confirmed to be intact. Following atrophy induction, iNOS expression (red) can be observed in the inner retina and remains present as the atrophy progresses into late stages (early and late evolution). iNOS expression decreases in the very late stages of atrophy (maturation). Outer retinal nuclear staining (blue) confirms the progressive deformation that increases over time. Negative antibody controls display no staining for either marker.

Figure 5.6 Retinal M1 TNFα expression changes over the course of atrophy progression. M1 TNFα expression (red), only appears in individual cells following atrophy induction and remains evident throughout the course of progression in the inner and outer retina. M2 CD163 (green) is also present at baseline in normal tissue, and persists throughout the stages of atrophy.

5.4.6 Some cells capable of displaying M1 and M2 markers simultaneously

During evolution of atrophy, we observe that some macrophages co-express both M1 and M2 markers (Figure 5.7). The same macrophages which express M1 marker iNOS are also expressing M2 marker CD163. This supports the notion that these cells are plastic, and that it is their dynamic change in phenotypes that may play a role in disease progression, and not simply an infiltration of a separate population of cells. Interestingly, cells displaying TNFα expression in the retina (Figure 5.6), appear to have distinct labelling of either TNFα or CD163, and do not express both markers simultaneously at least in the inner retina where this expression can be delineated, indicating multiple macrophage phenotypes are present in the retinal environment following atrophy induction, and supporting the notion of a complex innate immune response. In the outer retina, it becomes difficult to identify separate macrophage cells and their individual expression due to accumulation of multiple inflammatory cells in the retinal folds.
Figure 5.7 Simultaneous expression of M1 and M2 markers in retinal macrophages. During the evolution stage of atrophy macrophages in the retina present with markers of both M1 (iNOS, red), and M2 (CD163, green) polarization simultaneously. Nuclei are stained blue.

Figure 5.8 M1 cells confined within the deformed outer retina and subretinal space contribute to a sustained inflammatory response. TNFα (left) and MCP-1 (right) cells (red) are identified among the folds of the deformed outer retina and in the subretinal space in mature atrophy. These cells are not observed in normal outer retinal tissue at baseline.
5.4.7 **M1 cells trapped in the deformed outer retinal folds contribute to a sustained inflammatory response**

Staining of retinal tissue samples demonstrates that markers of M1 macrophages can be observed during late stages of atrophy in the NaIO₃ model (Figure 5.8). These cells are localized within the numerous retinal folds of the deformed outer retinal space. This was previously observed with neutral macrophage markers CD68 and Iba1 (Chapter 4), and it was determined that those cells contribute to the FAF signal on in vivo imaging. The current data suggests that the trapped cells have primarily an M1 phenotype, expressing markers of MCP-1 and TNFα. This suggests that the M1 inflammatory response is sustained, not acute, and that these trapped cells may contribute to progression of atrophy in the damaged retina and in vivo patterns of disease.

5.4.8 **Messenger RNA analysis confirms upregulation of M1 response during atrophy**

To confirm the tissue findings using gene expression and to evaluate a greater subset of M1 and M2 markers, we next performed quantitative PCR (qPCR) arrays to evaluate the M1/M2 response. PCR arrays were designed to target genes associated with M1/M2 polarization and those of the innate immune system. Individual fold-change values for all genes analyzed are provided in Supplementary Table 5.2. The combined average fold-change expression across all the M1 genes analyzed and all the M2 genes analyzed is displayed in Figure 5.9. By pooling 10 M1 inflammatory genes and 10 M2 anti-inflammatory genes and looking at the overall mean fold change we can observe a trend with a highly upregulated M1 response early in this model compared to baseline control, and a relatively stable M2 response over the course of progression. These dynamic changes in the M1 response can also be identified using volcano plots displayed in Supplementary Figure 5.5.

Analysis of select individual genes confirms these findings, showing dramatic early upregulation of cell surface M1 macrophage marker CCR2, M1 marker SOCS3, and M1 transcription factor STAT1 (Figure 5.10). M2 genes are evident at later stages of maturation and senescence, however typically not to the same extent. SOCS3 suppresses M2 signaling, thereby amplifying the M1 expression, and SOCS1 suppresses M1 signaling, amplifying M2 expression. The SOCS3 expression trends highly elevated throughout progression of the atrophy, decreasing only slightly towards the later stages. SOCS1 expression becomes significant at maturation and
senescence though at a very low level of expression compared to SOCS3. Similar pathways regulate M1 and M2 transcription factor expression via the STATs (Sica and Mantovani 2012). Likewise, a similar trend for the STAT transcription factor expression can be observed.

Figure 5.9 Mean mRNA expression of total combined M1 transcripts compared to total combined M2 transcripts over the course of atrophy progression identifies a greater overall M1 change. Fold change expression of ten M1 transcripts and ten M2 transcripts was pooled and the overall mean fold-change was determined. The trend shows a highly upregulated M1 response early in this model, and a relatively stable M2 response. RNA from n=3 independent samples of chorioretinal extracts was collected and custom qPCR arrays were ran. Data was generated using Qiagen RT² Profiler PCR Array Data Analysis software version 3.5.

Figure 5.10 M1 gene expression is upregulated early during atrophy and M2 gene expression is upregulated later. (a) Fold change expression of cell surface M1 marker CCR2, and M2 marker CD163. (b) SOCS expression of M1 marker SOCS3, and M2 marker SOCS1. (c) STAT expression of M1 marker STAT1, and M2 markers STAT3 and STAT6. Fold change \((2^{\Delta\Delta Ct})\) is the normalized gene expression \((2^{\Delta Ct})\) in the test sample (stage of atrophy progression) divided by the normalized gene expression \((2^{\Delta Ct})\) in the control sample (baseline). RNA from n=3 independent samples of chorioretinal extracts was collected and custom qPCR arrays were ran. Results were normalized against reference gene PPIH. Data was generated using Qiagen RT² Profiler PCR Array Data Analysis software version 3.5. *(P<0.05)
5.4.9 **ICG dye does not impact macrophage phenotype and does not induce inflammation**

Lastly, to confirm that ICG dye which we routinely use during in vivo imaging does not affect macrophage polarization, we performed cell staining for polarization markers on ICG-treated macrophages, and compared against positive and negative controls (Supplementary Figure 2). Due to the phagocytic ability of macrophages towards bacteria and other pathogens, we speculated there may be a potential for innate immune system activation following dye internalization if the dye is phagocytosed. No observed change in polarization markers was identified with ICG dye in vitro. ICG dye internalization does not activate the macrophage inflammatory response as measured by iNOS and CD163 expression. Cells were counted using ImageJ as expressing either CD163 for M2 or iNOS for M1, and compared to cells incubated with ICG. Percent (%) positive cells/field of view had a P>0.05, unpaired Student t-test. Data reported as mean +/- SEM.

Cells polarized towards M1 using LPS (1 ug/mL)/IFNγ (20 ng/mL) display positive expression for iNOS (red). Cells polarized towards M2 using dexamethasone (250 nM) display positive expression for CD163 (green). Control and ICG-labelled cells show no expression for either marker. To further confirm that cell phenotype isn’t adversely affected in vivo, tissue from ICG treated animals was stained for inflammatory markers. No changes in inflammatory cell populations similar to those typically observed following NaIO₃ were identified following ICG dye administration (Supplementary Figure 3). ICG did not cause migration of the cells into the outer retina, or activation of the cells by other means, as they maintained their morphology and spatial characteristics, unlike previously observed with NaIO₃. Together this data supports that ICG dye does not elicit the inflammatory response observed our NaIO₃ model.

5.5 **DISCUSSION**

The purpose of this study was to identify macrophage polarization in the NaIO₃ model of RPE and retinal atrophy, and to determine whether these macrophages may be associated with the progressive RPE loss that is evident in this model. We have shown that macrophages are present at sites of disease activity and may contribute to RPE loss, and that it is M1 macrophages that are likely responsible for this loss. This supports the presence of highly autofluorescent macrophages in this model, given that RPE cells contain a large amount of lipofuscin which may be passed on
to the macrophages if they phagocytose damaged RPE components. This phenomenon is supported by the evidence for macrophage autofluorescence after ingestion of POS components (Lei, Tzekov et al. 2012). Due to discrepancy in the literature about macrophage phenotypes across species such as mice, rats, and humans, we validated the markers used to stain tissue and determined a number of markers that successfully differentiated rat peritoneal macrophage in vitro under polarizing conditions (Gordon and Taylor 2005). We also focused on markers that have been identified in human specimens of dry AMD, in order to provide relevance and translational potential for this data (Cao, Shen et al. 2011).

We evaluated the time-course of M1/M2 expression in excised retinal and eyecup tissue at both the protein and mRNA level. Samples from NaIO₃-treated animals, evaluated at the same stages described by in vivo FAF imaging (Chapter 3), confirm that macrophages at baseline express an M2 phenotype. This is consistent with published reports of cytokine and chemokine expression in the retinal tissue, and with the notion that retina promotes an anti-inflammatory environment (Zamiri, Masli et al. 2006, Lau and Taylor 2009). In particular, healthy RPE are observed to suppress inflammatory activity (Zamiri, Masli et al. 2006, Lau and Taylor 2009). Conversely, with NaIO₃-induced RPE damage, there is a marked increase in both the total number of macrophages in the tissue, and in the proportion of M1 expression. Given this, it may be that the RPE are no longer capable of modulating alternative activation of macrophages in the tissue, and that the retinal microenvironment is overwhelmed with an M1 response, leading to propagation of inflammation.

Specifically, we observe extensive upregulation of MCP-1, CCR2 (the receptor for MCP-1), TNFα, iNOS, SOCS and STATs, amongst others. M1 markers predominate during induction and evolution, are present during maturation, and gradually decline towards senescence, though not back to baseline levels. At the same time an M2 phenotype upregulation can be observed at these later stages, though not to the same extent as the robust inflammatory M1 upregulation. In addition, we also report for the first time the presence of cells co-expressing both M1 and M2 markers in the retina, confirming both macrophage plasticity and the over-simplification of the M1/M2 paradigm. This also supports that macrophages which were originally M2 in the retina may take on an M1 phenotype during atrophy, and suggests that simply a systemic infiltration of new M1 macrophages into the damaged retina during AMD-like retinal atrophy may be an oversimplified view of the complex inflammatory response that takes place (Cruz-Guilloty,
Saeed et al. 2013). It points to an important role that the local tissue macrophage populations play in propagating this inflammatory response. This is supported by studies of other disease such as atherosclerosis, where a robust local proliferation response has been reported to play an important role (Robbins, Hilgendorf et al. 2013). As significant changes are noted in the expression of transcription factors reported to amplify or restrict M1/M2 polarization, it points to the importance of transcriptional networks during this response, and the potential for therapeutic regulation of the inflammatory response at the transcriptional level.

