Inflammation in Retinal Disease

Guest Editors: Scott M. Whitcup, Robert B. Nussenblatt, Susan L. Lightman, and David A. Hollander
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Inflammation in Retinal Disease

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1. Introduction

Ocular inflammation and its related complications are important causes of vision loss. Inflammatory processes have long been implicated in the pathogenesis and sequelae of non-infectious uveitis and understood to underlie the macular edema which may arise following even uncomplicated intraocular surgeries [1]. More recently, evidence has also arisen supporting a prominent role for inflammation underlying the pathogenesis of a wide array of retinal diseases, including age-related macular degeneration (AMD) [2], diabetic retinopathy (DR) [3], retinal vein occlusion (RVO) [4], and retinitis pigmentosa (RP) [5], and has suggested a role for anti-inflammatory therapies to potentially alter the severity and course of these disorders. The goal of this special issue is to highlight the latest understanding of the role of inflammation in retinal diseases, to address current questions and controversies, and to facilitate future research.

Traditionally, the eye has been considered an immune privileged site. Contributing to this immune privilege is the blood-retinal barrier which consists of both an inner barrier formed by the tight junctional complexes between retinal vascular endothelial cells and an outer barrier formed by the tight junctions between the retinal pigment epithelium (RPE) cells. Research over the last 30 years has demonstrated that mechanisms beyond tissue barriers contribute to ocular immune privilege and an immunosuppressive intraocular environment. In fact, the pigment epithelial cells which line the iris, ciliary body, and retina serve an immunomodulatory role through both the secretion of soluble immunosuppressive factors as well as contact-dependent mechanisms [6].

Vision is dependent on the exquisite and precise structure of the retina, and any process which significantly disrupts retinal architecture can have a profound impact on vision. The immune response, when controlled, is an adaptive response to restore homeostasis. Alterations in retinal homeostasis secondary to aging, metabolic abnormalities, altered vascular perfusion, or degenerative genetic conditions may initiate various inflammatory cascades. In all of these settings, a prolonged, dysregulated immune response may itself be pathologic, contributing to both the pathogenesis of retinal diseases as well as vision threatening complications.

2. Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a leading cause of irreversible vision loss in the western world. AMD can manifest as both a "dry" form (90% of cases) featuring geographic atrophy of the RPE which currently has no treatment as well as an exudative "wet" form (10% of cases) which is responsible for the majority of cases of vision loss due to choroidal neovascularization which may now respond to treatment with antivasular endothelial growth factor (VEGF) agents. While genetic, environmental, and metabolic factors may all be contributing factors, recent evidence supports a more central role for the immune system in the pathogenesis of AMD.

Aging is associated with a decrease in the number of RPE cells as well as the number of photoreceptors [7]. With aging, oxidative stress secondary to the accumulation of oxidized lipoproteins and free radicals in retinal and choroidal tissues may trigger a tissue adaptive response, recently described as
“para-inflammation,” in which cells of the innate immune system mount a low-grade inflammatory response in order to restore tissue homeostasis [8]. Sustained injury or chronic inflammation may lead to an imbalance in the local inflammatory response and contribute to 

Drusen, extracellular deposits located between the RPE and Bruch’s membrane, are most commonly seen in individuals over 60 years of age and represent the clinical hallmark of AMD. Though once considered simply to be waste products consisting of lipid and carbohydrate, drusen are now understood to also consist of byproducts of local active inflammation and complement activation (C3, C5a, and C9) [9]. The etiology of drusen and the progression of AMD are likely multifactorial, though a primary mechanism may be related to RPE cell injury. Injured RPE cells release cytokines and chemokines that recruit and activate choroidal dendritic cells. Dendritic cells may amplify the inflammatory process via cell to cell contact, immune complex formation, and complement activation leading to additional RPE cell damage, potentially producing a state of chronic inflammation [9].

In addition to elements of the complement system being found in drusen, both genetic and animal studies have also strongly supported a pivotal role of the complement system in the pathogenesis of AMD. While complement is active at a low basal level in the normal retina as a protective mechanism, alterations in the regulation of the complement system can trigger significant pathology. Strong associations with AMD have been identified in association with particular mutations in the complement factor H (CFH) protein (Y402H), a key regulatory component of the alternative pathway in distinguishing self from nonself [10–13]. Thus, a growing body of histopathological, preclinical, and epigenetic data now supports a key role of inflammation in the pathogenesis of AMD, a disease which was not classically described as inflammatory in origin.

3. Diabetic Retinopathy

Over 285 million individuals worldwide are estimated to have diabetes mellitus [14]. Diabetic retinopathy (DR), a common complication of diabetes, increases in prevalence with duration of disease. DR has traditionally been considered a disease of the retinal microvasculature and has been categorized into an early nonproliferative stage and an advanced, proliferative stage based on the natural history. The most common causes of vision loss in diabetics are diabetic macular edema (DME), typically seen early in the course of DR, and proliferative retinopathy.

The mechanisms by which high glucose levels directly lead to diabetic retinopathy have not been fully elucidated. Chronic hyperglycemia leads to a series of biochemical changes, including activation of protein kinase C, accumulation of polyols through the aldose reductase pathway, increased formation of advanced glycation end products (AGEs), and overproduction of free radicals. These metabolic changes increase proinflammatory cytokines, chemokines, and other inflammatory mediators that stimulate an influx of leukocytes and alter vascular permeability [15]. Elevated levels of interleukin 6 (IL-6), IL-8, tumor necrosis factor-α (TNFα), VEGF, interferon-induced protein-10 (IP-10), intercellular adhesion molecule 1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) have been demonstrated in eyes with DR [15].

Inflammatory processes may underlie many of the functional retinal vasculature alterations observed histologically in early diabetic retinopathy, such as pericyte loss, saccular microaneurysms, and occluded and degenerated capillaries. An increase in the attraction and adhesions of leukocytes has been observed in experimental models of diabetes within 1 week of disease onset [16]. This leukostasis is a direct result of the interactions between elevated expression of ICAM-1 on retinal vessels and the CD18 adhesion molecule on monocytes and neutrophils [16]. Increased leukocyte stiffness may also contribute to capillary nonperfusion [17]. Experimental models of diabetes in mice deficient in the genes encoding for ICAM-1 and CD18 have revealed fewer adherent leukocytes in the retinal vasculature, a reduced number of damaged endothelial cells, and less vascular leakage [3].

In addition to the leukocyte-mediated endothelial cell damage, increased vascular permeability leading to DME also arises due to conformational alterations in the tight junctional proteins. The tight junctions consist of over 40 different proteins and various inflammatory mediators, including VEGF, TNFα, protein-kinase-C, IL-1β, and IL-6, alteration of particular proteins via phosphorylation, redistribution, or alteration in content thereby reducing the endothelial barrier [18]. As inhibition of different inflammatory mediators has been shown to limit the degeneration of retinal capillaries characteristic of early stages of DR, continued investigations into the role of inflammation in the pathogenesis of DR are warranted.

4. Retinal Vein Occlusion

Retinal vein occlusion is the second most common ocular vascular abnormality, following diabetic retinopathy, resulting in vision loss. The occlusion may occur at or proximal to the lamina cribrosa of the optic nerve involving the central retinal vein or occur more commonly at an arteriovenous intersection involving a branch retinal vein. The origin of the occlusion likely stems from compression and local retinal vascular damage, followed by stasis and thrombosis. In some patients, inflammatory conditions may play a role in contributing to the vascular injury and thrombus formation [4]. Increased hydrostatic pressure proximal to the occlusion commonly leads to vascular leakage and subsequent macular edema, the most frequent cause of vision loss in the setting of RVO.

Vascular endothelial damage in the occluded vein may result in a low-grade, chronic inflammation and the production of inflammatory mediators that exacerbate and prolong the edema. A number of inflammatory cytokines and growth factors may be elevated in RVO patients, including IL-1α, IL-6, IL-8, MCP-1, platelet derived growth factor (PDGF) AA, and VEGF relative to control eyes [19–21]. These factors contribute to the transition from an acute to chronic inflammation, the recruitment of monocytes to the site of injury,
an increase in vascular permeability, and the development of ocular neovascularization. The severity of macular edema secondary to BRVO has been correlated with both elevated vitreous and aqueous levels of VEGF and IL-6 [22].

5. Retinitis Pigmentosa

Retinitis pigmentosa is a heterogeneous group of inherited retinal degenerative diseases which lead to photoreceptor cell death and severe vision loss. Clinically, RP is characterized by a pigmentary retinopathy, optic nerve pallor, progressive visual field loss, and nyctalopia. Additional clinical findings may include vitreous cells, posterior subcapsular cataract, and macular edema. Lymphocytes have been detected in the vitreous gel of RP patients, further characterizing the inflammatory nature of the vitreous cells [23]. While RP is now known to be primarily a hereditary disease caused by mutations in over 45 different genes, investigators have continued to examine the role of the immune system in the pathogenesis and progression of the disease.

It has been suggested that the observed immune responses are likely secondary to the release of retinal proteins by the underlying degenerative disease [24]. First, major differences in immune responses have not been detected across different subtypes of RP [24]. Secondly, it has often been in those patients with severe vision loss that significant cellular immune responses have been shown [25]. In a recent clinical study of RP patients, greater inflammation in the anterior vitreous correlated with worse VA as well as lower mean deviation on visual field testing [5]. Elevated proinflammatory markers, most notably MCP-1, have been detected in both the aqueous and vitreous [5]. MCP-1 is known to activate microglia as well as recruit monocytes, memory T cells, and dendritic cells to sites of injury. While the chronic inflammation in RP patients may be secondary to a primary genetic mutation leading to photoreceptor loss, the immune response to the shed proteins may subsequently exacerbate the retinal destructive processes in RP and other retinal degenerative diseases [26].

6. Conclusion

We believe that the papers included in this issue will offer readers a greater appreciation for the role of inflammation in a variety of retinal diseases, many of which were not traditionally considered to be inflammatory in nature.

Whitcup et al. summarize discussions from the 5th annual conference of the Arnold and Mabel Beckman Initiative for Macular Research by the Inflammation and Immune Response Task Force in which they review data supporting the dysregulation of immune response as a contributing factor to the pathogenesis of AMD and propose a series of experimental approaches to address unanswered questions.

In a mouse model of AMD, Cruz-Guilloty et al. demonstrate a link of AMD-like histopathological changes with the presence of macrophages in the outer retina during early stages of disease. The authors suggest that immune modulation may play a role in the future in either the prevention or treatment of patients with early signs of AMD.

Jain et al. address the evolving pharmacologic treatment options for DME, focusing on the multifactorial nature of the disease in their review of major studies of both corticosteroids and anti-VEGF therapies.

Deobhakta and Chang summarize the laboratory and clinical studies supporting the role of inflammation in the pathogenesis and clinical consequences of RVO. The authors also review the latest clinical studies of anti-inflammatory treatments for patients with macular edema secondary to RVO. Using ultra wide field fluorescein angiography, Tsui et al. report late peripheral retinal leakage in the fellow eyes in patients with BRVO and suggest that these findings may represent underlying systemic inflammation, hypertension, or bilateral BRVO.

Viringipurampeer et al. review the preclinical and clinical evidence linking inflammatory mediators to genetic retinal diseases, specifically RP and AMD, and summarize the latest anti-inflammatory interventional studies. The authors conclude that anti-inflammatory agents are likely to play significant roles in the future treatment algorithms of these diseases.

Schoenberger and Kim review the role of nonsteroidal anti-inflammatory drugs (NSAIDs) as inhibitors of the cyclooxygenase (COX) enzymes that catalyze the synthesis of prostaglandins. The authors review the scientific rationale and provide an update on the interventional studies that have been conducted with NSAIDs in postoperative cystoid macular edema, AMD, DME, and DR.

The ideas discussed in this issue should demonstrate that immune responses, while often beneficial in the acute setting, can have undesirable effects if they result in a state of chronic inflammation. Ultimately, as the roles of different inflammatory pathways in retinal diseases become more clearly elucidated, greater emphasis can be placed on new targets for future treatment options.

We would like to dedicate this special issue to Stephen J. Ryan, MD, who passed away on April 29, 2013. Dr. Ryan was an expert in retinal diseases and a leader in ophthalmology. He was the president of the Doheny Eye Institute from 1974 to 2012, the first full-time chairman of the University of Southern California (USC) Department of Ophthalmology, and the dean of USC’s school of medicine from 1991 to 2004 which later became the Keck School of Medicine. Dr. Ryan also was a member of the Institute of Medicine and a member of the National Advisory Eye Council and founded the National Alliance for Eye and Vision Research (NAEVR). Dr. Ryan devoted his career to understanding the pathogenesis of diseases of the retina including age-related macular degeneration. In addition to his own pioneering research, Dr. Ryan trained and educated countless scientists and clinicians around the world. It is therefore befitting that we dedicate this collection of manuscripts discussing the role of inflammation on the pathogenesis of retinal diseases to Dr. Ryan.
References


The Evolving Treatment Options for Diabetic Macular Edema

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1. Introduction

Diabetic retinopathy (DR) is the leading cause of vision loss in working-age adults. In 2002, there were estimated to be just over 13.5 million individuals afflicted with diabetes mellitus (DM) in the USA, or about 6% of the population. Since then, revised estimates for 2011 indicate that 25.8 million people have DM in the USA, of which 18.8 million are diagnosed and 7 million cases are undiagnosed [1, 2]. Approximately 28.5% of individuals with DM have some form of retinopathy; 4.4% of individuals are at risk of severe vision loss secondary to advanced disease. Present estimates indicate that the incidences of DM and DR are both significantly increasing with as many as 50 million or more individuals in the USA having DM by the year 2050, of which half are expected to have some form of retinopathy [1–5].

DR can be categorized into two broad groups: (1) nonproliferative diabetic retinopathy (NPDR) and (2) proliferative diabetic retinopathy (PDR). Within NPDR, patients are classified as mild, moderate, or severe; severe NPDR is based on at least one of the following findings: diffuse intraretinal hemorrhages in all quadrants, venous beading in at least 2 quadrants, or the presence of intraretinal microvascular abnormalities. Of the two broad categories, proliferative disease, while it is less common, results in more severe vision loss. In non-proliferative disease, the most common cause of vision loss is due to diabetic macular edema (DME). At present, individuals with DR in the USA have a prevalence of DME between 3 and 5%, with this percentage increasing with age [6].

A recent meta-analysis of 35 population-based studies pooling data from the USA, Europe, Asia, and Australia found that in individuals with DM the prevalence of any type of DR is 35%, with DME present in 7.5% and PDR present in 7.2% of individuals. These prevalence rates were found to be significantly higher in individuals with type 1 DM compared to type 2 DM [7]. In the USA, over 90% of individuals with DM are type 2 diabetics [8].

Summarizing the above data as it applies to the USA, at present, approximately 1.1 million individuals are at serious risk of sight-threatening vision loss from DR. Of these “at risk” individuals, DME is the major etiology of visual impairment or loss with approximately 900,000 individuals with active DME in the USA. A decrease in visual acuity (VA) is commonly used to assess the severity of DME. Fluorescein angiography (FA) has been used extensively to image and assess diabetic eye disease and is useful in the identification of specific areas to treat when using targeted macular laser photocoagulation. More recently, optical coherence tomography (OCT) has become the gold standard used to objectively assess and quantify DME; central macular thickness (CMT) is the most common OCT measurement used for comparative purposes in recent clinical trials. VA outcomes are the focus of this paper.

2. Inflammation and DME

DME is due to extracellular swelling typically in Henle’s layer of the macula caused by breakdown of the blood-retinal
barriers [3]. Previously, DME was defined as clinically significant macular edema (CSME) or not, and focal laser treatment was initiated only for CSME (defined as thickening of the retina at or within 500 microns of the center of the macula, hard exudates at or within 500 microns of the center of the macula, if associated with thickening of adjacent retina, or a zone or zones of retinal thickening 1 disc area or larger of which any part is within 1 disc diameter of the center of the macula) [9]. More recently, DME has been subcategorized into two main categories: (1) focal diabetic macular edema (fDME) and (2) diffuse diabetic macular edema (dDME). With advancements in retinal imaging and an increased armamentarium of treatment options, the terms fDME and dDME may be more clinically relevant. Center-involving diabetic macular edema (cDME) is also now commonly used to describe DME in which the central macula is involved.

As our knowledge of DME has advanced, we now know that the cause is multifactorial. Blood vessel damage plays a significant role in diabetics, both systemically and as related to the development of DME. Long-term hyperglycemia leads to vascular basement membrane thickening, nonenzymatic glycosylation, free radical formation, and pericyte death. These changes ultimately compromise the retinal vascular autoregulatory functioning leading to vascular dilation, increased capillary hydrostatic pressure, and microaneurysm formation [10]. The already weakened capillaries are further compromised due to the inflammatory changes known to occur in diabetics. The retinal vasculature of individuals with DM contains an increased density of leukocytes, which coincides with an increase in expression of ICAM-1 (intercellular adhesion molecule 1), also known as CD54 (cluster of differentiation 54) [11]. ICAM-1 can be induced by interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α). ICAM-1 activation leads to proinflammatory changes and increased vascular permeability due to damage of vascular endothelial cells via a FasL-mediated mechanism leading to further breakdown of the blood-retinal barrier [12]. Numerous cytokines and proinflammatory factors have also been implicated as having a role in DME, the most studied of which is vascular endothelial growth factor (VEGF) [13,14]. Table 1 lists the inflammatory factors which have been suggested to play a role in DME [15–23].

It is now well known that breakdown of the blood-retinal barrier results from compromised endothelial cell integrity. Osmotic fluctuations, due to hypertension and varying glycemic levels, increased vascular permeability, and capillary dropout, create an environment of inadequate blood flow to the retina. This retinal ischemia leads to the upregulation of VEGF, one of the most potent molecules in causing vascular permeability in humans [11]. VEGF mediates retinal vasculature hyperpermeability by opening endothelial tight junctions and inducing fenestrations. A compromised vascular endothelium secondary to ICAM-1 pathways in conjunction with damage caused by VEGF and other factors in the already weakened diabetic retinal vasculature precipitates a vicious cycle resulting in the inappropriate extravasation of intravascular contents.

While there is significant upregulation of proinflammatory factors in individuals with DME, there is also downregulation of antiinflammatory factors, in particular pigment epithelium derived growth factor (PEDF). Vitreous levels of the following proinflammatory molecules: VEGF, ICAM-1, interleukin-6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1) increase in individuals with DME, while vitreous levels of the antiinflammatory molecule PEDF may be significantly lower in diabetics with severe DME compared to those with only minimal or no DME [24]. Interleukin-8 (IL-8) levels are elevated in the aqueous of individuals with macular edema secondary to diabetes, but not retinovascular occlusive disease. Furthermore, IL-8 levels are not affected by the administration of intravitreal anti-VEGF or corticosteroid agents, indicating it could represent a new target in the management of DME [20].

### 3. Systemic Conditions and DME

Duration and control of DM play a major role in the development of DME. Individuals with a longer history of DM are at higher risk of developing DME as well as individuals with poor DM control (higher hemoglobin A1C concentrations) [3, 25]. Optimal hypertensive and DM control can delay and even prevent the onset of DME and vision loss.

The Diabetes Control and Complications Trial (DCCT) evaluated patients with type 1 (insulin dependent) DM for 6.5 years and demonstrated that intensive glycemic control reduced the risk of developing retinopathy by 76% (10.7% versus 33.2%, intensive versus conventional control groups, resp.) in those with no previous retinopathy and slowed the progression of retinopathy by 54% in those who had mild DR. The conventional group had a hemoglobin A1C of 9.1 versus 7.2 in the intensive control group. At the closeout of the DCCT study, 3.9% (intensive group) versus 7.7% (conventional group) developed CSME [26–28]. The Epidemiology of Diabetes Interventions and Complications (EDIC) Research Group followed patients for 4 years after conclusion of the DCCT and found that the benefits of intensive diabetes control persisted even with increasing hyperglycemia (hemoglobin A1C increased to 7.9 in the intensive group, compared with a reduction to 8.2 in the conventional group). After four years of follow-up in the EDIC study, 18% of the patients in the intensive-therapy group had a progression in DR compared to 49% of the patients in the conventional-therapy group. At the closeout of the EDIC study, 3.8% (intensive group) versus 13.3% (conventional group) developed CSME [29]. At 10 years after the conclusion of the DCCT study, both intensive and conventional groups had a hemoglobin A1C of 8, with 36% of patients in the intensive group demonstrating a progression of DR compared to 61% in the conventional group. In the intensive group, 9% developed CSME and 8.9% developed PDR compared to 19% developing CSME and 24.7% developing PDR in the conventional group [30].