Interestingly, this study of NaIO3-induced RPE damage show a timeline of M2-M1-M2 polarization, which to our knowledge is the first time this sequence has been reported in vivo in the eye. It points to the importance of M2 homeostatic macrophages present in the healthy retinal tissue, yet also recognizes that an M2 response may be associated with progressive damage, scar formation and fibrosis at later stages of chronic disease. It is known that recognition and clearance of apoptotic cells is anti-inflammatory and mediated by M2 associated genes and proteins such as IL-10, PGE2, and TGFbeta. It is also inhibited by genes such as TNFα, IL-6, IL-12 (Mondal, Ghosh-Roy et al. 2011). This suggests that debris clearance in the retina may be strongly dependent on this M2 function and that dysregulation of these processes may be either a result or cause of an M1 response, indicating that preservation of the M2 phenotype in the retina is important. However, robust M2 activation under appropriate conditions can also result in tumourigenic properties and promotion of vascularization (Gordon 2003, Ly, Bronkhorst et al. 2010, Dennaoui, Bronkhorst et al. 2011). As such, we propose that in modulating the inflammatory response in models of AMD, a downregulation of M1 response be prioritized over intensifying the M2 response.

Lastly we also confirm that ICG dye as used previously in our experiments (Chapter 2-4), does not play a role in polarizing the macrophage population. Photodynamic therapy has been adapted to use ICG encapsulated in nanoparticles to facilitate uptake by phagocytic cells (Gomes, Lunardi et al. 2006). These and other ICG containing particles such as liposomes are rapidly engulfed by macrophages (Turner, Moshkelani et al. 2012, Jeong, Lee et al. 2013). However, phagocytosis of particles can lead to initiation of a macrophage immune response in resting cells (Gordon and Read 2002). In this study, we show that systemic administration of ICG does not appear to elicit a pro-inflammatory response in phagocytic innate immune cells. These results confirm that ICG dye is not only safe, but also inert in retinal tissue. It also supports the evidence
that ICG dye is not simply phagocytosed by likely transported through lipoprotein receptors such as those for LDL (Vinegoni, Botnaru et al. 2011).

In summary, using the NaIO₃ model of RPE and retinal atrophy as a model of dry AMD, we suggest that the onset and expansion of patches of GA in late dry AMD reflects a shift from basal M2 polarization towards M1 polarization, with a resultant increase in macrophage infiltration and phagocytic activity. We further demonstrate that acute oxidative damage to the RPE initiates a chronic cascade of innate inflammation characterized by progressive changes in the M1/M2 profile, and is not simply an acute toxic insult leading to apoptosis or necrosis. Finally, we point out that preferential down-regulation of the M1 response, with relative preservation of the M2 response, could be a suitable therapeutic target for preventing the onset of patchy GA and reducing its expansion. The following chapter will focus on characterizing these effects.

**Supplemental Data**

**Supplemental Figure 5.1** Macrophage infiltration and progressive accumulation over time in the damaged RPE layer. Iba1 (red) single stained tissue sections of posterior eyecups over the course of atrophy show progressive immune cell accumulations over time.

**Supplemental Table 5.1** Macrophage markers tested in vitro on rat peritoneal macrophages.

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</tr>
<tr>
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**Supplemental Table 5.2 Macrophage polarizing conditions used to test and validate markers.**

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[Image showing baseline, induction, evolution, and maturation stages with TNFα and CD163 markers]
Supplemental Figure 5.2 TNFα expression in the choroid during stages of atrophy progression following NaIO₃. Profile of M1 marker TNFα, (red) in the choroid displays a similar profile as that observed in the retina. Macrophages are initially M2 at baseline displaying only CD163 (yellow). With atrophy upregulation of TNFα can be observed and is sustained throughout the stages of progression. The M1 signal is robust at the evolution stage and appears to overwhelm the CD163 signal, with some cells expressing both M1 and M2 staining at evolution and maturation.

Supplemental Figure 5.3 ICG dye internalization does not activate the macrophage inflammatory response in vitro. (a) Cells polarized towards M1 using LPS (1 ug/mL)/IFNγ (20 ng/mL) display positive expression for iNOS (red). Cells polarized towards M2 using dexamethasone (250 nM) display positive expression for CD163 (green). Control and ICG-labelled cells show no expression for either marker. ICG dye internalization does not activate the macrophage inflammatory response as measured by iNOS and CD163 expression. (b) Cells were counted using ImageJ as expressing either CD163 for M2 or iNOS for M1, and compared to cells incubated with ICG. Percent (%) positive cells/field of view had a P>0.05, Unpaired Student’s t-test, data is reported as mean +/- SEM. 89% of M1 polarized cells were iNOS positive, while less than 1% were positive in the control condition (*P<0.0001). Overall 46% of M2 polarized cells were CD163 positive, while less than 1% of the control cells were (*P<0.0001). Less than 1% of ICG-treated cells were positive for either marker (ns).
Supplemental Figure 5.4 Systemic injection of ICG dye does not activate the macrophage inflammatory response in vivo. Immunofluorescence of retinal and choroidal tissue shows inflammatory cells are negative for iNOS (red) and there is no observed upregulation of CD163 (green) compared to control tissue. Furthermore, the macrophages don’t change their spatial distributions.

Supplemental Table 5.3 Fold change and P-values for all genes analyzed at stages of atrophy progression. Genes at each time point were compared to baseline and normalized to PPIH reference gene. Fold change ($2^{\Delta\Delta Ct}$) and P-values (<0.05 considered significant) were generated from Ct values uploaded to Qiagen RT² Profiler PCR Array Data Analysis software version 3.5.

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PPIH Reference: 1.00
Supplemental Figure 5.5 Dynamics of gene expression patterns for M1 and M2 genes at each stage of atrophy progression. Volcano plots show upregulated and downregulated genes at each stage compared to baseline. M1 genes are highlighted in red, and M2 genes are highlighted in green. The y-axis displays –log of P-value, while the x-axis displays log2 value of fold change mRNA from treated group compared to control group. Significantly up or down-regulated genes are highlighted in gray (P<0.05). Volcano plots were generated using Qiagen RT² Profiler PCR Array Data Analysis software version 3.5.
Chapter 6

Modulation of the macrophage inflammatory response in the NaIO\textsubscript{3} model of dry AMD

6.1 ABSTRACT

With no current treatment for the late dry form of AMD, having established a preclinical model that phenocopies the clinical disease, and identified that macrophages play a role in this model, we elected to use macrophage-targeted modulation to determine the effects on atrophy progression. We have shown that the M1 response in particular plays a vital role in atrophy progression in this model, so we elected to use gadolinium chloride to deplete the macrophage population, and the investigational new drug bindarit to modulate the M1 response specifically. By depleting macrophages with intravitreal gadolinium chloride, we observed patchy preservation of the RPE and a reduced macrophage infiltrate. Administration of bindarit showed an improved effect over simply reducing the macrophage population, as it decreased the M1 pro-inflammatory response, analyzed both by tissue staining for M1 proteins iNOS, MCP-1 and TNF\textsubscript{α}, and gene expression of M1 transcripts. In addition, it preserved the RPE cell monolayer structure and overlying retina, both in tissue samples and as observed in vivo using FAF. Further, using an entirely novel modification of the NaIO\textsubscript{3} model with nitroglycerin demonstrated an expansion of the patch of atrophy, and validated the effect of bindarit to reduce not only atrophy development but also atrophy expansion in this model. Taken together this points to preclinical efficacy of bindarit as a potential novel treatment for dry AMD.

6.2 INTRODUCTION

There is currently no treatment for the millions of North Americans suffering from dry AMD, including severe GA. Clinical trials are hampered by the difficulty of not having suitable animal models that recapitulate clinical trial endpoints, and the difficulty in clinical trial design for this disease. Recently the size of the patch of atrophy in patients was introduced as a clinical trial endpoint, allowing to measure GA in dry AMD trials over time (Csaky, Richman et al. 2008). However no animal models exist where atrophy progression or expansion can be accurately
measured and where this expansion is quantifiable. Furthermore, while the non-cellular (complement) arm of the innate immune system is known to be vital in disease development, clinical trials targeting this response in late dry AMD have largely failed. This is likely due to complement genetics predicting the onset of dry AMD, rather than the course of progression of the GA phenotype (Klein, Ferris et al. 2010). Instead, GA patch expansion especially in severe cases is observed clinically to relate to increased hyperfluorescent FAF (Holz, Bindewald-Wittich et al. 2007). We now suggest that inflammatory macrophages may be responsible for these patterns of FAF, and so may be a preferential treatment target for GA progression.

Bindarit is a low molecular weight indazolic derivative that is a first-in-class transcriptional regulator originally designed to target inflammation associated with arthritis (Guglielmotti, D'Onofrio et al. 2002). Acting to alter the phosphorylation of a subset of p65 transcription factors, it prevents the phosphorylation of IκBα of the p65/p50 subunits, and their subsequent translocation to the nucleus where transcriptional activity takes place. Downstream this results in decreased binding of NFκB p65/p50 to the proximal promoter site and thereby reduced expression of MCPs in macrophages. During normal tissue homeostasis and low MCP expression, transcription is controlled primarily by SP1/AP-1 transcription factor binding, unlike under inflammatory conditions when the NFκB binding and subsequent canonical pathway becomes active (Mora, Guglielmotti et al. 2012). This differential expression under normal and inflammatory conditions provides an effective molecular switch that can be addressed pharmacologically. In a number of preclinical studies, bindarit was shown to reduce innate inflammation, inhibit toxin-induced colitis, and reduce neointima formation in vascular injury (Guglielmotti, D'Onofrio et al. 2002, Bhatia, Landolfi et al. 2008, Grassia, Maddaluno et al. 2009). In clinical trials, bindarit was shown effective in reducing lupus nephritis, diabetic nephropathy, and stent re-stenosis (Ble, Mosca et al. 2011, ClinicalTrials.gov 2016).

In humans, the MCP gene cluster is located on chromosome 17 and consists of MCP-1, -2 and -3. Rodents only possess the MCP-1 (CCL2) and MCP-3 (CCL7) genes but there is marked sequence homology in the 5’ regulatory region. The MCP family of genes plays a role in both the initiation and propagation of pro-inflammatory responses (Deshmane, Kremlev et al. 2009). As such MCP-1 is expressed by macrophages at the sites of inflammation where they initiate aggressive phagocytosis and recruit further macrophage activity with amplification of the M1 response. Having observed a robust macrophage, and specifically M1 response in our model of
dry AMD we employed three strategies to target this response: 1) using gadolinium (GdCl₃) to dampen the general macrophage response in order to observe the effect on atrophy, 2) using bindarit to selectively inhibit the M1 pro-inflammatory cascade in our model and observe the effect on atrophy progression, and 3) using an entirely novel method of expanding retinal damage using nitroglycerin (NTG) to observe effects of bindarit on atrophy expansion. Taken together, these studies present effective targeting of the inflammatory response to treat dry AMD in a preclinical model that represents clinical trial design.

6.3 METHODS

6.3.1 Animal Studies

All procedures were performed in accordance with the Canadian Council on Animal Care and with adherence to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. For all experiments, SD rats aged 6-10 weeks were kept at a 12 hour dark/light cycle, with food and water ad libitum. For evaluation, rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and pupils dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride. Eyes were conditioned repeatedly using GenTeal lubricating eye drops (Novartis, Canada) to prevent desiccation. Following completion of in vivo work, animals were humanely sacrificed by intracardiac injection of T61 (Intervet Canada, Whitby, Canada).

6.3.2 Sodium Iodate and Nitroglycerin Infusions

Sodium iodate (Sigma) was prepared fresh at concentrations of 25-45 mg/ml in 0.9% saline for each set of experiments, and injected to a final concentration of 25-45 mg/kg body weight using a 24 gauge catheter inserted into the tail vein. In a subset of animals that received an initial NaIO₃ injection, NTG (clinical grade, St. Michael’s Hospital) was infused at 2 µg/kg/min over 30 minutes using a tail vein catheter and an animal infusion pump system, and a second dose of NaIO₃ was injected subsequent to infusion. The second insult using NTG and NaIO₃ was given at the evolution stage of atrophy following the first insult.

6.3.3 Intravitreal Injections

Gadolinium chloride (GDCl₃, GAD, Sigma, 439770) was prepared as a 2.5 mg/ml solution in sterile saline and 2 ul was injected 24 hours prior to NaIO₃ injection in n=18 eyes for GdCl₃ and
14 eyes for saline control. To administer the intravitreal injection the eye was dilated as described earlier, and 0.5% proparacaine hydrochloride topical anesthetic (Alcaine, Alcon Laboratories, Inc.) was applied to each eye. Povidone-iodine 5% solution (St Michael’s Hospital) was used to disinfect the surrounding area, and sterile saline was applied periodically for lubrication. An injection site was made using a 30-gauge needle and a 33-gauge Hamilton microinjection needle was inserted into the injection site to deliver the solution. Bindarit solution was delivered intravitreally using the same method. Bindarit powder >99% purity (NuChem Therapeutics, Montreal, Canada) was thawed from -20°C to room temperature for one hour before dissolving in sterile injection water to a final concentration of 50 mg/ml. The solution was further diluted with saline to a final concentration of 0.01, 0.1, 2.0, 10 or 20 mg/ml for intravitreal injection. A total n=118 eyes were injected with bindarit or saline control. Ultimately 2 mg/ml was established to be an effective low dose, and 20 mg/ml as an effective high dose for further analysis.

6.3.4 In Vivo Imaging

FAF images were acquired using a commercially available cSLO (Spectralis, Heidelberg Retinal Angiograph, HRA-2; Heidelberg Engineering GmbH, Germany), in the 488/500 nm excitation/emission channel and the Eye Explorer Image Capture system, version 1.1.10. Animals were imaged at the evolution stage of atrophy following NaIO₃ injection, treated with either GdCl₃ (2.5 mg/ml), bindarit (2 mg/ml or 20 mg/ml) or saline vehicle control. For DNIRA imaging procedures were performed as previously described in earlier chapters. Briefly, ICG (Cardiogreen, Sigma) was injected at a dose of 2 mg/kg IV via tail vein catheter in animals that have previously received NaIO₃, and in vivo imaging was performed approximately 2 days later in the ICG angiography channel (795/810 nm excitation/emission) of the cSLO.

6.3.5 Histology

Hematoxylin and eosin (H & E) histology of paraffin-embedded tissue was performed as previously described, after fixation with Davidson’s fixative (Bancroft and Stevens 1990, McKay, Steele et al. 2009). Briefly, eyes were embedded in paraffin, and 5 µm sections were collected on glass slides. Sections were de-paraffinized and rehydrated, and stained with hematoxylin and eosin. Stained sections were viewed with a Nikon Upright E800 Microscope.
6.3.6 Whole Tissue Immunofluorescence

Following dissection of retina and eyecup (RPE) tissue, samples were blocked with 5% BSA/TBS, 0.1% Triton X-100 for 45 minutes at room temperature. Primary antibodies were added at a concentration of 5 µg/ml (unless specified otherwise) in blocking buffer, and incubated overnight 4°C. Samples were subsequently washed with TBST (TBS, 0.1% tween-20) for 5 x 1hr at room temperature followed by incubation with Alexa-fluor conjugated secondary antibody (1:400) overnight 4°C. Samples were washed with TBST for 5 x 1hr at room temperature and nuclei counterstained with To-Pro-3 (Life Technologies). Tissue was mounted onto 2-well Teflon printed slides using fluorescent mounting medium (Dako) and coverslipped. Images were acquired using a Leica TCS SL confocal fluorescent microscope (Leica Microsystems, GmbH, Wetzlar, Germany), with Leica Confocal Software Version 2.61. Primary antibodies used were as follows, mouse monoclonal anti-rat CD68 (AbD Serotec, MCA341R), mouse monoclonal anti-rat CD163 (Hycult biotech, HM3025), rabbit polyclonal anti-rat MCP-1 (PeproTech, 500-P76), rabbit polyclonal anti-Iba1 (Wako Pure Chemical Industries Ltd, 019-19741), rabbit polyclonal anti-iNOS (Abcam, ab15323), rabbit polyclonal anti-TNFα (Millipore, AB1837P), mouse monoclonal anti-rat RPE65 (Abcam, ab78036), rabbit polyclonal anti-ZO-1 (Life Technologies). These were compared against normal mouse IgG (Santa Cruz) or rabbit IgG (Abcam) controls. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (Life technologies) Alexa Fluor 555 goat anti-mouse (Life technologies).

6.3.7 RNA Isolation, QIAGEN RT² Profiler PCR Array and Data Analysis

Total RNA was isolated using 8 eyes at the evolution stage following NaIO₃ injection either with bindarit treatment (2 mg/ml or 20 mg/ml), or saline vehicle control, from rat retinas and posterior eyecups using Trizol Reagent (Life Technologies, USA). Briefly, after enucleation of rat eye balls, they were dissected in cold RNAlater Solution (Life Technologies, USA). The entire retina was then removed and stored in RNAlater solution, and RPE and choroid layers were scraped with a surgical scalpel blade and combined with the retina. Manufacturer’s protocol was followed for RNA extraction with the Trizol method. Total RNA was further purified with RNeasy MinElute Cleanup kit (Qiagen, USA). Their quantity and quality were checked with a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA).
cDNA was synthesized using RT² First Strand Kit (Qiagen, USA) with 2 µg of total RNA per reaction. Real-time PCR was performed using Qiagen 384-well Custom RT² Profiler PCR Array and RT² SYBR Green ROX qPCR Mastermix (Qiagen, USA) with 10 ng cDNA per well. The reactions were run on a ViiA 7 PCR system (Life Technologies, USA) with default cycling conditions. Differences in gene expression were analyzed using the Qiagen RT² Profiler PCR Array Data Analysis Software V3.5 Web Portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) based on ∆∆C_T method and normalized to Peptidyl-prolyl cis-trans isomerase H (PPIH) as a reference gene. Results are reported as fold-change (2-∆∆CT) normalized to baseline as 1-fold. P values were calculated based on a Student’s t-test of the replicate 2-∆∆CT values for each gene in the control group and treatment groups, and P values less than 0.05 were considered significant.

The primer sequences are proprietary. The amplicon size, Qiagen gene catalog number and primer reference position found below.

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6.4 RESULTS

6.4.1 Depletion of macrophages by GdCl₃ leads to regions of preserved tissue in vivo

In order to determine whether macrophage inhibition would preserve the tissue or reduce atrophy in this model, we used intraocular GdCl₃ prior to induction of damage with NaIO₃ to ablate the macrophage response. In vivo imaging during the evolution stage of atrophy shows that GdCl₃ administration results in regions of preserved retina and RPE using FAF and DNIRA, amongst the typical NaIO₃-associated damage (Figure 6.1). These areas of preservation show up as single or multiple islands of tissue and RPE cells within the overall damaged outer retinal layer.
Figure 6.1 Gadolinium chloride administration results in areas of preservation in vivo. Either single (a) or multiple (b) islands of preserved RPE can be detected within an otherwise damaged outer retinal layer, using both FAF and DNIRA imaging modalities in rodent eyes during the evolution stage of NaIO₃ atrophy with GdCl₃. ICG dye injections for DNIRA were administered 2 days prior to imaging.

Further analysis of excised eyecup tissue using AFM shows uniform patches of RPE cells in the eyes of animals treated with GdCl₃ compared to those treated with intraocular saline, where only the typical autofluorescent immune cells and RPE remnants are visible (Figure 6.2(a)). Immunofluorescent staining using RPE65 confirms patches of residual RPE cells among an otherwise damaged tissue where RPE are absent (Figure 6.2(b)). Enlargement of the RPE patches shows that the cells are elongated/deformed, and therefore not entirely healthy, but otherwise intact. This contrasted with intraocular saline treated animals where no such patches of RPE preservation are detected.

Figure 6.2 Depletion of macrophage population by GdCl₃ leads to regions of RPE preservation in tissue. (a) AFM imaging of excised eyecup tissue shows area of preserved normal RPE in the window of damage in a GdCl₃ treated animal, with underlying vasculature not visible due to uniformity of the RPE cells. Similar AFM imaging in a saline vehicle with NaIO₃ only shows immune cells/RPE remnants in the area of damage. (b) Immunofluorescent staining of tissue clearly delineates areas of RPE65 (green) cell preservation (left). Upon magnification (center), these cells are observed to be elongated/deformed, but intact. Similar areas of RPE65 staining are not observed in NaIO₃ with saline treatment.
**Figure 6.3 GdCl₃ diminishes the accumulation of CD68+ macrophages only.** (a) Intravitreal saline with NaIO₃ (left) shows CD68+ (green) accumulation in the damaged tissue, compared to intravitreal GdCl₃ with NaIO₃, where few/none CD68+ cells are observed. (b) No associated robust differences are observed in the tissue populations of Iba1+ cells (red). Areas away from RPE protection, in a GdCl₃-treated animal, numerous Iba1+ cells can still be identified where the monolayer is missing, and are comparable to saline vehicle control.

Further, fluorescent staining of macrophage populations demonstrates that while Iba1+ cells do not appear to be affected by intra-ocular GdCl₃, the accumulation of CD68+ macrophages in the RPE layer and subretinal space is diminished. Staining for macrophages using CD68 at atrophy evolution confirms an absence of the CD68+ macrophage population in the GdCl₃ treated animals, as little/none of these cells can be detected (Figure 6.3(a)). This compared to the saline control, where numerous CD68+ cells are observed to accumulate in the damaged RPE/eyecup. Staining for Iba1+ cells in the eyecup even in the GdCl₃ treated animals, in an area away from RPE protection where typical RPE atrophy is observed, numerous macrophages are evident, and the staining is similar across the GdCl₃ and saline treatment groups (Figure 6.3(b)). This
suggests that GdCl\textsubscript{3} primarily affects the CD68\textsuperscript{+} macrophages, likely infiltrating from outside the retinal and eyecup layers, while the resident tissue macrophages appear relatively unaffected.