The United Kingdom Prospective Diabetes Study (UKPDS) studied the effects of glycemic control on type 2 (non-insulin dependent) diabetics and found that intensive glycemic control was associated with a 25% decrease in microvascular complications and a reduction in the need for macular laser photocoagulation. The UKPDS also found
Table 1: Inflammatory factors suggested to play a role in DME.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Factor</th>
<th>Abbreviation</th>
<th>Clinical relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>[15]</td>
<td>Angiopoietin-1 and 2</td>
<td>Ang1/Ang2</td>
<td>Angiogenesis and neovascularization</td>
</tr>
<tr>
<td>[16]</td>
<td>Erythropoietin</td>
<td>Epo</td>
<td>Stimulates retinal endothelial cell proliferation</td>
</tr>
<tr>
<td>[17]</td>
<td>Hepatocyte growth factor</td>
<td>HGF</td>
<td>Stimulate: proliferation, migration, and invasiveness of retinal endothelial cells</td>
</tr>
<tr>
<td>[18]</td>
<td>High-sensitivity C-reactive protein</td>
<td>hsCRP</td>
<td>Possibly related to CSME and hard exudation</td>
</tr>
<tr>
<td>[19]</td>
<td>Insulin-like growth factor-1</td>
<td>IGF-1</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>[18]</td>
<td>Intercellular adhesion molecule 1</td>
<td>ICAM-1</td>
<td>Possibly related to CSME and hard exudation</td>
</tr>
<tr>
<td>[20]</td>
<td>Interleukin 6</td>
<td>IL-6</td>
<td>Vascular permeability</td>
</tr>
<tr>
<td>[20]</td>
<td>Interleukin 8</td>
<td>IL-8</td>
<td>Mechanism unknown, upregulated in DME but not macular edema from vascular occlusive disease</td>
</tr>
<tr>
<td>[20]</td>
<td>Monocyte chemoattractant protein 1</td>
<td>MCP-1</td>
<td>Leukostasis leading to hypoxia</td>
</tr>
<tr>
<td>[21]</td>
<td>Pigment epithelium-derived factor</td>
<td>PEDF</td>
<td>Antiangiogenic and antiinflammatory</td>
</tr>
<tr>
<td>[22]</td>
<td>Protein kinase C</td>
<td>PKC</td>
<td>Increases vascular permeability and contractility</td>
</tr>
<tr>
<td>[19]</td>
<td>Stromal-derived factor 1</td>
<td>SDF-1</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>[23]</td>
<td>Thrombospondins 1 and 2</td>
<td>TSP-1 and 2</td>
<td>Anti-angiogenic; inhibit endothelial cell proliferation and apoptosis</td>
</tr>
<tr>
<td>[20]</td>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
<td>Angiogenesis and vascular permeability</td>
</tr>
</tbody>
</table>

that intensive control of blood pressure (BP) had a 34% reduction in the risk of DR progression and a 37% reduction in diabetic microvascular endpoints, such as the need for retinal photocoagulation [31, 32].

4. Laser DME Treatment Paradigms

Until the early 1980s, there was no intervention available for the treatment of DME. A landmark prospective randomized study performed by the Early Treatment Diabetic Retinopathy Study (ETDRS) group found that grid macular photocoagulation decreased the risk of moderate to severe vision loss from DME by 50% compared to untreated controls over 3 years [33]. This was the standard of care for over 2 decades. Since the original ETDRS study, there has been evidence to support that a modified ETDRS laser technique has slightly better visual outcomes than a grid pattern of laser alone. In the modified technique, a light macular grid is performed in addition to the targeted treatment of microaneurysms with laser photocoagulation [34].

There is some pieces of evidence that very short duration focal macular laser photocoagulation and subthreshold micropulse diode laser treatments are just as effective as the modified ETDRS method of laser treatment for DME, but with less collateral damage, a lower risk of inducing choroidal neovascularization, and less likelihood of laser wound creep into the central fovea [35–37].

The goal of focal macular laser photocoagulation is preservation of VA and prevention of severe VA loss (≥15 ETDRS letters, or 3 Snellen lines of VA) over the long term. Visual acuity gains from focal laser treatment are frequently modest with most studies reporting that 40% of eyes gain between 0 and 5 ETDRS letters over a two-year period [38–41].

5. Pharmacological DME Treatment Paradigms

Corticosteroids were the first pharmacologic intravitreal treatment to be used for DME. Corticosteroids reduce vascular permeability of the retina; while their exact mechanism of action is not completely understood, they reduce production of arachidonic acid derivatives such as prostaglandins as well as inhibiting ICAM-1, TNF-α, and VEGF [3, 11, 37].

Triamcinolone acetonide has been the most widely used and studied corticosteroid in the treatment of DME [39, 42–44]. More recently, other formulations of corticosteroids have been studied and found to be effective in the reduction of DME, including a biodegradable dexamethasone implant (Ozurdex; Allergan, Irvine, CA), a time-released nonbioerodible surgically implantable reservoir of fluocinolone (Retisert; Bausch & Lomb, Rochester, NY), and a non-bioerodible injectable fluocinolone polymer (Iluvien; Alimera Sciences, Alpharetta, GA) [45–49]. None of the corticosteroids mentioned are currently Food and Drug Administration (FDA) approved for the treatment of DME. Table 2 lists the results of the major studies evaluating corticosteroids for the treatment of DME [39, 43, 46–48, 50].

Intravitreal triamcinolone acetonide has been used for the treatment of DME for a number of years. The effects are often short-lived, requiring frequent retreatment with the main side effects being cataract and glaucoma. In eyes with DME, use of both 2 mg and 4 mg doses resulted in over 50%
Table 2: Summary of major studies evaluating corticosteroids for DME.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study name</th>
<th>Follow-up</th>
<th>Type of DME</th>
<th>Type of study</th>
<th>Study methodology</th>
<th>Number of treatments</th>
<th>Mean ETDRS letter gains</th>
<th>Number of eyes</th>
</tr>
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<tbody>
<tr>
<td>[39]</td>
<td>DRCR protocol B: triamcinolone versus laser</td>
<td>36 months</td>
<td>CMT OCT ≥ 250 μm ciDME</td>
<td>Prospective, multicenter</td>
<td>Laser alone 1 mg triamcinolone 4 mg triamcinolone</td>
<td>3.1 4.2 4.1</td>
<td>5 0 0</td>
<td>115 93 98</td>
</tr>
<tr>
<td>[43]</td>
<td>Triamcinolone versus placebo for refractory DME</td>
<td>24 months</td>
<td>ciDME after ≥ 1 previous laser treatment</td>
<td>Prospective, multicenter</td>
<td>Placebo (sham IVI) 4 mg Triamcinolone</td>
<td>N/A 2.6</td>
<td>−2.9 3.1</td>
<td>29 31</td>
</tr>
<tr>
<td>[46]</td>
<td>Intravitreal implant for DME (Retisert)</td>
<td>36 months</td>
<td>CSME after ≥ 1 previous laser</td>
<td>Prospective, multicenter, Phase 2</td>
<td>0.59 mg flucinolone acetonide surgical implant Standard of care (observation or laser)</td>
<td>1</td>
<td>31% ≥ 15 letter gain</td>
<td>127</td>
</tr>
<tr>
<td>[47]</td>
<td>** FAME *(Iluvien)</td>
<td>36 months</td>
<td>CMT OCT ≥ 250 μm after ≥ 1 previous laser</td>
<td>Prospective, multicenter</td>
<td>0.5 μg flucinolone acetonide intravitreal insert 0.2 μg flucinolone acetonide intravitreal insert 1.3 IVI; ≥3 laser in 3.3% 1.2 IVI; ≥3 laser in 6.6%</td>
<td>1 1.3 1.2</td>
<td>7.1 8.1</td>
<td>270 276</td>
</tr>
<tr>
<td>[49]</td>
<td>*** Dexamethasone Drug</td>
<td>6 months</td>
<td>CSME after ≥ 1 previous laser</td>
<td>Prospective, multicenter, Phase 2</td>
<td>700 μg dexamethasone surgical implant 350 μg dexamethasone surgical implant</td>
<td>1 1</td>
<td>33.3% ≥ 10 letter gain 21.1% ≥ 10 letter gain</td>
<td>57 57</td>
</tr>
<tr>
<td>[50]</td>
<td>Dexamethasone drug Delivery system in vitrectomized patients</td>
<td>6 months</td>
<td>CMT OCT ≥ 275 μm with history of vitrectomy</td>
<td>Prospective, multicenter, Phase 2</td>
<td>0.7 mg dexamethasone IVI</td>
<td>1</td>
<td>3</td>
<td>56</td>
</tr>
</tbody>
</table>

*IVI: intravitreal injection.
**Specific number of laser treatments not stated.
***Specific letter gains not stated.
∧Trade name of medication used is indicated in parentheses ( ).
∧∧Primary endpoint was day 90 and 10 letter gain.
of eyes gaining ≥10 ETDRS letters (2 lines of Snellen VA), with the effects lasting for 16 and 20 weeks, respectively [42]. In 2-year follow-up of eyes with DME refractory to macular laser, eyes that received 4 mg of intravitreal triamcinolone acetonide gained 3.1 ETDRS letters compared to a loss of 2.9 ETDRS letters in the placebo group [43]. When comparing 2-year VA outcomes of focal macular laser alone to 1 mg versus 4 mg intravitreal injections of triamcinolone acetonide, it was found that laser was superior. Eyes treated with macular laser photocoagulation gained a mean of 2 ETDRS letters compared to a loss of 2 and 4 ETDRS letters in the 1 mg and 4 mg triamcinolone groups, respectively. At 3 years, the laser only group continued to fare better with a gain of 5 ETDRS letters compared to a 0 letter gain in both 1 and 4 mg triamcinolone groups [39, 44].

A Phase 2 clinical trial evaluating the safety and efficacy of a 0.59 mg surgically implanted fluocinolone acetonide intravitreal implant (Retisert) in eyes with DME found that VA gains of ≥15 ETDRS letters occurred in 16.8% of implanted eyes at 6 months and 31.1% of eyes at 3 years, compared to 1.4% at 6 months and 20% at 3 years in the macular laser group. The results were significant at the 6 month time point (P = 0.002) but not at 3 years (P = 0.16). The incidence of elevated intraocular pressure and cataract formation was much higher in eyes receiving the implant with 33.8% requiring incisional glaucoma surgery and 91% requiring cataract extraction compared to 0% and 20% in the standard of care group (observation or laser), respectively. Retisert is FDA approved for use in chronic, noninfectious uveitis [46].

A Phase 3 clinical trial evaluating the efficacy and safety of an intravitreally injected fluocinolone acetonide insert (Iluvien) in eyes with DME at low (0.2 μg/d) and high (0.5 μg/d) doses found VA gains at 3-years of ≥15 ETDRS letters in 33% and 31.9% of study eyes, respectively, while 21% of eyes in the sham injection group had a ≥15 ETDRS letter gain at 3 years (P = 0.030). Of treated eyes, 26% required more than one treatment over the 3 year period. Cataract surgery was required in 83.8% of eyes in the treatment groups compared to 27.3% in the sham group. The incidence of elevated intraocular pressure was much higher in the treatment groups with 4.8% (low dose) and 8.1% (high dose) of eyes requiring incisional glaucoma surgery compared to 0.5% in the sham group [47, 48]. While the 0.2 μg/d dose of Iluvien is approved for use in many European countries (Austria, the United Kingdom, Portugal, France, Germany and Spain), it has yet to be approved for use in the United States.

A Phase 2 clinical trial evaluating the efficacy and safety of a surgically implanted intravitreal dexamethasone delivery system in eyes with DME found that a 700 μg dose resulted in VA gains of ≥10 ETDRS letters at 90 days after implantation in 33.3% of eyes and 30% of eyes at 180 days. In the 350 μg group, ≥10 ETDRS letter gains were seen in 21.1% and 19% at 90 and 180 days after implantation, respectively. In the control (observation) group, ≥10 ETDRS letter gains were seen in 12.3% and 23% of eyes at 90 and 180 days, respectively. The only statistically significant difference between treatment versus control groups at day 90 was in the 700 μg treatment group (P = 0.007). There was no significant increase in cataract development between treatment and control groups. The treatment group did have a higher incidence of elevated intraocular pressure compared to the control group, but no incisional glaucoma surgery was required in any eyes study [49]. A Phase 3 study of an injectable form of this biodegradable implant (Ozurdex) is currently ongoing.

VEGF-A is believed to be one of the major mediating factors associated with the development of DR and DME. VEGF is a proinflammatory mediator and plays a pivotal role in vascular permeability. It is well known that VEGF levels are higher in diabetic eyes than in normal eyes [51]. At present, there are 4 medications available that target VEGF-A: pegaptanib (Macugen; Eyetech Pharmaceuticals, Palm Beach Gardens, FL, USA), bevacizumab (Avastin; Genentech, San Francisco, CA, US), ranibizumab (Lucentis; Genentech, San Francisco, CA, US), and aflibercept (Eylea; Regeneron, Tarrytown, NY) [40, 52, 53]. Table 3 lists the results of the major studies evaluating anti-VEGF agents for the treatment of DME [40, 41, 53–59].

Pegaptanib, a pegylated aptamer that targets the VEGF-165 isoform, when administered intravitreally every 6 weeks was found to be more efficacious than macular laser at 24 months, with ETDRS letter gains of 6.1 and 1.3, respectively [52]. Intravitreal bevacizumab, a full-length recombinant humanized antibody against all isoforms of VEGF-A, was found to be more effective than macular laser for persistent cDME at 24 months, with ETDRS letter gains of 8.5 and −0.5, respectively [40]. Neither pegaptanib nor bevacizumab is approved by the FDA for the treatment of DME though bevacizumab is widely used for this indication. Pegaptanib is FDA approved for the treatment of neovascular age-related macular degeneration (AMD).

In August 2012, ranibizumab, a recombinant humanized monoclonal antibody fragment that binds all isoforms of VEGF-A, was approved by the FDA for the treatment of DME at the 0.3 mg dose, administered monthly via intravitreal injection. Treatment with ranibizumab resulted in over 39% of eyes with visually significant DME gaining ≥15 ETDRS letters or more of vision compared to only 18% of control eyes (which were eligible for macular laser photoagulation based on protocol specific criteria). The overall gain in VA with monthly ranibizumab injections was 10.9 and 12 ETDRS letters in the 0.3 mg and 0.5 mg groups, respectively, compared to a 2.3 letter gain in the control group. Individuals with a hemoglobin A1C level ≤8 had a higher likelihood of a ≥15 letter gain than individuals with higher hemoglobin A1C levels. Results were sustained for 24 months with continued treatment [53].

The most recent anti-VEGF agent which has been introduced is aflibercept, previously known as the VEGF-Trap-Eye and is currently approved in the USA for the treatment of neovascular AMD and macular edema secondary to central retinal venous obstruction. Aflibercept binds both VEGF-A and placental growth factors 1 and 2, is delivered via intravitreal injection and is currently under study for the treatment of DME. Initial one year results demonstrate that over 40% of eyes with visually significant DME gained at least 3 lines of vision compared to 11.4% in the macular laser control group [58].
<table>
<thead>
<tr>
<th>Reference</th>
<th>Study name</th>
<th>Follow-up</th>
<th>Type of DME</th>
<th>Type of study</th>
<th>Study methodology</th>
<th>Number of treatments</th>
<th>Mean ETDRS letter gains</th>
<th>Number of eyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>[53]</td>
<td>RIDE</td>
<td>24 months</td>
<td>CMT OCT ≥ 275 µm</td>
<td>Prospective, multicenter, Phase 3</td>
<td>Sham 0.3 mg lucentis</td>
<td>1.6 laser 20.5 IVI; 0.7 laser</td>
<td>2.3 10.9</td>
<td>130 125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 mg lucentis</td>
<td>21.9 IVI; 0.3 laser</td>
<td>12 127</td>
<td></td>
</tr>
<tr>
<td>Note: rescue laser after month 3</td>
<td></td>
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</tr>
<tr>
<td>[53]</td>
<td>RISE</td>
<td>24 months</td>
<td>CMT OCT ≥ 275 µm</td>
<td>Prospective, multicenter, Phase 3</td>
<td>Sham 0.3 mg lucentis</td>
<td>1.8 laser 21.5 IVI; 0.8 laser</td>
<td>2.6 12.5</td>
<td>127 125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 mg lucentis</td>
<td>20.9 IVI; 0.8 laser</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>Note: rescue laser after month 3</td>
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</tr>
<tr>
<td>[54]</td>
<td>RESTORE</td>
<td>12 months</td>
<td>fDME and dDME</td>
<td>Prospective, multicenter, Phase 3</td>
<td>lucentis + sham laser</td>
<td>7 IVI</td>
<td>6.1</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lucentis + laser</td>
<td>6.8 IVI; 1.7 laser</td>
<td>5.9</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sham lucentis + laser</td>
<td>2.1 laser</td>
<td>0.8</td>
<td>111</td>
</tr>
<tr>
<td>[55]</td>
<td>READ-2</td>
<td>6 months</td>
<td>CMT OCT ≥ 250 µm</td>
<td>Prospective, multicenter, Phase 2</td>
<td>Lucentis alone</td>
<td>4</td>
<td>7.2</td>
<td>42</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>dDME and fDME</td>
<td></td>
<td>Laser alone</td>
<td>1.8</td>
<td>−0.4</td>
<td>42</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lucentis + laser</td>
<td>2 IVI; 2 laser</td>
<td>3.8</td>
<td>42</td>
</tr>
<tr>
<td>[56]</td>
<td>READ-2</td>
<td>24 months</td>
<td>CMT OCT ≥ 250 µm</td>
<td>Prospective, multicenter, Phase 2</td>
<td>Lucentis alone</td>
<td>9.3</td>
<td>7.7</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dDME and fDME</td>
<td></td>
<td>Laser alone; delayed lucentis</td>
<td>4.4 IVI; 1.8 laser</td>
<td>5.1</td>
<td>34</td>
</tr>
<tr>
<td>+18 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lucentis + laser</td>
<td>4.9 IVI; 2 laser</td>
<td>6.8</td>
<td>34</td>
</tr>
<tr>
<td>[57]</td>
<td>RESOLVE</td>
<td>12 months</td>
<td>CMT OCT ≥ 300 µm</td>
<td>Prospective, multicenter, phase 2</td>
<td>Lucentis Sham (no medication injected)</td>
<td>10.2</td>
<td>10.3</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Note: rescue laser for both groups</td>
<td></td>
<td>8.9 (sham treatments)</td>
<td>−1.4</td>
<td>49</td>
</tr>
<tr>
<td>[58]</td>
<td>DA-VINCI</td>
<td>12 months</td>
<td>CMT OCT ≥ 250 µm</td>
<td>Prospective, multicenter, Phase 2</td>
<td>Eylea (all arms combined)</td>
<td>9.3 IVI; 0.7 laser</td>
<td>9.7 to 13.1</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Laser alone</td>
<td>2.5</td>
<td>−1.3</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Note: rescue laser after month 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[59]</td>
<td>DRCR Protocol I: lucentis versus prompt or deferred laser</td>
<td>36 months</td>
<td>CMT OCT ≥ 250 µm</td>
<td>Prospective, multicenter</td>
<td>0.5 mg lucentis + prompt laser</td>
<td>12 IVI; ≥ 1 laser, 100%</td>
<td>6.8</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 mg lucentis + deferred laser</td>
<td>15 IVI; ≥ 1 laser, 46%</td>
<td>9.7</td>
<td>147</td>
</tr>
</tbody>
</table>
Given the results from studies with both corticosteroids and anti-VEGF agents, the goal in treatment of DME is now preservation and improvement in VA instead of just maintenance or reduction in the amount of vision loss as was the case with macular laser photocoagulation, the previous standard of care.

6. Combination Therapy for DME

Intravitreal pharmacotherapy has replaced macular laser photocoagulation as the gold standard in the care of DME. While it is quite successful in preventing vision loss from DME, and allowing for a significant number of people to realize a gain in VA, the burden of monthly intravitreal injections can become quite an encumbrance for patients, physicians, and the healthcare system as a whole due to high costs of medications, multiple physician visits, and potential complications from an invasive procedure. This has prompted studies to evaluate if combination therapies with both laser and intravitreal injections can be more efficacious than either treatment alone or if combination therapy allows for fewer treatments while maintaining VA gains. A large prospective, randomized, double-blinded study conducted by the Diabetic Retinopathy Clinical Research Network (DRCR) sought to answer this specific question. Eyes with DME were treated with focal macular laser photocoagulation alone, 0.5 mg of monthly ranibizumab + prompt focal macular laser, 0.5 mg of monthly ranibizumab + deferred focal macular laser (after week 24), or 4 mg of quarterly triamcinolone acetonide + prompt focal macular laser. After the first year, intravitreal medications were only administered as needed based on clinical examination. At the end of the 2-year study, it was found that ranibizumab + deferred focal macular laser was the superior treatment algorithm for eyes with visually significant DME. In the ranibizumab + deferred laser group 28% of eyes gained ≥15 ETDRS (mean gain = 9 letters); in the ranibizumab + prompt laser group 29% of eyes gained ≥15 ETDRS letters (mean gain = 8 letters); a median of 2 and 3 ranibizumab injections were required the second year for the deferred versus prompt groups, respectively. In the laser only group, 18% of eyes gained ≥15 ETDRS letters with a mean VA gain of 3 letters. In the triamcinolone + laser group, 22% of eyes gained ≥15 ETDRS letters, with a mean VA gain of 2 letters [60].