Finally, nuclear staining of the outer retinal layers confirms that the retina remains deformed even with GdCl\textsubscript{3} treatment, suggesting that the effect of NaIO\textsubscript{3} on the RPE and subsequently the retina is unaltered with GdCl\textsubscript{3} treatment (Figure 6.4). This also suggests that GdCl\textsubscript{3} depletion of only a subset of the macrophages has no effect on the NaIO\textsubscript{3} response, and that the GdCl\textsubscript{3}-affected macrophages only partially contribute to the progressive damage in this model. It should be noted that no deformation or inflammation of the RPE and retina is detected when GdCl\textsubscript{3} is administered on its own (Supplementary Figure 6.1).

![Figure 6.4](image)

**Figure 6.4** NaIO\textsubscript{3} effect on deformation of the rodent retina is independent of GdCl\textsubscript{3}. Outer retina remains deformed following damage to the RPE, and is comparable in both GdCl\textsubscript{3}-treated (left), and saline-treated (right) groups.

### 6.4.2 Bindarit prevents upregulation of the M1 inflammatory tissue markers and preserves RPE structure

In order to pursue a more targeted approach, and modulate specifically the active M1 macrophage population, we elected to use the drug bindarit in our model. Bindarit blocks the NFκB mediated transcription of the MCP-1 gene in macrophages, thus preventing upregulation of pro-inflammatory genes, including those of the M1 transcriptome, following an inflammatory stimulus (Figure 6.5(a)). As such, we elected to test this drug using a prevention (administration prior to NaIO\textsubscript{3} toxic insult), treatment (administration after NaIO\textsubscript{3} insult), and expansion (using a new model of atrophy expansion) paradigms (Figure 6.5(b)).
Figure 6.5 Bindarit mechanism of action and experimental design. (a) Bindarit targets specifically IκBα and NFκB phosphorylation and thereby translocation to the cell nucleus, where it subsequently initiates gene transcription of MCP-1 and related gene products in macrophages. (b) Studies using bindarit were carried out using a prevention (pre-treatment), treatment and expansion approach in the NaIO₃ model.

Immunofluorescent staining of excised posterior eyecup tissue confirms a decrease in MCP-1 expression within the damaged RPE layer when bindarit is administered before atrophy induction (Figure 6.6). As observed in the saline vehicle control, following NaIO₃ administration the RPE layer is damaged and MCP-1 signal accumulates among the remnants of the photoreceptor nuclei that transfer from the damaged and detached retina to the eyecup tissue during the dissection procedure. Compared to this, low and high dose bindarit shows a dose dependent decrease in MCP-1 signal, and an associated preservation of the RPE layer, as observed by the double nuclei staining of the RPE cells. High dose bindarit is comparable to baseline control tissue.
Figure 6.6 Intravitreal bindarit dose-dependently prevents NaIO$_3$-associated MCP-1 accumulation in damaged tissue. Compared to the saline vehicle treated, NaIO$_3$-treated animals (left), animals that received low and high dose bindarit showed a dose dependent decrease in MCP-1 (red) accumulation in posterior eyecup tissue, with high doses also preserving the structure of the RPE monolayer. High dose bindarit was comparable to the baseline control without NaIO$_3$ treatment (right).

Figure 6.7 Bindarit reduces pro-inflammatory mediators and preserves outer retinal structure. A dose dependent reduction of MCP-1 (a, red) and TNF$\alpha$ (b, red) accumulation can be observed in the deformed outer retinal tissue upon pre-treatment with bindarit at low and high dose prior to NaIO$_3$ challenge, with high doses preservation of the outer retina comparable to baseline control.

Similar results can be viewed in samples of excised outer retinal tissue (Figure 6.7). Inflammatory markers MCP-1 (a) and TNF$\alpha$ (b) both showed dose dependent decreased expression of these proteins when bindarit was administered before atrophy induction. In addition, a dose dependent preservation of outer retinal structure can be observed. Interestingly,
low dose bindarit showed a reduction in TNFα expression while the presence of inflammatory cells was still evident. This suggests that bindarit targets the pro-inflammatory response signaling in the cells that are already present in the tissue, while reduction of the immune cell infiltrate may be a subsequent effect of a reduction of the pro-inflammatory mediators.

![Image](image.png)

**Figure 6.8 Bindarit prevents expression of M1 marker iNOS in the retinal macrophages.** Retinal macrophages in the bindarit pre-treated arms only express the M2 marker CD163 (green), similar to baseline control, and show no expression of the M1 marker iNOS (red). By contrast in the intravitreal saline control, macrophages show upregulation of the M1 marker, and are capable of expressing both iNOS and CD163 simultaneously (yellow) as previously described (Chapter 5).

In the intravitreal saline vehicle, as previously described in Chapter 5, retinal macrophages are capable of expressing M1 marker iNOS (red) simultaneously with the baseline expression of M2 marker CD163 (green). Bindarit pre-treatment results in no simultaneous expression of the M1 marker iNOS in the retinal macrophages (**Figure 6.8**). Both high and low doses prevent the induction of iNOS expression in these cells below IHC detection limits. The cells continue to express the M2 marker CD163 (green) as under normal baseline conditions. Of note however, is that morphologically these cells do not appear to be ramified as in baseline control, but
amoeboid, suggesting that there is a presence of stimuli to activate the macrophages to an extent, but that these macrophages do not express a pro-inflammatory response.

Furthermore, pre-treatment with bindarit can preserve the RPE cells, and prevent the accumulation of Iba1+ macrophages in a dose dependent manner (Figure 6.9). Compared to saline vehicle, bindarit pre-treatment particularly at high dose prevents the loss of RPE65 signal that is associated with RPE cell function, and this signal is comparable to baseline control levels. This structural preservation of the RPE layer is also associated with a decrease in the amount of Iba1+ accumulation in this layer, suggesting that RPE monolayer stability is an important component of the extent of macrophage accumulation in the damaged tissue.

![Figure 6.9 Intravitreal bindarit dose-dependently prevents NaIO3-associated Iba1 cell accumulation and RPE degeneration.](image)

Compared to saline vehicle treated, NaIO3-treated animals (left), animals that received low and high dose bindarit pre-treatment showed a dose dependent increase RPE65 (green) expression. There was also an observed reduction in the NaIO3-associated accumulation of Iba1+ (red) cell infiltrate with bindarit. High dose bindarit preserved RPE structure to a greater extent than low dose bindarit, and was comparable to the baseline control animals (right).

### 6.4.3 Bindarit reduces upregulation of M1 gene expression during atrophy

To characterize the effect of bindarit on gene expression we performed quantitative PCR arrays to evaluate the relative M1/M2 response with bindarit treatment and pre-treatment in the NaIO3 model. Individual fold-change values for all genes analyzed is provided in Supplementary Table 6.1. Combined average fold-change expression across all the M1 genes analyzed and all the M2 genes analyzed is displayed in Figure 6.10. Pooling 10 M1 inflammatory genes and 10 M2 anti-inflammatory genes and looking at the overall mean fold change, there is an observed highly upregulated M1 response in the intraocular saline vehicle control, which is greatly
reduced with both low and high dose bindarit pre-treatment. The M2 response remains stable across the experimental arms. This dramatic difference in the dynamics of M1 expression between intraocular saline and intraocular bindarit pre-treatment can also be observed using volcano plots (Supplementary Figure 6.3).

Figure 6.10 Bindarit dose-dependently prevents the increase in total M1 gene expression. Average expression of total combined M1 genes compared to total combined M2 genes at the evolution stage of atrophy shows a greater overall M1 change in the saline treated arm compared to the low and high dose bindarit pre-treatment. Ten M1 transcripts and ten M2 transcripts were pooled, and the overall mean fold-change was determined. RNA from n=3 independent samples of chorioretinal extracts was collected and custom qPCR arrays were ran. Data was generated using Qiagen RT² Profiler PCR Array Data Analysis software version 3.5.

Individual gene expression analysis of primary target MCP genes supports the dose-dependent reduction in expression of M1 markers with drug pre-treatment (Figure 6.11). Gene targets Ccl2 (MCP-1) and Ccl7 (MCP-3) show a dose dependent reduction of gene expression at both doses of drug compared to saline vehicle control, supporting the druggable target of bindarit (Figure 6.11(a)). M1 markers SOCS3 and STAT 1 also show a reduction in gene expression with drug pre-treatment (Figure 6.11(b)), and a significant protection of RPE monolayer is confirmed, where a higher RPE65 expression is noted with bindarit pre-treatment compared to saline control (Figure 6.11(c))

6.4.4 Bindarit treats inflammation and RPE damage via its drug target MCP-1

The drug target validity is also evident in the treatment paradigm when bindarit is administrated 1 or 2.5hr following NaIO₃ (Figure 6.12(a)). At these timepoints, no in vivo changes can be observed in the NaIO₃ model, and no other genes, including the SOCS and STAT transcription
factors show a significant change in expression (Figure 6.12(b), Supplementary Table 6.1). However, the upwards trend for RPE protection is still evident at these early treatment timepoints, as the NaIO₃-associated reduction in RPE65 gene expression can be prevented (Figure 6.12 (c)).

Figure 6.11 Bindarit dose-dependently prevents upregulation of individual M1 genes and its drug targets. (a) Expression of rodent bindarit targets CCL2 (MCP-1) and CCL7 (MCP-3) shows a significant dose dependent decrease of these chemokines with bindarit pre-treatment, compared to saline control (b) Expression of M1 markers SOCS3, and STAT1, as well as STAT3 also show a dose dependent decrease with bindarit pre-treatment, compared to saline control. (c) Expression of RPE65 shows an upwards trend and a significant increase at the high dose of bindarit compared to control. Fold change ($2^{\Delta \Delta Ct}$) is the normalized gene expression ($2^{-\Delta Ct}$) in the test sample (NaIO₃ + IVT bindarit pre-treatment) divided by the normalized gene expression ($2^{-\Delta Ct}$) in the control sample (NaIO₃ + IVT saline). Data from n=3 independent samples of chorioretinal extracts was collected and custom qPCR arrays were ran. Results were normalized against reference gene PPIH. Data was generated using Qiagen RT² Profiler PCR Array Data Analysis software version 3.5. *(P<0.05)
Fig 6.12 Bindarit dose-dependently treats upregulation of drug targets subsequent to RPE atrophy. (a) Expression of rodent bindarit targets CCL2 (MCP-1) and CCL7 (MCP-3) is significantly reduced with bindarit treatment at 1 and 2.5 hr following toxin challenge, compared to saline control (b) SOCS and STAT marker remains relatively unchanged with bindarit treatment compared to control. (c) Expression of RPE65 shows an upwards trend towards RPE protection with bindarit treatment compared to control. Fold change \(2^{\Delta \Delta Ct}\) is the normalized gene expression \(2^{\Delta Ct}\) in the test sample (NaIO3 + IVT bindarit treatment) divided by the normalized gene expression \(2^{\Delta Ct}\) in the control sample (NaIO3 + IVT saline). RNA from n=3 independent samples of chorioretinal extracts was collected and custom qPCR arrays were ran. Results were normalized against reference gene PPIH. Data was generated using Qiagen RT² Profiler PCR Array Data Analysis software version 3.5. *(P<0.05)

6.4.5 Bindarit prevents evolution and expansion of atrophy in vivo

Finally, pre-treatment with bindarit can reduce GA-like atrophy in vivo in a dose dependent manner as observed using FAF (Figure 6.13(a)). Saline vehicle control presents with the typical reticular FAF pattern associated with NaIO3-induced retinal damage at the evolution stage of atrophy progression. Low dose bindarit pre-treatment presents with only a small portion of the fundus displaying bright FAF associated with atrophy, and high dose bindarit presents with a homogenous FAF background associated with healthy retina and RPE. This protection is confirmed with histological H&E staining where bindarit-treated eyes show the normal retinal
layer configuration with photoreceptor nuclei in dark blue in a uniform band above the outer segments and RPE cells (Figure 6.13(b)). This is compared to the saline vehicle where the outer nuclear layer is folded and POS are distorted, indicative of outer retinal damage. Histology of the typical NaIO₃-associated retinal damage has been reported in numerous studies over the years and is consistent with these observations.

As a final step to confirm the treatment efficacy for bindarit in vivo, we elected to create a new model using a secondary toxic insult to damage the retina further, compared to the NaIO₃-induced damage alone. To do this, we selected NTG, a potent nitric oxide generator and inflammatory mediator used in other non-ophthalmic preclinical models (Reuter, Bolay et al. 2001, Reuter, Chiarugi et al. 2002). Infusion of NTG at the evolution stage of atrophy along with second dose of NaIO₃ results in an expanded patch of atrophy into the region where the fundus was previously normal as observed on FAF (Figure 6.14(a)). Administration of bindarit alongside this secondary toxic insult prevents expansion of the atrophy (Figure 6.14(b)). Administration of a second NaIO₃ dose alone does not result in atrophy expansion, indicating that for the model to work a subsequent toxic insult is required alongside NaIO₃ (Figure 6.14(c), Supplementary Figure 6.4). These results validate bindarit’s preclinical efficacy as a potential treatment for dry AMD, and align with clinical trial design for GA progression.

Figure 6.13 Intravitreal bindarit dose-dependently arrests atrophy in vivo. (a) Intravitreal saline vehicle (left), shows the typical reticular FAF pattern associated with NaIO₃ damage at the evolution stage of atrophy. Low dose bindarit pre-treatment (center) shows a small amount of peripheral damage in the FAF, while high dose bindarit (right) completely protects the retina, showing no indication of damage in the fundus (b) Histological analysis using H&E staining confirms the in vivo findings, showing deformation of the outer nuclear layer in the saline arm (left), while tissue remains healthy and preserved with high dose bindarit pre-treatment (right).
Figure 6.14 Bindarit reduces patch expansion in vivo in a novel model of retinal damage. (a) FAF comparison of evolution versus maturation of stages of atrophy, imaged following a second dose of NaIO$_3$ together with NTG at the evolution stage to induce a patch expansion. Administration of intravitreal saline vehicle alongside the second toxin challenge does not reduce patch expansion. (b) Administration of bindarit prior to expansion of the patch of damage using a second dose of NaIO$_3$ and NTG at the evolution stage, shows no expansion of the patch of atrophy. (c) Attempts to induce patch expansion with a second dose of NaIO$_3$ alone does not result in changes in the area of atrophy between evolution and maturation stages.
6.5 **DISCUSSION**

We previously showed that the onset and expansion of patches of GA in late dry AMD reflects a shift from basal M2 polarization towards M1 polarization. This shift may result in an increase in macrophage recruitment and phagocytic activity causing progressive damage that is often associated with the rapid progression of GA in dry AMD. We stipulated that preferential down-regulation of the M1 response, with relative preservation of the M2 response, could be a suitable therapeutic target for preventing the onset of patchy GA and reducing its expansion. As such the objectives of this study were to use systemic NaIO\(_3\) alongside either intra-ocular GdCl\(_3\) to deplete the macrophage population, or the investigational new drug bindarit to preferentially down-regulate the M1 response in this model and determine their effects on atrophy progression in vivo.

We first determined the effect of macrophage depletion using the lysosomal disrupting agent, GdCl\(_3\). GdCl\(_3\) was previously shown to deplete macrophages in models of inflammatory lung disease, diabetes, and colitis (Keller, Paxian et al. 2005, Huang, Metlakunta et al. 2010, Du, Wang et al. 2014). It is reported to do so via induction of macrophage apoptosis, but may also affect the NF\(\kappa\)B pathway (Mizgerd, Molina et al. 1996, Du, Wang et al. 2014). In our model GdCl\(_3\) macrophage depletion alters the in vivo phenotype and partially rescues RPE loss. Intra-ocular delivery of GdCl\(_3\) reproducibly leads to regions of persistent RPE, seen as small patches of relatively healthy tissue within larger patches of RPE loss during the evolution stage of atrophy following NaIO\(_3\). Persistent RPE was detected both in vivo using DNIRA, and confirmed in wholemount posterior eyecup by IHC. This phenotype is not typically observed in animals treated with NaIO\(_3\) alone. However, GdCl\(_3\) appears to target only the CD68 infiltrating population, which is only a subset of the macrophages involved in the NaIO\(_3\) model, and thus only partially contribute to the resultant damage. This is likely why GdCl\(_3\) depletion shows only marginal tissue preservation.

Subsequently, we show that the drug bindarit can prevent the onset of GA and alters the inflammatory response in NaIO\(_3\)-treated rodent eyes. We demonstrate this effect both with the traditional NaIO\(_3\) model of retinal damage, and with a newly developed expansion model using NTG alongside a secondary NaIO\(_3\) dose to induce additional damage. We therefore show that bindarit prevents both the induction and the expansion of retinal atrophy, and works in both
prevention and treatment paradigms, validating the drug mechanism of action targeting the MCPs. This suggests it may be useful in clinical trials for GA, where not only the onset but also the expansion of atrophy is an important marker of disease activity. While the action of NaIO$_3$ and to an extent NTG is via an oxidative stress response, bindarit acts on a different set of factors to reduce the inflammatory response. Lin et al outlined that MCP-1 inhibition did not blunt oxidative stress in a model of hypertension, as such the drug’s effects are downstream of simply antagonizing the toxic insult invoked via NaIO$_3$ (Lin, Zhu et al. 2009).

In this model, we make the observation that differential utilization of the MCP gene cluster during normal and pathological conditions can influence the M1/M2 balance, and that MCP-1 is a key regulator of inflammation during retinal atrophy. MCP-1 has been referred to as a natural molecular switch, and may be the gene that ultimately regulates the balance between pro- and anti-inflammatory responses (Thompson, Karpus et al. 2008). By reducing overall MCP signaling, bindarit reduces 1) the number of inflammatory cells that accumulate in the damaged areas, and 2) reduces the M1 polarization of the cells that are already present in the areas of damage, thereby reducing overall inflammation in the model.

While there is an inherent limitation of using a monocyte chemoattractant as a blockade of the M1 response in that it becomes difficult to distinguish between a decrease in M1 polarization and simply a downregulation of the cellular inflammatory response, we acknowledge this caveat, and argue that it is extremely difficult to distinguish the two, since one is dependent on the other. This is due to the propagation loop of the M1 response, whereby the release of pro-inflammatory factors brings in more macrophages with time, which release even more pro-inflammatory factors (Deshmane, Kremlev et al. 2009). As such, it is the macrophages which themselves induce and propagate the M1 response, and their numbers increase this response. In this way under inflammatory conditions a decrease in M1 activity parallels in a decrease in overall macrophage activity. We observe this trend as we can see a typical upregulation of general macrophage factors such as Csf2 and CX3CR1 during model progression, and a significant dose dependent reduction with bindarit treatment (Supplemental Figure 6.3). In addition we can observe the downregulation of specific inflammatory markers such as iNOS and TNF$\alpha$, in areas where macrophages are still present but remain neutral or M2, suggesting those macrophages were localized to the tissue before the inflammatory insult. Furthermore, the relatively stable M2 response in the model supports the importance of specifically and individually targeting M1 with
drug treatment, and in our case we observe that bindarit leaves the M2 response relatively unaffected. Studies in other disease models have shown similar results, where upregulation of the MCP-1 pathway by local macrophages results in accumulation of iNOS+ and TNFα+ macrophages specifically, and that absence of MCP-1 can prevent local macrophage recruitment and disease (Huang, Wang et al. 2001, Dogan, Elhofy et al. 2008). This lends further support to our study on the local accumulation and upregulation of an M1 macrophage response in this model of dry AMD, and prevention of such a response using bindarit.

Expression of MCP-1 by damaged cells may play a central role in the initiation of this disease. RPE cells are reported to express MCP-1 and this can be dysregulated by toxic damage to the RPE such as that produced by cigarette smoke (Elner, Elner et al. 1996, Austin, Liu et al. 2009, Pons and Marin-Castaño 2011). RPE cells can also influence the phenotype of resident macrophages in the nearby choroidal vasculature, and it is known that RPE plays an important role in maintaining macrophage phenotype in the retina (Zamiri, Masli et al. 2006, Lau and Taylor 2009). MCP-1 is an important mediator of attracting macrophages to the site of injury, but in addition to that it can also induce apoptosis in photoreceptors (Nakazawa, Hisatomi et al. 2007). Remarkably, we observe that even high dose bindarit leaves the basal RPE levels of MCP-1 expression unaffected under normal conditions when administered on its own without NaIO3 (Supplemental Figure 6.2). Further studies by our group are underway to address whether bindarit could not only reduce macrophage-mediated inflammation, but could also reduce MCP gene and protein expression of isolated RPE cells in vitro under conditions of inflammation and oxidative stress.