A 2-year retrospective study evaluating bevacizumab versus bevacizumab + macular laser versus macular laser alone for eyes with DME found that the bevacizumab only group did better than the other groups with gains of 11.8 ETDRS letters compared to 8.2 and 4.8 ETDRS letter gains, respectively. There was no statistically significant difference between the bevacizumab and bevacizumab + macular laser group, but both these groups were statistically superior to the macular laser only group [61]. The retrospective nature of this study limits the conclusions that can be drawn, and the number of intravitreal treatments in the bevacizumab groups was not indicated.

Anti-VEGF agents have changed how DME is managed providing patients with significant VA gains that are sustainable with repeat injections. Combination therapy is an evolving field and further research is needed to determine how best to care for patients with DME. Given the multifactorial nature of DME, additional studies are necessary to evaluate the role of combination therapy of anti-VEGF agents with corticosteroids in an effort to alleviate the treatment burden of monthly dosing and to assess the efficacy in those individuals with persistent DME despite repeated anti-VEGF therapy. Macular laser photocoagulation still has a role in DME, particularly fDME; however, the optimal timing of when to initiate treatment needs to be further elucidated.

7. Other and Emerging Treatments for DME

The vitreous humor has been implicated as a cause of DME due to an increase in the concentration of factors affecting vascular permeability as well as the exertion of tractional forces on the macula [62]. The role of pars plana vitrectomy has been evaluated in the management of DME with mixed results with slightly more eyes gaining ≥10 ETDRS letters than losing the same amount (38 and 22%, resp.). The best outcomes were seen in eyes in which starting VA was lower and had an epiretinal membrane present prior to surgery (which was removed at the time of vitrectomy) [63, 64].

Use of pharmacologic therapy after vitrectomy in patients with persistent DME remains challenging as clearance of drugs is more rapid in vitrectomized eyes. In a retrospective study of 11 vitrectomized eyes with DME, 3 monthly injections of bevacizumab had no effect on mean VA or mean foveal thickness [65]. A single intravitreal injection of 0.7 mg dexamethasone (Ozurdex) in previously vitrectomized eyes with persistent DME demonstrated a VA gain of 6 ETDRS letters at week 8 and 3 ETDRS letters at week 26 [50]. In
a small prospective study evaluating vitrectomy + intravitreal bevacizumab and triamcinolone acetonide versus vitrectomy + intravitreal bevacizumab and triamcinolone acetonide followed by focal macular laser 2 weeks later in eyes with intractable dDME, VA gains of approximately 10 ETDRS letters were realized in both groups 1 year after treatment [66].

Due to the tractional component of the vitreous on the macula, induction of a posterior vitreous detachment (PVD) has shown some modest benefit in those with DME [67]. Ocriplasmin (Jetrea; ThromboGenics, Belgium) has been approved by the FDA for the treatment of vitreomacular adhesion and has some efficacy in inducing a PVD [67]. It is a serine protease which is injected into the vitreous and may have a beneficial role in the treatment for DME. Prospective studies to evaluate this are currently underway.

8. Conclusion

There has been an incredible advancement in the treatment of DME over the past 2 decades with the treatment paradigm changing from observation and macular laser photoacoagulation to intravitreal pharmacologic therapies of corticosteroids and anti-VEGF agents. Physician and patients are now pursuing gains in VA instead of maintenance or reduction in rate of visual loss from DME.

The future of DME has numerous treatment options available for physicians and patients to not only maintain vision but also improve and maintain sustained VA gains. The future is promising and will likely be comprised of a combination approach utilizing anti-VEGF agents, laser, and corticosteroids designed to address the multifactorial nature of the disease. Thanks to advances in our understanding and increased treatment options for DME, we are now able to better manage this condition for affected patients. While DME was often blinding in the past, we now are able to provide many of our patients with excellent and sustained vision, thereby allowing them to continue to be a part of the workforce. The future is promising, but it must be kept in mind that DM is a systemic disease and optimal glycemic and BP control are of paramount importance in both preventing and delaying the progression of both DR and DME. Communication and a team approach among primary care physicians, endocrinologists, and ophthalmologists will allow patients with DME to achieve and maintain long-term sustained VA gains.

References


Review Article

The Role of the Immune Response in Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries; with the aging population, the negative health impacts and costs of the disease will increase dramatically over the next decade. Although the exact cause of AMD is unknown, genetic studies have implicated the complement system as well as other immune responses in disease pathogenesis and severity. Furthermore, histologic studies have shown the presence of macrophages, lymphocytes, and mast cells, as well as fibroblasts, in both atrophic lesions and with retinal neovascularization. This review summarizes discussions from the fifth annual conference of the Arnold and Mabel Beckman Initiative for Macular Research by the Inflammation and Immune Response Task Force. These deliberations focused on the role of inflammatory immune responses, including complement, inflammasomes, adaptive immune responses, and para-inflammation, unanswered questions and studies to address these questions, and potential immune-related therapeutic targets for AMD.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of central vision loss in developed countries. The most recent data suggest that more than 3 million people in the United States will be affected by the disease by 2020 [1]. The disease affects the choriocapillaris, Bruch's membrane and the retinal pigment epithelium, with dysfunction and death of overlying photoreceptors. In addition to age, risk factors for the disease include both environmental and epidemiologic factors. Specific disease associations include smoking, light exposure, obesity, and race [2]. Recent genetic studies have implicated roles for the immune system, particularly abnormalities in the complement system, in disease pathogenesis, and severity. Although patients with AMD do not have signs of overt ocular inflammation, histologic studies have shown the presence of macrophages, lymphocytes, and mast cells, as well as fibroblasts, associated with both atrophic lesions and with neovascularization of the retina [3].

Importantly, the retina is a highly metabolically active tissue, with requirements to mediate photoreceptor turnover. As the retina ages, it may be less able to handle these metabolic requirements. Immunologically active deposits called drusen that contain lipids, complement, and other potentially
immune activating substances may act as additional triggers for immune responses in the eye. Other inflammatory initiators include oxidative stress and secondary mediators of inflammation such as cytokines. On the other hand, the retina performs well until late in life despite constant stress, suggesting that at least some of the inflammatory responses observed may be beneficial. Equally intriguing, although perhaps less well understood, is a renewed appreciation for the role of the adaptive immune response in the pathogenesis of AMD. Collectively, as a result of previous studies showing inflammatory cells associated with AMD and newer genetic studies implicating the innate immune system in developing the disease, there is heightened interest in studying the role of the immune response in AMD and in determining whether modulating the immune response could help treat the disease.

The extent to which innate and adaptive immune responses play roles in the pathogenesis of AMD, and the ability to target these pathways to effectively treat the disease, remains debatable. This may in part be due to the complexity of the immune response, the number of different inflammatory cell types and cytokines involved, and the kinetics of the inflammatory response. Further, it is as yet difficult to know whether immune responses are driven and controlled locally in the retina, or operate systemically, further complicating interpretations and the development of useful therapeutic approaches.

One key question, however, is whether this immune activation is always pathologic in AMD, or whether it can actually help preserve function and moderate damage at certain stages of the disease. The data support the idea that activated states confer protection. Resident CD200R myeloid cells in the retina are under tonic control by cognate interaction with CD200 [4, 5]. The tissue consequence of microglial activation is context dependent [6, 7]. For example, in photoreceptor neurodegenerative models, microglia do not contribute to the progression of disease despite being activated [8]. In more inflammatory scenarios, a recognized consequence of activated response is contributing toward immune regulation in an attempt to contain further retinal damage [9]. A chronic inflammatory state has also been identified in a number of nonocular diseases, including type 2 diabetes and cardiovascular disease. Could a low-grade immune response be helpful in some circumstances? The intriguing concept has been distilled and developed to infer that tissue stress or malfunction can induce an advantageous response, and has been referred to as para-inflammation [10]. Medzhitov hypothesized that a well-controlled “para-inflammatory” response could be beneficial by either protecting against infection or preserving function in diseased tissues. The experimental evidence and now the concept of para-inflammation have been further articulated and illuminated experimentally by Xu et al., who discuss the potential role of para-inflammation in the aging retina elsewhere [11]. Briefly, and discussed in more detail below, immune activation and recruitment of macrophages may be required to help process photoreceptor and RPE byproducts, thus controlling overt inflammation, tissue dysfunction, and cell death.

In January 2013, the fifth annual conference of the Arnold and Mabel Beckman Initiative for Macular Research was particularly focused on a common form of AMD, namely, atrophic macular degeneration. Meeting participants were divided into task groups devoted to discussing and brainstorming particular aspects of AMD, including one responsible for considering the role of inflammation and immune responses. This review arose in part from the discussions of that task group. Here, therefore, the role of immune responses in regulating or promoting tissue damage, including complement, inflammasomes, and para-inflammation, will be discussed, followed by a summary of the group’s thinking on potential research approaches and therapeutic targets.

2. The Complement System and AMD

The complement system is the most widely accepted pathogenic pathway of the immune system implicated in AMD. The genetic evidence from genome wide association studies (GWAS) and rare variant analyses indicate an overactive alternative pathway (AP). Multiple outstanding reports have detailed and reviewed this evidence at the genetic, RNA and protein levels [13, 14, 16–25]. Therefore, these data will primarily be summarized here—the underlying thesis being that excessive engagement of the alternative pathway is a key component in AMD pathogenesis.

In 2005, four GWAS demonstrated that approximately 50% of the inheritance in AMD could be accounted for by a single nucleotide polymorphism (SNP) in an exon encoding the regulator complement factor H (CFH) [26–29]. Moreover, this SNP in CFH at amino acid position 402—a tyrosine (Y) (major allele) or a histidine (H) (minor allele)—has a functional consequence. At sites of tissue injury, the risk variant 402H does not dampen the alternative pathway (AP) of complement activation as efficiently as 402Y [30–34]. While the complement system had been previously implicated in AMD [35–38], it was the GWAS-derived genetic data that cemented the relationship [26–29].

In Caucasian populations of European ancestry, the risk allele (402H) has a gene frequency of 0.3 to 0.4, and the more common allele (Y402) 0.6 to 0.7. The 402H allele is likely replacing the major one because in early life it provides a survival advantage against streptococcal infections [13, 39, 40]. Multiple bacteria and several groups of viruses impair the complement system by hijacking the host’s regulators (reviewed in [40]); for example, microbes bind CFH to their surface to inhibit complement activation. The CFH binding protein of group A beta hemolytic streptococcus has a lower affinity for 402H than for Y402. Consequently, the host’s complement system has greater activity against the pathogen if the host expresses 402H, thereby reducing the microbes’ ability to counteract the AP. CFH adheres to damaged eukaryotic cells and tissue debris via the same anionic (heparin) binding sites that microorganisms employ to attach it to their surface. Two and possibly as many as four such cellular and tissue binding sites are positioned along the linear CFH protein (Figure 1). An unintended consequence later in life of carrying 402H is that it does not bind as well as Y402 to debris in the retina. Differential binding of 402H versus
The complement hyper-inflammatory phenotype in AMD centers on the concept of an "overreaction," characterized by excessive amplification through the AP. Also, environmental factors further tip the balance in favor of more inflammation.

Multiple other CFH variants, both common and rare, influence risk of developing AMD [18, 20, 21, 53–55]. For example, the CFH 62I variant is protective, as is a 84 kb deletion of two CFH-related genes, FHR-1 and FHR-3. The simplest and most likely interpretation of these data is that these genetic changes enhance regulation of the AP by CFH. In contrast, a rare and defective CFH variant confers substantial risk (with high penetrance) for AMD [12]. This recent discovery of a rare variant in CFH with a large effect is probably just the beginning in terms of identification by targeted deep sequencing of highly penetrant mutations in regulators and components of the AP in AMD. Further, haploinsufficiency of C9 conferred a nearly 5-fold reduction in neovascular AMD in the Japanese population, where a nonsense mutation in the C9 gene is frequently found [56]. The interpretation here is that membrane attack complex is less active and thus is protective against retinal damage.

In addition to risk and protective variants of CFH and CFH-related genes, polymorphisms in AP components C3 [57, 58] and factor B [30, 58] are also associated with AMD. A consistent observation is that the protective variants result in less AP activity, whereas risk variants result in more AP activity. Genetic variants in Factor I, the protease employed by CFH to inactivate C3b, have also been associated with AMD by GWAS [59]. Taken together, these findings provide powerful evidence implicating overactivation of the AP predisposing to AMD; thus, common and rare variants in multiple members of a proinflammatory pathway of innate immunity—the AP—are associated with the same disease. Those that decrease function of the pathway are protective, and those that increase function create risk. Moreover, the variants have both independent and additive effects on the risk of developing AMD [25, 47, 48, 52, 58].

Other inhibitors of the AP include membrane cofactor protein (MCP; CD46), decay accelerating factor (DAF; CD55), and complement receptor one (CRI; CD35, the C3b/C4b or immune adherence receptor). MCP and DAF are widely expressed, whereas CRI has a more limited distribution. DAF and MCP are expressed on the cell surface, where they protect healthy cells from complement attack. MCP is expressed at a high level by RPE cells (particularly at the basal surface) and endothelial cells [24]. A decrease in MCP expression at this RPE location was observed in early AMD. CRI also has potent regulatory activity for AP C3 and C5 convertases. A surprising recent observation is CRI expression on the apical surface of RPE cells [24]. These observations concerning the expression and function of DAF, MCP, and CRI in the retina require further investigation. Although GWAS have not implicated DAF, MCP, or CRI in susceptibility to AMD, results of targeted next generation deep sequencing of these genes have not been reported.

A role for complement is further evident specifically in "wet" AMD. This severe condition is associated with choroidal neovascularization (CNV) [53], a process characterized by newly formed and leaky vessels invading the sub-retinal space. CNV is associated with fluid accumulation and retinal
detachment with loss of the underlying photoreceptors. One animal model of wet AMD is the laser-induced CNV model in rodents. The model is initiated by argon laser photocoagulation, which ruptures Bruch’s membrane and triggers complement activation [60]. In mice, the key role of the complement system in the development of CNV is well established. Using knockout and specific inhibitor approaches, it appears that the alternative pathway of complement is the key driver of CNV, in that the removing of the classical or lectin pathway has no protective effect [61, 62]. However, the alternative pathway alone is not sufficient to drive CNV, confirming its importance in the amplification loop [61]. With regard to effector functions, the anaphylatoxins C3a and C5a [60] are important in developing injury. In addition, the membrane attack complex (MAC) contributes to the development of CNV, as CD59−/− mice lacking the MAC regulator CD59 develop CNV at a higher level than control mice [63], and treatment with recombinant soluble CD59a-IgG2a fusion-protein [64] or gene therapy expressing soluble CD59 [65] both reduce CNV. The CNV model has also been successfully treated with the targeted murine CR2-factor H (muCR2-fH) protein, which consists of a domain which directs the regulatory domain of CFH to sites of complement activation [66], as demonstrated by systemic administration and evaluation of local CNV development [67]. Importantly, in each model evaluated, complement activation amplifies the generation of vascular endothelial growth factor (VEGF), which is strongly implicated in fueling the development of CNV and AMD [68].

3. Inflammasome Activation in AMD

The maintenance of the delicate balance between self and nonself regulates cellular homeostasis. However, during the aging process this system may be more vulnerable to a variety of noxious challenges that may activate host defense systems. The inflammasome is responsible for activation of many inflammatory processes. The inflammasome is a multiprotein complex, comprising of a sensor protein, the adaptor protein ASC (apoptosis-associated speck-like domain containing a caspase recruitment domain), and the inflammatory protease caspase-1. The assembly of the inflammasome signaling platform occurs due to conformational changes in the sensor protein, which in turn recruits caspase-1 to the complex and subsequently promotes the activation of caspase-1. Once activated, caspase-1 cleaves the inactive precursors of two proinflammatory cytokines, interleukin 1β (IL-1β) and IL-18, thereby generating mature forms which are then secreted from cells [69]. The inflammasome forming sensors are different receptor molecules, such as nucleotide-binding domain and leucine-rich repeat containing family pyrin (NLRP), which belong to the Nod-like receptor family of proteins. These include NLRP1, NLRP3, and NLRC4; or Absent In Melanoma (AIM 2), a receptor of the HIN (IFN-inducible nuclear proteins) family of proteins [70]. A growing body of evidence suggests that the NLRP3 inflammasome is clearly involved in host defense and autoinflammatory conditions, and is an integrator of cell damage and stress signals [71].

Activation of IL-1β by an inflammasome is required to efficiently control viral, bacterial, and fungal pathogen infections. However, excess IL-1β activity contributes to a variety of diseases [72]. The NLRP3 inflammasome has been shown to play a central role in the pathogenesis of autoinflammatory disorders; its activity has also been implicated in diseases such as Alzheimer’s disease, cancer, type II diabetes, and most recently AMD [71, 73, 74]. The classic pathology of AMD is multiple small or intermediate drusen in the macular area. In a recent study, drusen isolated from donor AMD eyes were shown to activate NLRP3 inflammasome, causing secretion of IL-1β and IL-18 [73]. The authors postulated that NLRP3 may be a sensor for drusen-induced inflammasomes, as NLRP3 has been shown previously to act as a receptor for “danger” signals such as amyloid-like structures. Because laser-induced CNV was considerably greater in NLRP3 knockout mice, but not IL-1R knockout mice, NLRP3 and IL-18 may have a protective role in the progression of AMD [73]. Further, CEP (carboxyethylpyrrole), a biomarker of AMD, was thought to prime the inflammasome. Interestingly, while CEP another complement component known to contribute to the inflammation and the pathophysiology of AMD [61], can also act as a danger signal that is, sensed by the NLRP3 inflammasome [75], CEP knockout mice develop CNV of similar size to control mice [61]. In addition, a recent study reported that lysosomal destabilization can activate the NLRP3 inflammasome in RPE cells [76].

Regulation of the NLRP3 inflammasome is poorly understood but probably involves the integration of signals from a number of stimuli, such as cellular damage and stress. It is now appreciated that inflammasome-dependent biological effects may be mediated not only by IL-1β and IL-18, but also by the multifaceted activities of caspase-1. Therefore, it is important to determine the mechanisms by which inflammasomes in RPE cells directly or indirectly modulate IL-1β activity that may lead to AMD. In chronically stressed states, where autophagy is increased, there may be secondary effects of protecting against inflammasome activation [77, 78]. Further understanding in context of drusen and RPE behavior may provide pathways to interrogate to maintain RPE function and health and attenuate inflammatory activation. Future studies to better understand how inflammasomes may be activated in AMD, and the molecular mechanisms involved in the assembly of the inflammasome signaling platform, may therefore lead to the development of novel therapeutic approaches for AMD.

4. Para-Inflammation in AMD

Inflammation, both acute and chronic, functions to control danger signals or to respond to pathogens to safeguard a host and maintain tissue health. Disturbances of homeostasis (e.g., infection, tissue injury, foreign bodies, but may also include stresses from aging) trigger inflammatory responses, the purpose of which are to remove or sequester the source of the disturbance and to allow the host to adapt to the abnormal conditions and return to a state of homeostasis. However, the spectrum of inflammation is broad. When appropriate,
inflammation can be both adaptive and protective. Conversely, the immune response also has significant pathological potential and can promote tissue damage and facilitate disease progression.

Medzhitov first introduced the idea of para-inflammation as a tissue adaptive response to noxious stress or malfunction that has characteristics intermediate between basal and inflammatory states [10]. Briefly, in the basal state, tissue-resident macrophages (principally retinal microglia and retinal perivascular macrophages or choroidal macrophages) may play a role to promote an adaptive change with short-term benefits, promoting tissue homeostasis. However, if the abnormal conditions are sustained, or if the tissue receives a “danger signal,” this can result in immune cell infiltration, which in turn can become maladaptive. Para-inflammation has characteristics that are intermediate between basal and inflammatory states. The purpose of normal para-inflammation is presumably to maintain tissue homeostasis and to restore tissue function. Nonetheless, if a tissue is exposed to prolonged stress or malfunction, para-inflammation can become chronic and promote disease progression. Dysregulated para-inflammation has been proposed to play an important role in the progression of diabetes, atherosclerosis, and obesity.

Similarly, dysregulated para-inflammation, which is especially relevant in aging tissues dependent on nonproliferative cells and characterized by very high metabolism and other oxidative stress (e.g., the macula), has also been postulated to contribute to the development of AMD [11]. In the aging retina, oxidized lipoproteins and free radicals are major causes of tissue stress and serve as local triggers for retinal para-inflammation. Para-inflammatory responses in the neuroretina may be reflected in microglial activation and subretinal migration, and (potentially) breakdown of blood-retinal barrier. At the retinal/choroidal interface, para-inflammation manifests as complement activation in Bruch’s membrane and RPE cells, and accumulation of microglia (and myeloid cells that have recently immigrated) in the subretinal space. In the choroid, para-inflammation may be characterized by increased thickness of choroid, increased macrophages, morphological abnormalities in choroidal melanocytes, mast cell activation and fibrosis.