Ultimately, the investigational new drug bindarit, reported to differentially down-regulate MCP mRNA transcription at times of tissue homeostasis versus active inflammation could be efficacious in the treatment of aggressive late dry AMD. Such treatment could reduce inflammation, and block or delay the demise of the RPE, providing a window of opportunity for recovery. It can further allow for the instillation of other therapies such as cell replacement, particularly if it can directly protect RPE cells in areas of surrounding inflammation. Finally, we point to a modified NaIO3 model as a superior alternative to study this disease efficiently and effectively in the rodent and potentially in larger animals, in order to de-risk translation to the clinical setting, better align with clinical trial design, and accelerate new treatments for dry AMD.
Supplemental Data

Supplemental Figure 6.1 GdCl₃ alone does not alter the RPE monolayer in vivo. (a) Fundus imaging using FAF and DNIRA demonstrates normal fluorescence associated with healthy RPE similar to baseline. (b) AFM of GdCl₃ treated retina (top) and eyecup (bottom) shows minimal cellular fluorescence, and healthy autofluorescent RPE respectively. (c) Immunofluorescence with RPE65 (top) and ZO1 (bottom) shows the normal structure of the RPE layer, and staining for Iba1 (top) and CD68 (bottom), shows no infiltration of macrophage populations into the RPE monolayer.

Supplemental Figure 6.2 Endogenous RPE MCP-1 expression remains unaffected with high dose bindarit. Granular expression of MCP-1 (red) can be observed in normal RPE cells, and this expression is unaffected in the cells even when high doses of bindarit are given (without NaIO₃ challenge).
Supplemental Table 6.1 Fold change and P-values for all genes analyzed with bindarit prevention and treatment approaches. Gene expression was normalized to NaIO₃ + IVT saline control, and compared to PPIH reference gene. Fold change (2^ΔΔCt) and P-values (<0.05 considered significant) were generated from Ct values uploaded to Qiagen RT² Profiler PCR Array Data Analysis software version 3.5.

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Supplemental Figure 6.3 Changes in dynamics of gene expression patterns of M1 and M2 transcripts with bindarit compared to intravitreal saline. Volcano plots show NaIO3 along with saline vehicle, low dose and high dose bindarit, all compared to baseline control. M1 genes are highlighted in red, and M2 genes are highlighted in green. The y-axis displays –log of P-value, while the x-axis displays log2 value of fold-change from treated group compared to control group. Significantly up or down-regulated genes are highlighted in gray (P<0.05). Volcano plots were generated using Qiagen RT2 Profiler PCR Array Data Analysis software version 3.5.
Supplemental Figure 6.4 Bindarit modulates gene expression of general macrophage markers. (a) General inflammatory phagocyte markers CX3CR1 and Cs2 are upregulated during atrophy progression compared to baseline. (b) Bindarit significantly downregulates macrophage markers at both low and high dose when compared to saline vehicle control at the evolution stage in the NaIO₃ model. RNA from n=3 independent samples of chorioretinal extracts was collected and custom qPCR arrays were ran, normalized to PPIH. Data was generated using Qiagen RT² Profiler PCR Array Data Analysis software version 3.5. *(P<0.05)
**Supplemental Figure 6.5 Reproducibility of the dual toxin expansion model.** Initial damage was induced using a medium dose of NaIO$_3$ to obtain a patch of tissue atrophy. A second toxic insult of either NaIO$_3$ alone (medium-high dose), or NaIO$_3$ + NTG was administered. Only 9.1% (3/33) of animals that received a second hit of NaIO$_3$ alone showed further changes in the retina in the FAF channel, while 90% (36/40) of animals that received a second dose of NaIO$_3$ along with NTG showed an expansion of the area of damage. Administration of bindarit at the time of the second toxic insult prevented the expansion in 100% of the animals (0% expansion; 0/20). Data presented as percentage of eyes with increased area of tissue damage as observed on FAF.
Chapter 7
Summary and Future Directions

7.1 SUMMARY OF THE WORK

The work presented in this thesis addressed four major objectives with respect to the development and characterization of a model of late dry AMD, imaging biomarkers for disease detection, and drug development for disease treatment.

The first was to develop and characterize a suitable in vivo model that recapitulates clinical phenotypes of late dry AMD observed in patients, namely GA and patterns of hyperfluorescent FAF. The second was to determine whether a macrophage response can be identified in this model, in particular in vivo using clinically relevant modalities. The third was to analyze whether macrophage polarization plays a role in this model and how the M1/M2 phenotypes change over time. And the last was to determine whether the macrophage response can be modulated and whether this would lead to any changes in polarization profiles and disease phenotype. To test our aims we first developed a rodent model of atrophy that phenocopies disease, developing patches of GA-like RPE/photoreceptor loss, and high-risk in vivo features. Further, because clinical quantification of GA has recently been adopted as a primary endpoint in advanced clinical trial, we developed DNIRA, a method using fluorescent ICG dye, to more clearly delineate the area of RPE loss in vivo in our rodent model thus potentially streamlining translation to the clinic. We used systemic delivery of NaIO₃, an RPE-specific toxin, and DNIRA to induce and visualize patches of RPE/outer retinal loss. In addition to DNIRA, we observed the concurrent changes that occur within and adjacent to these patches using the standard clinical method FAF, and have been able to characterize five stages of progression of FAF after NaIO₃ damage. We identified that in contrast to what is believed in human disease, hyperfluorescent FAF is found within areas of missing RPE, as observed on DNIRA, immunofluorescence and PCR in this model, and thus cannot simply be attributed to RPE lipofuscin.

This led us to address the source of the autofluorescence along with characterization of the macrophage response, and remarkably we have shown that the autofluorescent signal in this
model can be observed not only due to RPE cells, but also due to macrophage populations accumulating in areas of atrophy. We developed two techniques analogous to DNIRA – 1) Immuno-DNIRA and 2) Pulsed-DNIRA that enabled us to observe macrophages take up ICG dye and visualize them in vivo in the eye. We confirm that fluorescent macrophages observed with DNIRA signal correlate with areas of bright FAF, suggesting that these cells may contribute to the in vivo signal observed in patients. In addition, using immunofluorescent staining of retinal tissue we observed that the folded and convoluted retinal tissue creates grooves and curves in which macrophages are able to accumulate, forming 2D tissue patterns of macrophages interlaced within the damaged retinal tissue. These data support the observation that the patchy, reticular patterns of FAF observed in vivo at different stages of atrophy progression are due to fluorescent macrophages trapped in the retinal folds. They also provide the first clinicopathological correlate between 2D patterns of hyperfluorescent FAF and tissue pathology.

We then sought to determine whether evidence of macrophage polarization was present in the model. Having identified that M2 is the resting phenotype and M1 is the amplified response that takes place in the tissue immediately following, and during progression of NaIO₃ atrophy, we suggested that blocking the macrophage response, and specifically modulating the M1 response may lead to a decrease in atrophy progression. Administration of intravitreal GdCl₃ showed areas of preserved RPE and retinal tissue within patches of loss, accompanied by a decreased macrophage infiltrate; by contrast use of the M1-targeting drug bindarit produced a nearly complete protection of the tissue, suggesting that modification of macrophage behavior is a distinct and major component of the drug’s pre-clinical efficacy. This was observed with markers of M1 polarization such as iNOS, MCP-1 and TNFα, and M2 polarization such as CD163 in tissue, as well as SOCS and STATs transcription factor mRNA profiles. We further confirmed that bindarit works in the model by primarily acting on its drug target MCP-1, which is highly upregulated with induction of atrophy. Finally, modifying the NaIO₃ model by adding NTG as an additional oxidative stressor and pro-inflammatory agent, we show an expansion in the area of atrophy in vivo, which is not possible to incite using NaIO₃ alone, suggesting that an additional inflammatory stimulus is required for further propagating the atrophy. This expansion can also be prevented using bindarit, further supporting the drug’s preclinical efficacy as a potential new treatment for dry AMD, and aligning directly with current clinical trial design.
**Figure 7.1** provides an overview of the mechanism of bindarit action on the inflammatory response following an NaIO$_3$ insult. Macrophages are proposed to be attracted to the site of injury due to the release of MCP-1 chemokine from the injured RPE cells, which constitutively express it, as well as potential other DAMP signals. Immediately after NaIO$_3$ damage to the RPE, baseline M2 macrophages polarize to the pro-inflammatory M1 phenotype, due to the presence of elevated oxidative stress and RPE cell necrosis. Bindarit prevents this change in the macrophage population by preventing the inciting MCP-1 stimulus and preventing the amplification of the pro-inflammatory cascade in these cells, which typically results in an amplification of the response. This subsequently prevents the additional accumulation of macrophages at the site of damage and a strong M1-polarizing microenvironment, thereby preventing the M1 amplification loop.

**Figure 7.1 Proposed model of RPE injury, macrophage polarization and bindarit action in the NaIO$_3$ model.** Bindarit (B) influences MCP-1 gene expression in macrophages, thus reducing the initial signals that recruit macrophages, and modulates the macrophage M1/M2 profile once recruited, resulting in a reduction of the pro-inflammatory cascade and the overall M1 response.
7.2 LIMITATIONS

7.2.1 NaIO$_3$ Model

NaIO$_3$-induced toxicity has been criticized as a model of AMD because BM remains normal and devoid of classical drusen deposits within or external to it. It is also believed to be acute rather than chronic, and, as described in models of wet AMD, there is no observed rupture or break in BM, a presumed requisite for aberrant growth of CNV. In rodent models the route of NaIO$_3$ administration and the strain of mouse are important factors in determining the phenotypical outcome, as differences in any of the parameters will result in inconsistencies in the development of RPE/retinal damage (Enzmann, Row et al. 2006). It has been noted that IV administration of NaIO$_3$ at doses up to 90 mg/kg (close to lethal) had no effect on retinal function in swine (Noel, Fernandez de Castro et al. 2012). This may suggest that this toxin is an ineffective method of producing the desired retinal degeneration in some larger animals. Another significant limitation is that very early degenerative changes are difficult to capture due to the rapid action of the toxin on the cellular structures (Grignolo, Orzalesi et al. 1966). This is likely one of the main reasons NaIO$_3$ fell out of favour as a model for studying early and late dry AMD, where early changes are vital to prevent vision loss, and the disease has a prolonged chronicity. Having the primary effect take place so acutely can make it difficult to intervene with treatments. We note this limitation, as it precluded us from using bindarit treatment downstream of the first couple of hours, where the insult becomes too overwhelming to be effectively treated. However, in this study we have observed that while the initial toxic insult is acute, subsequent inflammation and tissue degeneration that follows, in both the RPE, photoreceptor layer, and as others have shown in the CC, takes place over the weeks, and even months following administration, leading to a progressive model of disease especially in smaller rodents such as rats. We suggest that active disease progression, for example at sites of expanding GA, may be an acute-on-chronic process characterized by high localized activity similar to that observed in our model. In addition, we suggest that using modifications to the NaIO$_3$ model, such as that with NTG, produces a more effective model for studying expansion of atrophy and therapeutic interventions for this disease.