Recent evidence, derived from the cybrid models of mitochondrial haplotypes into a mitochondrial DNA-null RPE cell line (ARPE19), showed that mitochondrial dysfunction may promote the progression and AMD [79]. The observed distinct polarization of energy cellular energy source and production suggest an approach with promise in further interrogating the influence on immune responses, including para-inflammation. The notion is that switching energy sources, which may be dependent on haplotype, influences the signaling pathways and thus phenotype of any subsequent immune activation of the cell. Further studies may increase our understanding of potential switch of energy sourcing, and the influence on immune activation of RPE that in turn will direct immune responses in cells (i.e., macrophages and choroidal mast cells) to deliver a trigger for progression of disease.

5. Adaptive Immunity in AMD

The role of adaptive immunity in AMD has received increasing attention. Whether adaptive immune responses relay pathogenic or regulatory functions, or are simply bystander effects, remains elusive. In support, there have been numerous reports suggesting involvement due to finding of autoantibodies in AMD patients, not least with the detection of anti-retinal autoantibodies [80, 81]. Whether they have a role as potential pathogenic mediators, or occur as bystanders, it remains to be determined if autoantibodies can act as a prognosticator or biomarker in AMD patients [82]. The search has been driven further with utilization of serum antigen arrays and 2-D gel electrophoresis. Specific targets such as RPB-3, aldolase C and pyruvate kinase IgG have been derived, and altered IgG/IgM ratios of anti-phosphatidylserine are associated with patients with AMD [83, 84]. Autoantibodies have been observed even when investigating responses to complement regulators, such as CFH [85]. The latter finding is enticing in that autoantibodies to CFH were unexpectedly lower in AMD patients, inferring a protective effect. Nevertheless, together there is increasing evidence of the presence of autoantibodies in AMD. The spectrum suggests secondary effects, and indeed also infers the potential of adaptive immune engagement. Consequently further searches for autantibodies, albeit possibly in only a small subset of patients, may be justified to determine whether there is a prevalent autoantibody signature.

More compelling data arises from mouse work. The data from Hollyfield et al. [43, 86] demonstrated that carboxyethylpyrrole (CEP) is present in AMD eye tissue, and mice immunized with this added oxidized product generated antibodies and exhibited pathology with some similarities with human AMD. Moreover, in experiments in RAG-deficient animals which lack B and T cells, no anti-CEP antibody was detected. Given the cell infiltrate noted around lesions, both T cell engagement and complement fixation were thought to contribute in this model to the loss of RPE and photoreceptors, and thus progression of AMD.

Most recently, novel observations of cytokine and T cell signatures from AMD patients have been published. First, an intriguing increase in IL-22 and IL-17 levels in serum from AMD patients was shown, supported by the further finding that C5a stimulated IL-22 and IL-17 from T cells [87]. Second, studies of twins and siblings found that the IL-17RC promoter is hypomethylated in AMD patients [88], further suggesting the involvement of adaptive immunity and TH17 cells, as well as potential effect on macrophages. Consequently, a testable hypothesis is that autoantibodies are present early in subsets of AMD patients, and are pathogenic. The notion that autoantibodies may create further complement-mediated damage, or activate myeloid cells to switch from protective para-inflamatory to pathogenic responses, may also be tested. Generation of autoantibodies (i.e., engagement of adaptive immune responses that are pathogenic) may tip the balance from para-inflammatory control, and create an environment that induces further loss of cells, angiogenesis, and an unremitting walk to late stage AMD.
6. Summary of BIMR Conference Discussions

Recent data suggest that dysregulation of immune response could contribute to the pathogenesis of AMD. However, there are a number of questions that remain unanswered. First, if para-inflammation is involved in the pathogenesis of AMD, when and how does a dysregulation of the immune response change from a protective role to a harmful process? Second, although genetic studies point to a role for the complement system and innate immunity in AMD, what role if any does adaptive immunity play in the disease? The group discussed a number of experimental approaches that could help address these questions.

6.1. Human Tissue Studies. Existing tissue banks may be used to interrogate immune response in AMD, with a particular focus on early events. Diseased and fellow eye tissue might first be graded using an established system \[89\], and then comprehensively characterized in terms of inflammatory cell contents and patterns, presence of complement and autoantibodies, and gene and protein expression profiles. It may be especially useful to compare tissue from different areas of an individual retina (e.g., in the fovea, adjacent to drusen, and “normal” tissue away from drusen).

6.2. Retrospective Clinical Studies. Existing medical records from large databases, such as those available from Medicare in the USA or anti-TNF-\(\alpha\) treatment registries in the UK, could be mined to gain insight into selected questions. For example, do patients on immunosuppressive therapies for rheumatologic diseases have a lower prevalence of AMD?

6.3. Prospective Clinical Trials. There are a number of randomized clinical trials examining immunomodulation as a therapy for retinal diseases including AMD. Results from these trials will help guide our knowledge about the role of the immune system in the disease. Examples of such informative trials include prior trials studying treatment with immunomodulators in other diseases, and trials in AMD using immunomodulators such as those that target C5 and other complement components, mTOR, or TNF-\(\alpha\).

6.4. Biomarkers. Identification of direct and/or surrogate biomarkers that are predictive or prognostic for disease susceptibility, disease progression, or treatment response will be beneficial to the study of AMD. Serum, plasma, PBLs, platelets, and aqueous humor could be obtained from patients enrolled in natural history studies, and at the same time, patients encouraged to consent to eventually donate eyes. Samples could be used to assess complement components, cytokines, carboxyethylpyrrole (CEP), and autoantibodies. Collectively, such studies may yield clinical/pathological correlations and genotype/phenotype relationships in individual patients.

6.5. Imaging Modalities. The development of new imaging modalities can detect the trafficking and function of immune cells in the retina and choroid (which may be transient). These tools, if designed to provide quantitative analyses of immune system functions such as the presence in the human eye of ongoing complement activation or specific cellular infiltration could then be applied to both direct imaging studies, particularly immunomodulatory treatments and bioenergetic evaluations, and to studies using transfer of ex vivo labeled cells.

6.6. Animal Models. Although there is no perfect animal model for AMD, preclinical models that reproduce specific aspects of early AMD (e.g., drusen, low grade chronic inflammation, and GA) need to be developed to ask specific questions about the role of inflammation, and to probe specific disease mechanisms. Although eyes in mice do not have macular structures and do have a distinct RPE morphology, an example of a model for para-inflammation may include deliberately inducing low grade inflammation in ob/ob or senescent mice, followed by addition of a systemic insult (e.g., light toxicity) to reproduce the hypothesized dysregulation of para-inflammation.

6.7. In Vitro Cell Biology Studies. Finally, there is interest in studies designed question whether changes in aging RPE and photoreceptors make them more susceptible to damage by dysregulated para-inflammation in AMD. For example, in vitro cultures of photoreceptors could be used to evaluate early changes in rods in AMD, or resistance to injury by cones in AMD. Cultures of RPE could be used to examine metabolic dysfunctions (e.g., mitochondrial dysfunction, haplotypes), and the responses of retinal and choroidal cells to cytokines released by such metabolic change. Of particular interest are epigenetic changes in such cells that may promote or protect cells in aging and AMD.

7. Conclusions

The prevalence of AMD will continue to increase as the population ages. Although we do not know the exact etiology of the disease, recent genetic studies have implicated the complement system in disease pathogenesis and severity. Other studies further support the hypothesis that the immune system is involved in the disease, in concert with or in addition to other factors such as environmental conditions and products of photooxidation (Figure 2). Importantly, understanding how immune responses initiate or exacerbate AMD will allow us to identify novel therapeutic approaches to the disease.

Conflict of Interests

Scott M. Whitcup is an employee of Allergan, Inc. John P. Atkinson is funded by Alexion Pharmaceuticals, and Bärbel Rohrer has received royalty payments as well as a sponsored research Grant from Alexion Pharmaceuticals. Michael Holers has received royalty payments from Taligent Therapeutics and Alexion Pharmaceuticals and has received sponsored research grants and served as a consultant to both companies.
**Putative RPE damaging agents**

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**Multifaceted pathogenesis of AMD**

- Excessive activation of alternative complement pathway, and of other proinflammatory pathways in patients carrying genetic variants

- Excessive retinal inflammation, in response to injury

- AMD

**Figure 2:** An integrated model of immune regulation of AMD.

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References


Inflammation in Retinal Vein Occlusion

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Retinal vein occlusion is a common, vision-threatening vascular disorder. The role of inflammation in the pathogenesis and clinical consequences of retinal vein occlusion is a topic of growing interest. It has long been recognized that systemic inflammatory disorders, such as autoimmune disease, are a significant risk factor for this condition. A number of more recent laboratory and clinical studies have begun to elucidate the role inflammation may play in the molecular pathways responsible for the vision-impairing consequences of retinal vein occlusion, such as macular edema. This improved understanding of the role of inflammation in retinal vein occlusion has allowed the development of new treatments for the disorder, with additional therapeutic targets and strategies to be identified as our understanding of the topic increases.

1. Introduction

Retinal vein occlusions (RVOs) are the second most common visually disabling disease affecting the retina, after diabetic retinopathy [1]. Obstruction of retinal venous flow leads to damage of the vasculature, hemorrhage, and tissue ischemia [2]. Occlusions affecting the central retinal vein, or central retinal vein occlusion (CRVO), affect the entire retina, while those affecting lesser tributaries of the venous circulation, the so-called branch retinal vein occlusion (BRVO), affect a portion of the retina. Despite the fact that the disease entity has been known to exist for over 100 years, current treatment options often still leave patients with clinically problematic visual disturbances and overall increased morbidity. RVO generally affects patients in middle age and the elderly population [2], and several studies have identified systemic risk factors, such as hypertension, diabetes, systemic vascular disease, glaucoma, and hypercoagulable states [3, 4].

Although proliferative vascular changes can cause significant morbidity (particularly due to subsequent vitreous hemorrhage and neovascular glaucoma), the main reason for decreased visual acuity in both CRVO and BRVO is macular edema [5]. As a result, elucidation of the causes of, as well as treatment for, macular edema has been at the center of large-scale studies on patients with RVO. While the causes for RVO are multifactorial, with local and systemic factors being identified as etiologic, most of the literature generally implicates vascular and inflammatory mediators as being particularly salient [6–8]. Prior to the advent of intravitreal drug delivery, treatment for macular edema for CRVO and BRVO was observation and grid laser photocoagulation, respectively, the latter of which resolved macular edema slowly even under optimal circumstances [9]. The subsequent creation of intravitreal medicines that block vascular endothelial growth factor (VEGF) and the intravitreal delivery of corticosteroids for RVO has led to better clinical outcomes overall [10]. While the focus of much of the literature is currently on the role of anti-VEGF medications in the treatment of RVO, the role of inflammation in both pathogenesis and treatment of RVO is equally exigent.

2. Pathogenesis of Inflammation in RVO

Both systemic and local inflammations have been hypothesized to play a significant role in the etiology of RVO. The predisposing systemic risk factors for RVO include hypertension, diabetes, dyslipidemia, and elevated plasma levels of homocysteine [11–13]. Atherosclerosis, a chronic, low-grade inflammatory condition, has been studied extensively in relation to RVO. Indeed, the systemic risk factors that predispose patients to RVO are also independently associated with
atherosclerosis [11, 13]. The initial pathological findings of this condition are composed of monocyte-derived macrophages and T-lymphocytes (purely inflammatory lesions) which later progress to thrombus and clot formation [14]. Results pertaining to the hypothesis of atherosclerosis as a risk factor for RVO have been mixed. Large population-based cross-sectional studies have found that, while the prevalence of RVO is fairly similar across ethnic groups, atherosclerotic disease and markers of inflammation, such as C-reactive protein, were not associated with the disease [15]. In addition, certain genetic polymorphisms that had been previously implicated in atherogenesis, inflammation, and coagulation did not show association with BRVO or CRVO [16, 17]. However, other reports have shown potential links between atherosclerosis (and by extension, systemic inflammation) and RVO. In particular, recent studies have shown that patients with RVO have an increased risk of asymptomatic ipsilateral carotid artery plaques, and those with BRVO often also have decreased aortic distensibility and elasticity, a finding frequently found in patients with atherosclerosis [18, 19]. In addition, pathological studies have shown an atherosclerotic retinal artery at the lamina cribrosa in some patients with CRVO [20].

Another mechanism by which systemic inflammation is proposed to lead to RVO is through the induction of systemic hypercoagulability. Many inflammatory chemokines/cytokines are prothrombogenic; for example, interleukin-1 beta, interleukin-6, and tumor necrosis factor Alpha all simultaneously upregulate tissue factor, which is a major activator of the extrinsic coagulation cascade pathway, and downregulate tissue type plasminogen activator, which disrupts fibrinolysis [21–23]. In particular, homocysteine, a plasma element found elevated in patients with chronic inflammatory conditions, such as atherosclerosis, as well as in patients with errors of protein metabolism (homocystinemia/homocystinuria), can cause adverse systemic thrombotic events. Patients suffering from grossly elevated plasma levels of homocysteine often develop deep vein thromboses, myocardial infarctions, carotid atherosclerosis, and stroke [24]. In a similar fashion to other inflammatory-mediated processes, proposed mechanisms of thrombosis include inhibition of plasminogen activator, inhibition of protein C activation, activation of Factor V, and the inducement of endothelial cell dysfunction [25–27]. Perhaps unsurprisingly, given the strong possible link between hyperhomocysteinemia and hypercoagulation, subsequent case control studies between patients with and without CRVO have demonstrated a robust correlation between CRVO and elevated plasma levels of homocysteine [28, 29]. However, other studies have rightfully pointed out that, given that elevated levels of plasma homocysteine are found in various other chronic inflammatory states, such as atherosclerosis, the association of homocysteinemia with RVO is likely multifactorial [30].

Local inflammation within the eye has also been implicated in the pathogenesis of RVO. In vivo assessment of the vitreous fluid in patients with RVO has demonstrated elevated levels of proinflammatory mediators and lower levels of anti-inflammatory cytokines [31, 32]. In particular, in a major study on inflammatory immune mediators in a group of vitreoretinal diseases, patients with RVO had elevated levels of interleukin-6, interleukin-8, and monocyte chemoattractant protein-1, and patients with CRVO had elevated levels of VEGF, all of which are considered highly proinflammatory [33]. In follow-up studies, patients with macular edema from both BRVO and CRVO were shown to have increased levels of soluble intercellular adhesion molecule-1 (proinflammatory) and decreased levels of pigment epithelium derived factor (anti-inflammatory) [34, 35]. Unsurprisingly, the literature suggests that for larger order vessel disruptions, such as those affecting the central retinal vein or a larger branch retinal vein ("major" BRVO), there are even higher elevations and reductions of the aforementioned pro-inflammatory and anti-inflammatory cytokines, respectively, as compared to smaller branch vessel disruptions [32, 36]. Of particular note is the fact that VEGF is classified as a pro-inflammatory cytokine; while VEGF is famously known for its central role in retinal angiogenesis, recent studies have revealed its role in permitting leukocyte infiltration into the retina—a key initial step in the inflammatory pathway [37, 38].

Macular edema itself has been shown to result from prolonged inflammatory states, such as those seen in uveitis [39]. While the exact mechanism for how inflammation actually causes macular edema is still unclear, the prevailing theory includes the instigation of pro-inflammatory cytokines that subsequently damage retinal cells, particularly retinal pigment epithelial cells, which leads to fluid leakage into the retina [15]. In addition, the retinal ischemia seen with RVO has also been postulated to lead to a pro-inflammatory milieu, with the added insult of increased vascular permeability partially due to a breakdown of the blood-retinal barrier [40]. Given these conditions, treatment options for RVO preventing inflammation were developed.

3. Treatment of Inflammation in RVO

While the mainstay of treatment for systemic inflammatory states has been oral or intravenous corticosteroids, this method of administration precluded their effective use for ocular conditions given the side effect profile of long-term steroid use. In addition, topical steroids do not penetrate the posterior segment of the eye in an efficacious manner [5]. However, injecting corticosteroids directly into the vitreous cavity allows for a targeted, high dose use of the medications for ocular inflammatory conditions with a low side effect profile. Currently, the major anti-inflammatory medications in use for the treatment of RVO are intravitreal triamcinolone acetonide (IVTA) and the newly developed dexamethasone intravitreal implant.

Triamcinolone acetonide is a synthetic glucocorticoid that has a potency that is five times that of cortisol and has been reported to remain in the eye for months to years after its initial injection [41, 42]. Initial use of IVTA for treatment of CRVO resulted in significantly improved anatomical changes within the macula [8, 43, 44]. As a result, the SCORE (Standard Care versus Corticosteroid for Retinal Vein Occlusion) trial was launched by the National Eye Institute. The study consisted of two multicenter, randomized
controlled clinical trials comparing the efficacy of IVTA versus standard of care for both BRVO and CRVO [45, 46]. The SCORE-BRVO arm placed patients in cohort groups which received 1 mg of IVTA, 4 mg of IVTA, or standard of care (macular grid laser photocoagulation). The results demonstrated no difference between the three groups in terms of visual outcome; however, there was an increased incidence of adverse side effects such as glaucoma, cataract, and injection-related problems in the IVTA groups relative to the laser group [46]. Expectantly, the adverse side effects were more pronounced in patients receiving the higher dosage of IVTA. As a result, the study concluded that for BRVO, macular grid laser photocoagulation should remain the gold standard for treatment. The SCORE-CRVO arm placed patients in cohorts similar to the SCORE-BRVO arm; however, the results demonstrated that both IVTA groups were superior to observation (standard of care for CRVO) in both visual acuity and anatomic resolution of macular edema [45]. These beneficial changes occurred as early as 4 months into treatment and persisted for 24 months. The study also demonstrated a reduced incidence of adverse side effects in the 1 mg IVTA group; as a result, this dosage has been preferred by some in the treatment of CRVO.

Given the partial success of temporary intravitreal corticosteroids, a method of delivering corticosteroids in a manner that obviated the need for multiple injections was developed. The dexamethasone implant is a biodegradable copolymer of both lactic and glycolic acids with micronized dexamethasone that gradually releases the dose of the steroid over a period of months via the pars plana [5]. The GENEVA trials were two phase III trials that tested the effect of dexamethasone implants (in the 0.35 mg and 0.7 mg dosages) versus sham injections in patients with BRVO and CRVO [47, 48]. The results for the BRVO study group were mixed; while there was a trend towards better visual acuity in the dexamethasone implant groups after 6 months, there was a statistically significant improvement of acuity in the dexamethasone implant groups after 3 months. A similar finding, though less in magnitude, was seen in the CRVO group. Patients tolerated the implant well, with a minority of patients developing medically manageable glaucoma and cataract [47]. Given the results of the GENEVA trials, some advocate use of the implant for patients with a relatively short duration of macular edema [48]. Others have suggested that the dexamethasone implant may be useful for less frequent occurrences of macular edema secondary to RVO, such as those occurring in postvitrectomized eyes with CRVO, and those with long-standing BRVO and chronic edema [49, 50].

However, considering that the pathogenesis of inflammation in RVO also includes VEGF as a key mediating cytokine, the advent of intravitreal anti-VEGF medications and their role in the treatment of RVO are especially salient. Ranibizumab is a monoclonal, humanized antibody fragment that binds to all VEGF isomers. Two randomized controlled trials were established to determine the efficacy and safety of ranibizumab in the treatment of RVO: BRAVO (BRVO) and CRUISE (CRVO) [51, 52]. In both BRAVO and CRUISE studies, patients with fovea involving macular edema within the prior 12 months were given monthly ranibizumab injections of either 0.3 mg, 0.5 mg, or sham injections. In the BRAVO study, patients who were not responding to treatment were eligible to receive rescue laser photocoagulation (standard of care) after 3 months. At 6 months of treatment, patients in the ranibizumab groups in both studies had significantly higher average gains in visual acuity, significantly higher proportions of patients gaining at least 15 letters of vision, and significantly lower mean foveal thicknesses relative to the sham injection group. In addition, patients maintained this vision with continued injections through 12 months; intriguingly, patients in the sham group who were subsequently given ranibizumab injections after the 6-month period enjoyed beneficial visual and anatomic changes—however, their final visual acuities were generally less than those in the ranibizumab groups, engendering a discussion on whether there was a visual penalty resulting from a delay in treatment [53, 54]. Similarly beneficial effects in smaller studies have been noted with another anti-VEGF antibody, bevacizumab; however, many of the studies also mention a high recurrence rate and relatively short-term efficacy [55–60].