The use of a chemically-induced model to study treatment alternatives for dry AMD also raised a concern of chemical antagonism. The first time NaIO$_3$ was used in the rabbit to study its effects on cysteine and glutathione residues, it was identified that both react with NaIO$_3$ in a test tube to
deactivate the toxin (Sorsby and Reading 1964). However, we and others have shown that bindarit does not play a role in reducing oxidative stress, the main effect of NaIO$_3$ (Lin, Zhu et al. 2009). This points to a role of the drug on downstream effects of the primary oxidative insult, and supports its role in targeting the subsequent inflammatory response.

A second inherent problem was that direct comparison of NaIO$_3$ damage to human populations is difficult, however our study strengthens the similarities by demonstrating the marked in vivo parallels using cSLO imaging, particularly FAF. It has been suggested that the mechanism of RPE cell death in this model differs from that of human outer retinal degenerative diseases (Hariri, Moayed et al. 2012). The major difference compared to AMD being the normal BM in the NaIO$_3$ model (Franco, Zulliger et al. 2009). NaIO$_3$ is a highly toxic compound and thus the human population doesn’t typically come into contact with it, however case reports of iodate toxicity in human eyes do exist. One such report describes vision loss following povidone-iodine pleurodesis where high concentrations of iodate in certain formulations resulted in postoperative visual loss (Wagenfeld, Zeitz et al. 2007). These patients developed marked atrophy of the retinal pigment epithelium. Another report describes retinopathy following potassium iodate overdose in a number of patients, resulting in decreased vision, reduced ERG signal, changes in pigmentation on fundus photography, and window defects on fluorescein angiography (Singalavaniya, Ruangvaravate et al. 2000). Despite the inability to study NaIO$_3$ effects in the patient population, these limited reports of iodate toxicity in patients are remarkably similar to the findings observed in the rodent models, as well as those in late stage dry AMD, strengthening the translation of our mechanistic findings to the clinical disease phenotypes.

Third, the proposed mechanism of direct damage to the RPE cells, and subsequent damage to the photoreceptors and inner retina, is challenged by studies that observed direct effects on the inner retina following NaIO$_3$ (Tao, Dai et al. 2013, Wang, Iacovelli et al. 2014). There is some evidence that NaIO$_3$ may affect the retina primarily, and in addition, rather than sequential, to the RPE (Kiryu, Yamamoto et al. 1992, Yamamoto, Kiryu et al. 1993). Some studies showed that dendrites and axon processes of neurons degenerate in the retina after NaIO$_3$ administration, however these studies have limitations, such as the reliance on late timepoints, and no changes detected in cell numbers of horizontal, amacrine and ganglion cells (Tao, Dai et al. 2013). Since work prior to this showed evidence of RPE changes as early as 6 hours, it is postulated that anything subsequent this is a consequence of the effect on RPE. Taken together, along with the
work presented here, the evidence remains in favour of NaIO₃ affecting primarily the RPE cells, with subsequent apoptotic effects on the photoreceptors. This is likely due to the proximity of these cells to the exposed toxin infusing from the vasculature, and/or due to the preferential damage to the RPE machinery (Refer to Figure 1.11). Parts of this mechanism still remain to be fully elucidated.

A potential final criticism of long term studies using this model is the spontaneous regeneration that has been observed to take place in some species, giving rise to patchy regions of focal RPE regeneration and photoreceptor recovery (Kiuchi, Yoshizawa et al. 2002). However, regeneration was observed primarily in early rabbit studies that used lower doses (Korte 1991, Korte, Moskowitz et al. 1994). We confirm that in SD rats, with administration of sufficient doses of toxin, the RPE atrophies to a point where regeneration does not take place. Regeneration may play a role when lower doses of the toxin are given, making the high dose insult a more useful approach for studying the mechanisms of propagating atrophy. Our data show that when NaIO₃ is given to rats at doses that affect the majority of the retina, degeneration progresses without recovery in the subsequent weeks and months. Furthermore, the neural retina is lost, the entire retinal structure is thinned and this is an irreversible process, with no recovery of the ERG signal (Baek, Liang et al. 2015).

Despite the earlier reported limitations of this model, we suggest that this body of work clearly demonstrates that NaIO₃-induced RPE loss is a powerful tool to study the pathogenesis of FAF, and for the first time demonstrates a spatial and temporal relationship between deformation of the retina, activity of the innate immune system, and in particular an M1 pro-inflammatory response, and patterns of 488 nm autofluorescence. We also show, for the first time, a model of dual oxidative and nitrosyl stress and RPE loss that mirrors clinical susceptibility and clinical trial design, in that regions of RPE loss can be quantitatively assessed and made to expand over time. Such expansion required serial observations that can only be made in vivo rather than by terminal histology. Finally, our cellular and molecular analysis utilizes markers and methods not previously applied to this model. In effect, we have converted an acute model of RPE loss to one of chronic immune dysfunction, having applied both novel and clinically-relevant imaging to its analysis.
Inflammatory Response and Macrophage Polarization

Some of the prevailing limitations to studying macrophage polarization is the requirement of a strong stimuli for complete polarization of macrophages towards distinct M1 or M2 phenotypes, that multiple different stimuli can elicit the same or similar phenotypes, and the evidence for the existence of multiple intermediary phenotypes and sub-phenotypes (Gordon 2003). On top of that, there exists a substantial heterogeneity of macrophage polarization across species including differences between mice, rats and humans (Gordon and Taylor 2005). This makes studying macrophage polarization inherently difficult, and it is the reason we limited our initial hypotheses to the two broad categories of polarization of M1 and M2. In addition, while we recognize that there are many markers that can be used to address questions of polarization, we measured those markers that were 1) relevant to our in vivo model as evidenced by their expression in the rat, 2) were available and could be validated, and 3) were most relevant to human patient populations as observed in the literature in studies of AMD samples.

We also recognize that there are a number of agents that in the literature are known to induce or block polarization (Sica and Mantovani 2012). For example, it has been recognized the PPARy agonists are important in M1 inhibition and M2 activation. As such, treatment with AngII Type 1 receptor blockers (ARB) that are known to be partial PPARy agonists can induce M2 activation, and in models of obesity, diabetes and atherosclerosis, results in phenotypic switch from M1 to M2 polarization. PPARy agonists inhibit MCP-1 and M1 associated pro-inflammatory markers (TNFα, IL-6, iNOS), and the inflammatory activation of macrophages. PPARy agonists are also associated with inducing macrophage phagocytosis, and clearance of inflammatory particles such as amyloid-beta by tissue macrophages (Elangbam, Tyler et al. 2001, Mandrekar-Colucci, Karlo et al. 2012).

In another study, the diabetic drug perindopril was found capable of improving visual function in diabetic patients by decreasing VEGF and increasing PEDF (pigment epithelium-derived factor), which is a potent inhibitor of angiogenesis. It also attenuates ROS generation via mitochondrial pathway and upregulation of PPARy (Zheng, Chen et al. 2009). As such it is evident that there are a number of avenues that remain to be explored for attenuation of M1 polarization and potential activation of M2 polarization, however we were cautious in our choice of drug as we did not want to stimulate the latter. We elected to focus on bindarit, as this study primarily
centered on the M1 inflammatory cascade and its inhibition. It is important to recognize that the activation of the M2 pathway may also be important in the pathogenesis of AMD, especially wet AMD since activated M2 cells are associated with angiogenesis in the eye (Apte, Richter et al. 2006, Dace, Khan et al. 2008). We did not want to tip the balance as far as to induce a wet AMD-like state in this model, which may or may not be possible. The M2 results that are presented in this body of work are complicated by the fact that there appears to be fewer M2 cells overall. Thus, although the M2 response was not the primary objective of this study, further work studying the M2 response may be challenging using the same methodology. This is due to the amplifying effects of the M1 cascade, and that reducing the M1 response also reduces the overall macrophage response.

7.3 FUTURE STUDIES

While the original NaIO₃ model has been around for a number of decades, much of the original information is based on very early studies with limited availability of techniques and equipment. There remains a multitude of avenues to be explored in the mechanistic and therapeutic insights of NaIO₃ induced retinal degeneration, and how this RPE damage is analogous to that observed in dry AMD. We believe that this model is beneficial for studying the consequences of RPE loss and the downstream progression of this acute effect. This type of long term progression comprising of the immune response, gene expression changes, and structural and functional alterations that take place over time may be more relevant than the immediate changes associated with the destruction of the RPE monolayer.

More extensive studies are also required to obtain a better understanding of the in vivo changes that take place in the retinal tissues. Only recently have fundus photography, cSLO and other imaging modalities been adapted to observe retinal changes in rodents in vivo. This type of observation is critical as it gives information on the progressive changes in the same animal over time, and strengthens the translational component of the work, since the only way to monitor patients in the clinical setting is using imaging modalities. With better OCT, cSLO, and fundus cameras now available and attainable, it will be important to continue characterizing the in vivo aspects of the disease and comparison to patient fundus imaging. Our study is missing the OCT component that would be beneficial to better establishing the 3D structural changes that take place.
Here we present a number of opportunities that may be interesting to pursue using the NaIO$_3$ model as a basis of inducing a dry AMD-like phenotype. A number of projects by our team are currently underway focusing on these elements.

### 7.3.1 **NaIO$_3$ as a Model of Reticular Pseudodrusen**

We suggest that the NaIO$_3$ model may enable studying RPD, a subtype of dry AMD that has been recently associated with a higher risk of progression and more severe complications. Classic drusen are the hallmark of early disease, and are found within BM or in the sub-RPE space. These are not located sub-retinally. More recent studies suggest that at least 50% and up to 90% of patients with non-neovascular AMD have sub-retinal drusenoid deposits or RPD (Curcio, Messinger et al. 2013). These are primarily located in the extrafoveal regions. Recent interest has focused on RPD because they are associated with high rates of late stage AMD disease (Klein, Meuer et al. 2008, Schmitz-Valckenberg, Alten et al. 2011). They are also associated with both myocardial infarction and stroke, which are predictors of early AMD and RPD (Tan, Mitchell et al. 2007). This may also be valuable in elucidating the similarities between AMD and atherosclerosis, another macrophage-mediated disease with components of lipid metabolism and oxidative stress. RPD are reported to emerge de novo, sparing the optic nerve head, and move up to the retinal periphery over time (Arnold, Sarks et al. 1995). On FAF imaging, the lesions are hypofluorescent and interspersed between slightly hyperfluorescent signal (Sarks, Arnold et al. 2011). RPD can also be detected in the NIR and RF channels, as well as by high-resolution OCT. Since we have observed deformations of the retina, and autofluorescent cells in the deformed subretinal space, we believe further studies using OCT may be useful in this model, and can help draw comparisons to the changes observed on OCT in RPD.