Given the beneficial treatment outcomes of both intravitreal steroid and intravitreal anti-VEGF medications, a few reports have attempted to ascertain whether a synergistic effect might exist. One study found no significant difference in outcome between patients with CRVO who only received bevacizumab versus patients who received both bevacizumab and triamcinolone [61]. Another study attempted to assess whether patients with RVO who received both bevacizumab and a dexamethasone implant (0.7 mg) had significantly better outcomes than those who received only the dexamethasone implant [62]. The patients in the combination group were given the dexamethasone implant 2 weeks after the first injection of bevacizumab. Most patients (65 percent) were being treated for BRVO. The primary outcome was the time required for reinjection based on existing OCT and visual data. While most patients gained vision, a small minority did not require a retreatment with an additional bevacizumab injection during the 6-month study. While the data suggests that there may be a synergy between anti-VEGF medications and steroids, further study is required.

4. Conclusion

RVO is a highly prevalent cause of vision loss in the world. While the causes for RVO are multifactorial, both local and systemic inflammations have been found to be highly contributory factors. Along with photocoagulation, medications that reduce the level of inflammation in the eye, specifically triamcinolone and the dexamethasone implant, have been shown to provide beneficial results for patients with certain forms of RVO. Coupled with the explosion of anti-VEGF medications, such as ranibizumab and bevacizumab, the treatment of RVO is destined to change. Further study of the role of inflammation in the pathogenesis and propagation of RVO will aid in the identification of therapeutic targets and the development of new treatment modalities for this disease.
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Research Article

Peripheral Fluorescein Angiographic Findings in Fellow Eyes of Patients with Branch Retinal Vein Occlusion

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Introduction. Branch retinal vein occlusion (BRVO) is a common retinal vascular condition that results in intraocular inflammatory changes. Ultra wide field fluorescein angiography (UWFFA) is a retinal imaging device that can capture peripheral retinal findings. The purpose of this study was to look for peripheral findings in the fellow eye of patients with BRVO using UWFFA. Methods. Retrospective imaging review of patients diagnosed with BRVO that had both eyes imaged with UWFFA. Images were graded for peripheral findings in other quadrants of the same eye as well as in all quadrants of the fellow eye. Results. Of 81 patients, 14 (17%) patients had late vascular leakage in a quadrant other than the BRVO distribution. Five (6%) findings were in the same eye, 8 (10%) findings were in the fellow eye, and 1 (1%) finding was in both the same eye and the fellow eye. Of these 14 patients, 11 (80%) patients had hypertension. Conclusion. Late peripheral retinal leakage in the fellow eye of patients with BRVO was detected in this cohort of patients with UWFFA. This novel finding may represent underlying systemic inflammation, hypertension, or bilateral BRVOs.

1. Introduction

Branch retinal vein occlusion (BRVO) affects approximately 1% of the population and can cause severe vision loss through macular edema, retinal neovascularization, and retinal detachment [1–4]. The disease is estimated to be bilateral in 5% of patients at presentation and become bilateral in 15% of patients over time [3, 5]. When bilateral and/or multiple, systemic vasculitis such as sarcoidosis, systemic lupus erythematosus, or Behcet’s disease may be the underlying etiology.

Atherosclerosis risk factors such as hypertension and hypercholesterolemia are thought to contribute to BRVO formation by causing arterial wall hardening and inflammation at arteriovenous crossing sites [6]. In theory, systemic risk factors should put patients at similar risk for BRVO in both eyes and it is unclear why the disease is typically unilateral. One explanation could be due to random and individual variation in arteriovenous crossing patterns [7].

There are several studies examining the vitreous of eyes that have suffered BRVO showing that there are increased inflammatory mediators when compared with control surgical patients. For example, vascular endothelial growth factor (VEGF), soluble intercellular adhesion molecule-1, interleukin-8, and interleukin-6 were among the inflammatory markers found to be elevated in the vitreous of BRVO eyes [8–10]. In these studies inflammatory markers were correlated to macular edema [10] and the size of BRVO [8]. However, vitreous samples were not taken before BRVO was detected nor was the vitreous of the fellow eye assessed as vitreous removal is invasive and there was no clinical indication. Therefore, it is not certain if the increase in inflammatory markers was a cause or effect of the disease.

Ultra wide field fluorescein angiography (UWFFA, Optos, Marlborough, MA, USA) is a retinal imaging technology that captures up to 200 degrees of the retina in a single picture and has been useful in detecting peripheral findings in a variety of retinal vascular diseases including diabetes, vein occlusions, and uveitis [11–13]. Prior work by our group studying peripheral retinal findings in BRVO using UWFFA found that peripheral nonperfusion and vascular leakage were detectable [11]. In a cohort of 80 patients, peripheral nonperfusion was associated with angiographic macular edema, while vascular leakage was not. The purpose of this current study was to assess peripheral findings on
fluorescein angiography in other quadrants of the same eye and in fellow eyes of patients with BRVO.

2. Materials and Methods

This retrospective imaging study was Institutional Review Board approved and carried out at the Jules Stein Eye Institute in Los Angeles, CA, USA. An imaging database was searched for patients with the diagnosis of BRVO who underwent UWFFA (Optos, Marlborough, MA, USA). Patients with another diagnosis or with concurrent other retinal diseases (i.e., age-related macular degeneration) were excluded. Patients with poorly controlled diabetes or signs of diabetic retinopathy such as intraretinal hemorrhage and venous beading were excluded. Patients with known inflammatory disorders such as sarcoidosis, systemic lupus erythematosus, and Behcet's disease were excluded.

Demographic data such as patient gender and age were collected. Significant past medical history such as hypertension, high cholesterol, and diabetes mellitus were noted. All images were reviewed with Vantage Review Software (Optos, Marlborough, MA, USA) and adjusted with zoom, gamma, and contrast to optimize image quality. The quadrant affected by a BRVO was recorded. Other quadrants of the same eye were assessed for peripheral findings. Similarly, all quadrants of the fellow eye were graded for peripheral findings.

3. Results

A total of 84 patients with BRVO were included in this study. Forty-nine (58%) patients were female and 55 (65%) eyes were OD. Average patient age was 60 years old (range 34–94 years; SD 13 years). Sixteen (19%) patients had no significant past medical history. Thirty-eight (45%) patients had hypertension only. Nineteen (23%) patients had hypertension and high cholesterol only. Five (6%) patients had hypertension and diabetes mellitus only. Four (5%) patients had hypertension, high cholesterol, and diabetes mellitus only. Two (2%) patients had diabetes mellitus only.

Thirteen (15%) BRVOs were macular and 71 (85%) were quadrantic. Of the macular BRVOs, 10 (77%) were superior and 3 (23%) were inferior. Of the quadrantic BRVOs, 40 (56%) were superotemporal, 25 (35%) were inferotemporal, 3 (4%) were inferonasal, and 1 (1%) was superonasal. Two (3%) patients had two quadrantic BRVOs in the same eye.

Three (4%) patients were known to have bilateral BRVOs at the time of presentation. Of the remaining 81 patients, 14 (17%) patients had late vascular leakage in a quadrant other than the BRVO distribution. Five (6%) findings were in the same eye, 8 (10%) findings were in the fellow eye, and 1 (1%) finding was in both the same eye and the fellow eye. The six eyes (7%) with leakage in the same eye all had leakage in a quadrant contiguous to the BRVO. Of the 14 patients with leakage in a quadrant other than the BRVO, 8 (57%) patients had hypertension only; 2 (14%) patients had hypertension, high cholesterol, and diabetes mellitus; 1 (7%) patient had hypertension and diabetes mellitus only; 1 (7%) patient had high cholesterol only; and 2 (14%) patients had no known systemic diseases.

4. Discussion

This study examined peripheral retinal changes in patients with BRVO in other quadrants of the same eye and in the fellow eye using UWFFA. In 81 patients, 14 (17%) patients had late vascular leakage in a quadrant other than the BRVO distribution. Two examples from our study are shown in Figures 1 and 2.
There are three explanations for detecting late peripheral leakage in 11% ($n = 9$) of fellow eyes. One possibility is that inflammation is not only a consequence of vein occlusion, but it is also a part of the pathogenesis of BRVO and patients with BRVO have higher levels of inflammation systemically. This hypothesis has been tested by examining gene polymorphisms related to inflammatory cytokines in BRVO patients and normal control patients [14]. In a study of almost 400 patients, 10 single nucleotide polymorphisms were assayed but were not found to be independent risk factors for BRVO suggesting that inflammation did not contribute to the pathogenesis of BRVO.

A multiethnic epidemiological study of over 6000 subjects considered the association of retinal vein occlusion (RVO) to traditional cardiovascular risk factors [1]. RVO was not associated with systemic inflammation, hematological abnormalities, or atherosclerosis but it was associated with hypertension and dyslipidemia. Given the high prevalence of hypertension in our group of patients, a second explanation for late peripheral leakage in the fellow eye is that it was a manifestation of hypertension.

A third explanation of bilateral leakage is that BRVO may be underrecognized as a bilateral disease and these areas of late peripheral leakage are actually small peripheral BRVOs that are otherwise undetectable with clinical exam and traditional imaging. This would be supported by the fact that the distribution of leakage was often in a venous distribution. Without coexisting macular edema or peripheral ischemia, small incidentally found BRVOs do not require treatment but the finding does warrant further observation to detect progression of disease.

Lastly, it is possible that these small areas of peripheral vascular leakage are not pathological and may be found in the general population. To our knowledge, there is no prior study establishing UWFFA findings in healthy patients without known ocular or systemic disease. In 1985, a study using traditional fluorescein angiography in 25 patients did not detect peripheral leakage in normal patients [15]. However, this was before UWFFA was available and likely incompletely captured the periphery.

Limitations of our study are related to its retrospective nature, sample size, and lack of a control group. Some patients had anti-VEGF treatment within two months of having UWFFA and this may have masked leakage in the treated eye. It is possible that patients with unexpected peripheral leakage do in fact have a coexisting systemic illness that was not documented in the chart. Furthermore, patients at a tertiary referral center may not represent the general population. Nonetheless, a subset of patients were found to have unexpected late peripheral leakage on UWFFA of possible clinical significance.

In conclusion, 11% of patients with BRVO in our population had unexpected late peripheral leakage in fellow eyes on UWFFA. It may represent an underlying inflammatory condition, hypertensive changes, or bilateral BRVO. Further studies to evaluate a larger population of BRVO patients in a protocol manner should be considered to further elucidate the significance of these findings.

### Conflict of Interests

Authors have no conflict of interests to declare.

### Acknowledgment

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### References

Research Article

Infiltration of Proinflammatory M1 Macrophages into the Outer Retina Precedes Damage in a Mouse Model of Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) is a major cause of blindness in the developed world. Oxidative stress and inflammation are implicated in AMD, but precise mechanisms remain poorly defined. Carboxyethylpyrrole (CEP) is an AMD-associated lipid peroxidation product. We previously demonstrated that mice immunized with CEP-modified albumin developed AMD-like degenerative changes in the outer retina. Here, we examined the kinetics of lesion development in immunized mice and the presence of macrophages within the interphotoreceptor matrix (IPM), between the retinal pigment epithelium and photoreceptor outer segments. We observed a significant and time-dependent increase in the number of macrophages in immunized mice relative to young age-matched controls prior to overt pathology. These changes were more pronounced in BALB/c mice than in C57BL/6 mice. Importantly, IPM-infiltrating macrophages were polarized toward the M1 phenotype but only in immunized mice. Moreover, when Ccr2-deficient mice were immunized, macrophages were not present in the IPM and no retinal lesions were observed, suggesting a deleterious role for these cells in our model. This work provides mechanistic evidence linking immune responses against oxidative damage with the presence of proinflammatory macrophages at sites of future AMD and experimentally demonstrates that manipulating immunity may be a target for modulating the development of AMD.

1. Introduction

Age-related macular degeneration (AMD) is the most common cause of legal blindness in the elderly population of developed countries with over 300,000 newly diagnosed patients per year in Europe and North America [1, 2]. It is widely believed that AMD starts with the insidious, slowly progressing "dry" form (dry AMD) and can later develop into the more severe "wet" AMD, which advances very rapidly and is characterized by abnormal development of blood vessels, a process called choroidal neovascularization (CNV) [3, 4] that affects the macular region of the retina and leads to loss of central vision. In turn, dry AMD without CNV can proceed to focal loss of the retinal pigment epithelium (RPE), termed geographic atrophy (GA), which is accompanied by loss of vision over these slowly expanding areas of RPE atrophy.
RPE loss or dysfunction renders the surrounding tissue vulnerable to damage by reactive oxygen species and loss of photoreceptor density ensues [6]. It has been recognized that accumulation of debris below the RPE in the macula, also known as drusen, is a risk factor for AMD. Identification of complement factor proteins in drusen from AMD eyes [7], coupled with genetic variation in several complement factor genes in AMD patients [8–13], collectively implicates inflammation as an important component in the pathobiology of this disease [14, 15]. For instance, it was recently demonstrated that complement factor H (CFH) serves a protective role in AMD by binding and inhibiting the inflammatory effects of the lipid peroxidation product malondialdehyde (MDA) [16]. Therefore, dissecting the initiating events of outer retinal inflammation during the early stages of AMD can lead to a better understanding of its pathogenesis. Such findings will enable the development of innovative immunotherapies that can prevent inflammatory damage to retinal tissue and loss of vision in a disease process that is estimated to increase in prevalence by 50% by the year 2020 [17].

In the past decade, several novel therapeutic agents have been identified as effective drugs to treat wet AMD, which delay new blood vessel formation and improve vision [18]. However, there is no effective treatment for dry AMD to date. In the pursuit of identifying the signals from the outer retina that initiate inflammation and possibly involve the immune system in AMD pathogenesis, we have evaluated immune responses to carboxyethylpyrrole (CEP), a protein modification that forms from an oxidation fragment of docosahexaenoic acid (DHA) [19], the most oxidizable of all long chain polyunsaturated fatty acids. Studies have shown that AMD donor eyes contain more CEP-modified proteins in the outer retina and drusen than in age-matched controls [7]. CEP-modified proteins and CEP autoantibodies are also more abundant in AMD plasma than in control samples [19, 20].

Since DHA is abundant in the outer retina [21], where the amalgamation of high oxygen tension and light provides an environment suitable for oxidative damage, our lab has previously developed a murine model in which mice immunized with CEP-modified mouse serum albumin (CEP-MSA) develop a CEP-specific immune response which correlates with dry AMD-like pathology when compared to age-matched controls, including CEP autoantibodies, complement deposition in the Bruch’s membrane, and RPE damage [22, 23]. To our knowledge, this is the first immune-mediated mouse model of dry AMD in genetically unmanipulated animals and stems directly from observations in human patients.

Another component of the immune system that has been implicated in AMD is the macrophage lineage [24, 25], although the specific role of these innate immune cells at different stages of AMD disease progression is still controversial. Within the retina, there are two sources of macrophages: (1) microglia, bone marrow-derived resident macrophages that are recruited to neural tissue during retinal development and provide immunosurveillance in the inner retina, and (2) circulating monocytes that can be recruited from the blood vessels to sites of inflammation when needed by specific chemokines and cytokines. Independent of the source, these cells can undergo a diverse program of differentiation depending on the microenvironment that ultimately dictates their effector functions [26, 27]. Macrophages activated in the presence of interferon-gamma (IFN-γ) become proinflammatory M1 macrophages, characterized by their production of tumor necrosis factor alpha (TNF-α) and interferon-alpha-2b (IFN-α-2b). On the other hand, macrophages activated in the presence of IL-4 differentiate into M2-type, marked by production of the immunosuppressive cytokine IL-10 and involved in tissue remodeling.

The CEP-MSA-induced changes in the outer retina that provide a model for AMD afford the unique opportunity to directly test the role of these cells in the disease process.

Our current study aims to characterize both the magnitude and kinetics of development of retinal lesions and macrophage involvement in the B6 mouse strain at various intervals postimmunization (p.i.) in young mice compared to age-matched controls, before extensive retinal lesions are observed. Here we extend our original study [22] by showing that CEP-MSA immunization leads, in aged (old) BALB/c mice, to the end stage cardinal feature of human dry AMD: loss of photoreceptor cells. This major damage is the result of a low-grade but significant inflammatory response in the retina prior to overt tissue damage, which can be quantified in young mice. We have identified M1 macrophages localized to the interphotoreceptor matrix (IPM) surrounding the photoreceptor outer segments in close proximity to the RPE. These changes occur in both BALB/c and B6 strains, but the kinetics are different; BALB/c mice are more susceptible at a younger age. We also detected elevated levels of the monocyte chemotactic protein Ccl2 in the retinas of CEP-immunized mice. Moreover, Ccr2−/− B6 mice immunized with CEP-MSA lack macrophage recruitment, and retinal lesion development is reduced or prevented. Since AMD is an age-related disease, defining the progression of inflammatory cell recruitment and development of AMD-like lesions at earlier stages of the disease is essential in order to map the character and timing of immune mechanisms that take place in our model and correlate with development of pathology. This work clarifies a long-standing question by defining a clear mechanistic path that explains the role of inflammation in AMD: M1 macrophages are key factors in dry AMD pathogenesis. We also provide experimental demonstration for the idea that regulation of immune responses (in this case, inhibition of macrophage recruitment) can be a target of therapy to prevent the development of AMD.

2. Results

2.1. Magnitude and Tempo of Lesion Development and Inflammatory Cell Recruitment. We have previously described AMD-like lesions in wild-type (WT) B6 mice immunized with CEP-MSA [22]. We now describe in detail lesions in CEP-MSA-immunized mice on the BALB/c background, which are inherently albino. Importantly, but also technically challenging, the severity of lesions increases with time after immunization, and it can take for up to 12–24 months to observe signs of geographic atrophy and photoreceptor cell loss, cardinal features of AMD.
Figure 1: Immunization with CEP-MSA leads to overt retinal degeneration in aged mice that resembles geographic atrophy with loss of photoreceptor cells, particularly in the BALB/c background. Histology of CEP-MSA versus naïve BALB/c or C57BL/6 eyes at different time points postimmunization. The RPE is located at the lower part of each image. The dark arrows show macrophage-like cells. CEP-MSA BALB/c mice show strong pathology since the early time points, starting with swollen RPE cells and leading to massive geographic atrophy at the late time point, including complete loss of the photoreceptor cells. The kinetics of pathology in CEP-MSA B6 mice is slower, but eventually there are focal lesions of the RPE such as vesiculation, as previously reported [21]. Representative images are shown (from 3–5 mice per group per experiment; two or three independent experiments were performed for each strain). INL: inner nuclear layer; ONL: outer nuclear layer; ROS: rod outer segment; RPE: retinal pigment epithelium. Images were obtained using a 63x oil objective, and the scale marker represents a 20 μm length.

(Figure 1, Supplemental Figure S1 available online at http://dx.doi.org/10.1155/2013/503725). The main purpose of the current study was to determine the earliest time at which significant differences between CEP-immunized and control mice could be detected. The reasoning behind this approach is to define the molecular and cellular mechanisms that take place at the initial stages of disease, before there are gross changes to the retina that could alter its function. These early time points could also be critical for therapeutic intervention.

Eyes harvested at 40–90 days p.i. were defined as of early recovery times, those harvested at 100–200 days p.i. were considered of intermediate recovery times, while those obtained after day 200 p.i. were considered of late recovery times. Focal lesions in the RPE and IPM consisting of vacuolization of individual or groups of cells, pyknotic RPE cells, hypertrophic RPE cells, and melanin engulfment by macrophages (only possible in B6 mice), as well as darkly stained nuclei of inflammatory (macrophage-like) cells in the RPE and IPM were counted (refer to Methods for detailed scoring parameters) (Figure 1). While significant damage can be observed in the retina of aged (12–24 months old) control mice (mainly thinning of the photoreceptor layer, Figure 1, Supplementary Figure 1), no GA pathology is seen in mice that did not receive CEP immunization or in younger CEP-immunized mice.

Our data was analyzed with a three-factor analysis of variance; the factors are (1) strain of mice (BALB/c versus B6), (2) immunization status (naïve versus immunized), and (3) time of recovery of tissue (early versus intermediate) (Figure 2). Pathology scores (defined as the summation of retinal lesions and cellular infiltration) were higher in BALB/c CEP-immunized mice at both early and intermediate recovery times but only higher in B6 CEP-immunized mice at the intermediate recovery time (Figure 2(a)). To have a representative and objective pathological score throughout the retina, we focused on the number of IPM-infiltrating cells present in close proximity to the RPE. Using routine histopathology, it is not possible to distinguish different types of monocyte lineage cells present in the outer retina (for example, resident microglia versus macrophages recruited from the circulation). For this reason in the description of the results to follow, we will simply refer to the nonneural cells in the IPM as macrophages, since microglia are distinguished mainly for their location within the central nervous system. Careful examination of the lesions at these early and intermediate recovery times demonstrated that a distinct population of macrophages is present in the IPM compartment near the RPE in these animals. Quantification in plastic sections showed that CEP-immunized BALB/c mice
have a significantly higher number of macrophages than age-matched naïve animals harvested at 40–100 days p.i. \((P = 0.023)\) as well as those harvested at 100–200 days p.i. \((P = 0.023)\) (Figure 2(b)). The same cellular quantification is observed with H&E staining of frozen sections (Figure 2(c)). When comparing IPM macrophages between the two time points, BALB/c mice showed an increase in the number of these cells in the early to intermediate recovery times in both immunized and naïve mice, but the magnitude was significantly higher in immunized mice \((P = 0.012)\). While CEP-immunized B6 mice harvested at early recovery times showed no significant differences in IPM macrophages when compared to naïve B6 mice, immunized mice harvested at intermediate time points had more IPM macrophages than age-matched B6 naïve controls \((P = 0.023)\). Therefore, the number of monocyte-lineage cells present in the IPM increases over time in CEP-immunized B6 mice to reach significant numbers by the intermediate recovery times.
2.2. Enhanced Immune-Mediated AMD-Like Pathology Is Specific to Genetic Background rather than Light Damage. Naïve BALB/c mice contained more macrophages in the IPM than age-matched naïve B6 mice ($P < 0.01$), and immunized BALB/c mice show higher number of IPM macrophages than immunized B6 mice as well ($P < 0.01$). This difference is also evident in the number of RPE lesions, which are higher in BALB/c mice than in B6 mice, regardless of immunization status. To test the possibility that photosensitivity in the BALB/c albino mice [28] could be responsible for the differences in outer retinal lesion development and macrophage presence when compared to B6 mice, we immunized albino B6 mice (B6(Cg)-Tyr<sup>−/−</sup>, or Tu<sup>−/−</sup> mice, which lack the tyrosinase enzyme) and compared them to age-matched naïve Tu<sup>−/−</sup> mice as well as to corresponding BALB/c and WT-B6-immunized and naïve mice at 40–90 days p.i. The results from these comparisons showed that both macrophages in the outer retina and development of retinal lesions observed in Tu<sup>−/−</sup> mice are comparable to WT B6 and do not show the enhanced cellular recruitment, RPE hypertrophy, and ROS vacuolization present in BALB/c mice at the early time points, regardless of immunization status (Figure 3(a)). Quantification analysis of pathological changes per section supports these observations (Figure 3(b)). This suggests that the differences in inflammatory cells present in the outer retina and AMD-like pathology observed between the BALB/c and the B6 strains are not due to photosensitivity in the albino mice because of lack of pigmentation, but instead they are specific to genetic background, possibly due to differences in the immune responses against CEP-MSA between the mice or to inherent differences in RPE function and/or local oxidative damage responses in the retina.

2.3. Inflammatory Cells Surround the Retina of CEP-MSA-Immunized Mice. Because inflammation associated with macrophages present in the IPM seems to play a role in the retinal pathology observed in CEP-MSA-immunized mice, we performed immunohistochemical (IHC) analysis using cell surface immune markers for the identification of the specific macrophages present in the retina, generally, and to the IPM region, specifically. A large number of CD45<sup>+</sup> (a pan-leukocyte marker) cells were observed in the choroid of immunized mice compared to controls (Supplementary Figure 2), indicating that CEP immunization leads to increased ocular inflammation. In terms of specific cell subsets, we observed only a few CD3<sup>+</sup> and CD19<sup>+</sup> cells in the outer retina, suggesting that both T cells and B cells are largely absent from the site of retinal lesions (data not shown). In contrast, there were substantial numbers of choroidal CD11b<sup>+</sup>, F4/80<sup>+</sup>, and CD68<sup>+</sup> cells surrounding the retinas of BALB/c CEP-MSA-immunized mice and not of naive controls (data not shown). Similar findings were observed in B6 immunized animals at late recovery times when lesions are present.

2.4. Proinflammatory M1 Macrophages Are Present in the IPM at Early Recovery Times in Mice Immunized with CEP-MSA. Immunofluorescence staining identified retinal CD11b<sup>+</sup>, F4/80<sup>+</sup>, and CD68<sup>+</sup> cells in mice immunized with CEP-MSA, suggesting the potential role of macrophages in the development of disease in this model. We were able to localize a significant number of these macrophages in the RPE and IPM using nuclear counterstaining with DAPI (Figure 4(a), Supplementary Figure 3). This correlates with the location of the macrophages identified by histopathology using toluidine blue and H&E staining that are mostly observed in proximity with vacuolization and lesions of RPE and photoreceptors. In order to determine the specific class and activation status of these macrophages, we performed intracellular stains for Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-12 (IL-12) production, which identify M1 macrophages, versus IL-10 production, a hallmark of M2 macrophage differentiation. We observed both TNF+ and IL-12+ cells within the IPM of CEP-MSA-immunized mice, but IL-10 staining was negative, indicating the presence of activated M1 macrophages in the pathological regions of immunized mice only (Figure 4(b)). To substantiate the IHC results, we performed mRNA quantification on IPM-infiltrating macrophages isolated by laser capture. These data confirmed that detectable levels of M1 marker genes (IL-6, TNF-α, and IL-1β) were observed only in CEP-immunized mice, whereas IL-10 expression was not detected (Figure 4(c)). Expression of Arg1, another M2 marker, was not elevated in CEP-immunized mice. Interestingly, we also observed CEP-associated increased expression of Ccl2, a monocyte chemoattractant that has been implicated in AMD, suggesting that the Ccl2/Ccr2 axis may play a role in CEP-induced pathology. These data strongly suggest that M1 macrophages are primarily associated with the lesions we observe in the outer retina and may be the main effectors of the inflammatory response observed in this model.

2.5. Macrophage Recruitment into the Outer Retina of CEP-MSA-Immunized Mice Is Necessary for the Induction of Disease. To directly test the role of macrophage recruitment into the IPM and development of outer retinal lesions we immunized Ccr2-deficient mice on the B6 background. These mice have a defect in chemokine signaling and show poor recruitment of inflammatory cells into sites of inflammation. In addition, it has been previously shown that disruption of chemokine signaling to macrophages in these Ccr2 knockout mice can have a deleterious effect on the integrity of the retina in aged mice (2 years and older) [29]. While Ccr2 deficiency did not affect the levels of anti-CEP antibody titers in young immunized mice (Figure 5(a)), Ccr2<sup>−/−</sup> animals showed no increase of IPM-infiltrating macrophages or outer retinal lesions in immunized mice compared to those in naive age-matched controls at late recovery times (Figure 5(b)). This observation strongly suggests that macrophages, mainly recruited by Ccl2, play a causative role in the induction of tissue damage in this model.

3. Discussion

Immunization of mice with CEP-MSA provides a valuable model to study dry AMD from an immunological perspective, helping to dissect the immune system’s role in
Figure 3: Immune-mediated cellular infiltration and development of outer retinal lesions are specific to genetic background and pigment independent. (a) Histology of CEP-MSA albino B6 mice compared to WT BALB/c and WT B6 at the early time point. Representative images are shown (from 3–5 mice per group per experiment; two or three independent experiments were performed for each strain). INL: inner nuclear layer; ONL: outer nuclear layer; ROS: rod outer segment; RPE: retinal pigment epithelium. Images were obtained using a 63x oil objective, and the scale marker represents a 20 μm length. (b) Quantification shows the lack of pathology at early time points in albino B6 mice, similar to WT B6 and in contrast to WT BALB/c mice. Mean values are shown; error bars represent S.D.
Figure 4: CEP-MSA immunization leads to M1 (TNF-α, IL-12 producing) macrophage recruitment and activation in the subretinal space. (a) Frozen sections followed by immunostaining and confocal microscopy. Surface marker stains were used to identify macrophages as the infiltrate cells. F4/80+ and CD68+ cells are absent from the RPE of naïve mice, but they are found in CEP-MSA-immunized BALB/c and B6 mice. (b) Intracellular stains for TNF-α, IL-12, and IL-10 production were used to determine the phenotype of the activated macrophages observed in CEP-MSA BALB/c mice at the intermediate recovery time. IgG2b was used as isotype control. Representative images are shown (from 3–5 mice per group per experiment; two or three independent experiments were performed for each strain). INL: inner nuclear layer; ONL: outer nuclear layer; ROS: rod outer segment; RPE: retinal pigment epithelium; CHO: choroid. The scale marker represents a 20 μm length. (c) Infiltrating macrophages from B6 mice were isolated by laser microdissection, and RNA was obtained for qPCR analysis of gene expression. Relative mRNA (in arbitrary units) was calculated using the $2^{-\Delta\Delta Ct}$ method with Actin as the calibrator gene. Transcripts for M1 marker genes (IL-1β, TNF, and Ccl2) were detectable in 3 out of 3 CEP-MSA-immunized mice but were not present in CFA age-matched controls ($n=4$). M2 marker expression did not correlate with CEP immunization: IL-10 was completely absent, whereas the levels of Arg-1 did not increase. Results are representative of at least two independent laser capture experiments.
not vulnerable to light damage [31]. Therefore, we believe that at least one major reason for the observed kinetic and quantitative differences is the number of inflammatory cells in the outer retinas of BALB/c mice. Whether there are significant differences in endogenous CEP levels in the retinas of these mice, inherent differences in RPE function and/or local oxidative damage responses in the retina, or the particular contribution of specific adaptive immunity pathways, is an aspect under current investigation in our laboratory.

This work also describes in detail the differences in subretinal macrophages between these two widely used mouse strains. While many macrophage-like cells are present in the subretinal space of young naïve BALB/c mice, we have not been able to successfully identify these cells based on surface marker expression. The true nature of these baseline retinal macrophages in BALB/c mice remains unknown. Importantly, we only found subretinal CD11b+/F4/80+/CD68+ macrophages in CEP-MSA-immunized but not naïve mice of either strain. A previous study has shown the presence of these macrophage-like cells in WT B6 mice but only after 20 months of age [32]. Because CEP-MSA immunization leads to the presence of these macrophages in younger mice, we believe that this is additional confirmation of the validity of our model in accelerating an endogenous aging-related process. Thus, the CEP model provides an ideal setting to study different subpopulations of retinal macrophages.

The controversial role suggested for macrophages in AMD stems primarily from the use of gene knockout mice as well as an acute model for choroidal neovascularization (CNV) that has been widely (and successfully) used to mimic wet AMD. For example, young macrophages inhibit CNV in the laser-induced model of wet AMD, but their antiangiogenic potential is reduced with age as they switch to an M2 phenotype [33]. More recently, it has been shown that microglia can induce RPE cells to produce proinflammatory cytokines and chemokines [36]. However, information is lacking to clarify the pathological role of macrophages at different stages of the AMD disease process, particularly at the time of onset of dry AMD before the transition to CNV. The presence of subretinal CD11b+/F4/80+/CD68+ macrophages in CEP-MSA immunized mice we show here is similarly reported in a recent

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**Figure 5:** Ccr2 chemokine receptor signaling is required for macrophage recruitment to the outer retina after CEP-MSA immunization. (a) Anti-CEP antibody titers were examined following immunization of WT versus Ccr2−/− B6 mice (n = 5). Naïve mice have no anti-CEP titers. Mean values are shown; error bars represent S.D. (b) Retinal pathology scores for the indicated groups at the late time point (day 200+ p.i.; n = 3). (*) denotes statistically significant difference (P < 0.05); ns: not significant. Data from one representative experiment was used for this analysis; similar results were obtained in a separate independent experiment.

A further complication involves the two different forms of AMD: macrophages could have different roles in dry versus wet AMD. It is important to stress that the laser-induced CNV model is a completely different system from our CEP model of dry AMD, and findings in one model will not necessarily be directly comparable to the other.

The initial evidence linking macrophages with AMD came from the analysis of mice deficient in macrophage chemokine signaling components (Ccl2−/− and Ccr2−/− mice) which show retinal defects similar to AMD with advanced age (2-year-old mice or older), including spontaneous CNV and “drusen” formation [29]. However, subsequent work by Luhmann et al. (2009) [32] revealed that these findings were in fact an artifact due subretinal macrophage accumulation and that any AMD-like pathology in Ccl2−/− mice was most likely due to aging alone. An additional problem with the knockout mice mentioned previously and their use as AMD models is the fact that these strains were found to include a known mutation (rd8) that by itself results in retinal degeneration [34, 35]. Therefore most, if not all, the previously published papers using these strains must be reevaluated in that context.

However, there is still acceptable evidence associating macrophages with AMD. For example, young macrophages inhibit CNV in the laser-induced model of wet AMD, but their antiangiogenic potential is reduced with age as they switch to an M2 phenotype [33]. More recently, it has been shown that microglia can induce RPE cells to produce proinflammatory cytokines and chemokines [36]. However, information is lacking to clarify the pathological role of macrophages at different stages of the AMD disease process, particularly at the time of onset of dry AMD before the transition to CNV. The presence of subretinal CD11b+/F4/80+/CD68+ macrophages in CEP-MSA immunized mice we show here is similarly reported in a recent

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paper by a different group [37]. In addition, we showed that these macrophages were M1 polarized. This suggests a strong causal link between the M1 macrophages and outer retinal lesions.

In the original publication of our model, it was suggested that macrophages were present as a result of tissue damage and were not likely to cause disease [22]. The rationale for this conclusion was the fact that many lesions occurred in the absence of these cells. However, that original paper did not go into detail on the characterization of these cells. Missing from the first study and addressed in this paper are three key parameters that now lead to the interpretation that there is a causal relationship between M1 macrophages and dry AMD-like pathology: (i) kinetics and magnitude (quantification) of macrophage infiltration into the IPM relative to lesion development; (ii) activation status of the observed macrophages; (iii) how are the cells being recruited? This current study provides evidence for the first time that the early involvement of M1 macrophages occurs in animals that are predisposed to develop retinal lesions. We also provide the mechanism for recruitment of these cells, as Ccl2 is elevated in retinas of CEP-immunized mice, and its receptor, Ccr2, is required for macrophage infiltration into the IPM.

While we cannot completely rule out at this time that the M1 macrophages present in the IPM of CEP immunized mice are actually microglia migrating from the inner retina, it is likely that these cells come from the blood because of the systemic nature of our immunization protocol; retinal microglia are present at their normal inner retina location in Rag-deficient mice that do not develop lesions upon CEP-MSA immunization [22]. Furthermore, this model relies on the endogenous accumulation of CEP adducts in the outer retina, which should occur at equivalent rates in immunized versus naïve mice, allowing resident microglia an equal access to the CEP antigen. A more definitive distinction of the original source of these cells awaits the development of microglia-specific and/or macrophage-specific markers. Regardless, our work confirms the critical role of bone-marrow-derived macrophages in the development of retinal degeneration and provides an excellent platform to further characterize this process.

As mentioned previously, we are aware that both Ccr2−/− and Ccl2−/− mice develop AMD-like pathology with age [29], even though the recent work by Luhmann et al. [32, 34] has challenged this notion, at least for Ccl2−/− mice. A major difference between these other studies and ours is that our model allows us to focus on the evaluation of relatively young animals following immunization with CEP-MSA, in contrast to the retinal lesions described previously that develop in the older knockout animals; we analyzed mice before 12 months of age, the naïve Ccr2−/− mice develop retinal pathology after 18–20 months. Therefore, it would be difficult to make a direct comparison with our study, but it provides the opportunity to explore new mechanisms that link immunity to AMD. Ccr2−/− mice do not lack ocular macrophages, just defective (or delayed) age-related recruitment (to the choroid). In fact, as shown by Luhmann et al. 2009 [32], “old” Ccl2−/− mice (which closely resemble the Ccr2 macrophage phenotype) have increased macrophage recruitment to the subretinal space (the same area in which we observe macrophage infiltration in CEP-MSA mice) when compared to wild type, showing that a defective Ccl2/Ccr2 axis does not necessarily, by itself, preclude retinal infiltration of macrophages. While there is certainly a possibility that the observed pathology in CEP-immunized WT mice may not be due to macrophages, we think the Ccr2−/− data in this paper answers that question: if macrophages did not play a detrimental role in our model (if the retinal lesions in CEP-MSA mice were macrophage independent) then we should have observed some pathology in the immunized Ccr2−/− mice, which we did not. Because the Ccr2−/− mice still develop CEP antibodies similar to WT (indicative of an effective adaptive immune response), the M1 phenotype of subretinal macrophages as well as the temporal relationship between macrophage infiltration and retinal lesions (macrophage recruitment precedes lesion development), we believe that our interpretation that macrophages are detrimental in our model is justified.

It is tempting to hypothesize that there could be two different populations of macrophages involved in the AMD disease process: one being early “harmful” M1 and the other being the “protective” late M2, which in turn may contribute to CNV (once disease has progressed sufficiently). We believe that our data is representative of the role of early M1 macrophages and provides a nice platform to study early events in the development of AMD. This does not exclude the idea that later cellular involvement may include M2 macrophages that could be important for resolution of disease, suggested in the published studies looking at aged Ccr2−/− knockout mice [29, 32]. In fact, it was recently shown in a retinal neuropathy injury model that IL-10-producing (M2) macrophages have a protective role [38]. The balance between M1 and M2 at different ages may actually dictate the damaged versus repaired tissue status of the retina. To support this notion, a recent paper analyzing human AMD eyes showed that AMD correlated with increased M1/M2 ratios, whereas normal aging eyes had more M2 macrophages [39]. In the context of the retina, CEP tilts the balance toward the M1 pathway for its role in inflammation-induced GA.

The enhanced presence of proinflammatory macrophages in our model offers new opportunities to investigate their role and function in AMD pathogenesis, as well as the immunological signals and inflammatory agents behind their activation and recruitment to the outer retina, a tissue historically thought of as an immunosuppressive environment. We believe that innovative immunotherapies that target the low-grade inflammatory responses at the early stages of our model can yield further promising information on the immune mechanisms that take place in response to oxidative damage in the retina.

4. Conclusions
An incomplete understanding of AMD pathogenesis prevents the development of effective therapies. Current understanding of AMD recognizes oxidative stress and chronic retinal inflammation as possible causative factors. Retinal
macrophages have been recognized to have a role in AMD, but their precise role (whether protective, damaging, or incidental) remains controversial. Using our AMD mouse model, we observed significant macrophage retinal infiltration that temporally preceded the onset of overt retinal pathology, suggesting a causative role for macrophages in retinal degeneration. Interestingly, mice with defective macrophage recruitment (Ccr2-deficient mice) lack macrophage retinal infiltrates and are devoid of AMD-like retinal pathology. This work uncovers an important and detrimental role for macrophages in the development of AMD. Such an understanding raises the possibility of exploring immune-modulating therapy for the treatment or prevention of retinal degeneration, especially in patients exhibiting early signs of disease.

5. Materials and Methods

5.1. Mice. BALB/c wild-type mice, C57BL/6 wild-type mice, and Ccr2−/− and B6(Tyr−/−) (B6-albino) mice were obtained from The Jackson Laboratory. All mice were housed in a room exposed to 300 lux (outside the cage) in a 12 hr dark/light cycle. Protocols for use of experimental animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Miami Miller School of Medicine.

5.2. Antigen. CEP-MSA was prepared from commercially available mouse serum albumin (Sigma-Aldrich), which was converted to CEP-modified MSA following previously published procedures [40].

5.3. Immunizations. The CEP-MSA immunization protocol has been described previously [22]. In summary, mice were primed by hind leg injections of 200 μg CEP-MSA in complete Freund's adjuvant (CFA; from DIFCO) at 6–10 weeks of age. Day 10 postimmunization (p.i.), the mice were challenged in the neck with 100 μg CEP-MSA in incomplete Freund's adjuvant (IFA; from DIFCO), followed by a final boost with 100 μg CEP-MSA in complete Freund's adjuvant (CFA; from DIFCO) at 6–10 weeks p.i. Anti-CEP antibody titers at days 40–60 p.i. were quantified by ELISA as previously described [22] and used to determine efficiency of immunization. All immunized mice were compared with age-matched naïve, sham-MSA, or CFA controls. There are no significant differences among the control mice (with low to undetectable anti-CEP titers) in terms of retinal pathology and are therefore used interchangeably, depending on experimental setup.

5.4. Histology. Eyes were harvested at early (40–90 days), intermediate (100–200 days), and late (over 200 days) recovery times postimmunization (p.i.). Right eyes were used for histology and were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M PO4 buffer (pH = 7.4) overnight and dehydrated in graded ethanol and propylene oxide. After polymerization in a resin mixture containing Polybed 812 (Polysciences) and Araldite 502 (Polysciences), semithin (0.7 μm) sagittal sections of each eye were stained with toluidine blue and analyzed for histopathology with light microscopy using a Zeiss microscope (equipped with an AxioCam digital camera) using a 63x oil-immersion lens.