On the other hand RPD may be a distinct age-related disorder found concurrently in patient with classic AMD that confers high risk of late complications. Other types of drusenoid deposits are reported in patients with Late Onset Retinal Degeneration (LORDs) and C1qTNF5 deficiency, with genetic defects implicating membrane function and RPE cell adhesion (Borooah, Collins et al. 2009, Vincent, Munier et al. 2012). These patients also present with scalloped patterns of FAF. As such, the NaIO$_3$ model may be useful in studying other retinal disease that are associated with, or present with similar phenotypic and mechanistic characteristics to dry AMD.
7.3.2 **PED in the NaIO₃ Model**

In all forms of early AMD, a disturbance of the epithelial barrier can often be observed. Pigment epithelial detachments (PED) can result in up to 42% progression to advanced AMD (Cukras, Agron et al. 2010). Vitelliform lesions, a sub-retinal accumulation of yellowish material in the subretinal space are reported in association with RPD (Zweifel, Spaide et al. 2011). Our model shows that geographic areas of damage present where chorioretinal barrier dysfunction takes place beforehand. This is evidenced by a transient fluid flux into the subretinal space as observed using angiography. It is then followed by the formation of folds in the retinal tissue likely due to detachment of the retina from the apical side of the RPE, where macrophages eventually get trapped, leading to areas of autofluorescence. We therefore suggest that this transient flux is an important early step in disease progression and merits further research. Early studies suggest that NaIO₃ may affect membrane permeability, adhesion, and membrane ion pumps but doesn’t suggest the specific mechanisms responsible for this (Nilsson, Knave et al. 1977, Ashburn, Pilkerton et al. 1980). Studies are underway to establish the mechanisms of these changes and the relevance to dry AMD, and are important for identifying the early processes that take place in the stages leading up to GA.

7.3.3 **Inflammatory Response in the NaIO₃ Model**

Until now, there has been a substantial lack of information about the immune response that takes place following the initial damage of NaIO₃. Some very early studies even went as far as to suggest that the macrophage response doesn’t play a significant (or any) role in this model (Grignolo, Orzalesi et al. 1966, Korte, Reppucci et al. 1984). At least in the rodent model we now know otherwise, and other studies support evidence of inflammatory cells following NaIO₃-associated damage (Kiuchi, Yoshizawa et al. 2002, Mendes-Jorge, Ramos et al. 2009). The differences observed in the rabbit may point to differences in the vasculature across animal species, and therefore different macrophage recruitment characteristics. The data presented in this study is limited to broad aspects of M1 and M2 macrophage polarization, but there remains a number of pathways that can be explored both upstream and downstream of these changes. Studying the M2 response may be an important future step in determining whether the NaIO₃ model can be used to obtain a wet AMD phenotype, which would show support that 1) the model has an even stronger association with the clinical disease, 2) macrophage polarization plays a
role in both dry and wet AMD, and 3) discovery of a link between the two effectively “polarized” subtypes of AMD.

More details of the molecular mechanism of the inflammatory response will help clarify the nature of the mechanistic changes that take place following the toxic insult, and whether these are truly secondary to the RPE damage. We know that the inflammasome complex plays an important role in both AMD and in other models of retinal disease (Ambati, Atkinson et al. 2013). We have observed upregulation of the genes of the NLRP3, IL-1b and IL-18, however these have not been analyzed in detail in this model as of yet. Further work to identify the mechanisms of inflammasome action and to better understand the DAMP signaling that plays a role in this model are underway to help uncover insights into the causes of the initiation and subsequent stages of dry AMD, as well as the other initiators of macrophage polarization.

There are a multitude of genes that remain to be characterized in the NaIO₃ model, considering the limited number of genes that have been analyzed to date in this study and others (Machalinska, Kawa et al. 2013). To further address the relevance of NaIO₃-induced RPE loss to dry AMD, it will be important to evaluate the mRNA, perhaps even microRNA, and where possible the protein products, of genes known to be associated with disease susceptibility, including those of the complement cascade such as CFH, and other complement proteins. Since the complement cascade, is an important susceptibility factor for the disease, it will ultimately be of value to uncover the link between macrophage activation and polarization, and the complement proteins that are believed to play a role in the disease. In particular, we are working to determine whether upregulation of propagators, or reductions in suppressors of the complement cascade is evident in this model. The final step in the complement cascade, the MAC, is an important complex that ultimately results in cell lysis at the site of complement activation (Bubeck 2014). This complex has now been identified in the CC of aging patients and those with AMD (Chirco, Tucker et al. 2015). It is essential to identify whether such a response plays a role in polarization of macrophage populations at the site of cell injury/lysis.

RPE is known to be the major source of CFH in the outer retina, but the protein is also found in outer segments and inter-photoreceptor space, likely due to its secretion there by the RPE (Smit-McBride, Oltjen et al. 2015). A study by Weismann et al used malonaldehyde (MDA), a lipid peroxidation product which is also found in drusen to test binding affinity to CFH. It was found
that the H402 polymorphism in AMD patients reduced the ability of CFH to bind MDA. CFH can block uptake of MDA-modified LDL particles by macrophages and the associated MDA-induced inflammatory response in mice, by binding the same epitope that is required for recognition by macrophages. Therefore when CFH binds MDA there is a decreased pro-inflammatory response (Weismann, Hartvigsen et al. 2011). This is a step towards deciphering the link between the role of complement and the activation of the inflammatory response in AMD. Further studies using lipid peroxidation products can address the nature of the macrophage response, and whether any differential phenotypes can be identified.

### 7.3.4 Novel Combination Models for Studying Dry AMD

The combination of NaIO₃ with other toxins is until now unexplored. However, a combination model may be an even better predictor of the disease mechanisms that play a role in dry AMD than NaIO₃ alone, since the disease is of a highly multifactorial nature. We combined NaIO₃ with NTG, a potent reactive nitrogen species (RNS) generator, and a stimulus capable of inducing inflammation in rodent models of migraine, where an upregulation iNOS was observed in macrophages (Reuter, Chiarugi et al. 2002). NTG generates NO and subsequent peroxynitrite (ONOO⁻) formation, which causes an increase in the oxidative load in the tissue (Sukhatme, Bouche et al. 2015). NO and ONOO- can damage organelles, causing toxicity and cell death (Wei, Chen et al. 2000, Wilcox 2005, Lubos, Handy et al. 2009).

The breakdown of NTG into NO is believed to be carried out primarily via the enzyme aldehyde dehydrogenase (DiFabio, Ji et al. 2003). Once the NO molecules are liberated they can move freely in tissues to serve a multitude of functions, including combination with O₂ radicals to form the highly toxic ONOO-. NO can also activate the NFκB pathway via p50/65 dimers resulting in their nuclear translocation and transcriptional activation of iNOS, IL-1β and IL-6 (Reuter, Chiarugi et al. 2002).

Interestingly, current research is looking at repurposing NTG as a drug to target cancer cells via its pro-apoptotic and immunomodulatory effects (Sukhatme, Bouche et al. 2015). This suggests that similar to NaIO₃, NTG may be an apoptosis-inducing toxin for RPE and retinal cells. Additional combination models incorporating toxins, oxidants, environmental variations such as light, and genetic modifications can also be explored. Much like the cellular and molecular mechanisms of dry AMD, gaps in the pathways that lead to NaIO₃-induced retinal degeneration
remain elusive despite decades of study. Since the disease is highly multi-factorial, one model, ie one genetic alteration or one toxic insult may not be sufficient to obtain many of its principal features and so, more focus should be placed on multifactorial models.

### 7.3.5 Clinical Trial Design and Translation

Studies correlate risk of GA expansion with differences in FAF patterns, the most aggressive being the banded and diffuse-trickling (Holz, Bindewald-Wittich et al. 2007). Overall, the more fluorescence, the faster GA is anticipated to expand, and areas of hyperfluorescence predict the future region of GA expansion. FAF imaging now drives clinical trial design, with patient enrollment protocols relying on patterns of hyperfluorescent FAF as part of inclusion criteria, and the quantification of hypofluorescent FAF as both inclusion criteria and endpoint analysis. Our model reliably reproduces these clinical findings, which have not been previously reported in any non-human species, and now provides the unique opportunity to determine their tissue correlate and test drug efficacy. This study further suggests that this model of atrophy progression and expansion can be reliably used to assess a response to treatment for diseases such as GA, where not only the onset, but also the expansion of atrophy is an important marker of disease activity.

We have now also been able to translate our DNIRA technique for observing RPE and macrophages in vivo, to clinical trials in patients. We have observed that the DNIRA patterns of atrophy in patients with dry AMD are much more extensive and complex than those that can be observed with FAF. These are now the subject of further study, and are a major step towards better understanding, diagnosis and treatment monitoring of dry AMD.

To further correlate clinical endpoints to the mechanisms presented in this model, it would be beneficial to measure cytokine and chemokine levels in the aqueous and/or vitreous of patients with dry AMD. MCPs, ILs, and other inflammatory mediators can be reliably measured in the ocular fluid specimens and have been used in the clinic for diseases such as diabetes, neovascular AMD and ocular melanoma (Yoshimura, Sonoda et al. 2009, Jonas, Tao et al. 2010, Nagarkatti-Gude, Bronkhorst et al. 2012). Some of these markers can even be used as predictors of severity of the disease (Jonas, Tao et al. 2010, Umazume, Usui et al. 2013), and therefore may be associated with severity of atrophic AMD as well. Though MCP-1 protein appears to be elevated in the eyes of AMD patients, it is not yet clear the role that it plays in dry AMD, but if drugs
such as bindarit can be used to target MCP mechanism in patients, measurement of cytokine and chemokine levels will be an important marker of drug efficacy.

Figure 7.2 Summary and platform of the work arising from this thesis.
7.4 CONCLUSION

In summary, this study sought out to overcome the two major limitations in dry AMD research: 1) Lack of suitable preclinical models and 2) lack of treatment alternatives. Using a translational approach, and by innovating on established techniques we were able to successfully characterize an in vivo rodent model of GA, the late stage blinding complication of dry AMD. By further characterizing the immune response and macrophage polarization in this model, we were able to target this response for therapeutic intervention (Figure 7.2). We maintained the focus on clinical translation by using clinically relevant imaging equipment, markers, and endpoints. The imaging techniques reported in this study have since been translated to the clinical setting, and we have correlated the inflammatory pathways in the NaIO₃ model of RPE atrophy to those that are observed to play a role in dry AMD. While this model does not reproduce all the features of the disease, it should be remembered that no animal model is a perfect replica of human pathology, but as in this case, can be sufficient for applying therapies and modifications that may lead to uncovering the pathways responsible for the clinical manifestations of disease. We believe that we have set the groundwork for further research using this model with the goal that a treatment for patients with dry AMD may ultimately be found.
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**Figure 1.7. Broad description of macrophage polarization and the phenotypes associated with M1 and M2 macrophage responses.** Adapted with permission from Macmillan Publishers Ltd.


**Figure 1.8. Transcription factors involved in M1 and M2 macrophage polarization.** Modified with permission from The American Society for Clinical Investigation.


**Figure 1.9 Mechanism of bindarit action.** Used with permission from Taylor & Francis.

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**Figure 1.10 Accepted models of AMD.** Used with permission from Elsevier.