5.5. Quantification of Lesions and Inflammatory Cells in the IPM. Each individual mouse in this study was scored for retinal pathology on a masked fashion, using 10 sections of the right eye with at least 25–30 μm intervals between each section. Scoring was divided in 2 subclasses: (1) the retinal lesion count represents the sum of RPE areas showing abnormal vesiculation, swelling, thinning, pyknosis, and cell lysis; (2) inflammatory cells were defined as dark nuclear stains of macrophage-like cells observed and counted only within the interphotoreceptor matrix compartment at the level of the photoreceptor outer segments and the apical border of the RPE. The overall pathology score for each eye is the sum of the two subclasses. The data is always presented as pathology (cells or lesions or combined) per section.

5.6. Statistical Analysis of Retinal Pathology. Our data was analyzed in the Biostatistics Department at the Bascom Palmer Eye Institute with a three factor analysis of variance; the factors are: (1) strain of mice (BALB/c versus B6), (2) immunization status ( naïve versus immunized), and (3) recovery time (early versus intermediate). A total of 3–5 mice were used in the analysis at each recovery time. At least two independent experiments were performed for each strain reported in this study. Repeat experiments with similar results were analyzed separately because of the use of independent batches of CEP-MSA.

5.7. Immunohistochemistry. Identification of inflammatory cells in the IPM was done by immunostaining of frozen sagittal sections from the corresponding left eye for each animal. Following enucleation, eyes were embedded in OCT compound (Sakura Finetek USA), frozen on dry ice, and 8 μm sections were cut using a cryostat (−20°C). Frozen sections were collected on microscope slides. The sections were fixed with 3% formaldehyde for 25 min then pretreated with a blocking solution containing 0.05% tween 20 and 3% bovine serum albumin in PBS for 1 h at room temperature to saturate nonspecific binding sites. The sections were then incubated 1 h at room temperature with the primary antibody diluted in PBS tween and 1% BSA. The following antibodies were used for surface stains: rat anti-mouse CD11b, F4/80, CD68, and CD45 (all from eBioscience). For intracellular staining, we diluted the primary antibodies in saponin buffer. The following antibodies were used for intracellular stains: rat anti-mouse TNF-α (BD Bioscience), IL-12p70 (Endogen), and IL-10 (BD Bioscience). Sections were then rinsed for 10 min in PBS tween and incubated with 1:2000 goat anti-rat Alexa Fluor 594 (Invitrogen) for 1 h at room temperature. The sections were washed three times for 10 min in PBS tween and for 10 min in PBS, coverslipped with Vectashield with DAPI for nuclear counterstaining (Vector Laboratory) and photographed in a Zeiss universal microscope (Carl Zeiss, Oberkochen, Germany) equipped for incident-light fluorescence and confocal microscopy.
5.8. Laser Capture Microdissection of Outer Retina Macrophages. CFA or CEP-MSA-immunized mice were euthanized in a CO2 chamber, and their eyes were harvested for tissue processing. Eyes were cryoprotected in 1.5% sucrose, embedded in Tissue-Tek OCT compound (Sakura Finetek, USA), and frozen. Cryostat sections, 12 μm thick, were mounted on PEN-membrane slides (Leica). Sections were then incubated in absolute ethanol and briefly stained for H&E. Single infiltrating macrophages in interphotoreceptor matrix were collected using Laser Microdissection System LMD6000 (Leica). Total RNA was extracted using the RNaseasy mini kit (Qiagen) and reversely transcribed with High-Capacity cDNA Archive Kit (Applied Biosystems). cDNA was preamplified with Taqman PreAmp Master Mix Kit followed by PCR amplifications of cDNA, using Taqman probe-based gene expression assay (Applied Biosystems). Relative mRNA (in arbitrary units) was calculated using the comparative quantitation method of relative quantity (2\(^{-ΔΔCt}\)), with actin as the calibrator for each gene of interest. Primer and probe sets were as follows: ActB, Mm00607939_s1, IL-1β, and Mm01336189_ml; TNF-α, Mm00443258_ml; IL-6, Mm00446190_ml; Ccl2, Mm00441424_ml; IL-10, Mm99999062_m1; Arg1, Mm00475988_M1.

Authors’ Contribution

V. L. Perez and J. G. Hollyfield initiated the research. F. Cruz-Guilloty and V. L. Perez designed the experiments and analyzed all the data. F. Cruz-Guilloty, A. M. Saeed, J. J. Echegaray, S. Duffort, A. Ballmick, Y. Tan, M. Betancourt, E. Viteri, G. C. Ramchellawann, and E. Ewald performed the research and analyzed data. W. Feuer performed statistical analysis. L. Hong, H. Wang, J. M. Laird, and R. G. Salomon prepared the CEP reagents. D. Huang and R. Wen contributed with analytical tools for histology. A. Sene and R.S. Apte performed the laser capture microdissection experiments. F. Cruz-Guilloty and V. L. Perez wrote the paper with input from J. G. Hollyfield and R. G. Salomon.

Conflict of Interest

The mouse model for dry AMD described in this study is protected for commercialization by SKS Ocular. J. G. Hollyfield, R. G. Salomon, and V. L. Perez are the inventors.

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References

Review Article

Targeting Inflammation in Emerging Therapies for Genetic Retinal Disease

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Genetic retinal diseases such as age-related macular degeneration and monogenic diseases such as retinitis pigmentosa account for some of the commonest causes of blindness in the developed world. Diverse genetic abnormalities and environmental causes have been implicated in triggering multiple pathological mechanisms such as oxidative stress, lipofuscin deposits, neovascularisation, and programmed cell death. In recent years, inflammation has also been highlighted although whether inflammatory mediators play a central role in pathogenesis or a more minor secondary role has yet to be established. Despite this, numerous interventional studies, particularly targeting the complement system, are underway with the promise of novel therapeutic strategies for these important blinding conditions.

1. Introduction

Inherited retinal diseases include some of the commonest causes of blindness in the developed world [1, 2]. Prominent examples included age-related macular degeneration (AMD), diabetic retinopathy, and the numerous monogenic conditions such as retinitis pigmentosa (RP), Stargardt’s disease, and X-linked retinoschisis. As well as causative/predisposing genetic abnormalities [3], in some cases (such as AMD), environmental factors such as smoking and diet have been highlighted [4, 5]. These aetiological factors have been associated with diverse abnormal biochemical pathways in the degenerating retina, for instance, oxidative stress [6]; lipofuscin accumulation in the retinal pigment epithelium [7]; abnormalities of the extracellular matrix [8]; mitochondrial abnormalities [9]; ischaemia with neovascularisation [10]; programmed cell death [11]. In particular in AMD and RP, inflammation has recently become a prominent member of this list of abnormal pathological pathways triggered by genetic retinal disease [12, 13].

2. Established Links between Inflammation and Genetic Retinal Disease

Early studies showed that autoantibodies can be detected in blood of AMD patients [14, 15] and that macrophages also accumulate in the choroid [16] which suggested that immune-mediated processes were involved in the pathogenesis of AMD. Renewed interest in inflammation in genetic retinal disease was, however, more recently triggered by the discovery of elements of the immune system and multiple proteins of the complement pathway within the drusen seen in AMD [17]. Although the exact pathophysiology of AMD remains largely unknown, accumulation of drusen is acknowledged as an early and major pathological hallmark of the disease, preempts damage in the retinal pigment epithelium, photoreceptors, and choroid leading to atrophic or neovascular complications.

Immunohistochemical studies of human retina highlighted that amongst other components, AMD drusen contained inflammatory mediators such as vitronectin,
immunoglobulin light chains, factor X, and complement proteins (C5 and C5b-9 complex). Importantly, it was also demonstrated that drusen displays intense HLA-DR immunoreactivity [17, 18]. This later finding complements other independent work suggesting and association between AMD and HLA-genotype [19]. It is, however, now recognised that the composition of drusen deposits is extremely heterogeneous amongst different patients and that many other components are also found in addition to inflammatory mediators. For instance, other studies have highlighted the appearance of oxidative protein modifications within AMD drusen including cross-linked species of tissue metalloproteinase inhibitor 3, vitronectin, and carboxyethyl pyrrole protein adducts suggesting oxidative stress as an etiological factor in AMD drusen formation [20].

The feasibility of linking immunity to AMD pathophysiology has also been suggested by the central role the retinal pigment epithelium (RPE) plays in both AMD pathogenesis [21] and ocular immune modulation [22]. Both in vivo and in vitro experiments have demonstrated that the RPE expresses both innate and adaptive immune receptors [22, 23]. In addition, RPE cells are known to secrete numerous cytokines, chemokines, and adhesive molecules including interleukin-6, interleukin-8, and immunosuppressive factors including tissue-necrosis factor-β, interleukin-11, and interferon-β [22].

Evidence suggesting a role for inflammation in AMD has, however, been strongly supported by molecular genetic studies. In particular, genes encoding components of the complement pathways have been associated with AMD. Strong associations have been demonstrated for alleles of genes encoding complement factor H (CFH) [24–26] which is a regulator of the alternate complement pathway, complement component C2/complement factor B (C2/CFB) [27], and CFH-related genes CFHR1 and CFHR3 [28]. Weaker associations have been linked to complement factor 1; complement C3 and complement component 1 inhibitor (SERPING1), a regulator of the classic complement pathway; chemokine C-X3-C receptor 1 (CX3CR1); and toll-like receptor genes TLR3 and TLR4 (with a role in the innate immune system) [29, 30]. It should be noted, however, that strong genetic associations between loci and AMD also exist, for example, to the PLEKHAI/ARMS2/HTRA1 region of chromosome 10q26 [29].

Most recently, however, there has been remarkable work linking nucleotide-binding domain and leucine-rich-repeat-protein 3 (NLRP3) and the “inflammasome” with the aetiology of ARMD [31–33]. The inflammasome is a term used to identify a collection of proteins that work together within cells with a common purpose, more specifically, a caspase-1 dependent multiprotein complex that has a key role in innate immunity. The inflammasome can be triggered by a number of stimuli including microbial pathogen-associated molecular patterns, bacterial toxins and most relevant to genetic retinal disease “damage-associated molecular patterns” (denatured nuclear or cytosolic proteins released from dying cells). This results in the upregulation of proinflammatory cytokines interleukin-1β and interleukin-18 [34, 35]. The inflammasome can be activated by three classes of immune sensors including the toll-like receptors, RIG-I-like helicases, and NLR proteins [36]. Currently, four inflammasomes based on NLRP3 have been characterised: NLRP1/NLRP1b [37]; NLRC4/IPAF [38]; NLRP3/NALP3 [39]; AIM2 [40]. Activation of the NLRP3 inflammasome has recently been reported in dry AMD drusen, by complement component C1Q or by carboxyethylpyrrole (during oxidative stress) [31]. In wet ARMD, activation of the NLRP3 inflammasome has been seen triggered by Alu RNA molecules (short chains RNA) [32]. Intriguingly, reflecting the diversity of immune responses, the most recent in vivo and in vitro studies have suggested that the NLRP3 inflammasomes may have a beneficial role in wet ARMD but a harmful effect leading to RPE cell death in dry AMD [31, 32].

Linking inflammatory mediators to the choroidal neovascularization seen in complicated AMD is also firmly established. In both preclinical and clinical studies, cellular components of immune immunity including macrophages, lymphocytes and neutrophils have been found to be significant components of choroidal neovascular complexes [16, 41, 42]. Additionally, inflammatory cytokines such as interleukin-6 and interleukin-8 have also been identified in the aqueous humor of AMD patients suffering from choroidal neovascularisation [43].

While the term “retinitis pigmentosa” as coined by Donders in 1857 is generally considered a misnomer, a role for inflammation and immunity in the pathogenesis of the disease has significant merit. The earliest studies to suggest this showed elevated IgM in six out of ten RP patients [44]. Other early studies also suggested that retinal (probably photoreceptor) autoantibodies could be found in the systemic circulation of RP patients [45–48]. Immune reactivity in RP was also established by exposing lymphocytes and leucocytes from blood samples of RP patients to human and bovine retinal antigens [49]. However, results have been complicated by the fact that immune reactivity appears to vary amongst RP patients possibly reflecting the genetic heterogeneity of the disease. Studies suggested that circulating immune complexes could be detected in less than 50% of RP patients [50]. This was, however, reported as significant since it correlated with statistically significant reductions of circulating complement factors C3 and C4 and a significant reduction in time taken for RP patient sera to achieve 50% hemolysis of sheep red blood cells [50]. A link with HLA status has also been reported in RP patients [51], and vitreous samples from RP patients have been shown to contain many immune system cells such as various types of lymphocytes [52].

More recent studies have highlighted the activation of microglia in RP retina preceding photoreceptor cell death. Activation of microglia results in many biochemical events including the release of cytokines and chemokines [53]. In rd mice (a homozygous nonsense phosphodiesterase-beta subunit gene mutant), it has been shown that prior to peak photoreceptor cell death, there is an upregulation of mRNA of proinflammatory factors: monocyte chemoattractant proteins 1 and 3; macrophage inflammatory proteins 1alpha and 1beta; regulated on activation normal T-cell expressed and secreted (RANTES); tumor necrosis factor-alpha [54]. Microglia activation and upregulation of proinflammatory
markers has also been demonstrated to precede peak photoreceptor cell death in rd10 mice (homozygous missense phosphodiesterase beta subunit mutant) [55].

Although direct biochemical assessment of retina from human RP patients is difficult, more detailed recent studies have searched for signs of inflammatory cells and humoral inflammatory factors in aqueous and vitreous humor from RP patients [13]. Slit lamp examination revealed that cells could be visualised in the anterior chamber 37% (190) and the vitreous of 61% (313) of 509 RP eyes (up to 30 cells identified in a 1 × 9 mm vertical slit-lamp field). It was not explained however how it was concluded that these were inflammatory cells (and not for instance, pigmentary cells). The study did, however, also report multiplex ELISA data suggesting significantly increased protein levels of cytokines (interleukin 6) and chemokines (interleukin-8, monocyte chemoattractant protein 1, and thymus activation-regulated chemokine) in the aqueous. Much more notably, in the vitreous of these RP patients, significantly elevated levels of chemokine (inducible protein-10, and thymus activation-regulated chemokine) were found [13].

### 3. Therapeutic Targets

Numerous therapeutic strategies are emerging in the treatment of genetic retinal disease. These include cell-based therapies [56]; gene therapy [57]; electronic retinal replacements [58]; molecular-based approaches such as neuroprotection [59] and antiangiogenesis [60]. With an established role for inflammatory mediators in the pathogenesis of both AMD and RP, it would therefore be rational to investigate anti-inflammatory approaches. However, despite numerous animal models for RP [61], no universally recognised animal model for AMD yet exists, and only approximations modeling aspects of the disease are available [62]. This has limited preclinical research into AMD treatment. In addition, it is as yet unclear what role relative to other pathogenic mechanisms inflammation plays in disease pathogenesis. Anti-inflammatory approaches, for example, might have little impact on disease if inflammation is a secondary effect or a minor contributor to pathogenesis. To some extent, therefore, the importance of inflammation in AMD and RP can be validated through quantification of the effect of anti-inflammatory therapeutics.

There are currently 846 listed clinical trials (http://www.clinicaltrials.gov/) focused on AMD of which 51 are specifically targeting inflammation. There are also 78 clinical trials listed for RP although none are focused on anti-inflammatory therapeutics. Studies targeting inflammation in AMD and RP may be subdivided into broad approaches targeting multiple components of inflammation or more specific targeting of complement activation, for instance.

#### 3.1. Broad-Based Anti-Inflammatory Studies in AMD

Corticosteroids have been used in retinal disease for many years because of their general anti-inflammatory effect, generally up-regulating the expression of anti-inflammatory proteins and suppressing the expression of proinflammatory factors [63, 64]. Although their use in uveitis and retinitis is well established, corticosteroids are now being considered in AMD. Many studies have looked at the use of intravitreal dexamethasone in the treatment of neovascular complications of AMD, mostly as an adjuvant therapy in combination with photodynamic therapy, intravitreal anti-VEGF therapy, or in multimodality approaches [65, 66]. A sustained release dexamethasone (such as Ozurdex, Allergan, Inc., Irvine, CA) would seem to be the logical option in such an adjuvant approach. One single-masked, randomized control study in 243 eyes reported work comparing intravitreal ranibizumab plus sustained release dexamethasone with ranibizumab plus sham. Results suggested a reduction in the need for multiple ranibizumab injections and an increasing interval between injections although combination therapy was associated with raised intraocular pressure requiring treatment in 16% [67, 68]. A number of other clinical trials are now assessing dexamethasone as an adjunct in the treatment of wet AMD, and full reports are awaited (http://www.clinicaltrials.gov/, NCT0162746; NCT00793923; NCT00390208).

Other approaches include Iluvien, an intravitreal implant containing fluocinolone acetonide packed in a nonbiodegradable polyamide tube [69]. An ongoing phase 2 clinical trial is currently recruiting patients to look at the efficacy of this intravitreal implant in inhibiting geographic atrophy progression in AMD (http://www.clinicaltrials.gov/, NCT00695318).

Rapamycin, a drug originally designed as an antifungal agent, has recently been shown to be a potent immuno-suppressive, anti-inflammatory, and antiangiogenesis agent by inhibiting the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase. Recent work in the senescence-accelerated OXYS rat has shown some inhibition of the spontaneous retinopathy phenotype seen which models age-related macular degeneration in some respects [70]. A phase 2 study has been launched by National Eye Institute to determine whether repeated intravitreal rapamycin can slow progression of geographic atrophy (http://www.clinicaltrials.gov/, NCT01445548). Glatiramer acetate is another broad immunomodulatory agent that upregulates specific suppressor T-cells and suppresses inflammatory cytokines. Intravenous glatiramer acetate in dry AMD patients has been shown to reduce drusen load on optical coherence tomography imaging [71]. In the "wet" form of AMD, anti-inflammatory agents have been found to be moderately successful in controlling choroidal neovascularization. For example, intravitreal triamcinolone acetonide and infliximab, an antibody of tumor necrosis factor α (TNF-α), have shown positive effects in treating CNV in patients and animal models [72, 73].

#### 3.2. Focused Targeting of the Complement System in AMD

The area in which inflammatory mediators in genetic retinal disease is being most intensively investigated, however, is in inhibiting activation of the complement system in AMD patients [74]. These studies have focused on both
antibody dependent and independent complement inhibition trials.

FCFD4514S is a recombinant, humanized monoclonal antibody Fab fragment antibody targeted to block the complement factor D, an early rate-limiting enzyme in the activation of the alternative complement pathway. Current phase 2 clinical trials are assessing its use in geographic atrophy of the alternative complement pathway. Current phase complement factor D, an early rate-limiting enzyme in the activating antibody Fab fragment antibody targeted to block the complement factor 5 to block subsequent downstream anaphylatoxin activation and the formation of membrane attack complexes. It is considered by some to be the most likely approach since it preserves production of C3a anaphylatoxin and C3b production required for opsonisation and clearing of harmful immune complexes [64]. Intravenous eculizumab is currently under trial for dry AMD (http://www.clinicaltrials.gov/, NCT00935883).

An example of studies focusing on nonantibody approaches to modify complement activation is POT-4, the first complement inhibitor to be assessed in AMD. This molecule is a cyclic peptide that reversibly binds to C3 preventing its activation to C3a and C3b, thus blocking the classic pathway and the lecithin pathway as well as the alternative complement pathway. Intravitreal depot injections are being assessed in patients with wet and dry AMD (http://www.clinicaltrials.gov/, NCT00473928).

Other nonantibody approaches include small molecule peptidomimetic C5a receptor antagonists currently being considered for AMD [75]. An alternative approach that may avoid the general consequences of inhibiting complement activation is studies focusing on replacing abnormal complement factor H alleles. TT30 is a recombinant fusion protein being used to replace defective complement factor H [76] and is being considered for use in AMD trials. Modification of complement activation may also be of benefit in wet AMD. In a laser-induced mouse model of choroidal neovascularization, intravenous administration of “CR2-fH,” a recombinant form of complement factor H linked to complement receptor 2, inhibited neovascular growth [77].

3.3. Broad-Based Anti-Inflammatory Studies in RP. Corticosteroids have been routinely used to treat the macular edema seen in late RP with mixed success [78]. Most recently, however, a sustained-release dexamethasone implant (Ozurdex) has been used in a small cohort of RP patients with macular oedema, suggesting some structural and functional benefits [79]. Also recently, N-acetylcysteine, an orally bioavailable antioxidant, has been used in rd10 mice [55]. It was shown by TUNEL staining that a reduction in photoreceptor cell death was associated with a strong suppression of expression of cytokines interleukin 1β and tissue necrosis factor-α and chemokines monocyte chemotactic proteins 1 and thymus activation-regulated chemokine [55]. In another study, fluocinolone acetonide has been conjugated with dendrimer particles (a hydroxyl-terminated polyamidoamine dendrimer-drug conjugate nanodevice) to target outer retina activated microglia [80]. They showed that after intravitreal administration in the Royal College of Surgeons rat model of RP (homozygous Mertk mutant), four weeks later, there had been significant preservation of outer nuclear layer thickness (indicative of photoreceptor survival) and in electroretinogram b-wave response. In addition, it was shown that this was associated with a reduction of activated microglia in the retina [80].

4. Conclusion

Considerable evidence now exists linking inflammatory mediators to genetic retinal diseases such as AMD and RP. It is, however, still unclear whether this is a central or pivotal role [81]. Preclinical and clinical trials suggest that inhibiting inflammatory mediators can have some therapeutic benefit, but further ongoing trials are needed to demonstrate the true impact of this approach. The benefits of inhibiting inflammation in genetic retinal disease might, for instance not be so clear-cut. In recent clinical trials, ciliary-derived neurotrophic factor (CNTF, a neuroprotective growth factor) has been shown to provide some inhibition of degeneration in both dry AMD [82] and RP [83]. Studies in mouse retina, however, have suggested that CNTF induces expression of proinflammatory genes in retinal Müller cells [84]. This would seem counterintuitive, and its clinical relevance has yet to be determined. A role for anti-inflammatory agents, as stand-alone monotherapies or as adjuvants (for instance, in combination with neuroprotective strategies or anti-VEGF therapies), is certain to be prominent feature of future research into the treatment of retinal disease.

Conflict of Interests

There is no conflict of interests related to this paper.

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Review Article
Nonsteroidal Anti-Inflammatory Drugs for Retinal Disease

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are used extensively in ophthalmology for pain and photophobia after photorefractive surgery and to reduce miosis, inflammation, and cystoid macular edema following cataract surgery. In recent years, the US Food and Drug Administration has approved new topical NSAIDs and previously approved NSAIDs have been reformulated. These changes may allow for greater drug penetration into the retina and thereby offer additional therapeutic advantages. For example, therapeutic effects on diabetic retinopathy and age-related macular degeneration may now be achievable. We provide an updated review on the scientific rationale and clinical use of NSAIDs for retinal disease.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly prescribed classes of medications and are routinely employed for their analgesic, antipyretic, and anti-inflammatory properties. NSAIDs are potent inhibitors of cyclooxygenase (COX) enzymes and thereby the synthesis of pro-inflammatory prostaglandins (PGs). In ophthalmology, topical NSAIDs are used to stabilize pupillary dilation during intraocular surgery and to treat allergic conjunctivitis and postoperative inflammation, pain and cystoid macular edema (CME) [1]. The therapeutic efficacy of topical NSAIDs for these aforementioned conditions has been well established [1, 2]. There is also increasing evidence that PGs play a role in the pathogenesis of diabetic retinopathy and age-related macular degeneration (AMD) and recent years have seen more studies examining the therapeutic role of NSAIDs for these disorders [1]. The intent of this paper is to focus on the potential application of NSAIDs to treat retinal disease.

2. Nonsteroidal Anti-Inflammatory Drugs

NSAIDs are a class of medications that lack a steroid nucleus and inhibit COX enzymes [1]. COX enzymes catalyze the production of five classes of PGs: PGE$_2$, PGD$_2$, PGF$_{2\alpha}$, PGI$_2$, and Thromboxane A$_2$. Two main isoforms of COX, COX-1 and COX-2, exist [3], and a third (COX-3) remains largely uncharacterized [4]. COX-1 contributes to normal physiological processes and is expressed in the gastrointestinal tract, kidneys, platelets, and vascular endothelium [1]. COX-2 is an inducible enzyme that is upregulated during pain, fever, and inflammatory responses, but is also expressed in some systems under normal conditions. COX-2 is the predominate isofrom in retinal pigment epithelium (RPE) cells and is up-regulated in the presence of proinflammatory cytokines [5]. COX-2 has an important role in angiogenesis and has been implicated in choroidal neovascularization (CNV) and proliferative diabetic retinopathy (PDR) [1].

PGs are an important class of inflammatory mediators that are biosynthesized from membrane bound arachidonic acid. Within the eye, PGs disrupt the blood-ocular barrier, increase vasodilation, and facilitate leukocyte migration [1]. They also interact with and amplify many other soluble mediators including vascular endothelial growth factor (VEGF) [1, 6, 7]. As a result, their inhibition has favorable effects on intraocular inflammation and retinal edema [8].

2.1. Formulations. Several topical NSAIDs are commercially available for ophthalmic use, including ketorolac, diclofenac, nepafenac, bromfenac, and flurbiprofen. Dosing varies from daily (Bromday, bromfenac 0.09%, ISTA Pharmaceuticals) to four times daily (Acular, ketorolac 0.5%, Allergan, Inc).
Ketorolac is reported to be the most potent inhibitor of COX-1, while bromfenac and amfenac are the most potent inhibitors of COX-2 [9–13]. Bromfenac may be 3 to 18 fold more potent of an inhibitor of COX-2 than diclofenac, ketorolac and amfenac (the active metabolite of nepafenac) [9, 12], but this attribute has not been consistently reported [13]. Furthermore, the relative importance of COX-1 versus COX-2 inhibition in ocular disease remains unproven [1].

2.2. Aqueous Levels. Several studies have measured intraocular NSAID levels in humans after topical application. After a single application, peak aqueous drug levels are detectable for: diclofenac 0.1% (82 ng/mL; 2.4 hour peak), flurbiprofen 0.03% (60 ng/mL; 2.0 hour peak), nepafenac 0.1% (205.3 ng/mL; peak 30 minutes), amfenac (70.1 ng/mL), ketorolac 0.4% (57.5 ng/mL; 60 minutes), and bromfenac 0.09% (25.9 ng/mL) [13, 14]. Acuvail (Allergan, Inc) is a newer preservative-free formulation (0.45%) of ketorolac dosed twice daily that has been reported to achieve a much higher peak aqueous concentration after a single application than older formulations but as of yet has not been tested in humans [15]. More frequent and continued dosing leads to even higher aqueous levels. Twelve doses over two days of ketorolac 0.4% and nepafenac 0.1% result in reported aqueous levels of 1079 ng/mL of ketorolac and 353.4 ng/mL of amfenac [16], which far exceed reported inhibitory concentration 50 (IC50) for COX-1 and COX-2 enzymes for both NSAIDs: ketorolac (COX-1, 5.3 to 7.5 ng/mL; COX-2, 33.9 to 42.5 ng/mL) and amfenac (COX-1, 35.6 to 63.6 ng/mL; COX-2, 0.51 to 38.1 ng/mL).

2.3. Vitreous Levels. In contrast to aqueous drug levels, there is a paucity of human studies measuring NSAID levels in the vitreous after topical application. A single study measured vitreous drug levels in patients who received ketorolac 0.4% four times daily, bromfenac 0.09% two times daily, or nepafenac 0.1% three times daily for three days before vitrectomy surgery [17]. Vitreous levels of ketorolac, bromfenac, and amfenac were reported as 2.8 ng/mL, 0.96 ng/mL, and 2.0 ng/mL, respectively, but only ketorolac resulted in significantly lower vitreous PGE2 levels compared to placebo. Aqueous and vitreous concentrations of NSAID would likely have a direct effect on anterior (ciliary body and iris) and posterior (retina and choroid) PG production, respectively.

3. Postoperative Cystoid Macular Edema

Cystoid macular edema is the accumulation of extracellular fluid within the retina due to leakage from dilated capillaries. It is the most common cause of vision loss after cataract surgery [1], and was first described over a half-century ago [18]. Its incidence has been reported to be as high as 9–19% on fluorescein angiography (FA) and 41% on optical coherence tomography (OCT), but clinically important CME is far less common [19–21]. Inflammation has been implicated as a main cause of postoperative CME [1] and numerous studies have examined the role of NSAIDs for the treatment of acute and chronic CME and its prophylaxis.

3.1. Acute and Chronic CME. CME associated with cataract surgery may be treated early (less than 6 months) or late (6 months or more) following its diagnosis [1]. These two groups are distinguished as acute and chronic CME. The efficacy of topical NSAIDs in treating both conditions has been reviewed in great detail elsewhere with general consensus, despite the paucity of well-designed studies, that treatment with NSAIDs is beneficial (reduces macular edema and may improve vision) at least over the short-term [1]. Recently, Warren et al. evaluated the adjunctive use of nepafenac 0.1%, diclofenac 0.1%, ketorolac 0.4%, bromfenac 0.09%, or placebo in 39 patients for 16 weeks in addition to intravitreal triamcinolone and bevacizumab for treatment of chronic CME [22]. Both adjunctive use of nepafenac and bromfenac resulted in greater reduction of retinal thickness at 12 and 16 weeks but only nepafenac led to a significant improvement in vision. Similarly, in a retrospective, uncontrolled study, nepafenac 0.1% improved retinal thickness and visual acuity in patients with chronic, recalcitrant CME [23].

3.2. Prophylaxis of CME. Numerous studies have evaluated NSAIDs for prevention of postoperative CME following cataract surgery. Only pertinent well-designed studies are reviewed here. A randomized, double-masked, placebo-controlled trial by Flach et al. reported that prophylactic use of ketorolac 0.5% was effective in reducing angiographic CME in aphakic patients without the use of corticosteroids [24]. A multicenter, prospective study compared the effects of topical diclofenac 0.1% versus fluorometholone (FML) 0.1% on prevention of CME in eyes undergoing modern, small-incision phacoemulsification [25]. Five weeks after surgery, angiographic CME was present in 5.7% of diclofenac-treated eyes and 54.7% of FML-treated eyes. FML has limited intraocular penetration; therefore, these results may approximate the effectiveness of diclofenac as compared to placebo. A more recent randomized, masked comparison of topical ketorolac 0.4% plus corticosteroid versus corticosteroid alone demonstrated a significantly reduced rate of CME with combination treatment in low-risk patients after cataract surgery [26]. However, the absolute incidence of definite or probable CME was low in both groups (2.4% for corticosteroid group; 0% for ketorolac/corticosteroid group) and there was no difference reported in visual outcomes. The results of this latter study question the cost-effectiveness of routine prophylactic treatment with both a corticosteroid and NSAID for patients at low risk for CME. On the other hand, routine use in patients with diabetes or uveitis who are at higher risk of developing postoperative CME may be warranted [27].

The use of a topical NSAID and corticosteroid together is sometimes reported to be “synergistic” in the literature. This clinical impression of synergy remains unproven and would seem unlikely given the fact that both drug classes have overlapping mechanisms of action [8]. Synergy is defined as two or more agents working in combination to produce an effect that could not be obtained by either agent alone. A classic example of synergy involves penicillin and aminoglycoside antibiotics where use of both antibiotics in
combination significantly lowers the IC$_{50}$ of each antibiotic for a given microorganism. Although a large, randomized, prospective study demonstrated that ketorolac 0.5% was more effective than dexamethasone sodium phosphate 0.1% solution in facilitating reestablishment of the blood-aqueous barrier after surgery, differences in drug formulation and intraocular concentration preclude any conclusions about synergy [28]. Furthermore, although many prospective studies have confirmed that the combination use of a NSAID and corticosteroid is superior to a corticosteroid alone for CME and visual improvement after intraocular surgery, these findings can be explained by an additive effect of a second anti-inflammatory agent.

3.3. CME after Vitreoretinal Surgery. Several studies have assessed the therapeutic benefit of NSAIDs for the prevention of CME after vitreoretinal surgery. A prospective, randomized, placebo-controlled trial reported that topical ketorolac 0.4% reduced both retinal thickness (9%) and total macular volume (6%) but neither outcome reached statistical significance [29]. Schoenberger et al. reported that topical nepafenac more rapidly reduced macular volume in patients undergoing epiretinal membrane surgery, but this effect was not observed by another study using nepafenac [30, 31].

4. Age-Related Macular Degeneration

CNV is the most common cause of severe vision loss in patients with the wet (neovascular) form of age-related macular degeneration (AMD) [32–34]. AMD is the leading cause of blindness in the United States and will affect nearly 8 million Americans by 2020 [32]. Many patients with AMD have moderate vision loss (20/50 to 20/100) in the better eye that results in quality-of-life measurements that are 32% below normal and similar to patients with severe angina or hip fractures [33]. An increasing percentage of patients with AMD suffer severe vision loss (20/800) which results in a 60% reduction in quality of life and is similar to a patient who is bedridden due to a catastrophic stroke.

It is now firmly established that VEGF is a principle mediator of CNV. While VEGF inhibitors have been an important advance in treating neovascular AMD, they do not slow down the underlying disease process. Moreover, VEGF is essential for normal homeostasis of retinal cells and its chronic inhibition may therefore be undesirable [35]. Consequently, it is clear that strictly inhibiting VEGF neither addresses the multifactorial pathogenesis of CNV nor the underlying cause of VEGF induction. Instead, a growing body of scientific evidence indicates that inflammation plays a central role in CNV [36, 37]. A better understanding of inflammatory mediators of VEGF induction may therefore provide an opportunity to develop preventative strategies.

In this regard, COX-2 can be detected in human choroidal neovascular membranes [38] and considerable scientific evidence indicates that COX is a promoter of angiogenesis [39, 40]. Patients who regularly take NSAIDs have a 40–50% reduction in mortality from colorectal cancer and a distinguishing feature of colorectal tumors is high expression of COX [41]. Pharmacologic inhibition of COX appears to reduce VEGF expression in cultured human RPE cells and suppresses VEGF in both trauma- and ischemia-induced models of retinal angiogenesis [42–44]. In a variety of experimental systems, inhibition of COX-2 suppresses angiogenesis. In vitro studies have demonstrated that PGE$_2$ increases VEGF expression in cultured Müller cells and agonism or antagonism of the PGE$_2$ receptor EP$_4$ increases or decreases VEGF production, respectively [42].

4.1. Animal Studies. Animal studies have consistently shown that NSAIDs reduce or inhibit CNV. Kim et al. have demonstrated that both topical and intravitreal ketorolac significantly reduces angiographic leakage and retinal levels of PGE$_2$ and VEGF in an animal model of CNV [45, 46]. Furthermore, CNV was significantly reduced in COX-2 null mice after laser-induction, an effect that could be explained by reduced retinal VEGF [47]. Other investigators have also independently reported similar observations with administration of topical or oral NSAIDs [48, 49].

4.2. Clinical Studies. In contrast to more robust evidence in animal studies, clinical evidence demonstrating a consistent therapeutic benefit of NSAIDs for AMD is lacking. A cohort of patients with rheumatoid arthritis was prospectively followed and found to have a low prevalence of AMD [50], presumed to be due to long-term administration of anti-inflammatory medications, and a large retrospective study reported decreased rates of CNV among AMD patients taking aspirin [51]. In contrast, no association between systemic NSAIDs and five-year incidence of age-related maculopathy was observed in the Blue Mountains Eye Study [52].

Studies investigating topical NSAIDs for exudative AMD (Table 1) [53–58] have also reported conflicting results. A randomized, controlled study reported no additional benefit in regards to vision or lesion size with combination treatment with diclofenac and photodynamic therapy for subfoveal CNV [55]. Two retrospective studies also showed no benefit with the addition of topical bromfenac or nepafenac to intravitreal anti-VEGF agents in patients with persistently active exudative AMD [53, 54]. In contrast, two prospective, randomized, controlled clinical studies reported favorable effects of topical bromfenac with respect to retinal thickness and reduced number of anti-VEGF treatments. Flaxel et al. investigated combination treatment with topical bromfenac 0.09% for new or recurrent exudative AMD [57]. Patients received monthly intravitreal ranibizumab (IVR) for four months, followed by as needed treatment and were randomized to either combination treatment with bromfenac or monotherapy. There was no observed difference in regards to vision or number of injections between groups, but there was a significant difference in favor of combination treatment in reduction of central macular thickness (−81.56 microns, combination group; −42.50 microns, IVR group). In an independent study by Gomi et al., combination treatment with bromfenac 0.1% and IVR significantly reduced the number of anti-VEGF injections needed compared to IVR monotherapy [58].
Advanced retinopathy is a significant cause of vision loss in diabetic patients, affecting about 75,000 new patients in the United States every year [59]. Diabetic macular edema (DME) is the most common cause of vision loss in diabetic patients, affecting one million people in the United States every year [60]. Proven preventable measures for DME include lowering of high blood pressure and strict control of blood glucose [61,62].

5. Diabetic Macular Edema and Diabetic Retinopathy

Diabetic retinopathy (DR) is the most frequent cause of legal blindness among working-aged individuals in developed countries [59]. Diabetic macular edema (DME) is the most common cause of vision loss in diabetic patients, affecting about 75,000 new patients in the United States every year [60]. Proven preventable measures for DR include lowering of high blood pressure and strict control of blood glucose [61,62] but a growing body of scientific evidence supports a pathogenic role of inflammation [63].

In support of this, a number of pro-inflammatory cytokines are consistently elevated in the vitreous of patients with advanced stages of DR [64–66] and treatment with NSAIDs prevents or delays its progression in animal models. Recent work from our group has demonstrated elevated levels of PGE2 in vitreous samples taken from patients with PDR which correlate with vitreous levels of VEGF and provides support for a pathogenic role of PGs in DR [67].

5.1. Experimental and Animal Studies. In both experimental and animal models, PGs induce VEGF production [45,68] with subsequent development of vascular leakage and retinal neovascularization [69]. In cultured Müller cells, agonism or antagonism of the PGE2 receptor EP3 increases or decreases VEGF production, respectively, in a dose-dependent manner [42]. Retinal cells consistently upregulate COX and PGs [43,70] in DR and PGE2 is increased by 40% in the retinal vasculature of diabetic rats [70]. Topical nepafenac 0.1% significantly inhibits diabetes-induced retinal microvascular disease and treatment with celecoxib reduces retinal VEGF expression and vascular leakage in streptozotocin-induced diabetic rats [71,72]. Administration of other NSAIDs (nepafenac, aspirin, meloxicam) has also been reported to inhibit diabetes-induced retinal microvascular disease and prevent early DR [71,73].

5.2. Systemic Therapy. The therapeutic benefit of systemic NSAIDs for DR has been evaluated in a few clinical studies. It was first observed a half century ago that rheumatoid arthritis patients taking salicylates had a reduced incidence of DR [74]. This observation was later examined in two large multicenter clinical trials, the Early Treatment Diabetic Retinopathy Study (ETDRS), which examined the effect of 650 mg aspirin on advanced DR [75], and the Dipyridamole Aspirin Microangiopathy of Diabetes (DAMAD) Study [76], which tested the impact of 990mg aspirin in patients with more advanced DR in ETDRS, a significant effect was seen in the DAMAD study, where higher doses of aspirin were found to slow the development of retinal microaneurysms. This latter observation is supported by a randomized 3-year pilot study where the NSAID sulindac prevented development and progression of DR [77]. Similarly, a recent prospective, controlled trial conducted by the National Eye Institute...
5.3. Topical Therapy. There are uncontrolled case reports reporting anatomical and visual improvement with topical NSAIDs for DME. Hariprasad et al. described several patients reporting anatomical and visual improvement with topical

NSAIDs [79]. This provides further support for anti-inflammatory based therapies [79].

5.4. Intravitreal Therapy. Four studies have evaluated intravitreal diclofenac or ketorolac for DME (Table 2). Soheilian et al. investigated the safety and efficacy of a single intravitreal injection of diclofenac (500 mcg/0.1 mL) in five eyes with DME [83]. After eight weeks, VA improved in two eyes, worsened in two eyes, and remained stable in one eye, while mean central macular thickness (CMT) was actually worse than at baseline. Elbendary and Shahin compared intravitreal diclofenac (500 mcg/0.1 mL) to intravitreal triamcinolone (4 mg/0.1 mL) in the treatment of diffuse DME in a randomized study [84]. CMT decreased in the diclofenac group from 419.8 microns at baseline to 323.5 microns at one month and 271.1 microns at three months. There was no difference between the two groups in CMT, final VA, mean line improvement, and percent of eyes with improved VA. Reis Ado et al. treated twenty patients with bilateral DME refractory to laser therapy [85]. One eye received intravitreal ketorolac (500 mcg/0.1 mL), while the other served as a control. At one month, there was a significant improvement in VA in the treated eyes relative to controls, but there was no change in foveal thickness or macular volume. Maldonado et al. treated 25 patients with DME refractory to laser with a single injection of ketorolac (3000 mcg/0.1 mL). At one month, 28% of patients had an improvement in VA of at least five letters, while there was no significant difference in macular thickness [86].

6. Conclusions

Although there is good collective evidence that topical NSAIDs treat and prevent CME after cataract surgery, the long-term visual benefits of this practice remain unknown since CME can resolve spontaneously. It is now well established that inflammation plays a pathogenic role in AMD, DR, and DME, but clinical data demonstrating a therapeutic effect of NSAIDs for these diseases is limited and derived mostly from small, retrospective or uncontrolled studies. Despite considerable scientific rationale, there is insufficient evidence to recommend using NSAIDs to treat these conditions until more compelling clinical data emerges.

**Conflict of Interests**

The authors declare no conflict of interests.

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